Dear Friends and Colleagues:

On behalf of the Abstract Committee, let me welcome you to HUPO2009 World Congress and to Toronto. Thanks to your enthusiastic support and contributions, we have put together an excellent and comprehensive program covering a wide-spectrum of proteomic science from 36 countries. This Proceeding Book provides all programmatic information as we have it when we go to press, including the program overview, the over 920 abstracts that you submitted and sorted according to the 33 categories that you had chosen, as well as information on invited speakers and Young Investigator Awardees. We are pleased to be able to put together an oral program that comprises some 150 presentations, most of which are selected from the abstracts that you submitted.

We thank the editorial efforts of Dr. Peipei Ping and her associates, as well as the production teams of Clinical Proteomics for bringing our concept of a Proceeding Book to fruition.

Thank you again for your enthusiastic support. Have a great conference!

Professor K.W. Michael Siu

Chair of Abstract Review Committee

On behalf of HUPO 2009 Organizing Committee

HUPO20009 World Congress Organizing Committee

Christoph Borchers, PhD
Director, Genome BC Proteomics Centre
University of Victoria, Canada

Michael Siu, PhD
Director, Centre for Research
in Mass Spectrometry, York University, Canada

David Fenyo, PhD
Senior Research Scientist
The Rockfeller University, USA

Peipei Ping, PhD
Professor and Director
Physiology and Medicine
UCLA School of Medicine, USA

Peter Liu, MD
Scientific Director, ICRH
Professor of Medicine and Physiology
at the University of Toronto, Canada

John Yates, PhD
Director, Mass Spectrometry Lab
Department of Chemical Physiology
The Scripps Research Institute, USA

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The Multicultural Canadian Experience Welcomes You at HUPO2009 World Congress.

Important Dates for HUPO2009 World Congress Events:

Friday, September 25th, 2009.

- CNPN Symposium.

The 2009 CNPN Symposium is to be held on Friday, September 25th, 2009 from 8:30am-5:00pm at the Westin Harbour Castle Hotel. This 2009 CNPN Symposium is sponsored by the Canadian National Proteomic Network (CNPN). It will be co-chaired by Professor Juergen Kast and Professor John Marshall. This symposium is expecting 300 attendees.

Saturday, September 26th, 2009.

- HUPO Education Day Program.

The Education Day Program of HUPO2009 is scheduled to take place on Saturday, September 26th, 2009. HUPO2009 Education Day Program is co-chaired by Professor Angelika Gorg, Professor Thierry Rabilloud and Professor Ole Jensen. Detailed Program is online (www.hupo2009.org).

- HUPO Scientific Initiative Workshops.

Professor Gil Omenn, Chair of the HUPO Initiative Committee, has coordinated the organization of all Initiative workshops for HUPO2009 World Congress. Detailed Program is online (www.hupo2009.org).

- International Forum of Proteomics.

The 1st International Forum of Proteomics is scheduled to take place on Saturday, September 26th, 2009, from 9:00am to 5pm. This event is co-chaired by Professor Michael Siu, Professor Daniel Chan, and Professor Peipei Ping. Detailed Scientific Program is online (www.hupo2009.org).

- Proteomic Gala.

The 1st Proteomic Gala is scheduled to take place on Saturday, September 26th, 2009, from 7:30pm to 10:00pm. This event is co-chaired by Professor Christoph Borchers and Professor Peipei Ping. Detailed Scientific Program is online (www.hupo2009.org).

Sunday, September 27th, 2009.

- HUPO Clinical Day Program.

The Clinical Day Program of HUPO2009 is scheduled to take place on Sunday, September 27th, 2009. HUPO2009 Clinical Day Program is co-chaired by Professor Denis Hochstrasser and Professor Jan Schnitzer. Detailed Program is online (www.hupo2009.org).

- Proteomics Technology Development Symposium.

This symposium is co-organized by Professor Christoph Borchers and Professor John Yates. Detailed Program is online (www.hupo2009.org).

- HUPO Council Meeting and Council Dinner.

For detailed information on HUPO Council Meeting, please contact Dr. Pierre Legrain at Pierre.Legrain@cea.fr. For detailed information on HUPO Council Dinner, please contact info@HUPO2009.org.

- Dinner Cruise Sponsored by Brukers.

A dinner cruise is sponsored by Brukers following the Proteomics Technology Development Symposium. This is available for registered HUPO2009 attendees only. Please see detailed information online (www.hupo2009.org).

Monday, September 28th, 2009.

- HUPO2009 World Congress Opening Ceremony and Reception.

The opening ceremony for HUPO2009 is scheduled to take place on Monday, September 28th, 2009 from 5:00pm to 7:30pm. It includes two keynote lectures, welcoming address, entrainment program, and reception.

Tuesday, September 29th, 2009.

- HUPO Member General Assembly is scheduled from 6:30pm to 7:30pm.

Wednesday, September 30th, 2009.

- HUPO2009 World Congress Closing Ceremony and Banquet is scheduled from 5:00pm to 10:00pm.

HUPO2009 Pre-congress Programs and Events

HUPO Scientific Initiative Workshops
Saturday, Sept 26th, 2009

Chaired By

Gil Omenn

Professor and Director
Internal Medicine and Human Genetics
University of Michigan, USA

HUPO-sponsored Scientific Initiatives

HLPP	HBPP
PSI	HAI
PPP	HGPI
HUPO CVI	DBI
Stem Cell	HKUPP

Human Proteome Projects

HUPO Education Program
Saturday, Sept 26th, 2009

Co-Chaired By

Thierry Rabilloud

CNRS Permanent Researcher
DRDC/BECP
CEA-Grenoble, France

HUPO Education Program
Saturday, Sept 26th, 2009

Co-Chaired By

Ole Noerregaard Jensen

Professor and Director
Biochemistry and Molecular biology
Syddansk, University, Denmark

HUPO Clinical Day Program
Sunday, Sept 27th, 2009

Co-Chaired By

Denis Hochstrasser

Professor and Chairman
Head of Pathology Department
University of Geneva, Switzerland

HUPO Clinical Day Program
Sunday, Sept 27th, 2009

Co-Chaired By

Jan Schnitzer

Professor and Scientific Director
Vascular Biology Program
PRISM, US

HUPO2009 graciously acknowledges the generous support by:

Agilent Technologies
Thermo SCIENTIFIC
BIO-RAD
BRUKER
AB applied biosystems
DIONEX
GenomeCanada
GenomeBritishColumbia
CIHR IRSC
UVIC GENOME BC Proteomics Centre
PROTEOMICS
PROTEOMICS Clinical Applications

HUPO2009 Pre-congress Programs and Events

1st International Forum of Proteomics

Award Ceremony and Lectures

Saturday, September 26th, 2009
9:00 am – 5:00 pm

Award Lecture By

Michael Snyder

Professor and Director
Genetics
Stanford School of Medicine, US

Award Lecture By

Ralph Bradshaw

Professor and Deputy Director
Mass Spectrometry Facility
UCSF and MCP, US

Award Lecture By

Fuchu He

Professor and Director
Beijing Institute of Radiation Medicine
Chinese National Center of
Biomedical Analysis, China

1st Proteomic Gala

Where world leaders meet to better human health

Saturday, September 26th, 2009
7:30 pm – 10:00 pm
The Westin Harbour Castle
Toronto, Canada

Enjoy an evening of discussion on science, society,
and our environment with philanthropic visionaries, policy makers,
community leaders from the private and the public sector,
patients, physicians, and world-renowned scientists
from North America and around the world.

Come and join us at the 2009 Gala event, be part of a global effort
to raise public awareness of proteomic sciences,
to build knowledge base for biology and medicine, and
to develop concepts and initiatives towards better health.

Keynote lecture by an internationally distinguished leader committed to the
advancement of science, global health and medicine;
announcement of the top 10 most influential icons and milestones in proteomic science,
selection made by the proteomic community.

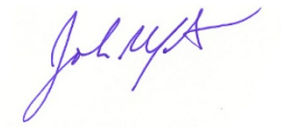
Banquet and Live Entertainment

A Message from the Chair:

We would like to congratulate the awardees for their excellent research and participation in the HUPO2009 World Congress. A total of 117 awards were provided to support young investigators from 28 countries/regions. We wish the continued success of the young investigators in proteomic sciences.

Our sincerely appreciations for those provided generous support for the HUPO2009 Young Investigator Awards, Genome Canada, Genome British Columbia, Thermo Scientific and Agilent Technologies. In parallel, our special thanks go to HUPO2009 Keynote Lecture Speakers and many Invited Speakers, who made personal contributions to support the HUPO2009 Young Investigator Award.

I wish you sincere congratulations and a successful meeting.



Professor John R Yates III
Chair of HUPO2009 Young Investigator Awards Committee

GenomeCanada Thermo SCIENTIFIC Agilent Technologies GenomeBritishColumbia

List of HUPO2009 Young Investigator Awardees

Name	Affiliation		Name	Affiliation
Cecilia Ng	Australia		Graham Gibson	Canada
Albert Lee	Australia		Geraldine Walsh Ph.D	Canada
Rommel Mathias	Australia		Elizabeth Clemis	Canada
Sibasish Dolai	Australia		Rachel.Kozlowski	Canada
Naveid Ali	Australia		Philipp Lange, Ph.D	Canada
Muhammad Rizwan	Austria		Pitter Huesgen	Canada
Klaus Kratochwill, MSc	Austria		Xi Simon Wang	Canada
Ruth Daniels, Ph.D	Belgium		Jonathan B.Béland	Canada
Annelies Vanheel	Belgium		Jean-Philippe Gagne	Canada
Christopher Hughes	Canada		Valerie Poirier	Canada
Amelia Nuhn	Canada		Liangliang Sun	China
Nicholas Carruthers	Canada		Qiang Shan	China
Lei Li	Canada		Haidan Sun	China
Leslie Mitchell	Canada		Li Zheng	China
Mohamed Abu-Farha	Canada		Lian Zhao	China
Huadong Liu	Canada		Tine Engberg Thingholm	Denmark
Jean-Philippe Lambert	Canada		Fidel Anaya	England
Ruijun Tian	Canada		David Trudgian, Ph.D	England
C.Y. Chan,	Canada		Dhilia Udie Lamasudin	England
Chris Hao Pu	Canada		Gina Lynn Eagle, Ph.D	England
Xuan Ding	Canada		Andrew Dowsey	England
Parveen Sharma, Ph.D	Canada		Martin Barrios-Llerena, Ph.D	England
Nancy Fang	Canada		Budin Ghyslain	France
Brett Eyford	Canada		Somayyeh Fahiminiya	France
Morteza Razavi	Canada		Olivia Baessler	Germany
Nan Wang	Canada		Xiaobo Yu, Ph.D	Germany
Tomonori Kaneko	Canada		Yan Luo, Ph.D	Germany
Cordula Klockenbusch, Ph.D	Canada		Stella Sun	Hong Kong

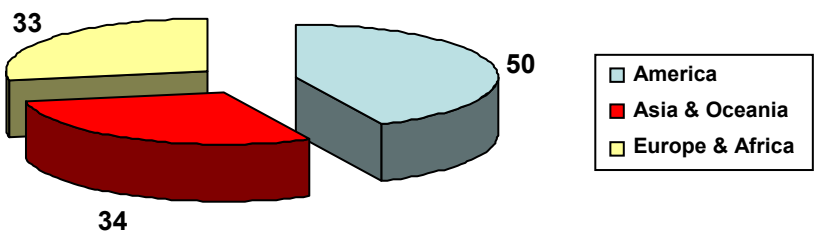
Name	Affiliation
Xu Zhi, Michelle	Hong Kong
Satyendra Chandra Tripathi	India
Poonam Gautam, Ph.D	India
Ravindra Varma Polisetty	India
Amol Suryawanshi, Ph.D	India
Tanu Bajaj	India
Muzafar Ahmad Macha	India
Mahdyie Jadaliha	Iran
Aisling Robinson	Ireland
Ciara McManus, Ph.D	Ireland
Elisa Maurizio	Italy
Domenica Scumaci, Ph.D	Italy
Rena Sano	Japan
Sameh Magdeldin, Ph.D	Japan
Venugopal Balakrishnan	Malasyia
Magalie Boucher	New Zealand
Håvard Loftheim	Norway
Therese Solstad Saunders	Norway
Nikolaus Oberprieler, Ph.D	Norway
Saadia Zahid	Pakistan
Rizma Khan	Pakistan
Samreen Riaz, Ph.D	Pakistan
Igor Popov	Russia
Dmitry Avtonomov	Russia
Ilya A. Agron	Russia
Karthik Narasimhan	Singapore
Patricia Mathabe	South Africa
Suereta Fortuin	South Africa
Sandro Argüelles Castilla, Ph.D	Spain
Fernando de la Cuesta	Spain
Kah-Wai Lin, M.D	Sweden

Name	Affiliation
Olena Zakharchenko Ph.D	Sweden
Loïc Dayon, Ph.D	Switzerland
MiaoHsia	Taiwan
Wei-Chi Ku	Tawain
Chih-Ching Wu Ph.D	Tawain
Chun-I Wang	Tawain
Ting-Yu Lin	Tawain
Wararat Chiangjong	Thailand
Rattiyaporn Kanlaya, Ph.D	Thailand
Huan He	USA
Christine Vogel	USA
Gurudutt Pendyala, Ph.D	USA
Daniel Boutz	USA
John M. Asara, Ph.D	USA
Yan Li, Ph.D	USA
Anastasia Yocum	USA
Brendan Faherty	USA
Hyungwon Choi	USA
Tao Liu, Ph.D.	USA
Timothy Clough	USA
Aleksey A. Nakorchevskiy, Ph.D	USA
Gurkan Bebek, Ph.D	USA
Estelle Glory	USA
Judith Coppinger	USA
Bryan Fonslow	USA
Amit Kaushal	USA
Sarah Warburton	USA
Gabrielle Giese	USA
Ning Deng, Ph.D	USA
Christopher I. Murray	USA

Awardees Grouped by Country/Region

Country/Region	Number	Country/Region	Number	Country/Region	Number
Australia	5	India	6	Singapore	1
Austria	2	Iran	1	South Africa	2
Belgium	2	Ireland	2	Spain	2
Canada	29	Italy	2	Sweden	2
China	5	Japan	2	Switzerland	1
Denmark	1	Malasyia	1	Taiwan	5
England	6	New Zealand	1	Thailand	2
France	2	Norway	3	USA	21
Germany	3	Pakistan	3		
Hong Kong	2	Russia	3		

Awardees Grouped by Continent



Keynote Speakers in HUPO2009 Plenary Sessions

P1 Lecture
On Monday AM By

Mathias Uhlen

Professor and Vice President
Microbiology
KTH, Sweden

P2 Lecture
On Monday AM By

Jan Schnitzer

Professor and Scientific Director
Vascular Biology Program
PRISM, US

P3 Lecture
On Monday PM By

Cathy Costello

Professor and Director
Mass Spectrometry Resource
Boston University, US

P4 Lecture
On Monday PM By

John Yates

Professor and Director
Chemical Physiology
TSRI, US

P5 Lecture
On Tuesday AM By

Jack Greenblatt

University Professor
Medical Research
University of Toronto, Canada

P6 Lecture
On Tuesday AM By

Mark Baker

Professor and Chair
Proteomics and CEO of APAF
Macquarie University, Australia

P7 Lecture
On Tuesday PM By

Carol Robinson

Professor and Director
Churchill College
University of Cambridge, UK

P8 Lecture
On Tuesday PM By

Christoph Borchers

Professor and Director
Genome BC Proteomics Centre
University of Victoria, Canada

P9 Lecture
On Wednesday AM By

Ruedi Aebersold

Professor and Director
Molecular Systems Biology
ETH-Zürich, Switzerland

P10 Lecture
On Wednesday AM By

Donald Hunt

Professor
Chemistry and Pathology
University of Virginia, US

A Human Protein Atlas for Profiling Cells, Tissues and Organs

Mathias Uhlen

School of Biotechnology, AlbaNova University Center, Royal Institute of Technology (KTH), Stockholm, Sweden

The new version 5.0 of the Human Protein Atlas (www.proteinatlas.org) contains 8,000 validated antibodies targeting 6,800 genes corresponding to approximately one third of the protein-encoded genes in humans. The portal contains more than 6 million high-resolution images generated by immunohistochemistry and confocal microscopy. The antibodies have been generated to regions of low homology (1) and the long-term objective is to generate paired antibodies towards the protein targets with separate and non-overlapping epitopes (2). Pilot projects have been initiated to also generate recombinant affinity reagents (3) and a community-based portal for sharing antibody and antigen validation data has recently been launched (4) as well as a pilot version of a Rodent Brain Protein Atlas (5). We have used the human protein atlas as a discovery tool to find potential biomarkers for cancer diagnostics.

1. Berglund et al (2008) Protein Science 17, 606-613.

2. Rockberg et al (2008 Nature Methods, 5(12):1039-45

3. Uhlen et al (2008) Nature Methods 5 (10): 854-855.

4. Björling and Uhlen (2008) Mol Cell Proteomics 7(10): 2028-37

5. Mulder et al (2009) Mol Cell Proteomics, in press.

Note: The full version of this abstract can be found at the section of *Human Proteome Projects* within this proceeding book (abstract #H121).

Proteomic Imaging of Endothelium and Caveolae for Targeted Penetration into Single Organs and Solid Tumors

Jan. E. Schnitzer

Proteogenomics Research Institute for Systems Medicine, San Diego, CA

Hundreds of disease biomarkers have been discovered. Accomplishing noninvasive targeted imaging and pharmacodelivery using biomarkers is challenged by *in vivo* barriers limiting access inside most tissues. For example, vascular endothelium prevents the penetration of intravenously injected biological agents into tissue where they can be effective. By integrating tissue subfractionation, subtractive proteomics, bioinformatic interrogation, antibody generation, expression profiling, and various imaging modalities, we can identify and actually validate the subset of biomarkers that are targetable *in vivo*. Tissue microenvironment extensively influences endothelial expression. Antibodies to select endothelial and caveolar proteins can not only very rapidly target a specific organ or tumor *in vivo* but also penetrate deep into the tissue. This greatly enhances the targeting of drugs, nanoparticles, gene vectors, and radioisotopes to facilitate disease imaging and treatment. This “organellar proteomic imaging” strategy creates a unique delivery system with many clinical opportunities to diagnose and treat a wide variety of diseases.

Note: The full version of this abstract can be found at the section of *Organelle Proteomics* within this proceeding book (abstract #O125).

Top-down Analysis of Native and Crosslinked Proteins to Locate Modifications and Investigate 3-Dimensional Structures

Catherine. E. Costello

Boston University School of Medicine, 670 Albany Street, Rm 511, Boston, MA 02118-2646 USA.

In biological systems, most proteins undergo one or more forms or post-translational modification; these changes strongly affect protein localization, interactions and turnover. In addition, multiple sequence variants can occur, and proteins associate into functional complexes. Proteolytic digestion of protein mixtures risks the loss of valuable information, since critical details regarding the extent and interrelationships of these important features is lost. We are pursuing top-down and crosslinking as methods to extract the maximum information from intact proteins that are identified as significant in proteomic investigations and are applying new strategies and software tools for this purpose. Top-down sequencing of native and crosslinked proteins has been carried out online and offline, on LTQ-Orbitrap, FT, Q-o-TOF and ion trap MS systems and multistage MS^n, including in-source fragmentation, CID, IRMPD, ECD and ETD. The spectra have been interpreted using the Boston University Protein IDentifier – Top Down (BUPID-TD), and publicly available programs.

For the crosslinking experiments, we employed BS^3, a homobifunctional crosslinker that contains an amine-reactive *N*-hydroxysulfosuccinimide (NHS) ester at each end of an 11.4-A spacer arm. Conditions for the crosslinking reaction were carefully optimized to minimize the number of crosslinks, to focus on the most significant through-space interactions. Proteins of particular interest to our investigations of oxidative stress in cardiovascular disease, *e.g.,* hemoglobin, and/or systemic amyloidoses, *e.g.,* transthyretin, were chosen as the central objects of the research; other well-characterized proteins were used for method development and evaluation of results. Our results are consistent with published X-ray crystallographic data. When fully developed, the method should be applicable to novel proteins and sequence or post-translationally modified forms of known proteins, which occur in very small amounts and have no X-ray data.

Acknowledgements:

This project was funded NIH-NCRR grants P41 RR10888, S10 RR15942, S10 RR20946 and S10 RR25082 and by NIH-NHLBI contract N01 HV28178. We thank Bruker for loan of the amaZon and access to the solariX.

Note: The full version of this abstract can be found at the section of *Structural Proteomics* within this proceeding book (abstract #S213).

Driving Biological Discovery Using Quantitative Mass Spectrometry

John R. Yates

Department of Chemical Physiology, The Scripps Research Institute, LaJolla, CA, USA

A component to understanding biological processes involves identifying the proteins expressed in cells as well as their modifications and the dynamics of processes. Several major technologies, but especially mass spectrometry, have benefited from large scale genome sequencing of organisms. The sequence data produced by these efforts can be used to interpret mass spectrometry data of proteins and thus enables rapid and large-scale analysis of protein data from experiments. Advances in multi-dimensional separations as well as mass spectrometry have improved the scale of experiments for protein identification. This has improved the analysis of protein complexes, and more complicated protein mixtures. Quantitative mass spectrometry can be used to study biological processes such as protein-protein interactions, development or the effects of gene mutations on pathways. Recent studies on the interactions of the Cystic Fibrosis Transport Regulator as it progresses through the folding pathway will be presented. Metabolic labeling of whole organisms can now be readily accomplished using 15N labeled proteins as a food source for studies of *in vivo* biology. The use of this method in combination with Shotgun proteomics was used to measure protein and phosphoprotein expression in organelles over several developmental time points in the rat brain (P1 to P45).

Note: The full version of this abstract can be found at the section of *Quantification* within this proceeding book (abstract #Q155).

Protein Complexes and Functional Pathways in Yeast and Bacteria

Jack Greenblatt

Banting and Best Department of Medical Research, University of Toronto, Toronto, ON, M5S 3E1, Canada
Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada

We used TAP-tagging, affinity-purification and mass spectrometry (APMS) to sort the *S. cerevisiae* and *E. coli* soluble proteins into complexes and recently extended the yeast PPI network to encompass membrane proteins. Yeast complexes have been assigned to functional pathways using synthetic genetic interaction analysis in an E-MAP version of the SGA approach for genes related to yeast nuclear processes, and we have developed an analogous method, eSGA, for *E. coli*. The co-functionality of the proteins in yeast membrane-associated protein complexes is being tested by comparing protein complexes with genetic interactions and assessing the effects of membrane proteins on the morphology of intracellular compartments. Our *E. coli* PPI network has also been integrated with systematic genomic context inferences to derive a probabilistic network of functional inferences and assign ~57% of the functional orphan proteins to discrete functional neighborhoods. Analogous APMS and RNAi approaches could be used to functionally classify human proteins.

Note: The full version of this abstract can be found at the section of *Assembling the Parts: Whole Cell Modeling* within this proceeding book (abstract #A205).

Membrane Proteomics: Putting the Peptide Jigsaw Back Together

Mark S Baker

APAF, Dept. Chemistry and Biomolecular Science, Macquarie University, NSW. Australia

Introduction Proteomics promises delivery of comprehensive coverage but in membrane proteomics problems of protein solubility and low copy number have made this difficult.

Our recent data on membrane protein:protein interactomics, membrane proteomics peptide IPG-IEF "shotgun" approaches with label-free quantitation and glycan analyses on cancer cell lines, human embryonic stem cells and from liver membranes from cancer-bearing animals are discussed in the context of APAF's attempts to comprehensively increase membrane proteome coverage.

Methods & Results Using a benign tumour bearing mouse model and "shotgun" peptide IPG-IEF proteomics with label-free quantitation we have examined reproducible, significant changes in the liver proteome.

In control animal experiments, tryptic peptides isolated through methanol-assisted digestion showed optimal peptide coverage of ectodomain-containing membrane proteins, where ~95% were concentrated into 3 main pI range peaks and ~2,000 non-redundant proteins (513 predicted to have between 1 and 19 transmembrane domains) were identified (including most (42) rat CYP450 protein family members). Using pI as a filtering tool enhanced identification reliability.

In cancer-bearing mice, major changes in glycosylation pathways, drug metabolism and clearance (e.g., multi-transmembrane solute carrier family proteins), cell surface receptors, 11 electron transport chain complex I subunits and fatty acid catabolic enzymes were observed.

Subsequently, glycan analyses on Triton-X114 phase partitioned membranes from cancer-bearing mice closely paralleled changes anticipated from glycosylation enzyme changes.

We have deployed additional methods to map embryonic stem cell lines (with various monogenic diseases) membrane proteomes and to membrane changes when the malignant HCT116 colon cancer line is transformed to a benign counterpart (through antisense uPAR suppression.

We report how immunoprecipitation proteomics can identify true uPAR-interacting proteins involved in the "metastasome'. Subsequent detailed analyses of the exact sites of protein interaction between uPAR and the integrin $\alpha v\beta 6$ [using overlapping peptide array binding blots and ELISA-type peptide competition assays] suggest that some very interesting biology may be associated with these interactions. This may have implications for cancer sufferers as both cancer lynchpin proteins uPAR and $\alpha v\beta 6$ are known to be independent poor prognostic indicators of patient outcome, but little is know regarding outcome when they are co-expressed in cancers.

Innovative aspects

- Collectively, our data demonstrate that combination(s) of methods including "shotgun" peptide IPG-IEF, glycoprotein enrichment, glycan analyses and membrane protein complex immunoprecipitation can result in deep, comprehensive mammalian membrane proteome coverage.
- Studies like these are providing vital new information regarding complications involved in cancer treatment (e.g., cachexia) , changes in glycoprotein biosignatures associated with disease and proteins that may drive the process of malignancy as well as potentially being novel therapeutic targets.

References

1. Saldanha RG, et al., Proteomic Identification of Lynchpin Urokinase Plasminogen Activator Receptor Protein Interactions Associated with Epithelial Cancer Malignancy. J Proteome Res, 2007 6, 1016-28.
2. Lee, A, et al., Rat liver membrane glycoproteome: enrichment by phase partitioning and glycoprotein capture. J. Proteome Res, 2009; 8:770-81.
3. Chick JM, et al., Liver Membrane Proteome Dynamics in Cancer Bearing Mice, Mol. Cell Proteomics, submitted July 2009.

Note: The full version of this abstract can be found at the section of *Assembling the Parts: Whole Cell Modeling* within this proceeding book (abstract #A204).

Mass Spectrometry and its Role in Structural Biology

Carol V. Robinson

Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK

Standard proteomics techniques are unable to describe the stoichiometry, subunit interactions and organisation of assemblies since many are heterogeneous, present at low cellular abundance and frequently difficult to isolate. Using methods designed to maintain non-covalent complexes within the mass spectrometer we show how we can provide definitive evidence of interacting subunits based on the masses of complexes and subcomplexes generated by perturbation both in solution and gas phases. Structural models will be presented for a number of oligomeric protein complexes including the human U1snRNP and eIF3 complexes.

Developments in mass spectrometry have added a further dimension to the study of protein complexes. Using ion mobility mass spectrometry we have been able to add spatial restraints to our models with measurements of collision cross–sections.

Very recently we have had a considerable breakthrough which has enabled us to preserve intact membrane complexes in the gas phase . This enables us to establish lipid and nucleotide binding and to define the stoichiometry and post translational modifications within the intact transmembrane regions of a number of complexes.

Note: The full version of this abstract can be found at the section of *Structural Proteomics* within this proceeding book (abstract #S214).

Dynamic Processes in Proteins and Protein Interactions Studied by Mass Spectrometry Combined with Protein Chemistry

Christoph H. Borchers

University of Victoria/Genome BC Proteomics Centre, 3101-4464 Markham Street, Vancouver Island Technology Park, Victoria, BC V8Z 7X8, Canada

The combination of tertiary structure selective chemical modification in proteins (like acetylation of lysine residues), mass spectrometric approaches, and molecular modeling methods has been used to determine structure-function relationships in proteins. One main focus of our research is to combine photochemical reactions, H/D exchange and chemical crosslinking of proteins with mass spectrometric methods. This concept has proven useful for identification of target proteins and components of protein complexes, structural characterization of protein-ligand and protein-biomolecule interactions, and elucidation of dynamic processes in proteins. The photochemical reactions of proteins used for this purpose can be classified as photoaffinity labeling, photo-crosslinking, or time-resolved photochemical reactions. The purpose of the research is to develop and apply these approaches to the study of proteins involved in a number of disease states and biochemical and pharmaceutical processes. Photoaffinity labeling, combined with mass spectrometric approaches and molecular modeling, is being used to determine the drug binding structures of HIV integrase, an important drug target in AIDS research. Determination of drug binding structures is essential for understanding the mechanism of binding to the inhibitor, and to provide information useful for generating more efficient anti-HIV drugs.

For the concept of crosslinking combined with MS to structurally elucidate protein-protein interaction, novel crosslinker and crosslinking strategies are being developed. In particular, crosslinkers and strategies are developed towards the enrichment, detection and identification of inter-peptide crosslinks. Their feasibilities have been shown with numerous model complexes and first applications to address biological and biomedical questions including the elucidation of interaction sites of membrane/cellular adaptor protein complexes. Within this research area, our special interest is to study the topology of the anaphase-promoting complex (APC), a multi-protein ubiquitin ligase involved in cell cycle regulation that is essential for the metaphase to anaphase transition and exit from mitosis using chemical crosslinking and mass spectrometry.

Note: The full version of this abstract can be found at the section of *Proteomics of Macromolecular Complexes* within this proceeding book (abstract #P224).

Mapping and Measuring Proteomes

Ruedi Aebersold

Institute of Molecular Systems Biology, ETH Zurich, and faculty of Science, University of Zurich, Zurich, Switzerland

The human genome project has taught us that a complete map -in the case of the genome project the complete genomic sequence - along with computational tools to navigate the map, represent invaluable resources for experimental and theoretical biologists. A main consequence of such a complete map is that all the biological processes have to be explainable with the components that constitute the map.

For technical and conceptual reasons the generation of complete maps has been more challenging in the field of proteomics. One of the main difficulties has been the definition of a suitable end point, e.g. definitive proof that all the proteins in a sample have been identified. To overcome this limitation, we have chemically synthesized multiple proteotypic peptides for each ORF in the genome of the yeast S. cerevisiae and generated reference fragment ion spectra for these peptides, thus generating for the first time a complete map of a mass spectrometry observable proteome.

In this presentation we will discuss experimental and computational challenges related to the generation of complete proteomic maps and demonstrate the value of the generated reference spectra libraries for shotgun as well as selected reaction monitoring (SRM) based targeted proteomics strategies that will transform proteomics from perpetual proteome re discovery to accurate proteome measurement.

Note: The full version of this abstract can be found at the section of *Human Proteome Projects* within this proceeding book (abstract #H122).

Innovative Mass Spectrometry Technology for the identification of Cancer Immunotherapeutics

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At present, the most effective treatment for metastatic melanoma is adoptive T-cell therapy (ACT). In this approach, tumor-infiltrating lymphocytes (TIL) are isolated from resected tumor and expanded ex vivo. After the patient's immune system is ablated by a combination of chemotherapy and total body irradiation, the TIL plus cytokine (IL-2) are re-infused. Objective (tumor skrinkage) and complete responses for this therapy in a recent clinical trial of 25 late stage patients with metastatic melanoma were 72% and 16%, respectively. Tumors the size of grapefruit disappear in a matter of months. Reported here are efforts to identify class I and class II MHC phosphopeptide antigens that are presented (1-10 copies/cell) as a result of dysregulated signal transduction pathways in cancer cells and that will make it possible to employ adoptive T-cell therapy for many additional human cancers.

Note: The full version of this abstract can be found at the section of *Mass Spectrometry Innovations* within this proceeding book (abstract #M123).

HUPO2009 Scientific Sessions at A Glance August 12th, 2009

Day	Highlighted Session Metro Ballroom	Parallel Session 2 Room Pier 2+3	Parallel Session 3 Room Pier 4	Parallel Session 4 Room Pier 5	Parallel Session 5 Room Pier 9
Monday **Sept 28th, 2009**					
AM (8:00-9:30)	Keynote Lectures: P1 and P2				
AM (10:00-12:15) **Co-Chairs**	Proteomics Tech Bill Hancock TBA	NCRR Workshop Doug Sheely Susan Old	Systems Biology Daniel Figeys Jeffrey Smith	Computation & Databases Igor Martin McIntosh	MS in Biomedical Applications Young-Mok Park Helmut Meyer
PM (1:30-4:30) **Co-Chairs**	Blood-Based Proteomics Daniel Chan John Marshall	Struct. & Chem. Proteomics Juergen Kast Gerold Schmidt-Ulms	NHLBI Workshop Aebersold Srinivas	Array Based Technologies Ravi Sirdeshmukh Mike Snyder	Membrane Proteomics Mark Baker TBA
PM (5:00-6:30)	Keynote Lectures: P3 and P4				
Tuesday **Sept 29th, 2009**					
AM (8:00-9:30)	Keynote Lectures: P5 and P6				
AM (10:00-12:15) **Co-Chairs**	Proteoomics Publications Ralph Bradshaw Peipei Ping	NIDA Worshop Ciborowski Fox	Protein Markers TBA TBA	Protein / Protein Interactions Anne-Claude Gingras Andrew Emili	Microbes & Model Org. Gerald Spaeth Mike Tyers
PM (1:30-4:30) **Co-Chairs**	Tissue Proteomics Liang Li Michael Siu	Antibody-Based Approaches Mathias Uhlen Christoph Borchers	Interface of Genome-Proteome Rolf Apweiler TBA	Posttranslational Modifications Michael Moran John Kelly	Young Investigator Awards John Yates Joshua LaBaer
PM (5:00-6:30)	Keynote Lectures: P7 and P8				
Wednesday **Sept 30th, 2009**					
AM (8:00-9:30)	Keynote Lectures: P9 and P10				
AM (10:00-12:15) **Co-Chairs**	Thermo Symposium Amy Zumwalt John Yates	Human Biol. & Diseases Thomas Kislinger Anthony Gramolini	Cancer Proteomics Xiaohong Qian Ab Guha	Biomarkers Ken Evans Ranju Ralhan	Cardiovascular Proteomics Peter Liu Jérôme Garin
PM (1:30-2:30) **Co-chairs**	Plenary Panel Discussion: Challenges and Opportunities of Proteomics Srinivas & Yates, and Panel TBA				
PM (3:00-4:00) **Co-chairs**	HUPO Awards Maxey Chung & Pierre Legrain				
PM (4:00-5:00) **Co-chairs**	Plenary Panel Discussion: HUPO Scientific Initiatives and Human Proteome Projects Omenn & TBA, and Panel TBA				
PM (5:00-5:30)	Closing Ceremony				
PM (7:00-10:00)	Congress Banquet				

Information for Poster Presentations:

For abstract authors preparing their posters for presentation, please note the following instructions.

- Posters are to be made in dimensions 4 ft x 4 ft.
- Please identify the designated category of your submitted abstract in the Table of Contents for Abstracts (next page). Example, Cancer Proteome Biology and Medicine (I and II) is C201-C299 and C601-606.
- Please identify your abstract number shown in the abstract pages (in the pages following each category). Additional author and abstract information are tracked in the abstract author index (located at the end of all abstracts in the following pages).
- Please note the presentation schedule according to your abstract category and abstract numbers shown in the table below in this page.
- Poster presentations are expected in the following schedule:

  Monday, Sept 28th, 2009: 8:00 am – 5:00 pm
  Tuesday, Sept 29th, 2009: 8:00 am – 5:00 pm
  Wednesday, Sept 30th, 2009: 8:00 am – 1:00 pm

Poster Presentation Schedule According to Abstract Categories and Numbers:

Following the instructions above, note the category and number of your submitted abstract; use the following table to find the time and location for your poster presentation. Example, C205, in the category of Cancer Proteome Biology and Medicine by Takuya Yamashita, et. al., is scheduled for poster presentation on Monday, Sept 28th, 2009 in the Harbour Ballroom at the Westin Harbour Hotel.

	# of Poster Spots		Session Categories and Abstract # for Posters Scheduled		
			Monday (Sept 28th ,2009)	**Tuesday (Sept 29th, 2009)**	**Wednesday (Sept 30th, 2009)**
Frontenac Room	92		A101-A176 A201-A205 C601-C606	C501-C552 E101-E110 G101-G124 I301-I303	P301-P339 P401-P411 S101-S112 S201-S214 S301-S311
		Total	87	89	87
Metro West	100		B101-B144 C101-C104 C301-C324 C401-C417	H101-H122 I101-I133 I201-I207 K101-K112 M101-M126	Q101-Q155 S401-S443
		Total	89	100	98
Harbour Ballroom	100		C201-C299	M201-M206 N101-N104 N201-N224 O101-O125 O201-O207 P101-P105 P201-P224	S501-S528 T101-T144 Y101-Y106
		Total	99	95	78

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Array Based Proteomics

(A101 – A176)

A101

ATP Triggered Cytokine Profiles in P2X7 receptor polymorphism individuals

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Introduction
Understanding biological diversity of individual single nucleotide polymorphisms (SNPs) and its physiological functions yields an insight into the origins of individual traits and its role in disease susceptibility and development. In recent years, the advances of genomic and proteomic technologies enable to study the function of individual SNPs in a systematic manner. P2X7 receptor plays an important role in the initiation of the innate immune response. Therefore, SNPs in the P2X7 gene may influence this function in a variety of infectious and inflammatory diseases. P2X7 polymorphism can affect P2X7 receptor activity and initiate a pro- or non inflammatory response. To date, the knowledge of P2X7 polymorphism and its physiological functions is still limited. Thus the systematic investigation of cytokines triggered by P2X7 polymorphism is a promising approach to achieve a better understanding of individual's immunological states. This will finally enable a novel avenue for the treatment of inflammatory disease in personalized medicine.

Methods
518 healthy donors were screened for four loss-of-functions and one gain-of-function SNPs of the P2X7 gene, and linkage disequilibrium analysis was performed. P2X7 pore activities were determined in cells harbouring single or multiple SNPs. Finally, in order to investigate the influence of P2X7 polymorphism on the human immune state, Peripheral Blood Mononuclear Cells (PBMCs) were stimulated with 1mM ATP in the presence of T cell stimulators (PHA, CON-A, PWM and anti-CD3 IgG2a, and anti-CD3 IgG1) at 37°C for 96hs. PBMCs from healthy donors with homozygous mutated 1513A→C genotype were compared to wild type P2X7 harbouring PBMCs. The cell culture supernatants were analyzed for cytokine profiles with the quantitative xMAP technology for 13 cytokines and 8 soluble fragments of transmembrane receptors.

Results
SNP analysis of the P2X7 gene showed that the distribution of homozygousity and heterozygosity for 1513A→C is 3.7% and 31.5%, respectively. The frequency of heterozygous and homozygous mutated P2X7 genotype for 489C→T is 45% and 20.7%, respectively. P2X7 pore activity analysis confirms that the polymorphic 1513A→C mutation of P2X7 plays a key role when occurred with other genotypes, e.g. 489C → T. The results of the cytokine profiling revealed that the SNP 1513A→C genotypes of P2X7 can change the individual immune states by decreasing the release of IL-1b, IL-18 and secondary cytokines e.g. IL-6, IFN-g, TNF-a, TNF-R1, and IL-10.

Innovative aspects
- Identifying within a healthy population four loss-of-function and one gain-of-function P2X7 genotypes
- Analyze the influence of the P2X7 genotypes on the pore activity in single and coordination way
- Compare the cytokine expression of PBMCs derived from individuals carrying homozygous mutated 1513A→C genotype and wild genotype. This allows to define the immunological states of the individual patients with different P2X7 genotypes

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(1) L. Kruglyak, The road to genome-wide association studies. Nat Rev Genet. 2008. 9(4): 314-8
(2) EM Schneider, et al, Role of ATP in trauma-associated cytokine release and apoptosis by P2X7 ion channel stimulation. Ann N Y Acad Sci. 2006. 1090: 245-52
(3) MF Lister, et al, The role of the purinergic P2X7 receptor in inflammation. J Inflamm (Lond), 2007. 4: 5

A102

MALDI-TOF Profiling of Breast Cancer Sera for Pattern Analysis

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Introduction:
Blood samples hold a great diagnostic potential to identify the markers for early detection of cancer. Recently developed proteomic workflows in conjunction with MALDI-TOF have shown great promise in reading the changing molecular patterns in the blood, demonstrating that such profiles can discriminate diseased samples from controls. In the present work, we highlight the MALDI-TOF profiling of Enchant/Magnetic bead enriched low abundant protein/peptide fraction from breast cancer sera to distinguish the benign from invasive cases.

Methods:
The breast cancer serum (20µL) samples were first processed in duplicates with Enchant multi-affinity protein separation kit (Pall Scientific) to remove the high abundant proteins, albumin and IgGs using a 96 well filtration plate. The flow through fraction from each sample was split into three equal parts and the proteins in each part were enriched with a different type of functionalized magnetic beads (ClinProt) for MALDI-TOF profiling. The magnetic bead (HIC8, IMAC, WCX) enriched fractions were profiled on Bruker Ultraflex MALDI-TOF for pattern analysis. The SpecAlign tool was used for MS data processing (alignment, baseline subtraction and binning data points) and a rule learning (RL) [1, 2] algorithm was applied on the processed data to generate the rules for class discrimination.

Results:
To enrich the potential diagnostic markers from breast cancer sera, we employed a combination of high abundant protein depletion and magnetic bead (MB) enrichment steps for serum sample profiling. Out of the three functionalized MBs that were used to fractionate peptides from each sample, only HIC8 and WCX fractionations gave consistent and good feature rich MALDI-MS profiles for pattern analysis. To examine the discriminative features that distinguish the benign and invasive profiles, the processed MALDI-TOF profiles (HIC8 & WCX) from both types of cases were exported from SpecAlign and the replicate datasets from each sample were averaged. Then, RL [1, 2] combined with an efficient Bayesian discretization (conversion of values for each *m/z* to a set of discrete ranges) method [3] was applied to perform classification. From the preliminary analysis of HIC8 profiles, RL yielded 13 rules with a panel of 12 MS peak markers. This set of markers fits the entire training data (64) almost perfectly except for one misclassification though10 fold cross-validation on the datasets showed only 64% performance. Generation of blinded serum datasets is underway to test the RL model.

Innovative Aspects:
- Proteomic workflow that combines the high abundant protein depletion and protein fractionation using MBs for MALDI – TOF profiling of low abundant serum protein fraction.
- Application of rule learner algorithm for class prediction.
- Discriminative marker model development for early detection of breast cancer.

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A103

IDENTIFIED RECTAL CANCER PATIENTS THAT RESPOND TO NEOADJUVANT TREATMENT

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Introduction. In patients with loco-regionally rectal cancer pre-operative chemo-radiation therapy (CRT) improves disease free and overall survival.
However, only a portion of patients will respond to CRT, with a pathological complete response of 15-30%. Unfortunately, at present there is no reliable technique for predicting clinical or pathological complete tumor response after CRT, to confer major optimization of current treatment regimens. The activation state of kinase-driven signal networks contains important information relative to cancer, including response to treatment. Reverse phase protein microarray (RPPA) technology, provides a unique opportunity to understand the signalling network.
Methods. We performed RPPA on human tissue biopsy to identify a phosphoproteomic profile associated with response to CRT. Fifteen rectal cancer samples with different response rates, collected during endoscopic procedures before CRT, were collected at the Clinica Chirurgica II in Padova (I), and at the CRO-IRCCS National Cancer Institute, Aviano, Italy (III), and immediately snap frozen. Using laser capture microdissection we were able to isolate about 15,000 pure cancer epithelium from each sample. The cells were lysed and subjected to RPPA analysis. Using this technique, we measured the phosphorylation level of nearly 81 key-signalling proteins. Molecular network analysis was performed using commercially available software.
Results. We indentified 4 protein kinases whose phosphorilation leves were significantly different between the group of good vs bad responders: CHK2, GSK 3α/β, and β cathenin had higher phosphorilation levels, while PDK1 had lower phosphorilation levels. Interestingly GSK 3α/β, β cathenin and PDK1 below to the same signalling pathway (PI3K-AKT). Based on our results we can suppose that the PI3K-AKT pathway could be one of the keys to achieve response to neoadjuvant treatment in rectal cancer patients. Obviously these results require future validation in larger studies. The identification of potential prognostic and predictive markers that can stratify patients who could benefit most from neoadjuvant treatment is a fundamental step in the management of patients affected by locally advanced rectal cancer in term of quality of life and medical resources. Moreover, identification of theranostic targets have the potential to pinpoint new therapeutic strategies for the non-responsive population.

A104

Transcriptome and proteome profiling of high- and medium copied proteins ofChr-18.

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Introduction
The human proteome is one of the most complex system in molecular biology. Roughly estimated number of all protein species expressed in cells or tissues can be calculated as: ("number of expressed genes" + "number of gene splice variants") x "average number of PTMs per protein". Following this formula and "number of gene splice variants" equal to 0.8 per gene (1) and "average number of PTMs per protein" equal 50 (2), the number of all proteins species expressed in the HepG2 cell line is close to 13300. Here we apply the proteomic approach for determination of number of expressed protein species in HepG2 cells.

Methods
Total RNA for the microarray analysis was isolated from ≈ 1mln cells. Following the first round of reverse transcription, the amplified cDNA underwent a second round of amplification and fluorescent labeling with Cyanine 5-CTP or Cyanine 3-CTP. The microarray chips were scanned with a Agilent confocal laser scanner.
The reversed-phase separations of proteins were performed on Agilent 1100 LC system using a mRP-C18 column. The separations were archived by combined multisegmented and linear elution gradient. HPLC fractions were automatically collected by time into plastic tubes, combined based on UV absorbance at 280 nm in four fractions and dried in a SpeedVac. Each fraction was trypsin digested and subjected subsequent 2D LC-MS/MS analysis.

Results
Methods of "shotgun" proteomics and whole genome transcriptome analysis were applied for determination of number of protein species expressed in human hepatocyte HepG2 cell line. According to obtained data, at least 7333 genes were expressed under conditions of the cell line culturing. Among them 148 belonged to coding regions of 18th chromosome.
Experiments at proteome level were performed using 3D LC-MS/MS. This method includes separation of whole cell lysate by macroporous RP-C18 chromatography onto 5 protein fractions with following "shotgun" proteomics analysis of trypsin digested mRP fractions. As a result 3552 proteins were identified in the hepatocyte cell line. Among those 3552 proteins 76 belong to 18th chromosome, that consist 48% of all expressed genes.

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A105

"Host tissue damage" signal ATP impairs IL-12 and IFNγ secretion in LPS stimulated whole human blood

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Introduction
Critical illness is associated with tissue damage, inflammation and the development of immune dysfunction. Leukocyte reprogramming occurs causing insufficient production of pro-inflammatory cytokines upon subsequent stimulation. We hypothesized that extracellular nucleotides in concentrations similar to those found near injured and ischaemic tissues will modulate cytokines secretion. In this regard, we chose to examine the effects of various nucleotides in a standardized *ex vivo* LPS stimulated whole blood cytokine production assay to investigate whether they may play a role in leukocyte reprogramming during critical illness.

Methods
The whole blood model ILCS characterized by leukocyte count and cytokines response stability was previously described[1]. In brief, 1mL of peripheral venous blood was collected to multifunctional tubes supplied pre-filled with a cell culture medium (RPMI 1640), supplemented with heparin as anticoagulant and LPS (10 ng/mL). The ILCS tubes firstly served as syringes to draw blood (from 14 healthy volunteers), which is thus immediately stimulated by LPS (without or with ATP at 20, 100, and 500 μM concentrations) for the first experiment and thereafter ILCS tubes served as non-adherent culture vessels. In the second and third experiments ATP, ADP, the $P2X_7$ receptor agonist BzATP (also $P2X_1$, $P2X_2$ and $P2Y_{11}$ receptor agonist), the non-hydrolysable ATP analogue $ATP\gamma S$ and the potent non-selective adenosine A2 agonist CV1808 were added similarly as described[2]. The concentrations of cytokines in the samples were determined using multiplex cytokine kits (9-plex, Bio-Rad).

Results
Cytokine production in non-stimulated whole blood - addition of 100 μM ATP to non-stimulated cultures improved leukocyte survival (74% vs 54%) but had only negligible impact on cytokines production. Effects of various ATP concentrations on cytokine production in LPS stimulated whole blood - leukocyte survival rate was not influenced by either 20, 100 or 500 μM ATP and was similar to LPS stimulated ILCS without added ATP (77, 70, 75% vs. 70%). In contrast, ATP significantly reduced the secretion of several cytokines. Both IFNγ (1688 vs. 4571 pg/mL) and IL-12(p70) (27.5 vs. 41.4 pg/mL) productions were reduced with 100 μM ATP (Fig. 1). Effects of ADP, BzATP, $ATP\gamma S$ and UTP on cytokine production in LPS-stimulated whole blood - neither of the nucleotides led to any significant change in the levels of IL-2, IL-10 or MCP-1. In contrast, ADP, BzATP and $ATP\gamma S$ but not UTP significantly inhibited IL-12(p70) and IFNγ secretion. Effects of A2 receptor agonist CV1808 on cytokine production in LPS-stimulated whole blood - CV1808 has reduced IL-12(p70) (114.8 vs. 36.1 pg/mL) as well as IFNγ (5583 vs. 1070 pg/mL) secretion suggesting that activation of A2 receptors could be partially responsible for the ATP and ADP effect.

Innovative aspects
- Immune dysfunction associated with critical illness is characterized by decreased TNFα, IL-12 and IFNγ production by leukocytes, extracellular nucleotides (e.g. ATP, ADP, etc.) might contribute to its pathogenesis.

References
(1) Schmolz *et al.* (2004). Exp Gerontol 39:667-71.
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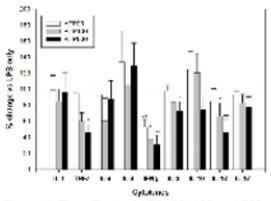

Figure 1. The effects of ATP (20, 100 and 500 μM) on LPS stimulated cytokines secretion in whole human blood from seven healthy volunteers (aged 23-60, mean 42.4 years) incubated for 24 hours. Data are expressed as percentage of LPS stimulated controls without ATP; * p<0.05, ** p<0.01.

A106

Bioinformatic approach form bottom-up proteotyping

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Introduction
Proteotyping is a new approach in proteomics aimed at mining the genotype information at the protein level by unravelling unique amino acid signatures – proteotypes. Proteotype encompasses post-translational modifications, spliced products and single amino acid polymorphisms. According to UniProt KB it is about 5 SAPs per protein for human proteome. In additional about 10 splice variants per gene is predicted. Combination of amino-acid polymorphisms, alternative splicing and PTMs constitutes a challenge of proteotyping in Human Proteome Project.

Methods
Annotated human-related mass-spectrometric data was downloaded from PRIDE web-based database. In addition, home-made mass-spectra were processed too. Scheme of the experiment is shown on Fig. 1. At the first stage mass-spectra were searched by Mascot against human portion of SwissProt database. XML-reports of Mascot identification were processed and the lists of proteins identified in each experiment were created. For each of identified proteins UniProt KB was requested for protein sequences and information about amino-acid polymorphisms. It allows us to create sub-databases which contain allelic sequences of identified peptides. Sub-database was appended to the SwissProt database and the second Mascot identification was performed. Comparison of results of first and second Mascot identification allows us to identify protein variants in MS experiments.

Results
From the HUPO Liver Proteome Project 4 mzData files were downloaded. According to PRIDE annotation, these were LC-ESI-MS/MS qTOF spectra collected on human liver cytoplasm samples. Sample preparation included SDS-PAGE, slicing and in-gel digestion by trypsin. From initial identification totally 272 proteins hits were reported by Mascot. For identified proteins there were 1880 SAPs in UniProt KB. The extended database containing 1880 additional allelic sequences was bound to Mascot and second iteration of MS-identification delivered us 21 SAPs. Of them 20 corresponded to the substitutions with mass-shift lower than 1 Da and thus were discarded. One remaining SAP corresponded to the allelic variant of Human hemoglobin with the substitution of Alanine to Glutamatic acid in 23rd position (Feature identifier VAR_003101 according to UniProt, known as "flatbush" [1]).

Innovative aspects
- Iterative protein identification scheme

References
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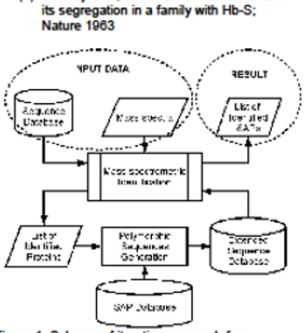

Figure 1. Scheme of iterative approach for "bottom-up" proteotyping.

A107

Surfactant-Assisted Shotgun Methods for Membrane Proteome Analysis

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Introduction
Membrane proteins play an important role in biological processes. Currently, about 70% of all known pharmaceutical drug targets are membrane proteins. Due to their importance, there is growing interest in analyzing the membrane proteome. MS analysis of membrane proteins is challenging for their hydrophobic properties (1). In this study, we characterized the integral membrane proteome of E. coli by using SDS, ALS and urea, respectively, and compared SDS, ALS and urea for their relative efficiency in solubilization and proteolysis as well as recovery of membrane proteins.

Methods
The integral membrane fraction was extracted from E. coli K12 culture. SDS, ALS and urea were used to solubilize the protein pellet, separately. Reduction and alkylation were carried out based on standard protocol (2). Trypsin digestion was conducted at 37°C overnight after 10 fold dilution. SDS PAGE was used to compare the digestion efficiency using SDS, ALS and urea. After acidification and centrifugation, the SDS-assisted fraction was loaded into SCX chromatography on an Agilent 1100 HPLC, individually, in order to remove detergent. The SDS removal efficiency was examined by using Pyrolysis GC. The protein component of ALS-assisted, SDS-assisted and urea-assisted fraction was analyzed by using RPLC-ESI Q-TOF.

Results
Proteins were resolved by 1D SDS PAGE and silver stained. Similar gel image patterns were observed suggesting that membrane proteins extracted by solubilization in different surfactants had similar protein composition. However, in the case of proteolysis, sample preparation using ALS showed more efficient digestion than the membrane proteins solubilized in SDS.
From SDS-assisted fraction, 38% of the sample amount was recovered after SCX cleanup step. Clearly, the sample cleanup step caused serous sample loss. We profiled the peptides in the sample by using LC-ESI MS/MS. In total, 1589 peptides, corresponding to 477 proteins, were identified. For ALS-assisted fraction, we directly subjected the digest into mass spectrometry after desalting and 2332 unique peptides and 554 unique proteins were identified. Each peptide sample was analyzed by using precursor ion exclusion strategy until saturation (3). A total of 627 proteins were identified by merging the data of the SDS-assisted fraction and ALS-assisted fraction. We are in process of analyzing the urea fraction and examining the SDS removal efficiency.

Innovative aspects
- Comparison of extraction methods by using SDS, ALS and urea to solubilize membrane proteins.
- Identification of membrane proteins of E. coli by RPLC-MS/MS.

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A108

Exploring protein networks using epitope-tagged knock-in cell lines and affinity-purification mass-spectrometry

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Affinity purification mass spectrometry (AP-MS) provides a powerful and widely used method to study protein-protein interactions not only because AP-MS complements the binary interactions between proteins detected by technologies such as yeast two hybrid (Y2H) and fluorescent resonance energy transfer (FRET), but also because it provides a convenient and efficient means of high throughput screening for protein interactions [1, 2]. Protein interaction mapping whereby over-expression of bait proteins (from constitutive promoters) in cultured cells is combined with AP-MS has been used to investigate the binding partners of these target proteins and has been successfully applied in many verification assays for protein interactions [1]. However, these exogenous proteins, especially when expressed above physiological levels, may have toxic or other unintended effects on the cultured cells and consequently alter the protein complexes *in vivo* [2]. Recently, epitope-tagged knock-in cell lines have been reported to facilitate studies including western blot, immunoprecipitation and immunostaining [3]. Thus, the combination of these knock-in cells lines for tagged protein expression and AP-MS will provide a novel platform for protein-protein interaction studies. In our study, we used both over-expression and a knock-in cell-line to investigate the MRE11 protein network. MRE11, a key player in the MRE11-Rad50-NBS1 (MRN) complex, plays a crucial role in meiotic recombination and DNA repair. Both known and novel components were identified from replicated AP-MS experiments. Aside from enabling the discovery of novel interactions, using both over-expression and knock-in expression provides a unique dataset for comparison of these two methods, allowing us to investigate both the advantages and limitations of using tagged knock-in cell lines with AP-MS in exploring protein networks.

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A109

Mapping Phosphotyrosine Signaling Network by Peptide and Protein arrays

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Introduction: Protein tyrosine kinases (PTK) play pivotal roles in cellular function by modifying the activity, localization and/or signaling potential of target proteins, and accordingly, aberrant kinase activation often leads to cellular transformation. A signal initiated by a PTK is frequently passed on to downstream molecules by modular interaction domains such as the Src-homology 2 (SH2) domain that have been evolved to recognize a phosphoryrosine (pTyr) residue embedded within specific sequence motifs. The SH2 domain represents the largest family of pTyr-binding modules as the human genome encodes 120 such domains. Deciphering specificity of the human SH2-ome and mapping how they are coupled to physiological phosphotyrosine sites are key to understand the phosphotyrosine signaling network in a cell and how aberrant changes in this network leads to cancer.

Methods: We used an Oriented Peptide Array Library (OPAL) approach to define the phosphotyrosyl motif recognized by an SH2 domain[1]. This OPAL binding profiles allowed for the construction of position-specific scoring matrices (PSSM) that were used for prediction of SH2 domain-ligand interactions and SH2 signaling networks[2,3]. To map the phosphotyrosine signaling network that regulates an immune response, we developed a novel bi-directional array screening approach, in which pTyr-peptide probes were used to screen an SH2 domain array (ie. forward screen) and an SH2 domain was used to screen a pan phosphotyrosyl peptide array (ie. reverse screen). Interactions identified by both screens were considered positive and were further validated by binding experiments in cells using the corresponding full-length proteins.

Results: Using the OPAL approach, we determined the ligand binding specificity of 80 SH2 domains. This has allowed the classification of the SH2-ome according to specificity (Fig. 1A). Besides recapitulating known motifs for certain SH2 domain, a number of novel binding motifs have been identified, which is exemplified by the BRDG1 SH2 domain that selects for a Ile or Leu residue at the +4 position relative to the pTyr residue. The structure of a BRDG1-peptide complex revealed a novel binding pocket lined by aromatic residues to specifically accommodate an Ile or a Leu residue. Based on the OPAL binding patterns, we developed SMALI (or scoring matrix-assisted ligand identification, accessible via http://lilab.uwo.ca), a web-based program for predicting binding partners for SH2-containing proteins[2]. By combining SMALI prediction with peptide array-target screening (PATS)[4], we identified a signaling network mediated by a group SH2 domains selected to cover the specificity space of the SH2-ome. This SH2-interaction network intersects significantly with reported interactions for the corresponding SH2-containing proteins and may therefore provide a framework upon which the functions of SH2 domains can be systematically explored. Moreover, by screening SH2 domain arrays produced in the lab using peptide probes and reversely screening pTyr-peptide arrays using purified SH2 domains, we identified the first phosphotyrosine signaling network involved in immune regulation. Network analysis indicates that, while some SH2 domains are promiscuous and signal downstream a variety of immunoreceptors, others are rather specific. The distinct signaling potentials of different SH2 domains provide a basis for the design of specific inhibitors that target a specific SH2 domain or network inhibitors that block the function of a group of SH2 domains.

Innovative aspects

-OPAL is a powerful approach by which to determine the ligand specificity of a modular interaction domain as demonstrated here for SH2.
-SMALI provides a useful platform for accurate prediction of SH2-partner interactions and for systematic analysis of the SH2 interactome.
-A novel bi-directional peptide and protein array screening approach minimizes false positives and allows for the identification of a high-confidence phosphotysine immune signaling network.

Acknowledgement

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A110

The SH2-domain pilot project - protein array analysis of all generated binders

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Introduction
In March 2008, a workshop was organized by the Structural Genomics Consortium, the Human Protein Atlas program and the EU Proteome Binders network program (1). Scientists and funders from more than 20 countries met to discuss and investigate the possibility of systematically generating renewable protein binders. A pilot project was formed, based around the generation of several different types of affinity reagents directed to human proteins containing the SH2-domain.

Methods
A protein array format (2) routinely utilized within the Human Protein Atlas project (3) to validate the specificity of 150 monospecific antibodies per week, was converted into a platform for the analysis of all binders generated towards the SH2-domain containing proteins. Protein arrays containing 6000 spots in 14 identical subarrays with 432 proteins in each where spotted. Approximately 100 of the spots corresponded to SH2-domain containing proteins. These where both protein epitope signature tags (PrESTs) from the Human Protein Atlas project and folded domains from Structural Genomics Consortium.

Results
The results will be presented from the specificity analysis of various types of affinity reagents that have been produced from academic research centers throughout the world; Stockholm, Chicago, Toronto, Melbourne, Braunschweig, Cambridge and Beijing. In total have approximately 400 binders, i.e. msAb, mAb, scFv, Fab, been analyzed, generating 200.000 data points of antigen-antibody interactions.

Innovative aspects
- All participating labs have generated binders with good specificity
- Validated renewable targets have been generated for 91% of the investigated targets
- The highly multiplexed protein array format is a suitable platform for analysis of specificity
- The community within the large scale production of affinity reagents is ready and able to generate renewable protein reagents.

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A111

Proteomic screening discovers a critical protein/pathway regulated by ID4 in mammary gland development and tumorigenesis

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Introduction
The ID (Inhibitor of DNA binding) family of basic helix-loop-helix proteins regulates cell proliferation and differentiation in many different developmental pathways (1). From gene expression array studies (2), we found that ID4 is highly induced during mammary tumorigenesis in the MMTV-Wnt-1 transgenic mouse model. Therefore, we decided to determine the function of ID4 in mammary gland development and tumorigenesis

Methods
In this study, we surveyed the expression pattern of ID4 at puberty and in adulthood, and studied the impact of ID4 loss on mammary gland development and response to estrogen and progesterone stimulation and on mammary tumor development and survival. And we used the Luminex xMAP technology and characterized protein changes in a set of six groups of samples from mouse mammary tissues that express wild type or knockout mutant ID4 gene with or without the treatment of estrogen or estrogen plus progesterone.

Results
We found that ID4 knockout mice had impaired mammary gland development phenotype including a less developed ductal tree with fewer side-branches at different age and a less proliferative response to stimulation by exogenous estrogen or progesterone. In order to elucidate the mechanism of ID4 in mammary gland development and tumorigenesis, we used the Luminex xMAP technology and characterized protein changes in a set of six groups of samples from mouse mammary tissues that express wild type or knockout mutant ID4 gene with or without the treatment of estrogen or estrogen plus progesterone. We have analyzed these samples on a total of 70 proteins, including phospho-proteins for signal transduction pathways, proteins involved in cell adhesion and apoptosis, and cytokines and cytokine receptors. ANOVA (ANalysis Of VAriance) identified 17 proteins that were different among these groups. These proteins include proteins that have not previously been identified as estrogen and/or progesterone targets and those that showed differential responsiveness to these hormones upon ID4 knockout.

In our above proteomic screening, we found that phospho p38MAPK was expressed at an increased level in ID4-null mammary glands in comparison to the age-matched ID4-wild type mammary gland. Based on this, we discovered that inactivation of p38MAPK restored proliferation in these ID4-null mammary cells.

Furthermore, ID4 nullizygosity suppressed hyperplastic ductal expansion initiated by the transgenic expression of the Wnt-1 oncogene, probably due to failing to maintain cell viability. In accord, ID4 knock-down in a tumor cell line cultured from this transgenic model induced apoptosis that could be mostly prevented by p38MAPK inactivation.

Innovative aspects
Taken together, these data demonstrate that ID4 controls mammary gland development and tumorigenesis through suppressing the p38MAPK activity and that the Luminex xMAP technology is a valuable discovery tool for screening biological samples to identify targets for further functional characterizations.

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A112

The Fc Receptor Supramolecular Complex From RAW Macrophages

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Introduction
Cell surface receptors found on the plasma membrane are key targets for modulating cellular responses. Hence, there is an urgent need to capture and elucidate activated receptor complexes from the surface of live cells. However, isolation and characterization of cell surface receptors and the identification of the ligand-receptor-protein complex is challenging. A novel method, Live-cell Affinity Receptor Chromatography (LARC) has been recently shown to capture the Fc receptor complex from the surface of live cells with the use of microbeads coated with the receptors ligand, IgG, before analysis by LC-MS/MS alongside numerous controls (1). However, the number of proteins shown to be specifically associated with the Fc receptor was quite large. Hence the use of data and literature mining algorithms and computer modeling programs such as iHOP (2), and Cytoscape (3) were used to confirm similar interactions in heterologous experimental systems.

Methods
RAW 264.7 macrophages derived from mice were obtained from ATTC and grown in Alpha Modification of Eagle's Medium supplemented with 5% Fetal Bovine Serum in an incubator at 7% CO2 at 37°C. The Fc receptor supramolecular complex was isolated by LARC with incubation of live cells on ice with receptor-ligand-coated microbeads before warming, disruption, and isolation of the beads followed by LC-ESI-MS/MS as described (1).

Results
The LARC method yielded a total of approximately 250 proteins identified specifically with the Fc receptor complex and Fc receptor-mediated phagocytosis (1). The proteins specifically associated with the activated Fc receptor included specific isoforms of kinases, phosphatases, phospholipases, adaptors, scaffolds, GTPases and their associated regulators such as GAP and GEF proteins, cytoskeletal proteins and proteins of unknown functions as shown in the accompanying tables. The proteins identified by the LARC method contain most of the protein types that are known to, or have been predicted to be, associated in the Fc receptor complex or the downstream phagocytosis pathway (1). For greater certainty, we filtered the list of specifically associated proteins through iHOP and visualized the results with Cytoscape under the limiting condition that the specific mass spectral data was supported by at least four known protein-protein interactions. The analysis revealed a network of interacting proteins that included not only the known Fc association effectors but also novel proteins and isoforms. The resulting list of specifically identified proteins was associated with the Fc receptor complex and IgG-mediated phagocytosis. The Mass spectroscopy results and network analysis indicated that the main interactors of the Fc receptor complex included tyrosine kinases (Syk, Src, Ptk2), lipases (PLCγ1, PLCβ4), GTPases (Rab11a) and phosphatidylinositol kinases (Inpp5d).

Innovative aspects
An activated receptor supramolecular complex was captured from the surface of a live cell.

Capture of the Fc receptor complex by LARC shows good agreement with previous biochemical and genetic evidence.

Many specific isoforms and novel proteins associated with Fc receptor were discovered and shown to agree with previous protein-protein interaction data.

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A113

Towards a next generation array-based plasma profiling

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Introduction
The Human Protein Atlas is a publicly available database (www.proteinatlas.org) that has been set-up to allow a systematic, antibody-based exploration of the human proteome by expression and localization profiles of proteins in a variety of human tissue sections and cell lines (1).
This resource produces an extensive range of affinity reagents are validated with various tools, such as multiplexed assay systems (2), to offer tools for approaches such as antibody microarrays to discover biomarkers in plasma.

Methods
For the systematic analysis of serum/plasma an antibody suspension bead array has been developed (3) to study biotinylated specimen. This microtiter plate-based workflow combines optimized buffer, sample labeling and sample treatment conditions to analyze the samples without any prior purification steps for the removal of excess labeling substance. With this current approach, 96 tests are performed in parallel on each of the 96 samples.

Results
The bead array-based assay system, detecting proteins into sub ng/ml levels, has been employed to evaluate an undirected approach for biomarker discovery by selecting antibodies based on binding specificity and concentrations instead being driven by previously known disease relevance. Antibody performance was assessed in terms of reproducibility, variability and signal intensity levels to reveal that a majority of the 96 capture reagents qualified for serum/plasma analysis. In a proof-of-concept study with 80 plasma samples of a prostate cancer related cohort, two antibodies indicated that their target proteins were differentially expressed. While Western blot analysis revealed abundance as discriminator for one, differences in the other protein profiles were found to be based on the antigen's posttranslational modifications.

These results suggest that the presented procedure holds the great potential to perform a proteome-wide, undirected biomarker discovery. A future approach would consequently aim at to screen larger numbers of serum/plasma samples from different disease and control cases with antibodies that have been selected based on validation scores rather then antigen relevance. Such a multi-disease cohort setting would promote to collect interesting targets to be validated in subsequent, single disease studies.

Innovative aspects
- Undirected biomarker discovery
- Two dimensional multiplexing
- Antibody-based plasma profiling

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A114

Integrated Functional Proteomics Research of Human Epididymal Secretary Sperm-Binding Proteins

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Introduction
Approximately 10-15 percent of couples are infertile, and among these couples, male factor infertility accounts for approximately 50 percent of causes. There are 80 millions infertile couples in the world, and 2 million added every year. Current research of human male reproductive lagged far behind other biology researches, and only 35 human epididymal expressed proteins in the main database(approximately 20 proteins were studied).Due to the limited knowledge of sperm maturation, infertility diagnosis and treatment for men based on molecular biology findings remains under-explored.

The epididymis is an essential male reproductive organ in mammalian. Human spermatozoa originate from testis, but its maturation must travel through the epididymis. Then, the spermatozoon acquires motility, ovum organization, activation and fertility maintenance. Such process was performed by the combination and action between spermatozoon and the secreted proteins from the epithelium of epididymis duct. Therefore, functional proteome research of human epididymis has an important clinical meaning.

Methods
In order to comprehensively demonstrate the human epididymal proteome involved in spermatozoa maturation, we applied 2D-PAGE, 2D-DIGE and mass spectrometry (The 4800 Plus MALDI TOF/TOF™ Analyzer) on the analysis of the human epididymal proteome, including the epididymal proteins profiling of the different aging groups (new-born, young and old), the proteins profiling of the secreted epididymal fluids, and the difference proteins profilings of the epididymis of different regions (caput, corpus or cauda). Furthermore, epididymal luminal proteins were further confirmed and studied by Westen Blot, Immunohistochemistry, Indirect immunofluorescence (LSCM) and sperm function analysis.

Results
Through large scale proteome analysis of normal male epididymis. Epididymal tissue protein spots (1720) and luminal protein spots (405) were identified. The numbers of unique epididymal and luminal proteins were 485 and 248, respectively. Bioinformatics analyses were performed on the data to assess predicted subcellular locations and involvement in metabolic pathways. Furthermore, 40 proteins were cloned and polyclonal serum was raised against recombinant proteins. Other 600 polyclonal antibodies were produced against the specific polypeptide. Immunofluorescent analysis showed about 180 proteins were located on the different site of mature sperm. Functional analysis showed 60 proteins may be involved in the process of motility and fertilization.

Innovative aspects
This study generated a comprehensive proteome profile in fertile human epididymal tissue and luminal fluids, providing a reference map for further investigations into epididymal protein expression.

Display a global epididymal sperm-binding protein family, which showed unimaginable binding and functional polymorphism.

These datasets can be applied to male infertility diagnosis and treatment, as well as to the development of contraceptive drug

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A115

"X!TANDEM TO SQL PARSER PERMITS ANALYSIS OF HuPO BLOOD DATA BY SAS"

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Introduction
A standardized SQL database could provide mass spectral results to a general statistical analysis system. The LC-ESI-MS/S experiments from the HuPO blood consortium was correlated by X!TANDEM against the proteins of ENSEMBL and posted on the GPM file transfer site (1). Each of the 611 .xml files contains information regarding proteins, peptides, parent and fragment ions. The results of mathematical modeling, and information on the correlation parameters employed, must also be collected and archived in the same relational database. An X!TANDEM-to-SQL parser was written in vb.net to parse the many .xml files of the HuPO blood protein data into an SQL database. The inherent functions of the SQL database system were then used to render the data non-redundant. The resulting .mdb tables were imported in to the generic Statistical Analysis System (SAS) for transformation, sorting, analysis and visualization.

Methods
The data was rendered non redundant with respect to proteins and peptides and calculations performed within the SQL database. The operating system was Windows server 2003 with Windows SQL server 2005 installed and configured for mixed mode authentication. The open source code for the parser was written within Microsoft Visual Studio 2005 in VB.net. The Parser was tested against X!TANDEM version 2006. Most manufactures of Protein Mass Spectrometers allow the user to export the results of an experiment in a number of output formats. One popular format being the matrix generic format or .mgf file. Correlation engines such as X!TANDEM may be designed to import these .mgf files. The researcher sets the search parameters for the process of identifying what peptide fragments may have been recorded in the MGF files. In larger studies a number of mass spec runs may be generated, some of which could be controls while others may represent collected clinical samples. The parser assumes that search result files will be grouped into directories where all files in that directory are related in some meaningful way. For example, control runs might be in one directory while the corresponding clinical samples might be in a discrete directory. Accordingly the parser is designed to import search engine result files on a directory by directory basis, and as part of this process populates the database tables.

Results 1200
A redundant list of 247,077 proteins were correlated by X!TANDEM that was collapsed to a set of 13,379 distinct proteins, with 34,956 distinct peptides and 39 potential contaminants by SQL. Some proteins were detected by as many as thousands of redundant peptides or as few as one peptide. We calculated that 6875 distinct proteins were only represented by a single distinct peptide, 2866 proteins showed 2 distinct peptides, 1319 proteins showed at least three distinct peptides and 3020 distinct proteins had more than three associated peptides. Hence as the unique peptide count increased from as few as 1 to as many as 25, the number or distinct proteins declined from as many as 6875 to as few as 10 proteins. By sequence analysis we estimate that about 9208 of the proteins identified by HuPO have not been previously detected. About 1964 proteins were observed by at least one other group, and about 1132 proteins were observed by two other groups. Many peptides that showed expectation scores of 0.095 were associated with proteins that showed highly significant expectations scores in aggregate. Thus, well known blood proteins that showed overwhelming low protein expectation scores in toto also often showed many individual peptides with expectation scores as large as 0.095. Hence it seems clear that many of the peptides that did not show low expectation values were still correct. The analysis of the data indicated that X!TANDEM is a robust means to analyze LC-ESI-MS/MS data at the level of proteins or peptides. We conclude that the bulk of the results of the HuPO blood project show good fidelity with authentic blood proteins using X!TANDEM correlation algorithm provided by the GPM.

Innovative aspects
-X!TANDEM to SQL parser permit standardization in the collection of the LC-ESI-MS/MS data.

-Analysis of proteomic data using a Statistical Analysis System (SAS).

-Smooth and continuous distribution of peptide and protein data with a most data showing expectations values together of E-2 or less.

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A116

Proteome Signature of Superior Temporal Gyrus and Effects of Anti-psychotic Drug Treatment in Non-human Primates

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Introduction
The superior temporal gyrus (STG) is comprised of primary and secondary (association) auditory cortices and is considered the primary substrate of auditory signal processing and speech. In recent years, it has been the subject of multiple studies in the pathophysiology of schizophrenia. Neuroimaging studies show an inverse correlation between the STG volume and the severity of hallucinations. In this study, we investigated the STG membrane proteome from rhesus monkeys treated chronically with 1st and 2nd generation antipsychotics at different dose levels. By coupling iTRAQ® reagent labeling with tandem mass spectrometry, we can demonstrate proteome changes that occur in monkey STG membrane after anti-psychotic drug treatment

Methods
To study the proteome signature and to determine the effects of antipsychotic administration, rhesus monkeys were administered atypical antipsychotic clozapine (2.6mg/kg, b.i.d.; n=10), typical antipsychotic haloperidol- low dose (0.07mg/kg, b.i.d.; n=10) and –high dose (2mg/kg, b.i.d.; n=4) for 180 days and compared with controls (n=10). STG membrane proteins were harvested and pooled from monkeys, and then digested with trypsin. The samples were then iTRAQ reagent labeled and separated with strong cation exchange chromatography into 30 fractions. Each fraction was further separated by running a 90 minute reverse phase LC gradient and spotted directly onto stainless steel MALDI plates. Samples were then analyzed using a AB SCIEX TOF/TOF™ 5800 system.

Results
A survey scan was performed on every sample spot and up to 30 precursors from each spot were then automatically selected using predefined criteria for subsequent MS/MS analysis. A total of approximately 8000 MS/MS spectra were acquired. Database searches were done using ProteinPilot™ software 3.0 with Paragon™ and Pro Group™ algorithms against the NCBI non-redundant database. Approximately 5000 distinct peptides and approximately 700 proteins were identified with confidence (1% global FDR). Given the multivariate nature of the collected data, Principal Components Analysis (PCA) was used to determine relationships between the variables. The current study documents the first proteomic signature as well as the effects of chronic antipsychotic administration on protein expression in the primate STG.

Innovative aspects
- The comprehensive assessment of Proteome signature of STG
- Study the effects of anti-psychotic drug treatment in Non-human Primates

A117

Protein Biomarkers in Radioresistant Cancer Cells Identified using Antibody Microarrays

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Introduction
The current standard for treating early-stage breast cancer is conservation surgery and adjuvant radiotherapy (RT). RT is also the main modality for treating early stage head and neck cancers. Resistance to RT may contribute to local recurrence. We aimed to explore the presence of protein markers indicative of RT resistance through developing in vitro models of radioresistant breast cancer and oral cancer cell lines and proteomic analysis using antibody microarray technology.

Methods
Novel cancer cell lines resistant to radiotherapy were established by exposing parental cell lines (3 breast cancer: MCF-7, MDA-MB-231, T47-D and 2 oral cancer: PE/CA-PJ41, PE/CA-PJ49) to fractionated external beam radiation. A dose response curve at the end of treatment demonstrated a statistically significant increase in radioresistance in the novel sublines.
We utilized the Panorama ™ Antibody Microarray-XPRESS Profiler725 (Sigma-Aldrich) protocol to analyze proteins extracted from the paired cell lines. Protein lysates were labelled with Cy3 and Cy5 fluorescent dyes, applied to a nitrocellulose-coated glass slide containing 720 antibodies and analyzed using GenePix™ Pro 4.1 and Acuity 4.0 software (1).

Results
Differential expression between parent and radioresistant cell lines was demonstrated in 43 proteins from the 3 breast cancer cell lines (9 proteins in ≥2 cell lines simultaneously), and 21 proteins in the 2 oral cancer cell lines (3 proteins in both cell lines simultaneously). Importin alpha was the only protein which demonstrated differential expression in both beast and oral cancer cell lines. The isoform Importinα-1 was demonstrated in MCF-7 and T47D, while Importinα-3 was demonstrated in PE/CA-PJ41 and PE/CA-PJ49. The proteins identified are currently undergoing further confirmation using immunoblotting and immunohistochemistry.

Innovative aspects
- The use of a novel high density AbMA has successfully identified a number of protein targets which may be associated with a radioresistant phenotype.
- These proteins require further study to validate the results.
- High density AbMA's potentially offer a powerful new proteomic technique to allow the global analysis of many proteins simultaneously. These could be invaluable in the identification of candidate biomarkers which may be involved in radioresistance.

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A118

Dynamic Proteomics Profile of Mesangial Cells in Response to High Glucose Conditions

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Title: Dynamic Proteomics Profile of Mesangial Cells in Response to High Glucose Conditions

Introduction: Diabetic nephropathy(DN) is a major long-term complication of diabetes. Hypertrophy of glomerular mesangial cells (GMC) is an earliest morphologic alterations in the kidney after the onset of diabetes. Since GMC are pivotal in the glomerulus in both normal and pathological states, and are an accepted in vitro model for events that occur in the development of DN, we sought to study the proteome level changes induced in GMC by high glucose(HG), a diabetes mimicking stimulus. This study was designed to investigate dynamic proteomics profile of mesangial cells in response to high glucose condition.
Methods: Primary glomerular mesangial cells were cultured as previously described[1]. Cells were cultured in 5.4 mM normal glucose (NG) and 30 mM high glucose (HG) for 0, 8, 16, 72hour and 25 day respectively. Cell total proteins were prepared from NG and HG. Those Proteins were resolved by two-dimensional gel electrophoresis. Imagemaster software and statistical methods were combined use of t-test and false discovery rate (FDR) to analyze the HG-induced protein spot dysregulation. which expression changed in expression over twofold were subject to MALDI-TOF-MS and Mascot search engine analysis. The identified proteins were classified by GoMiner™, and GO expression were anylysed by BIGO, protein interact imported from IntAct Web Service Client visualized by cytoscape and analyzed by MCODE.
Results: 28 differential protein spots were found between NC and HG group. Among those in 8, 16h, 2 were (Rcn2, Hspb1) upregulated, and 24 downregulated (Tcp1, Wdr1, Cct2, Eno1, Adss, Eef1g, Idh1, Pcbp1, Akr1b1, rCG53488, Pgam1, Psma6, Psmb7, Prdx6, Gstp1, Stmn1, Sod1, Krt2-7, Hmgb1, Serpinb9). In 72h, 2 downregulated (Psma6, Hmgb1). In 25 days, 2 upregulated (Pdia3 and Idh3a) over twofold.
The differential expressed proteins mostly located in cytosol, nucleus, cytoskeleton, mitochondria and endoplasmic reticulum. Their molecular functions were mainly bound with protein, nucleic acid, ion, and they have the activity of antioxidant, oxidation-reduction, hydrolytic enzyme and isomerase. They primarily involved in the biological process such as cell communication, cell cycle, apoptosis, proliferation and metabolic process of carbohydrate, DNA, RNA, protein, oxygen and active oxygen. Gene ontology(GO) over expression and protein interaction analysis reveal that those protein were not only highly linked with already known diabetic nephropathy process such as glucose metabolism, oxidative stress and molecular chaperones, but also highly linked with proteasomes protein degradation.
Innovative aspects: 1. Proteasomes dysfunction might play an important role in hypertrophy of mesangial cells in response to high glucose conditions.
2.We newly found PCBP1, HMGB1, Psma6, Psmb7, Prdx6, Rcn2 and Serpinb9 changed in the mesangial cells response to high glucose conditions, which might provide new clues about the etiopathogenisis of diabetic nephropathy.
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A119

Characterization of alpha-amylase produced from bacteria isolated from soil at NWFP (Pakistan)

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Fifty soil samples from different areas of NWFP (Pakistan) were collected and processed for total count and spore count. Hundred isolates were screened for the production of α-amylase. Twenty five strains that showed clear zone on starch plate were isolated, purified and characterized. Genomic DNA of twelve strains was extracted and 16S RNA gene was amplified and sequenced for identification. Different media were tried for the production of α-amylase at laboratory scale. Thermostability and activity of enzyme was determined. Extracellular enzyme produced was partially purified with ammonium sulphate. Purified α-amylase was analyzed by polyacrylamide gel electrophoresis and Fast Protein liquid chromatography.

A120

Elafin is a biomarker of graft versus host disease of the skin

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Introduction Allogeneic hematopoietic cell transplantation (HCT) is the most potent immunotherapeutic treatment for hematologic diseases, but its practical use is impeded by acute graft versus host disease (GVHD). But GVHD diagnosis relies almost entirely on the presence of clinical symptoms of one of three target organs, the skin, gastrointestinal (GI) tract, or liver. Thus, we performed discovery and validation experiments to identify a panel of biomarkers with diagnostic and prognostic value for GVHD[1]. Yet, there are no biomarkers that are specific to any of the three target organs of GVHD.
Methods In an initial discovery study, a large scale quantitative proteomics analysis was performed comparing plasma pooled from ten patients with GVHD of the skin to plasma pooled from ten patients with no GVHD. In a validation set of 492 patients that consisted of 159 cases samples drawn from patients at the time of GVHD onset who present with skin GVHD and controls were samples from 223 patients who never developed GVHD taken at similar time points or 57 patients who present with GI GVHD or 53 patients who present with a non-GVHD skin rash. Logistic regression models tested the association of elafin levels with the severity of GVHD, transplantation-related-mortality (TRM), and overall survival (OS).
Results Of 631 proteins quantified based on differential stable isotope labeling, 66 proteins were increased 2 fold in skin GVHD while 6 could be measured by ELISA in individual samples. Elafin, an epidermal proteinase inhibitor that is found in inflamed epidermis[2], was identified as the best candidate biomarker. We therefore measured levels of elafin (expressed hereafter as mean±SEM pg/ml) in the validation set. Elafin levels were two fold higher in plasma from patients with skin GVHD as compared to plasma from patients without GVHD, with biopsy proven GI GVHD and with non-GVHD rash (7899±478 vs. 3934±125 vs. 4178±305 vs. 4212±372, p<0.001), respectively. We next analyzed whether elafin levels provided prognostic information regarding stage of GVHD skin, transplant-related-mortality and overall survival. We divided the patients into 2 groups using a threshold level of elafin that provided 85% specificity (5500 pg/ml). The group with the high levels of elafin developed more severe skin GVHD (p<0.001), experienced greater TRM at 2 years: 32% (95%CI, 19-43%) vs. 14% (95%CI, 8-20%) (p<0.001), and experienced lower OS at 5 years: 31% (95% CI, 23-41%) vs. 54% (95% CI, 48-61%) (p<0.001).

Innovative aspects
1. The first innovation is the use of state-of-the-art proteomic technology including the intact protein analysis system that allows me to discover novel proteins for GVHD in an unbiased manner.
2. The second innovation is the identification and validation of organ specific proteins in the circulating plasma.
3. The third innovation is the relevance of the discovered proteins to clinical outcomes including long term survival.

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A121

Global Proteome Survey Using Antibody-Based Arrays And Mass Spectrometry

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Introduction
Antibody microarrays is a rapidly evolving affinity-proteomic technology that recently has shown great promise in clinical applications (1, 2). Miniaturized arrays can be printed with numerous antibodies harboring pre-determined specificities, capable of targeting complex proteomes (2). However, the resolution of such profiling effort correlate directly to the number of antibodies included, which today is a key limiting factor (2). To overcome this bottleneck and to be able to perform global proteome profiling, we propose to interface antibody arrays with MS-based read-out in a conceptually new method denoted Global Proteome Survey (GPS) (3).

Methods
We have designed an entirely new class of antibodies denoted context-independent motif specific (CIMS) antibodies. By taking on a bioinformatics approach, we have defined sets of short peptide motifs, 4 or 6 amino acids long, where each motif is present in up to a few hundred different proteins, using the human proteome as model system. We have then selected binders specific for these peptides using our human recombinant scFv antibody library, composed of 2 x 10^{10} of members and microarray adapted by molecular design, as probe source. Next, the binders were immobilized in an array format, multiplexed plate format or column format and used to capture and enrich motif-carrying peptides from the digested (trypsinated) proteome(s). The captured peptides were then detected and identified (and quantified) using MS and tandem-MS based readout.

Results
In this proof-of-concept study, we have designed 27 selection peptide motifs, shared among 2,144 proteins (perfect match) or 57,434 proteins (allowing one residue to wobble). Based on these motifs, we have selected 91 readily renewable scFvs, i.e. CIMS antibodies, of which 14 were characterized in this study. Immobilized CIMS antibodies were exposed to synthetic peptide mixtures or crude digests from either human colon tissue or mouse liver homogenate. Any specifically bound peptides were then eluted, and detected and identified (and quantified) using MS or MS-MS. The results showed that the CIMS antibodies were capable of binding and enriching numerous peptides (proteins) harboring the corresponding selection motif, even when targeting crude digests originating from different species. The CIMS antibodies were found to recognize a linear epitope composed of three to four conserved residues, while the identity of the neighboring residues was more flexible. The limit of detection was found to be in the 0.40 pmole peptide range. Hence, the results showed that the GPS method could be used to probe crude proteomes in a specie independent and sensitive manner using a limited set of probes.

Innovative aspects
- We have demonstrated proof-of-concept for a novel method for global proteom analysis, denoted Global Proteome Survey (GPS), by combining the premium features of antibody arrays (affinity proteomics) and mass spectrometry.
- By using CIMS antibodies, a limited set of renewable antibodies can be used to target a significant part of the proteome.
- The GPS platform has the potential to become a key discovery technology for high-throughput analysis of complex proteomes in health and disease in a specie independent manner.

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A122

Comparative proteomic profile of 36-week Old OLETF and LETO Renal Cortex Might Reveal a Role of Protein Post Translation Modification In Diabetic Nephropathy

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Title: Comparative Proteomic Profile of 36-week Old OLETF and LETO Kidney Cortex Reveals a Role for Protein Post Translation Modification In Diabetic Nephropathy

Introduction: Diabetic nephropathy(DN) is a leading cause of end-stage renal disease in many countries. OLETF rat is a spontaneous animal model of non-insulin-dependent diabetes mellitus, which is considered as a useful model in the study of pathogenesis of DN because renal abnormalities of OLETF rat are similar to those observed in diabetic patients[1]. LETO rat originated from the same colony of Long-Evans rat with OLETF rat, but it hasn't shown the diabetic syndrome.In the study, proteomic analysis was performed to screen for global changes of renal cortex protein expression in OLETF and LETO rat.
Methods: 7 male OLETF and LETO rats were used in this experiment. OLETF rats in this experiment develop hyperglycemia, a progressive increase in urinary protein excretion, glomerulosclerosis. At 36 weeks OLETF and LETO rats was sacrificed, and the renal cortical total membrane and cytosolic fractions were respectively prepared. Those Proteins were resolved by two-dimensional gel electrophoresis. And Imagemaster software, statistical methods combine use of t-test and false discovery rate (FDR) to analyze the HG-induced protein spot dysregulation, whose expression changed over twofold were subject to MALDI-TOF-MS and Mascot search engine analysis. The proteins identified in this study were classified by GoMiner™
Results: Compared with LETO, in cytosolic fractions, 9 protein spots down-regulated (identified as Gapdh, Qdpr, Fah, similar to Protein C14orf159 mitochondrial precursor, Ephx2, Alb, Acadsb, Clybl) and 8 up-regulated (identified as Qdpr, Ephx2, Fthfd, Tf, Naprt1, Idh1, Fah, Cryab); In membrane fractions, 11 protein spots were down-regulated (identified as Uqcrfs1, Nrn1l, Pbld, LOC683519, Calb1, Acadsb, Dmgdh, Etfb)and 10 up-regulated(LOC683519, Qdpr, GMPK, Etfb, Uqcrfs1, Fah, Dmgdh, Gpx3, cytokeratin 8 polypeptide). The differential expressed proteins of OLETF and LETO mostly located in cytosol, mitochondria and cyto plasmic vesicle. Their molecular functions were mainly bound with ion, vitamin and protein. They have the activity of oxidation-reduction, electron carrier, transporter and antioxidant. They were primarily involved in the biological process such as cell communication, cell apoptosis, cell response to stress and cell metabolic process such as aromatic compound, oxygen and reactive oxygen species. MALDI-TOF-MS results suggest Ephx2 and Uqcrfs1 in different sports stands for different phosphorylation state.
Innovative aspects 1. Ephx2 and Uqcrfs1 post translation modification newly link with pathogenesis of type 2 diabetic nephropathy. 2. Some of these changed proteins or their modification like P55, Ephx2 and Uqcrfs1 were newly found to be involved in pathogenesis of type 2 diabetic nephropathy, which would provide us new clues about the etiopathogenisis of diabetic nephropathy.
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Normal Control (LETO) Diabetic nephropathy (OLETF)
Figure 1. Ephx2 were phosphorylated in normal LETO rat renal cortical (left) and the phosphorylations were lost in type 2 Diabetic nephropathy OLETF rat .

A123

Pre-validation of Biomarker Signature for Pancreatic Cancer Using Recombinant Antibody Microarrays

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Introduction
Pancreatic cancer is today the 4th leading cause of cancer-related deaths with a 5-year survival of less than 4%. There is a significant clinical need for cancer-specific biomarkers enabling early diagnosis by a non-invasive screening procedure. We have developed a state-of-the-art recombinant antibody microarray technology platform for high-throughput protein expression profiling of crude, non-fractionated serum samples. The aim of this study was to examine whether a candidate serum biomarker signature for pancreatic cancer, recently discovered by us, could be pre-validated using our antibody microarrays.

Methods
Crude, directly biotinylated serum samples from 147 patients diagnosed with pancreatic cancer and pancreatitis, and healthy control individuals were analyzed on our human recombinant scFv antibody microarrays. 160x8 microarrays were fabricated on black polymer Maxisorb slides using a non-contact printer. The arrays were processed in a protein array workstation and visualized using a confocal microarray scanner. The intensity of each spot was quantified and the data was scaled and normalized. Differentially expressed analytes were identified using Wilcoxon test. A Support Vector Machine was trained using the leave-one-out cross validation procedure and used to classify the samples as cancer, panreatitis or healthy.

Results
First, using a training set composed of 64 samples, the results showed that we have identified a condensed biomarker signature composed of 22 non-redundant serum analytes able to distinguish between pancreatic cancer and healthy controls with a sensitivity and specificity of 91% and 93%, respectively. The ROC-curve displayed an area under the curve (AUC) of 0.99. Next, this candidate biomarker signature was successfully pre-validated on an independent test set, composed of 24 cancer patients and 30 healthy controls, giving an AUC value of 0.97. Further, have identified the first candidate biomarker signatures capable of distinguishing between pancreatic cancer vs. chronic pancreatitis (AUC of 0.84), autoimmune pancreatitis (AUC of 0.99), acute pancreatitis (AUC of 0.97), or all pancreatitis patients and healthy controls combined (AUC of 0.88) with high specificity and sensitivity. Taken together, we have successfully pre-validated the first candidate serum biomarker signature for pancreatic adenocarcinoma.

Innovative aspects
- We have pre-validated the first serum biomarker signature for pancreatic adenocarcinoma.
- We are well on the way to develop the first blood-based diagnostic tool for pancreatic adenocarcinoma.
- Antibody-based microarrays provide unique opportunities for biomarker discovery and disease diagnostics.

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A124

Quantitative determination of the Amyloid-beta peptide with Asp7 in different isomeric forms

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Introduction
Transition of the amyloid-ß peptide (Aß) from its native monomeric conformation into insoluble fibrillar beta-sheet aggregates, which form the extracellular amyloid plaques, is believed to be a key event in the pathogenesis of Alzheimer's disease (AD). Since the full-size Aß is hard to work with due to its aggregation in aqueous solutions, here we present some MS approaches in which instead of the full-size Aß only its zinc-binding domain (residues 1-16, Aß16) is used as a convenient model for investigating the role of isomerization of the Asp7.

Methods
Linear ion traps (Finnigan LTQ) combined with collision induced dissociation (CID), Fourier Transform Ion Cyclotron Resonance mass-spectrometry combined with electron capture dissociation (ECD) and collision induced dissociation (CID) methods (Finnigan LTQ FT and Bruker Apex Qe mass-spectrometers) were used for quantitative analysis of solutions of synthetic Aß16 peptides containing normal and isomerized forms of the Asp7 residue in different concentrations. Calibration curves for quantitative measurements were measured using sets of solutions with peptide concentrations in the range from 5 nM to 1mkM.

Results
High quality calibration curves for different mass-spectrometers and fragmentation methods were obtained. Detailed analysis of the dependence of the calibration curve from different parameters, like the absolute concentration of peptides, AGC and parameters of the ion transport systems and fragmentation settings was carried out. Standard errors of determination of the relative abundances of the isomerized forms in binary solutions were also measured and analysis of their dependence on the relative and absolute concentrations of the peptides in solutions and parameters of the detection and fragmentation systems was performed. Method sensitivity was also determined for different instruments (FT ICR, Ion Traps) in different scan modes (MRM, SRM, Full scan).

Innovative aspects
Method of amyloid-ß peptide (Aß) quantitation using mass spectrometry is developed. An analysis of factors affecting the analytical capabilities of this method of quantitative analysis is carried out.

A125

The Fc Receptor Supramolecular Complex From Human Neutrophils

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Introduction
Human neutrophils are one of the first lines of innate cellular defense against invading pathogens but also respond to immunoglobulins provided by the adaptive immune system. Thus, the Fc receptor on the plasma membrane of neutrophils is a key target of autoimmune and oxidative cellular responses. The Fc receptor from the surface of white blood cells, and other phagocytic cells has been the focus of intense scrutiny at the cellular, biochemical and genetic levels. Recently, Live-cell Affinity Receptor Chromatography (LARC) has been shown to capture an activated receptor supramolecular complex from the surface of live macrophage cells for identification by LC-MS/MS, with superior sensitivity compared to immuno-precipitation, and the results were confirmed by Western blots, immuno cytological staining, expression of GFP constructs, the use of dominant negative mutants and silencing RNA (1). Data or literature mining algorithms and computer modeling programs such as iHOP (2) and Cytoscape (3) may be used to look for similar interactions in heterologous experimental systems.

Methods
Neutrophils were isolated from 100 ml whole blood by dextran sedimentation, centrifugation through a discontinuous percoll gradient, followed by re-suspension in NaCl experimental medium. The neutrophils were incubated with ligand coated microbeads on ice before washing away unbound beads. The cells were warmed to activate the receptor complex prior to disruption with a french press. The ligand microbeads bound to the activated receptor complex were isolated with a sucrose gradient by centrifugation and analyzed with 300 micron ID C18 column with Decca XP-100 ion trap using the SEQUEST software as previously described: Proteins specific to the activated Fc complex, that had at least three different, fully-tryptic peptides with a cumulative unified score of 2400 or more using BIOWORKS were accepted (1).

Results
LARC was used to capture the activated Fc receptor supramolecular complex from the surface of live human neutrophils. LC-ESI-MS/MS identified hundreds of proteins to be specifically associated with the activated Fc receptor supramolecular complex. The Fc receptor associated proteins were found to not only be in close agreement with previous biochemical and pharmacological experiments, but also revealed many novel proteins and isoforms associated with the Fc receptor pathways. These proteins were assembled into a network model of Fc receptor protein signaling in human neutrophils by computation using IHOP (2) and cytoscape (3).

From the unprocessed data, nearly 900 proteins were confidently identified from Fc supramolecular complex bound to the IgG-coated LARC beads. Background proteins that were found to non-specifically bind the uncoated control beads and IgG-coated control beads were subtracted. Several members of the Fc receptor family, their binding proteins or associated factors were specifically detected on the LARC beads. LARC beads also specifically bound co-receptors including serpentine receptors, channels, kinases, phosphatases, phospholipases, phospho-diesterases, monomeric and trimeric G proteins along with their activation and exchange factors or effectors. In addition, there were adaptors, scaffolds and an array of cytoskeletal and membrane proteins that support and organize the Fc supramolecular complex. Filtering the data using iHOP (2) and Cytoscape (3) revealed a set of about 300 proteins specific to the Fc receptor that showed at least three previously documented interactions The network model revealed the central proteins in the regulation of Fc receptor signaling that included factors which are well established in Fc mediated phagocytosis by previous biochemical and genetic evidence including specific isoforms of SRC, SYK, PLC, PLD, PI3K, RAS, PKC, ERK, P38, JNK, PAK, DOCK, ELMO as well as many novel proteins.

Innovative aspects
The first demonstration that an activated receptor supramolecular complex may be captured from the surface of a live, human primary-cell.

Capture of the Fc receptor complex by LARC shows good agreement with previous biochemical and genetic evidence.

Many novel protein and specific isoforms associated with Fc receptor were discovered and shown to agree with previous protein-protein interaction data.

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A126

Automated Electron Transfer Dissociation of Large Peptides and Medium Size Proteins in a QTOF instrument on an LC time

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Introduction
Reversible phosphorylation is known to be one of the most important functions in eukaryotic cells, but its detection and characterization is often difficult. In most cases the conventional CID-MS/MS (collision induced dissociation) just generates the neutral loss of phosphoric acid, resulting in missing information about its binding site and sometimes in low further fragmentation of the peptide chain itself. Therefore, Electron transfer dissociation (ETD) became the preferred method for the analysis of common post-translational modifications (PTM) in proteins. ETD keeps the labile PTM bonds intact by generating a prompt dissociation at the amino acid backbone and allows therefore the facile localization of the PTM.

Methods
ETD is well established on ion trap instruments due to the fact that the reaction has to take place in a trapping device: an excess of radical anions - generated in a negative chemical ionization source – is added to multiply charged peptide cations there.
However, the high resolution and accuracy of orthogonal time-of-flight (oTOF) instruments would largely improve the data quality and extend the possible applications of ETD to larger peptides and medium-size proteins.
Here, we demonstrate ETD-QTOF with a variety of PTM and top-down sequencing applications which are done in direct LC coupling, i.e. on the LC timescale.

Results
Major steps to enable LC applications of ETD in QTOF instruments are the adaption of ETD reaction times to several kHz repetition rate of the TOF analyzer, automated precursor ion selection taking into account the charge state of the analyte ions and optimized ETD sequence timing.

Innovative Aspect
ETD-QTOF for online experiments on an LC timescale.

A127

Mucin Gycoforms Associated with Subtypes of Pancreatic Cancer Cells

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Introduction. Alterations to protein glycosylation are prevalent in pancreatic cancer and are associated with poor prognoses. Secreted proteins with altered glycosylation are thought to have mechanistic roles in disease progression, but those roles have not been clearly defined. Insights into the functions of altered glycoforms may be gained by characterizing the cell types and conditions that produce them. Pancreatic cancer cells are known to be a heterogeneous mixture of cells in varying states of differentiation from highly-differentiated, epithelial-like cells to migratory, mesenchymal-like cells. The mesenchymal-like cells are more migratory, drug resistant, and indicative of a poor prognosis. We hypothesized that the glycans secreted by mesenchymal-like cancer cells differ significantly from those produced by epithelial-like cells. **Methods.** We investigated this question by examining the glycans on mucins—proteins with known cancer-associated glycan alterations—secreted by cultured cancer cells (either mesenchymal-like or epithelial-like) and by cells induced to undergo an epithelial-mesenchymal transition (EMT). In addition, we are examining secretions from xenografts of primary tumors that were sorted according to differentiation status. We used a novel antibody-lectin sandwich array (ALSA) platform[1-3] as well a variety of LC-MS methods. **Results.** The use of the ALSA platform revealed consistent alterations to mucin glycans associated with a transition to the mesenchymal state. We identified alterations to N-glycans that were not previously recognized on mucins. These glycoforms also appear on mucins in the blood of pancreatic cancer patients. **Innovative aspects.** This work shows the potential importance of altered glycoforms in contributing to cancer aggressiveness, and it shows it shows the value of novel methods of profiling both protein and glycan levels. Further defining the glycoforms associated with sub-populations of tumor cells could provide information about the mechanisms used by mesenchymal-like cancer cells in disease progression.

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A128

Effort of green tea catechins on copper-assisted human serum amyloid P using mass spectrometry

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Introduction

Human serum amyloid P component (SAP) monomer is glycosylated with a single N-linked site at Asn 32. SAP is a particular interest because SAP has been found to be associated with amyloid fibrils in the amyloidosis, e.g. in Alzheimers disease[1]. Green tea is considered as potential pharmacological activities for prevention and therapeutics in several diseases. Metal-chelating properties of green tea polyphenols are well known. Catechin polyphnols have been demonstrated to act directly as radival scavengers. Copper as an essential trace element is presented in the diet. In the present study, SAP is incubated with fresh green tea solution after binding Cu^{2+}, digested it with trypsin and then characterized the effect of green tea metal-chelating on two distinct SAP forms.

Methods

SAP was incubated at room temperature for two days with a 200-fold excess of $CuCl_2$ solution in 50 mM Tris pH 8.0. After incubation, the excess $CuCl_2$ was removed by a Micro BioSpin chromatography column previously equilibrated in 50 mM ammonium bicarbonate. After binding Cu^{2+}, the sample was incubated with fresh green tea solution in 50 mM ammonium bicarbonate at different incubation time or concentration. Excess green tea solution was removed by a Micro BioSpin chromatography column and digested with trypsin at an enzyme-to-protein ration of 1:10 in 50 mM ammonium bicarbonate containing 10% acetonitrile at 37 ℃ for overnight. The peptides were desalted by a C18 ZipTip. All experiments were done on a Q-TOF Global Ultima (Waters, Micromass) with a nanoES source.

Results

We have previously reported that Ca^{2+} was an effective metal ion leading to two distinct forms (form 1 and form 2) in the presence of Ca^{2+}, which appear the difference in their resistance to tryptic digestion. The two forms in the presence of Cu^{2+} was also examined not only to approach whether Cu^{2+} induce the formation but also to determine the effect of chelators of green tea on the Cu^{2+}-dependent forms since green tea catechins possess well-established copper-chelating properties.

To determine the effort of green tea catechins on Cu^{2+}-dependent human serum amyloid P in the presence of Cu^{2+}, Cu^{2+}-dependent human serum amyloid P was incubated with green tea solution (1 μl) at different incubation time from 1 hour to 5 days. The spectra after 1hour and 1 day of green tea catechins shows a graduate decrease in relative intensity of a series of ions corresponding to two distinct forms. After 3 days of green tea catechins, all ions corresponding respectively to form 1 and form 2 are completely converted into tryptic peptides and small glycopeptides. As the time of the green tea catechins reaction is increased up to 5 days, no ions corresponding to form 1 and form 2 are visible in the mass spectrum. The glycopeptide complexes are digested completely into tryptic peptides. Cu^{2+}-dependent human serum amyloid P was also incubated with green tea solution at different concentrations. Similar observations have been made in a graduate decrease in relative intensity of a series of ions corresponding to two forms at lower Cu^{2+} concentration and completely conversed into tryptic peptides and glycopeptides at a higher Cu^{2+} concentration, indicating that green tea catechins with the Cu^{2+} affect the two forms in protecting from tryptic digestion.

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A129

Organellar Proteomics: Analysis of Proteins in Castor Bean (*Ricinus communis*) Leucoplasts

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Introduction

Castor beans store triacylglycerides (TAG) in the endosperm of seeds. Fatty acids required for TAG anabolism are synthesized in leucoplasts an endosperm organelle. Castor bean oil has wide industrial applications, being used as raw material for biodiesel production. Then, an effort to screen enzymes that are present in the metabolic pathway of fatty acids synthesis will have great importance in castor bean biotechnology projects. This work aims both at the isolation of leucoplasts (a specialized plastid) and at a high-throughput screening of its proteins by proteomics analysis.

Methods

Plastids from the endosperm of developing castor oil seed at stage 4 (20 days after anthesis – DAA) were isolated by differential and Percoll density gradient centrifugation [1]. The protein extraction protocol of enriched organelle and of leucoplast-rich fractions was according to [2, 3]. Transmission electron microscopy (TEM) was used to examine organelle constitution. Two-dimensional gel electrophoresis (2D-PAGE) was employed for protein mapping. Protein patterns of each isolated fraction were compared, the spots were excised, digested and analysed by tandem mass spectrometry (MALDI-TOF-TOF) and identified by peptide mass fingerprinting (PMF) and non-processed data search (MS/MS ion search) using Mascot™ and non-redundant NCBInr as search engine and database, respectively.

Results

TEM showed that leucoplast-rich fraction also contained mitochondria and small vacuoles. Highly reproducible proteomic reference maps between pH 3-10 and 4-7 were obtained. The reference map detected ca. 1100 spots. Approximately 160 spots, selected based on quantity or relative volume and rate of expression, were removed for identification by mass spectrometry. Key enzymes that belong to the anabolic pathway of fatty acids in leucoplasts, e.g. beta-ketoacyl-ACP synthase, 3-ketoacyl-ACP dehydratase, were identified. Furthermore, other plastid and mitochondrial proteins, as well as primary metabolism, ATP synthesis, HSPs, and storage proteins were also present in this fraction.

Supported by: CNPq, CAPES, Faperj, Funcap

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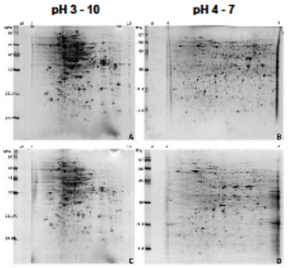

A and B - enriched organelle fraction
C and D - leucoplast-rich fraction

A130

Protein Identification Using SELDI Technology with MALDI TOF-TOF Analysis

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Introduction

Many methods exist to evaluate biological samples for potential Biomarkers, but identification of these markers is often elusive. Using SELDI ProteinChip arrays, direct identification using a Bruker Ultraflex III Mass Spectrometer was demonstrated for peptides of up to 4,000 daltons. For proteins, 3-stage,enrichment (prior to in-gel tryptic digest) was monitored at each stage via SELDI methodology and analyzed on an Ultraflex III ToF-ToF. Enrichment of serum proteins was achieved using three chromatographic media, IMAC, Q, and Reverse Phase. Verification was achieved using adrenocorticotropic hormone 1-24 (ACTH), spiked into serum.

Methods

Serum proteins too large for direct sequence analysis (>4 kDa) were enriched using anion exchange (Q), metal affinity (IMAC-CuS04), and reverse phase (PLRP-S) media. Crude serum was applied to Q resin, and sequentially eluted using a stepwise pH gradient. Eluted fractions were applied to IMAC resin and eluted stepwise by increased imidazole concentration. Fractions were concentrated and de-salted on reverse phase media. SDS-PAGE was conducted on all RP fractions, and bands of interest were selected for tryptic digest followed by identification on the Bruker Ultraflex III.

Peptides small enough for direct identification were enriched on a SELDI ProteinChip array. In some cases peptides were enriched prior to SELDI using the previously mentioned media and also ProteoMiner technology.

Results

Identification of native proteins using SELDI coupled with MS/MS technique has a high rate of success. Small fragments of proteins were identified directly from the surface of a SELDI CM10 array following profiling of human serum. The matrix used was SPA. This demonstrates direct compatibility of one sample preparation for profiling and ID, for species which are present on the surface in sufficient concentration.

TOF-TOF analysis on the Bruker Ultraflex III was performed on intense peaks using laser fragmentation to identify albumin, apolipoprotein, and spiked ACTH fragment 1-24. Pre-treating samples to enrich proteins of interest will enhance capabilities to identify lower abundant species directly from SELDI surfaces.

Serum proteins were successfully identified after enrichment and purification using selected chromatography media and SDS-PAGE. It was found that both visible coomassie stained and unstained bands could be used for in-gel digest and subsequent MS/MS analysis and identification. Fragments of albumin, transthyretin, Ig Kapa chain, apolipoprotein, hemoglobin, and lysozyme were identified.

Dominant peaks were selected from SELDI arrays for subsequent MS/MS analysis to demonstrate proof of principal for direct identification from a SELDI arrays. Selected bands from SDS-PAGE were also dominant bands.

Innovative aspects

The use of SELDI technology in direct TOF-TOF identification of serum proteins has been demonstrated.

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A131

Macromolecular Protein Complex Characterization via an Open-modification CXMS Pipeline

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Introduction

Chemical cross-linking and mass spectrometry (CXMS) is a powerful method for investigating molecular interfaces in protein complexes. While use of CXMS has been reported for many small protein complexes, its success in characterizing macromolecular assemblies remains limited. We reported an open-modification CXMS pipeline to identify crosslinked peptides from LC-MS/MS datasets [1] that relies on accurate mass tandem mass spectra. Here we combine this pipeline with an alternative database search strategy to characterize multi-protein macromolecular assemblies.

Methods

T3SS needle complexes (NC) from Salmonella typhimurium (consisting of 12-24 copies each of PrgH, PrgK, InvG and 100-150 copies of PrgI) and procapsids from bacteriophage lambda (consisting of 420 copies of gpE, 12 copies of gpB and 10-12 copies of gpC) were purified and cross-linked with EDC and BS2. Cross-linked complexes were denatured and digested with trypsin after reduction/alkylation. Resulting peptide digests were analyzed on an LTQ-Oribtrap. Data-dependent ion selection was triggered on ions ≥4+ charge-state, followed by tandem MS acquisition in the Orbitrap analyzer. Database searches were performed using Popitam (SIB, Switzerland) in an "open-search mode" or with xComb (in-house) and Phenyx (Genebio, Switzerland) in a "combinatorial mode".

Results

Using the method outlined above, inter- and intra-protein interactions were identified between components of NC. Measured mass accuracy of ±5ppm for precursor and fragment ions was used to confirm the presence of cross-linked peptides. Interactions identified between NC components were consistent with their currently accepted membrane orientations. Biological relevance of the identified interactions was investigated by biotinylation of intact and denatured NC. Surface accessibility mapping of lysine residues using biotinylation was consistent with CXMS results. Distance constraints provided by CXMS are being used for molecular modeling to predict the structure and assembly of NC. The power of our approach is further exemplified by successful elucidation of the protein interactions involved in procapsid assembly in bacteriophage lambda. These results clearly demonstrate the utility of our CXMS pipeline for characterizing multi-protein assemblies.

Innovative aspects

- Characterization of chemical cross-links in macromolecular multi-protein complexes
- Improved detection of low abundant cross-linked species
- Specific assignment and validation by complimentary search strategies (open-search and combinatorial-search mode)

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A new strategy for large scale quantitative phosphoproteomics of low amount of material. A132

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Introduction
There are still many challenges to phosphoproteomics including enrichment of phosphopeptides, loss of phosphopeptides during sample preparation, fragmentation and quantification of phosphorylated peptides from complex biological samples. *In vivo* studies employing primary cells are often performed in low amounts of material imposing even higher demands on phosphoproteomics strategies. Here we present a new strategy for quantitative phosphoproteomics using on-TiO2 iTRAQ labelling combined with SIMAC (1) and HILIC chromatography. We apply the strategy on cytokine stimulated pancreatic beta cells.

Methods
Phosphopeptides were extracted from tryptic digests of proteins originating from IL-1B and TNFα stimulated pancreatic primary betacells at various time points using an optimized TiO_2 enrichment procedure employing 1M glycolic acid and 5%TFA as "non-phosphopeptide excluders". After phosphopeptide binding, the phosphopeptides were labeled with the iTRAQ 8 plex directly on the TiO2 beads. After labeling the eluted mixed phosphopeptides were further separated using a combination of SIMAC (1) and HILIC chromatography. The phosphopeptides were analyzed on an nano-LC-LTQ-Orbitrap XL using multistage activation (MSA) and electron transfer dissociation (ETD) combined with high energy C-trap dissociation (HCD) MS/MS.

Results
The labeling efficiency was observed to be >90% for the phosphopeptides bound to the TiO_2 beads. The standard deviation was found to vary significantly depending on the experimental setup. Performing the enrichment in a batch-mode resulted in a much lower standard deviation, similar to that obtained when iTRAQ labeling was performed prior to phosphopeptide enrichment. Labeling of phosphopeptides directly on TiO_2 beads has several benefits; (i) much less iTRAQ reagent is used for large scale experiments, (ii) iTRAQ is not compatible with a large variety of reagents, whereas TiO_2 purification is very tolerant towards most reagents (2) so interfering reagents can be efficiently removed by TiO_2 chromatography prior to iTRAQ labeling. (iii) the reagent does not bind to TiO_2 and can therefore be removed efficiently prior to the elution of the phosphopeptides.
The method will be combined with our SIMAC and HILIC methods for phosphopeptide separation and applied to characterizing the phosphoproteome of cytokine stimulated primary pancreatic beta cells. Initial experiments using this strategy have successfully quantified >2000 phosphopeptides from <300ug of EGF stimulated HeLa cell extract.

Innovative aspects
- Optimized iTRAQ labelling of phosphorylated peptides in a complex mixture.
- Large scale quantitative phosphoproteomics of primary cells.
- Biological use of quantitative ETD and MSA measurements.

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Phosphoproteomics of human breast epithelial cells in response to treatment with TGFbeta and Heregulin-alpha1/EGF A133

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Introduction
Protein phosphorylation is one of the most important PTMs in cancer. A non-controlled activation of oncogenic kinases is often a strong pro-tumorigenic factor. IMAC-based phosphoproteins enrichment technique has been developed in our group (1). This technique provides high yield of phosphoproteins which is required for a comprehensive study. The objective of this study is to identify proteins which are common targets of TGFbeta and EGF, and explore their role in tumorigenesis.

Methods
We use MCF10 and MCF7 cells under treatment with TGFbeta and HRGalpha1 in combinations. MCF10A are immortalized but not tumorigenic, and MCF7 are tumorigenic cells. IMAC was used to enrich for phosphoproteins from treated and control cells. 2D-GE, gel image analysis and MALDI TOF MS were used to identify proteins. Systemic analysis of identified proteins was performed using various tools, such as Ingenuity and FunCoup (ingenuity.com, funcoup.sbc.su.se). Validation studies include identification of phosphorylation sites in selected proteins, generation of phosphorylation site-mutants, and exploration of their role in breast tumorigenesis.

Results
We have generated 2D gels of cells treated with TGFbeta and EGF alone and in combination. More than 100 differentially phosphorylated proteins have been detected. The emphasis is on identification of proteins which have TGFbeta+EGF specific pattern of phosphorylation, as compared to proteins which are affected by TGFbeta or EGF only (Fig 1).
We identified more than 50 proteins affected by combined treatment with TGFbeta and EGF. In-depth studies of identified proteins are expected to provide insights into mechanisms of cooperation of EGF and TGFbeta regulatory systems in breast cancer. This study is currently ongoing. Results of phosphoproteome profiling of combinatorial treatment of human breast epithelial cells with TGFbeta and EGF/HRGalpha1 will be presented.

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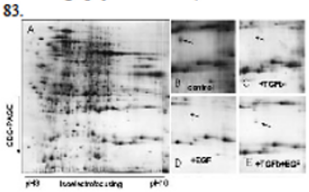

Figure 1. Image of representative IMAC 2D gel of MCF7 cells is shown (A). Inserts of IMAC 2D gels show an example of changes in protein phosphorylation/expression in IMAC 2D gel upon treatment of MCF7 cells with TGFbeta1 (C), EGF (D) and TGFbeta1+EGF (E). (B) Image of the IMAC 2D gel insert of a control not-treated cells. Arrows show migration position of a phosphorylated protein, which was observed in control cells and in cells treated with both TGFbeta1 and EGF, but not in cells treated with only TGFbeta1 or EGF.

Automated Multiple Round Searches for Peptide/protein Identification A134

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Introduction
One of the challenges researchers face in mass spectrometry-based proteomics investigations is that there will often be a number of spectra that remain un-interpreted due to the unsuspected modifications or unknown peptide sequences, etc. To increase the coverage, a two-round search may be launched in the identification process in order to accelerate the job when looking for many variable modifications or nonspecific cleavages. However, manual input of parameters for the second round is typically required (for example, with Mascot). In addition, users need to guess possible modifications. We proposed an automated multiple round search approach that automatically generates possible PTMs, correct precursor mass, and identifies unknown peptide sequence. Preliminary test show its promising results for this approach.

Methods
1. First round search: this is typical tag-based database search. Sequence tags were obtained via de novo sequencing for each spectrum.

2. Find PTMs not defined in the first round: We observed that dataset usually contain the pairs of peptides with modifications and without modifications. By aligning identified peptides which contain peptides without modifications and un-identified peptides which contain peptides with unsuspected modifications, a set of pairs were selected. Then, PTMs are further identified by comparing mass difference for each pair.

3. Homology search: The de novo sequences of spectra not identified in above two rounds are performed homology search against identified proteins. In this process, the error of precursor mass or peptide sequence not in the database will be handled.

Results
The method was implemented it in Peaks suite. Spider was used for homology search and was tested with MS/MS data sets from two samples. First sample is six protein mixture (LC Packings) digested with trypsin and alkylated with iodoacetamide. The other was from a complex protein mixture from C. elegans. All spectra were acquired on a LTQ Orbitrap XL. The peptide mixtures (2 ul injected) were separated via Surveyor LC equipped with MicroAS autosampler (Thermo Fisher Scientific) using a reversed phase peptide trap (100 um inner diameter, 2 cm length) and a reversed phase analytical column (75 um inner diameter, 10 cm length, 3 7m particle size, both Nanoseparations, NL), at a flow rate of 250 nl/min. A gradient of 5 - 30% acetonitrile in 90 minutes was used.

Two data sets were analyzed with our automated multiple round search against SWISS-PROT database with PTMs of Carboxymethyl on Cys and Oxidation on Met. For the data set of six protein mixture, 900 MS/MS spectra were collected along with 2076 survey scans. 596 spectra were identified in the first round. The coverage is 66.2%. In second round, the PTMs of deamidation on Asn and Gln were automatically selected. 35 more spectra were identified. With homologue search, 8 more spectra were identified, and the total coverage was 71%.

For the data set of complex peptides of C. elegans, 19969 MS/MS spectra were obtained along with 12771 survey scans. 10044 spectra, 959 spectra and 79 spectra were identified with respect to first, second and third round. In the second round, the PTMs of deamidation on Asn and Gln, Glycosylation (hexose) on Ser and Thr, and Acetylation on Lys were automatically selected.

The experiment results showed that our multiple round search approach increased the coverage of protein/peptide identification. The identification process is fully automated.

Novel Aspects
Automated multiple round search

New multiplexed detection technologies for protein Interaction arrays A135

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Introduction
Protein interaction arrays are showing real promise as higher throughput tools in functional proteomics (1). Normally an assortment of capture proteins are deposited on the array, which is then challenged with a second protein, usually tagged so that the interactions can be determined. This often requires isolation of the protein of interest and chemical labelling, and can only be used for determination of interactions of a single species. We have developed methods for multiplexed reading of arrays using both optical (QDot) or mass spectrometric methods, and compared these to conventional methods.

Methods
Home made arrays were generated by piezoelectric spotting onto ultrathin nitrocellulose coated slides. Three optical methods for detecting interactions were compared: a) Streptavidin coated QDot655 (Invitrogen), b) alexa647 conjugated streptavidin, and c) alexa647 conjugated antibody, using three different scanning technologies (Typhoon, ScanArray Plus and Dyversity). Methods for conjugating antibodies directly to QDots were developed and the detection of interactions with these conjugates also tested. For mass spectrometric reading of arrays, arrays were challenged with partner proteins, washed and the protein spots digested with trypsin. The arrays were then analyzed by overlaying with matrix and analysis by mass spectrometry on an Ultraflex III (Bruker).

Results
Arrays were produced on a home made ultrathin nitrocellulose surface that showed minimal background fluoresecence and allowed sensitive detection of protein interactions. Comparison of three methods of detection showed that QDots were the most sensitive methods, if excited at the optimum wavelengths (200nm). However, most array readers are unable to excite at UV wavelengths, so Alexa647 conjugated streptavidin was the most versatile detection method. QDots were also shown to work on commercial arrays (ProtoArray, Invitrogen). The advantage of QDots was shown to be the ability to multiplex analysis by using antibodies conjugated to different coloured QDots, and this was also shown to work in Western Blots. Mass spectrometric reading of arrays was facilitated by on-array trypsin digestion, followed by MALDI-MS screening and MSMS identification of interacting proteins using and Ultraflex III (Bruker). High quality MSMS spectra were obtained using a 10 micron laser spot, which allowed many MSMS spectra to be collected from a single spot. MS imaging of precursor ion masses were then used to monitor arrays rapidly and sensitively.

Innovative aspects
- Multiplexed QDot based measurement of interaction on protein arrays.
- Optimized detection methods for protein interactions on arrays.
- Mass spectrometric methods for detecting protein interactions on arrays.

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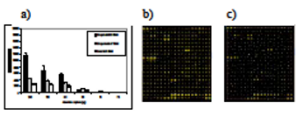

Figure 1. a) Intensity of signal from array probed with strep-QDot, strep-Alexa and antibody-Alexa conjugates, b) and c) Protoarray probed with RKIP and detection with b) StrepAlexa647 and c) strepQDot655.

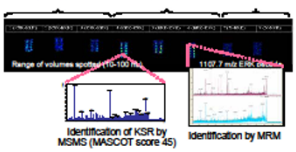

Figure 2. MS and MSMS identification of interaction between KSR and ERK using MS iamaging, MS and MSMS methods.

A136

RAId_deNovo: using de novo statistics to combine search results from multiple scoring functions and more

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Introduction
Combining search results from different peptide identification methods is impeded by the lack of a universal statistical standard. Although it is feasible to translate the score or heuristic E-value reported by any method into the textbook-defined E-value via calibration (1), one may lose spectrum-specific statistics and a recalibration is needed when major changes in experimental setup occur. Using score histograms from all possible de novo peptides, RAId_deNovo gives correct E-values, thus creating a calibration-free protocol for accurate significance assignment and combining search results.

Methods
Given a spectrum and a scoring function that sums local contributions, RAId_deNovo computes via dynamical programming, see Fig 1, the score histogram of all possible peptides, of which there may be nonillions and each peptide may be weighted according to its amino acid composition. From this histogram, the P-value of a given score can be determined and the E-value equals the P-value times the number of peptides within the allowed mass range in the database searched. For a given spectrum, different scoring functions assign different E-values to the same candidate peptide. Converting those E-values into database P-values, we use Fisher's formula to calculate the combined database P-value (2), from which the combined E-value is inferred.

Results
Using experimental spectra to query random databases, we find RAId_deNovo reports accurate E-values. Implemented in RAId_deNovo are five scoring functions: RAId_DbS, Hyperscore (similar to X!Tandem), Weighted Peak Count (similar to MS-Tag), XCorr (similar to SEQUEST), and K-score (similar to the K-score plug-in for X!Tandem).

If one specifies only the mass, RAId_deNovo returns the total number of de novo peptides within a prescribed mass error. One may input a spectrum and a list of peptides, and select a number of scoring functions provided, RAId_deNovo then assigns for each peptide in the list P-values corresponding to multiple scoring functions and calculates the combined P-value. One may also upload the output files from search engines such as Mascot, SEQUEST, and X!Tandem. Here, RAId_deNovo reranks peptides using the combined E-values.

RAId_deNovo can be used as a search engine capable of using multiple scoring functions simultaneously. Receiver operating characteristic (ROC) analysis shows that RAId_deNovo performs comparably with existing search engines when tested with profile/centroid spectra. Weblink: http://www.ncbi.nlm.nih.gov/CBBresearch/qmbp

Innovative aspects
- Assigning peptides' significances from de novo statistics, RAId_deNovo can bypass calibration while combining search results from multiple scoring functions.

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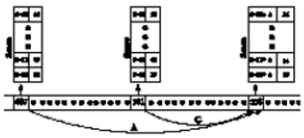

Figure 1. Strip down version of the de novo mass grid. For illustration, the grid is made 1Da apart and the peptide weighting (3) by elemental composition is omitted. Each grid contains a score histogram, updated by backtracking. For example, at the mass grid 558, the local score contribution from evidence peaks in the spectrum is assumed to be Δ. Looking back to mass grid 501/487, that is 57 /71 Da less than 558 Da, one knows that by growing a glycine/alanine residue the partial peptides reaching mass index 501/487 will advance to grid 558. Therefore, at mass index 558 the score histogram is the superposition of score histograms associatd with the other twenty lighter mass grids corresponding respectively to the twenty amino acids.

A137

Profiling With SELDI ProteinChips for Identification of Biomarkers with TOF-TOF Methodology

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Introduction
Profiling often detects differentially expressed proteins comparing *eg* diseased to control donor samples. Many methods exist to evaluate biological samples for biomarker candidates, but identification of these markers may be elusive. Direct TOF-TOF analysis of SELDI arrays profiled with human serum resulted in identification of species up to 4kDa.
Profiles of different bird organ tissues on different SELDI surfaces achieved profiling results with similar spectra on Bruker and SELDI systems up to 40kDa. This additionally demonstrates a simple and quick method for investigating proximal tissues.

Methods
ID: Pooled human serum was spiked with ACTH Fragment 1-24 at 0.5 µg/mL. Crude serum was bound and washed on anion exchange (Q) column with fractionation with pH gradient, followed by bind and wash on metal affinity fractionation column (IMAC-Cu) and fractionation with imidazole concentration gradient. All Q and IMAC fractions were enriched and de-salted using Reverse phase resin (PLRP-S). Processed samples were profiled on SELDI CM10 ProteinChip arrays using the Bruker Ultraflex Mass Spectrometer. Proteominer treated plasma was also profiled. Dominant species were further analyzed by TOF-TOF for direct identification.
Profiling: different Zebra finch bird organs were sectioned and printed onto SELDI surfaces both directly and in the presence of binding buffer. Chips were profiled on the Bruker Autoflex Mass Spectrometer and Bio-Rad PCS.

Results
Profiled serum samples were compared on the SELDI PCS-4000 system and Bruker Ultraflex, and very similar spectra were obtained in the mass region of interest, 1,000 to 10,000 dalton. Dominant peaks were further analyzed by TOF-TOF with laser fragmentation on the Ultraflex system. TOF-TOF data was analyzed in Mascot data base with a high degree of success. ACTH Fragment 1-24 was enriched in IMAC fractions and identification was successful from CM10 profiling directly using TOF-TOF analysis on the Ultraflex. Fragments from albumin and apolipoprotein were identified from CM10 profiling of ProteoMiner extracts.
Profiling of bird organ samples: this is a way of looking at proteins from proximal tissue with a simple sample preparation methodology compared to tissue lysing or imaging. The profiles obtained are demonstrably reproducible from the same tissue type. Profiles are different for different chip types and different organ types. Samples give similar profiles up to 20kDa on the Autoflex and the PCS.

Innovative aspects

Combination of SELDI sample preparation and high-resolution Mass spectrometry for serum and plasma

Easy sample preparation for proximal tissue investigation

A138

RAId_DbS: mass-spectrometry based peptide identification web server with knowledge integration

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Introduction
Existing scientific literature is a rich source of biological information such as disease markers. Integration of this information with data analysis may help researchers to identify possible controversies and to form useful hypotheses for further validations. In the context of proteomics, individualized proteomics era may be approached through consideration of amino acid substitutions/modifications as well as information from disease studies. Integration of such information with peptide searches facilitates speedy, dynamic information retrieval that may benefit clinical laboratory studies.

Methods
We have integrated from various sources annotated single amino acid polymorphisms (SAPs), post-translational modifications (PTMs), and their documented disease associations into one enhanced database per organism (1). We have also augmented RAId_DbS (2) to take into account this information while analyzing a tandem mass spectrum. In principle, one may choose to respect or ignore the *correlation* of SAPs and PTMs within each protein. The former leads to targeted searches and avoids scoring of unnecessary SAP/PTM combinations; the latter explores possible SAPs in a controlled fashion. To facilitate new discoveries, RAId_DbS also allows users to conduct searches permitting *novel* polymorphisms as well as to search a knowledge database created by the users.

Results
From analyzing the Aurum dataset (3) using different search spaces, we confirmed that the number of true positives found at a false positive threshold may decrease if the search is done in a larger search space, i.e., with novel SAPs and/or annotated SAPs/PTMs unconditionally enabled (Fig 1). This indicates that it is not productive to search with the annotated SAPs/PTMs and novel SAPs enabled all the time. We recommend the user to turn on these features conditionally. For example, if a spectrum does not receive any significant hit from a regular search, one may then allow the annotated SAPs/PTMs. If the search still returns no significant hit, one may then turn on novel SAPs in the search.
So far, we have finished constructing enhanced databases for 17 organisms. The webserver link to RAId DbS and the enhanced databases is http://www.ncbi.nlm.nih.gov/CBBResearch/qmbp/

The relevant databases and binaries of RAId DbS for Linux, Windows, and Mac OS X are available for download from the same web page.

Innovative aspects
- RAId_DbS is augmented to integrate annotated PTMs, SAPs and disease information extracted from existing databases or specified by the user.

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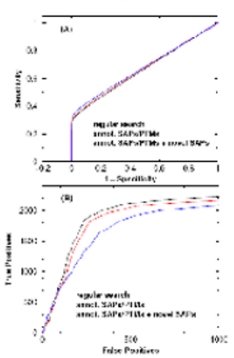

Figure 1. Receiver operating characteristic (ROC) analysis of augmented RAId_DbS using the Aurum dataset are shown in two ways. Panel A shows sensitivity versus 1-specificity, while panel B shows the number of true positives versus the number of false positives.

A139

Development of an Electron Transfer Dissociation capable Ultra High Resolution Orthogonal Quadrupole Time of Flight Mass Spectrometer

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Introduction
Electron Transfer Dissociation (ETD) is rapidly becoming an accepted dissociation technique for the characterization of post translation modifications. ETD is most commonly implemented on relatively slow, low mass accuracy trapping instruments such as 3-D or 2-D ion traps. In contrast, an ESI-quadrupole time of flight mass spectrometer was recently introduced that is capable 40,000-60,000 resolving power and mass accuracies of less than 2 ppm, at an acquisition rate of 20 spectra per second. Orthogonal TOF is traditionally a beam-type instrument, whereas ETD typically requires a trapping mode of operation. This presentation will describe the development and characterization of an instrument that merges the trapping ETD technique with the continuous-beam ultra high resolution (UHR) quadrupole-TOF (qTOF).

Methods
The main instrument used for this work is a modified Bruker maXisTM quadrupole TOF. Fluoranthene reagent ions were generated in an nCI source and are orthogonally injected between a pair of octapoles; which along with the nCI source were adapted from an HCT ETD IITM ion trap. Reagent and analyte ions were mass selectively transmitted through a mass resolving quad and trapped in a hexapole collision cell. After the ETD reaction, product ions are transferred to a cooling cell where they are stored and extracted to the TOF analyzer. While ions are extracted, the next ETD experiment is ongoing in the collision cell thus maximizing duty cycle. Experiment timing, potentials, and rapid polarity switching were achieved with custom electronics.

Results
The main instrument parameters which can affect the ETD reaction are the collision cell RF voltage, the collision gas pressure (and type), and axial storage conditions. Both ion traps and linear quadrupoles have shown that the RF amplitude can have an impact on the ETD performance. In the hexapole device where the fields in the center are weaker the RF voltage should presumable have less of an impact. Results suggest that over a wide range of RF voltages (1 kVpp) there is minimal impact to the ETD reaction. The collision gas in any trapping device serves to condense the ion cloud. It is therefore suggested that a higher collision gas pressure should improve the overlap of the ion clouds thus further improving ETD. The data shows that at high collision gas pressures ETD efficiencies are improved. With respect to speed, the Results show that 2.5 to 4 ETD MS/MS spectra can be acquired per second. Additional optimization should allow for even faster acquisition rates. Standard samples Substance-P, Melittin, Beta Caseine, and Ubiquitin all showed sequence coverages of 80-100% depending on the number of proline residues, with mass accuracies < 2ppm on all product ions. It can also be shown that the product ion spectra have sufficient signal to noise for proper peptide identification in a single spectrum (i.e. no averaging). The TOF analyzer had a resolving power exceeding 40,000. In the case of Ubiquitin, with very complex ETD product ion spectra, this high resolution and mass accuracy was more than sufficient for identification. The work thus far shows a novel ultra-high resolution quadrupole TOF that is now capable of rapid ETD experiments with sufficient resolving power for ETD MS/MS analysis of < 10 kDa proteins.

Innovative Aspect: Electron transfer dissociation on an Ultra High Resolution TOF with 40,000 resolving power achieving rep.-rates of several hertz.

A140

Antibody Capture and Identification of APP Fragments by Mass Spectrometry

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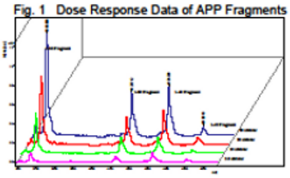
Introduction
Antibodies specific to human Amyloid Precursor Protein Fragments were immobilized to a glass coated aluminum substrate for use in capture of these fragments from biological samples applied directly to the modified surface. Optimal binding and wash conditions were developed, and calibration was demonstrated for four fragments of APP using linear mode TOF mass spectrometry. Identification of APP was achieved by TOF-TOF fragmentation directly from the array surface of fragment 1-33.

Methods
Glass coated aluminium SELDI ProteinChip substrates were coated with 3-Aminopropyltriethoxy silane, followed by reaction with 1,1 carbonyldiimidazole. 6E10 antibody was immobilized to this surface by application at 0.25 mg/mL in PBS overnight at 4 Degrees C, followed by PBS wash. Artificial CSF was spiked with APP fragments 1-33, 1-38, 1-40, and 1-42 each at 2.5, 10.0, 20.0 and 50.0 nMolar. Each level was applied on two spots of the surface. 60 minutes binding was followed by exhaustive washing with PBS with and without detergents, then desalted with HEPES. CHCA EAM in aqueous 50% acetonitile, 0.5% TFA was applied to the washed and air dried surface.
The prepared array was interrogated using a Bruker Autoflex Mass spectrometer summing 500 shots per sample. The 50 nMolar level was chosen for MS-MS analysis on a Bruker Ultraflex III mass spectrometer with identification using the Mascot data base.

Results
Dose response was achieved over a molar concentration of 2.5 nMolar to 50 nMolar for four fragments of APP simultaneously detecting by TOF mass spectrometry. The data supports quantitative capability for fragments of APP using TOF Mass Spectrometry. Consistent results were observed from 2 separate measurements at each level from different array regions. Using TOF-TOF with LIFT fragmentation on a Bruker Ultraflex III Mass Spectrometer identification of APP was achieved using fragment 1-33. The artificial CSF used contained bovine serum albumin at 0.3 mg/mL. The identification was carried out directly from the array prepared for dose response, with no additional sample purification steps. Sensitivity of the method is better than 2.5 nMolar for all four fragments tested, but the limit has not been established.

A second spike was prepared using the four fragments spiked into 20% human serum in PBS at 50 nMolar per fragment. Identification was repeated with this sample on the Bruker Ultraflex III using TOF-TOF LIFT fragmentation analysis. The results are consistent with ACSF data.

Fig. 1 Dose Response Data of APP Fragments



Innovative aspects
Immobilized antibody capture surface for quantitative analysis using TOF Mass Spectrometry

Simultaneous calibration of multiple fragment recognized by the same antibody

TOF-TOF identification of captured peptides from an antibody modified surface.

A141

Whole Proteome Tumor Antigen Arrays for Serological Assay of Melanoma and Non-melanoma Skin Cancer Patients Utilizing a Low Cost Microarray Scanning System

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Prioritization of candidate biomarkers is critical to any protein biomarker development effort. Currently, there are few tools to focus efforts on the most clinically relevant biomarker candidates for analysis and validation with MS, immunoassay, or other techniques. In this study, a two-dimensional liquid-phase protein fractionation method (proteome partitioning) was used to generate whole proteome arrays and applied as a "front-end" assay for prioritizing downstream biomarker research. In proteome partitioning, proteins from tumors or cell lines are first separated into fractions by chromatofocusing then subsequently separated by hydrophobicity. The resulting individual fractions are printed on an APiX array and screened with patient samples to identify autoantibodies that are present. The proteins are detected by a gold catalyzed, silver deposition (analogous to silver staining electrophoresis gels) optimized for microarrays. The microarrays are then scanned on a low cost scanner (APiX GR8). In this study, proteome partitioning was used to produce four 864-feature microarrays by fractioning four sets of primary cell lines isolated and expanded from the lymph nodes of two melanoma patients. Sera were obtained from normal, late stage, and early stage melanoma patients, as well as from patients with precancerous lesions participating in a clinical trial. These sera samples were applied to the arrays and IgG binding was detected and quantified. The method allows for rapid identification of target fractions containing potential biomarkers using autoantibody signatures, effectively allowing the immune system to select fractions for subsequent protein identification by western blot and mass spectrometry analysis using fractions retained for later analysis.

A142

Altered Glycosylation Pattern of Total Serum IgG in Patients with Rheumatoid Arthritis analyzed by MALDI-TOF MS

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Introduction
Glycosylation of human serum immunoglobulin is affected by different parameters, e.g. age, disease, etc.. Variations in the composition of the glycan moiety can dramatically influence antibody activity. Rheumatoid arthritis (RA) is a chronic autoimmune disease known to be characterized by changes in the glycosylation pattern. Here, we present a simple method based on graphite purification and MALDI-TOF MS to investigate the relative distribution of N-glycans of total serum immunoglobulins.

Method
Total serum of patients and controls were digested with PNGase F. Released N-glycans were applied on graphite loaded filter plates equilibrated with 0.1 % TFA, Bound glycans were eluted off using 30% ACN/0.1 % TFA. Eluates were prepared with DHB (10 mg/ml in 50 % ACN/5 mM NaCl) on AnchorChip2000™ targets.

Results
The acquired reflector spectra comprised about six to ten peaks in the mass range of 1000 to 3500 Da. Subjecting these masses to a Glycomod database search (http://www.expasy.org/tools/glycomod/) facilitated the correlation to defined N-glycans. The three most prominent peaks at 1485, 1647 and 1809 Da formed a series of fucosylated biantennary complex glycans deriving from immunoglobulin G. Calculation of the peak areas and subsequent normalization allowed for the relative quantitation of the extent of galactosylation. Analyzing the different patient groups (healthy, RA OA) the averages for the mono-galactosylated form were 1.7, 0.9 and 1.1, respectively. Correspondingly, the averages for the di-galactosylted form were 0.9, 0.3 and 0.5 for healthy donors, RA and OA patients. In healthy individuals the mono-galactosylated glycan is the main glycan form. Patients with RA showed a relative increase of agalactosylated glycans compared to healthy individuals.

Innovative Aspect
Using glycosylsation patterns as biomarkers for Rheumatoid Arthritis in a direct analysis of patient serum

A143

Ion Funnel Transmission Increases Number of Protein Identifications in Complex Proteomics Samples

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Introduction
Although ion traps are used as workhorses in life science due to their high spectral rate and unmatched MS/MS sensitivity, there is a high demand to further push their limits. An improved ion transfer e.g. using funnel-ion guides allows for a substantial increase of ion flux, however, as the benefit of the higher ion flux depends strongly on the ratio of accumulation time and scan duration[1], it is crucial to couple funnel inlets with fast scan rates to achieve best performance. To test this approach, peptide identification from complex mixtures using LC-autoMSn is investigated as key application: In complex samples only a part of all precursor ions is selected for fragmentation thus any reduction in duty cycle is important.

Methods
All measurements were carried out on a modified Bruker ETD II ion trap system in which a two-stage funnel ion-guide results in 10-times higher ion flux. Via an improved control of the non-linear ejection process and the trap environment faster scan rates as well as a higher mass resolution are readily achieved. As a sample of high complexity digests of Escherichia coli cells were analyzed using 90 min nanoLC gradients at a flow rate of 300nl/min. Depending on fragmentation type (CID or ETD) trypsin or Lys-C were used to obtain peptides most suitable for the respective technique. Peptide identification was performed with Mascot search algorithm and results validated using decoy searches.

Results
We initially validated the total 10fold increase in ion transmission resulting from the funnel interface with infusion and flow injections of reserpine. Signal to noise values for MS were unaffected, indicating that chemical background remained comparable to the skimmer nozzle interface. For MSMS signals a 10fold better signal to noise could be obtained.
Using E. coli digests the numbers of identified peptides and proteins depending on sample amounts were analyzed. Several concentrations from 5-500 ng protein on column were investigated: The largest improvement –up to twofold- was observed for the smallest amount, whereas the benefit for higher sample concentrations was lower, as the ion accumulation time has a minor proportion in this case.
The combination of ion-funnel interface and fast scan rates allows for more information from limited sample amounts. If sample availability is less critical the increased MSMS sensitivity enables further fractionation of the sample or repeated investigations using information from previous runs.

Innovative Aspect: The benefit of increased ion flux in combination with fast scan rates is demonstrated for peptide identification in complex samples.

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Identification of mosquito salivary gland proteins and determination of parasite infection

A144

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Introduction
Mosquito salivary glands are organs specialized in the production of a mixture of molecules that facilitate blood feeding by the lubrication of mouthparts and the inhibition of homeostasis. This is relevant for Malaria research since the Plasmodium sporozoites invade salivary glands and are injected with the saliva into vertebrate hosts during blood feeding.

Methods
To get insights into the proteomics of mosquito salivary glands, the organ was isolated from mosquitos and the proteins separated by 1D-PAGE after cell lysis. The gels were silver stained, bands were cut from the gel and subsequently digested using trypsin. The bands were analyzed by nanoLC-ESI-QTOF-MS/MS on a Bruker micrOTOF-Q II system and all the data transferred into the ProteinScape database system for further data analysis.

Results
As expected, in the course of this project, a number of mosquito proteins specific for salivary glands could be identified yet surprisingly not only mosquito-originating proteins were identified but also three proteins originating from the nematode parasite Brugia malayi, which was not expected in the sample. This parasite causes the disease Elephantiasis and the combination of 1D-PAGE and nLC-MSMS analysis of the salivary glands allowed the identification of the parasite infection in the mosquitos. Since no P. falciparum related proteins were identified, it can be regarded as a confident identification of the parasite infection, accidential identification of three different proteins from a known mosquito parasite can be regarded as very unlikely.
ProteinScape performs handling and processing of the data from multiple LC-MS/MS datasets in a fast, convenient and automated manner and allows for automated decoy validation of the results. This removes a major bottleneck in complex proteomics experimental setups with multiple separations, multiple types of MS analyses and the use of multiple search engines

Innovative Aspect
Use of combined data from different instruments from increasing proteome coverage

Pushing toward to next generation Ion-Trap-MS: consequent adoption of newest technology in ion formation, transfer and trapping.

A145

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Introduction
Radio-frequency Ion-Trap mass spectrometers have achieved a leading position in life science applications. This is mainly due to their flexibility, robustness, the extraordinary multiple MS/MS capabilities and to the unmatched MS/MS sensitivity at high duty cycle. The commercial availability of ETD strongly increased their use in detailed structure elucidation of proteins and peptides. Especially for these applications there is a strong demand to resolve the isotopic structure of highly charged ions over a large seamless m/z-range at analytical feasible scan-speed and sensitivity.
Preparing Ion-Traps for the expanding ultra-fast chromatography, for the requirements of top-down ETD/PTR workflows and to sharpen their position with respect to alternative techniques it is crucial to push the limits of their key performance factors.

Method
The presented development is based on an HCTultra ETD II Ion-Trap system. The introduced two-stage funnel ion guide results in a substantially increased ion flux and MS/MS sensitivity. The improved control of the non-linear ejection process and the trap environment supports faster scan modes as well as a higher mass resolution. The increased spectral rate can even be maintained during positive / negative ion mode switching due to a novel zero-dead time ion inlet system. The performance factors (sensitivity, resolution, scan rate and polarity switching) have been investigated systematically in several applications: peptide identification in complex matrices, clinical screening applications, and top down ETD/PTR workflows.

Results
Based on the predicted improvement in ion transfer yield due to the incorporated dual stage ion funnel the modified Ion-Trap MS achieves one decade improvement in MS/MS sensitivity, measured at a level of 250 fg Reserpine injected. Taking advantage of the 10-times increased ion flux and increased scan speed, high protein identification yields can be achieved at much lower sample amounts for example in the analysis of complex samples like complete organism extracts. The increased duty cycle allows selecting more of the generally incompletely covered precursor ensemble. The further enhanced trap-control allows doubling the highest scan speed while still meeting the resolution specifications of standard HCTultra ETD II for the Ultra scan mode with a peak width of less than 0.6 Th. The system is furthermore able to clearly resolve the isotopic pattern of six-fold charged ions at a scan speed 5-times higher than the former Maximum Resolution scan mode, making this resolving power available for a routine, wide m/z-range, full spectrum acquisition, as required for the ETD-based detailed structure elucidation of peptides or proteins larger the 10 kDa. In alternating polarity acquisition mode a level of 15 spectra/s is surpassed, especially suitable for small-molecule applications like rapid drug and metabolite identification and quantification in body liquids in conjunction with ultra fast chromatography.

Innovative Aspects:
Consequent combination of ion formation, ion transfer and control of trap environment: pushing applications beyond their current limits.

The Role of Increased Resolution and Scan Speed of Ion Traps for Top-Down Proteomics with ETD/PTR

A146

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Introduction
Dedicated MS/MS-techniques for top-down proteomics are electron-induced fragmentation processes like electron capture or electron transfer dissociation. However, the analysis of ETD MS/MS data of highly charged proteins can be rather complicated, because a plethora of multiply charged and overlaid fragment ions can be expected. The complexity of the ETD MS/MS-data is significantly reduced when the initial ETD-step is followed by a subsequent proton transfer reaction (PTR) reducing the charge states of the multiple charge fragments. For common ion trap instruments, the charge reduction step is typically optimized leading to singly and doubly charged fragments. An increase in resolving power of the MS-instrument is strongly demanded if highly charged ETD-fragments (z > 4) have to be analyzed

Methods
All measurements were carried out on a modified Bruker ion trap. An improved control of the non-linear ejection process and the development of the trap environment support faster scan modes as well as a higher mass resolution. The previously purified proteins were introduced into the ion trap with offline nanospray. ETD/PTR of the isolated protein is performed with reagent anions dedicated for either ETD or PTR. The formation of the different reagent anion is accomplished from only one neutral compound by altering the voltage of the negative chemical ionization source.

Results
We investigate the role of the increased resolution of a modified ion trap mass spectrometer on the top-down sequence analysis of larger peptides and proteins with ETD/PTR. The unmatched resolution of common Bruker ion traps at scan speeds of 8,000 Th/sec is ideally suited for the identification of fragment ions with charge states equal or less than z = 3. For the sequence analysis of intact proteins with ETD/PTR MS/MS, the subsequent charge reduction step with PTR has to be tuned for generating mainly singly, doubly and triply charge fragments.
One major drawback of the charge reduction step is the decrease of the total ion signal after the PTR-step. With each proton transfer the m/z of an ETD-fragment will be shifted towards higher masses. Any highly charged ETD-fragment will get out of scope if the final m/z of the ETD-fragment exceeds the maximum accessible m/z of the instrument.
The higher accuracy of the resonant ejection process of the modified ion trap system allows a fivefold faster scan speed compared to the established maximum mode. With the resulting resolving power of the modified ion trap instrument ETD-fragments having five or even six charges are isotopically resolved (peak width ~ 0.1 Th). The scan speed above 4000 Th/sec and the high charge capacity of the modified ion trap system enables the fast identification of ETD-fragments within a seamless scan (scan range 100-3000). We will present the impact of the increased resolution on the sequence analysis of intact proteins (molecular weight up to 16 kDa). With the increased resolution we were able to sequence a purified biomarker protein which has been previously discovered via MALDI imaging of breast cancer tissues.

Innovative Aspect: Increasing resolution and scan speed of ion trap mass spectrometer enhances the sequence analysis capability of top-down proteomics with ETD/PTR

Sequence characterization of monoclonal antibodies based on a combined LC-MS/MS approach powered by sophisticated bioinformatics

A147

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Introduction
Antibodies represent one of the most important classes of protein drugs. In accordance to common pharma QC requirements, recombinant proteins products need to be characterized in detail. This includes an as complete as possible confirmation by experimental data of the expressed protein sequence as well as the in-depth characterization of modifications.
In this paper we describe exemplarily the sequence characterization of a recombinant antibody based on a combined strategy involving multiple enzymatic digests in conjunction with LC-MS/MS analyses performed on various MS/MS platforms. Seamless integration and analysis of the resulting data as well as final result compilation is demonstrated employing a unique database software illustrating the crucial importance of sophisiticated bioinformatics tools to the efficiency of in-depth protein characterization tasks.

Methods
This study was carried out on a recombinant monoclonal IgG. The reduced and alkylated antibody (with and without chain pre-separation by PAGE) was enzymatically digested using four different proteases: trypsin, chymotrypsin, GluC and LysC, respectively. nanoLC-MS/MS analyses of the obtained digests were performed using various types of MALDI and ESI mass spectrometers, including TOF/TOF, high capacity ion trap, ultra-high-resolution qTOF and FTMS instruments.
All LC-MS/MS data was archived and further analyzed in the ProteinScape database featuring ProteinExtractor, a sophisticated algorithm developed for efficient compilation of data obtained from multiple LC-MS/MS experiments into a combined, non-redundant and comprehensive result.

Results
The ProteinScape database software employed here allows the user to build up in an intuitive fashion a customized, flexible project structure mirroring the workflows that have been applied in the lab (i.e. LC or gel based separation methods involved, proteases used, etc.).
The application of a combined analytical approach as described here results in high amounts of mass spectrometric data. Approximately 20 LC-MS/MS datasets obtained from the various digests and MS/MS platforms, respectively, were imported and organized in ProteinScape, and were then matched against the antibody chain sequences considering expected modifications, especially terminal modifications like N-terminal pyroGlu. The sequence information obtained from these individual experiments was then further compiled into a detailed, comprehensive, non-redundant project report integrating all acquired data irrespective of the workflow and MS instruments used. This compilation step was performed very efficiently applying ProteinScape´s unique ProteinExtractor algorithm. As a result, nearly 100% MS/MS sequence coverage was achieved for both, the antibody´s light and heavy chains. The compiled overall result is displayed in ProteinScape by means of easy-to-navigate sequence viewers, protein and peptide tables, respectively, which allow to overview on a glance the protein coverage achieved at any stage of the experiments, and, at the same time, enable direct access to annotated raw data of single peptide MS/MS spectra for critical evaluation.

Innovative aspects
In-depth sequence characterization of recombinant antibodies; combined LC-MS/MS strategy; integrative power of bioinformatics; ProteinExtractor algorithm; result compilation; efficient data mining

Key words
Recombinant proteins, antibodies, protein drugs, pharma, quality control, sequence characterization, LC-MS/MS, sequence coverage, bioinformatics

A148

Generation, Validation and Publication of Proteomics Results Following Publications Guidelines

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Introduction
Several initiatives have emerged during the last 2 years that try to establish standards to the Proteomics research community. Results are e.g. the HUPO PSI data formats (such as mzML) or minimal information requirements (such as MIAPE) and publication guidelines of Proteomics journals such as MCP, Proteomics and others. In addition, initiatives provided first platforms on the web to accept data along with publication of proteomic data, such as PRIDE.
Here, we present a software workflow in a study that provides for all requirements listed in those initiatives.

Methods
We analyzed the effect of TGF beta on a human lung carcinoma cells in a SILE quantitation experiment. The lysates of treated and untreated cells were labeled using ICPL and separated by means of SDS PAGE. Bands were excised and proteins were digested. The extracted peptides were submitted to RP LC-MS/MS runs. Following separate protein database searches the identification results of all gel bands have been integrated into an overall protein list by the ProteinExtractor algorithm. The results have been stored in a project-oriented database (ProteinScape) and in this way were accessible for automatic and manual result validation.

Results
Following a Decoy validation and quantitative analysis, our bioinformatics platform collected the relevant results and exported them into dedicated report files. These contained the relevant information in the required formats to submit it to journals along with the publication draft.
In an automatic result validation, (1) two search engines (Mascot, Phenyx) that produce partly overlapping peptide lists, and (2) the automatic acceptance of proteins in the list below a given false positive rate was used. This is done based on the peaklists that are stored in the database and the peptides identified by the search engines. For manual result validation, the ProteinScape client program contains an LC-MS survey viewer that shows the parent masses together with the raw data. Furthermore, uncertain matches can be manually assessed on the respective MS/MS profile spectra. Thus, ProteinScape helps to discover those proteins that need to be addressed in the first place, and it provides the tools necessary for a manual revision.

Innovative aspects
- Integrative database platform for various kinds of MS data
- combination of automated and interactive validation tools
- automatic report generation following author's guidelines of the major journals

A149

Quantitation of Phosphopeptides Using Chip-Based TiO_2 Enrichment and MRM Mass Spectrometry

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Introduction
Quantitation of protein phosphorylation is of great interest as reversible protein phosphorylation plays an important role in cell signaling pathways. For the extracellular signal-regulated kinase ERK1, two key regulatory phosphorylation sites have been identified as the neighboring T202 and Y204.[1] The degree of phosphorylation of these two sites is very important to the overall function of Erk1 in the signal transduction pathway. In this study, an MRM assay was developed using a re-usable microfluidic chip designed for the selective enrichment of phosphopeptides.

Method
ERK1 protein (Millipore) was digested with trypsin resulting in a tryptic peptide that contains these two phosphorylation sites, Four peptides labeled at the valine residue with ^{13}C were synthesized (Figure 1). The 4 peptides are 1) without phosphorylation, 2) with single phosphorylation at T202, 3) with single phosphorylation at Y204, and 4) with double phosphorylation at both T202 and Y204. The Agilent HPLC-Chip was coupled to a 6430 triple quadrupole mass spectrometer and both the phosphorylated peptide standards and the Erk1 tryptic digest were injected on the Phosphochip, a re-usable microfluidic chip with an enrichment column consisting of titanium dioxide particles sandwiched between reverse-phase materials for on-line selective phosphopeptide enrichment.

Results
In a preliminary study, the Erk1 phosphopeptides were analyzed using the HPLC-Chip in reverse phase mode only. The selection of the MRM transitions was critical because of the similarity of the sequences. For two of the peptides, the most sensitive MRM transition was identical and thus chromatographic separation of the target peptides was required for accurate quantitation. The quality of the labeled phosphopeptide standards was assessed to confirm purity (97.6 – 100%). Calibration curves were created from the analysis of human plasma digest spiked with the ^{13}C labeled peptides. The four calibration curves all had excellent linearity, with correlation coefficients better than 0.995. The LOQ was determined to be 500 amol and limit of detection was determined to be 50 amol. The percentage for each phosphorylation form in the activated Erk1 sample was determined to 59% of T202 and 55% of Y204 (Table 1).

These results will be compared to those obtained from the Phosphochip with enrichment of the phosphopeptides.

Innovative aspects
- On-line enrichment of phosphopeptides using a microfluidic chip
- MRM-based assay of phosphopeptides using a microfluidic chip based interface

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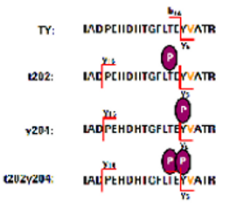

Figure 1. Sequence and product Ions for the monitored peptides.

Peptide	% Molar ratio	RSD (n=9)
TY	20%	0.13
t202	25%	0.15
y204	21%	0.12
t202y204	34%	0.08

Table 1. Quantitation of phosphorylation forms present in the activated Erk1 sample.

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Immunoproteomic assay of cell wall proteins of *Sporothrix schenckii*

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Introduction. *Sporothrix schenckii* is the etiological agent of sporotrichosis, a subcutaneous mycosis and an emerging disease in immunocompromised patients. Dimorphism of *S. schenckii* is controlled by temperature and pH. Accordingly, at 37 °C and at basic pH it assumes a yeast morphology, which is the parasitic form isolated from infected tissues, whereas at 28 °C and an acid pH it develops into the mycelial, saprobic phase.

The cell walls surface proteins of *S. Schenckii* are major elicitors of host immune responses during sporotricosis. The use of immunoproteomics (dimensional electrophoresis followed by immunoblotting) allowed us to investigate the reactivities of antibodies present in sera from mice to antigens of *S. schenckii* yeast. The identification of all these antigens would be useful for the development of diagnostic strategies.

Methods. To obtain the yeast phase, the pH of the medium was adjusted to 7.2 and flasks were incubated on a rotary shaker at 120 rpm at 37 °C. After six day, cells were harvested by low speed centrifugation and washed twice with buffer 50 mM Tris/HCl, pH 7.5, resuspended in the same buffer and disrupted with glass beads (0.55 mm in diameter) by alternate periods of breakage (30s) in a MSK cell homogenizer (Braun Melsungen, Germany). The cell homogenate was centrifuged at 1300 g for 15 min at 4 °C to separate the cell wall fraction which was exhaustively washed with buffer until a clear supernatant was obtained.

The cell wall fraction obtained was used for extraction of proteins as described for Elorza *et al.*, (1985). Briefly, cell walls were heated in boiling water for 10 min with 2% SDS, the extract was centrifuged to sediment the wall residue. The supernatant was recovered and proteins were precipitated with 70% ethanol. The proteins were analized using dimensional electrophoresis (2D-PAGE) and also by Western blot in immobilized pH gradient strips (pH 3-10; 7 cm).

Results. The use of a 2D-PAGE joined with Western blotting with sera from mouse allowed the detection of more than 7 immunoreactive proteins. Some of these proteins had different isoforms.The most reactive protein was a protein of 70 kDa in the acidic part of the gel (pI 4.1). The analysis of the antibody studied and the comparison between the antibody response in infected and noninfected mouse showed that the infection by *S. schenckii* produces changes in the antibody response which may be of relevance in the serodiagnosis of sporotrichosis. The technique used resolved the isoforms of several antigens.

Innovative aspects
- This work reports the usefulness of two-dimensional gel electrophoresis followed by Western blotting in the identification of the major *S. schenckii* antigens.

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A151

Application of the cancer/testis antigens chip onto screening biomarkers of lung cancer

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Introduction
Detection of autoantibody against tumour-associated antigens (TAAs) is one of effective approaches to explore the protein biomarkers related with diseases. Cancer/tesits (CT) antigens are widely accepted as a member of TAAs. For instance, some CT antigens were frequently detected in the patient sera suffered from non-small cell lung cancer (NSCLC) (1), suggesting that the autoantibodies against the CT antigens are potentially useful for lung cancer diagnosis as well as prognosis. There are, however, still a number of controversy reports regarding the CT autoantibodies in sera due to lack of systematic investigation to these antigens. To globally survey the CT autoantibodies, herein we established a protein chip with low density and high specificity against the CT antigens, and employed it onto screening the biomarkers related with lung cancer.
Methods
Using the software of DNAMAN (version 5.2.2), all the protein sequences of CT family were filtered to define the minimal proteins which are able to represent all the CT antigens. If a CT antigen with full length over 1.5Kb, it was designed as the truncated recombinant base on antigenic analysis of Kolaskar's method. All the selected and designed CT antigens were expressed in *Saccharomyces cerevisiae* or *E.coli* followed by purification with affinity chromatography such as GST and Ni-NTA agarose. For protein ship generation, the CT recombinant proteins and 5 Non-CT proteins were spotted on poxy slides with duplication. The serum samples, including 40 normal and 80 NSCLC, were collected from the Beijing Oncological Hospital. The samples were randomly divided to two groups, a training set with 80 sera and a testing set with 40 sera, which had the same proportion of healthy and NSCLC serum. The informative antigens are selected based on the rank of the SNR score to build classifier following by tested its accuracy in the testing set. A non-parameter method of k-nearest neighbour (k-NN) was employed to classify the samples and run 50 times to estimate the model accuracy.
Results
From all the CT proteins, a total of 69 CT proteins are unique to represent the CT antigen. With RT-PCR screening the cDNA library, 73 CT genes were amplified and further adopted for construction of expression vectors. For the CT protein chips, a total of 81 recombinant proteins were spotted onto proy slides (0.25ng--1ng/antigen), including 73 CT, 5 non-CT and 4 controls (Human IgG, Mouse IgG, GST, NUS). To test the quality of the CT protein chip, anti-GST and anti-NUS polyclone antibodies coupling with Cy5-labeled goat anti-rabbit IgG as the secondary antibody were loaded onto to chips. With exception of three proteins without GST or NUS tag, all of the antigens on the chips released positive fluoresoent signals.
In the quality control experiments, the coefficient variations (CVs) of human IgG were 17.6% within slides and 21.6% among the different slides, indicating that the serum quality collected from patients and controls were comparable for further analysis. In the screening experiment with the clinical samples, 30% (23/73) CT antigens, such NY-ESO-1, MAGEA1, SPANXC and so on, were detectable in many NSCLC sera (Figure 1); whereas number of control samples contained the silent signals. Top 12 antigens with high SNR scores were selected to build a classifier. With k-NN method (k=4), 80 samples were used for computer training to establish a prediction model, and the rest of 40 samples were taken to evaluate the prediction accuracy. After 50 runs, the values of accuracy, sensitivity and specificity upon the model were 0.634±0.06, 0.889±0.10 and 0.462±0.15, respectively.
Innovative aspects
- To generate a CT protein chip.
- To screen the autoantibodies against CT in lung cancer serum.
- To identify some CT antigen as the potential biomarkers for clinical application.

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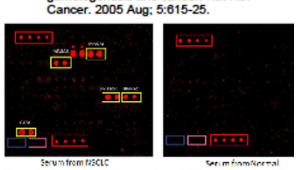

Figure1. Representative results of CT antigen protein chip. Chip was probed with 1:400 dilutions of patient or control sera followed by Cy5-labeled goat anti-human detection antibody prior to scanning. Yellow box indicates reactive antigens. Blue box and pink box indicates GST Nus tag respectively, while red box indicates marker features (the former of four spots are human IgG and later are mouse IgG respectively) used to orient the arrays and normalization.

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MALDI imaging: Interpretation of gastric cancer MALDI images by hierarchical clustering

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Introduction
MALDI imaging draws more attention in the clinical research community, mass spectrometrists are increasingly requested to perform these analyses. The resulting datasets can only be interpreted in the histological context by a pathologist. Usually, the mass spectrometrist is asked to provide the results in an accessible format that allows a quick and intuitive analysis by the pathologist. A quick way for analyzing MALDI imaging data based on similarity of spectra rather than on individual mass signals is highly desirable. Here, we present hierarchical clustering as a tool to interpret MALDI imaging results and compare them to the histology in an interactive way.

Method
Tissue sections (human gastric cancer and mouse organs) were cut on a cryostat and thaw mounted on conductive, ITO-coated glass slides. The MALDI matrix was applied with an imagePrep station. The mass spectra were subjected to hierarchical clustering. Images were reconstructed by selecting nodes in the dendrogram and assigning a color to the respective mass spectra (pixels).

Results
Hierarchical clustering is a technique that sorts the mass spectra in a dendrogram according to overall similarity. It is useful to select individual tree nodes in the full dendrogram and to highlight the respective spectra on the image. This allowed a semi-supervised interactive annotation of the datasets. The results were generally in good agreement with the histology and allowed in some cases even the assignment of histological structures invisible in individual ion images. Compared to the analysis of MALDI imaging results in a tedious and time-consuming peak-by-peak fashion, the hierarchical clustering allowed a complete annotation of the images in a few minutes.

Innovative Aspect
Hierarchical clustering as an effective, very fast post-processing method for MALDI Imaging

A153

The Prognsotic of IL-2 Level for Patients with TNM stage I Hepatocellular Carcinoma after Hepatectomy

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Introduction
To investigate the prognostic value of Th1 and Th2 cytokines in hepatocellular carcinoma (HCC) patients after resection.

Patients and Methods
We analyzed 12 cytokine protein expression levels in noncancerous hepatic tissues from 133 stage I HCC patients using enzyme-linked immunosorbent assay (ELISA). Prognostic value of these cytokines and other clinicopathologic factors was evaluated by Cox regression and Kaplan-Meier analysis. All statistical tests were two-sided.

Results
Th1 cytokines IL-2 and IL-15 of nonrecurrent group were statistically significantly higher than that of recurrent group. However, there were no statistically significant differences between the two groups with respect to Th2 cytokines. Survival analysis by the Kaplan–Meier method showed that the low IL-2 group had a significantly shorter period for recurrence when compared to the high IL-2 group (p=3.48E-8). A univariate analysis revealed that IFN-γ, IL-2, TNFα, IL-15, IL-4 and IL-5 were significantly associated with recurrence. IL-2 was far superior (P=7.16E-7) to other variables (P=0.006 to 0.049). The multivariate Cox regression model for recurrence showed IL-2 was independent predictor for recurrence (P=1.84E-4) and survival (P=0.024), whereas none of IL-15, IL-5, IFN-γ, TNFα and IL-4 were independent predictors of recurrence or survival. However, interestingly, the results of linear regression analysis revealed that all of them were significantly positively correlated with IL-2.
IL-2 was independent predictor for recurrence and survival of stage I HCC patients. Th1-dominant immune status of the tumor-surrounding tissue microenvironment is associated with reduced recurrence and improved survival in HCC after resection. IL-2 might eventually guide personalized therapies.

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Computational Analysis of Human Blood Proteins and Peptides by SQL Database

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Introduction
It has been previously suggested that there is little agreement on the proteins of human blood as determined by comparison of accession numbers. In contrast to accession numbers that may change over time, the blood peptide and protein sequences themselves are immutable and portable identifiers that may be compared between libraries and datasets. The results of twelve studies of human blood proteins and/or peptides identified by LC-MS and correlated against a disparate array of genetic libraries were parsed and matched to proteins from the human RefSeq and ENSEMBL databases. The reported protein sequences were organized into an relational database with RefSeq numbers, full protein sequences, up to five unique peptides in order of prevalence along with the peptide count for each protein.

Methods
RefSeq is a non-redundant database of human transcripts together with their gene ID numbers, and Gene Ontology (GO) annotations that were parsed into a SQL database. The International Protein Index (IPI), conceived as an integrated database for proteomics experiments, was built as a complete and non-redundant (NR) compilation of the Swiss-Prot, TrEMBL, ENSEMBL and RefSeq databases. Structured Query Language (SQL) and Basic Local Alignment of Sequence Tool (BLAST) were used to analyze the data at the level of protein and peptide sequences. SQL or BLAST was used to acquire descriptive information from similar or identical sequences in current databases where required. Twelve sets of publicly available proteins and/or peptides by LC-MS/MS of blood that were fractionated and analyzed by different methods were assembled together in SQL to be summarized and compared (1). One way to avoid ambiguity is to map the sequences identified to the full-length proteins of a relatively non-redundant set of transcripts using SQL and BLAST (2).

Results
The results here and elsewhere indicate that LC-MS/MS correlation of single blood peptides is strongly non-random. In total 75,432 peptides were correlated by the various groups, enough to randomly assign peptides to all the proteins in RefSeq more than twice. Yet instead the results showed two or more peptides were assigned to a set of only about nine thousand proteins with a set of more than four thousand proteins with 5 peptides. This represent far more proteins than can be confirmed by present independent mass spectrometric or immunological methods.

The overall conclusion is that LC-MS of blood is tractable but that a multiple sample fractionation schemes and correlation algorithms are required to make a comprehensive study (1-3) The simplest interpretation that accounts for all data and requires no arbitrary assumptions may be that the groups differed not only in separation strategies, experimental setup and correlation algorithms/parameters but also enzymatic degradation and sampling of peptides. The protein and peptide database would be useful in designing immunological or MRM experiments to test the proteins predicted to be in blood to date by LC-ESI-MS/MS.

Innovative aspects
-LC-ESI-MS/MS and correlation of human blood is not a random process.

-SQL and BLAST show independent agreement on some nine thousand proteins from human blood.

-LC-ESI-MS/MS of human blood has robust sensitivity to proteins as low as 1 ng/ml.

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A155

In-depth characterization of the N-glycosylation of recombinant monoclonal antibodies by means of MALDI and ESI mass spectrometric analysis

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Introduction
Antibodies represent one of the most important classes of protein drugs. According to pharma QC requirements, recombinant glycoprotein products need to be characterized in detail regarding both, the aminoacid sequence and the N-glycosylation pattern.
We describe here the in-depth characterization of the N-glycosylation present in a recombinant monoclonal antibody involving two complementary MS based analysis techniques:

- accurate mass measurement of the intact antibody and the glycosylated heavy chain, respectively, performed on an ESI-ultrahigh-resolution (UHR)-TOF instrument
- LC-MS/MS analysis of N-glycopeptides obtained from the enzymatically digested antibody heavy chain employing offline LC-MALDI-TOF/TOF coupling

Methods
A monoclonal IgG was analyzed here. Part of the antibody (AB) sample was reduced and alkylated to release the glycosylated heavy chain. The reduced/alkylated sample was separated by SDS-PAGE, and the gel separated AB heavy chain was then subjected to digestion using trypsin as an enzyme.
LC-MS analyses of the intact antibody and the released heavy chain were performed on an ESI-UHR-TOF instrument applying a short LC gradient for sample cleanup. The tryptic in-gel digest of the AB heavy chain was analyzed by LC-MS/MS employing offline LC-MALDI-TOF/TOF coupling. From the resulting MALDI data, N- glycopeptides were traced using dedicated software tools. N-glycopeptides representing various glycosylation isoforms were structurally confirmed by interpretation of the according MALDI-MS/MS spectra.

Results
Employing an ESI-UHR-TOF instrument, the mass accuracy achieved in the measurement of the intact antibody and the released AB heavy chain, respectively, was below 2ppm. This high resolution and accurate mass data enables instant, high confidence assignment of the most abundant glycosylated isoforms of the expressed AB, including the characterization of major processing artefacts resulting from undesired overalkylation occuring in the course of the release of the AB chains.
An even more detailed profile of the glycosylation heterogeneity is obtained from LC-MALDI-TOF/TOF analysis of N-glycopeptides obtained from a tryptic in-gel digest of the AB heavy chain. The contained glycan moiety reduces the specific retention of N-glycopeptides on a reversed phase column in a way, which leads to the formation of a distinct N-glycopeptide region on the resulting LC-MALDI-MS map, appearing well separated from most of the remaining non-glycosylated peptide load. This helps to assign various series' of N-glycopeptides carrying different peptide moieties (due to partial missed trypsin cleavage) and reflecting in detail the heterogeneity of the attached glycan structures (fucosylated, non-fucosylated, complex and high-mannose type, respectively). The identity of the various proposed glycopeptide series' was confirmed by corresponding MALDI-MS/MS data. MS/MS spectra of candidate N-glycopeptides were traced automatically out of the entire LC-MALDI-MS/MS dataset applying a software algorithm which selectively targets the unique fragmentation pattern occuring in MALDI-MS/MS spectra of N-glycopeptides.

Innovative Aspects
In-depth characterization of N-glycosylation in antibodies; accurate mass ESI measurement of intact protein; LC-MALDI-TOF/TOF analysis of N-glycopeptides from antibody digest

Key words
monoclonal antibodies; protein drugs; pharma; N-glycosylation; characterization; quality control; accurate mass; intact antibody; ESI-UHR-TOF; N-glycopeptides; LC-MALDI-TOF/TOF; glycosylation profile

A156

Mining the human placenta proteome ≥ 5000 proteins deep using CID/ETD on a novel ion trap mass spectrometer

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Introduction
In order for proteomics to realise its potential as a genomic science, it is important to be able to measure a proteome as completely as possible. Novel technology developments are providing important steps towards this goal and mass spectrometry based peptide sequencing in particular is showing great promise in this regard. In order to expand our knowledge about proteins expressed in human placenta – a primary human tissue of embryonic origin - we applied a combination of protein and peptide separation techniques in conjunction with CID and ETD on a novel ion trap instrument to identify several thousand human proteins. Our data represents the most complete protein atlas available for primary human tissues to date.

MethodPost-delivery human placenta was obtained with written consent from a healthy female. Total protein extraction was performed using 6 M guanidine HCl, 1% v/v Triton X-100. Protein extracts were separated by 1D SDS gel electrophoresis and each lane cut into 18 regions. All samples were digested with trypsin. Peptides were separated by nanoscale reversed phase liquid chromatography (Bruker Easy-nLC) coupled online to an ion trap mass spectrometer (Bruker). Each protein digest was measured eight times (four gas phase fractions, each by CID and ETD). Tandem mass spectra were searched against the International Protein Index (IPI) database using Mascot and protein identifications were accepted at a false discovery rate of <5%.

Results
In order to identify as many proteins as possible from human placenta, we chose a multi-tier analytical approach. First, proteins were extracted under fully denaturing conditions which ensures that e.g. integral membrane proteins are readily solubilised. Second, we separated intact proteins by 1D-SDS PAGE in order to provide a medium resolution protein separation dimension. Third, peptides were separated by high-resolution reversed phase liquid chromatography. Fourth, the mass spectrometer was operated in the gas phase fractionation mode in order to increase the depth at which ions can be picked for tandem MS. Fifth, complementary CID and ETD spectra were acquired for all samples in order to maximise information content and data quality of peptides with charge states ranging from 2+ to 5+. Confining the full scan m/z range enabled the acquisition of high resolution peptide mass spectra ensuring that the charge states of peptide precursors selected for fragmentation are unambiguously known. The improved scan speed of the instrument and a novel ion transfer line provided for a high duty cycle and sensitivity thus maximising the number of acquired high quality tandem mass spectra per unit time. Each of the elements of this multi-tier approach contributes to improved proteome coverage and collectively enabled the identification of several thousand proteins from human placenta, an improvement of a factor of at least two over traditional MudPit approaches. Gene ontology classification of the identified proteins indicates that all major cellular compartments and functional categories are represented and numerous proteins are identified that are specific for this embryonic tissue.

A157

Systemically interpret methylation codes by CHiMPA and permutation array

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Introduction
Histone methylation, in company with histone acetylation and DNA methylation, plays an important role in the regulations of gene transcription and cell activity[1]. As known as histone codes, more than eight modifications appeared in histones. Compare with other modifications, methylation on lysine or arginine is more complicated. In each candidate site, there are three different modification states which indicate different biological output. Here we provide a Comprehensive Histone Methylation Peptide Array (CHiMPA) which detected most known interactions and identified many novel interactions either in known or unknown methylation binding proteins.

Methods
To generate the methylation binding map of reading proteins, modifications focused on methylation, acetylation and phosphorylation sites in human histones were collected from literatures. As an example of non-histone proteins, the tumor suppresser p53 was chose to collect its C-terminal modification sites. The lysine methylation sites were used to generate peptides library with non-, mono-, di- and tri- methylation states. The sequences of peptides were generated with six residues flanked in both directions of the modification sites. Labeled with biotin, all peptides were predefined by correctness and concentration, and then were incubated with neutravidin separately before being printed onto the aldehyde slides. The arrays were subjected to far western probe by GST fusion proteins to generate the binding maps with GST as control (Figure 1).

Results
As one of the most researched cases, Hp1beta-chromo binding with H3K9 methylations, accompany other interactions such as 53BP1-Dtudor recognizing H4K20me2 or p53K382me2, JMJD2A-Dtudor interacts with H4K20me3 or H3K4 methylation were detected in our data. Moreover, some novel binding sites of known methylation binding proteins were showed. HP1beta-chromo might also binding with H3K23 methylation, 53BP1-Dtudor has the ability to interact with H3K18me2 or p53K382me2, JMJD2A-Dtudor was found binding to many sites like H1K25me3, H3K18 methylations H3K23 methylations and p53K382 methylations. Mpp8-chromo has strong binding with H3K9 and H3K23 di- and tri-methylation peptides, which has similar binding Kd and trend for different modification states with HP1beta-chromo. Many of the interactions were verified by fluorescence polarization binding study. Although the dissociation constants (Kd) value has no linear correspond with binding z-score detected in the chip, most high z-score interactions were found positive in FP experiment. Based on the permutation arrays, HP1beta-chromo binding specificity was defined and prediction was successfully matched with CHiMPA data, which indicate this strategy can be used to find novel non-histone methylation binding targets (Figure2).

Innovative aspects
- A strategy to systemically interpret histone methylation codes and define the reading specificity of readers was established.
- Many novel interactions were found from both known and unrevealed methylation binding proteins.

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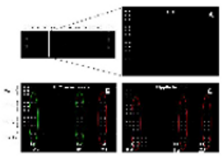

Figure 1. Chromo domains binding on CHIMPA slides. Green circled means the known interaction and new interaction circled with red. Binding profiles for the GST (A), the HP1βchromo domain (B) and the Mpp8 chromo domain (C) are shown.

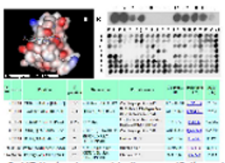

Figure 2. HP1beta-chromo binding specificity probed by H3K9me3 permutation. A: The complex structure of H3K9me3 and Drosophila HP1 chromo domain (1KNE). B: Truncated H3K9me3 peptides and permutation array were probed by HP1 β chromodomain. C: Z-score matrix derived from permutation array was used to predict the potential binding nuclear proteins. H1K25 and H3K23 peptides were verified from the prediction.

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Quantitative Proteome Analysis of Colorectal Cancer by Acetylation Stable Isotope Labeling Method

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Introduction
Colorectal cancer (CRC) is one of the most commonly diagnosed cancer and is the third leading cause of cancer-related death worldwide. Early metastasis and postoperative recurrence are the leading cause of poor prognosis and also an important lethal factor. It is important to search candidate biomarkers for early diagnosis and prognosis evaluation of CRC so as to guide the treatment and improve the outcome of patients. The development of proteomics technology provides a powerful tool for CRC biomarker study.

Methods
In this study, laser capture microdissection (LCM) were used to obtain interested cells from 20 CRC and paired normal mucosa tissues. The differential proteins between the microdissected tumor cells and normal mucosa epithelia were analyzed by acetylation stable isotope labeling coupled with fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). Western blot and immunohistochemistry (IHC) were used to assess the expression of differential proteins.

Results
In total, 873 reliable proteins were quantified and 137 proteins were differentially expressed by at least 2-fold, including 67 proteins up-regulated and 70 down-regulated in cancer. Three differential proteins transgelin-2, SLC12A2 and Rab-10 were validated by Western blot and the results were consistent with acetylation stable isotope labeling analysis, which shows the reliability of strategy applied in our study. According to GO analysis, CRC related differential proteins covered a wide range of subcellular locations and involved in many biological processes. It means that our methods have better protein coverage and may become an effective strategy for oncoproteomics study. Among the differential proteins, the significant up-regulation of transgelin-2 in CRC was further analyzed in 120 paired CRC and normal mucosa by immunohistochemistry (IHC). The positive expression rate of transgelin-2 in CRC tissues was significantly higher than normal mucosa (85% vs. 16.67%) (P<0.05) which indicates transgelin-2 may be a novel marker predicting tumorigenesis of CRC.

Innovative aspects
- Application of LCM combined with a quantitative proteomic strategy based on acetylation stable isotope labeling for the first time in searching novel markers of CRC.
- Identifying transgelin-2 as a novel potential marker for predicting tumorigenesis of CRC.

A159

High-throughput Screening, Isolation and Analysis for Glycan-binding proteins

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Introduction
Specific protein-glycan interactions underlie many aspects of important biological processes, for example, cell differentiation, cell adhesion, immune response, trafficking and tumor cell metastasis, which occurs through glycans of glycoprotein and glycolipid, as well as polysaccharide displayed on cell surfaces and glycan-binding proteins (GBPs). Here a strategy has been utilized for the screening and purification of GBPs based on carbohydrate microarrays and magnetic particle technology by the underivatized sugars that efficiently immobilized on hydroxyl-functionalized surface of glass and magnetic particles by formation of glycosidic bond with the hemiacetal group at the reducing end of the suitable carbohydrates via condensation (1,2). The methodology could work together with the glycan microarrays for screening and purification of the important GBPs from complex protein samples.

Methods
In the first step, the glycan microarrays with mono-, di- and oligosaccharides based on above method were fabricated and used to screen proteins from healthy human sera. In the second step, the mannose was selected according to the signal intensity on the glycan microarrays. The mannose was coupled to hydroxyl-functionalized surface of the magnetic particles by formation of glycosidic bond. The magnetic particle-mannose conjugates were used to purify the mannose-binding proteins (MBPs) from healthy human sera. The fractionated MBP population was displayed by SDS-PAGE and further analyzed using 2-D gel electrophoresis.

Results
The fabricated glycan microarrays were used to screen proteins from healthy human sera diluted at 1∶100. The result was shown in Fig.1. The mono-saccharides (mannose, glucose and Galactose), disaccharides (lactose and Cellobiose), oligosaccharides (Lewis trisaccharide, NGA3 Glycan, NGA4 Glycan, NA4 Glycan, Man-9 Glycan and NA3 Glycan) and glycogen showed the stronger fluorescent signal on the glycan microarrays. The magnetic particle-mannose conjugates were used to purify the MBPs from healthy human sera. The fractionated glycan-binding protein population was further analyzed using 2-D gel electrophoresis (Fig.2). The preliminary result showed that a total of 83 protein spots were found by 2DImageMaster software.
In conclusion, our method allows rapid screening and isolating the GBPs from proteomic samples for subsequent downstream analyses, such as 2-D gel electrophoresis or mass spectroscopy in order to characterize protein topographies responsible for driving molecular mechanisms involved in sugar complexation.

Innovative aspects
- Novel simple strategy for the high-throughput screening and purification of GBPs
- An integrated tool for search GBPs related disease

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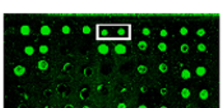

Figure 1. The fabricated glycan microarrays used to screen proteins from healthy human sera. The sera were diluted at 1∶100. The mannose was marked with the white frame.

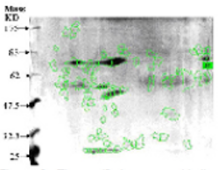

Figure 2. The purified mannose-binding proteins of healthy human sera were analyzed by 2-D gel electrophoresis (pI3-10).

Increased Sequence Coverage in Complex Protein Digests by Consecutive, Targeted LC-MSMS runs with both CID and ETD

A160

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Introduction
Highly complex protein digests cannot be analyzed in one single run as not all tryptic peptides will be triggered with an automated MSMS approach. Additionally, many post translational modifications are low abundant and therefore often not detected in a first run. However, for a second injection the same sample amount is needed. Using the RePlay module a second analysis is possible from the same first injection. When data from the first run are processed in between, the second analysis can be used for a targeted MSMS approach using different fragmentation techniques like CID and ETD. Thus, the 'replay run' of the same sample can improve protein and peptide coverage, can increase identification of post translational modifications, and improve analytical confidence.

Methods
A standard nanoLC is coupled to the RePlay module (Advion BioSciences). After column separation the flow from a 75 µm column at a typical flow rate of 250 nL/min is split, so that part is directed to the mass spectrometer for analysis whilst the remainder flows to a capillary tube where it is stored. After the direct LCMS run, the flow is switched and the portion stored in the capillary is analyzed ('replay run'). CID and ETD data are acquired on a novel ion trap mass spectrometer (Bruker Daltonik) with increased sensitivity

Results
A short column between the storage capillary and the mass spectrometer refocuses the stored peaks in the second analyses so that width and intensity is identical to the initial run. When the first run is finished, the data are processed due to their sample origin. Examples of combining exploratory and targeted analysis are shown by targeting PTM's, excluding peptides identified in previous run or using different fragmentation techniques:
- For posttranslational modifications like glycosylation or phosphorylation, data from the first run are screened for PTM specificic fragmentation patterns to identify modified peptides. These peptides are investigated in the second run by targeted ETD and CID experiments.
- For protein ID data are submitted to a Mascot data base search. Peptides identified with a significant ion score are excluded for the 'replay run' to get additional low abundant signals selected for MSMS. As a result protein ID rate and protein sequence coverage increase.

Innovative Aspect: Ultra sensitive protein characterization by "doubled" LC/MS/MS runs with exclusion lists in CID and ETD.

Resolution and Accuracy at Speed – a New Technology Base for Axial MALDI - TOF

A161

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Introduction
Major analytical workflows like LC-MALDI, MALDI-Imaging or bacterial identification require highest mass accuracy and resolution at short acquisition time as they involve the acquisition of a great number of spectra. MALDI-TOF has the inherent advantage that, unlike cyclotron technologies, short acquisition times do not compromise accuracy and resolution. However, expanding a TOF system to higher repetition frequencies is not as straight forward as it may appear. Therefore all commercially available instruments are limited to 200 Hz so far. We have developed a new MALDI-TOF/TOF instrument that achieves 1 kHz operation speed in TOF and TOF/TOF mode of operation and simultaneously doubles the resolution previously reported - with a mass accuracy in the sub–ppm range.

Methods
A prototype ultrafleXtreme MALDI-TOF/TOF was used for all measurements. It is equipped with a 1 kHz smartbeam II solid state laser, an ion source that can be cleaned by laser induced heating in approx. 10 min in order to reduce instrument downtime in all high throughput applications such as tissue imaging or LC-MALDI.
Proteins for Top-Down sequencing (ADH1, G3P) were obtained though the ABRF-ESRG research group study, all model proteins were purchased. A novel calibration software, that was developed based on the Simion model of the instrument provides now sub-ppm accuracy with internal standards.

Results
For proteomics applications such as LC-MALDI and PMFs, a mass accuracy of 1 ppm was achieved with internal calibration, at mass resolutions in the 20,000-40-000 range across the entire peptide mass range. This provided increased sequence coverage from PMFs of model proteins such as BSA and higher identification scores.
Also the ability to read out N- and C-terminal sequences from intact proteins by Top-Down sequencing was improved by the higher resolution. Isotopic resolution was maintained, e.g. for 35 kDa proteins such as ADH1 and G3P to Top-Down fragments until 9 kTh. Increased resolution and sensitivity enabled the automated acquisition of top-down sequencing spectra using in-source decay (ISD) with 1,5-diaminonaphthalin matrix: 2000 shot spectra were acquired in 2 sec.
The simultaneous improved performance characteristics of MALDI-TOF provides now new capabilities from Top-Down to Bottom-Up proteomics work and molecular imaging applications to the detailed characterization of small molecules even after TLC-MALDI analysis.

Innovative aspects
- First MALDI TOF/TOF system with simultaneous kHz operation in MS and MS/MS modes of operation
- >40 k resolving power on Axial MALDI at <1 ppm accuracy
- Top-Down protein sequencing spectra acquired automatically in ~2 sec.

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Minimum Bias Analysis for vaccine QC/QA and biomarker discovery

A162

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Introduction
The Label Free Intrinsic Imaging (LFII; 1) approach has been used in a wide variety of proteomic studies and has gained adherence particularly in the analysis of vaccines and in protein biomarkers. LFII is being applied in the analysis of small molecules, peptides (2), viral titre, analysis of nucleic acids, including sequencing and in protein conformational studies. LFII is a methodology, which introduces the minimum possible bias into proteomic analysis and has therefore an important role where quantifiable correlation or anomaly analysis is important.

Methods
LFII's growing influence in proteomics comes in part through the inherent minimum bias approach on which it is founded: by eschewing the use of chemiluminescent labels, a whole class of systematic errors associated with the interaction of the label with the protein is avoided. The core methodology builds on the use of differential optical depths associated with Beer-Lambert time-resolved absorption in a separating medium, in which quantitative analysis of the analyte takes place over (typically) hundreds of thousands of correlated images separated in space and time (Fig. 1). Space-time correlation tools developed in particle physics allow the identification and removal and biases produced by unwanted thermal, chemical or optoelectronic gradients.

Results
We demonstrate the way LFII minimum bias analysis is based on simple and highly robust principles, and show the use of LFII in highly quantitative and reproducible (Fig. 2) minimum-bias analyses of Sub-Unit Vaccines, Antibodies and the up-regulation of biomarkers. As a consequence, LFII's minimum bias approach results in inherent advantages in the areas of most significance to proteomics:
- the increasingly pressing need for standardisation in methodologies and measurements
- the growing significance of correlations between genomic and proteomic data
- the relative degree of and correlation between up- or down-regulated protein markers
- the need to make parallel analyses in miniaturisable systems
- the avoidance of hypothesis-driven analyses, which may bias a researcher towards an assumed conclusion.

Innovative aspects
LFII's innovation comes from the
- Simplicity of the approach used, with effectively just the Beer Lambert law at its heart; the resultant raw small signal to noise at an individual electropherogram level is over come by:
- The complementary data aggregation approaches developed which bring back sensitivities while avoiding extraneous biases
- The results which allow minimum-bias correlation and anomaly studies to be undertaken across the broad spectrum of proteomics.

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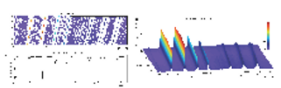

Figure 1. Schematic of equiphase (EVA) map and single pixel electropherogram and enhanced imaging due to the vertexing process, the detector also measures the intensity of the signal. This is shown by the color of the signal, changing from green through yellow to red at its most intense.

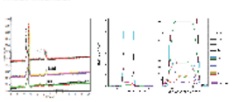

Figure 2. (Left) Overlay of GST processed data for 10, 2 and 1µg/ml α-lactalbumin. (Right) Overlay of EVA processed data for 200, 100, 50, 20, 10, 5, 2 and 1µg/ml α-lactalbumin.

Approach for isotopic distribution deconvolution in mass spectra of peptide compounds

A163

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Introduction
Modern algorithms of isotopic clusters deconvolution in mass spectra are based on principally empirical approaches. The most widely used in proteomic applications is Horn-transform. It is based on the idea of averagine, where one can evaluate the number of "averaged" amino acids and therefore the number of carbon atoms to build theoretical isotopic distributions in order to find the best fitting. This method works fine, unless it encounters overlapping distributions of 16O/18O labeled peptides. Here we present the algorithm, based on the numerical solution of system of nonlinear equations.

Methods
We solve the system of non-linear equations, which relates number of carbons, monoisotopic masses and abundances for compounds giving a contribution to the isotopic cluster. Taking into account that isotopic distribution of carbon containing molecule is binomial each peak intensity distribution in a spectrum could be represented by a system of non-linear equations. There are 3l unknowns in the system where l is the number of compounds comprising isotopic cluster, among unknowns are the abundances of these compounds, the numbers of carbons per compound n, and the ordinal number k of the first visible peak regarding monoisotopic peak.

Results
Developed method has been tested on different isotopic patterns, when, for instance, two compounds have the same monoisotopic mass. They have been resolved, and also the criteria of applicability were obtained. There are 3l arguments, but one can effectively decrease it to 2l. In the case of compounds that have less, than 500 carbons, one of the arguments (k) will be equal to zero; therefore in the small mass region there are 2l effective arguments. With heavier masses, when the first visible peak contains one or more 13C-isotopes, the cluster becomes much richer, and it's already enough to solve the system with 3l arguments.
The first modeled was the situation, when two compounds fall in one distribution at the m/z about 3000, therefore there are over 130 carbons. It makes possible to observe about 6 peaks, and one needs only 4 arguments (2l effective arguments) to solve the system. Two other possibilities, when one has fewer or more carbons (45 and 600), have also been modeled: in the first case there is the coincidence of the number of observed peaks in the spectrum with the needed number of equations in the system. In the second case there are 14 peaks, 10 more than arguments.

Innovative aspects
- The new method to deconvolute isotopic clusters
- Possibility to resolve overlapping clusters from 18O/16O peptide mix.

A164

Secretome-Based Identification of Potential Head and Neck / Oral Squamous Cell Carcinoma Markers

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Introduction: The development of cancer involves tumor cells and the dynamic host microenvironment (1). The cancer itself or its microenvironment could be sources for potential cancer markers that are shed into the blood or other bodily fluids. To date, molecular markers are not available for the diagnosis / prognosis of head and neck / oral squamous cell carcinoma (HNOSCC). Availability of blood-based markers would significantly enhance the ability to detect HNOSCCs, thereby improving its management in early stages.

Methods: To determine which of the potential markers may be secreted, we identified proteins secreted by head and neck cancer cell lines (SCC4, SCC38, HSC-2, and AMOSIII). Cells were cultured in conditioned serum-free media (CM) for 24-48 hours and CM was collected. Proteins were identified using using shotgun proteomics via nanoLC-tandem mass spectrometry (LC-MS/MS). SignalP and SecretomeP software was used to determine the probability of secretion for these proteins. Further, verification of the potential markers was performed in the sera obtained from patients suffering from HNOSCCs and a healthy control using Western blotting. Ingenuity pathway analysis (IPA) revealed the protein networks and canonical pathways involved.

Results: 323 non-redundant proteins were identified in the CM of HNOSCC cells. Of these, 164 proteins were found to be secreted. Proteins were considered secreted if they satisfied at least one of the following four criteria: (1) they were extracellular/membrane-bound; they were (2) classically (SignalP) or (3) non-classically (SecretomeP or exosomes) secreted from HNOSCC cells; (4) they were reported to be detected in blood plasma. Importantly, 6 of the secreted proteins have not previously been demonstrated to be associated with any forms of cancer and could, therefore, potentially be of significant interest. The other 148 proteins have previously been linked to cancer, 35 of which have been reported to be overexpressed in HNOSCC tissues. These 35 proteins, therefore, warrant further investigation as potential cancer markers for HNOSCCs. Western blot analysis showed overexpression of PCM70, PCM93, and PCM94 proteins in serum samples of HNOSCCs, underscoring the importance of these potential cancer markers. IPA revealed involvement of most of the secreted proteins in cellular processes such as proliferation, apoptosis, hypoxia signaling, cell migration, and glycolysis/gluconeogenesis, which are known to be associated with cancer development.

Innovative aspects:

- Secretome analysis holds the promise of developing biomarker based non-invasive methods for better diagnosis / prognosis of HNOSCCs.

References:

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A165

Mass Spectrometric and Peptide Chip Epitope Analysis of Autoantigens and Application to Diagnostics

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Introduction
Rheumatoid arthritis (RA) is one of the most significant chronic diseases affecting 1-2% of the human population. Autoantibodies against the RA33 autoantigen are early markers for RA in patients, however, specificity is about 35% only [1]. The aim of the model study is to characterize the epitope of a monoclonal anti-RA33 antibody using mass spectrometric epitope mapping. Afterwards, the here developed approach has been applied for studying epitope profiles from individual patient sera. Once identified, the epitope regions alone shall be used for patient screening using peptide chips in order to eliminate false positive results and in order to enable patient stratification according to individual epitope pattern.

Methods
RA33 was purified by HPLC, the intact protein was characterized mass spectrometrically and partial sequence was determined by ISD-MS. SDS-PAGE analysis was performed to show homogeneity of the protein. In-gel digestion was carried out with multiple enzymes (Asp-N, trypsin). Subsequently, peptide mass fingerprint analysis (PMF) was performed. RA33 was then subjected to BrCN degradation. The fragments were analyzed by SDS-PAGE and epitope carrying regions were identified by Western blots. Protein fragments in bands from SDS-PAGE were excised, digested in-gel with trypsin and analyzed by MALDI-ToF and MALDI-QIT-ToF mass spectrometry. BrCN degradation products were separated by HPLC. Major fractions were subjected to ESI Q-ToF MS and MALDI-ToF MS, and to dot blot analysis for detection of antigenic fragments. Peptide chips contained 15-mers of RA33 peptides with a frameshipft of 6. Incubation with patient sera was performed in 1:100 dilution.

Results
Recombinant RA33 eluted as a single fraction from HPLC, showing high homogeneity of the affinity-purified product. Mass spectrometric analysis of the intact protein yielded in a molecular mass of 34166 Da (M_r (avg.): 34132). ISD-MS analysis enabled determination of the C-terminal partial sequence comprising aa233-aa303. SDS-PAGE analysis revealed the presence of a single strong band, in agreement with HPLC. In-gel digestion with trypsin and Asp-N, respectively, resulted in high sequence coverage (98 %) by peptide mass fingerprinting (PMF). For confirmation of PMF assignments, selected peptide ion signals were subjected to MS/MS and MS^3 sequencing.

HPLC purified recombinant RA33 was subjected to BrCN degradation. The fragment mixture was analyzed by MALDI-MS in linear mode and proved complete degradation. Subsequently, BrCN fragments were separated by SDS PAGE. All 13 bands were subjected to in-gel trypsin digestion in order to assign partial sequences to the BrCN fragments in each band. Then the protein bands were analyzed by Western blot using a monoclonal anti-RA33 antibody. Bands 1-9 stained positive and, thus contained the epitope region. From the here obtained results, it can be concluded that the epitope is located in region aa79–aa163. Additionally, BrCN degraded RA33 fragments were separated by HPLC. Ten major fractions were collected and screened for immunogenicity using dot blot reactions with the monoclonal anti-RA33 antibody. Fractions 8 and 9 stained positive. Analysis of HPLC-separated fractions by MALDI-ToF-MS and ESI-Q-ToF MS enabled determination of partial RA33 sequences in each fraction. As a result we demonstrated that the epitope lies directly C-terminal to Met79. These results match precisely with peptide chip analyses, where the epitope region had been determined as aa79-aa84.
RA patients whose sera samples reacted positive for RA33 showed a distinctively different response pattern than that obtained with the monoclonal antibody on peptide chips. The same procedure as described above showed that the main epitope regions in patients are (i) N-terminal - consistent with the literature - and (ii) C-terminal. The C-terminal epitope region (aa240-aa255) has not been described before and enables for the first time to sub-classify patients according to their epitope pattern. Robust stratification of patients is the key for targeted therapy – a goal to be reached in "Individualized Medicine" approaches. We are now in the state of elucidating, whether groups of patients with similar epitope peptide patterns shall receive similar treatment.

Innovative aspects
Mass spectrometry combined with peptide chip analysis enables epitope identification in autoantigens with the potential for patient stratification.

References
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A166

Rapid Protein Validation Assay Development Using Intelligent Software and Acquisition Strategies

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Introduction: Targeted peptide quantitation is a rapidly growing application within proteomics mass spectrometry. As more extensive protein panels need to be monitored in a targeted way across multiple samples, higher throughput is becoming essential. The need for rapid assay development, higher multiplexing and more robust assays are some of the key challenges. In this work, the combination of unique workflows on the hybrid triple quadrupole - linear ion trap mass spectrometer and automated software has been used to automate and simplify creation of highly multiplexed MRM assays (>1000 MRM per analysis).

Methods: Tryptic digest of yeast and E. coli samples were separated by reversed-phase HPLC on an Tempo nanoLC System. MRM analysis was performed on an hybrid triple quadrupole linear ion trap MS system (AB/Sciex). By using the MRMPilot™ Software coupled with the MIDAS™ workflow acquisition strategy, all MRM transitions to the proteins of interest were designed. Iterative MRM analysis was performed which provided rapid refinement of MRM parameters without requiring synthetic peptides.

Results: Identification of yeast proteins was performed using ProteinPilot database search engine and served as the starting point for assay development. MRMPilot software was used to create MRMs to quantify 100 proteins using specific criteria yielding a total of 1500 MRMs divided into 6 MIDAS Workflow (MRM triggered MS/MS) methods (~250 MRMs/method). As a result, both MRM data and ion trap MS/MS of each peptide can be obtained. A final list of ~1200 MRMs was created using MRMPilot and used to generate a single MRM method utilizing time scheduling. Triplicate analysis was performed to test for reproducibility of the peak areas, 82% of the peak areas had %CV < 20 (without any internal standard). This workflow took only 4 days to complete (data analysis and processing) substantially improving upon previous manual strategies. Future work involving differential expression studies of samples from e.coli will be performed using mTRAQ labelling and global internal standard (GIS) as additional strategies for improving efficiency of assay development. The results from these studies will be presented.

Innovative aspects:
Unique workflow using hardware and automated software to automate and simplify creation of highly multiplexed MRM assays containing >1000 MRMs.

A167

Antibody Colocalization microarray (ACM): A scalable chip for multiplexed and quantitative protein profiling

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Introduction: Robust, high sensitivity, high throughput multiplexed protein profiling technologies (*e.g.* protein chips) are still missing and constitute a bottleneck to the widespread adoption of proteomics. Antibody (Ab) based assays could not be scaled easily because they suffer from interferences due to the cross-reactivity of Abs and non-specific binding; when directly labelling a sample, it is not possible to differentiate between a "real" binding, or interference. ELISAs (sandwich assays) overcome this problem with a matched pair of Abs: the capture Ab serves the "purification" of an analyte, and the detection Ab discriminates against interference. In a microarray format, sandwich assays do not perform as well because the Abs readily cross-react with other Abs of the array in a combinatorial manner. As a result, quality, multiplexed Ab-based assays (chips or beads) are typically limited to less than 30 analytes. Here we present a new Ab microarray format, called Ab colocalization microarray (ACM) that overcomes these challenges and is scalable because matched Ab pairs are physically colocalized.
Methods: ACM requires two spotting rounds for capture and detection Abs with absolute alignment <30 µm. A special pin spotter with alignment mechanisms was custom built (Gesim) and optimized. Capture Abs were spotted (400 mg/ml) on nitrocellulose slides (Grace bio-labs). After a blocking step (1% BSA-PBST) the slides were incubated with dilution series of spiked PBS and serum (10%) in 16 compartments. Upon re-alignment of the slides, biotinylated detection Abs were spotted (colocalized), and incubated. Next, the slide was incubated with fluorescently labeled streptavidin. Binding was detected using a fluorescent scanner.
Results: Many challenges needed to be solved for ACM, notably: (i) alignment, (ii) interference due to 2nd contact spotting and (iii) incubation with sub-nanoliter volumes of spotted detection Abs. Pins were selected because inkjet did not allow precise colocalization. Nitrocellulose slides were compared to epoxy slides with custom-made nano-compartments, and found to be superior. The 3D matrix of the nitrocellulose helps distribute the binding and wicks the liquid. The optimal conditions detection antibody spotting are a 10 µg/ml concentration in PBS (consumption is reduced 100 fold compared to cocktail) with 10% glycerol and 80% humidity. To date, we identified 30 Ab pairs that work well on this platform. As an illustration, we show ACM with dilution series of 12 analytes in PBS and serum with widely varying concentrations, Fig. 1. For the best Abs (*e.g.* IL8), good signals are obtained below < 1 pg/ml. Some of the antibodies used in ACM have already been found to cross-react when used in a conventional antibody microarray with a cocktail (not shown).

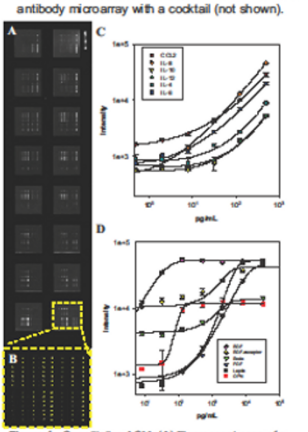

Figure 1. Quantitative ACM. (**A**) Fluorescent scan of a 16 colocalization Ab microarray used to detect 12 spiked proteins with 8 dilutions in PBS (left) and serum (right). (**B**) Close-up view of one array. Each column corresponds to 6 replicate spots of analyte or positive control (4 6-spot columns). Negative controls are not visible. (**C**) Binding curves for 6 high sensitivity assays (all cytokines) and (**D**) 6 other proteins measured simultaneously in spiked serum. The range of the assays is being adjusted based on these curves.

Innovative aspects & conclusion

- ACM is versatile because addition or removal of a matched pair of Abs to an ACM is independent of all other pairs on the array.
- ACM allows parallel screening of Ab pairs.
- ACM combines the sensitivity and selectivity of ELISA with the multiplexing ability of microarrays, and to our knowledge, constitutes the first quantitative proteomics technology that can be scaled (parallelized) without compromising assay quality.

High density protein microarray chip for biomarker discovery

A168

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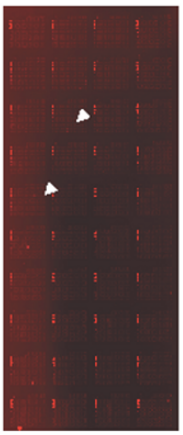
Introduction
The accomplishment of human genome sequencing project leads the biological science research to a new era. One of the major challenges for modern biological research is to study biological function at the whole proteome level. With its high sample density and multiplexed experimental settings, the protein microarray is the perfect technology for this purpose. Technology development has demonstrated that it is possible to spot whole human proteome on a single glass slide. However, the greatest challenge and difficulty in assembling protein microarrays is still the provision of high quality functional proteins for whole human proteome. In this research, OriGene produced more than 10,000 human recombinant proteins in HEK293T cells, and spotted them on a single Schott nitrocellulose glass slide. One of the important utilities for this high density protein microarray chip is for disease biomarker discovery.

Methods
We shuttled more than 15,000 druggable ORF cDNAs into OriGene's proprietary Eukaryotic expression system and produced more than 10,000 C-terminal myc- and Flag-epitope tagged human proteins in a human cell originated HEK293T cell line. The target protein expressions for all these ORF cDNA clones have been fully validated with anti-Flag western blot analysis. Aushon 2470 solid pin microarray printer was used to spot these overexpression lysates on a Schott nitrocellulose slide. To examine the quality and consistency of our lysate protein microarray chip, the chips were extensively tested under different experimental conditions.

Results
Our results showed that the protein spots printed on the slide exhibit high uniformity. The expression level of each individual recombinant protein within overexpression lysate can be accurately quantified by our myc-Flag tagged reference standard. Our preliminary data demonstrated that this high density microarray chip can be utilized for various research fields, including novel antibody decoding, and tumor biomarker discovery (Figure 1).

Innovative aspects
- High density lysate protein microarray chip
- Novel application for tumor biomarker discovery



Breast cancer serum

Figure 1. OriGene lysate microarray chip can be used for tumor biomarker discovery. The OriGene lysate microarray chips were immunostained with sera from breast cancer patient or normal female donor with age matched (Not shown). The cancer patient specific autoantigens are highlighted with white arrows.

High Sensitivity Biomarker Detection Using Protein NanoArrays

A169

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Introduction:
The development of miniaturized protein nanoarrays will be essential for the pursuit of personalized medicine, allowing for the prediction, diagnosis and treatment of human disease, also known as disease proteomics. Current conventional mass spectrometry-based proteomic efforts have only been remotely successful in the effort to discover new biomarkers of disease systems; thus, new emerging protein technologies are necessary for the discovery of new biomarkers, needed per disease system. The advantages of miniaturization have long been realized for nucleic acid analysis, and are being further miniaturized in the field of proteomics and protein profiling. We report here on our progress of fabricating sub-micron antibody nanoarrays, consisting of feature sizes ≤ one micron, resulting in enhanced sensitivity in the detection of biomarkers compared to conventional microarrays.

Methods:
NanoInk has developed an instrument based on DipPen Nanolithography, a new direct-write spotting technology which generates micron to sub-micron sized features of biomolecules on solid surfaces. The innovative instrument can generate dot arrays over millimeter-scale areas with nanometer resolution in mass production. This printing technique offers significant advantages over current microarray printing technologies that suffer from poor spot to spot reproducibility in terms of size, shape, and density. Recent patterning experiments of antibodies, as well as screening for their biological activity, yielded high-quality results with excellent uniformity and repeatability of features within and between arrays. Each of our nano-spots occupies only a small fraction of the surface area occupied by a single spot in a conventional microarray, still maintaining a sufficient surface area to bind sufficient numbers of antibodies or antigen. Furthermore, preliminary studies in our labs have indicated that the decreases spot size can actually concentrate, or recruit, low abundance biomarkers.

Results:
As a proof-of-concept studies, we created two nanoscale assays, one for the detection of prostate-specific antigen (PSA) a widely used biomarker for prostate cancer screening and the second assay is based on the detection of cytokines through the immobilization of multiple anti-cytokine antibodies similar to an ELISA-based assay. We used our process to generate miniaturized capture antibodies arrays for the specific test at the nanoscale. These arrays were used to capture their corresponding specific antigen followed by fluorescent detection using fluorescent labeled antibodies and high resolution fluorescent microscopy. These nanoarrays were found to have better sensitivity, improved binding kinetics over conventional microarrays, with detection levels of 100 to 10 fg/ml. Currently, we are trying to push the sensitivity below 1 fg/ml. The improved level of detection is due to the generated nanoscale capture biomolecules which behave as both more specific and more sensitive probes, resulting in the reduction of the amount of low abundance analytes required for detection by enriching the target molecule onto a nanoscale spot. Miniaturization as well as multiplexing of biological assays will be essential in modern medicine's quest for personalized medicine. Such promising nanotechnologies will enable multiple diagnostic measurements from small amounts of blood, hopefully making it possible to someday differentiate health and disease from a systems biology perspective.

Lifestyle: a key modulator of selective protein damage in skeletal muscle mitochondrial proteome

A170

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Introduction: Skeletal muscle presents clear age associated deterioration in structure and function [1]. At a cellular level, mitochondria are one of the organelles most affected by aging. The underlying mechanisms are usually related with enhanced oxidative stress. It is assumed that physical activity attenuates age-related deleterious effects, although the mechanisms are not completely known. To better understand the mitochondrial functional alterations of aging muscle and how they are affected by lifestyle, we performed a global search of the potential mitochondrial protein targets for carbonylation.

Methods: C57BL/6 mice mitochondria from young (2 months), old (25 months) active and sedentary, aged with or without access to running wheels, respectively, were isolated from skeletal muscle. Histochemical staining of BN-PAGE respiratory complexes was used to evaluate functionality differences between experimental groups. To assess the mitochondrial proteome differential susceptibility to carbonylation, we have performed 2-DE oxyblots by derivatizing IPG strips with DNPH between IEF and molecular weight separation, followed by electrotransfer to nitrocellulose membrane and immune detection of carbonyl derivatives [2]. Coupling this with Maldi-TOF/TOF protein identification we have identified the preferential targets of protein oxidation.

Results: Old active mice presented a notorious boost on functionality, while there was a clear decline in old sedentary mice mitochondria. On the other hand, carbonyl content was found increased in sedentary mice. While some proteins were found carbonylated in the three groups, such as, ATP synthase α chain, citrate synthase, VDAC 1, aconitate hydratase and creatine kinase, other proteins were only found carbonylated in aged mice. These latter include Complex III iron sulphur subunit; MnSOD, aspartate aminotransferase, creatine kinase, malate dehydrogenase. Furthermore, ATP synthase D chain, Complex I MLRQ subunit, cytochrome c and cytochrome c1 were only found carbonylated on aged sedentary mice. The preferential targeting of OXPHOS subunits to sedentaryness-related carbonylation might explain the observed differences in functionality.

Innovative aspects:
- Identification of OXPHOS subunits as specific targets of oxidative damage;
- Association between sedentariness and increased protein damage, namely in OXPHOS subunits;
- Evidence of lifelong physical activity as a powerful mechanism to prevent selective carbonylation of OXPHOS subunits and promote up-regulation of the antioxidant system, resulting in improved functionality.

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Specificity screening of affinity binders using high-end antigenic diversity exposed on nanoarrays

A171

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Introduction: Proteins are non-tractable to amplification by PCR. As a consequence, producing large numbers of recombinant proteins is likely to remain highly cost and time intensive into the foreseeable future. As such, the use of protein arrays has yet to become as common place as their nucleic acid cousins. This *status quo* must be redressed if meaningful progress is to be delivered with respect to the: a) Validation of biomarkers; b) Confirmation of affinity binder specificity; and / or c) Verification of the presumed targets of drugs and biotherapeutics within the human body, i.e. in the absence of substantial cross-reactivity. In all the aforementioned cases, access to extensive antigenic diversity is critical.

Methods: In order to redress this situation, recent efforts have focused on nano-gridding in order to dramatically reduce the cost of generating biochips from high-cost or precious starting-material, namely recombinant proteins, reverse arrays & biobank samples[1]. In parallel, these same nano-deposition techniques are being combined with DNA to Protein Array (DAPA) *in situ* protein synthesis technique[2], as a means to display extensive and addressable antigenic diversity reminiscent of the human proteome (Indicative of, but not necessarily identical to it). Examples of both nano-gridding and DAPA across some 30 SCOP protein-fold families will be detailed.

Results: A Bioplume spot volume of ≈ 20-200 fl can in theory produce some 10^{10} - 10^{11} spots from just 2µl of sample. Current version biochips contain 14 replicate sub-arrays per microscope slide and demand the use of 0.06µl-0.07µl of starting-point affinity reagent (diluted at 1/1000) per sub-array. Longer term, up to 64 identical sub-arrays per microscope slide is envisaged, each containing up to 15,000 spots. These steps will allow high-throughput and low cost monoclonal antibody supernatant (or other affinity binder) screening for target recognition in the absence of cross-reactivity, whilst consuming negligible reagent volumes. Spot-on-spot deposition can still further reduce the amount of sample employed. However, here, gridding times become a major issue. We are currently working towards a standardized array for international distribution specifically designed for screening affinity binder specificity. Parties interested in trialing these biochips free-of-charge should contact the author.

Estimates of cross-reactive binding with respect to a significant portion of the whole human proteome can be derived from a combination of *in vitro* wet laboratory screens and *in silico* projections of 3D conformational epitopes contained on a standard 1000-protein nano-array[3]. The utility of combining *in silico* conformational epitope information with wet lab observations will be exemplified.

Innovative Aspects:

- Cost-effective use of valuable bioresources, e.g. biobank stocks
- Affordable screening of monoclonal antibodies, biotherapeutics & other affinity binders
- Biomarker validation in the presence of high-end antigenic diversity

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A172

High Throughput Cell-Based Studies and Protein Microarrays for Biomarker and Target Discovery

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Self assembling protein microarrays arrays can be used to study protein-protein interactions, protein-drug interactions, search for enzyme substrates, and as tools to search for disease biomarkers. In particular, recent experiments have focused on using these protein microarrays to search for autoantibody responses in cancer patients. Several bona fide autoantibody responses, such as responses to p53, have been detected, and a pilot study of responses to 7500 full length human proteins in 50 breast cancer patients and 50 controls has identified over 700 candidate proteins with more frequent responses in patients. These experiments show promise in finding antibody responses that appear in only cancer patients.

A173

Rapid production of custom protein arrays for multiplexed assays

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Introduction
Protein arrays are becoming a central proteomics technology providing means to investigate protein interactions and functions as well as protein expression level and protein modifications. However lack of high-throughput method for protein expression and purification remains a bottleneck in advancing this technology. Here we present a new integrated approach for creating protein arrays that combines *in-vitro* protein expression system with covalent, site specific and oriented immobilization method which allows for rapid capture of proteins onto a glass slide without any prior purification.

Methods
The HaloTag protein is a 33 kDa engineered derivative of bacterial hydrolase that forms a covalent bond with its ligand (1). In the current study we expressed nine different proteins fused with HaloTag protein in *E.coli* or in cell-free protein expression systems. Two of the nine proteins were model proteins GST and Protein G. The other proteins were: p65, p53, cJun, cFos, R1a, βGal and PKA kinase. To create protein arrays, lysates containing HaloTag-fusion proteins are incubated for 1.0hr on a glass slide coated with HaloTag ligand activated hydrogel. Binding of HaloTag-fusion proteins to the ligand is, oriented covalent and specific. The hydrogel coating of the slides minimizes the non-specific binding.

Results
All proteins fused to HaloTag could be expressed and directly captured either from cell free expression systems or lysed *E.coli* cultures even at low concentration (<10µg/ml). Cell-free protein expression systems have the advantage that they are easy to use, rapid, and ideal for high-throughput formats. Using purified HaloTag-GST and HaloTag-Protein G we show that the amount of protein captured is higher than the conventional covalent attachment that uses lysines at the protein surface and requires highly purified proteins. In addition, proteins captured using HaloTag are oriented for maximum functional activity. Captured fusion proteins were tested for interactions with different potential binding partners and positive protein-protein interactions were detected with excellent signal to noise ratios. We also demonstrate that HaloTag fusion proteins expressed in cell-free systems are stable when exposed to multiple freeze-thaw cycles. Hence for long term studies requiring reproducible results, lysates containing HaloTag fusion proteins can be aliquoted and frozen and used to create "array-on-demand" just before their use.

Innovative aspects
- Specific and covalent attachment of proteins onto glass slide directly form a crude lysate
- Oriented attachment improves efficiency of capture and functionality of captured proteins

References
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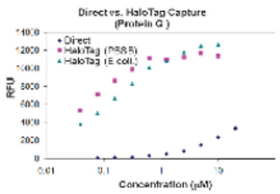

Figure 1. HaloTag-protein G fusion proteins were captured on HaloLink slides and detected using fusion specific antibodies. Blue diamonds show direct capture of pure HaloTag-protein G fusion through lysines; pink squares show HaloTag mediated attachment of pure HaloTag-protein G and green triangles show HaloTag mediated attachment of HaloTag-protein G directly form E. coli lysate.

A174

Proteomic-Cell Biology Unravels HIV-1 Hijacking of Tunneling Nanotubes for Intercellular Spread in Monocyte-Derived Macrophages

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roduction
nneling nanotubes (TNT) are cytoskeletal bridges that pport intercellular exchanges of receptors, lysosomes d mitochondria. HIV-1 transmission by such ercellular connections facilitates cellular transmission. chanisms of TNT formation and pathogen immune cape has generated intense interest following failure HIV-1 vaccine trials.

thods
determine the mechanisms underlying the formation and viral trafficking linked to the TNT still and time se confocal imaging, transmission and scanning ctron microscopy were employed. Antibodies and emical inhibitors of motor and cytoskeletal proteins re used to facilitate studies of intercellular viral nsmission. Proteomics techniques determined the chemical composition of TNT and vesicular cargoes opted for viral transport.

sults HIV-1-infected human monocyte-derived crophages (MDM) establish direct cell-cell contacts ough TNT networks. HIV-1 induces vesicle sorting d turnover. Co-cultivation of uninfected with virus-ected cells results in intercellular contacts extending er 700 um. Electron and confocal microscopy showed IT tethering at perinuclear/Golgi regions of connected lls rich in vesicular compartments. These were nfined by the TNT limiting membranes and showed cumulation of adherence junction components along ntact sites. Cytoskeleton-disrupting drugs including ochalsin D, nocodasole demonstrated a central role of tin in TNT formation. Proteomic analysis collected m infected MDM showed the presence of early, late d recycling endosomes, vesicle trafficking mponents including Rab GTP-ase family proteins ab2a/5a/7, 11) and myosins along the TNT. These says also identified HIV-1 proteins, in the TNT, such Tat, Rev, Gag and gp120. Confocal imaging showed IT support active and bi-directional vesicle exchange an average velocity of 1.5 um/s. These events were companied with the co-localization of Env and Gag h vesicle-cargo motor proteins and were actively nsported from the infected to uninfected target cells. ibition of myosin II by blebbistatin and butandiene noxime with cytochalasin D/nocodasole abrogated vement of HIV-1 containing TNT-associated vesicles.

Innovative aspects
1. Proteomic and structural characterization of TI and secretory microvesicles in HIV-1 infect macrophages have not been previously describ These were crucial in the functional investigation direct intercellular vesicle and virus traffick pathways along the TNT.
2. These data provide new evidence that HIV-1 m escape immune surveillance by inducing b structural rearrangements (TNT formation) a membrane turnover (vesicle sorting) in human MD Importantly HIV-1 uses such processes for dir transport from infected to the target uninfected cell

A175

Immunoassays in multiplex for biased and unbiased proteomic research

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Introduction

Within the last decade, microarray technology has been adapted to analyze the expression and function of proteins. Different analytical systems are being developed that are likely to evolve into key technologies for a detailed protein profiling of biological samples (1-3).

Immunoassays in multiplex for biased proteomic research

However, the degree of multiplex assays is limited to approximately 20 analytes per assay - the so-called "Gold´s wall" Incompatible assay formats and the inherent problem of antibodies' cross-reactivity decrease the sensitivity of a multiplex versus a singleplex test. We have identified a simple and efficient way to overcome these current limitations of multiplexing sandwich immuno-assays. Protein microarray assays are performed under ambient analyte conditions. According to Roger Ekins' ambient analyte theory, the number of formed antibody-analyte complexes is small compared to the total amount of analyte in the solution. This is due to the low number of capture molecules in a microspot. Therefore the actual concentration of the analyte is nearly unchanged when performing a microspot-based immuno-assay. This fact was the basis for the development of our workflow to perform bead-based sandwich immunoassays sequentially. Paramagnetic color-coded beads and a magnetic bead handler allowed an automated set-up and the sequential performance of various bead-based multiplexed sandwich immunoassays. Examples will be given which demonstrate the capability of sequentially analyzing the expression of cell signaling proteins and their degree in phosphorylation in the same sample material from the same cavity. Sequentially performed multiplex sandwich immunoassays reuse the same sample several times. Therefore the sample is not limiting, and non-compatible assays become compatible. This workflow enables the analysis of several dozens of parameters from the same sample in a sequential mode.

Immunoassays in multiplex for unbiased proteomic research

However, this scenario allows only a biased protein profiling approach. The current demand in discovery proteomics for antibodies is still unmet. The availability of appropriate antibodies limits current immunoaffinity-based methods for detecting and quantifying target proteins. In contrast to the one antibody-one analyte approaches, we have developed the Triple X Proteomics (TXP) approach, which uses one antibody for the enrichment of several dozens of analytes.TXP antibodies, which recognize short terminal peptide sequences, are generated (4). These TXP antibodies can be used for affinity enrichment of classes of signature peptides from a tryptic digested sample. These enriched peptides share the same common 3 to 4 terminal amino acid motif. Subsequently peptides can be accurately identified according to their molecular weight and peptide mass fingerprint. Here proteins are detected on peptide level and not as intact molecules. First concept studies will show that a variety of TXP antibodies can be generated and applied for a targeted and unbiased protein analysis. The TXP strategy offers the possibility to use a small number of group-specific capture antibodies to increase analytical resolution. The TXP approach has the potential to cover a wide range of any given proteome with a minimal set of antibodies.

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A176

Matrix metalloproteinase (MMP) activity assay using an array-based spectral SPR biosensor

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Introduction

MMPs participate in extracellular matrix degradation and play a central role in many normal physiological processes such as embryogenesis, normal tissue remodeling, wound healing, and angiogenesis and in pathological situations such as atheroma, arthritis, cancer, and tissue ulceration (1). The proteolytic activity of MMPs has been predominantly determined by zymography, ELISA and FRET-based approaches, but these assays are complex, time-consuming and/or expensive. In this study, we present an array-based SPR biosensor system using gelatin arrays for a high-throughput analysis of MMP-3 activity.

Methods

We prepared gelatin arrays by immobilization of intact gelatin on amine-modified gold arrays (Fig. 1). To analyze MMP-3 activity in a high-throughput manner, we applied various concentrations of cMMP-3 onto gelatin arrays, and the arrays were analyzed by the line-scanning mode of the spectral surface Plasmon resonance (SPR) biosensor (2). MMP activity was determined by detecting of SPR wavelength shift caused by gelatinolysis. The gelatinolytic activity of MMP-3, which was determined by SPR biosensor, was further supported by molecular imaging with atomic force microscopy and fluorescence-based protein arrays. To investigate inhibitory effects of TIMP-1 and GM6001, MMP inhibitors, various concentrations of TIMP-1 and GM6001, mixed with 5 μg/ml cMMP-3, were applied to gelatin arrays and the resulting arrays were analyzed by the line-scanning mode

Results

Digestion of gelatin layers on gold arrays with MMP-3 was investigated with SPR spectroscopy. Gelatinolytic activity of MMP-3 was dose-dependent with the evident increase in gelatin digestion at 50 ng/ml cMMP-3 ($p < 0.01$), and almost saturated at 20 mg/ml cMMP-3 (Fig. 2). Then, we investigated inhibitory effects of TIMP-1 and GM6001, MMP inhibitors, on MMP-3 activity using the array system. TIMP-1 and GM6001 inhibited MMP-3 in a dose-dependent manner. The half maximal inhibitory concentrations (IC_{50}) of TIMP-1 and GM6001 were 338 and 300 nM, respectively. Therefore, this new assay system using a spectral SPR biosensor is simple, and high-throughput, and is likely to have a strong potential for inhibitor screening, activity-based proteomics as well as diagnosis of human diseases.

Innovative aspects

- A novel array-based SPR system for MMP-3 activity assay
- a high-throughput analysis of MMP-3 activity
- A strong potential for MMP-3 inhibitor screening and activity-based proteomics

References

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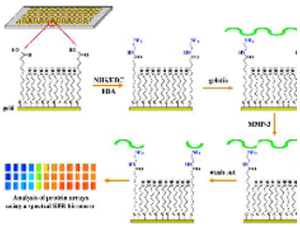

Figure 1. Schematic diagram of MMP-3 activity assay on gelatin arrays using a spectral SPR biosensor.

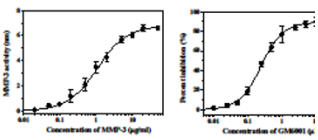

Figure 2. Dose-dependent MMP-3 activity and its inhibition by GM6001 on gelatin arrays.

Assembling the Parts Whole Cell Modeling

(A201 – A205)

A201

Interactions of Actin in RAW 264.7 Macrophages from the Fc Receptor Complex and *in vitro* Protein-Protein Analysis

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Introduction
Actin interactions are fundamental for the cytoskeleton, cell morphology, mobility and signal transduction. There is a need to examine protein-protein interactions *in vivo* and *in vitro* using physiological salts and organic acids that are directly compatible with mass spectrometry. However, the use of physiological salts that will preserve interactions will necessarily also result in greater background binding. Hence, there is a need for comparison to control experiments to account for non-specific binding. Since the LC-ESI-MS/MS technique is so sensitive, large data files are generated and hence computational methods will be required to reveal specifically bound proteins from protein-protein interaction experiments.

The assembly of the Fc receptor supramolecular complex requires a foundation of actin and other cytoskeletal proteins. A plasma membrane sub-domain forms around the activated receptor where proteins and signalling factors recruit and accumulate. The inner bilayer of the plasma membrane, the actin cytoskeleton and local membrane potentials are all required to assemble the receptor complex. The proteins of the Fc supramolecular complex may be captured by Live Cell Affinity Chromatography (LARC) (1). Hence the true "positive" interactions of actin may be faithfully read off from the specific results of the LARC experiment with the Fc receptor's ligand IgG. The established interactions of actin from the literature and LARC will be invaluable to adjudge the veracity of putative *in vitro* protein-protein interactions under different binding and elution conditions.

Methods
RAW 264.7 macrophages derived from mice were obtained from ATTC and grown in Alpha Modification of Eagle's Medium 1x (AMEM Multicell, Wisent Bioproducts, St-Bruno, Quebec, Canada) supplemented with 5% Fetal Bovine Serum in an incubator at 7% CO_2 at 37°C. The actin- driven Fc receptor supramolecular complex was isolated exactly as described (1).

Results
Alpha, beta, and gamma actin and many related actin isoforms were observed in the Fc receptor complex captured from live cells. Similarly, HS1 and Annexin I, II, IV, V and VI and other actin binding proteins were observed *in vivo* from LARC.

However, actins and their interacting proteins were also observed *in vitro* on polystyrene beads incubated with crude extracts of RAW macrophages. Actin also readily bound to NHS affinity chromatography beads in the absence of anti-actin antibodies.

NHS affinity chromatography using Actin, anti-Actin, anti-Annexin I, II, IV, V and anti-HS1 revealed the presence of more than 50 proteins not found in control beads alone that showed expectation values p(x) of 0.0001 (E-4) or less by LC-MS/MS. Annexin II and V and the haematopoietic specific protein HS1 (also called Lyn substrate) were prominently observed in isolated Fc receptor complexes and not observed in control beads incubated with crude extracts of RAW macrophages. Annexin II and V as well as G3BP, FLT1, FARP2, STARD13, RAB25 and FRAP1 were observed to bind actin in affinity chromatography experiments using LC-MS/MS.

The actin binding proteins of the Fc receptor complex established by LARC and the literature may be used as an absolute standard and positive control for actin interactions. Many of the putative actin interactions were confirmed by network modeling, Western blot, cytological staining or expression of GFP fusion proteins. The actin specific proteins were assembled into an interaction network using iHOP (2) and Cytoscape (3) in agreement with the literature but with the detection of novel complex members.

Innovative aspects
-Actin interacting proteins defined *in vivo* and *in vitro* by mass spectrometry.

-Novel actin interactions were detected and confirmed by independent biochemical methods.

-Interaction network in agreement with previous literatures as determined by iHOP and Cytoscape.

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A202

Whole proteome N-terminal peptide isolation and analysis from M. tuberculosis H37RV

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Introduction
Current DNA sequencing technology allows for the complete sequencing of an entire bacterial genome in a few days. Bioinformatic analysis of the genome allows for the predication of many proteins. Accurate information on alternative start sequences, and post translational modifications to the N-terminus of the proteins, whether from in vivo or ex vivo proteolytic processing, are needed to correctly map out these genes and their associated protein functions. Using a modification to the technique used by MacDonald et. al.(2006), we have been able to work with BL3+ organisms which require additional procedures to ensure safe handling. In addition, we have used a simple "in cassette" processing procedure that allows for sequential reagent addition/exchange. This procedure acetylates all primary amines, capping any n-terminal sequences, and after proteolytic digestion, removes any free amines generated by trypsin on an amine reactive column. High resolution mass spectrometry along with iterative and orthogonal fragmentation modes then generates data on as many sequences as possible.

Methods
Whole TB was lysed in GuHCl, bead beaten, and boiled. After reduction and alkylation the bug proteins were dialyzed in Slidalyzer cassettes against sodium bicarbonate. Cassettes were removed from the dialysis buffer and proteins were acetylated by injecting sulfoNHS-acetate into the cassette. After further dialysis into triethylammonium bicarbonate the proteins were digested with trypsin and the digested proteins run over an immobilized NHS ester column.
Eluted peptides were analyzed using Nanospray LC-MS/MS on an LTQ-Orbitrap with CID and ETD (Trap and Orbitrap) and HCD (orbitrap) with sequential targeted runs using an inclusion list generated with SIEVE. These sequential SIEVE inclusion list runs maximize the effectiveness of longer duty cycle MS2 events.
Results
Using an iterative LC-MS/MS approach to analyze the samples allows for inclusion lists to specify peptide precursor ions for alternative fragmentation modes. Initial raw data was analyzed by SIEVE with sequence searches performed with SEQUEST against a modified H37RV translated database. Unidentified frames were then rerun on the LTQ-Orbitrap using an orthogonal fragmentation mode, and a time dependent inclusion list. Novel N-terminal modifications (other than acetylation) are identified by searching the MS2 fragment data for sequence tags and comparing to a limited database containing only putative protein start sequences.
Innovative aspects
- Yields information about N-terminal start sequences, in vivo proteolytic processing of proteins as well as decreasing proteome complexity. "One Pot" processing. Simplified proteome analysis.

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A203

Two-dimensional native/SDS PAGE proteomic approach for analysis membrane protein complexome under proximately physiological conditions in *Escherichia coli*

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Introduction
Cell biochemical processes are practically performed by complexome, defined as a set of protein complexes, or interactome, the network of interacting protein complexes. Therefore, the identification and characterization of protein complexome of cells are essential for comprehensive understanding and global vision of their functions, which might be the ultimate goal of life sciences research.

Methods
Native-PAGE, by improving resolution with a little modification, combination with SDS-PAGE (2-D native/SDS PAGE) was firstly used as a renew technique for detection of membrane protein subcomplexome of *Escherichia coli* under close proximately physiological conditions.

Results
Total 97 well visualized protein spots were labeled and corresponding spots were excised from the gels and identified by MALDI-TOF/TOF tandem mass spectrometry. Out of them, 94 protein spots were successfully identified, and they represented 75 unique proteins annotated in Swiss-Prot database.
Subcellular localization of identified proteins was analyzed combined by PSORTb 2.0.4 software and annotation of the proteins from the Swiss-Prot database. Of all proteins, 36 (48%) were membrane proteins (27 inner membrane proteins and 9 outer membrane proteins), 6 (8%) were periplasmic proteins and 28 (37%) were cytoplasimic proteins. 5 (7%) proteins location were still unknown to date. The reason that cytoplasimic and periplasmic proteins detected from the membrane fractions is probably that these soluble proteins were bond to membrane proteins and thus were co-precipitate with them.
In total, 21 lines which contain more than two different proteins aligned perfectly were identified by global analysis of the 2-DE map. However, only 17 lines are represented heteromeric protein complexes by protein function analysis, owing to proteins aligned in a line are not consequentially components of a complex.

Innovative aspects
- To present a first characterization of membrane protein complexome of the *E. coli* with application of 2-D native/SDS-PAGE combination with mass spectrometry.
- Two-dimensional gel system with optimization can be expanded for studying protein-protein interactions to help us understand the function of protein complexes within living cells.

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Membrane Proteomics: Putting the Peptide Jigsaw Back Together

A204

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Introduction

Proteomics promises delivery of comprehensive coverage but in membrane proteomics problems of protein solubility and low copy number have made this difficult.

Our recent data on membrane protein:protein interactomics, membrane proteomics peptide IPG-IEF "shotgun" approaches with label-free quantitation and glycan analyses on cancer cell lines, human embryonic stem cells and from liver membranes from cancer-bearing animals are discussed in the context of APAF's attempts to comprehensively increase membrane proteome coverage.

Methods & Results

Using a benign tumour bearing mouse model and "shotgun" peptide IPG-IEF proteomics with label-free quantitation we have examined reproducible, significant changes in the liver proteome.

In control animal experiments, tryptic peptides isolated through methanol-assisted digestion showed optimal peptide coverage of ectodomain-containing membrane proteins, where ~95% were concentrated into 3 main pI range peaks and ~2,000 non-redundant proteins (513 predicted to have between 1 and 19 transmembrane domains) were identified (including most (42) rat CYP450 protein family members). Using pI as a filtering tool enhanced identification reliability.

In cancer-bearing mice, major changes in glycosylation pathways, drug metabolism and clearance (e.g., multi-transmembrane solute carrier family proteins), cell surface receptors, 11 electron transport chain complex I subunits and fatty acid catabolic enzymes were observed.

Subsequently, glycan analyses on Triton-X114 phase partitioned membranes from cancer-bearing mice closely paralleled changes anticipated from glycosylation enzyme changes.

We have deployed additional methods to map embryonic stem cell lines (with various monogenic diseases) membrane proteomes and to membrane changes when the malignant HCT116 colon cancer line is transformed to a benign counterpart (through antisense uPAR suppression.

We report how immunoprecipitation proteomics can identify true uPAR-interacting proteins involved in the "metastasome'. Subsequent detailed analyses of the exact sites of protein interaction between uPAR and the integrin αvβ6 [using overlapping peptide array binding blots and ELISA-type peptide competition assays] suggest that some very interesting biology may be associated with these interactions. This may have implications for cancer sufferers as both cancer lynchpin proteins uPAR and αvβ6 are known to be independent poor prognostic indicators of patient outcome, but little is know regarding outcome when they are co-expressed in cancers.

Innovative aspects

- Collectively, our data demonstrate that combination(s) of methods including "shotgun" peptide IPG-IEF, glycoprotein enrichment, glycan analyses and membrane protein complex immunoprecipitation can result in deep, comprehensive mammalian membrane proteome coverage.
- Studies like these are providing vital new information regarding complications involved in cancer treatment (e.g., cachexia) , changes in glycoprotein biosignatures associated with disease and proteins that may drive the process of malignancy as well as potentially being novel therapeutic targets.

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Protein Complexes and Functional Pathways in Yeast and Bacteria

A205

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We used TAP-tagging, affinity-purification and mass spectrometry (APMS) to sort the *S. cerevisiae* and *E. coli* soluble proteins into complexes and recently extended the yeast PPI network to encompass membrane proteins. Yeast complexes have been assigned to functional pathways using synthetic genetic interaction analysis in an E-MAP version of the SGA approach for genes related to yeast nuclear processes, and we have developed an analogous method, eSGA, for *E. coli*. The co-functionality of the proteins in yeast membrane-associated protein complexes is being tested by comparing protein complexes with genetic interactions and assessing the effects of membrane proteins on the morphology of intracellular compartments. Our *E. coli* PPI network has also been integrated with systematic genomic context inferences to derive a probabilistic network of functional inferences and assign ~57% of the functional orphan proteins to discrete functional neighborhoods. Analogous APMS and RNAi approaches could be used to functionally classify human proteins.

Blood-based Proteomics

(B101 – B144)

B101

Efficient depletion of albumin and IgG from human plasma using new prepacked columns

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Introduction: One of the major obstacles during analysis of human plasma or serum is the presence of high abundant proteins obscuring the analytical signals from less abundant proteins. Human serum albumin and IgG constitute more than 60% of the total protein content in plasma and the effect of sample preparation using the HiTrap™ Albumin & IgG Depletion column is shown. The prepacked 1 ml LC column contains media with highly specific affinity ligands coupled to Sepharose™ High Performance and is designed for larger volumes of undiluted human plasma. The media is also available in SpinTrap™ format.

Methods: Human plasma samples were applied to HiTrap Albumin & IgG Depletion columns. The non-bound fraction, containing the depleted sample, was collected and the amount of remaining albumin and IgG was determined by ELISA analysis. The study involved tests for reproducibility during repeated runs and comparisons with a 1 ml Albumin/IgG Depletion Cartridge from another supplier.
To evaluate the effects on analytical resolution, non-depleted and depleted samples were analyzed by MS and 2-D PAGE. The 2-D PAGE was carried out by Difference Gel Electrophoresis (DIGE) where parallel run and labeling of the samples with different fluorescent dyes made it possible to significantly determine the differences in protein abundances.

Results: Sample preparation of 150 µl human plasma using HiTrap Albumin & IgG Depletion resulted in >95% human serum albumin depletion and >90% IgG depletion. High reproducibility during repeated runs on the same column was achieved. The degree of depletion was significantly higher compared with results obtained using the depletion cartridge. In addition, the HiTrap depletion procedure was notably faster for HiTrap Albumin & IgG Depletion since no dilution of the sample is required and the flow rate of 1 ml/min is maintained also during sample application.
The efficiency of albumin and IgG depletion was estimated by MS- and 2-D PAGE analysis. Enhanced visualization of other proteins with isoelectric points and/or molecular weight similar to albumin and IgG, respectively, was clearly demonstrated. In addition, the depletion allowed for a relatively higher load of less abundant proteins, enabling detection through stronger signals and also of a larger number of proteins.

Innovative aspects:

- A fast and efficient protein depletion approach
- Designed for depletion of large sample volumes, up to 150 µl, of undiluted human plasma

B102

Alterations in Protein Composition of Plasma in Patients Suffering from Hypertrophic Cardiomyopathy

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Introduction
Hypertrophic cardiomyopathy (HCM) is an inherited disease affecting myocardium and is frequently associated with high risk of sudden death, particularly in young (1). In this study, we conducted iTRAQ quantitative analysis of plasma proteins collected from both patients suffering from HCM (n=22) and the controls (n=23) in order to extend the group of markers potentially associated with the presence of this pathology (2).

Methods
The plasma samples were collected by venipuncture using BD P100 Vacutainer system (Becton Dickinson). After immunoaffinity depletion of the 14 most abundant proteins (Agilent) the pooled samples were digested and labeled using the iTRAQ probes (Applied Biosystems). In the first dimension of the proteomic workflow, the labeled peptides were isoelectrically focused on an IPG strip, pI 3-10 (GE Healthcare). The gel was cut into 34 uniform pieces and upon extraction from each slice, the peptides were separated using RP-HPLC and spotted in time dependent fractions onto a MALDI plate. Each LC fraction was analyzed on a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems). Proteins with altered concentration in patients' plasma which are known to be potentially involved in the pathophysiology of HCM were further tested using antibody-based approach with each collected sample separately.

Results
Due to the enormous concentration range of proteins the analysis of plasma belongs to the most challenging proteomic studies. Despite this fact, the combination of IEF with LC-MALDI approach allowed us identification of approximately 200 plasma proteins, and by implementing the iTRAQ technology into the workflow, we were also able to quantify the majority of them. However, although we depleted 14 most abundant proteins we did not reach the most valuable segment of plasma - very low abundant proteins - using our LC-MALDI experiment. Confirmation of the most interesting findings from iTRAQ analysis is currently under way.

Innovative aspects

- To the authors' knowledge, this study represents the first iTRAQ proteomic data focused on plasma markers of hypertrophic cardiomyopathy in human
- Several proteins found with altered concentration between patients a controls seem to be linked to the pathophysiology of HCM and thus are currently being analyzed in more detail
- Most of the alterations in detected protein concentrations seem to be a consequence of hypolipidemic and antithrombotic treatment

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B103

A proteomic and systems biology approach to dissect adenine as the contributing factor in human plasma for the antifolate resistance

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# These three authors contributed equally to this work. * corresponding author

Introduction
In current comparative proteomics, the combination of 2-DE and MS has become the most utilized workflow in tasks of protein target discovery. Systems biology is another fast growing inter-disciplinary subject that focuses on the systematic study of complex interactions in biological systems. In this study, with the aid of proteomic techniques and systems biology analysis, we identified the factor in HP contributing the antifolate resistance, and investigated the possible mechanism. This factor, which was confirmed as adenine, provides a new perception of salvage pathway in current cancer chemotherapy.

Methods

- Cell culture experiments
- 2-DE experiments
- MS protein identifications
- Systems biology analysis
- Evaluation of the antifolate resistance and measurement of the LD_{50} of antifolates
- Retrospective survival analysis of the methotraxate (MTX) treated patients

Results

1. Proteomic approaches revealed that in the presence of antifolate, aminopterin (AMT), cell proliferation related proteins such as glyceraldehydes 3-phosphate dehydrogenase and heterogeneous nuclear ribonucleoproteins A2/B1, as well as anti-apoptosis proteins such as heat shock protein were found up-regulated in the HP culture medium, but not in the fetal calf serum (FCS) culture medium. In contrast, cell death related proteins such the activator of apoptosis harakiri and prohibitin were found down-regulated in SP2/0 cells growing in the HP culture medium.
2. By employing Ingenuity Pathway Analysis (IPA) software to analysis the differentially expressed profiles of identified proteins, a DNA repair and/or energy production event was suggested for the HP treatment.
3. Factor(s) conferring the antifolate resistance was found heat stable, and with a molecular weight less than 3 kDa.
4. Taken together, it leads to the observation that the additive in the prepared HP, presumably adenine, rather than other intrinsic factors in HP, confers the antifolate resistance to the SP2/0 cells. Our results imply that with the external supply of nucleotide salvage materials cells may be more proliferative in the more energetic state. ,
5. A retrospective investigation for the survival rate of patients treated by the less toxic antifolate, methotrexate (MTX), also suggests an unnoticed role of adenine in current medicine.

Innovative aspects
A realization of translational medicine in cancer therapy by utilizing proteomic and systems biology approaches.

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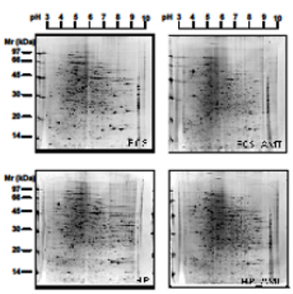

Figure 1. 2-DE analysis of the proteomes of SP2/0 cells in the four experiment sets.

Alterations in Cellular Proteome and Secretome upon Differentiation of Monocyte to Macrophage: Insights into Biological Processes

B104

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Introduction
Monocyte and macrophage are important cells involved in immune response and inflammatory processes. Monocytes circulate in the bloodstream and migrate to various tissues where they can differentiate to macrophages. However, molecular basis of biological processes involved in this cellular differentiation remains unclear. This study was therefore to investigate alterations in cellular and secreted proteins upon differentiation of monocyte to macrophage.

Methods
Macrophage was differentiated from U937 human monocytic cell line by treatment with 100 ng/ml phorbol myristate acetate (PMA) for 48 h (**Figure 1**). Cellular and secreted proteins extracted from PMA-treated cells (macrophages) were compared with those of untreated cells (monocytes) using 2-D gel electrophoresis (n=5 gels/group; stained with Deep Purple fluorescence dye). Image Master 2D Platinum (GE Healthcare) software was used for matching and analysis of protein spots in 2-D gels. Differentially expressed spots were identified by Q-TOF MS and/or MS/MS analyses. All of the altered cellular and secreted proteins were then subjected to global protein network analysis using Ingenuity Pathways Analysis tool (*http://www.ingenuity.com*).

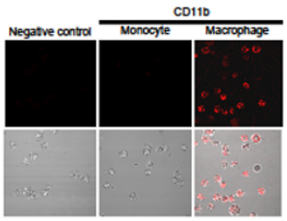

Figure 1: Expression of CD11b surface marker upon differentiation of U937 monocytic cells to macrophages by induction with 100 ng/ml PMA for 48 h.

Results
Quantitative intensity analysis revealed 81 and 67 spots whose levels were significantly altered in cellular proteome and secretome, respectively, of macrophages compared to those of monocytes. These differentially expressed proteins were subsequently identified by Q-TOF MS and/or MS/MS analyses. The altered levels of elongation factor-2, α-tubulin and heat shock protein 90β were confirmed by Western blot analysis. Global protein network analysis showed that these altered proteins were involved in cell death, lipid metabolism, cell morphology, cellular movement, and protein folding.

Innovative aspects
- Our data may provide some insights into molecular mechanisms of biological processes upon differentiation from monocytes to macrophages.

Proteomic analysis revealing GNB1 and isoform of talin related to HIV infection

B105

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Introduction
With the rapid emergence of the immunodeficiency virus (HIV)/AIDs pandemic and rapid development of multiple drug-resistant strains of virus, the comprehensive elucidation of HIV-1-mediated effects on host cellular networks is urgently needed for rational therapeutic targets. In this work, we performed the differential proteomes of peripheral blood mononuclear cells (PBMCs) from HIV-positive patients and healthy controls.

Methods
31 HIV-positive patients without anti-virus treatment and 40 healthy donors were enrolled in this research. Peripheral blood mononuclear cells (PBMCs) were isolated from 5 mL blood by ficoll density centrifugation. Proteins from PBMCs were extracted and separated by two-dimensional gel electrophoresis (2DE) (1). After ImageMaster software analysis, the differentially expressed proteins spots were cut, trypsin digested and identified by ESI-Ion trap mass spectrometry. The mRNA expression of 10 differentially expressed proteins was analyzed by real time RT-PCR. The expression of GNB1, talin-1 and Filamin-A was further validated by western-blot analysis from pooled and individual samples. Cytoscape software was used to draw a protein-protein interaction of HIV and identified host cell proteins.

Results
About 1100 protein spots were detected in silver-stained 2DE gels. 20 differentially expressed spots with 1.5- or more –fold difference (figure 1A) were successfully identified by HCT with more than four peptides, 4 of which were simultaneously identified by MALDI-TOF/TOF. These identified proteins include Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1(GNB1), Talin-1 (TLN1), Filamin-A (FLNA), Vinculin (VINC), et al. These identified proteins were involved in various cellular functions including binding (7 proteins), enzyme (4 proteins), signal transduction (3 proteins) and immune response (one protein). Of which, 6 genes were found to be statistically different between HIV-positive patients and healthy donors in the mRNA level through real time RT-PCR. The mRNA expression of TLN1 and VINC was up-regulated for 1.5 and 1.6 fold, which was agreed with the results from 2DE. The expression level of filamin-A, talin-1and GNB1 was verified by western blot analysis (figure 1B and C). Furthermore, a wide protein-protein interaction network was affected in HIV/AID patients. For example, the host protein interaction net such as VCL-TLN1-ACTG1-FLNA was found to be interacted with nef and pol (HIV function proteins), and obviously up-regulated in HIV/AID patients. The similar result was observed in ENO1-LDHB-PK3 network which interacting with rev, gp41 and gp120 (figure 2).

Innovative aspects
- Proteome analysis revealing GNB1, isoform of talin-1 and filamin-A to be affected by HIV.
- A wide protein-protein interaction network was found to be affected in HIV-positive patients.

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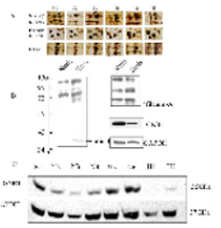

Figure1 A. The partly magnified images of five protein spots in 2DE. B. Western blot confirmation of talin-1, filamin-A and GNB1 in HIV-positive and healthy donors. C. WB of GNB1. From left to right, healthy 1, 2, 3, 4, 5 and 6, and HIV-positive patients 1 and 2.

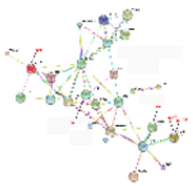

Figure 2 Protein-protein interactions of the identified proteins with HIV function proteins. The identified differential proteins were highlighted with "", HIV function proteins were marked with red.

Liquid chromatography-mass spectrometry metabonomic and proteomic technology for discovering new biomarkers of liver failure caused by Hepatitis B Virus

B106

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Introduction
It is estimated that approximately 350 million people worldwide and 120 million in China alone are infected with hepatitis B virus (HBV). Liver failure is the most emergent clinic complication which leads to high mortality. To look for new biomarkers for predicting liver failure, LC/MS-based metabonomic and proteomic strategies were used to analyze metabolites and proteins from plasma of HBV infected patients with different status of disease progression including hepatitis and liver failure.

Methods
For metabolomic analysis, metabolites from patients with liver failure (26), hepatitis (10) and healthy controls (16) were analyzed by LC-MS, followed by principal component analysis (PCA) [1] to select differential compounds. Four differential compounds were analyzed through multiple reaction monitoring (MRM) in 167 clinical samples to select possible biomarkers and through an animal model experiment to exclude the influence of medicines taken by analyzed patients. Lastly, two verified compounds were purified and identified through ESI-Q-TOF (Bruker Company).
For proteomic analysis, plasma proteins from liver failure and healthy controls were analyzed by 2DE-MS according to our previous work [2]. One differential protein was verified by ELISA experiment.

Results
For metabonomic analysis, a LC/MS-based metabonomic platform was presented which combined the discovery of differential metabolites through PCA with the verification by MRM. A pattern recognition profile that had significantly difference between liver failure, hepatitis and healthy control ($P < 0.05$) was obtained. Four different peaks with m/z of 414.5, 432.0, 520.5 and 775.0 were verified by MRM experiment in 40 clinical samples (Figure 1) and detected not to be induced by the medicines taken by patients through rat models. Through 167 clinical subjects including healthy donors, hepatitis and liver failure verification, compound with m/z of 414.5 might be a new biomarker of liver failure with specificity and sensitivity of 95.7% and 86.5% in predicting liver failure. The qualitative analysis of 414.5 will be carried.

For proteomic analysis, 25 differential protein spots were identified including 11 non-redundant proteins such as Alpha-1-antitrypsin precursor, and fibrinogen alpha chain precursor. Alpha-1-antitrypsin precursor was verified to be up-regulated in failure liver through ELISA and consistent with the result from 2DE.

Innovative aspects
- A set of LC-MS methods for selecting metabonomic biomarkers was established and verified in nearly 200 samples with liver disease.
- Animal model experiment was used to exclude the influence of medicines.
- A possible new metabolite biomarker (m/z of 414.5) and protein biomarker (Alpha-1-antitrypsin precursor) for liver failure diagnosis were verified.

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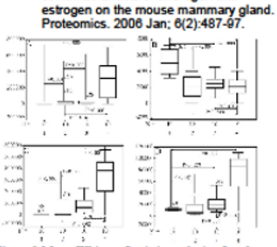

Figure 1 Mann-Whitney Statistic analysis of peak area of m/z 775.0 (A), 520.5 (B), 414.5 (C) and 432.0 (D) through MRM analysis in human plasma. 1, 2, 3 and 4 represents healthy controls, hepatitis, cirrhosis and liver failure.

IDENTIFICATION OF PLASMA PROTEOMIC BIOMARKERS IN HEREDITARY BREAST CANCER

B107

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Introduction
Breast cancer (BC) is a leading cause of death among women. Familial history of BC represents a major risk factor and germ-line mutations in BRCA1/2 genes account for most of the hereditary breast and/or ovarian cancers. Gene expression profiling studies have disclosed a specific molecular signature for BRCA1/2-related breast tumors as compared to sporadic cases.
Plasma plays a central role in clinical diagnosis and it is expected that, when reliable biomarkers will be available, proteomics will provide robust information for early diagnosis and individualized treatment of patients.
The aim of the study was the development of a comprehensive approach to identify biomarkers in plasma samples of subjects affected by hereditary breast cancer.

Methods
Plasma sample were collected in according with HUPO plasma proteins guidelines, and depleted of seven high abundant proteins using the LC Plasma 7 Multiple Affinity Removal Column (Agilent Technologies).
Two distinct approaches for global protein profiling were applied: i. Protein labeling with isotope-coded affinity tags (ICAT) followed by tandem mass spectrometry (MS/MS), ii. two-dimensional polyacrylamide gel electrophoresis (2D PAGE) coupled with MS/MS. Validation of data was performed by western blotting analysis.

Results
The integration of different techniques allowed us to detect tumor-specific changes in the plasma proteome of BC patients and healthy family carriers sharing the same BRCA1 gene founder mutation (5083del19).
We identified a set of biomarkers capable to distinguish BC affected individuals carrying the BRCA1 founder mutation from healthy carriers; additional analysis of plasma from healthy controls led to the identification of prognostic markers able to define, among the healthy carriers, a subset of individuals with an increased risk of eventually developing BC. Moreover, data obtained from this experiment were used to create, by using a computational systems biology-driven approach, an in silico network pathway of proteins in which the candidate markers are involved.
Imaging-based screening strategies have made a tremendous improvement in the identification of breast tumours; even though, a great number of women is still diagnosed at a late stage, including those with a hereditary imprinting. Gene testing approaches are expected to reveal the carrier status in families with altered BC predisposition genes. The real challenge will be to develop tools able to discriminate, among healthy carriers, those who will never develop cancer from those who will.
A serum-proteomics-based approach, like the one we propose, holds great potential in providing an answer to this dilemma, i.e. who is going to get cancer and when.
Obviously, only a few of the potential biomarkers identified so far will probably turn out to be clinically effective, but we are confident that the strategy developed will open the way to more comprehensive studies aimed at improving early diagnosis in hereditary cancer.

Innovative aspects
- Integration of two distinct quantitative and qualitative approaches
- Creation of network pathway of proteomics data

B108

Development of a Rapid Serological Test for the Determination of Strongyloides Infection

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Introduction
Strongyloidiasis is the disease caused by infection of the intestinal nematode, *Strongyloides stercoralis* with worldwide distribution, particularly in tropical and subtropical regions. Current serological tests use heterologous Strongyloides antigen derived from parasite extract to detect serum antibodies by ELISA, which is both time consuming and prone to false positive results. Here, we describe a rapid, label-free method of determining Strongyloides infection using a diffraction-based optical biosensor technology.

Methods
The dotLab System (Axela, Inc) was used to detect serum antibodies from patient samples. In brief, biotinylated NIE recombinant protein from L3 stage Strongyloides larvae was immobilized on avidin-coated biosensors and incubated with 10 ul of patient sera yielding a real-time binding curve of the antibody-antigen interaction. The sensors were then probed with anti-human IgG antibody to amplify binding signal. Samples tested were from: 1) Strongyloides positive patients; 2) healthy individuals; and 3) patients with other parasitic infections.

Results
The ratio of anti-human IgG antibody binding signal to Biotinylated NIE binding was used to determine antibody titer in test serum samples. The assay performed at a sensitivity >90% and specificity >90%. Total assay time was approximately 30 minutes, making it useful for future point-of-care applications where rapid diagnosis is required.

Innovative aspects
- High sensitivity/specificity serological assay for Strongyloides infection with potential point-of-care applications.

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Serum Proteins indicating Remission Status in Patients with Acute Myeloid Leukemia

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Introduction
The response criteria of complete remission in acute myeloid leukemia (AML) are presently based on the morphology and number of blood cells. However, it is often difficult to apply these criteria due to the inadequate quality of bone marrow samples and the loss of the cellular morphology. Here, we investigated whether the BM aspirate (serum) of AML patients contains any proteins that reflect the responsiveness of the disease throughout chemotherapy by using a multidimensional liquid chromatography-differential gel electrophoresis (MDLC-DIGE) technology.

Methods
First we performed multidimensional liquid chromatography-differential gel electrophoresis protein profiling of two pooled serum samples, taken both prior to (PreCR) and after chemotherapy (CR), from 33 patients who had achieved CR. This proteomic analysis revealed two differentially expressed proteins, complement factor H (CFH) and apolipoprotein H (ApoH). Then, ELISA assay of CFH and ApoH was performed for the sera from AML patients in various states, including PreCR and CR, as well as for samples taken prior to (PreNR) and after chemotherapy (NR) for patients who failed to achieve CR (*i.e.* non-remission) and from healthy controls (Normal).

Results
Comparison of the CFH and ApoH levels among combinations of the two groups (PreCR *vs.* CR- p_{ApoH}<0.01, p_{CFH}<0.01; PreNR *vs.* NR- p_{ApoH} >0.05, p_{CFH} >0.05; PreNR *vs.* PreCR- p_{ApoH} >0.05, p_{CFH} >0.05; NR *vs.* CR- p_{ApoH}<0.01, p_{CFH}<0.01; and CR *vs.* Normal- p_{ApoH}<0.01, p_{CFH}<0.01) revealed significant differences between the recovered (CR and Normal) and non-recovered groups (PreCR, PreNR, and NR). The results of the present study suggest that CFH and ApoH are good markers for distinguishing between CR and NR in AML patients.
Two different explanations can be given, depending on whether CFH and ApoH are being produced by malignant or normal cells. Stirewalt *et al.* reported the results of a microarray analysis of gene expression levels in AML patients[1].Their data indicate that the levels of CFH and ApoH gene expression in patients are neither decreased nor increased in comparison to those in normal controls. These observations suggest that not tumor-related factors but host factors may determine the CFH and ApoH levels in AML patients who are in CR or NR.

Innovative aspects
- Clinically, all the patients showing morphologic CR with incomplete blood count recovery (CRi) had high levels of CFH and ApoH
- Modified the three-dimensional IPAS (intact-protein analysis system)[2] by applying emPAI (exponentially modified protein abundance index) analysis and individual protein quatitation.

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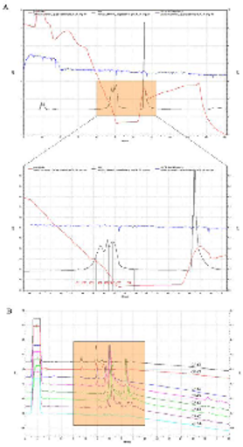

Figure 1. CF (A) and then RP (B) separations of the mixture of Cy3-labeled (pooled PreCR) and Cy5-labeled (pooled CR) proteins.

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Selection and Validation of Human Liver Cancer Plasma Biomarker Candidates

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Introduction
The human plasma directly shows healthy status, because it contains lots of proteins that are secreted from tissues to a body. But, the complex human plasma proteome has dynamic protein concentration range. So, it is very difficult to analyze the low abundant proteins that are related to disease by using the recent proteome analysis technology. Many of proteins between normal plasma and disease plasma are differently post-modified. In this work, some peptides that were quantitatively different between control plasma and cancer plasma were selected and validated MRM(multiple monitoring reaction). Steric hindrance effect by glycan using alpha 1-acid glycoprotein(AGP) was verified.

Methods
The seven abundant proteins were depleted by immunoaffinity chromatography (MAR column, 4.6 x 50 mm, Agilent). For selection peptides, depleted and digested proteome were quantitated three times for label free method by Q-TOF mass spectrometry(Waters, UK). The raw data were identified and quantitatively analyzed using Protein expression system(Waters, UK). Selected peptides of pooled normal plasma and another HCC plasma were monitored for validation were analyzed by LTQ/FT(ThermoFinnigan, U.S.A.). The result were statistically analyzed by PCA(principle component analysis). PNGase F and trypsin digested AGP and trypsin digested AGP were quantitatively analysis using spiked stable isotope labeled peptide that is related to N-linked glycosylation site in AGP by LTQ/FT.

Results
Most of identified proteins were quantitatively similar in protein level without protein separation, but some peptides that are related to N-linked glycosylation were quantitatively different in peptide level between normal plasma and disease plasma(Figure 1). So, some peptides were selected for liver cancer biomarker panel candidates. Panel candidate peptides' quantitative pattern is important. The degree of modified glycosylation in proteins are different because of steric hindrance effect of glycan between control plasma and cancer plasma. So, trypsin activity will be not similar. Selected peptides were validated by MRM(multiple reaction monitoring) and the statistical result clearly classify normal group and HCC group. Alpha 1-acid glycoprotein(AGP) standard (that include one of selected peptides) was used for verification of steric hindrance effect of glycan. In trypsin digested AGP, N-linked glycosylation related peptide was not detected. But, in PNGase F and trypsin digested AGP, N-linked glycosylation related peptide was shown.

Innovative aspects
- Selected peptides are related to N-glycan site and can be analyzed by LC/MS/MS
- Steric hindrance effect of glycan is verified by AGP

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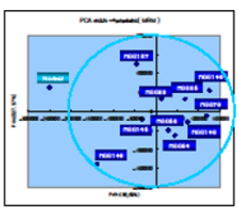

Figure 1. Principle Component Analysis(PCA) result from selected peptides shows that normal group and liver cancer plasma group are clearly classified.

B111

Early Protein Signatures of Diabetic Nephropathy: Moving toward Epidemiological Proteomics

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(Make certain you underline the presenting author's name)

Introduction We believe that the most reliable biomarkers for a human condition will most likely be discovered from large, clinically well-characterized human sample cohorts. By applying a series of discovery technologies with classic univariate and independent component analysis (ICA), we are searching for early molecular signatures of diabetic nephropathy in a cross-sectional cohort of patients with type 1 diabetes with incipient and overt nephropathy.
Methods Plasma from the GENEX cohort of patients was analyzed on an individual basis using a series of top-down approaches. High throughput anion exchange and hexapeptide bead library treatment were combined with SELDI-TOF-MS while the same samples were also subjected to a strategy involving two complementary bead types prior to MALDI-TOF-MS. All resulting data underwent a rigorous quality check and the remaining features were reduced using univariate statistics and the ICA signal reduction algorithm. Peak identification was established using tandem mass spectrometry and/or immunoprecipitation.
Results The study revealed a series of potential biomarkers that varied in size from 1 – 100 kDa. The use of several combinations of approaches provided more candidate biomarkers than any single strategy. The MALDI-TOF-MS results were stronger in the smaller mass range (1-10 kDa) while SELDI-TOF-MS proved superior in the range of 5-30 kDa. A study using a bottom-up approach is currently being performed as well. As with other top-down approaches, most of the identified proteins are acute phase reactants, lipoproteins or involved with the complement system. While the proteins we have identified are not specific for diabetic nephropathy we do believe that they will be important in better defining a patient's biological state and, therefore, a general risk for disease susceptibility. Chronic low grade inflammation, as measured by CRP levels, is a risk factor for cardiovascular disease and we postulate that the same is true for diabetic complications. The standardized methods established here, especially magnetic bead fractionation and MALDI-TOF-MS, will be carried forward to the analysis of much larger cohorts that allow an epidemiological analysis of risk factors for type 2 diabetes.
Innovative aspects
- Employed multivariate feature reduction in clinical proteomics
- Combined several different processing and analysis strategies
- Methods are basis for epidemiological proteomics

Verification of Protein Biomarker Candidates in Blood: An MRM-Based Approach

B112

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Introduction:
Protein biomarker candidates must be quantitatively verified before they can progress to clinical validation. For measuring proteins quantitatively in a complex sample matrix such as plasma, MRM surpasses other analytical methodologies in terms of specificity, sensitivity, as well as multiplex capability. Here we demonstrate the steps involved in optimizing MRM workflow for verification of potential biomarkers in human plasma. The processes include protein extraction, digestion, optimization of MRM transitions, liquid chromatography, and quantification.

Methods:
After protein extraction, plasma was spiked with trypsin-digested dynamic range mixture of 48 proteins. Trypsin digests were analyzed by nano-flow LC/MRM using a reversed-phase C_{18} column coupled online to a hybrid triple quadrupole-linear ion trap mass spectrometer (QTRAP 4000). MRMPilot was used to select Q1/Q3 pairs through experimental data and *in silico* digestion. MRM transitions were then experimentally tested for robustness and specificity. Those proteins whose peptides showed poor robustness and were deemed to be non-specific were re-optimized using a larger number of peptides per protein. Using this approach, three optimization runs were required for 200 MRM transitions. Quantitation was done using MultiQuant software.

Preliminary Results:
Protein extraction using depletion and precipitation were compared for robustness and reproducibility. The test case employed two proteins, 1 peptide per protein and two transitions per peptide. The first part of the comparison tested reproducibility over 3 LC/MRM experiments (using the same depleted and precipitated samples). CVs of absolute peak area ranged from 20% for precipitation to over 60% for depletion. Retention time CVs were 2% for precipitation and 9% for depletion.

Summary:
The success of MRM quantification relies on many factors. These include sample processing, LC separation, and Q1/Q3 selection and optimization. Once these parameters are optimized, one LC/MRM experiment can be used to reliably quantify as many as 48 proteins 200 transitions, having a wide dynamic range, with CV<=20%.

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Low Abundance Endogenous Peptides of Normal Human Serum with Aqueous-Based Extraction from the Acetonitrile Pellet and Analysis by C18 LC-ESI-ion trap

B113

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Introduction
The endogenous peptides from human serum were precipitated with proteins in acetonitrile and extracted with acidified water before separation by reversed phase liquid chromatography and analyzed using C18 LC-ESI with a Paul ion trap. Peptides from a variety of high abundance proteins and many low abundance proteins were confidently identified. Multiple peptides and even overlapping peptides were observed from many proteins that would not be expected to show high concentrations in the blood.

The clear identifications of low abundance endogenous polypeptides from blood by ESI based mass spectrometry is a major step and precondition towards the discovery of peptides that vary between physiological states.

Methods
Serum was pre-fractionated by precipitation in acetonitrile followed by extraction of the pellet in organic solvents and water prior to drying, collection over preparative C18 chromatography, and further fractionated over an analytical C18 column coupled to an Agilent 1100 series capillary pump with an acetonitrile gradient prior to analysis by ESI ionization MS and MS/MS. Peptides were ionized via a metal needle electrospray head into a Thermo XP100 ion trap. Endogenous peptides that may be generated by C terminal or N terminal exo-peptidases were searched under no enzyme conditions [X][X] using the X!TANDEM algorithm on separate personal computers and the results subsequently assembled in an SQL relational database. The spectra were analyzed by X!TANDEM with –1 D and +3 Da as the tolerance of the parent mass and 0.4 Da error in the fragments. The peptides identified against forward human libraries were compared to those of reverse human, and Bos Taurus, Danio rerio and Arabidopsis proteins. Detection of peptides from the same types of proteins were confirmed by Qq-TOF with no enzyme correlation using the Paragon algorithm.

Results
Experiments using the arbitrary control of reversed library, or heterologous library, searching indicated that endogenous peptides assigned to proteins with aggregate expectation scores of log-16 or lower by X!TANDEM and at least three different peptides were reliably detected from serum. Proteins closely associated with cell growth, gene expression or development were detected in serum with scores lower than log – 16, with multiple peptides, and often displayed overlapping peptides from the same site in the apparent low abundance protein.

Endogenous peptides from coagulation factors, collagens, serum amyloid, zinc finger protein, bromo domain protein, erythroid differentiation factor, extracellular matrix proteins, mixed lineage leukemia and many other evident low abundance peptides were apparently detected by ion trap. The presence of these and many other low abundance peptides were confirmed with high mass accuracy by LC-ESI-Qq-TOF. In addition, the presence of the parent proteins were confirmed by tryptic digestion and LC-ESI-ion trap. Taken together the low total expectation scores, the multiple peptides from each parent molecule, the often overlapping distribution of peptides, the confirmation of parent mass and the near complete agreement with previous trypsin digest experiments all seem to confirm that peptides from biologically significant molecules may be directly detected from serum by mass spectrometry with only a simple preparative step of acetonitrile precipitation and extraction with aqueous based buffer prior to LC-MS/MS.

Innovative aspects
-Novel, rapid and effective method to extract low abundance endogenous peptides from serum.

-X!TANDEM scoring parameters against reverse and control libraries for no enzyme peptides

-Biologically interesting peptides associated with cancer were confirmed by three methods.

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Comprehensive analysis of short peptides in sera from patients with IgA nephropathy

B114

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Introduction

IgA nephropathy (IgAN) is the most common form of primary (idiopathic) glomerulonephritis in the world. Although renal biopsy is essential for diagnosis of IgAN, the biopsy is rather invasive and sometimes dangerous for patients. Accordingly, novel biomarkers for diagnosis and monitor of IgAN should be established. In this study, we analyzed short peptides in the serum of untreated IgAN patients and healthy donors by MALDI-TOF mass spectrometry, and IgAN-related peptides were searched using a multivariate data analysis tool, orthogonal-partial-least-squares-discriminate analysis (OPLS-DA).

Methods

Serum samples from 26 patients with untreated IgAN and 25 healthy donors were tested. Short peptides with molecular weight of ~7kDa were purified from the serum samples by magnetic bead-based weak cation exchange (Bruker Daltonics), and detected by MALDI-TOF mass spectrometry. Peak area data of the detected peptides were analyzed by OPLS-DA to calculate models for the separation between the IgAN patients and the healthy donors. The discriminative performance of OPLS-DA models was evaluated based on the four parameters, sensitivity, specificity, R2Y, and Q2 according to a 7-fold full cross-validation. Variable importance in the projection (VIP)-plot and "S"-plot were generated to select candidate biomarker peptides that were mainly responsible for the separation of the two groups.

Results

By purification using magnetic bead-based weak cation exchange and MALDI-TOF mass analysis, total of 97 peptides peaks were detected in the tested serum samples. The profile of all the peptide peaks generated an OPLS-DA model that discriminated the IgAN group and the healthy group completely (sensitivity = 1.0, and specificity = 1.0) with a high R2 value (0.92) and a high Q2 value (0.861). Among the 97 peptides, 25 peptides were considered to contribute to the IgAN / healthy discrimination significantly (VIP > 1.0). Further, use of the profile of only 6 peptide peaks (5 increased and 1 decreased in the IgAN group) was found to effective for the discrimination of the two groups.

In conclusion, we demonstrated that the serum peptide profile was a useful tool to discriminate IgAN from healthy conditions, of which the 6 peptides may be candidate serum markers for the diagnosis of IgAN and may be related to pathogenesis of IgA. Further investigation of the biomarker candidate peptides may help understanding of etiology or pathophysiology of IgAN.

Innovative aspects

- Serum peptidomics of IgA nephropathy using magnetic bead-based weak cation exchange and MALDI-TOF mass spectrometry.
- Application of orthogonal-partial-least-squares-discriminate analysis (OPLS-DA) to the serum peptidomics data.
- Novel biomarker candidates for the diagnosis of IgA nephropathy

A proteomic approach to identify biomarkers for naturally occurring regulatory T cells

B115

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Introduction
Regulatory T cells (Tregs) maintain peripheral tolerance and immune homeostasis by their ability to suppress activation of other immune cells. So far the mechanisms behind this critical function are relatively poorly understood. To define a protein expression profile and identify potential candidates involved in immune suppression, we compared the protein expression profiles in Tregs vs. effector T cells (Teffs) from human blood. Candidate proteins were identified in several subcellular compartments and subjected to phenotypical verification and functional testing in Treg suppression assays.

Methods
Tregs and Teffs were pre-enriched from human buffy coats by enriching for CD4+ cells. To ensure highest possible purity of cells, Tregs and Teffs were isolated by FACS (Fluorescence Activated Cell Sorting) prior to subcellular fractionation, SDS-PAGE, in-gel trypsination, chemical labeling, sample preparation and analysis by LC-LTQ-Orbitrap MS (Fig. 1). Mass spectrometric data were searched against the Swiss Prot human proteome database using MASCOT as the search engine. Quantitative analysis was performed using MSQuant. Candidate proteins were further validated by RT-qPCR, flow cytometry, western blot analysis and functional analysis by the Treg suppression assay (2).

Results
We have identified approx. 30 proteins that seem to be uniquely or highly expressed in the Treg population as compared to Teffs. Our mass spectrometric data were confirmed by flow cytometry and western blot analysis on a selected set of proteins, in addition to RT-qPCR on all our candidates. Interestingly, most of the identified proteins revealed only marginal differences on mRNA expression level, thus confirming the importance of a proteomic approach rather than a microarray/RNA-based approach in the search for Treg biomarkers.
Several of our candidates were identified as transmembrane proteins defining so far uncharacterized Treg subsets. Furthermore, functional studies on these subsets suggest that one of these membrane proteins splits the Treg population into two functionally distinct populations. Cells expressing the protein at a high level were highly suppressive, while the low expressing cells displayed little or no suppressive ability.

Innovative aspects

- Purification and isolation of primary T cells by FACS prior to subcellular fractionation ensures highest possible purity of cells and reduces complexity prior to proteomic analysis
- Identification of novel Treg biomarker whose cell surface expression correlates highly with immune suppression
- Identification of novel markers for Treg subpopulations

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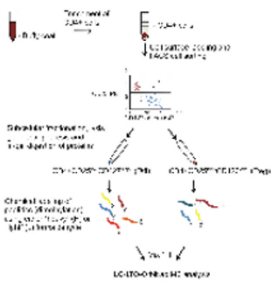

Figure 1. Work flow of the strategy applied to identify Treg biomarkers

B116

Protein Profiles of Peripheral Blood Mononucleocytes are Useful for Discrimination of Ulcerative Colitis from Crohn's Disease

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Introduction Ulcerative colitis (UC) and Crohn's disease (CD) are two major disorders of intractable inflammatory bowel diseases (IBD). In early phases of IBD, it is sometimes difficult to discriminate between UC and CD. Thereby, it would be beneficial if biomarkers to distinguish between UC and CD are established. We thus here comprehensively analyzed proteomes of peripheral blood mononucleocytes (PBMCs) from UC and CD patients to find protein profiles that can discriminate between the two diseases. Further, we tried to elucidate relationship between clinical parameters of the diseases and the PBMC-derived protein profiles.

Methods Proteins, extracted from PBMCs, were separated by 2D-DIGE. Intensities of the separated protein spots was compared among UC patients (n=17), CD patients (n=13), and age- and gender-matched healthy subjects (n=14). The protein spots with significantly different intensities among the UC, CD and healthy groups were selected by ANOVA. They were subjected to a multivariate analysis using SIMCA-P+ to discriminate between UC and CD. Intensities of all the spots were used for the multivariate analysis to predict the clinical parameters.

Results We obtained in total 547 protein spots common to the UC patients, the CD patients and the healthy subjects. 276 protein spots with significantly different intensities among the UC, CD and healthy groups were selected from the 547 spots by ANOVA. The OPLS analysis of the 276 protein spots established the discrimination between the UC group from the CD group (R^2=0.994, Q^2=0.462). Further, use of 58 protein spots selected by the reliability and the magnitudes of the spots improved the discrimination ability (R^2=0.948, Q^2=0.566). A similar analysis of the PBMC-derived protein profile achieved excellent prediction of clinical parameters in the case of UC as followings: R^2=0.960, Q^2=0.660 for the disease duration; R^2=0.776, Q^2=0.760 for the disease activity; R^2=0.961, Q^2=0.821 for distribution of the intestinal lesions; R^2=0.945, Q^2=0.909 for CRP and R^2=0.980, Q^2=0.595 for responses to the treatments. Taken together, the proteomic analysis of PBMCs would be useful for the differential diagnosis of UC and CD. In UC, the proteins which contributed to generation of the clinical parameter-associated protein profiles may play important roles in the pathophysiology of the disease.

B117

Large-scale Proteomic Discovery of Candidate Biomarkers Predictive of Trauma Patient Outcomes

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Introduction
The host response to injury is a collection of pathophysiological processes that depend critically upon the regulation of the innate immune system. High throughput quantitative proteomics is promising for profiling potential abundance changes involved in the innate immune response. Although current clinical proteomics studies have mainly focused on plasma/serum, the circulating leukocytes represent another set of easily accessible samples for such studies. Enriched leukocyte populations are particularly suitable for studying the immuno-inflammatory response and for discovering potential markers predictive of clinical outcomes such as severe trauma and new targets for therapeutic interventions. However, the analysis of clinical leukocyte samples has generally been challenged by the sensitivity and reproducibility of current proteomics technologies due to the often very small sizes of available samples.

Methods
Venous blood samples from subjects suffering severe trauma (i.e., major car accidents) were collected directly into 8-mL CPT™ cell preparation tubes (BD, NJ). Samples were collected at seven time-points from 1 to 28 days after admission. Leukocytes (monocytes and T-cells) were specifically isolated from each subject. The individual cell samples were processed using a trifluoroethanol (TFE)-based protocol with trypsin. For quantitative analyses of individual samples, an ^{18}O-labeled universal reference peptide sample was generated and spiked into each clinical sample to enable ^{16}O/^{18}O based quantitation. All peptide samples were analyzed by LC-MS and MS/MS on an LTQ-Orbitrap instrument, and peptides were identified using an accurate mass and time (AMT) tag strategy.

Results
In this work we present the first of its kind large-scale LC-MS based population proteomics application for discovery novel protein candidate biomarkers predictive of clinical outcomes of severe trauma patients. High-throughput quantitative LC-MS analyses were performed for ~1500 longitudinal clinical blood leukocyte samples (T-cell and monocyte) from ~100 severe trauma subjects. Preliminary results revealed interesting information regarding signalling pathways related to host response and ~25 proteins were found as potential candidates predictive of multiple organ failure based on data from early time-points.

The initial AMT tag databases for each cell type were created by 2D-LC-MS/MS analysis of both control and trauma samples. For quantitative analysis of individual clinical samples, we applied the ^{18}O-labeled "universal" reference strategy so that all analyzed samples over the span of two years can be quantitatively compared with the same labelled reference sample spiked into each patient sample. The reproducibility of this high throughput quantitative approach was evaluated for both instrumental and sample processing variations. The results revealed that the main variations for analytical replicates were introduced by the cell isolation process. Average CV's of ~15% and ~25% for relative peptide abundances were observed for the analytical replicates of clinical samples, while CVs of ~40% were observed among control samples (i.e., healthy subjects). Our results demonstrate that the ^{18}O-labeled "universal" reference strategy is an effective enabling approach for large-scale quantitative proteomics where sample analyses require a long period of time.

Innovative aspects
- The first of its kind large-scale population proteomics application illustrates the potential for discovering novel protein predictors of disease outcomes.
- The ^{18}O-labeled "universal" reference strategy as an enabling approach for large-scale quantitative proteomics where analyses require long period of time.

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B118

Low molecular weight serum protein analysis using acetonitrile precipitation in rheumatoid arthri

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Introduction
Rheumatoid arthritis (RA) is a chronic auto-immune disease, characterized by development of synovitis. Application of proteomic technologies enables large-scale profiling of proteins biomarkers in tissues and serum from rheumatoid arthritis patients for diagnosis and prediction in the clinical setting. Serum potentially carries an archive of important informations in which low abundant proteins make up about 1% of the entire human serum proteome (Tirumalai et al). It is therefore imperative to deplete the level of abundant proteins in the characterization of serum by proteomic analyses.

Methods
Samples of peripheral blood were collected form 50 newly diagnosed active RA patients, 25 males and 25 females, aged 40.3 +/- 10.78, with a disease course of 5.36 +/- 1.9 months, and 40 years of age and sex matched healthy persons. The samples were divided into 10 groups containing 5 samples of patient serum and 5 samples of healthy serum to undergo. Low-molecular-weight proteins (LMWP) were enriched by ACN fractionation method (Kay et al). Low molecular weight serum proteins were separated using ACN fractionation by one dimensional SDS-PAGE followed by two-dimensional gel electrophoresis. After comparative proteomic analysis, the different protein spots were identified using matrix-assisted laser desorption ionization-time of flight-ionization-mass spectrometry (MALDI-TOF-MS).

Results
Fig.1 shows the fractionation pattern of serum lower molecular weight proteins. The PDQuest analysis of the gels showed that the expression level of apolipoprotein A-I (ApoA-I), IgG heavy chain fragment and transthyretin chain B of the RA patients were significantly higher than those of the healthy controls. Expression level of a cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) was found to be increased 3 times in patients serum which functions as a T cells activator (Kremer et al). Altered expression was also observed by MALDI-MS analysis of identified proteins.

Innovative aspects
- Effective enrichment of lower molecular weight proteins from serum of rheumatoid arthritis patients and healthy controls using ACN based fractionation.

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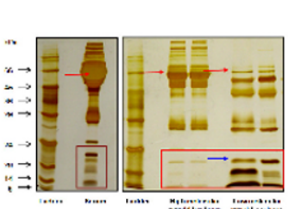

Figure 1 Fig. 12% SDS-PAGE analysis of two replicate serum extractions using the ACN fractionation method.

B119

Diagnostic Cerebrospinal Fluid Proteome Profiling for Multiple Sclerosis

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Introduction
Multiple Sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) of putative autoimmune etiology (ref.1). Early discrimination between MS and Neuromyelitis optica (NMO) is important, as optimum treatment for both diseases may differ considerably. Although finding of anti-aquaporin 4 (AQP4) autoantibody in NMO patients' serum (NMO-IgG) spawned a new diagnostic criteria, there is a claim that NMO-IgG plays a primary role in the pathogenesis of NMO is still in doubt. For the development of a novel diagnostic criteria, here we adopted an affinity bead-based purification technique following mass spectrometry (ClinProt system) to investigate the protein patterns present in the cerebrospinal fluid (CSF) from patients with MS, NMO and other inflammatory and degenerative CNS diseases.

Methods
Magnetic bead-based purification followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), ClinProt system, was employed to profile human cerebrospinal fluids (CSF) proteins and peptides in a total of 107 samples from patients with definite MS (remission and relapse), Neuromyelitis Optica entity (remission and relapse), primary progressive multiple sclerosis (PPMS) and MS with a long cord lesion (LCL-MS). As a control, amyotrophic lateral sclerosis (ALS) and other inflammatory neurological diseases (OIND) were enrolled in this study. All study participants gave their written informed consent. We used a reagent set of chemically coated magnetic beads, reversed phase (C8) and α- cyano-4-hydroxycinnamic acid as the matrix solution. The eluted samples were analyzed by *Autoflex II* with an aid of robotics (Bruker Daltonics). The criteria for peak detection were: signal-to-noise ratio >5, 2-Da peak-width filter, and maximum peak number of 200. The pretreated data were graphed as spectra and evaluated by statistical analysis using theThe *ClinProTools* and *BioTyper* software (Bruker Daltonics).

Results
Multiple components from CSF samples were detected as clear signals in the mass range of 0-20kDa (Figure 1). Obtained data are graphed as columns representing normalized peak intensities and further analyzed by a multivariate statistical analysis including principal component analysis (PCA). The results showed a differential distribution of samples from MS and NMO both in relapsing stage. Samples from PPMS were not separated effectively in the same analysis. Interestingly, samples from LCL-MS in relapsing phase were well classified into NMO entity, of which trend was retarded in a remission stage. In conclusion, the application of magnetic bead-based separation combined with MALDI-TOF-MS technique for CSF samples holds the potential to advance our understanding of the biochemical basis of MS and NMO entity.

Innovative aspects
- ClinProt system, applied into CSF proteomics gives reproducible profiles that can differentiate diseases as well as the clinical stages of the CNS disease.
- Using a multivariate statistic, CSF samples from MS in relapsing and NMO in relapsing phase can be clearly discriminative. With this criteria, CSF samples from LCL-MS and PPMS are re-classified.

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Figure.1 Typical ClinProt spectrum using a C8 magnet beads : the *m/z* range of 1000-15000.

Acknowledgements
We thank Dr.Keiko Tanaka for analyzing serum anti aquaporin-4 antibody for our patients. We also thank Mr. Atsuhiro Kanzaki for excellent statistical assistance and discussions.

B120

Sensitive Peptidomics Profiling of Native Plasma Samples by Efficient Tandem Mass Tag Labeling

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Introduction
Native peptides and polypeptides carry a relevant portion of proteomic information. However, the analysis of native peptides in plasma is challenging due to its high complexity and sensitivity towards artificial proteolysis. As a consequence, it has been difficult to differentiate analytical artifacts from truly regulated peptides. We have developed a novel peptidomics workflow by using tandem mass tag (TMT) labeling for the quantitative measurement of peptides. We introduce a multi-step extraction protocol for peptides aiming to measure low abundant molecules such as hormones and cytokines.

Methods
Native human plasma samples were labeled using TMTsixplex reagents under optimized conditions. Afterwards, the extraction of peptides was achieved by using an optimized multi-step procedure deploying a combination of membrane ultrafiltration, cation exchange as well as multiple reversed phase solid phase extraction steps. Peptides were measured by means of LC-MS/MS analysis using various electrospray MS instrumentations. Data were preprocessed, integrated and annotated using a specialized peptidomics bioinformatics workflow. Quality control was done by spiking the plasma samples with reference peptides enabling a validation of the analytical platform by MS, MS/MS and ELISA measurements. The efficiency of protein depletion was monitored by means of an albumin ELISA.

Results
Native plasma samples were labeled after reduction and carboxamidomethylation under denaturing conditions. It was possible to extract the peptides in an efficient manner while depleting proteins to be able to increase the total loading capacity of the analytical column. We show by protein determination and ELISA that the efficiency of protein depletion after ultrafiltration, cation exchange and reversed phase extraction is 99.8 % while retaining more than 75% of spiked peptide standards. With this the loading capacity of the LC column could be extended from sub-microliters to a plasma equivalent volume of up to 100 microliters using conventional ToFToF or QTOF MS-instruments. Consequently, for low abundant peptides of 1 pmol/liter concentration the total amount on the column is 100 attomoles, which can be quantified efficiently by many modern ESI instruments. The results show the richness of the native human peptidome and enable the creation of a comprehensive peptidomics database of low abundance peptides defining novel landmarks.

Innovative aspects
- Labelling of whole plasma proteome to allow for reference-control by TMT-labelling
- Controlled depletion of proteins to allow processing of 100µl plasma per LC-MS/MS analysis
- New level of sensitivity accessible for quantitative plasma proteomics

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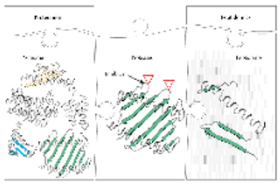

Fig.1: The richness of the proteome is to a large extent generated by proteases which convert proteins in peptides. A peptidomics technology therefore provides the opportunity to monitor the activity of proteases and protease inhibitors. In plasma proteomics the removal of proteins is key to address low concentrated peptides.

B121

Circulating Lamin B1 (LMNB 1) Biomarker Detects Early Stages of Liver Cancer Patients

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Introduction
Hepatocellular carcinoma (HCC) is a major liver malignancy possessing a high mortality rate and is particularly prevalent in China and Asia (1). While early stage HCC can be readily treated by surgery, the asymptomatic features of HCC during the course of neoplastic development and malignant progression evade conventional diagnosis. Therefore, HCC patients all too often tend to be diagnosed at advanced disease stages and endure poor clinical outcomes. We employed a proteomic expression profiling approach to identify biomarkers for early stages of HCC and subsequently assessed the clinical feasibility of a novel marker in plasma.

Methods
Frozen liver tissues from a retrospective cohort of 75 liver patients (39 HCCs, 20 cirrhosis and 16 non-diseased subjects) were subjected to proteome-wide expression profiling by 2-DE. Differentially expressed proteins were characterized by MALDI-TOF/TOF, and confirmed by immunoblotting, RT-PCR and immunohistochemistry. RT-PCR was used to further analyze the abundance of selected biomarker at mRNA level in a separate cohort of 63 plasma samples (35 HCCs, 16 liver cirrhosis, 12 healthy individuals).

Results
We successfully identified lamin B1 (LMNB1) protein to be significantly upregulated in HCC tumors. LMNB1 functions in nuclear envelope lamina and possesses a transcriptional co-regulatory activity having an important role in DNA replication, cellular ageing and stress responses. Clinically, while the expression level of lamin B1 correlated positively with tumor stages, tumor sizes and number of nodules, the upregulation of lamin B1 level was generally high in the HCC cell lines. The present study further demonstrated circulating LMNB1 marker in plasma could detect early stages of HCC patients by RT-PCR, with detection sensitivity of 76% and specificity of 82%.

Innovative aspects
- Lamin B1 is a potential marker that acts to detect early stage HCC.
- Overexpression of lamin B1 is positively correlated with the tumor development.
- The finding of a detectable quantity of *lamin B1* mRNA in the plasma of early stage HCC patients.

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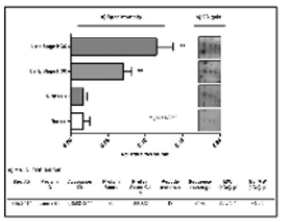

Fig. 1. Lamin B1 protein detects HCC of different tumor stages. Overexpression of lamin B1 was characterized by 2-DE proteomic profiling in different stages of HCC tumors compare to the non-malignant controls (normal and cirrhosis).

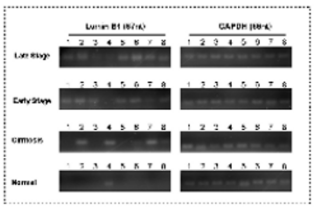

Fig. 2. Circulating *lamin B1* mRNA expression in human plasma by RT-PCR. Representative DNA gels of *lamin B1* and *GAPDH* RT-PCR products. The images

B122

Human Uremic Erythrocytes. A Role for the Membrane Proteome

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Introduction
The patients with chronic kidney disease in haemodialysis present low quality erythrocytes that show higher rigidity. So, their life time is reduced compared to erythrocytes from healthy subjects. On the other hand, it is not clear the influence of erythropoietin (EPO) administered during dialysis in the quality of these cells. We hypothesize that the membrane structure may play a role in the flexibility properties of erythrocytes. In the present work we investigate possible differences in the membrane proteome of uremic patients with and without dialysis (administered or not with EPO) comparing them to healthy individuals by DIGE technology.

Methods
Four individuals per group (control, chronic kidney disease stage V not in dialysis and dialyzed patients) were selected. Erythrocytes were isolated from plasma after maturation at 4°C for three days. They were washed three times and lysed in a total of five cycles in order to isolate the membrane fraction. Membrane proteins were solubilized in 7M urea, 2M thiourea, 4% CHAPS and purified on chromatographic minicolumns prior to CyDye DIGE fluor saturation labeling.

Results
The DIGE analysis was performed with a total of 12 gels: 3 groups (control, chronic kidney disease stage V not in dialysis and dialyzed patients) and 4 biological replicates per group, with a number of detected spots in the range 740-810. Among them, 30 confirmed spots were differentially expressed ($p<0,05$) between at least two of the three groups investigated, most of them in the high molecular range. In particular, 21 spots were differentially expressed between the control and dialyzed patients (10 were upregulated; 11 downregulated); 10 spots were differentially expressed between the control and chronic kidney disease stage V not in dialysis patients (2 upregulated; 8 downregulated) and 11 were differentially expressed between dialyzed and not in dialysis patients (5 upregulated; 6 downregulated). The PCA Anova analysis was able to classify the samples in the three groups investigated.
Identification and validation of the differentially expressed proteins is currently ongoing.

Innovative aspects
- A possible role for the membrane proteome of uremic erythrocytes is investigated.
- The influence of EPO is also investigated by comparing dialyzed patients (EPO administered) and not in dialysis uremic patients (non-EPO administered).

B123

A Multidimensional Separation Strategy for Proteomic Profiling of Prostate Cancer Sera

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Introduction
Human serum is frequently used for the discovery of new disease markers, or studying protein expression patterns in human biology. It is a challenge to characterize the serum protein content due to its protein complexity. The ability to characterize a complex protein sample by mass spectrometry depends on the power of the separation techniques employed prior to the MS analysis. Here we introduce a combination of affinity depletion, off-gel electrophoresis and reverse phase separation to reduce the sample complexity with great fractionation capability in analysis of serum samples from prostate cancer patients and healthy subjects.

Methods
Sera of prostate cancer patients were obtained from Bioreclamation inc. Prostate cancer and control serum samples were first depleted of thirteen high abundant proteins by an Agilent multiple affinity removal column. The depleted sera were further fractionated in-solution by isoelectric point using an Agilent 3100 OFFGEL fractionator (OGE). The OGE fractions were then separated by a reversed-phase chromatography using a high-recovery superficially macroporous column (mRP). The OGE and mRP separations for each sample type were plotted in a 2D map using Agilent visualization software to enhance identification of protein expression differences among the two sample groups. RP fractions with 2D map differences were in-solution digested, and subjected to LC/MS/MS analysis using HPLC-Chip/ 6520 QTOF.

Results
In this study, we demonstrate a multidimensional gel and label free approach for studying and visualizing proteomes of prostate cancer sera. 2D intact proteome visualization maps of depleted serum samples from a healthy control and prostate cancer patient were compared and contrasted. A select number of fractions among each group based on band intensity changes, were analyzed for protein identification. The differences in protein expression levels will be reported.
The presented separation techniques and methods exhibited high sample load tolerance and enabled excellent peak capacities. Additionally, the methods delivered exceptional resolution, and enabled high protein recovery and demonstrated good run to run reproducibility [1-3]. This work flow shows as a proteomic platform for greatly reducing sample complexity for comparative studies of human disease states.

Innovative aspects
- Sample complexity reduction by affinity depletion and intact protein prefractionation with off-gel isoelectric focusing and high recovery reverse phase chromatography
- Two dimensional visualization maps for comparing, contrasting and analyzing complex proteome

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Proteomic and Glycomic Characterization of Glycoproteins for Prostate Cancer Detection

B124

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Introduction
There is a crucial need to discover novel biomarkers to improve the specificity of prostate cancer detection. To achieve this goal, quantitative proteomic profiling can be used to identify changes in protein expression levels between prostate cancer and non-cacer groups in sera. Furthermore, changes in glycosylation patterns of the glycoproteins can potentially further improve the specificity of cancer detection if the specific glycosylation forms are associated with cancer development. Thus, combined proteomic and glycomic patterns can potentially improve the specificity of cancer detection.

Methods
With the rapid developments in glycoprotein isolation and stable isotope labelling technologies, mass spectrometry-based proteomic analysis is able to quantify and identify thousands of glycoproteins in a high-throughput fashion. The identified glycoproteins with high prostate-specificity, cancer specificity, and serum detectability are selected as candidate serum markers. To profile glycosylation patterns of the glycoprotein candidates between cancer and non-cancer groups, a two-step analytical platform, including a high-density lectin microarray and an electrochemiluminescent-based lectin-antibody immunoassay, was developed. Both steps are able to provide sensitive, reproducible, and high though-put analyses.

Results
We have previously identified glycoproteins specifically expressed in prostate cancer tissue using glycopeptide isoltaion[1] and LC-MS/MS[2]. Eight glycopeptides were selected for quantitative analysis using synthetic heavy-isotope-labeled peptides, and 5 of the 8 glycopeptides were quantified in patient sera[3]. Furthermore, the 5 targeted proteins were analyzed in other major cancer sera to determine the cancer type specificity. Four of the 5 proteins highly expressed in sera from prostate cancer patients including PSA were selected as candidate serum markers for prostate-specific detection.

Further, to determine the glycosylation changes associated with cancer, the 4 candidate glycoproteins were first extracted from patient specimens and detected by a high-density lectin arrays to globally profile protein glycosylation patterns. The lectins that have different binding ratios to the glycans of candidate glycoproteins were then selected as targets. The ECL-based immunoassays were developed with LOD down to ng/mL of PSA in serum and CV < 5%. The ECL-based immunoassays were used to verify the glycosylation changes in tissue and serum samples in the validation study.

Innovative aspects
- Absolute quantification and high-throughput proteomic analysis using glycoprotein isolation
- Ultra-sensitive and high-throughput glycomic analysis in clinical specimens
- Combination of both proteomic and glycomic analyses for prostate cancer marker discovery
-

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Global Proteomic and Bioinformatic Strategies for the Analysis of Human Platelets

B125

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Introduction
Platelets are key components of the haemostatic system, involved in arrest of blood loss following injury. Released into the blood stream through budding of megakaryocytes, platelets are small anucleate fragments, but contain low mRNA levels enabling limited protein translation. These features make MS-based proteomics one of only a few techniques available for the comprehensive molecular analysis of platelets. This study used high resolution MS followed by in-depth bioinformatic analysis using tools at the Global Proteome Machine to explore platelet protein complement and higher organization.

Methods
Whole blood was drawn from the antecubital vein of healthy human volunteers into 0.15% acid-citrate-dextrose and platelets were isolated and washed by centrifugation. Platelets or Jurkat or HEK293 cells were lysed in 1X TX-100 lysis buffer. Approximately 250 µg protein/sample was separated by 1D SDS-PAGE, Coomassie-stained and cut into 40 bands. Standard tryptic in-gel digestions, with no reduction or alkylation, were performed followed by nano-HPLC MS/MS analysis on an Orbitrap or FT-ICR (Thermo Scientific). Identification of proteins was performed using the X!Tandem algorithm at the Global Proteome Machine (www.thegpm.org). Further analysis using GPM-linked tools including the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were conducted.

Results
This fast, unbiased proteomic approach led to an average of nearly 1,400 high confidence protein identifications from n=4 separate experiments, greater than the estimated 1,100 proteins identified in all previous platelet proteomic studies combined (1). The datasets were found to be consistent, with 570 proteins found in all datasets. Using these large datasets, two GPM-linked tools were used to explore platelet features: GO, which categorizes proteins based on molecular function, cellular component or cellular process, and KEGG pathways mapping. Known platelet characteristics, including a lack of ribosomal proteins and under-representation of pathways relating to transcription, were readily confirmed using both GO and KEGG, and features were found to be consistent across datasets. To determine the validity of using these tools to infer specific information regarding organizational features and/or function of platelets, comparisons to similar-sized datasets from nucleated cells (Jurkat and HEK-293) were conducted. These revealed features unique to platelets, or over-/under-represented in platelets, including pathways related to metabolism, cytoskeleton and organelle compartments.

Innovative Aspects
- 1D SDS-PAGE separation followed by high resolution mass spectrometry resulted in large datasets allowing the most extensive proteomic analysis of platelets to date
- This approach highlights the utility of the GPM, the largest curated repository of MSMS data, to aid in-depth systems biology-style studies, providing access to higher organizational features of biological systems using tools such as gene ontology and pathways analysis

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Plasma proteome profiling in Fetal Growth Restriction

B126

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Fetal growth restriction (FGR) is a pregnancy complication in which normal gestational growth is not maintained. This occurs due to a lack of nutrient and/or substrate supply to the fetus, brought about by maternal, fetal, or placental disease conditions. FGR infants have greatly increased risk of perinatal death or metabolic health complications in later life. Insufficient availability or transfer of nutrients across the placenta is a major hallmark of the disease. Although its etiology is considered to be diverse, little is known of the biochemical factors that contribute to the disease. Uncovering protein changes associated with the disease will indicate patho-biological factors underlying FGR that will support a better understanding of the disease manifestation. Quantitative proteomic profiling of plasma by 2-D gel electrophoresis was therefore undertaken. Maternal plasma collected at delivery from FGR pregnancies with birthweight <3rd percentile and gestational age matched (28 – 36 weeks) healthy pregnancies were compared. To maximize the protein spot resolution changes, an immunoaffinity-based depletion technique that removed interfering proteins albumin and IgG from plasma was used. ELISA revealed 93 ± 1.4% of albumin and 94 ± 1.5% of IgG was removed, leaving 38 ± 1.2% residual protein. Depletion allowed improvement in resolution to ~1300 protein spots from ~600. Comparison of high resolution profiles of depleted FGR plasma samples to control (N=24), revealed 14 proteins of interest to be significantly altered. These proteins include decreased expression of Serotransferrin, Complement Factor B, Haptoglobin, and IgA. Proteins with increased expression include Hemopexin, Apolipoprotien α-II, α-1-antichymotrypsin, β-Hemoglobin, Clustrin, Apolipoprotein CII, Apolipoprotein D, and Alpha-1-acid glycoprotein. Many of these proteins are liver secreted proteins involved in either lipid or iron metabolism and transport. Further studies may determine if the reduction in the levels of these substrate carrying proteins may be contributing to the nutrient deprivation of the fetus. Using a HepG2 hepatic cell culture model of the FGR liver in hypoxic conditions, we are currently seeking to corroborate hepatic secretion changes of the protein changes seen in plasma. Identification of novel protein expression changes in FGR will lead to a better understanding of the underlying causes of the disease, and may underpin novel clinical interventions.

Serum Proteomics of Kawasaki Disease based on Multiprotein Complex Analysis

B127

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Introduction
Kawasaki disease (KD), an acute febrile illness of unknown etiology, is characterized by diffuse vasculitis that affects infants and young children. Its most common and serious clinical feature is the involvement of the coronary arteries, resulting in aneurysmal changes that can lead to stenotic lesions or myocardial ischemia associated with late mortality (ref.1). Biomarkers to predict disease outcome is the most urgent clinical necessity as well as to investigate its etiology. While serum is the most generally informative proteome from a medical standpoint, its complexity and enormous dynamic range make serum the most difficult specimen to be dealt with. Here we established multiprotein complex analysis method for human serum using two dimensional Blue Native /SDS Gel electrophoresis (2D-BN/SDS) and evaluated its diagnostic potentials for KD.

Methods
Here we collect blood samples from patients diagnosed as KD with an informed consent of their parents under the guidelines of ethical committee of the hospital. Blood sampling was performed from 17 patients before and after intravenous immunoglobulin (IVIG) treatment on the clinical day 3.41+1.50 and 7.88+1.54 (Mean+SD) respectively. Serum samples obtained were kept in -80 degree before analysis. One micro liter of serum was run on a 2D-BN/SDS gels. For the first dimension, 5–15% gradient gels cast on the Bio-Rad protean minigel system (Bio-Rad, Hercules, CA) and were run overnight at 4 °C. Then the second dimension was run on a 15% gel with SDS condition. Proteins were stained with CBB reagents and stored at 4 °C before analysis. The data analysis was conducted by computational gel evaluating system and each protein/peptide identification was performed by MALDI-TOF-MS (Shimadzu Co.).

Results
We have generated a standard protocol for diagnostic 2D-BN/SDS PAGE for human serum. Multiprotein complex in serum was separated on this diagnostic 2D-BN/SDS gels and all the peptides/proteins were identified. Especially, hemoglobin-haptoglobin (Hb-Hpt) complex revealed most definitive inter-individual differences reflecting their haptoglobin genotype. With this map, we have evaluated dynamic changes of multiprotein complex in serum of the KD patients before and after IVIG therapy. Some of the acute phase proteins, C-reactive protein (CRP) and Serum amyloid A protein (SAA), were down regulated after IVIG treatment. The way intravenous immunoglobulin affect Hb-Hpt complex formation should be clarified according to its phenotypes of the complex formation. In conclusion, serum proteomics of KD based on multiprotein complex analysis can yield a basic database for understanding pathogenesis as well as biomarker signatures for clinical course of the diseases.

Innovative aspects
- A standard protocol for 2D-BN/SDS PAGE for human serum was established. Hb-Hpt complex in human serum reveals the most profound inter- individual differences.
- CRP and SAA were down regulated in the serum of the patients with KD after IVIG treatment.
- IVIG effect on serum proteomics should be clarified according to Hb-Hpt complex formation.

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B128

Identification of universal vaccine candidates against Gram-negative bacteria by the combination of heterogeneous antiserum-based immunoproteomics with bacterial challenge

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Introduction
Bacteria are still one of the prevalent causes of life-threatening microbial infections. Vaccine prevention is the best choice, but development of universal vaccines against variety of bacterial serotypes and species is a very tough task due to the difficulty to find broadly cross-protective antigens among these pathogenic bacteria. Recently, development of a broadly protective vaccine rather than serotype-specific vaccine against all disease-causing serotypes has become the goal of the new vaccine strategy. Another universal vaccine against primary pathogens *Shigella* and ETEC (enterotoxigenic *Escherichia coli)*, which are the causes of diarrhoeal diseases and cause more deaths per year among children under five than malaria and AIDS together, is planning.

Methods
A new approach was developed to identify universal vaccine candidates from outer membrane (OM) proteins of *Vibrio alginolyticus* against infections by different genera and families of Gram-negative bacteria. This approach is based on the combination of heterogeneous antiserum-based immunoproteomics with bacterial challenge after immunization. The shared epitopes were suggested by bioinformatics.

Results
Out of the 35 proteins resolved in a OM-sub proteome 2-DE gel, four OM proteins were recognized by not only homogeneous antiserum, but also other genera and families of heterogeneous antisera. The shared epitopes were suggested by bioinformatics. There were 5, 4, 7 and 6 epitopes, including 2, 2, 2 and 3 helixes, respectively, in VP1061, VA0764, VAP0248 and VPA1186. These helix epitopes were utilized as primer sequences for blast analysis in all species of bacteria released in NCBI since helical epitopes showed better immunogens. Moreover, genes of the four OM proteins were cloned, and their recombinant proteins were used to immunize carps for investigation of their protective ability and their cross-protective spectrum and activities. The carps immunized by VA0764 and VA1061 could be against infections not only by *Vibrio alginolyticus*, but also by *Aeromonas hydrophila* and *Pseudomonas fluorescens*.

Innovative aspects

- To shed lights into the identification of broadly cross-protective antigens.
- To suggest a vaccine strategy to develop effectively universal vaccines against microbial infections.

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B129

A NEW SERUM MARKER FOR ENDOMETRIOSIS

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Endometriosis is a gynecological disease characterized by the presence of endometrial lesions outside of the uterus. It is the main cause of infertility in women at reproductive age. The physiological changes of endometriosis are abnormal tissue growth, invasion, and adhesion phenomena, which are similar to those seen in timorous tissues. The etiology of endometriosis is not well understood. There is no efficient biomarker for diagnosis, unless the invasive laparoscopy or laparotomy operation is applied to confirm the occurrence of endometriosis. We previously have found a1-antitrypsin (A1AT) was increased in serum of women with endometriosis by proteomic analysis, suggesting that A1AT can be a potential serum marker for diagnosis of endometriosis. A1AT is a glycoprotein that inhibits many serine protease by forming a stable, inactive complex with its target protein. In this study, we investigated the correlation of the expression of A1AT with endometriosis. The 42, 55, 60, 90, and 120 kDa of A1AT were present in patients with endometriosis. The 55, 60 and 90 kDa forms were glycosylated. Additionally, sialyl Lewis X glycol-epitope was expressed significantly in serum of patients with endometriosis. Finally, the sensitivity and specificity of the antibodies against the potential markers were investigated.

Keywords: endometriosis, proteomics, serum marker

B130

Determination of Candidate Biomarkers in Sera from Three Malignancies and Control Groups Using Isotopic Labels and Multiple Reaction Monitoring.

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Introduction
Proteins and peptides in the bloodstream have potential value in clinical diagnostics. Levels of specific proteins may be affected by several malignancies and these proteins could be monitored in multiple cancer types by mass spectrometry. Sera from patients with endometrial carcinoma (EmCa), head-and-neck/oral squamous cell carcinoma (HNOSCC) and glioblastoma multiforme (GBM) were analyzed by targeted tandem mass spectrometry alongside sera from control groups. A group of candidate biomarkers previously identified in tumor tissue were examined (1, 2).

Methods
Twenty of the most abundant serum proteins were first removed from the samples by immunoaffinity chromatography (Sigma, St. Louis, OH, USA). The remaining proteins were digested with trypsin, labeled with isotopic mass tags (mTRAQ, Applied Biosystems, Foster City, CA, USA), combined, and then separated by strong cation exchange chromatography using a step gradient from 10 to 500 mM KCl. The resulting fractions were separated by C18 reversed-phase nano LC and analyzed by multiple reaction monitoring on a linear ion trap mass spectrometer (Applied Biosystems / MDS Analytical Technologies, Concord, ON Canada). The C18 LC retention times and the MRM transitions provided high-confidence identifications of the proteins via multiple peptides, each with multiple transitions. The relative abundance of each peptide in the control and cancer sera was determined based on the peak areas of the respective mass tag ions.

Results
Proteins previously identified in tumor tissues as candidate biomarkers of the various cancers were observed in the serum extracts. In some instances, such as PCM 67, as many as 3 peptides each with at least three distinct transitions were detected in sera from an EmCa patient. These were further confirmed by Mascot searches of the MRM-triggered enhanced product ion scans. A number of other potential markers for GBM, such as PCM 216 and PCM 217, were also detected in sera from GBM patients. In the majority of these instances they were detected with two peptides of each candidate biomarker with PCM 216 being detected using transitions specific for four independent peptides. Differential expression of several candidate biomarker proteins was apparent when the cancer and control groups were compared.
Collectively the results are a confirmation of our long-held position of the feasibility of the tissue-to-serum approach for biomarker discovery. Ongoing experiments are aimed at quantifying these potential markers in order to provide a means of establishing threshold levels of these proteins in normal and the respective disease sera.

Innovative aspects

- Proteins previously identified as candidate biomarkers in tumor tissue were measured in blood by mass spectrometry
- Immunoaffinity chromatography of specific candidate biomarkers was not required for their detection in sera
- Isotopic labelling with mTRAQ reagents allowed relative quantification of multiple proteins between blood samples

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B131

Cross-Sample Analysis of MRM-Based Plasma Protein Expression for Optimizing MRM Assays

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Introduction
Twin studies are well recognized as powerful avenues to biological insight. In this study, relative expression levels for 37 proteins were measured using MRM-based quantitative assays on plasma samples from a set of 20 human twins. While these measurements will eventually be mined for biological conclusions in the context of the greater study, here we take advantage of the special attributes of a twin study to assess and improve the quantitative quality of the MRM assays using early results. General conclusions applicable to all MRM-based quantitative assays will be drawn from this work.

Methods
Plasma samples from 20 pairs of twins were identically collected, depleted of the top 7 proteins, reduced and alkylated, and digested with trypsin. Pooling aliquots of each of the 40 samples provided a study-averaged global internal standard (GIS), which was labeled with heavy mTRAQ™ reagent, while each sample was labeled with light mTRAQ reagent. Each sample was mixed 1:1 with GIS and analyzed by 1D RP-HPLC using MRM on a triple quadrupole linear ion trap mass spectrometer in random order, blinded of twin associations. A total of 109 peptides covering 37 proteins were monitored with 872 MRM transitions (436 heavy/light MRM ratios). The analysis of all 40 samples was repeated on three different instruments.

Results
To explore assay performance across samples, twin correlations were computed at the ratio, peptide, and protein level. Restricting to the 16 proteins with one or more Pearson r>0.7 at any level, unreliable MRM ratios and entire peptides were apparent. For these proteins, 46 of 168 ratios and 11 of 40 peptides had r more than 0.25 lower than the maximum r for the respective protein. For some proteins, the trends across samples indicated by different peptides were very consistent but the ratio magnitudes were shifted apart. Revised analysis using a preliminary attempt to correct for this bias by normalizing the peptide log means across samples to zero and excluding unreliable assays yielded dramatic improvements. The median 95% uncertainty error at the protein level decreased by almost 60%, driven by increased average consistency among peptides within proteins.
These results highlight that the reliability of any MRM assay is not well determined until it is used over a range of relevant samples. Some MRMs or whole peptides will not be robust to the variation encountered across samples, and the initial assay should have a sufficient excess of measurements to allow for some removals.

Innovative aspects

- MRM-based protein expression measurements of plasma from twin pairs.
- Strategies to refine MRM assay quality by analyzing samples over a range of biological variation.

Assessment of Plasma Proteome Stability

B132

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Introduction Many of the studies examining the stability of plasma and serum have focused primarily on the analysis of the low molecular weight proteome of serum using MALDI-TOF mass spectrometry; however, information regarding the overall global integrity of proteins, particularly in plasma, has not been previously described (1). Here we report our on-going efforts to characterize changes in the plasma proteome using shotgun proteomic analysis for the development of quality control metrics used to evaluate sample viability for biomarker discovery and verification assays.

Methods Blood was collected in EDTA collection tubes and plasma isolated at 4 °C or room temperature after a period of 0, 4 hr, 24 hr, 72 hr or 1 week. Aliquots of each plasma sample were analyzed using MALDI mass spectrometry. Spectra were analyzed using univariate statistical tests and building multivariate predictors. Additional plasma samples were collected and subjected to 0, 1, 2, 3, 5, 10, or 25 freeze-thaw cycles. All plasma samples were digested using trypsin and analyzed using reversed phase LC/MS/MS. Tandem spectra were identified using a semi-tryptic database search using Myrimatch and results filtered using IDPicker with a 5% FDR. Shotgun datasets were analyzed using MonsterMod to identify modifications and cleavage products resulting from such processing and handling conditions. Statistical analysis of LC-MS/MS datasets was carried out using an in-house software tool, QuasiProto. LC-MRM-MS analysis was performed on all plasma digests to measure levels of truncated peptides derived from fibrinogen and complement C3f.

Results Time and temperature have a very significant effect on the form of the produced MALDI spectra; however, the most notable observations were visual trends in changes of intensity values of several peaks with changing temperature and/or time. Initial shotgun proteomic analysis indicates the overall numbers of peptide and protein identifications are similar between each time and temperature point and between freeze-thaw cycles. MonsterMod software identified no significant truncations resulting from cleavage of fibrinogen or complement C3f. Analysis of plasma samples subjected to various collection conditions followed by tryptic digestion and reversed-phase liquid chromatography mass spectrometry revealed minimal changes in proteins of plasma collected in EDTA tubes with only modest degradation of those proteins previously shown to be highly susceptible to cleavage (i.e. fibrinogen, complement C3). Changes in the global distribution at the peptide level, both tryptic and semi-tryptic, in stored plasma are not detected upon digestion and LC-MS/MS analysis. Our results indicate that degradation occurring during storage is randomly distributed, and while some proteins may display higher instability, most proteins are relatively unaffected under such conditions.

Innovative aspects
Study examining changes in global plasma proteome due to extreme handling conditions using mass spectrometry-based platform for development of quality control metrics.

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Validation Study of Plasma Collection and Processing in the Presence of Protease Inhibitors.

B133

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Introduction
One of the biggest challenges in blood based protein biomarker studies is the accessibility to high quality biological samples. The implementation of standard operating procedures for blood collection, processing and storage is critical to minimize any bias introduced by preanalytical variability and to generate good quality data (1). The addition of protease inhibitors at the time of blood collection has been shown to confer some plasma stability when samples are processed shortly after collection (2). However, in the clinical setting it is not unusual to delay the processing of blood samples for several hours due to time, equipment or staff constraints. The goal of the present study is to investigate whether protease inhibitors can confer stability at the protein level to blood samples processed at different time points after collection.

Methods: We collected blood from five fasting healthy volunteers in tubes containing either k-EDTA or k-EDTA plus protease inhibitors (BD P100 tubes). Tubes were processed immediately (time 0) or left on the bench for 2 hours and 6 hours at room temperature and processed thereafter. Strict SOPs were implemented for the collection, processing and storage of these samples. Aliquots of plasma obtained from each tube type and time point were used to look at protein levels using different proteomics platforms. Levels of low abundant cytokines were measured using the 27-plex cytokine assay from Bio-Rad. We also performed a quantitative proteomic analysis using iTRAQ technology following depletion of the top 12 most abundant plasma proteins. Finally, we analysed a panel of 45 mid and high abundant plasma proteins using MRM/MS and a cocktail of 45 isotopically labeled peptide standards.

Results: We first analysed the changes in cytokine levels across different time points for blood collected in kEDTA and P100 tubes. There was a significant trend showing an increase in cytokine levels with longer processing delays when blood was collected in k-EDTA tubes with fold changes ranging from 1.5 to 3.3. Although there was a decreasing trend with time for some cytokines collected in P100 tubes, the fold differences were below 2 suggesting that cytokine levels are better maintained across time in P100 tubes. At baseline time=0, we detected 5 more cytokines in the P100 tubes than in the kEDTA tubes, indicating that the presence of protease inhibitors effectively prevented early degradation of 18% of tested plasma cytokines . Analysis by iTRAQ resulted in the identification of 214 different proteins in two separate iTRAQ runs (only peptides with confidence at least 80% were included and at least 2 peptides per protein). Thymosin like 4, actin cytoplasmic 2, thrombospondin 1 and platelet basic protein are some of the proteins that we detected at higher levels in P100 tubes when compared to kEDTA in both experimental runs; fold changes ranged from 2.4 - 19 fold. We will be presenting further details on the analysis of iTRAQ and MRM experiments assessing the effects of protease inhibitors on mid and high abundant plasma proteins.

Innovative aspects

The results from our study are an independent validation of BD P100 tubes using different proteomics technologies.

Identification of specific low and high abundant plasma proteins stabilized by the use of protease inhibitors.

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Multiprotein Complexes in Serum Proteomics by 2D BN/SDS Gel Electrophoresis

B134

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Introduction
Serum is the most generally informative proteome from a medical standpoint and at the same time, its complexity and enormous dynamic range make serum the most difficult specimen to be dealt with. In addition, low molecular weight peptides or proteins may be excreted through glomerular filtration system in a very short time range. Thus, targeting multiprotein complex in serum may be an attractive way of elucidating pathogenesis of various diseases. Here we established method for multiprotein complex in serum using two dimensional Blue Native / SDS Gel electrophoresis (2D BN/SDS) and evaluated its diagnostic potentials by applying into animal models.

Methods
We collect murine serum samples from two experimental models; 1) Acute brain ischemia model (ref.1) and 2) Experimental Autoimmune Encephalomyelitis model (EAE) (ref.2). Both experiments were performed on SJL/J mouse background. Blood sampling was performed from orbital plexus in both experiments and serum was obtained in the following time point 1) time 0, 1, 3, 6, 12, 24, 37, 58 and 117 hours after brain ischemia and 2) at day 0, 10 and 17 and more after immunization. One microliter of serum was run on a 2D BN/SDS gels. For the first dimension, 5–15% gradient gels cast on the Bio-Rad protean minigel system (Bio-Rad, Hercules, CA) and were run overnight at 4 °C. Then the second dimension was run on a 15% gel with SDS condition. Proteins were stained with CBB reagents and stored at 4 °C before analysis. The data analysis was conducted by computational gel evaluating system and protein/peptide identification was performed by MALDI-TOF-MS (Shimadzu Co.).

Results
We have generated a standard protocol for diagnostic 2D BN/SDS PAGE for serum samples. Applying this method, we have mapped all the peptides/proteins on this diagnostic 2D gels as a basic database of mouse serum. In the next step, abundance of each peptides/proteins was plotted according to its time course for both of the animal studies and have determined its annotation. As a result, multiprotein complex in serum shows a dynamic change on this diagnostic 2D gels and this was beautifully moved in a time course dependent manner. Especially, hemoglobin-haptoglobin (Hb-Hpt) complex revealed most dynamic change in both an acute brain ischemia and an experimental autoimmune encephalomyelitis models. By contrast, the complement system was shifted in terms of the complex formation in the later part of an acute brain ischemic model.

Innovative aspects
- A standard protocol for 2D BN/SDS PAGE for serum was established.
- All the protein/peptides were mapped onto this diagnostic platform of serum 2D BN/SDS PAGE.
- Hb-Hpt complex in serum revealed the most profound dynamic changes in both an acute brain ischemia and an experimental autoimmune encephalomyelitis mouse models.

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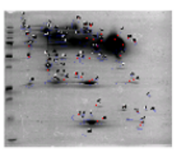

Figure 1. Standard 2D-BN/SDS PAGE panel for serum proteomics. Each spots were identified by MALDI-TOF-MS.

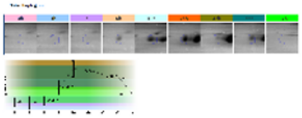

Figure 2.Haptoglobin-hemoglobin complex in mouse serum revealed a dynamic change in the time course of acute brain ischemic model.

Plasma Proteome Changes in Rats Exposed to Cold Sea Water Environment

B135

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Introduction
The purpose of this study was to establish a model as severe hypothermia in disaster at sea and identify the changes of plasma proteins by two-dimensional polyacrylamide gel electrophoresis (2-DE) and mass spectrometry.

Methods
Ten male Wistar rats were randomly divided into 0, 2, 4, 6, 10 hours groups. The rats were immerged vertically below armpit in 15°C seawater after anesthesia. The blood was collected from inferior caval vein at different times. Then plasma was prepared and purified by the serum protein mini kit to eliminate high-abundance proteins (IgG and albumin) and salts. The sample was separated by 2-DE and differential proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), then analyzed in database of NCBI and Swiss-prot.

Results
The core temperature of rats decreased rapidly in 15°C seawater, 19°C after 30 minutes, and 16°C stably after 120 minutes, indicating severe hypothermia occurs. Pulmonary SP-A, SPA3K and TIMP-3 were identified as hypothermia associated proteins, which levels were increased apparently in plasma accordant with immersing time. The study of plasma proteome changes in cold seawater immersed rats may contribute to the understanding of molecule mechanism of hypothermia injury, which to provide scientific datum for the research of pathomechanism and treatments of patients suffering from sea accidents.

Innovative aspects
- Pulmonary SP-A, SPA3K and TIMP-3 were identified as hypothermia associated proteins
- These proteins may contribute to the understanding of molecule mechanism of hypothermia injury, which to provide scientific datum for the research of pathomechanism and treatments of patients suffering from sea accidents.

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B136

Quantitative analysis of chaotropic & solvent effects on the trypsin digestion of human plasma

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Introduction
The MS based quantitation of proteins based on peptides assumes trypsin digestion is complete and that the resultant peptides are equimolar surrogates for their parent protein. Plasma, an extremely complex and highly proteinaceous sample, is frequently used in quantitative proteomics analyses of disease. The potential clinical utility of MRM based protein assays will require reproducible, highly efficient and complete sample digestion methods. We have therefore quantitatively characterized the trypsin digestion kinetics of 45 plasma proteins using 11 different digestion protocols.

Methods
We have previously developed a panel of 45 stable isotope labeled peptides (Lys[$^{13}C_6$] or Arg[$^{13}C_6$]) that we used to quantify 45 physicochemically diverse (hydrophobic, heavily glycosylated, disulfide bonds) proteins of medium (L-selectin) to high concentration (transferrin) in human plasma (1). Various chaotropic reagents (SDS, urea, guanidine HCl, sodium deoxycholate), and solvents (methanol, acetonitrile, trifluoroethanol) alone and in combination were used during trypsin digestion. This standard peptide mixture was added to samples of plasma digests collected at time points (0.5, 1, 2, 4, 6, 9 & 23 h) during trypsin digestion. Trypsin digests were desalted and concentrated by SPE prior to analysis by LC-MRM/MS using multiple reaction monitoring (MRM) on a triple quadrupole MS.

Results
We have previously presented a preliminary optimization of plasma trypsin digestion efficiency using both heat and chemical denaturation with SDS, urea, and sodium deoxycholate (NaDOC). In this on-going study we have focused on characterizing both stronger chaotropic reagents (GnHCl) and solvents (MeOH, TFE, ACN) to aid/improve the accessibility of trypsin to denatured plasma proteins while maintaining trypsin activity.
Recent data suggests that trypsin digestion efficiency equivalent to that obtained with NaDOC can be obtained with post-reduction and alkylation solvent denaturation alone. To ensure efficient and complete reduction, we have combined solvent denaturation with NaDOC denaturation. Both kinetic profiles of digestion time courses and technical reproducibility of each method will be presented. While improvement of overall digestion efficiency could be achieved with certain proteins, complete digestion of others was never attained. Our results suggest digestion protocols should be tuned/selected specific to the target proteins of interest with complete trypsin digestion often possible in <120 min.

Innovative aspects
- Novel MS compatible plasma digestion protocol
- Combined detergent and solvent denaturation improves digestion efficiency
- Quantitative reference for optimal specific protein digestion protocol

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B137

Hepcidin-22 produced in urinary space

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Introduction: Hepcidin, a key regulator of iron metabolism, is expressed in the liver, distributed in blood, and excreted in urine. In previous report, molecular peaks of hepcidin-25, -22 and -20 are detected in urine (Tomosugi,2006) and it is speculated that urinary hepcidin could pass through gromerular basement membrane because of its small molecule. Recently, it is reported that hepcidin is also produced in the kidney (Kulaksiz,2005) and the heart (Merle,2007). We hypothesized that cleavage of hepcidin in urinary space could provide insight into the early pathophysiology of human interstitial nephritis (IN).

Methods: Here, we used surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) with IMAC-30 distinctive serum and urine proteomic patterns of patients with IN. MALDI-TOF/TOF (AXIMA, SHIMAZU) analysis utilizes only 0.1 ml of urine samples were extracted with trichloroacetic acid. The extract was evaporated to dryness and target molecules were analysed by MALDI-TOF/TOF.

Results: In the normal urine, about 20 peaks are expressed in almost all volunteersâ€™ urine profiling by SELDI. The profiling pattern showed almost same patterns during 12 months in one volunteer, and the peaks at 2192 and 2789m/z were excreted at trace levels. In urine from patients with IN, these peaks were expressed highly compared with their sera and the peak at 2436m/z was detected only in the urine. When the peaks at 2192, 2436 and 2789m/z in extracted urine samples were analyzed by MALDI-TOF/TOF, same patterns of 8 repeated peaks were detected from each peak. Each interval was 32m/z which means the size of sulfur molecule which is constructed in disulfide binding. These findings imply that these fragments were induced from one precursor; hepcidin-25 which has 4 disulfide bindings. Hepcidin-22 may be cleavage from hepcidin-25 or prohepcidin by endopeptidase activated by IN and could work in inflammation and innate immunity.

Innovative Aspects:
1. hepcidin-22 as biomarker for IN.
2. 8 peaks with 32 m/z interval from hepcidin.
3. innate immunity in urinary space.

B138

Glycoproteomics and Glycomics: A New Paradigm for Plasma Biomarker Discovery

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Cancer is a heterogeneous group of diseases. It can be subdivided into different subtypes based on morphological and molecular features. Biomarkers have the potential to distinguish different subtypes of cancer which will be the key prognosis factors for treatment options. Despite the advances in cancer treatment, many patients are still developing resistance to the therapy. New biomarkers are needed to select patients for targeted therapy.

Glycoproteins and their glycosylation patterns are the most common features on the surface of cancer cell. First, extracellular proteins, including cell surface proteins, transmembrane proteins, and secreted proteins, are mostly glycosylated. They are the easily accessible blood based biomarkers as cancer diagnostics. Second, glycosylation changes are the universal features of malignant transformation and tumor progression. Glycosylation patterns are affected by cellular environment where the glycoproteins are synthesized including the expression of glycosylation related enzymes and other genes responsible for substrate synthesis. The availability of the total human genome sequence, application of gene expression analysis, DNA methylation profiling, and proteomic techniques to identify glycosyltransferases and related genes have accumulated much knowledge on the transcriptional background of cancer-associated glycan alteration. However, unlike proteins, which are generally encoded by a single gene, glycosylation of a protein is produced by the concerted action of many related genes, making it difficult to predict the cell-surface glycans based on current gene expression data. Therefore, quantitative glycoproteomic and glycomic analyses of extracellular proteins from tumors with different subtypes and clinical outcomes will allow us to identify glycoproteins and glycans that can be used to identify patients likely to benefit from certain therapy. The cell surface glycoproteins can potentially be used as novel therapeutic targets to increase the response rate for cancer treatment.

For this study, we developed glycoproteomic and glycomic technologies using mass spectrometry and protein arrays for the analysis of clinical specimens with high sensitivity, throughput, and reproducibility. We assembled a multidisciplinary team of scientists with expertise in cancer biology, pathology, clinical oncology, statistics and epidemiology. We design the study and applied our novel proteomic and glycomic approaches for the analysis of clinical specimens to identify glycoproteins and glycosylation patterns associated with different subtypes of tumors. These glycoproteins and glycans can be served as new candidate markers for cancer prognosis to stratify patients for different treatment options as well as potential therapeutic targets for a new paradigm in cancer therapy.

B139

Blood-based exopeptidase activities as biomarkers for cancer.

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Human cells produce some 550 proteases that have greatly differing biological roles. Proteases have also been implicated in disease, notably in cancer where they promote both tumor progression and suppression. Examples of peptide degradation have unexpectedly been observed in recent serum oncopeptidomics studies whereby subsets of peptides provided class discrimination between patients with different types of solid tumors and control individuals without cancer. All relevant peptides sorted into nested sets of sequences, each the result of exopeptidase activities that confer cancer-type-specific differences superimposed on the proteolytic events of *ex vivo* clotting. We speculated that exopeptidase activities might serve as biomarkers for cancer. There are an estimated 86 human exopeptidases, 71 of which could potentially contribute to the observed serum peptide degradation patterns. I will describe exopeptidase activity assays that can measure *de novo* peptide breakdown in large numbers of biological samples. The metabolites are quantitated (c.v. ≤15%) and the results subjected to kinetic and/or statistical analysis. Preliminary analyses of patient samples and mouse models of prostate cancer suggest potential diagnostic applications. Our studies are additionally guided by proteomic and biochemical analyses of secreted exopeptidases using cell culture models representative of invasive and non-invasive prostate cancer.

B140

The human plasma proteomic response to severe burn

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Introduction
The molecular mechanisms of severe burn injury and the human body's systemic response are poorly understood. A subset of patients who survive their initial injury will later experience adverse outcomes, such as multiple organ failure or even death, due to host inflammatory response. Here we describe a proteomic study of plasma of burn patients with different outcomes and healthy control patients to both better understand the body's proteomic response to burn, and to identify temporal markers of multiple organ failure within burn groups.

Methods
In this work we obtained plasma from 22 burn patients, each at two timepoints, and 10 healthy control subjects. The first timepoint was taken close to initial admission, and the second timepoint corresponds to time of peak multiple organ failure injury. Samples underwent tryptic digest, followed by liquid chromatography and Fourier-transform ion cyclotron resonance mass spectrometry (LC-FTICR MS). Peptide identification was achieved using an Accurate Mass and Time (AMT) tag approach (1). We have developed normalization and inference approaches to identify proteins that are significantly differentially expressed between burn and control patients from the peptide level data.

Results
By comparing the plasma of burn patients with healthy controls, we identify a set of 51 proteins that are significantly enriched or depleted in the plasma of burn patients. Using Ingenuity Pathway Analysis, we discovered that thirty-eight of these fifty-one proteins are known extracellular proteins. Furthermore, this proteins set was significantly enriched for acute phase response proteins, coagulation proteins, and complement proteins, as would be expected in proteins involved in burn response.

We also identified a preliminary set of proteins that show a consistent and significant relative change in burn patients from early admission to time of peak injury. These markers, if validated in a larger set, could permit earlier identification of patients at risk for multiple organ failure (MOF).

Innovative aspects
- One of the first and largest plasma proteomic studies of inflammation and the host response to severe burn
- Novel methods to determine significantly differentially expressed proteins from differentially expressed samples
- Identification of plasma proteins that characterize the human response to burn, and possible predictors of multiple organ failure

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Figures
a)

b)

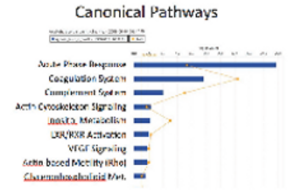

Figure 1. a) Cellular localization and b) Pathway enrichment for proteins identified as significantly associated with the host response to burn.

B141

Validating serum protein profiling for early stage breast cancer diagnosis in a large case control study

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Introduction
Serum protein profiling by mass spectrometry is a promising diagnostic tool for early detection of cancer and other human diseases. The overall aim of the current study was to validate a previously published breast cancer case-control study (1) in order to discriminate early stage breast cancer patients from healthy controls.

Methods
Pre-operative serum samples obtained from 184 early stage breast cancer patients and 236 healthy controls were used to generate matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) protein profiles. Early stage tumor refers to cases with a tumor size less than 20 mm and negative lymph node status.
Serum samples were processed using ClinProt magnetic beads with IMAC charged with copper as the affinity surface (Cu(II)-IMAC (Profiling kit MB-IMAC Cu, Bruker Daltonics, Leipzig, Germany) with adjustments as previously described (2).
For MALDI-TOF MS analysis a Bruker Ultraflex (Bruker Daltonics, Bremen, Germany) was used.
A total of nine mass spectrometric protein profiles were obtained per serum sample and PrepMS (3) were used for peak extraction.

Results
We were able to identify 537 peaks representing an "overall protein profile". Among these 537 peaks a total of 145 peaks were found significantly different ($p<0.01$) when comparing the intensity between cases and controls using Wilcoxon rank sum test. Furthermore, when controlling the false discovery rate with the Benjamini-Hochberg method, 103 peaks were still significant at the 0.01 level.
In order to construct a diagnostic rule these 145 mass values were combined into 46 clusters based on their Spearman correlation
In the current study we were able to retrieve 38 of 72 previously published discriminatory peaks (1).

Innovative aspects
- Discriminatory protein patterns could be demonstrated between early stage breast cancer patients and controls.
- We provide validation of results obtained in a previous published breast cancer case-control study.
- The current study adds evidence to the potential usefulness of proteomics as a diagnostic tool for early detection of breast cancer.

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B142

The Human Proteome Project 2009
Analysis of the raw serum peptidomic pattern of glioma

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Introduction
Glioma is a unique lethal disease which is difficult to diagnose at early stage and even more difficult to cure. Human serum contains a large number of peptides which are "recordings" of the cellular and extracellular events that take place at cancer-tissue microenviroment. The analysis of charateristics of glioma specific raw serum peptidomic pattern may be used as ideal biomarkers for the early diagnosis and prognosis.

Methods
In this study, serum samples were collected and preceded to analysis of high throughput MALDI-TOF/TOF mass spectrometer. The analysis method is based on the hydrophobic bead capture peptides without digestion. The integrated application for the data variance analysis was performed to describe the glioma specific raw serum peptidomic pattern.
The data analysis strategy for the variance analysis of the cancer specific serum peptidomic patterns included 2 steps. In the first step, Clinpro™ software was used to get common peak list masses in all the measured samples. In latter way, an optimal subset of variables was selected by using the newly proposed CARS method and pattern recognition equation was established through PLS-DA analysis.

Results
The difference of serum peptidomic patterns were compared in 75 serum specimens including 32 glioma patients and 43 normal controls. Using Clinpro™ software, 105 normalized peaks were detected in these two groups. Glioma specific serum peptidomic pattern was characterized by 9 peptides which could distinguish glioma from normal with high sensitivity and accuracy. Meanwhile, pattern recognition equation was established:
$Y=-0.7367X_1-0.5071X_2-0.6482X_3+0.6753X_4+0.6231X_5-0.7229X_6-0.7491X_7+0.5349X_8-0.5164X_9$. Using 10-fold cross validation, the accuracy was 0.760 and the AUC of ROC curve was 0.832 before variable selection. But after variable selection, the accuracy and the AUC of ROC curve were all improved obviously which achieved 0.920 and 0.923 separately. This differentially expressed raw serum peptidomic pattern might be useful as molecular indicators of glioma diagnostic value. In addition, we obtained 5 peaks kept same change trend at different phases of clinical pathology of glioma. Among these, the peak intensity of 2952.72, 4209.96 and 7929.96 was positively correlated with the degree of malignancy and that of 3096.41, 5805.52 was negatively related.

Innovative aspects
- Integrated application of data analysis strategy for pattern recognition
- Characterization of Glioma specific raw serum peptidomic pattern

References
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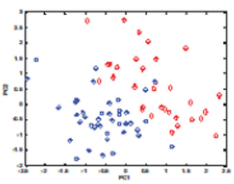

Figure 1. The score plot of glioma (red) and normal control (blue) groups by partial least squares-discriminant analysis (PLSDA) analysis using the selected 9 peaks.

B143

The Human Proteome Project #X
Analysis of Serum Peptidomic Patterns for Nasopharyngeal Carcinoma Diagnosis

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Introduction
Serum contains a large number of peptides which are "records" for the cellular and extracellular events that take place at the level of the cancer-tissue microenvironment. They could be a rich source of cancer-specific diagnostic information. Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in China, which is difficult to be diagnosed at early stage. In this study, serum peptidomic pattern of NPC was investigated to seek biomarkers and establish diagnostic model.

Methods
Serum specimens were collected before treatment following the HUPO protocols and avoiding more than two freeze/thaw cycles. Affinity purification of serum with WCX magnetic beads and matrix-assisted laser desorption ionization time-of-flight/ time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis were used to screen potential NPC markers. The data was analyzed by using Clinpro™ software and the newly proposed competitive adaptive reweighted sampling (CARS) method. The pattern recognition equation was established and verified through partial least squares-linearity discriminant analysis (PLS-LDA).

Results
Serum peptidomic patterns of NPC were compared in 172 serum specimens including 121 NPC patients without treatment and 51 normal patients as controls. By data analysis, 133 normalized peaks were detected in these two groups, and 8 peptides were selected as the NPC specific serum peptide pattern which could distinguish NPC from normal with high sensitivity and accuracy. At the same time, the pattern recognition equation was established:
$Y=0.2548X_1-0.5415X_2-0.2096X_3+0.8948X_4+0.7942X_5+0.3421X_6+0.3907X_7+0.2006X_8$ (X_1:2714.16Da, X_2:2723.35Da, X_3:2952.98Da, X_4:3216.70Da, X_5:3890.58Da, X_6:4281.81Da, X_7: 5390.37Da, X_8:5753.26Da). Using 10-fold cross validation, the accuracy and the AUC of ROC curve were 0.983 and 0.9860. The sensitivity and specificity achieved 0.9835 and 0.9804 separately. This differentially expressed serum peptidomic pattern might prove to be useful molecular indicators of NPC diagnostic value.

Innovative aspects
- Integrated application of data analysis strategy for pattern recognition.
- Characterization of NPC specific serum peptidomic pattern for diagnosis.

References
(1) Petricoin EF et al, The blood peptidome: a higher dimension of information content for cancer biomarker discovery; Nature Reviews Cancer. 2006, 6, 961-967
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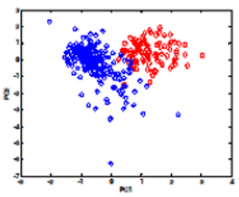

Figure 1. The score plot of normal (red) and NPC (blue) groups by PLS-LDA using the selected 8 peaks.

B144

Toward the Proteome of the Human Peripheral Blood Eosinophil

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Toward the Proteome of the Human Peripheral Blood Eosinophil

Introduction: Eosinophils are granular leukocytes that play active roles in inflammatory diseases and in immunoregulatory processes. Many diseases associated with eosinophils, such as asthma, allergic inflammation, and eosinophil esophagitis, affect a large part of society and are responsible for large amounts of health care spending. The characterization of the eosinophil proteome is thus important so as to characterize a comparative basis toward the understanding of eosinophil-related pathologies. This study is the first time that eosinophils from normal donors are studied via a gel-based proteomics approach.

Methods Eosinophils were isolated from nonsmoking donors who showed neither asthmatic nor allergic symptoms and did not demonstrate significant elevation of the eosinophil activation marker CD69. Cellular lysates were resolved using 2D SDS-PAGE and visualized with Sypro Ruby fluorescent stain. 2D gels were analyzed using Nonlinear Progenesis SameSpots software and selected protein spots were robotically picked and analyzed by MALDI-TOF/TOF mass spectrometry (MS). We also used Calbiochem's ProteoExtract® Subcellular Proteome Extraction Kit methods to reduce sample complexity. In addition, Ingenuity Pathway Analysis software was utilized to evaluate the relevant biological functions of the MS identified proteins in the dataset.

Results We identified 3,141 proteins with significant Mascot expectation scores ($\leq 10^{-3}$) and 434 proteins were unique. Of this group, 278 proteins were proteins not previously reported to be expressed by eosinophils. Many eosinophil proteins were phosphorylated and actin was especially abundant when compared to other cells. Ingenuity Analysis results identified proteins in the dataset whose function was especially relevant to eosinophils, namely, immunologic and inflammatory diseases, immune response, immune and lymphatic system development and function, and respiratory disease.

Innovative aspects:

- This study comprises the first comprehensive proteomic dataset of proteins expressed in normal eosinophils.
- This dataset will serve as a valuable reference for future studies in which we and other groups will compare differential protein expression in various pathologies associated with eosinophils.

C.elegans and D. melanogaster Proteomics

(C101 – C105)

C101

Phosphopeptide isolation from *Caenorhaditis elegans* using the CE PrEP, PCT, and PhosphoScan Technologies

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Introduction. So resilient is the exoskeletal cuticle of *Caenorhabditis elegans* that specimens aboard the Space Shuttle Columbia were the only survivors of the disastrous crash [1]. Nematodes are also resistant to disruption at high hydrostatic pressure and an LD_{50} of 7,400 psi is estimated. Although 100% mortality is observed at 35,000 and 45,000 psi, Trypan blue staining shows permeability of less than 30% of nematode cuticles [2]. Complete disruption of this cuticle is necessary for reproducible and unbiased protein recoveries. This facilitates phosphoproteomic analyses as exemplified by the isolation of target peptides of src-1 kinase activity. **Methods.** The Pressure Enhanced Processing (PrEP) Kits were developed in combination with Pressure Cycling Technology (PCT) for the complete disruption of nematodes and the destruction of cuticles enabling unprecedented protein yields using either native buffers (PBS, TBS, or HEPES) or high stringency reagents (chaotropes, detergents, and reducing agents). For biomass processed with the CE PrEP Kit, nematodes and their exoskeletons were obliterated as observed microscopically. Proteins were reduced, alkylated and trypsin digested and the resulting peptides were incubated with anti-phosphotyrosine antibodies coupled to Protein G agarose beads provided in the Phosphoscan Kit (Cell Signaling Technologies). **Results.** The absorbed phosphotyrosine-containing peptides were eluted and analyzed by LC tandem mass spectometry. The phosphoproteomes of N2 wild type and src-1 RNAi knock outs were compared.

References:

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C102

Proteomic Analysis of *O*-GlcNAcylation during dauer formation in *Caenorhabditis elegans*

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Introduction
Modification of proteins at serine or threonine residues with N-acetylglucosamine, termed *O*-GlcNAcylation, plays a regulatory role in many model organisms. Here, we investigated the mechanism by which *O*-GlcNAcylation regulates an entry into dauer in *Caenorhabditis elegans*. *O*-GlcNAcylation-induced alterations in proteome were analyzed by performing two-dimensional electrophoresis (2DE). To identify the potential target proteins that have been differentially *O*-GlcNAcylated during dauer formation, we specifically labeled *O*-GlcNAc with fluorescent dye that was followed by 2DE analysis. Taken together, these data could provide evidence that high *O*-GlcNAcylation enhances dauer formation. *This study was supported by a grant from the Korea Health 21 R&D project, Ministry of Health and Welfare of Republic of Korea [A030003 to YKP]*

Methods
We performed daumone assay to check whether *O*-GlcNAcylation affect dauer formation process. We performed daumone assay with *O*-GlcNAc cycling mutants. We also used *O*-(2-Acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate (PUGNAc, *O*-GlcNAcase inhibitor) for specifically enhancing the *O*-GlcNAcylation in vivo. *O*-GlcNAcylation-induced alterations in proteome were analyzed by performing two-dimensional electrophoresis (2DE) with lysates of N2 and *oga-1(ok1207)*. To identify the potential target proteins that have been differentially *O*-GlcNAcylated during dauer formation, we specifically labeled *O*-GlcNAc with fluorescent dye that was followed by 2DE analysis.

Results
We confirmed that *ogt-1(ok430)* mutants deficient in *O*-GlcNAc transferase activity exhibited a dauer-defective phenotype, whereas *oga-1(ok1207)* mutants deficient in *O*-GlcNAcase, catalyzing *O*-GlcNAc removal exhibited a dauer-prone phenotype with synthetic daumone. Consistent with these findings, treatment of low amount of daumone and PUGNAc, *O*-GlcNAcase inhibitor, enhances the frequency of dauer entry. *O*-GlcNAcylation-induced alterations in proteome were analyzed by performing two-dimensional electrophoresis (2DE) with lysates of N2 and *oga-1(ok1207)*. Seven differentially expressed spots were analyzed using LC/MS. The identities of these proteins suggest that *O*-GlcNAcylation influences stress resistance, protein folding and mitochondrial function. To identify the potential target proteins that have been differentially *O*-GlcNAcylated during dauer formation, we specifically labeled *O*-GlcNAc with fluorescent dye that was followed by 2DE analysis from which 5 candidate proteins were selected. The identities of these candidates suggest that *O*-GlcNAcylation may regulate a shift in cytoskeleton and protein turnover during dauer formation.

Innovative aspects
- An optimized daumone assay with various mutants and chemicals
- 2DE analysis of highly *O*-GlcNAcylated mutants
- Specific *O*-GlcNAc labelling and its subsequent analysis for identification of target proteins

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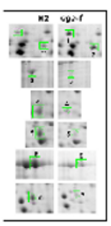

Figure 1. High O-GlcNAcylation induces proteome change. The lysates of N2 and *oga-1* were resolved by 2 DE. Various spots are differentially expressed upon high *O*-GlcNAcylation. The selected spots were differentially expressed more than 2.5 fold. Spot IDs were indicated for each spot. The identities of spots are as follows: 1: F15E11.12, 2: F15E11.13, 3: DAF-21, 4: RPL-7A, 5: CPL-1, 6: H28O16.1, 7: NUO-2.

C103

The SILAC fly allows for accurate protein quantification *in vivo*

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Introduction
The fruit fly *Drosophila melanogaster* is one of the most widely used small animal models in biology. Until now, most biological studies in *D. melanogaster* employed genetic approaches while proteomic studies are still underrepresented. A recent large scale effort provided a first catalog of the *D. melanogaster* proteome (1) and ^{15}N labeling was established for protein quantification (2). However, quantification with both of these methods has limited accuracy. SILAC (stable isotope labeling with amino acids in cell culture), which is a powerful approach to precisely quantify differences in protein expression levels (3), has not been established in this model so far. Here we present stable isotope labeling with amino acids in *D. melanogaster*, which allows for accurate protein quantification *in vivo*.

Methods
Unlabeled *D. melanogaster* embryos were spread on a culture dish containing a minimal medium supplemented either with "light" $^{12}C_6{}^{14}N_2$ L-lysine or "heavy" $^{13}C_6{}^{15}N_2$ L-lysine labeled *Saccharomyces cerevisiae*. Adult *D. melanogaster* were lysed in RIPA buffer containing SDS and protease inhibitors. Proteins were separated on a Bis-Tris gel followed by Lys-C digestion. Peptides were extracted and analyzed by liquid chromatography – tandem mass spectrometry on a LTQ-Orbitrap hybrid mass spectrometer. Data processing was performed using MaxQuant software. False discovery rates were estimated based on a target-decoy strategy using a protein database from flybase.

Results
First, a lysine auxotrophic *S. cerevisiae* strain was grown in medium containing heavy L-lysine. To ensure high labeling efficiency, total protein extracts from yeast were analyzed by LC-MS/MS. Based on the 100 most intense proteins, incorporation rate of proteins with heavy L-lysine was 97.1% (± 2.4). Next, unlabeled *D. melanogaster* larvae (F_1) were fed with heavy L-lysine *S. cerevisiae*. Hatched *D. melanogaster* showed an incorporation rate of 95.4% (± 2.7). Furthermore, incorporation rate of adult F_2 was 96.4% (± 2.0) indicating that labeling efficiency is almost maximal in F_1. Analysis of a 1:1 mixture of heavy and light L-lysine – labeled adult F_1 *D. melanogaster* revealed a normal distribution of heavy to light ratios indicating that heavy L-lysine labeling does not affect protein expression levels. Bicoid and Nanos, which form gradients along the anterior-posterior axis in *D. melanogaster* embryos, either activate or repress downstream targets that are important for body axis formation. We are currently comparing Bicoid and Nanos mutant strains with wildtype *D. melanogaster* to measure relative protein expression of Bicoid and Nanos downstream targets along the anterior-posterior axis.

Innovative aspects
- Establishment of stable isotopic labeling with amino acids in *D. melanogaster*

References
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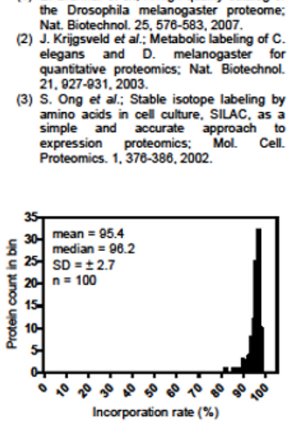

Figure 1. Labeling efficiency of adult *D. melanogaster* of the F1 generation. Graph shows heavy L-lysine incorporation rates in percent of the 100 most intense proteins.

C104

Elucidation of DAF-16 Cofactors in *C. elegans* using a Quantitative GFP-Immunoprecipitation

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Introduction

DAF-16, the *C. elegans* ortholog of FOXO1A, is the essential downstream target of the insulin/insulin-like receptor DAF-2. DAF-16 is a forkhead transcription factor which mediates most of the signaling from DAF-2. DAF-2 regulates dauer formation, metabolism, reproduction, stress resistance, development, and senescence. Elucidation of DAF-16 binding partners will clarify how DAF-16 can influence such a diverse number of cellular processes. The likely cofactors or other transcription factors which interact with DAF-16 will shed light on its roles in diabetes and aging in humans and drive future therapeutic intervention.

Methods

Green fluorescent protein (GFP) is the most frequently used fusion protein for tracking protein localization and expression in *C. elegans* and is often used as a crude handle for protein complex purifications, but suffers from high background purification levels. We have developed a quantitative proteomics procedure to increase the confidence and selection of *bona fide* cofactors of DAF-16 using the DAF-16::GFP fusion. Modified growth conditions for previous quantitative proteomics of *C. elegans* grown on ^{14}N and ^{15}N *E. coli* lawns were used.[1] DAF-16::GFP expressing worms in N2 and *daf-2* background were grown on either ^{14}N or ^{15}N *E. coli*, respectively. Worms were harvested as young adults, lysed using a French press, mixed in equal protein ratios, immunoprecipitated (IP) using a GFP antibody after preclearing, digested with trypsin, and analyzed using MudPIT on a LTQ-OrbiTrap. Protein ratios were compared to elucidate different protein binding partners of DAF-16 from the two genetic backgrounds and from background binding proteins.

Results

A DAF-16::GFP fusion has previously been shown to be sequestered and inactivated in the cytosol under normal conditions, while active and nuclear in a *daf-2* mutant.[2] However, only a few binding partners have been determined such as FTT-2 and SIR-2.1.[3] Our quantitative comparison allowed for differentiation of the cofactors of the two genetic conditions from background binding proteins. The majority of proteins identified by MudPIT were non-specifically pulled down, indicated by equal quantitative ratios corresponding to the two genetic conditions. Very high or low outlying quantitative ratios allowed for differentiation of new *bona fide* DAF-16 cofactors which will be validated by reverse immunoprecipitation, genetic mutation, and RNAi. This method should be applicable to elucidating binding partners of other GFP fusion proteins of interest in *C. elegans*.

Innovative aspects

- Elucidation of new DAF-16 interactors in the wild type and *daf-2* mutant
- Removal of background during GFP-IP using quantitative proteomics

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Cancer Proteome Biology and Medicine

(C201 – C606)

C201

Novel biomarker candidates for well differentiated thyroid cancer

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Introduction
Thyroid tumor diagnostics is a challenging field, as the initial standard approach, namely fine needle aspiration biopsy (FNAB), is inconclusive in about 15-20% of cases (1). To overcome this obstacle, thyroid oncology research has shifted its focus in discovering molecular biomarkers that will facilitate a safer and timelier diagnosis. Proteomics, in particular, has been gaining ground as a promising research field for the discovery of such markers.

Methods
Cytosolic protein extracts from frozen thyroid tumor tissue (follicular thyroid adenoma or FTA, follicular thyroid carcinoma or FTC and papillary thyroid carcinoma or PTC), as well as from frozen normal thyroid, were separated by two dimensional electrophoresis (2DE). In total 39 gels were scanned and image analysis was performed by using PDQuest. This software was also used to assess significant quantitative differences in protein expression between the different tumor groups. Those significant proteins were excised from the gels, digested by trypsin and the peptides produced were purified and processed by MALDI-TOF-MS for protein identification. The final step in this procedure is to validate these results by Western Blot and/or immunohistochemistry.

Results
On average approximately 1200 protein spots were separated in each gel included in the study. After meticulous review of the image analysis results, we came up with 92 protein spots with statistically significant differences in expression between FTA and FTC. Among these, annexin V and HSP90 seem to be overexpressed in FTA. For the comparison between FTC and PTC 70 protein spots differed significantly with e.g. cathepsin B being among those overexpressed in PTC.

Innovative aspects
- One of the few proteomic studies in thyroid cancer
- The first study to include subcellular protein fractions from FTA, FTC, PTC and normal thyroid tissue

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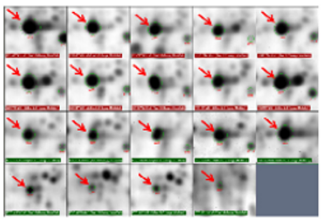

Figure 1. The protein spot corresponding to annexin V (red arrows) as reviewed by image analysis software in a series of 19 gels. The upper ten panels (red) indicate the FTA group, while the lower nine panels (green) indicate the FTC group.

C202

Functional Proteomic Analysis of Active Serine Proteinases in Ovarian Cancer Ascites Fluid

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Introduction:
Activity-based probes (ABPs) have become major tools for monitoring proteinase activity (1). Using these probes, we have recently developed a highly sensitive ELISA method to quantify the enzymatically ACTIVE (vs. immuno-reactive) form of a tumour-derived serine proteinase, kallikrein-related peptidase 6 (KLK6), in ovarian cancer ascites and have shown that its concentration would be sufficient to trigger tumour signaling (2). The present study aims to identify other active tumour-derived proteinases that may regulate tumour tissue function and may be of therapeutic importance.

Methods:
Isolation of active serine proteinases from ovarian cancer ascites fluid was performed with a biotinylated activity-based probe as bait (Fig. 1). Optimization of the method was performed by KLK6 quantification with our activity-based probe ELISA (2). Immobilization of the biotinylated complex was done with streptavidin-conjugated magnetic beads and was followed by on-bead trypsin digestion and enzyme identification by LC-MS/MS. Isolation of active proteinases complexed to proteinase inhibitors in ascites fluid was also performed using inhibitor-specific antibodies. The immobilized complex was subjected to protein identification as above. Identified enzymes were assigned to molecular and biological pathways using bioinformatic tools and publicly available protein databases (3).

Results:
Our activity-based probe proteomics protocol confidently identified the KLK6, which was added to the ascites fluid prior to ABP incubation. Furthermore, other endogenous proteinases, proteinase-related proteins and proteinase inhibitors were identified within the ovarian cancer ascites fluid proteome. More specifically, our list included serine proteinases of the complement system, thrombin, coagulation factors, KLK10 and plasma kallikrein, which corresponded to about 15% of the total number of proteins identified. All of the identified proteinases have been previously associated either directly or indirectly with carcinogenesis.
Estimation of the proteinase enzymatic activity with the help of fluorogenic substrates revealed that the overall activity of serine proteinases in ovarian cancer ascites is low (< 1 nM of trypsin-like activity). We, therefore, aimed to identify the proteinases that are active but sequestered by inhibitor complexes in ovarian cancer ascites fluid. Our preliminary analysis of potential KLK6 inhibitors in ascites fluid has revealed high molecular weight complexes of the enzyme with α2-macroglobulin and α1-antitrypsin.

Innovative aspects:
- Successful coupling of serine proteinase activity-based probes with sensitive and high-throughput tandem mass spectrometry; the method can be applied to other enzyme classes or cancer types.
- Identification of ACTIVE serine proteinases and POTENTIALLY ACTIVE proteinases in inhibitor complexes in ovarian cancer ascites fluid.
- Assignment of these enzymes to ovarian cancer-related pathways.

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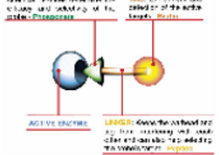

Figure 1. Typical structure of an activity-based probe (modified from Ref. 1). The probe contains three groups: (a) a warhead, which reacts with the active site of the proteinases and in this study contains a diphenylphosphonate group, (b) a linker region, which in the case of the present probe contains two amino acids (proline and lysine) to assist in the selectivity of the probe for binding to serine proteinases with trypsin-like activity, and (c) a tag that allows immobilization and visualization of the probe-proteinase complex.

C203

Identification of lymphoma-associated vascular accessible antigens using DeepQuanTR

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Introduction
The identification of biomarkers discriminating healthy and diseased tissues while being easily accessible from the bloodstream is a crucial requirement for the design of site-directed drug delivery approaches. Here we describe the in vivo protein biotinylation of a complex mouse model of cancer and the identification of novel targets for antibody-based lymphoma therapy using two-dimensional peptide mapping and DeepQuanTR. Candidate biomarkers abundantly expressed in lymphoma lesions while being absent in normal tissues have been identified and were confirmed by in vivo targeting experiments.

Methods
The label-free relative quantification of vascular accessible proteins after in vivo protein biotinylation (1) of a complex mouse model of lymphoma has been performed using the two-dimensional peptide mapping technology in combination with DeepQuanTR (2). A panel of nine differentially regulated proteins has been characterized by immunofluorescence on normal and diseased mouse tissues. Three promising candidate antigens (an endothelial, a stromal and a tumor cell surface expressed antigen) have been further validated by in vivo targeting studies. Finally, two independent monoclonal antibodies against an endothelial antigen were intravenously injected into a syngeneic mouse model of the disease to investigate their potential of tumor-growth inhibition.

Results
The in vivo biotinylation of healthy mice and mice bearing disseminated B cell lymphoma resulted in an efficient labeling of accessible proteins in all analyzed lesions and the corresponding normal tissues. Protein extracts of liver lesions, lymph node metastases and spleen metastases as well as their healthy host tissues were used for label-free relative quantification applying the two-dimensional peptide mapping technology in combination with DeepQuanTR. The proteomics analysis resulted in the identification of about 700 proteins, of which more than 100 were upregulated at least ten-fold. The immunofluorescent characterization of nine selected candidate biomarkers confirmed their preferential expression in diseased compared to normal mouse tissues. In order to validate the accessibility of these antigens for antibody-based lymphoma therapy, commercial antibodies against three candidate biomarkers were intravenously injected into mice bearing the analyzed B cell lymphoma, confirming selective in vivo localization. Finally, a therapy experiment targeting an endothelial antigen with monoclonal antibodies resulted in a significant reduction of tumor growth (3).

Innovative aspects
- Identification of vascular accessible antigens in a complex lymphoma model
- Large scale, label-free quantification study using DeepQuanTR
- Therapeutic effect after targeting of an endothelial antigen

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C204

Complementary Quantitative Proteomics reveals an AP-4 complex repressing *HDM2* expression

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Introduction

AP-4 is a ubiquitously expressed transcription factor which can either activate or repress target gene expression through recruiting other co-factors binding to the promoter region. Here we demonstrate E-box mediated *HDM2* repression by AP-4 in p53- and HDAC-independent manners. To unravel the role of AP-4 in *HDM2* repression, we applied DNA affinity purification followed by complementary quantitative proteomics to identify unknown AP-4 associated complex. Our data suggests that AP-4 participates in a transcriptional regulating complex at the *HDM2*-P2 promoter in response to DNA damage.

Methods

An E-box in *HDM2*-P2 promoter responsible for transcriptionally repressing *HDM2* was identified by electrophoretic mobility shift assay, chromatin immunoprecipitation, and luciferase reporter assay. Two biotinylated oligonucleotides containing either wild-type or mutant E-box were synthesized and conjugated onto strepavidin-coated magnetic particles. Nuclear extracts from HCT 116 cells were incubated with two synthesized DNA-beads. Proteins containing putative AP-4 complex and other contaminating proteins enriched and labeled with either cICAT or iTRAQ methods. After LC-MS/MS analyses, identification and quantification of candidate AP-4 protein complex was performed by Mascot followed by ASAPRatio and Multi-Q (1).

Results

We first demonstrated AP-4 binds to an E-box of *HDM2*-P2 promoter and represses *HDM2* transcription. Next we performed DNA-affinity purification using the identified E-box followed by cICAT and iTRAQ labeling methods, and identified a previously unknown E-box-bound AP-4 protein complex containing 75 components. The two labeling methods complementarily quantified differentially AP-4-enriched proteins, including the most significant recruitment of DNA damage response proteins, followed by transcription factors, corepressors, and histone-modifying proteins. Specific interaction of AP-4 with CTCF, SP1, and HDAC1 (an AP-4 corepressor) was validated using AP-4 truncation mutants. Importantly, inclusion of trichostatin A did not alleviate AP-4-mediated repression of *HDM2* transcription, suggesting a previously unidentified HDAC-independent repression mechanism. In contrast, our study suggested that transcription repression occurs via coordination with other transcription factors, histone methyltransferases, and/or nucleosome remodeling SWI/SNF complex. Our data also suggest that AP-4 participates in a transcriptional regulating complex at the *HDM2*-P2 promoter in response to DNA damage.

Innovative aspects

1. Identification of an E-box of *HDM2*-P2 promoter responsible for AP-4 mediated *HDM2* transcriptional repression.
2. Demonstration of utility of complementary quantitative proteomics for characterizing protein complex from single-step affinity purification.
3. Implication of potential role of AP-4 in regulating *HDM2* expression in response to DNA damage.

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Figure 1. Possible mechanisms of AP-4-mediated *HDM2* transcription repression revealed by complementary quantitative proteomics.

C205

Development of an anti-tumor blood vessel specific antibody using phage antibody library

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Introduction

Tumor blood vessels are vital for tumor growth[1]. Therefore, these blood vessels are potential targets for anti-cancer therapy. The purpose of this study is to develop anti-tumor endothelial cell (TEC) antibodies for delivering anti-cancer agents or drugs. To achieve this goal, we utilized the phage antibody display library method to create monoclonal antibodies *in vitro*. Accordingly, we developed anti-TEC antibodies from an antibody phage display library[2] prepared using the Fv genes amplified from the mRNAs isolated from the TEC-immunized mice.

Methods

TEC model: Human umbilical vein endothelial cells (HUVEC) cultured in Colon26 carcinoma-conditioned medium (Colon26 CM-HUVEC) were used as model TECs.

phage antibody library: The single chain antibody fragment variable (scFv) phage display library was prepared by amplifying the Fv genes from the mRNA derived from the TEC-immunized murine splenocytes.

Selection of anti-TEC specific antibodies: Anti-TEC antibodies were selected by cell panning against Colon26 CM-HUVEC with subtractive panning against normal HUVEC.

Evaluation of the selected antibodies: DNA sequences of the isolated antibody genes were analyzed. The binding specificities of the phage antibodies were assessed in vitro by using ELISA and Western blotting (WB), and in vivo by analyzing their accumulation on the tumor tissue.

Results

The size of the phage antibody library prepared from the mRNA of the TEC-immunized mice was approximately 1.3×10^7 CFU. To select and enrich for the phages displaying the anti-TEC antibodies, cell panning was performed first using the Colon26 CM-HUVEC followed by subtractive panning using the normal HUVEC. After five cycles of panning, the affinity of bound phage clones increased approximately 10,000 folds. Subsequently, clones isolated from the post-panning output library were tested for their antigen-specificity by ELISA and WB. One of the scFv phage clones showing antigen-specificity recognized only Colon26 CM-HUVEC in vitro, and when injected into the Colob26 bearing mice, this clone accumulated more on the tumor surface than the wild type phage. These results suggest that the isolated an antibody and this clone's target molecule could be potentially useful for novel anti-tumor therapies.

Innovative aspects

- Preparation of a phage antibody library using mRNAs from TEC-immunized mice
- Selection of an anti-TEC antibody from the phage antibody library

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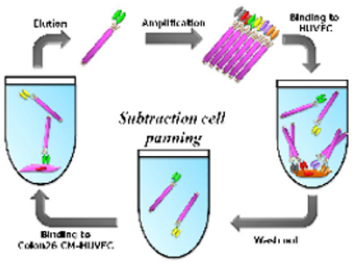

Figure: Subtraction cell panning

C206

PRA1 knockdown-associated dysregulated proteins in nasopharyngeal carcinoma cells

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Introduction

The Epstein-Barr virus (EBV)-encoded oncoprotein latent membrane protein 1 (LMP1) majorly contributes to EBV-associated malignancy, including nasopharyngeal carcinoma (NPC). We have previously identified prenylated Rab acceptor 1 (PRA1) as a new cellular interacting partner for LMP1 promoting LMP1's intracellular trafficking and signaling (1). Herein we demonstrated that LMP1 expression led to PRA1 sequestration to LMP1 exhibition sites.

Methods

To elucidate the effect of PRA1 sequestration and assess the role of PRA1 in LMP1-mediated tumorigenesis, we generated PRA1-knockdown NPC stable clones, which exhibited a change in cell morphology and an increase in cell motility, and sought to identify proteins differentially expressed in these knockdown cells by means of isobaric mass tags (iTRAQ) labeling combined with multidimensional liquid chromatography-mass spectrometry (LC-MS/MS).

Results

We discovered a panel of proteins which showed consistent dysregulated expression in PRA1-knockdown clones and represent several functional categories including lipid metabolism/transport (such as FABP5 and CAV1) and cell adhesion/migration (such as LAMC2, ITA6 and ITB4). The protein expression level was verified using Western blotting (Fig 1) and quantitative RT-PCR. Immunofluoresence microscopy demonstrated altered localizations of ITA6, ITB4 and CAV1 in conjunction with intracellular cholesterol accumulation in PRA1-knockdown clones and these alterations was phenocopied by treatment with a cholesterol transport inhibitor, U18666A. Ectopic PRA1 expression could alleviate the dysregulated expression of selected proteins initiated by PRA1 knockdown or U18666A treatment, implicating the involvement of PRA1 in regulation of these proteins. Consistently expression of these proteins was dysregulated under LMP1 expression, associated with an increase in intracellular cholesterol accumulation and in cell motility, suggesting that PRA1 knockdown at least in part resembled the spatially functional hindrance resulted from LMP1 sequestration of PRA1.

Innovative aspects

- Proposing a PRA1-involved linkage between lipid transport and cell migration
- Providing new insights into the molecular etiology of NPC, namely effects of LMP1-PRA1 interaction

References

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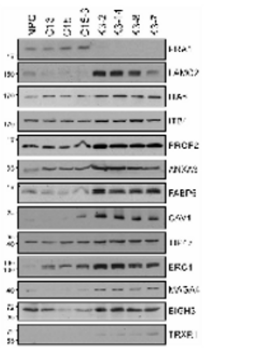

Figure 1. Validation of proteins identified to have differential expression from iTRAQ data. Equal amounts of protein lysates from four PRA1-knockdown clones (K3-2, K3-7, K3-8, and K3-14), three mock clones (C15, C15-1, and C15-3), and parental NPC cells were applied to Western blot analysis with antibodies as indicated. α-Tubulin was used as the loading control.

C207

HnRNP-K Is a Novel Binding Protein of YAP in Hepatocellular Carcinoma

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Introduction

Yes associated protein (YAP) is a negatively regulated downstream target of Hippo signaling pathway, and functions as a transcriptional co-activator involved in the regulation of cell growth, proliferation and apoptosis (1). Our recent findings have shown that YAP is a bona fide oncogene in mouse liver carcinoma model (2), and clinically associated with poor survival and tumor recurrence of HCC patients (3). We also find overexpression of YAP oncoprotein in human HCC tissues and predominately accumulated in the nucleus. Nuclear translocation of YAP effects multiple target gene activation relating to cancer cell proliferation and survival. This study employed the proteomic method to identify YAP-binding protein(s) that plays a pivotal role in the nuclear functions of YAP.

Methods

The total cell lysate from MIHA-YAP cells (YAP over-expressing MIHA cells) and Hep3B cells (endogenous high expression of YAP) were immunoprecipated by using a rabbit polyclonal antibody against YAP to obtain the binding complex. Then the complex was subjected to 1-D SDS-PAGE gel electrophoresis and visualized by silver staining (Fig 1). The antibody only and rabbit IgG were used as controls. Four distinctive protein bands were trypsin digested and analyzed by MALDI-TOF MS/MS (Fig 2). The identified protein (hnRNP-K) was cloned into pEGFP-N1 plasmid and tagged with GFP. The binding domain of YAP with hnRNP-K was further confirmed by using co-IP and double immunofluorence staining with YAP different domain deletions. The localization of YAP and related target genes were investigated by western blotting and qPCR after transient transfection of hnRNP-K.

Results

Heterogeneous nuclear ribonuclear protein K (hnRNP-K), an RNA- and DNA-binding protein was identified in the immunoprecipated YAP complex. Co-IP showed that hnRNP K interacts specifically with the proline-rich amino terminus of YAP protein. Both the hnRNP K and YAP proteins were localized in the tumor nucleus and ectopic expression of hnRNP K could further enhanced the YAP targeted genes expression.

Innovative aspects

- hnRNP-K is known frequently overexpressed in many cancers, but the molecular mechanism is unknown. We identify hnRNP-K as a potential partner with YAP oncogene in HCC, providing a novel insight of hnRNP-K in carcinogenesis.
- hnRNP-K binds to the proline-rich domain of YAP oncoprotein that sequestrate nuclear YAP and activate multiple target genes causing tumor proliferation and survival.

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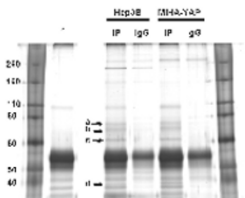

Fig. 1. The immunoprecipated YAP complexes were visualized by 1-D SDS-PAGE gel electrophoresis and silver staining. Four distinctive protein bands (a, b, c and d) were found in both complexes from MIHA-YAP and Hep3B cell lysate. (1: Antibody only control)

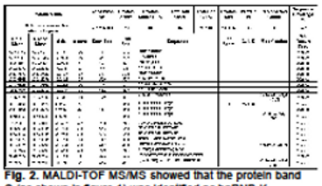

Fig. 2. MALDI-TOF MS/MS showed that the protein band C (as shown in figure 1) was identified as hnRNP-K.

Human Liver Proteomics between Early and Late Recurrence of Hepatocellular Carcinoma

C208

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Introduction
Half of hepatocellular carcinoma (HCC) cases early recur within two years after the curative resection. However, we may observe late or no recurrence in certain HCC cases. We suppose that the difference between early and late recurrence is due to two mechanisms: one is intrahepatic metastasis of the primary HCC which is related to the degree of malignancy of the primary HCC. Another mechanism of HCC recurrence is multi-centric carcinogenesis from non-tumorous liver and not recurrence by original primary tumor. To find signature for the recurrence of HCC, we, in this study, obtained tumorous and non-tumorous tissues from HCC cases with early and late recurrence after the first curative resection, and compared protein expression profiles of liver tissues between the two recurrence groups.

Methods
We studied a total of 41 cases of hepatitis C virus-positive HCC. Our subjects were restricted to the early stages of HCC. They were all solitary, and multiple HCC cases were excluded. They also had no metastasis to lymph nodes and other organs. The tumor was less than 6 cm in diameter. The 2D-DIGE of 32 tumorous and 27 non-tumorous samples was performed. 11 of the 32 tumorous samples (less than 12 months to recurrence) are referred to as an early recurrence group, while 10 samples (more than 48 months to recurrence) are a late group. As for non-tumorous samples, 15 and 12 samples were an early (less than 24 months to recurrence) and a late recurrence group (more than 48 months to recurrence), respectively. Protein spots with statistically differential expression between the two groups of early and late recurrence were detected by the Progenesis SameSpots software, and identified by MALDI-TOF MS or LC-MS/MS after in-gel digestion with trypsin, followed by the Mascot search.

Results
1623 protein spots were detected in 32 tumorous samples, of which 51 were differentially produced between the two groups. Clustering analysis of 21 tumor samples using 51 proteins resulted in 2 groups in which one sample was misclustered. 10 up- and 8 down-regulated proteins in the early recurrence/tumor group were identified by MALDI-TOF MS. Malignant tumor markers previously suggested were up-regulated in the early recurrence tumor group. Anti-oxidative stress and anti-angiogenesis proteins were up-regulated in the late recurrence tumors group. Thus, we suggest a set of biomarkers from tumor tissues which may be useful for predicting recurrence. On the other hand, 1513 protein spots were detected in 27 non-tumorous tissue samples. We subdivided the 27 samples into 16 cases of chronic hepatitis and 11 cases of liver cirrhosis to analyze the recurrence-related proteins in each category. 60 protein spots were differentially expressed in chronic hepatitis with early vs. late recurrence, and 32 spots were detected in liver cirrhosis. Nonetheless, we examined whether these protein spots were shared between chronic hepatitis and liver cirrhosis. However, none was. Therefore, different mechanisms are involved in recurrence between chronic hepatitis and liver cirrhosis, and recurrence-related markers are also identified from non-tumorous liver tissue.

Innovative aspects
- Signature for the recurrence of HCC determined by 2D DIGE of liver tissues in comparison between early and late recurrence
- A set of predictor proteins for the HCC recurrence

Proteomic approach of multiple parallel chromatographies for identification of tumor related antigens.

C209

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Introduction
Autoantibodies have been described in various cancer patients to be useful for cancer diagnostics, prognostics, and potential targets for immunetherapy (1). Serological proteome analysis (SERPA) combining 2-DE is a classical platform to identify tumor-associated antigens in the serum samples of cancer patients (2). But, it often hardly distinguishes a true antigen out of complex proteome identified from a single protein spot in a 2-D gel. To overcome this problem, we employed multiple parallel chromatographies to identify a true antigen.

Methods
A breast cancer cell line Hs578T was used as an antigen source. The protein extract from Hs578t was splitted into four, and each aliquots was fractionated by MonoQ (MQ), Phenyl Superose (PS), or isoelectric focusing chromatography (IEF), or by using an OFFGEL IPG strip (pH 3-10) separately. Four different fractionations were resolved by SDS-PAGE. To test this method, commercial antibodies specific for ARPC2 and PTEN were utilized as the primary antibodies, and the identical band sets identified from the four chromatographies, based on the molecular size, were excised and analyzed by LC-MS/MS. In application step, four different fractionations were immunoblotted with pooled healthy or cancer plasma as a primary antibody (Figure 1).

Results
We identified ARPC2 from the western blot bands of the four different chromatographies and it was the only common protein. but failed to identify PTEN probably since that the cellular PTEN level is too low to be detected by LC-MS/MS.
To identify immunoreactive cancer proteins, the overexpressed bands were digested by trypsin, and analyzed by LC-MS/MS. By this method, we identified two tumor-associated antigens which overlapped in 4 parallel fractionations. There also existed five candidate antigens which overlapped in 3 of the four chromatographies. In conclusion, our method is useful for accurate identification of antigens from complex mixtures as far as they are detectable under the current MS technology

Innovative aspects
- The candidate list of tumor/disease-related biomarkers can be narrowed down by removing false positives.
- Various applications of column separations off line in combination with LC-MS/MS technology for accurate and precise identification of differentially expressed proteins and other biological components.

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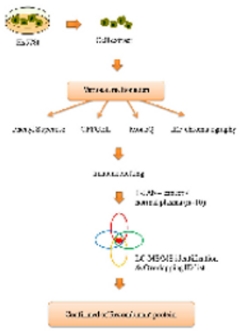

Figure 1. Scheme of identification of breast cancer-associated antigens by multiple parallel chromatography and LC/MS/MS.

Over-expression of Glyoxalase I in Human Gastric Carcinoma determined by the proteomic approach

C210

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Introduction
Gastric cancer is the fifth leading cause of cancer-related death in Taiwan. Histomorphologically, "intestinal" and "diffuse" are the two primary gastric cancer types. Identification of gastric cancer related factor is essential for diagnosis and prognosis. The expression level of glyoxalase I (GLO1) is increased in colon cancer, prostate cancer and breast cancer (1). But the GLO1 expression level in gastric cancer is still unknown. This study attempts to identify novel putative diagnostic or prognostic markers using proteomic approach.

Methods
The two-dimensional (2D) gel electrophoresis following matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF MS) analysis following real-time quantitative RT-PCR (Q-RT-PCR) and immunohisto-chemistry (IHC) was used to determine the aberrantly expressed protein in gastric cancer tissues. The clinico-pathological correlations and prognostic significance of these aberrantly expressed proteins were evaluated.

Results
Via 2D gel electrophoresis and MALDI TOF/TOF MS of gastric cancer tissues compared with normal stomach tissues, 271 proteins were identified from 418 selected spots on 2D gels. Real-time Q-RT-PCR data for GLO1 expression is presented as the ratio of GLO1 signals in tumor tissue to that in non-cancerous adjacent tissues. Protein GLO1 is significantly up-regulated in 54.9% (T/N>1.5, 49/71) of gastric patients (Table 1). Expression levels of GLO1 were further verified in several patients via Western blot analysis. Figure 2A shows an intestinal-type and Figure 2C a diffuse-type tumour. The immunostaining was most marked (dark brown color) in the cancer cells, and low levels were observed in the stroma cells or fibroblasts in gastric cancer tissues. Weak staining was observed for GLO1 in the normal gastric epithelial cells (Figure 2B and D). The 70 of 76 gastric tumor tissues have high GLO1 expression more than normal tissues.

Innovative aspects
- A putative novel biomarker in gastric carcinoma
- GLO1 is specifically overexpressed in gastric tumor tissues.

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	Cases	percentage
T/N > 2	32	45.07%
2>T/N > 1.5	7	9.86%
1.5>T/N >1	9	12.68%
T=N	2	2.82%
T<T/N	21	29.58%
Total case	71	100%

Table 1. The Q-RT-PCR determined GLO1 mRNA expression levels in the tumor tissue divided by its matching noncancerous mucosa.

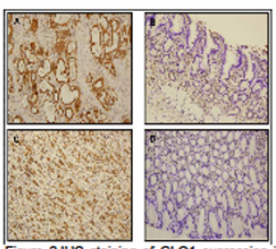

Figure 2.IHC staining of GLO1 expression in normal or gastric cancer tissues. (A) intestinal type; (C) diffuse type; (B) and (D) adjacent normal tissues.

Over-expression of Gelsolin in Human Cervical cancer determined by the proteomic approach

C211

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Introduction
Carcinoma of the uterine cervix is still the most prevalent female genital cancer worldwide. Identifying biomarkers is essential to improve patient survival. The clinicopathological correlations and prognostic significance of these aberrantly expressed proteins were evaluated to determine the novel cervical cancer biomarkers. Gelsolin is an actin binding protein that involved in many cellular function such as cell migration, invasion and apoptosis. (1). In this study, we used proteomic approach to identify the biomarker and its significance in cervical cancer.

Methods
We used two-dimensional (2D) gel electrophoresis following matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF MS) analysis. The over-expression of proteins was confirmed by using real-time quantitative reverse-transcription polymerase chain reaction, Western blot and immuno- histochemical analysis. The clinico-pathological correlations and prognostic significance of this aberrantly expressed protein were evaluated.

Results
Two-dimensional gel electrophoresis and subsequent MALDI-TOF mass spec-trometry analyses led to the identification of 31 differentially expressed proteins. In this study, expression of gelsolin is significantly up-regulated in 60 % of cervical patients analyzed by the quantitative PCR either in squamous cell carcinoma (SCC) or adenocarcinoma (AD). The immunohistochemical analysis shows that the expression of gelsolin were higher in the cervical cancers than normal (Fig 1). The gelsolin expression in tumor tissues was 2.14-fold increased compared with that expressed by adjacent non- cancerous mucosa (Fig 2).

Innovative aspects
1. The gelsolin is a secreted protein and highly expressed in cervical cancer.
2. The gelsolin may be used as a biomarker and therapy target in cervical cancer.

Reference
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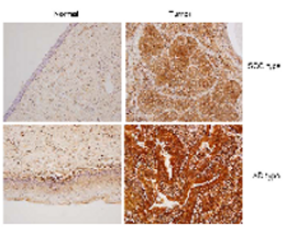

Figure 1. Cervical cancer tissues and normal tissues were stained with gelsolin by immumohistochemistry. The tissues were divided into SCC and AD.

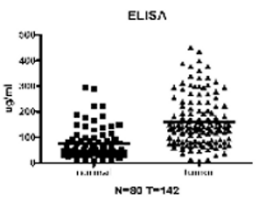

Figure 2. The quantification of serum gelsolin was detected by ELISA. The serums were from healthy donors and cervical cancer patients.

C212

Nucleophosmin as a prognostic biomarker of Ewing sarcoma revealed by proteomics

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Introduction
Ewing sarcoma (ES) is the second most common primary malignant bone tumor in children and adolescents. Intensified first-line chemotherapy regimens could improve clinical outcomes as compared to conventional chemotherapy. However, intensified therapy may result in serious toxicity, including fatal gastrointestinal toxicity, grade 3 or 4 infections, and severe myelosuppression. Approximately two-thirds of patients with localized ES are cured with conventional therapy alone. Therefore, the identification of prognostic factors may lead to the development of risk-adapted treatment strategies for ES.

In this study, we performed a proteomic study using biopsy samples from ES patients. We found that nucleophosmin (NPM) expression significantly correlated with progression of ES (1).

Methods
This study firstly included a total of eight frozen incisional biopsy samples taken before treatment at the time of diagnosis from eight ES patients. From the eight cases included in the study, three patients were alive and continuously disease free in the follow-up period of at least three years from diagnosis and five patients were dead of disease within two years from initial diagnosis. All five patients in the latter group developed distant metastases within seven months from initial diagnosis.

Protein samples were prepared from the tumor tissues and subjected to two-dimensional difference gel electrophoresis (2D-DIGE) (2) with an internal control sample. Protein expression profiles were compared between the different patient groups. The proteins corresponding to the protein spots with different intensity between the two groups were identified by mass spectrometry. The results were validated by immunohistochemistry in additional 34 cases.

Results
The protein expression profiles produced using 2D-DIGE consisted of 2364 protein spots among which we identified 66 protein spots whose intensity showed more than two-fold difference between the two patient groups. Mass spectrometric protein identification demonstrated that the 66 spots corresponded to 53 distinct gene products.

Pathway analysis revealed that 31 of 53 proteins, including NPM, were significantly related to bone tissue neoplasms ($p<0.000001$).

The prognostic performance of NPM was evaluated immunohistochemically on an additional 34 ES cases. Following univariate analysis, NPM-positivity ($p<0.01$) and clinical stage (presence of metastatic disease at diagnosis) ($p<0.01$) significantly correlated with shorter overall survival. No other factors examined, including the tumor size, age at diagnosis, sex, and primary site were associated with overall survival. Multivariate analysis performed on NPM staining and clinical stage, identified as significant prognostic factors by univariate analysis, revealed that both significantly correlated, as separate variables, with overall survival.

Innovative aspects
- These results establish NPM as an independent prognostic marker for ES patients.
- Measuring NPM in biopsy samples before treatment may contribute to the effective management of ES.

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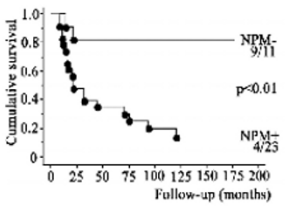

Figure 1. Kaplan-Meier curves for overall survival are illustrated for all patients by NPM positivity. Statistical significant differences in overall survival period were observed between the NPM positive and negative cases ($p<0.01$, log-rank test).

C213

Identification of proteomic differences between squamous cell carcinoma of the lung and bronchial epithelium

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Introduction
Lung cancer has both in Europe and the US a high incidence. Most of the patients have a poor prognosis, the median survival is shorter than 20 moth. Diagnosis and treatment of the disease are difficult because of tumor subtypes' heterogeneity and late diagnosis. Therefore early detection of the disease using biomarker proteins may lead to improved treatment outcomes. In our study we compared microdissected tissue from lung squamous cell carcinomas and bronchial epithelium. Proteins exhibiting a higher expression level in cancerous tissue are candidates for biomarkers which might help to improve diagnosis and treatment of lung cancer.

Methods
After manual microdissection of bronchial epithelium and lung squamous cell carcinoma tissue, we used protein fluorescence dye saturation labelling in combination with two dimensional differential in gel electrophoresis (2D-DIGE) to separate protein species. Image analysis revealed differentially expressed proteins which were subsequently identified by mass spectrometry (MALDI-MS and nano-HPLC/ESI-MS/MS). For validation of differentially expressed proteins tissue microarrays were used.

Results
We compared the proteome of microdissected cells from normal human bronchial epithelium (n=7) and squamous cell carcinoma tumors (n=14) of histopathological grade G2 and G3. 2D-DIGE analysis and subsequent MS based protein identification revealed that 32 non redundant proteins were differentially regulated between the respective tissue types. Cluster analysis of differential protein spot data demonstrated that based on protein expression data not only a separation of tumor from non-tumor tissue but also stratification between tumor grades is possible. Furthermore immunohistochemical validation of five proteins showed to be differentially expressed proteins was carried out. The experiment on tissue microarrays containing tissue specimen of 55 patients, including normal bronchial epithelium, squamous cell carcinomas, adenocarcinomas and large cell carcinomas confirmed the data from the DIGE-analysis. Additionally we could find cytokeratin 17 to be significantly higher abundant in G2 grade compared to G3 grade squamous cell carcinomas in both the gel based and the immunohistochemical analysis. Therefore this protein might be used as a marker for stratification between different tumor grades.

Innovative aspects
- Identification of biomarker candidate proteins
- Proteome analysis of 5 µg of sample protein from microdissected tissue

C214

Determining the Mechanisms of Action of a COX-2 Inhibitor in Mesothelioma using Antibody Microarrays

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Introduction
Malignant pleural mesothelioma (MPM) is a rare but highly aggressive disease which is increasing in incidence. Currently, treatment options for these patients are limited and prognosis is extremely poor. The incidence of MPM has been predicted to rise dramatically within the next 20 years due to the number of people who were exposed to asbestos in the workplace before 2000. We have identified COX-2 inhibitors which have a cytotoxic effect in mesothelioma cells (1, 2) and have investigated the mechanism of effect using antibody microarrays.

Methods
The mesothelioma cell lines MSTO-211H and NCI-H2052 were lysed in triplicate in Laemmli buffer and COX-2 expression was assessed by western blotting using two separate antibodies (#SC-19999 Santa Cruz Biotechnology and #ab52237 Abcam). Cells were treated with the specific COX-2 inhibitor DuP-697 (Tocris Bioscience #1430) in a dilution series (0, 1, 2.5, 5, 10, 25, 50, 100, 250 µM) in triplicate and cell proliferation was assessed using an MTT assay. Assays were performed with 24, 48 and 72 hour incubation periods and the IC_{50} values were calculated. The mechanism of effect of DuP697 was analysed in the cells using the Panorama XPRESS Profiler725 Antibody Microarray (Sigma-Aldrich #XP725).

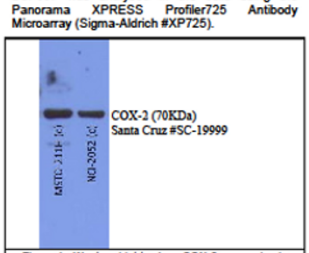

Figure 1: Western blot to show COX-2 expression in MSTO-211H and NCI-H2052 mesothelioma cell lines

Results
The mesothelioma cell lines MSTO-211H and NCI-H2052 showed strong COX-2 expression by western blot in all replicates and with both antibodies tested (see figure 1). MTT results indicated that the specific COX-2 inhibitor DuP-697 has cytotoxic effects on the two mesothelioma cell lines MSTO-211H and NCI-H2052. The IC_{50} values were established at 72hrs (see figure 2). The IC_{50} values at 72hrs were used to assess the mechanism of action of DuP697 in treated vs. untreated cells using the Panorama XPRESS Profiler725 antibody microarray. Results show a number of differentially expressed proteins which maybe related to the actions of the COX-2 inhibitor DuP697. Proteins related to apoptosis were shown to be down-regulated in treated cells. These results may give a greater understanding into the cytotoxic nature of the COX-2 inhibitor and its mechanisms of effect.

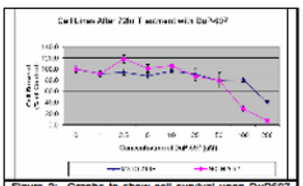

Figure 2: Graphs to show cell survival upon DuP697 dosage after 72hrs in MSTO-211H and NCI-H2052 mesothelioma cells

Innovative aspects
- We have utilised the antibody microarrays to investigate mechanisms of action of novel therapeutic strategies in mesothelioma.
- The identification of proteins and cellular pathways which are affected by COX-2 inhibitors will lead to increased efficacy of therapy in mesothelioma and will highlight novel drug targets.

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C215

Cdk-dependent phosphorylation regulates localization and interaction of DDK with chromatin

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Introduction Aneuploidy and cancer may developed if DNA replication were not tightly regulated. To prevent this disaterous consequence, a mechanism has evolved to ensure that a mitotic cell replicates its entire genome only once per cell division. The Dbf4-Cdc7 (DDK) kinase is an essential part of this replication control mechanism. DDK acts as a licensing factor for DNA replication at individual origins of DNA replication. Based on DDK's role in activating DNA replication, novel drugs have recently been developed (1). Despite the extremely important role of DDK in tumorigenesis, the regulation mechanism of Cdc7 itself is largely unknown.

Methods An integrated mass spectrometry and site-directed mutagenesis approach were carried out to map the phosphorylation sites on the primary Cdc7 sequence. The functional significance of Cdc7 phosphorylation was also determined using a Cdc7 overexpression system.

Results We found that the phosphorylation of Cdc7 is cell-cycle dependent, with a peak of phosphorylation during G2/M. Our data are consistent with the notion that: (1) Cdc7 phosphorylation is Cdk1-dependent, as inhibition of Cdk1 leads to the decrease in phosphorylated Cdc7 (pCdc7); (2) Cdc7 phosphorylation may be relevant to its ability to interact with chromatin; and (3) pCdc7 is associated with the nuclear export factor CRM1, suggesting that phosphorylation may play a role in facilitating nuclear transport.

Innovative aspects
- Offers mechanistic insight into the regulation of an essential kinase during the cell division cycle.
-Suggests that Cdc7 may have novel roles outside of the initiation of DNA replication.
-Provides a basis for the continued analysis of structure-function relationships of Cdc7 within and beyond the context of canonical DDK function at the chromatin

References:

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Phosphoproteome analysis of breast cancer signaling regulated by an EGFR1/HER2 kinase inhibitor

C216

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Introduction
Overexpression and/or hyperactivation of human epidermal growth factor receptor 1 (EGFR1, HER1) and HER2 promote tumor growth in breast cancer cells. An EGFR1/HER2 dual kinase inhibitor, lapatinib, has been shown to exhibit excellent specificities [1] and is currently in the market for clinical treatment of breast cancer. Although the selectivity of lapatinib has been well characterized *in vitro*, its downstream effect on signaling pathways *in vivo* is poorly understood. To date, an *in vivo* profiling strategy using immobilized kinase inhibitors in coupled with mass spectrometry has been reported to characterize kinase inhibitors [2]. In this strategy, however, the captured proteins are generally limited to kinases and therefore it is difficult to overlook the entire signaling network affected by the inhibition of target kinases. In this study, to investigate the *in vivo* mechanism of the molecular-targeting drug in the signaling network, we measured global phosphorylation dynamics of SKBR3 human breast cancer cells regulated by lapatinib using highly efficient phosphopeptide enrichment technique [3] together with SILAC-based quantitative phosphoproteomic approaches.

Methods
In all experiments, SILAC-based quantitation was performed. The SILAC-labelled SKBR3 cells were treated with lapatinib (0.1 μM and 1 μM) for 1, 5, 10 and 60 min. Phosphopeptide enrichment was performed based on aliphatic hydroxyl acid-modified metal oxide chromatography (HAMMOC) [2], and the purified phosphopeptides were analyzed by nanoLC-MS/MS using an LTQ-Orbitrap.

Results
Using this unbiased phosphoproteomic approach, we have quantified 4,505 unique phosphorylation sites (pS/pT/pY; 3494/806/205) on 1,830 proteins. It was found that kinases, phosphatases, receptors, metabolic enzymes and numerous functional proteins (~20% of the identified sites) were regulated at least 2-fold by lapatinib treatment. We also observed the down-regulated phosphorylation of EGFR1/HER2 pathway molecules including EGFR1, HER2, SHC1, SOS1 and ERK1/2 with the time- and dose-dependent manners. In addition, we have identified a number of novel phosphorylation sites on the EGFR1/HER2 pathway molecules, *e.g.*, we found 8 pS/pT sites on HER2, which would affect the downstream pathway. For the signal network analysis, we selected approximately 60 protein kinases, which are not the known EGFR1/HER2 pathway molecules, and examined their phosphorylation dynamics data. As a result, ~60% kinases were regulated at least 2-fold. By mapping the drug-induced phosphorylation changes in the identified molecules, this approach will provide additional insight into the *in vivo* mechanism of the drug in the signaling network and potential off-targets of the drug.

Innovative aspects
- The first temporal and global phosphoproteome analysis of signaling inhibition *in vivo* by a molecular targeting drug.
- Identification of novel phosphorylation sites of the signaling molecules on EGFR1/HER2 pathways including pS and pT on HER2.

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Identification of Cancer Specific Secreted Proteins by Integrative Proteomics and Bioinformatics Analysis

C217

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Introduction
Aiming at the identification of cancer specific secreted proteins we have used an integrative proteomics and bioinformatics approach to characterize the secreted proteomes of several representative lung cancer cell lines. In this study, we first identified the secreted proteins by label-free or SILAC (Stable Isotope Labeling with Amino acids in Cell culture) based comparative proteomic analysis of secreted and total cell lysate proteins (1). Then we examined the mRNA levels of the identified proteins using public microarray data. In our preliminary study, several secreted proteins with differentially mRNA expression patterns were identified.

Methods
Lung cancer cell lines A549, H1299, CL1-0, and CL1-5 were cultured in light and heavy SILAC media. The secreted proteomes in serum free media and total cell lysates were collected and digested with trypsin. The resulting peptides were analyzed by reversed phase C18 LC coupled LTQ-Orbitrap MS. The SEQUEST algorithm was applied to search against a non-redundant database containing the forward and reversed (decoy) sequences of human and bovine proteins. The relative abundance of proteins were estimated by peptide counting or the ratio of light- and heavy-SILAC medium labelled peptides using the ASAP software (2)

Results
Approximately 254, 382, 807 and 981 proteins were identified in A549, 1299, CL1-0 and CL1-5, respectively, secreted proteomes and more than 1,000 each in the total cell lysate samples. Comparative analysis of secreted and cell lysate proteomes indicated a number of proteins are more predominant in secreted or cell lysate samples. An examination of microarray data suggested several differentially expressed proteins such as the MMP1 and annexin are present in the secreted proteomes.

Innovative aspects
- An alternative approach which identify biomarker starting from proteomics analysis and then examine differential expression pattern using public microarray data
- We can discriminate the residual bovine proteins in the serum free conditioned medium via SEQUEST search of both human and bovine database

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Circulating Vimentin as a Novel Serological Marker for Detection of Small (≤ 2cm) Hepatocellular Carcinoma

C218

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Introduction
The prevalence of hepatocellular carcinoma (HCC) is increasing worldwide mainly owing to the advanced stages of the malignancy at diagnosis, no effective treatment and lack of reliable surveillance programs (1). Curative surgery is more effective when the tumors are still small, hence early detection favors a low recurrence rate and prolonged survival (2). Nevertheless, conventional radiological and serological methods are not sensitive or accurate enough to detect HCC smaller than 2 cm in size. Therefore, new tools are urgently needed for the diagnosis of early HCC.

Methods
76 human liver tissue samples (10 HCCs ≤ 2 cm, 30 HCCs >2 cm, 20 cirrhosis and 16 normal livers) were profiled by 2-dimensional gel electrophoresis. Differentially expressed proteins were selected and identified by MALDI-TOF/TOF, and confirmed by immunoblot and qPCR (3). Biomarker performance was evaluated by ELISA in sera from a separate cohort of 152 HCC patients (44 HCCs ≤ 2 cm and 44 HCCs >2 cm) and control subjects (27 cirrhosis and 37 normal livers).

Results
We identified vimentin as a candidate biomarker for small HCCs by proteomic profiling and confirmed its overexpression in small HCCs by qPCR and immunoassay. There was significantly more soluble vimentin in serum samples of patients with HCC of all sizes compared to the non-neoplastic controls. Receiver operating characteristic analysis showed superior performance of circulating vimentin (40.91% sensitivity and 87.50% specificity) to alpha-fetoprotein (AFP) (400 ng/ml) in detecting small HCC at a cut-off of 245 ng/ml. Combined diagnostic performance of vimentin and AFP at optimized cut-offs enhanced the detection sensitivity and specificity to 80.97% and 91.44%, respectively.

Innovative aspects
- Here we provide the first evidence that vimentin is overexpressed in HCC as biomarker for early detection.
- Different from other studies, we find vimentin is present in HCC patient's serum samples.
- Soluble vimentin is a potential serological marker alone or in combination with AFP for detection of small-size (≤ 2 cm) HCC.

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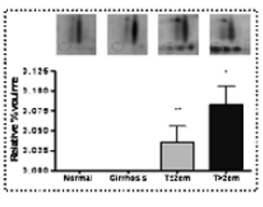

Fig. 1. Overexpression of vimentin in HCC tumors of all sizes. 2-DE analysis shows elevated vimentin expression in different size HCC tumors compared to normal and cirrhosis. Spot intensities are represented in relative % volume.

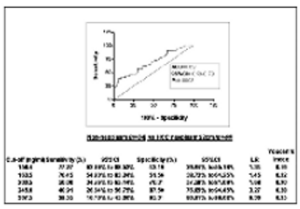

Fig. 2. ROC curves of circulating vimentin in separating non-neoplasm from small HCCs. Descriptive statistics illustrate the optimized cut-off (ng/ml) value for the ideal diagnostic performance of circulating vimentin.

Degradation Profile of Metastasis Hepatocellular Carcinoma for Apoptosis related Network study

C219

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Introduction
For cancer metastasis, apoptosis is an important biological processing for invasion and migration. During apoptosis, caspases cleave cellular substrates to break down and package the apoptotic cell for removal. Proteolysis is a key regulatory process that promotes the (in)activation, translocation, and/or degradation of proteins. As such, There is a considerable interest to study the degradation profile of metastasis HCC for studying apoptosis related networks or pathways.

Methods
Two human HCC cell lines of different metastatic potentials, MHCC97L and HCCLM6, were cultured at standard condition and collected 1×10^7 cells to lysis. 200 μg of cytosolic protein was separated via a 12%SDS-PAGE gel and cut into 20 parts based on the quantity of protein bands. Bands were subjected to in-gel trypsin digestion. The digested peptides of each part were further separated and identified with RPLC-LTQ-Orbitrap system. Raw MS/MS data were searched using the SEQUEST algorithm using a concatenated target/decoy variant of the IPI human_3.45 database. The identification results were analyzed with PROTOMAP reported by Dix[1]. Combined with IPA bioinformatic tool, several related networks and pathways were found out.

Results
Combined with SDS-PAGE gel and RPLC-MSMS technology, total 4766 proteins were identified in MHCC97L or HCCLM6. In this result, 225 proteins had confirmed information of degradation. Based on the types of cleavage fragments, these proteins were clustered into four groups. The numbers of proteins had C-terminal fragments, N-terminal fragments, N- and C-terminal fragments and multiple/internal fragments were separately 68, 84, 2 and 71. With IPA analysis, these proteins were enriched in TP53-central network related with cell death and TNF-central network related with immune response. They were both important networks and pathways with cell apoptosis. From the biological readout, we found several important proteins such as BID, NCL, HSP90B1,HSPA8 and their cleavage fragments changed in different HCC cell lines of metastatic potentials. How did the degradation effect functions of these proteins, active or loss, was still a mystery. The further experiments of biological verification are on going.

Innovative aspects
- Study degradation profile of metastasis HCC in the first time
- Using IPA analysis, find apoptosis related networks and pathways in HCC metastasis
- Study how degradation act on HCC metastasis

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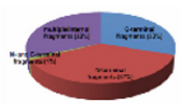

Figure 1. Percentages of different cleavage fragment types.

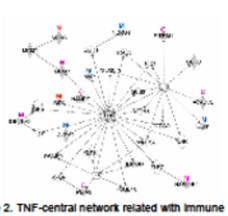

Figure 2. TNF-central network related with immune response. The characters labeled on the proteins were different fragment types. N,C and M is separately N-terminal, C-terminal and multiple fragments. The red or blue color meant the degradation only occurred in lower metastatic cell line MHCC97L or higher metastatic HCCLM6 cells. And the purple color meant the degradation occurred in both cell lines.

C220

Deciphering the Degradome of Matrix Metalloproteinases in Breast Carcinogenesis

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Introduction
Where conventional proteomics and genomics approaches aim to identify a maximum number of genes and proteins present at a given time, they typically lose information on their activity. Here we describe a targeted analysis of the abundance, activity and substrates of proteases in breast cancer with different metastatic potential.
For the first time, proteolytic cleavage events in complex proteomes are quantified by iTRAQ™ labelling of enriched protein N-termini. Furthermore we make use of intensity based quantification models to calculate quantification confidences.

Methods
Culture supernatants from T47D cells were incubated with either no protease, protease A, protease B or both at the same time. The samples were labelled using 4plex iTRAQ™, pooled, and subsequently digested, separated by SCX chromatography at low pH, and collected fractions analyzed by LC-MS/MS.
Natural N-termini and N-termini derived from basal proteolysis present iTRAQ™ reporter ion intensities of 1:1:1:1 whereas neo-N-termini resulting from proteolytic activity of the added protease generated a reporter ion in the respective channels only. N-termini that could only be generated by synergistic activity of both proteases resulted in only one reporter ion in the respective channel. In addition negative selection preserved the identification of naturally blocked (e.g. acetylated) N-terminal peptides.

Results
Using CLIP-CHIP™ expression analysis of all proteases and protease inhibitors in mouse mammary gland tumors derived from 67NR, 66cl2 and 4T1 breast cancer cell lines revealed a set of 15 genes to be specifically up regulated in tumors showing high metastatic potential. Among them Matrix Metalloproteinase 10 (MMP) and Matrix Metalloproteinase 13 showed the most profound increase and were analyzed by TAILS (Terminal Amine Isotope Labeling of Substrates) using enriched protein N-termini and 4plex iTRAQ™ labeling.
After peptide identification and subsequent evaluation based on intensity dependent quantification confidence factors (2) we were able to identify 226 proteins to be cleaved by MMP-10 and 43 cleaved by MMP-13. A total of 45 proteins were cleaved in a synergistic way. In addition, all cleavage events identified in single protease treated samples had been identified in the double protease treated samples as well nicely confirming the robustness of TAILS.
In conclusion we could identify a set of proteases selectively up regulated in tumors of high metastatic potential and reveal several so far unknown *in vitro* substrates for MMP-10 and -13.

Innovative
- Selective enrichment of N-terminal peptides using a novel amine reactive polymer
- Multiplex TAILS is a novel powerful quantitative proteomic technique for protease substrate discovery in complex samples
- TAILS allows identification of substrates for multiple proteases in a single assay

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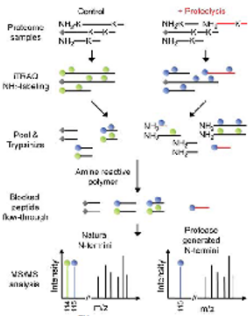

Figure 1. iTRAQ™-TAILS strategy for the identification of protease substrates in complex samples.

C221

Investigating Biomarkers using Glioma Cell Lines and Plasma from Glioma Patients

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Introduction
Gliomas are primary brain tumors of the central nervous system; astrocytomas being the major histotypes. Surgery is the main treatment mode and prognostic methods pre and post surgery remain a great challenge. Tumor cells in culture release proteins of various functional categories. With an objective to explore this possibility with glioma cell lines, we have analyzed the proteins from conditioned media of high-grade astrocytoma cell lines by SDS PAGE - LC-MALDI MS approach. We then examined the presence of select protein candidates in blood plasma.

Methods
High-grade glioma cell lines HNGC-2, U87MG and U373MG were cultured in DMEM supplemented with serum followed by their incubation in serum free conditions for 24 hours. Cell viability was tested by trypan blue staining and western blot for alpha-tubulin. The conditioned media were harvested, lyophilised and proteins analyzed by LC-MALDI approach. Select proteins from the list were further examined for their presence in the conditioned media or the blood plasma by Western blot analysis.

Results
SDS-PAGE analysis of the conditioned medium of HNGC-2 glioma cell line indicated release of proteins from the cells. Cell viability tests confirmed minimal cell death during incubation in serum free culture conditions. MS and MS/MS data was searched against HumanRefSeq and modified NCBI databases using Mascot search engine, resulting in the identification of 776 peptides, which mapped to 662 unique proteins. Gene Ontology annotation of 315 of the proteins from the list revealed the presence of proteases, signaling proteins, extra cellular matrix, transporters, growth factors and many of the binding proteins (Fig 1A). Studies with Protein secretomes of U87MG and U373MG cell lines are under progress.
Glial fibrillary acidic protein (GFAP), a 50kDa intermediate filament protein is specific to astrocytes - the cells of the gliomas. Western blot analysis using GFAP polyclonal antibody revealed GFAP species - 40kDa and smaller in the conditioned media of these cells (Fig 1B). We are presently examining the presence of GFAP fragments in the plasma obtained from mice carrying HNGC-2 xenografts. Preliminary experiments also indicated presence of these GFAP species in a small set of clinical plasma samples from Glioma patients (Fig 1B). These observations are consistent with our earlier theory on GFAP proteolysis in glioma tissues in grade related manner and release of the protein fragments into body fluid such as blood plasma (1).

Innovative aspects
- Tumor cell secretome studies have the potential of complementing observations made with clinical tissues.
- GFAP detected by us and by others (2) in the plasma of glioma patients may be a proteolytic product formed in the tumor tissue and released out of the cell and may have prognostic application.

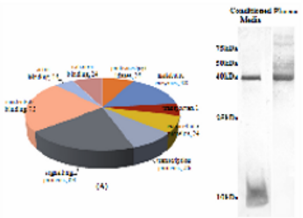

Figure 1: (A)-Functional classification of HNGC-2 glioma cell secretome; (B)-Western analysis of GFAP in conditioned media and patient plasma using anti-GFAP polyclonal antibodies.

References
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C222

STRIPAK: A Novel PP2A Complex Identified by High-Density Mass Spectrometry

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Introduction: In an effort to identify novel protein complexes that control cell growth, we used iterative affinity purification coupled to mass spectrometry (AP/MS) in human cells to identify a high-density interaction map surrounding the Protein Phosphatase 2A (PP2A) catalytic subunit – a potent tumour suppressor and central mediator of signalling pathways that control cell proliferation. This approach confirmed previously identified PP2A-containing complexes and facilitated the identification of a novel PP2A assembly referred to as the STRIPAK (Striatin-interacting phosphatase and kinase) complex (1).

Methods: Single affinity purifications were performed on Flag-tagged baits that were stably expressed in HEK293 cells (2). Flag-tagged proteins were purified and the immunoprecipitate was eluted in ammonium hydroxide and lyophilized. Samples were resuspended in ammonium bicarbonate and digested with Trypsin. Digested samples were resuspended in Formic Acid/Acetonitrile and loaded onto capillary columns packed with Magic 18AQ. Tandem MS data was obtained in a data-dependent mode on a ThermoFinnigan LTQ. Acquired RAW files were searched with the MASCOT search engine against the human RefSeq database. Identified interactors were, in turn, processed for AP/MS to resolve complex components. Specific interactors for all baits were highlighted by excluding proteins identified in Flag-tag AP/MS datasets.

Results: STRIPAK contains the PP2A catalytic (PP2Ac) and scaffolding (PP2A A) subunits, the striatins (PP2A regulatory B"" subunits), the striatin-associated protein Mob3, the novel proteins STRIP1 and STRIP2 (formerly FAM40A and FAM40B), the cerebral cavernous malformation 3 (CCM3) protein, and members of the Germinal Center Kinase III family of Ste20 kinases. STRIPAK also establishes mutually exclusive interactions with either the CTTNBP2 proteins or a subcomplex consisting of the sarcolemma-associated protein (SLMAP) and the small, related coiled-coil proteins SIKE and FGFR1OP2 (Fig. 1).

Innovative aspects:
- First high-density proteomic survey to link both kinases and phosphatases to the CCM family of proteins
- Use of high-density AP/MS data to predict mutually-exclusive complex formation

References:
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Fig.1. STRIPAK associates mutually exclusively with the CTTNBP2 proteins or the SLMAP/SIKE subcomplex

C223

Cancer Degradomics: Semaphorin 3C Processing by ADAMTS1 and ADAM17 Affects Cell Migration

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Introduction
Metalloproteases play a complex role in tumor progression. Proteomic approaches to identify the array of substrates of a given metalloprotease (degradome), can help to unveil its role in tumor growth and metastasis. Here we describe a proteomic screening using two complementary approaches, 2D-DIGE and SDS-PAGE LC-MS/MS SILAC analysis, to identify new substrates of the metalloprotease ADAMTS1, released to the conditioned medium of invasive mammary tumor derived MCF7 cells overexpressing the protease.

Methods
MCF7 mammary cancer cells were engineered to conditionally express ADAMTS1 using the Tet-Off system. Cells grown in conditions were the expression is induced or switched off were differentially labelled by SILAC. Glycoproteins released to the conditioned media of each of the two cell cultures were purified by lectin affinity chromatography. Samples of the two conditions were compared by the 2D-DIGE methodology, or pooled and run on a 1D SDS-PAGE gel. The gel lane was then cut into 20 fractions, and each one was digested and analyzed by RP-LC-MS/MS on a HCT ion trap mass spectrometer. Protein identification and quantification of relative abundances was performed using ProteinScape and WARP-LC, an integrated software platform for proteomic data processing and analysis.

Results
The DIGE approach led to the identification, among others, of Semaphorin 3C (S3C) as a new potential substrate of ADAMTS1, together with Thrombospondin-1, a known substrate of the protease.
A total of 3163 and 2697different peptides, from 749 and 728 individual proteins, were identified and quantified, respectively, in two complementary SILAC screenings, in which isotopic labelling was swapped. A number of known substrates of ADAMTS1 were identified in the analysis, such us Thrombospondin-1 and Dystroglycan. In addition several new candidate substrates of the protease were identified, including S3C.
Processing of S3C by ADAMTS1 was further validated and characterized, showing the protease-dependent release of a 95 kDa S3C fragment into the medium. Additionally, we showed that S3C is also processed by the protease ADAM17, resulting in a different fragment of S3C. Cell migration assays showed that the different fragments of S3C have different chemoattracting properties. Semaphorins, first described as axon guidance factors, have also been related to tumor progression. Our results show that metallo-proteases can regulate cell migration through Semaphorin processing in cancer cells.

Innovative aspects
- Proteomic screening for specific substrates of ADAMTS1 metalloprotease in mammary cancer cells.
- Use of dual-swapped SILAC labelling scheme to effectively control for false positive differential proteins.

C224

Development of MRM-MS methodology to measure peptide biomarkers in the prognosis of prostate cancer.

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Introduction: Prostate cancer is currently the leading type of cancer in American men. Diagnosis of asymptomatic prostate cancer is by digital rectal examination (DRE) in combination with the serum measurement of prostate specific antigen (PSA). Unfortunately, PSA may not discriminate benign hyperplasia from malignant disease resulting in high false-positive rates. Additionally, PSA has also shown high false-negative results.[1] Therefore, new biomarkers that differentiate indolent from aggressive disease and recognize patients for which treatment would be suitable are greatly needed.

Methods: Several putative biomarkers previously characterized by orthogonal genomic and transcriptomic methodologies were chosen for multiplexed peptide MRM-MS methodology development. Whole cellular protein extract from immortalized benign and cancerous prostate cell lines and additionally human biopsy tissue from benign hyperplastic, localized tumor, and metastatic prostate cancer were obtained. Total protein extract was quantified with bicinchoninic acid (Pierce Biotechnology) and 50 μg of protein from each sample was separated by 1D SDS-PAGE. Twenty-four equal sized gel bands were excised and subjected to in-gel digestion. Extracted peptides were reconstituted with isotopically labeled peptide standards prior to on-line reverse phase LC-MRM-MS (1200 Series HPLC-CHIP, 6410 Triple Quadrapole, Agilent Technologies).

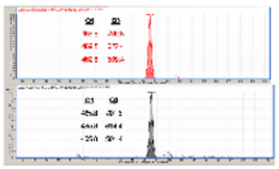

Figure 1: MRM-MS analysis of peptide biomarker QTSILIQK of SPINK1 protein. This peptide was measured only in 22Rv1 cell line as expected from orthogonal methodologies. Top panel; extracted ion chromatogram of spiked internal standard heavy peptide. Bottom panel; extracted ion chromatogram of light. i.e., endogenous peptide.

Results: Three transitions for each stably isotopically labeled peptide, 27 total transitions, were developed empirically. Cross talk for each of the transitions within the mass spectrometer was tested by injecting only heavy internal standard and monitoring for both light and heavy transitions, total 54 transitions. Standard curves with spiked internal standard were developed for each of the nine peptides in a background of whole cell protein lysate from VCaP cells prepared as stated above. Six immortalized cell lines representing different aspects of prostate disease were measured for absolute quantification of the nine peptide markers. As expected and verified by orthogonal methodologies, only cell lines positive for a particular marker were detected by this MRM-MS methodology.

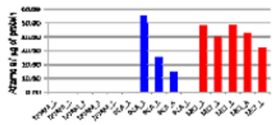

Figure 2: Quantitative MRM-MS results of peptide biomarker TEILNQEWK of EZH2 protein. In benign hyperplasic tissue, the peptide was not detected, while increased levels are seen in both local and metastatic disease prostate tissue.

While only five biopsy tissue samples of each type have been completed by abstract submission, peptide markers show promising results for discriminating benign hyperplasic disease from localized cancer (EZH2 and AMACR) and localized cancer from metastatic disease (SPINK1 and TMPRSS2). Additionally, both PSA peptide markers track one another and correlate clinically. These results suggest that this multiplexed MRM-MS based assay may provide more discriminatory information for the prognosis and treatment of prostate cancer.

Innovative aspects:

- Multiplexing different peptide markers increases discriminatory information for the prognosis of and treatment of prostate cancer.

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C225

Global Proteome Analysis of DNA Mismatch Repair reveals novel aspects of MMR biology

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Introduction
Deficiencies in DNA mismatch repair (MMR) systems occur in HNPCC and in 10-15% of sporadic colorectal cancers (CRC). Loss of function mutations in MMR genes lead to genetic instability, which contributes to tumor formation. Widespread mutations at the genome level presumably give rise to alterations in protein expression profiles; however, little is known about the effects of MMR deficiency on the proteome. Such analyses could lead to new insight into biological pathways involved in the MMR phenotype of identify mutant peptides that could be used as markers of MMR-deficient tumors.

Methods
We carried out global shotgun proteomic analysis of four MMR proficient CRC cell lines (HT-29, COLO-205, CaCo2, SW480) and four MMR deficient CRC cell lines (HCT-15, HCT-116, LoVo, LS174T) Tryptic peptides from whole cell lysates were separated by isoelectric focusing (IEF) and analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) on a linear ion trap quadrupole (LTQ) mass spectrometer. Resulting spectra were searched against protein databases using the Myrimatch search algorithms (1) and proteins were identified using parsimonious assembly with IDpicker (2).

Results
When data from all cell lines were assembled together, 5047 protein groups were identified (4.8% protein FDR). Unsupervised hierarchical clustering analysis of proteomic data showed that the CRC cell line proteomes cluster together primarily according to MMR status. Analysis of protein networks by MMR status by Ingenuity Pathway Analysis (IPA) identified differentially expressed proteins in pathways relating to protein trafficking, DNA repair and RNA transport. Further investigation of group-wise differences identified a small number of proteins that differentiated by MMR status, with notably increased spectral counts for DNA repair proteins in MMR-proficient cell lines (MSH2, RAD50B, MRE11 and others). We further examined protein expression levels for genes known to be mutated in these cell lines. These genes include BAX, LMAN1, and the mismatch repair proteins MSH2 and MSH6. BAX was not detected in MMR deficient cell lines, LoVo or LS174T cells, which are known to possess a BAX frameshift mutation, but was detected in all wildtype BAX-expressing cells. Furthermore, MSH2 and MSH6 were not detected in LoVo cells, which express mutant forms of these genes.

Innovative aspects

- One of the largest dataset generated allows systems biology description of phenotypes
- Biological replicates show high reproducibility in the shotgun identifications
- Known changes in individual proteins are detectable using the global shotgun approach
- In addition, we identified novel protein networks involved in MMR

References

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C226

Proteomic Profiling of Colon Cancer Cell HCT116 Overexpressing Frataxin

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Introduction
Glycolysis is the preferred route of glucose metabolism in tumor cells even in oxygenated conditions (Warburg effect). The mechanism involved and association of a strong preference for glycolysis in tumourigenesis however remain to be elucidated. Friedreich's ataxia is a hereditary disease caused by a reduced expression of the protein, frataxin. Frataxin plays a role in mitochondria function especially in the modulation of ATP production. Recent studies had shown overexpression of frataxin increased oxidative phosphorylation and inhibited tumor growth (1).

Methods
Vivid Colors™ pcDNA™ 6.2 / EmGFP-Bsd / V5-DEST Mammalian Expression Vector (Invitrogen) containing frataxin gene was transfected into HCT116 colon carcinoma cell line. Three stable cell lines overexpressing frataxin were established. Cell proliferation assay was performed using crystal violet stain. Cell motility was measured using wound healing assay. Resistance of the cells to reactive oxygen species (ROS) were determined by treating cells with tert-butyl hydroperoxide (TBHP). Isobaric tags for relative and absolute quantification (iTRAQ) were used to compare the proteome profiles of the three frataxin-overexpression cell lines with HCT116 transfected with the vector alone. The iTRAQ labelled peptides were separated and analyzed with an LC-MALDI platform.

Results
The frataxin-overexpression cell lines showed significant decrease in proliferation as compared to the vector-alone control. However, no significant difference in cell motility was observed by wound healing assay. The frataxin-overexpression cells showed significant higher resistance to TBHP treatment, indicating enhanced tolerance to oxidative stress. A total of 232 proteins were identified using iTRAQ LCMS quantitative profiling. From these 51 proteins were significant altered in all three frataxin-overexpression cell lines. Gene ontology classification based on cellular function revealed metabolic proteins and tumorigenic proteins as the major groups of proteins that were altered.

Innovative aspects

- Global proteome profiling of the reverse metabolic transformation of cancer cells.
- Further the understanding of the metabolic transformation and tumorigenesis.

Reference

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C227

The application of plasma proteomic patterns for detection patients with lung adenocarcinoma

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Introduction: Lung cancer is the leading cause of cancer-related death worldwide among both males and females, with more than 1 million deaths annually. Most patients present at advanced stage at their initial diagnosis. To improve lung cancer management and survival, there is a great need to develop screening and early diagnosis strategies that are sensitive, specific, and noninvasive; tools predicting prognosis to optimize treatment and avoid overtreatment; and tools identifying potential therapeutic targets. Blood proteome analysis assumes that tissue perfusion of tumors or host responses contribute to modification of circulating proteins or peptides. Because it is noninvasive, easy, fast, and amenable to repetitive measurements over time, this approach appears very appealing to researchers addressing the discovery of biomarkers. Adenocarcinoma (AC) is the most common type of lung cancer, accounting for 30-35% of all cases. The aim of this study is to explore the application of plasma matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) proteomic patterns to distinguish patients with lung adenocarcinoma from healthy individuals.
Methods: The EDTA plasma samples have been prefractionated using magnetic beads kits functionalized with weak cation exchange coatings. We compiled MS protein profiles for 74 patients with lung AC (stages III-IV) and compared them with profiles from 189 healthy controls. The control group was subdivided into a preliminary set (125 controls) for model generation and a second validation set (64 controls). The AC group was also subdivided into a training cohort with 48 patients and test cohort with 26 patients for validation. Peptide profiles were acquired with an Ultraflex II mass spectrometer in linear positive mode. The MALDI-TOF spectra were analyzed statistically using *ClinProTools*™ bioinformatic software.
Results: In dependence on the sample used up to 385 peaks/spectrum could be detected in a mass range of 1000 – 20000 Da. About 184 of these proteins had statistically differential expression levels between AC and control plasma ($P < 10^{-5}$). Compared to controls, 6 peaks ($p<10^{-5}$) are increased in AC and 178 peaks are decreased. The series of the peaks were automatically chosen as potential biomarker patterns in the training set. They allowed to discriminate plasma samples from healthy control and samples from AC patients (sensitivity – 90%, specificity – 95% for genetic algorithm) in external validation test.
Innovative aspects: These results suggest that plasma MALDI-TOF MS protein profiling can distinguish AC patients from normal subjects with relatively high sensitivity and specificity, and the MALDI-TOF MS is a potential tool for the screening of lung cancer.

C228

Quantitative Proteomics Study of Breast Cancer Cell Lines Isolated from a Single Patient: Discovery of TIMM17A as a Breast Cancer Marker

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Introduction
Breast cancer is the most common cancer types in women in China, and second most in the United States. The proteins as well as the mechanisms that involve in the progression of breast cancer still elude researchers around the globe. 21T series cell lines, consist of normal, primary tumor and metastatic cell lines, were isolated from a single breast cancer patient, representing a progressive breast cancer model that might help reveal the mystery of breast cancer progression. We set out to thoroughly investigate the quantitative changes of the proteins among this series of cell lines to discover biomarkers that may contribute to the initiation and progression of breast cancer.

Methods
Three cell lines from the 21T series were grown in SILAC labeling media, where NT cells (the primary tumor cells) were labeled with D_3-Leucine, and 16N (normal) cells and MT2 (metastatic) cells were labeled with D_0-Leucine. The labeled cells were lysed and mixed in pairs (16N:NT; NT:MT2), then separated with SDS-PAGE. Each gel lane was cut into pieces, followed by in-gel digestion and LC-MS/MS analysis. The spectra data were searched against human IPI database (version 3.35) using SEQUEST program. The protein quantitation was achieved by using the Bioworks V2.0 software. The resulting list of proteins with relative abundance information was analyzed for important pathways that involve in the tumorigenesis and metastasis of breast cancer. Several potential markers were validated using Q-PCR, western blot and immunohistochemistry in cell lines and tumor tissues. TIMM17A was cloned into pcDNA3 vectors and functional analyses were performed by transfecting gene expression vectors into MDA231 breast cancer cells.

Results
Using a stringent search and filtering criteria, we identified a total of 1266 non-redundant proteins, among which 1230 had quantitative information. Deregulated proteins were obtained using statistical analysis of normal-distributed log-transformed ratio values. Gene ontology analysis revealed that proteins involved in metabolic processes were the most deregulated in both tumorigenesis and metastasis. Interaction network analysis indicated that ERBB2 signaling played a critical role in tumorigenesis. Other than known markers that have been identified and verified, such as ERBB2 and E-cadherin, novel markers, including BRP44L, MTHFD2 and TIMM17A, were found to be over-expressed in 21T breast cancer cells and verified in additional breast cell lines. mRNA expression analysis as well as IHC analysis in breast cancer tissues indicated that expression level of TIMM17A was directly correlated with tumor progression, and survival analysis suggested that TIMM17A was a powerful prognosis factor in breast cancer. More interestingly, over-expression and siRNA knock down experiments indicated an oncogenic activity of TIMM17A in breast cancer. Our study provides a list of potential novel markers for breast cancer tumorigenesis and metastasis using a unique cell model. Further studies on TIMM17A as well as other markers on the list may reveal mechanisms that result in more effective therapeutics for cancer treatment.

Innovative aspects
- A progressive breast cancer model isolated from a single patient was used.
- Statistical significance analysis based on distribution of the ratio, not on the ratio itself.
- Novel markers, e.g. TIMM17A, was identified, validated in clinical samples, and functionally verified in cell lines.

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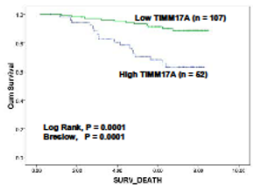

Figure. Kaplan-Meier survival analysis of breast cancer patients with microarray data. High level of TIMM17A expression had much worse prognosis.

C229

Enrichment of tumorigenic stem-like cells in biopsy spheroids from colorectal cancer

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Introduction
In some tumors, including colorectal cancer (CRC), only a small population of cells has tumor initiating potential. Identification and characterization of colorectal tumorigenic cells may provide novel diagnostic biomarkers and targets for therapy.
We have developed a culture model that enriches for stem cell-like cells from primary tumor biopsies(1). Here, we apply this three-dimensional spheroid culture model to colorectal cancer tissue and use it in a large scale proteomics study, to identify novel markers of colorectal tumorigenic cells.

Methods
Tumor fragments were obtained at surgery with the informed consent of patients with colorectal carcinoma. Tumor spheroids were compared to corresponding biopsies in a 'geLC-MS' (gel-liquid chromatography-mass spectrometry) experiment. 30ug of proteins from each sample were separated on a 10% polyacrylamide gel, the gel was stained and sliced in equal parts. Proteins were *in-gel* digested, extracted and injected in a nano-LC LTQ-FT system. MS/MS samples were analyzed using Sequest 3.3 (ThermoFinnigan, Waltham, MA, USA). Expression of selected proteins of interest was validated using immunohistochemistry.

Results
Using a 'geLC'-MS approach, 1366 proteins were identified (at least 2 peptides sequenced, C.I. ≥ 99%) across the samples of the three patients. With the spectral counting quantification of the relative abundance in protein expression 342 proteins were found regulated in 3 patients (± 1.5 fold, p<0.05). 169 were upregulated, and 173 were downregulated in the spheroids compared to the corresponding tumor tissue. The list of non-redundant proteins was searched for gene ontology (GO) annotation and KEGG pathway functional annotation of the regulated proteins using FatiGO and IPA. Top significant biological processes terms included: Cell adhesion, Cell Communications, Response to stress. Top significant molecular function terms included: Catalytic activity, Protein binding and Oxidoreductase activity, while top significant cell component terms included: Organelle, Non-membrane-bound organelle, Receptor complex. Expression of several proteins, including those from the Wnt signalling pathways (Rac1), were validated by immunohistochemistry on colorectal tumor tissue and corresponding spheroids.

Conclusion
Enrichment of proteins from non-canonical/planar cell polarity (PCP) ***'wnt signaling pathway'***, as well as proteins from ***'colorectal cancer pathway'*** in the cells forming colon tumor spheroids, compared to the colon cancer tissue, is consistent with an enrichment of colorectal cancer stem cells (Co-CSC) in the spheroids. This points out potential new marker candidates for Co-CSC involved in the deregulation of cancer stem cell signaling that are not a part of the CD marker system.

Innovative aspects
- spheroid model,
- spectral counting quantification,
- non-CD markers for Co-CSC

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C230

Elucidation of Protein Signaling Mechanisms in Cell Mobility Studies

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Introduction
Characterization of both the spatial and temporal signalling mechanisms of cellular systems is critical for the understanding of cellular function. This is particularly evident in the study of cell mobility, specifically in cancer metastasis and cell migration where morphological polarization occurs. Here we review previous efforts and present current results and studies of analysis approaches for multiple cell mobility systems of interest, i.e. cell pseudopodia extension and retraction, neurite extension and retraction, and osteocyte dendrite outgrowth.

Methods
Protein samples were isolated from various cell sources, COS-7 cells, MLO-Y4 osteocytes, and N1E-115 neuroblastoma cells. Physical separation of the cell body from the cellular extensions was accomplished utilizing microporous filter membranes in transwell growth chambers. Isolated proteins were fully denatured, reduced, digested and analyzed by LC-MS/MS. For quantitative studies, proteins where labelled using either an ^{18}O strategy or label free approach. Analysis was performed utilizing high mass accuracy LTQ-Orbitrap instrumentation. Phosphoproteome analysis utilized a modified IMAC approach. Peptides were identified based upon searching primarily with SEQUEST against the respective human/mouse IPI database coupled with high mass accuracy and/or elution time information.

Results
Previous studies coupling quantitative proteomic and phosphoproteomic approaches with the differential purification of cell body and pseudopodia extensions have revealed spatial protein abundance relationships mapped across signalling pathways. We have now extended the current approaches to further characterize the signalling pathways and protein localization for both the pseudopodia extension and retraction phases of chemotaxis. Additionally, further application of this approach includes the study of osteocyte dendrite outgrowth and regulation under lysophosphatidic acid (LPA) stimulation. Proteome characterization of both osteocytes and their dendritic membrane extensions has recently been performed and has resulted in the identification of key components in the dendrite outgrowth including cytoskeletal elements and adaptors, adhesion molecules, and proteins involved in motility and establishment of cell polarity.

Innovative aspects
- Application of unique cell culture transwell techniques with quantitative proteomic approaches.
- Proteomic characterization and elucidation of key cell migration and extension mechanisms.

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C231

Identification of proteins in lymph node metastasis by antibody proteomics technology

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Introduction
Because lymph node metastasis of cancer is one of the most important prognostic factors, many researchers are trying to develop diagnostic and therapeutic methods for such pathology. In this regard, we have established "antibody proteomics technology", which accelerates the discovery of proteins useful for diagnosis and therapy of cancer. Here, we have applied antibody proteomics technology to searching tumor lymphatic endothelial cells for expression of proteins related to lymph node metastasis.

Methods
Tumor lymphatic vessel model: Human primary lymphatic endothelial cells were cultured in conditioned medium (CM) prepared from a low-metastatic human lung cancer cell line (RERF-LC-MS), a high-metastatic cell line (RERF-LC-KJ) or a normal respiratory tract epithelial (NHBE) cells.
In vitro invasion assay: Cells were added to the upper chambers of an invasion assay plate and incubated for 72 hr. Invasive cells were stained with calcein-AM and detected by fluorescence.
2D-DIGE analysis: Cell lysates of tumor lymphatic vessel models were labeled with Cy3 or Cy5 and subjected to 2-dimensional differential gel electrophoresis.
Immunohistochemical staining of tissue microarray (TMA): After being blocked with 5% BSA, TMA slides were incubated with antibody and stained by the Envision+ system.

Results
Initially, the invasiveness of cells in two tumor lymph vessel models was tested in vitro. Lymphatic endothelial cells cultured in CM from RERF-LC-KJ cells were more invasive to basal membrane than those cultured in CM from RERF-LC-MS cells. To search for lymph node metastasis-related proteins, the differential proteome among the three types of lymphatic endothelial cells was analyzed by 2D-DIGE. This analysis identified 14 proteins that were over-expressed in lymph endothelial cells cultured in CM from RERF-LC-KJ cells. These candidate biomarker proteins were then verified by TMA analysis. Consequently, we identified a protein that is expressed both on lymphatic endothelial cells in lung cancer tissues and tumor cells, but not on lymphatic endothelial cells in normal lung tissues. The protein identified might be a novel drug target in the therapy of lymphatic metastasis of cancer.

Innovative aspects
- We attempted to identify a novel protein specific to tumor lymphatic endothelial cells by antibody proteomics technology.

C232

In silico screening and verification of *APC-p21* crosstalk in the Apc$^{(min/+)}$ Colon Cancer Mouse Model

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Introduction
Colorectal cancer is the third most commonly diagnosed cancer and the third leading cause of cancer death in the US. Adenomatosis Polyposis Coli (APC) mutations play a role in almost 90 percent of these colon cancers. The Apc$^{(min/+)}$ mouse model develops tumors in the intestine due to a mutant allele within the APC gene. Moreover, the p21$^{(-/-)}$ mouse model was observed to carry rare polyps. While both alterations in the same mouse create a very aggressive model of cancer (1), it is still unclear how APC and p21 physically communicate with each other to result in this emergent phenotype.

Methods
We map known pathway proteins to their functional annotations to capture underlying characteristics of pathways (2). These are then used to search for likely signaling pathway segments. *Association rule* mining, a procedure to collect data attributes that are statistically related in the underlying data, is used to discover patterns from known pathways, such as those found in KEGG. Given proteins from an extended and purified protein-protein interaction network, we utilize the acquired *association rules* and then search for pathway segments that describe APC-p21 signaling. We count *association rules* on each path connecting the target proteins and identify significant paths to form hypotheses for colon cancer signaling, which can be verified *in vivo*.

Results
Assuming that these target proteins are correlated with each other in terms of tumorigenic phenotype, the effect should be reflected at the level of expression as well as in the interaction patterns of these proteins. Hence, if these proteins are statistically associated with available high-throughput data, we should observe these relationships and eventually form hypotheses about the functional relationships of these proteins in terms of pathways.

The *in silico* pathway screening algorithm generated putative paths connecting APC and p21, as shown in the Figure. The proteins along these "crosstalk" paths are hypothesized to be mediating the synergy observed when APC and p21 are mutated or lost simultaneously. If an increase in cellular proliferation and cell cycle progression are observed when APC and p21 are knocked down simultaneously, and the crosstalk proteins are responsible for this synergy, then knockdown of one or more of these crosstalk proteins should reduce proliferation & cell cycle progression to levels observed in single mutants.

All of the intermediary proteins identified and shown in the Figure are oncogenes, several of which are supported by literature as being novel mediators of the Apc-p21 network. For instance, Pim1 is a well-characterized serine/threonine kinase in prostate cancer – inhibiting p21 and promoting cell growth. Pim1 is expressed in murine colon tumors, but its role is not yet known. Src, as another example, is a well-studied kinase whose levels or activity are frequently increased in human colorectal cancers and colorectal cancer cell lines (3). Though Src is rarely mutated, it plays an important role in mediating cellular proliferation and adhesion and, hence, is hypothesized to "integrate" many signals in colorectal cancer. These targets support the synergy identified between APC and p21, and can be verified via *in vivo* experiments.

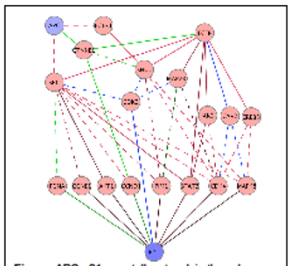

Figure: APC-p21 crosstalk network in the colon cancer mouse model. The proteins that are connecting these targets, APC and p21 are colored pink. Dashed edges indicate predicted interactions via the computational framework used; solid edges indicate known (i.e. published) interactions.

Innovative aspects
- Integration of proteomics and genomics
- Computational hypothesis generation
- Novel Apc-p21 crosstalk network in colon cancer

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C233

Quantitative Phosphoproteome Analysis of the Hedgehog Signaling Pathway

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Introduction

The Hedgehog (Hh) signaling pathway directs pattern specification during development in most animals[1]. Aberrant activation of the Hh pathway is implicated in several cancers, including basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma[2]. While well characterized in *Drosophila* where it was first identified, Hh signaling in mammals remains incompletely understood. In an effort to identify novel members of the Hh signaling pathway, a quantitative phosphoproteome analysis of Hh-stimulated mouse cells was undertaken.

Methods

Hedgehog responsive mouse mesenchymal cells (S12 cells[2]) were stimulated with 100 ng/ml of Sonic Hedgehog (Shh) ligand and harvested after 0 min, 5 min, 30 min, and 180 min. Simultaneously, unstimulated S12 cells were grown in medium containing stable isotope-labeled $^{13}C_6$-L-Lysine and $^{13}C_6$,$^{15}N_4$-L-Arginine. Cells were lysed with hypotonic buffer and isotope-labeled lysates were mixed with each time point in equal amounts and digested with trypsin. 2 mg of material for each time point was loaded onto a TSKGel Amide-80 HILIC column (Tosoh Bioscience) and separated into 12 fractions. Phosphopeptides from each fraction were enriched by titanium dioxide (GL Sciences) and subsequently analyzed on an Orbitrap mass spectrometer (Thermo Scientific) equipped with a nanoACQUITY UPLC (Waters).

Results

Combining HILIC with titanium dioxide phosphopeptide enrichment, 3,482 unique phosphopeptides were identified across 4 time points of Shh stimulation with a false positive rate less than 0.5%. HILIC fractionation of the trypsin-digested lysates prior to titanium dioxide phosphopeptide enrichment reliably separated unphosphorylated and phosphorylated peptides along the HILIC gradient (Figure 1). Due to the nature of data-dependent acquisition on a mass spectrometer, only ? phosphopeptides were identified by MS/MS spectra in all 4 time points of the experiment. Requiring MS/MS spectrum identifications prohibited quantification of many phosphopeptides across the time course experiment, so a custom quantification algorithm was developed that is capable of quantifying peptides without MS/MS identifications based on the retention time, precursor masses, isotope labeling mass shift, and isotope distributions of the peptides identified in other time points or replicate analyses within the same experiment. This data set provides quantitative abundance profiles for thousands of phosphopeptides responding to Shh stimulation that are now being validated for their involvement in the Hh pathway.

Innovative Aspects

(1) The first large-scale quantitative phosphoproteome analysis of the Hh signaling pathway
(2) Development of a custom algorithm for cross-quantification of phosphopeptides without requiring MS/MS identification
(3) Application of HILIC combined with titanium dioxide yielding several thousand phosphopeptides

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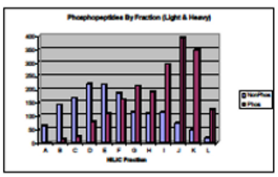

Figure 1 Separation of titanium dioxide enriched peptides across fractions of a hydrophilic interaction chromatography gradient following 2 hours of Shh stimulation (non-phosphorylated peptides in blue; phosphorylated peptides in purple)

C234

Protein profiles of gastric cancer paraffin-embedded (PFFE) samples using MudPIT analysis

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Introduction
Gastric cancer is a very serious neoplastic disease naturally resistant to many anticancer therapies. Despite its decreasing trend in incidence, especially in the western world, gastric cancer still remains a large cause of cancer-related death on a worldwide basis. In this context, we used Multidimensional Protein Identification Technology (MudPIT) (1) for describing protein profiles of formalin-fixed paraffin-embedded (FFPE) gastric tissues, which provides vast archive samples, thus representing a good alternative or integration to newly obtained fresh tissues.

Methods
FFPE gastric tissues, mucosa and tumour samples, were stored for long time at room temperature after being used for diagnosing and staging tumors by histopatological evaluation. The same samples were used for proteomic analysis. Proteins were extracted from paraffinized tissue sections using the principle of heat-induced antigen retrieval technique (2), digested with trypsin and resulting peptide mixtures were analyzed by means of MudPIT methodology. Specifically, peptides were separated onto SCX (using 10 salt steps) and reversed-phase columns, and directly detected by means of a linear ion trap. Data handling was performed by means of Bioworks 3.3.1, based on SEQUEST engine, for the identification of peptide sequences and the related proteins.

Results
As part of our scientific interests, we have performed proteomic investigations of FFPE tissues obtained from a gastric cancer bio-bank. Specifically, paraffin samples from tumor and no-tumor gastric mucosa from the same patients. have been analyzed.
The shotgun approach, based on MudPIT methodology, permitted to identify more than 500 proteins for each sample and each protein list has been plotted on a virtual pI vs MW virtual map, as shown in Figure 1. The home-made *MAProMa* software allowed semi-quantitative evaluation of the differentially expressed proteins (normal vs tumor) based on label-free approach (3). Some of these proteins were previously described by other authors, but new ones were characterized and they need to be validated using alternative methods, such as ELISA. Correlation of proteomic patterns with histopathologic, immunohistochemical and molecular features is under way in a series of gastric cancers with defined clinical outcome.
In addition, as *H.pylori* has been shown to induce gastric carcinogenesis, the acquired MS spectra were correlated with protein database of this bacterium, thus allowing to identify some of these proteins inside mucosa and tumor tissues.

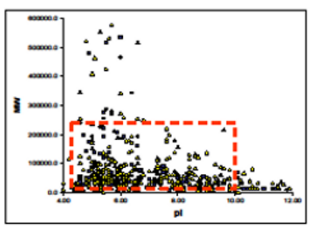

Figure 1 – Typical 2D map of identified proteins from MudPIT analysis of FFPE gastric cancer sample (the red box corresponds to the typical pI and MW ranges for gel electrophoresis)

Innovative aspects
- Characterization of proteins in FFPE gastric tissues using shotgun approach
- Evaluation of relative differential expression of protein based on label-free approach
- Identification of *H. pylori* proteins in gastric tissues

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C235

Phosphoproteomic profiling of Met / K-Ras signaling in colorectal cancer cells

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Introduction
Colorectal cancer (CRC) is the second leading cause of death from cancer overall[1]. *KRas* mutations are the most frequently observed in CRC, occurring in ~50% of all cases[2]. Activation of receptor tyrosine kinases, including Met, is also common in CRC[3]. Met and KRas share common downstream signaling pathways as well as divergent ones; however, co-activation of both Met and KRas could result in preferential over-activation of specific downstream cascade(s). We therefore hypothesize that Met and KRas co-operate to activate novel tumourigenic and metastatic signaling pathways.

Methods
We used the DLD1 CRC cell line model. DLD1 cells contain a heterozygous K-*ras* activating mutation. These cells express a relatively low level of Met. A DLD1 derivative, DKO4, has its mutant K-*ras* allele inactivated by somatic recombination. We used a label-free mass spectrometry (MS)-based proteomics examination of protein-phosphotyrosine (pY). For this study, DLD1 and DKO4 cells that overexpress Met were lysed, then phosphopeptides were purified and analyzed by LC-MS/MS on an LTQ-Orbitrap mass spectrometer. Peptide quantification was achieved by using both peptide ion spectral counting and normalized extracted ion current methods.

Results
In a constitutively active Met background, K-Ras causes quantitative changes in tyrosine phosphorylation of proteins involved in cell-cell adhesion, indicating a possible role for Met and K-Ras co-activation in epithelial mesenchymal transition (EMT) of CRC cells and identifying possible targets of future therapeutic intervention in CRC progression.

Innovative aspects
- Novel use of model of Met/K-Ras constitutive activation
- Promising identification of pathways activated by Met/K-Ras co-activation

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C236

Analysis of Oncogenic NPM-ALK Induced Phosphoproteins: New Insights into Lymphomagenesis

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Introduction
Analplastic lymphoma kinase-postive anaplastic large cell lymphoma (ALK$^+$ ALCL) was a subtype of T/null-cell non-Hodgkin lymphoma (1). 80% of ALK+ALCL tumors are caused by nucleophosmin (NPM)-ALK tyrosine kinase (2). Previous studies show that NPM-ALK promotes ALK$^+$ALCL through aberrant phosphorylation of downstream substrates. We have comprehensively identified phosphoproteins from NPM-ALK transformed GP293 cells and nontransformed GP293 cells, revealing global phosphoproteome changes induced by oncogenic NPM-ALK, by combining a sequential affinity purification method with LC MS/MS analysis.
Methods
NPM-ALK tyrosine kinase domain was mutated by mutating Tyr-338, Tyr-342, Tyr-343 to phenylalanine (FFF mutant) using site-directed mutagenesis. Wild type NPM-ALK and FFF mutant was separately transfected into GP293 cells. The cell lysate was collected after 24 hours of transfection. The cell lysate was digested by trypsin. Phosphopeptides were enriched by Fe^{3+}-IMAC and eluted by using 250mM acetic acid/30% acetonitrile. The eluate was then loaded onto pTyr or pY-antibody beads. The pY peptides were further enriched by pY-antibody and the eluate was directly subjected to RPLC-ESI QTOF MS/MS analysis. The flow-through containing pSer and pThr peptides was fractionated by strong cation exchange chromatography. The individual phosphopeptide fractions were then analyzed by RPLC-ESI MS/MS.
Results
Western blot analysis showed the functional expression of NPM-ALK in NPM-ALK transfected GP293 cells and loss of tyrosine kinase activity in the FFF mutant NPM-ALK transfected cells. By combining the sequential affinity purification strategy and LC MS/MS analysis, we identified 1758 phosphoproteins, 4292 phosphopeptides (3814 pThr and pSer, 451 pTyr) including 3540 phosphorylated sites from the NPM-ALK transfected GP293 cell lysate. From the NPM-ALK FFF mutant transfected cell, we identified 1570 phosphoproteins, 3665 phosphopeptides (3466 pThr and pSer, 199 pTyr) and 4799 phosphosites. Subsequently, 548 putative NPM-ALK induced phosphoproteins were identified in this work. Most of previously reported NPM-ALK induced phosphoproteins were found in our list. To further verify those putative NPM-ALK induced phosphoproteins, we are in process of performing immunoprecipitation and antibody array analysis, which will provide additional evidence for the NPM-ALK induced phosphoproteome changes. We believe these results will put new insight into the ALK-mediated lymphomagensis.

Novel aspects
- Identification of phosphoproteome changes induced by oncogenic NPM-ALK
- Reveal potential therapeutic targets for treatment of anaplastic large cell lymphoma

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C237

Phospho-Proteomic Interrogation of Neuroblastoma Tumor Initiating Cell Signaling Pathways

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Introduction
Neuroblastoma (NB) is the most common extra-cranial solid tumor in children with a survival rate of less than 30% for high-risk patients. Primary human NB tumor initiating cells (TICs) or cancer stem cells were shown to be highly enriched in tumorgenic capacity, with as few as 10 cells forming NB tumors in immuno-compromised mice [1]. Proteomic studies of cancer stem cell biology and signaling pathways are still in their infancy. The purpose of this study is to uncover signaling effectors driving tumor initiating cell survival and proliferation that can be targeted for novel therapeutic approaches against NB.
Methods
To screen NB-TIC signaling pathways, we surveyed the phospho-tyrosine containing subset of the cellular proteome. Primary cell cultures of TICs isolated from bone marrow aspirates of patients in both clinical remission and relapse states were utilized for the screen.
We applied an antibody-based phosphotyrosine affinity-purification method to enrich for phospho-tyrosine containing peptides. Phospho-peptides were identified by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) using an LTQ-Orbitrap system. Expression of proteins of interest and their phosphorylation status was verified by western blotting. The physiological roles of kinases of interest in tumor initiating cells were probed by ligand stimulation and small molecule inhibitors.
Results
We identified over 250 phosphorylated proteins including 31 protein kinases across TICs derived from bone marrow aspirates of three patients with high-risk disease. We detected components of several receptor tyrosine kinase signaling pathways, including those of fibroblast growth factor receptor (FGFR1) and insulin receptor, as well as B-cell receptor (BCR) signaling effectors including activated SYK, BTK and LYN kinases. The expression of SYK, LYN and other kinases downstream in the BCR pathway were confirmed by western blotting across different TIC patient lines. To determine the role of these kinases in NB-TIC survival and proliferation, we treated TICs with the SYK inhibitor BAY-61 and the LYN/Src family inhibitor SU6656. Both inhibitors induced cell death in a concentration-dependent manner in three NB-TIC lines (Figure 1).

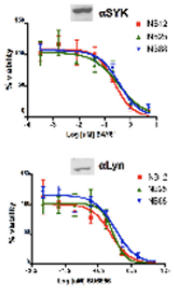

Figure 1: Lyn/Src Family member inhibitor SU6656 and SYK inhibitor BAY61-3606 are cytotoxic to NB-TICs derived from remission (NB25) and relapse (NB12, NB88) samples

We hypothesize that NB-TICs express active components of the B-cell signaling pathway that mediate their survival in the bone marrow niche. We suggest that targeting hematopoietic survival pathways of tumor initiating cells from the bone marrow, which have thus far not been predicted to play a role in this neural malignancy, may provide new drug therapies for NB.
Innovative Aspects
- First phospho-proteomic study of primary pediatric tumor initiating/cancer stem cells
- Uncovered novel hematopoietic signaling pathways in a neuronal malignancy
- Data suggests a functional role for Syk and Src family signaling in Neuroblastoma-TIC survival that may be exploited for novel NB therapies

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C238

Search for tumor-related biomarker proteins by antibody proteomics technology

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Introduction
Disease proteomics, which systemically analyzes and recognizes differentially expressed proteins in healthy and diseased tissue samples, is a potentially useful method for identifying biomarkers. Thus far, however, very few biomarker proteins have been discovered using this approach. The critical issue that remains to be solved is the efficient identification of useful biomarker proteins from numerous candidates. To circumvent this problem, we have developed "antibody proteomics technology" that can screen the biomarker proteins by comprehensively creating antibodies against each candidate.

Methods
2D-DIGE analysis: Cell lysates prepared from breast tumor cells (SKBR3) and normal cells (184A1) were labeled with Cy3 or Cy5 and then separated by IEF-PAGE and SDS-PAGE.
Isolation of monoclonal antibody: Candidate proteins were extracted from the gel pieces of 2D-DIGE, immobilized onto a nitrocellulose membrane and then used as target antigens in scFv-phage enrichment and affinity selection.
Immunohistochemical staining of tissue microarray (TMA): After blocking with 5% BSA, TMA slides were incubated with scFv-phages and stained by the Envision+ system.
***In vitro* invasion assay**: MCF-7 cells transfected with the candidate gene were added to the upper chambers of wells and incubated for 72 hr. The invasive cells were stained with calcein-AM and assayed for fluorescence intensity.

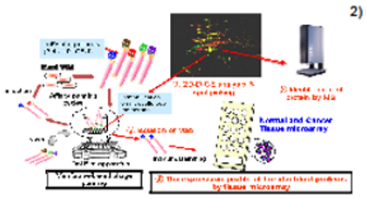

Figure Schematic illustration of screening tumor-related proteins using antibody proteomics technology

Results
Cell extracts derived from breast tumor cells (SKBR3) and normal cells (184A1) were analyzed by 2D-DIGE[1]. Following an *in vitro* scFv-phage affinity selection procedure[2], monoclonal scFvs binding to 20 differentially expressed proteins in tumor cells were successfully isolated within a few weeks. The expression profiles of the identified proteins were then determined by TMA analysis using the scFv-phages. As a result, we identified three breast tumor-specific proteins and one lymph node metastasis-related protein. In order to analyze their function, we investigated the effect of expressing each candidate on cell invasion using an *in vitro* assay. Gene-transfected cells were found to be significantly more invasive. Our data demonstrates the utility of an antibody proteomics system for discovering and validating tumor-related proteins in pharmaceutical proteomics. Currently, we are analyzing the functions of the identified proteins to use them as diagnostic markers or therapeutic targets.

Innovative aspects
- Identification of three novel breast tumor-specific proteins.
- Development of an antibody proteomics system useful for the discovery of novel biomarkers.

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C239

Knocking down 14-3-3 zeta sensitizes oral cancer cells to 5-fluorouracil treatment in vitro

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Introduction

Despite advancements in treatment strategies, the prognosis of head and neck / oral squamous cell carcinoma (HNOSCC) patients has not improved markedly. Major emphasis is being laid on designing new therapeutic strategies for more effective disease management. Recently, using iTRAQ-labeling and LC-MS/MS, we identified a panel of biomarkers, including 14-3-3 zeta and 14-3-3 sigma in HNOSCCs and oral pre-malignant lesions (1, 2). Further, retrospective analysis of survival data revealed poor prognosis for HNOSCC patients showing increased expression of both these biomarkers in their resected tissues (3). Herein we report preliminary results on determining the potential of 14-3-3 zeta as a molecular target for the therapy of oral cancer.

Methods

Transient transfection of siRNAs targeting 14-3-3 zeta was performed using LipofectAMINE 2000 in oral cancer cells (SCC4). Transfection efficiency and specificity of siRNA mediated downregulation of 14-3-3 zeta was determined by transfecting SCC4 cells with fluorophore (Alexa-488) labeled negative control siRNA. Cells were harvested for further analysis (Western blot/ cell viability assays / FACS) after 48-72 hours. Further, to determine the effect of down-regulating 14-3-3 zeta expression on chemosensitization in oral cancer cells, transfected and control SCC4 cells were treated with chemotherapeutic agent, 5-fluorouracil (5-FU), after 48 hours of siRNA transfection in a dose dependent manner for another 24 hours. Cell viability was determined using MTT assays. Flow cytometery analysis (FACS) was carried out using Propidium Iodide (PI) staining.

Results

Transfection efficiency of > 90% was achieved using LipofectAMINE 2000 in SCC4 cells as revealed using fluorophore (Alexa-488) labeled negative control siRNA. Western blot analysis showed >70% decrease in the expression of 14-3-3 zeta protein at a dose of 100nm for 48 hours in siRNA transfected SCC4 cells in comparison to control cells. SCC4 cells transfected with negative control siRNA (i.e. scrambled siRNA sequence showing no homology to any known mammalian sequence) showed no effect on expression of 14-3-3 zeta protein. Cell viability assay showed 45% cell death of SCC4 cells transfected with 14-3-3 zeta siRNA (100 nM, 48 hours) implicating the role of 14-3-3 zeta in proliferation of oral cancer cells. These findings were validated by FACS analysis. Importantly, 5-FU treatment of 14-3-3 zeta siRNA transfected SCC4 cells showed increased cell death at lower doses in comparison to cells treated with 5-FU alone suggesting increased sensitivity of oral cancer cells with reduced expression of 14-3-3 zeta, towards chemotherapeutic agents like 5-FU.

Innovative aspects

- Strategies aimed at suppressing the expression of 14-3-3 zeta protein may have a therapeutic benefit in HNOSCC patients.

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C240

Hepatocellular Carcinoma: Expression Profiling at Membrane Proteome Level

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Introduction
High incidences of community-acquired hepatitis C virus infection that can lead to the progressive development of chronic active hepatitis, liver cirrhosis and primary development of hepatocellular carcinoma occur throughout the world. Chronic infection with hepatitis C virus is known to be a risk factor for hepatocellular carcinoma (HCC). The molecular analysis about the healthy and HCC diseased liver should lead to a better understanding of the interaction and functions of biomolecules in liver tissues.

Methods
To identify the proteins involved in HCC carcinogenesis, we used one and two dimensional electrophoresis to study the differentially expressed proteins in tumor sample. Liver biopsy samples were immediately washed completely with ice cold PBS at 4°C and membrane obtained after performing subcellular fractionation. Membrane proteins were separated by 2-DE, performed with IEF as the first dimension using Multiphor II (Amersham Biosciences) and SDS-PAGE as the second dimension. Gels were visualized with silver staining method.

Results
The expressed proteins revealed interesting results which contributes to the evaluation and understanding of HCV infection leading to cirrhosis. Membrane proteins of the HCV biopsy samples were extracted and analyzed on SDS-PAGE and 2DE. The electrophoresis pattern of membrane protein separated on SDS PAGE and visualized by silver staining revealed 19 components. Two dimensional electrophoresis patterns using narrow range IPG strips revealed significantly up and down regulated proteins differentially expressed as compared to control liver samples. Interestingly, more of the acidic proteins as compared to the basic protein were differentially expressed.

Innovative aspects
- First of its kind of study in Pakistan on HCC patients.
- Identification of high molecular weight membrane associated proteins, differentially expressed in HCC samples.
- Identification of novel membrane proteins may helps to reveal the possible mechanism of virus entry and HCC progression mechanism.

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Acknowledgement
We thank Professor Dr. Abid Azhar, Co-Director Karachi Institute of Biotechnology and Genetic Engineering, University of Karachi, for providing the working facilities. We are indebted to Dr. Viqar, Dr. Samina, Jinnah Post Graduate Medical Centre, and Dr. Rubina Ghani, Molecular laboratories for kindly providing HCC biopsy samples. This work was supported by a grant from the Higher Education Commission, Pakistan, No. 20 -725/ R&D/07.

C241

Identification of Putative Biomarkers for Ovarian Cancer from Human Ascitic Fluid

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Introduction
Ovarian cancer is the most lethal gynecologic malignancy, partly due to its late-stage diagnosis when prognosis is poor[1]. Despite substantial research, only one biomarker is in clinical use (CA125), lacking sensitivity and specificity esp. for early detection[2]. The identification of reliable biomarkers for early diagnosis, prognosis and monitoring is thus imperative. In this study, we use shot-gun proteomics followed by comprehensive data mining to identify a panel of putative biomarkers from patient ascites samples. Furthermore, we devise a MS-based method for quantification of candidates.

Methods
Ascitic fluid samples from five patients with serous adenocarcinoma were immunodepleted of twelve high-abundance plasma proteins, concentrated, and digested. Samples were subjected to on-line 12-step MudPIT, and OFFGEL fractionation into 12 fractions (pH 3 -10) followed by 1D-RP-LC-MS/MS on a LTQ-Orbitrap. MS-data was searched with two algorithms (X!Tandem, SEQUEST) and confident identifications combined. Data mining was performed in Protein Center software suite. Method development for MS-based quantification was performed on a TSQ Quantum Ultra.

Results
Analyses of five ascitic fluid samples with two powerful shot-gun proteomics platforms led to the identification of 677 unique proteins, the largest inventory of the ascites proteome to date. After computational comparison to other published data sets from proteomic and microarray studies on ovarian cancer as well as in-house proteomic data on expressed prostatic secretions (EPS), we present a list of 31 candidate proteins – including some previously proposed markers, underlining the feasibility of our mining scheme – that encourage further evaluation (**Fig. 1**). Since the rapid, paralleled quantification of putative biomarkers seems a promising and reasonable approach for future biomarker research, we then developed a selected reaction monitoring (SRM-MS) method for six candidates in ascites samples. This multiplexed method will allow for further scrutinizing of the occurrence of these in patient samples in different contexts (FIGO stage, recurrence, chemotherapy resistance etc). Our study also serves as a proof of method for future biomarker research, in which a discovery phase could be followed by the multiplexed analysis of a whole panel of intriguing candidates.

Innovative aspects
- Most comprehensive coverage of ovarian cancer ascites proteome
- Multiplexed analysis of a panel of putative biomarkers in biological samples

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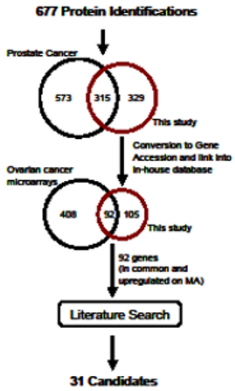

Figure 1: 677 proteins were identified by proteomic analyses of ascitic fluid; Comparison to studies on prostate cancer, microarrays (MA) of ovarian cancer and other studies on ovarian cancers yields a panel of 31 putative biomarkers.

C242

The Glioblastoma Exosomal Proteome: Modulation by Wild-type p53

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Introduction Cultured glioblastomas cells (U87 MG) have been shown to express high levels of galectin-1 as mediated with p53 treatment.(1) Also, exosomes have been shown to export galectin-1 to extracellular space under the same conditions (2) Thus, a full exosome proteomic study on empty vector and wtp53 treated samples is desired. Proteomics was performed by nano-LC FT-ICR MS. The results are highly confident protein identifications after proper use of informatics for false discovery rates. Confident results translate into semi-quantitative analysis for confident cellular communication assignment.

Methods Exosomes were harvested from ~1x10^7 U87 MG cells after treatment with empty vector or wild-type p53. Proteins from the two treatments were separated by SDS-PAGE. Sixty four gel slices (32 for each cell treatment) were subsequently in-gel digested with trypsin prior to separation by nano-LC. Top-5 data dependent MS/MS was performed with a modified LTQ FT-ICR equipped with a 14.5 T superconducting magnet. RAW files were extracted with multiplierz (3) prior to searching against the IPI human protein database with MASCOT (2 ppm search tolerance). Results were then validated by Scaffold (Proteome software) or extracted from MASCOT with multiplierz for further data mining. False discovery rates were determined with both the protein prophet and reversed database models.

Results Because of the limited amount of sample, SDS-PAGE separated the proteins prior to trypsin digestion and nano-LC MS. From the first pass proteomic analysis, more than 300 proteins were identified. The mass accuracy for all identified peptides within the data set was 280 ppb rms. The major functional groups to which these proteins belong include structural proteins, chaperones, signal transduction proteins, MHC class I and II proteins, and enzymes related to glycolysis. Of those 300 proteins, 27 protein identities were determined to be unique to the empty vector-treated exosomal fraction. For the wt-p53 treated samples, 52 proteins, including CD97, CD109, CD166 (ALCAM), niban, and kynureninase, were uniquely identified. The end goal is to expand the analysis to generate highly confident relative protein quantification of the differently treated glioma cell lines. The relative quantification values will facilitate the creation of protein networks to provide important clues about the role of exosomes in cellular crosstalk and the dynamic process of p53 mediation.

Innovative aspects
- Proteomic analysis of glioma exosomes in response to p53 modulation
- High mass accuracy MS data (300 ppb rms) with external calibration
- High confidence protein identification with two independent false discovery rate models

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C243

Clinical significance of TC21/R-Ras2 overexpression in Oral Carcinogenesis

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Introduction
The limited effectiveness of therapy for patients with advanced stage Oral cancer or recurrent disease is a reflection of an incomplete understanding of the molecular basis of head and neck carcinogenesis. Conceivably, improvement in the ability to identify in an early stage and to predict malignant progression of OSCC lesions would lead to more effective treatment and reduction of morbidity and mortality. In search of novel molecular targets, our laboratory reported increased levels of TC21 transcripts in clinical samples of oral squamous cell carcinomas (OSCCs) using Differential Display RT-PCR(1).

Methods
Immunohistochemical analysis of TC21 was carried out in 120 leukoplakias [with no dysplasia (62 cases) or with dysplasia (58 cases)], 83 OSCCs and 30 non-malignant tissues obtained from research tissue bank. Each section was scored semi-quantitatively on the basis of intensity and percentage positivity of the epithelial cells and total score was obtained by adding the scores of percentage positivity and intensity. The immunohistochemical data were subjected to statistical analyses using the SPSS 10.0 software (Chicago). Sensitivity and specificity were calculated and quantified using receiver operating characteristic (ROC) analysis. The relationships between TC21 protein expression and clinicopathological parameters were tested using Chi-Square and Fischer's exact test. Follow-up studies were analyzed by Kaplan-Meier analyses. One sided p-values were calculated and p≤0.05 was considered to be significant. These results were corroborated by western blotting. Co-immunoprecipitation assays were carried out to determine association of TC21 with PI3-Kinase, ERK2, 14-3-3ζ and 14-3-3σ A.

Results
Immunohistochemcal analysis shows predominant TC21 expression in nuclei of epithelial cells in leukoplakia (90/120, 75%) and OSCCs (66/83, 79.6%). Forty six of 62 leukoplakia with no dysplasia (74.2%) and (44 of 58) 75.9% leukoplakia with dysplasia showed increased expression of TC21 protein. Of the 30 normal tissues analyzed, 28 cases (93.3%) showed no detectable or faint immunostaining of TC21 protein in epithelial cells. TC21 nuclear expression increased progressively from normal tissues to leukoplakia and frank malignancy ($p < 0.001$) suggesting its utility as a plausible diagnostic marker for OSCCs. Receiver operating characteristic (ROC) analysis showed that the area-under-the curve (AUC) values were 0.895, 0.885, and 0.919, while the positive predictive values (PPV) were 95.8, 95.6, and 97.1, for nuclear immunostaining for normal versus leukoplakia with no dysplasia, leukoplakia lesions with dysplasia, and cancer, respectively. Immunoblotting confirmed over expression of TC21 protein in oral lesions in comparison to normal tissues. Using co-immunoprecipitation assays, we also showed interaction of TC21 with Erk2, PI3-kinase, 14-3-3ζ and 14-3-3σ proteins in oral cancer cells and tissues indicating involvement of TC21 in novel signaling pathways. In conclusion, our findings suggested that TC21 may serve as an important potential molecular marker for early diagnosis and as a poor prognosticator of OSCCs. Thus, it may serve as potential candidate for molecular targeted therapy of OSCCs.

Innovative aspects

TC21/R-Ras2 is a novel potential therapeutic target for oral cancer.

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New Approaches to Quantitative Western Blotting

C244

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Introduction
Multiplex fluorescent detection dramatically increases the quantitation possibilities compared to traditional chemiluminescent Western blotting. Chemiluminescence signals have limitations in stability and signals will decrease over time. Fluorescence properties include high sensitivity, broad dynamic range and stable reproducible signals. We show the advantages of fluorescent Western blotting for simultaneous detection of up to three targets, detection of targets of same Mw without stripping and re-probing and 2-D Westerns. We also show new approaches such as triplex, 3-layer probing, 3-color detections.

Methods
Samples were separated by 1-D electrophoresis or 2-D electrophoresis (small gels, 8 X 7 cm) and transferred onto Hybond LFP™ (low fluorescent PVDF) membranes and blocked in ECL Advance™ blocking agent for optimal fluorescence detection. The membranes were probed with primary antibodies followed by secondary CyDye™ conjugated ECL Plex antibodies or other CyDye conjugates (Cy 5 Strepatavidin and biotinylated anti-rabbit antibody, Cy5 Sheep anti-biotin antibody). Deep Purple was used for total protein staining to confirm the transfer efficiency and for quantification of amount loaded protein per lane. The membranes was dried and scanned for Cy™ 2, Cy 3, Cy 5 and Deep Purple signals using a fluorescent imager (Typhoon™ or Ettan™ DIGE Imager). The images were then analyzed using ImageQuant™ TL software.

Results
Fluorescent western blotting offer high sensitivity, broad dynamic range and stability of signals. This makes it optional for high sensitive and accurate quantitative Western blotting. Here we show how fluorescent western blotting can be used for simultaneously detection of up to three different proteins on the same blot at the same time as well as detection of proteins of the same molecular weight at the same time without stripping and re-probing. We also show how fluorescent Western blotting with 3 layer probing is used to obtain increased sensitivity and possibility to detect very low abundant proteins. With Deep Purple protein staining prior ECL Plex western blotting it is possible to detect the total protein amount and the target protein at the same time.

Innovative aspects
- Fluorescence Western blotting offers stable signals, highest sensitivity and broadest dynamic range enables accurate quantification.
- Simultaneous detection of up to three target proteins as well as proteins with same Mw without stripping and re-probing.
- 3 layer detection increases signal intensity and possibility to detect very low abundant proteins.

References
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Triplex - Detect three proteins simultaneously

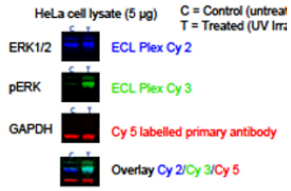

Figure 6. 1-D Western blot of UV Irradiated and control H cells. ERK1/2 was targeted with ECL Plex Cy 2, phosphorylated ERK 1/2 (pERK) with ECL Plex Cy3 and loading control, the house- keeping protein GAPDH prote were detected with a primary antibody labeled with Cy 5 (antibody labeling kit from GE Healthcare). All three prote were detected simultaneously in a triplex fluorescent detection. Triplex detection which is very advantageous fo detection of ±PTMs and in addition relates the signals to a house-keeping protein. This is very useful to monitor if the levels of total protein pool (modified and none modified) is changed during the experiment.

Mining the Secretome of 11 Breast Cell Lines To Identify Novel Breast Cancer Biomarkers.

C245

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Introduction
Breast cancer is the most frequently diagnosed cancer in women and the second leading cause of cancer death in the United States [1]. While early diagnosis relies on mammography screening the sensitivity varies from only 62.9% to 79% [2]. Furthermore, the absence of a clinically useful molecular biomarker [3] highlights the need for the development of additional non-invasive diagnostic methods for the detection of early stage breast cancer. In this study, the proteomes of 11 cell lines (9 cancer, 2 normal) were analysed with LC MS/MS for the discovery of novel breast cancer biomarkers.

Methods
A "bottom-up" proteomics approach and a two-dimensional LC-MS/MS strategy on a linear ion trap-orbitrap were utilized to identify the secretomes of nine breast cancer cell lines (HCC-1428, BT483, MCF-7, MDA-MB-231, HCC-1143, HCC-38, SK-BR-3, HCC-202, UACC-812). Three cell lines for each breast cancer type (oestrogen/ progesterone receptor positive, triple negative, HER2/neu amplified) were selected, in order to capture the heterogeneity of this disease. The conditioned media were processed (reduction, alkylation, tripsinization) and peptides were fractionated by cation exchange chromatography. All the samples were run in triplicates and results were searched using MASCOT and X!Tandem with the IPI 3.46 human forward and reverse database. Scaffold was used to cross-validate Mascot and X!Tandem results.

Results
Over 800 unique proteins were identified in the conditioned media of each cell line, resulting in more than 4000 proteins identified from the eleven breast cell lines. The reproducibility of protein identification between replicates of the same cell line was approximately 70-80%. We developed an in-house software to analyze and compare the proteomes of the different cell lines. We identified the common and unique proteins among the breast cancer cell lines (of both the same and different type) and we compared these with the secretome of the normal breast cell lines. We also determined cellular localization and functions of the identified proteins and we focused on the extracellular and membrane proteins which constituted approximately the 25% of the total proteins. More than 100 proteins were found to be uniquely expressed by the cancer cell lines. Moreover, we compared these proteins with the proteome of nipple aspirate fluid from breast cancer patients and approximately one third were found in common. Further studies are ongoing to investigate the potential use of these molecules as breast cancer biomarkers.

Innovative aspects
- The most extensive proteomics analysis of nine breast cancer cell lines and two normal breast cell lines to reflect heterogeneity of breast cancer
- The first study to compare proteome of breast cancer cell lines with nipple aspirate fluid proteome

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Quantitative Phosphoproteomics of Glioblastoma Cancer Stem Cell Differentiation

C246

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Introduction: Cancer stem cells (CSCs) are hypothesized to provide a repository of cells in tumor cell populations that are refractory to chemotherapeutics developed for the treatment of differentiated tumor cells. A glioblastoma cancer stem cell line (NSC11) differentiates from a pluripotent state to a progenitor cell-like state when stimulated with a STAT3 phosphorylation inhibitor (WP1193). We applied a novel phosphoproteomic workflow to determine pathways of NSC11 differentiation.

Methods: Six different treatments of ~$2x10^7$ NSC11 cells (each) were studied: vehicle, IL-6 or WP1193 and IL-6 stimulation, and cell culture conditions with normoxia or hypoxia. Cells were lysed in the presence of protease and phosphatase inhibitors. Proteins were enriched in a phosphoprotein enrichment column, reduced, alkylated and enzymatically digested. Peptides were isotopically labelled with Thermo Scientific Isobaric TMT6-plex tags and combined into one sample. Phosphopeptides were enriched by HILIC chromatography and TiO2 prior to analysis in a Thermo Scientific LTQ-Orbitrap Mass Spectrometer. Protein identification was performed and relative peptide quantification was performed simultaneously by comparison of the abundance ratios of the reporter ions from the TMT® tags. Pathway analysis of normalized quantitative data was performed with Ingenuity Pathway Analysis software.

Results: Nearly 2,000 proteins were quantified in two replicated studies. The largest number of significant protein changes was observed during hypoxic conditions compared to normoxic conditions with IL-6 stimulation. Functions associated with the quantitated phosphoproteins included 11% transcription factors. In addition to studying how protein expression levels change, it is clear that differential phosphorylation at specific sites of the same protein occurs as a result of the various treatments. The high sensitivity of the assay allows for detection and quantification of phosphorylated peptides from key proteins such as transcription factors. Because large numbers of changes in peptide phosphorylation patterns are measured in the assay, it will be possible to assign by computational methods kinases that are activated in the intracellular pathways, even if the kinases are not directly measured. About 15% of the quantitated phosphoproteins were linked to the IL-6 pathway. About 70% of remaining proteins could be assigned to other interlinked networks that were regulated by treatments and differential effects of the treatments were observed in those networks and canonical signaling pathways. Quantification of pathway changes in NSC11 cancer stem cells due to six different conditions showed expected pathway changes in the IL6/STAT3/HIF1α loop but also provided novel knowledge of other pathways changed in response to treatment and peptide phosphorylation patterns in response to treatments. The assignment of phosphoprotein responses currently not defined in the literature by high-level statistical analysis will provide further insights into the behavior of the NSC11 cells during differentiation to progenitor-like cells.

Innovative aspects
•Phosphoprotein analysis of NSC11 CSCs
•Determination of differentiation pathways in CSCs
•Identification of potential secondary biomarkers of cancer stem-like character

Large-scale Tumor Proteomics Pinpoints a Specific Molecular Signature for High-risk Triple Negative Breast Cancer

C247

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Present diagnostic procedures and therapeutic strategies for breast cancer rely on the detection and targeting of specific marker molecules to classify the tumour and to design an efficient therapeutic strategy. Examples for such markers/targets are the oestrogen receptor (ER), progesterone receptor (PgR), and the Erb2/Her2 molecule, a receptor tyrosine protein kinase that is overexpressed in a subset of breast cancers. There are number of treatment options for tumours that express the ER, PgR or Her2 receptors, however up to 20 percent of the newly diagnosed cases belong to a subset, which is negative for all three of the receptor molecules. Triple negative breast cancer is more prevalent in pre-menopausal women, women of African and African-American origin, and is associated with worse prognosis because the therapeutic options are limited compared to receptor-positive cases. Clearly, there is need for more research focussed on establishing new molecular markers and potential drug targets for triple negative breast cancer. To address this need we undertook a large-scale quantitative proteomics study utilizing recently described tumor proteomics approach (1, 2). We found that a group of proteins that regulate or respond to activation of the interferon gamma signalling pathway are strongly overexpressed in metastatic triple negative breast tumours in comparison with non metastatic tumours of the same receptor status and grade (Fig. 1). Quantitative invasion assays utilizing cultured breast cancer cells demonstrated that interferon gamma treatment enhances the invasiveness of the cells. Thus our data from large-scale tumor tissue profiling and model system analysis supports the hypothesis that interferon gamma modulates the ability of triple negative breast cancer cells to invade surrounding tissue and suggest a possible new approach to finding useful drug targets for treating triple negative breast cancer. This presentation will discuss the discovery and validation of the proteins identified as a molecular signature for invasive triple negative breast cancer and the mechanistic models that can explain how these proteins contribute to the heightened invasive potential in triple negative breast cancer.

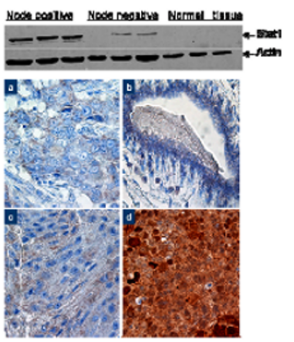

Figure 1. Stat1 protein in triple-negative breast tumours. **Top**: Western blotting of 3 node-positive tumours, 3 node-negative tumours, and 3 normal tissue samples. Mouse monoclonal anti-Stat1 antibody was used. **Bottom**: immuno-hystochemical staining of Stat1 protein in normal tissue (**b**), a Stat1-negative tumour (**c**), and a Stat1-positive tumour (**d**). Secondary antibody-only staining of the Stat1-positive tumour tissue is shown in panel **a**. Statistical analysis of the western blotting data for Stat1 expression by Fisher's exact test resulted in a p-value =0.0055.

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C248

Quantitative Analysis of C/EBPα Transcription Complexes in Leukemia

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Introduction
Acute myeloid leukemia (AML) remains a highly lethal malignancy with limited therapeutic options. Mounting evidence indicates that impaired function but not complete absence of the transcription factor C/EBPα leads to consecutive accumulation of myeloid progenitors and blast crisis in AML. Recent studies demonstrated that oncogenic Flt3 signaling leads to phosphorylation on serine 21 (pS21) of C/EBPα with concomitant impairment of myeloid differentiation [1]. We hypothesized that assembly of transcriptionally active protein complex is governed by pS21-regulated protein associations.

Methods
To test this hypothesis we established tet-inducible affinity tagged C/EBPα expression system in myeloid cells with constitutive Flt3 activity. Treatment of these cells with Flt3 inhibitors modulated pS21 in a dose-dependent manner. Next, advanced quantitative proteomics methodology, including true nanoflow LC coupled with multidimensional RP/RP fractionation and on-column iTRAQ stable isotope labeling, was used to monitor remodeling of C/EBPα protein complexes as a function of pS21. siRNA methods were used to abrogate expression of interactors modulated by pS21 to determine their functional role in transcription of myeloid-specific genes. Promoter assay and ChIP were performed to show that interactors regulate C/EBPα transactivation potential and binding to C/EBPα-target promoter.

Results
Our data represent by far the largest catalog of C/EBPα interactors, including more than 100 proteins involved in chromatin organization, transcriptional modulation, and cell cycle regulation. Furthermore, our quantitative proteomics data demonstrate that 1) C/EBPα interacts with proteins genetically linked to leukemia; and 2) many of these interact with C/EBPα in a phosphorylation-dependent manner. Knock-down of newly-identified, leukemia-associated interactors DEK and SET reduced the ability of C/EBPα to drive expression of granulocytic target genes. In addition, DEK and SET bind to C/EBPα-target promoters and enhance C/EBPα binding to its DNA binding site. Our data demonstrate that phosphorylation on serine 21 modulates the association of C/EBPα with protein partners that are functionally relevant for myeloid differentiation. These data show direct links between tumor suppressor C/EBPα and oncogenes DEK and SET. Our ability to quantitatively monitor multiple leukemia-related gene products in the context of C/EBPα protein complexes provides valuable insight into the mechanisms by which oncogenic kinase activity disrupts transcription and leads to leukemogenesis.

Innovative aspects
- Biological mechanism driven by proteomics-based discovery
- Quantitative analysis of protein complexes responding to molecular switch
- Target protein validation in myeloid differentiation models

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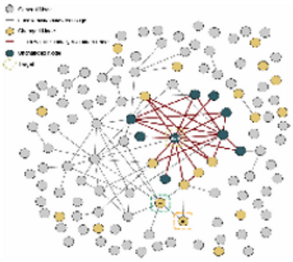

Figure 1. C/EBPα putative partners' network. The edges reflect HPRD, STRING, DAVID and Ingenuity data base annotated protein-protein interactions assessed by various experimental approaches. Functionally validated protein partners are highlighted with squares.

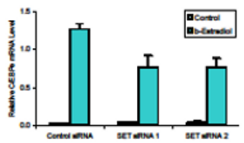

Figure 2. Depletion of novel C/EBPα partner SET decreases C/EBPα capacity to induce the transcription of granulocytic gene C/EBPε in myeloid cells.

C249

2-D DIGE analysis of Multicellular Tumor Spheroids (MTS) in evaluation of cancer treatment

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Introduction
Positron emission tomography (PET) imaging is widely used in clinical diagnostics of tumors and detection of metastasis, but can also be used for evaluation of response to treatments. In preclinical cancer research, the *in-vivo* like Multicellular Tumor Spheroid (MTS) is increasingly used as model system for tumors. In this study we show how 2-D DIGE analysis can be used for analysis of differences in protein expression as a result of drug treatment of MTS. New suitable biomarkers for development of new PET tracers and drug targets can be identified. Combining 2-D DIGE PET imaging results can be used to develop cancer diagnostics and treatment.

Methods
Untreated (control) and Doxorubicin treated MTSs were lysated in lysis buffer (7M urea, 2 M thiourea, 30 mM tris, 4 % Chaps pH 8, 5).
Each samples and an internal standard (mix of all samples), containing 50 µg protein, were labeled using CyDye DIGE Fluor minimal dyes (Cy3, Cy5 and Cy2). Control samples, Cy5 or Cy3 labeled, are mixed with the treated samples, Cy3 or Cy5 labeled. Cy2 labeled internal standard is added to all samples. The mixed samples are loaded onto the anode of Immobiline DryStrip precast IEF gel with pH range 3-11 (non-linear gradient; NL). Separation was performed using EttanIPGphor III IEF system followed by second dimension separation on new precast DIGE Gels. DeCyder 2D software v 7 were used for analysis of protein abundances in the different samples.

Results
In this study we used 2-D DIGE analysis to show differences in protein expression in MTS's as a result of drug treatment. Analysis and evaluation of data in DeCyder 2D software v 7 show differentially protein expression between the samples and the control and treated samples are clustered into separate groups. Data showing this will be presented.

Innovative aspects
- 2-D DIGE can be used to compare MTS's and patient tissue protein profiles
- 2-D DIGE can be used to identify possible new biomarkers for development of new PET tracers.
- A combination of 2-D DIGE and PET analysis may increase possibilities to optimize and improve diagnosis and treatment of cancers.

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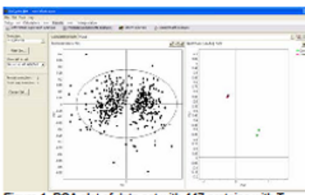

Figure 1. PCA plot of data set with 447 proteins with T-test < 0.05. Control and Treated samples (Spot maps) clustered into separate groups.
Evaluation in EDA module, DeCyder 2D v 7

C250

Identification of Alternative Splice Isoforms in Mouse Model for Human Breast Cancer

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Introduction
Alternative splicing generates protein diversity without significantly increasing genome size. In cancers, there are examples of every kind of alternative splicing, including the use of alternative individual splice sites, alternative exons, and alternative introns. We have devised a proteomic informatics approach to identify alternative splice variants of both known and novel proteins. In this paper, we present the alternative splice variant analysis done on the tandem mass spectrometric data from tumor and normal mammary tissue of HER2/Neu-driven breast cancer in the mouse.

Methods
The approach searches mass spectrometric data against our non-redundant database created with three-frame translations of mRNA sequences from ECgene and Ensembl mouse databases. The search results were processed with the Michigan Peptide to Protein Integration method [1]. Sequence analysis of peptides matching to the final integrated protein list was done against the latest Ensembl, NCBI-nr databases and mouse genome. All peptides matching to protein sequences or protein-coding regions are known peptides, while high-quality non-matching peptides are novel isoforms. Only those peptides that derived from genes with multiple transcripts were retained as they are peptides from alternative splice variants. Finally, we randomly selected 15 novel peptides and validated them by Q-RT-PCR.

Results
We identified a total of 892 distinct alternative splice variant proteins from normal and tumor tissue specimens; 461 proteins were found exclusively in tumor samples. Among these tumor-specific variants, 157 proteins had peptides that did not align perfectly to known protein or protein-coding sequences. These novel peptides include peptide sequences with deletion of exonic regions, inclusion of intronic regions, or presence of UTR regions. Peptide sequences from intronic regions suggest the occurrence of an alternative splice site in the intron. The peptide sequence identified as GSGLVPTLGRGAETPVSGAGATRGLSR matched to an intronic region of sox-7 protein. The Q-RT PCR validation showed a 9-fold increase in mRNA expression in tumor samples when compared to normal tissue. Amino acids TVI from the peptide TVIMPHSYPALSAEQKKELSD that matched to aldolase C are from the 5'UTR region of this gene, which imply an alternative translation initiation site. An 18-fold increase in the mRNA expression of this peptide was observed in tumor samples. We also identified variants of such genes as pyruvate kinase, creatine kinase, and fibulin 2, all reported to play roles in breast cancer mechanisms.

Innovative aspects
- Identified tumor-specific known and novel alternative splice variants
- Some of these novel alternative splice variants may contain regulatory elements that play important roles in tumor mechanisms.

References
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C251

Proteomics of a Novel Human Prostate Cancer Cell line and the effects of EGCG

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Introduction:
Epigallocatechin gallate, a naturally occurring polyphenolic antioxidant found in green tea, has been shown to sensitize prostate cancer cells (LNCaP) to apoptosis. Little is known about development and progression from benign to metastatic prostate cancer disease. What is lacking is a human derived progression model for the disease. This study utilizes such a model, derived from T+E2 treatment of BPH-1+rUGM tissue recombinants. Non-tumorigenic ($PHEC_{NT}$), tumorigenic ($PHEC_T$) and metastatic ($PHEC_M$) cell lines were grown in culture and treated with EGCG or control. Effects were studied by looking for differences in expression using 2D-PAGE and mass spectroscopy.

Methods
2D Liquid Fractionation and Enzymatic Digestion: PF 2D fractions were separated and digested via trypsin similar to a method previously described (1). **2D PAGE**: Two dimensional gel electrophoresis was performed using BioRad 17cm strips and 3-10 amphyolytes. **Mass Spectrometry**: Mass spectra of digested proteins were acquired on an Autoflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Billerica, MA) or a Thermo LCQ or LTQ ion trap mass spectrometer (Thermo Corporation, San Jose, CA) or a MicroTOFQII (Bruker Daltonics, Billerica, MA) .

Results
Our results produced many proteins of interest, most of which have been previously associated with PRCA, such as calmodulin, stathmin, calreticulin, 78kDa glucose regulated protein, α-enolase, BMP2, and HSP90. In addition, we observed several steroidal co-regulator proteins that either enhance or repress the steroidal receptors, such as calreticulin, BAG-1, high-mobility group protein 2, and AR-associated protein 160 (ARA160). Further, we have observed several proteins that have yet to be associated with prostate cancer, such as vacuolar membrane ATPase, heterogeneous nuclear ribonucleoprotein A1, zinc finger CCCH-type containing 11A protein, N-acetylglucosamine-1-phosphate transferase and nucleophosmin. The three cancer states appear to be consistent with specific time points of growth: $time_0$ is non-tumorigenic; $time_{8\ weeks}$ is tumorigenic, and $time_{16\ weeks}$ is metastatic.

Innovative aspects
- Novel proteins identified that may be related to prostate progression
- Novel cancer cell line that produces all three forms of cancer
- Novel drug testing system

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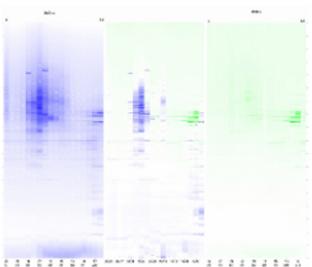

Figure 1 there are PF2D spectra of $PhEC_{NT}$, $PhEC_T$ in the right and left panels, respectively The middle panel of figure A and B are the comparative spectra.

C252

Identification of signalling pathways involved in primary chemotherapy resistance of breast tumours

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3 School of Medicine, Centre for Medicine and Oral Health, Griffith University, Gold Coast Campus, Australia

Introduction

Development of drug-resistance tumours is a global problem in clinical oncology. Patients with large locally advanced tumours show a poor or partial response to primary chemotherapy consisting of Taxol/AC (Adriamycin and Cyclophosophamide)(1).Beast cancers are routinely subcategorised on clinical stage, cellular morphology and on immuno-hisotchemical markers. We aim to identify signalling proteins and pathways including tyrosine kinases and phosphatases,that may drive resistance to primary chemotherapy and may lead to the discovery of novel prognostic markers and new targets for therapeutic intervention.

Methods

Tissues prior to (core biopsies) and following primary chemotherapy (surgical specimens) were collected and categorised into pathological responses (Progressive, no response, 30%, 80% response, and complete response). Tissues will be lysed and enriched for phosphoproteins by immunoprecipitation using 4G10-conjugated Agarose A beads. Tyrosine phosphorylated (pTyr) proteins will be eluted and separated in the first dimension by isoelectric focusing (IEF) using immobilised pH gradient 3-10 and in the second dimension using 2Dgels. Proteins showing consistent differences in pTyr will be subjected to MS identification using MALDI-TOF and database sequence identification. Validation of selected proteins will be by western blot and by IHC.

Results

We have successfully optimised protein extraction from fresh frozen human breast tumour core biopsies and post surgery tissue. Enrichment of tyrosine phosphorylated proteins from small amounts of breast tissue (core biopsies) by immunoprecipitation using 4G10 has also been achieved (Figure 1and Figure 2). Different methods of eluting tyrosine phosphorylated proteins following immunoprecipitation (Figure 1 Lane 8, 9,10) (that are compatible with 1st and 2nd dimension separation) have been investigated and optimized. Recovery of tyrosine phosphorylated proteins using these methods has been monitored using silver staining (Figure 1) western blot with 4G10 (Figure 2) and with the phosphoprotein stain, ProQ Diamond. Conditions for separation of pTyr proteins in the first dimension using IEF and the second dimension using 2Dgels have been established and clinical samples are ready to be analysed. Results using tissue from primary chemotherapy patients will now be generated and presented at this conference.

Innovative aspects

- The use of fresh human breast tissue to enhance the understanding and prediction of poor responsiveness of breast tumours to agents including Taxol, Adriamycin and Cyclophosophamide.
- Pivotal for the development of assays to screen and identify drug-resistant and responsive patients which may result in avoiding harsh and expensive unnecessary chemotherapy treatments.
- Initiate a new kind of phospho-proteome profiling and may result in specific patient centered drug regimes.

References

(1) Data from Chelmsford and Essex Breast Unit.

Figure 1

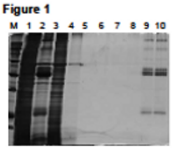
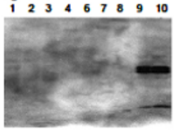


Figure 2



Enrichment of Tyrosine phosphorylated proteins from breast core biopsies (n=3) by immunoprecipitation with 4G10. Figure1. Silver staining of pTry enrichment process and **Figure 2.** Western blot using Mab 4G10. M = marker; Lanes 1= Input lysate (1/50 of input); 2 =pre-clearing with Protein A Agarose beads (all beads); 3 = lysate after 4G10 IP (1/50); 4-7= washes (1/50); 8 =Elution with Phenyl Phosphate (1/30 of elution fraction); 9 = Elution with 8MUrea and 2%CHAPS (1/50);10 = Elution with 2.5%SDS(1/10)

C253

A Rapid Method for Biomarker Evaluation & Validation: Application on Prostate Cancer Marker

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Introduction

Biomarkers in drug development and diagnostics have expanded across a broad range of applications from research tools during early discovery to surrogate endpoints in the clinic. Due to the development of advanced proteomics, the number of publications on experimental biomarkers has extended into the several thousands each year. However, only a small number of these markers will achieve clinical success. Biomarker evaluation and validation constitute critical steps in biomarker development. We developed a rapid method, EDGE*TEST, for biomarker evaluation and validation using our Edge™ technology, a stepwise density based extraction technology in combination with a unique statistical algorithm.

Methods

Twenty four patients suspected of being positive for prostate cancer (PCA), as determined by prior clinical evaluation of their PSA and/or PCA3*plus* scores, as well as by digital rectal examination, were chosen by their urologist for this study. A total of 12 needle core biopsies from each patient were collected for pathological evaluation. PSA and PCA3*plus* analyses were also done on each of the patients. Two additional biopsies from the right gland and the left gland were taken from random locations for this study. Samples were homogenized then fractionated into four subcellular density fractions using *Edge 200 Separation System*. All fractions from each sample were subjected to western blot using the GRP78 marker. Results were analyzed statistically to give clustered relative distribution ratio profiles of GRP78 for each patient.

Results

It has been reported that glucose-regulated protein GRP78 is related to several human cancers (1). A recent study has demonstrated that GRP78 plays a crucial role in the development of prostate cancer by promoting cancer cell proliferation, mediating oncogenic signaling and protecting cancer cells against cell death resulting from the stress of tumor development (2). Our study shows that the ratios of the relative percent of GRP78 contained in two specific fractions within a sample are different for patients having cancerous and benign biopsies, with tight clustering of data for the cancer samples (Fig 1). The data indicates that the ratio of the relative percent of GRP78 between two specific fractions has a high predictive value in assessing whether a patient will show cancerous or benign biopsies.

This study demonstrated that EDGE*TEST provides (i) a simple and reproducible technique for subcellular fractionation of tissue homogenates/cell lysates, (ii) a unique statistical method for evaluation of potential biomarkers, and (iii) the opportunity for generating data for further clinical validation.

Innovative aspects

- The EDGE*TEST method—which is based on Edge technology, DOES NOT require internal or external standards.
- Since the method relies on the ratio between internal fractions of the sample, the method is independent of sample amount.
- The EDGE*TEST method only needs two needle biopsies, and provides more sensitivity and more specificity than the traditional PSA test and the genetic PCA3 test.

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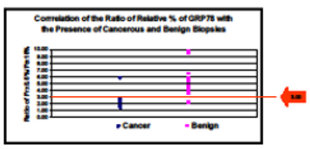

Figure 1.The clustered distribution of the ratios of relative percent distribution of GRP78 in fraction 8.5% vs. fraction 15% in a group of patients with cancerous and benign biopsies

C254

Exploration of the Cathepsin D Protein Complexes in Senescent Pathways

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Introduction

Cathepsin D (Cath D), a lysosomal aspartyl proteinase, is highly expressed and secreted by cancer cells (1). It has been reported that Cath D plays multiple roles during tumorigenesis, such as stimulating tumor cell proliferation and metastasis, and inducing outgrowth of fibroblasts and angiogenesis (2). However, the mechanisms for these events remain un-elucidated. Based on our early observation, the proteomic investigation revealed that Cath D was involved in lung cancer and tumor cell senescence. The nude mouse model transplanted the cells with high Cath D provided additional evidence that this protein was able to provoke tumorigenesis. Herein, we further inquire how Cath D participates in senescent development and which proteins would interact with it to form the functional complexes in signaling pathways.

Methods

We first generated two inducible cell models by transfections of pTER-Cath D to T-RE Hela cells and pcDNA4/TO-Cath D to T-RE 293cells, in which Cath D expression was knockdown or over-expressed by addition of Doxycyclin. Upon the cell models, we systematically examined cyto-characteristics, such as cell growth, apoptosis, senescence, and the ability of tumorigenesis in nude mice. To explore the proteins interated with Cath D, we immunoprecipitated the Cath D protein complexes by the antibody against Cath D, separated them by gradient SDS-PAGE, and identified them by MALDI-TOF/TOF MS. We then used CoIP and Confocal to confirm the interactions between Cath D and one of the interacted proteins, GRP78.

Results

As compared of the control cells without the treatment of Cath D expression, we recognized that the over-expression of Cath D could attenuate cell senescence, enhance cell growth and stimulate tumorigenesis. Contrarily, the knockdown of Cath D could induce cell senescence, slow cell growth and reduce tumorigenesis. When the antibody against Cath D was employed to immunoprecipitate the protein complexes of Cath D in T-RE 293 cells with high Cath D, GRP78 was found to be one with high affinity to Cath D. In the T-RE Hela cells that GRP78 was knockdown due to transfection of RNAi vector, pTER-GRP78, the cells exhibited significantly increased senescence, meanwhile the formation of Cath/GRP78D complex was gradually attenuated. Furthermore, we extended the study into p53 pathway involved in senescence and defined the roles of Cath D/GRP 78 in this signaling track. Our data has first clarified that involvement of Cath D in senescence was likely through formation of its complexes.

Innovative aspects

- Cath D can involve in cell senescence and interact with GRP78.
- The complex of Cath D/GRP78 may play a role in cell senescence.

References

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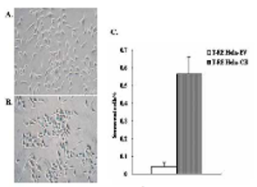

Figure 1. The knockdown of Cath D induced cell senescence. SA-β-gal activities were detected in T-RE Hela-EV (A) and T-RE Hela-CR (B) cells treated with 2ug/ml Dox for 24h. (C) The percentage of senescent cells in T-RE Hela-CR cells was statistically higher than that in T-RE Hela-EV cells.

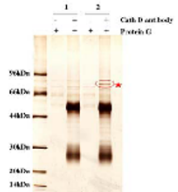

Figure 2. Immunoprecipitation of Cath D from T-RE 293-EV (1) and T-RE 293-CD (2) cell lysates. The immunoprecipitated proteins were separated by gradient SDS-PAGE from 8% to 15%. The significant differential band was labeled by a star sign and identified as GRP78.

C255

Capturing Protein-Protein Interactions of the MAPK pathway scaffold KSR

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Introduction

Kinase Suppressor of Ras (KSR) is a key component responsible for the integration of diverse growth and differentiation signals across eukaryotic cells. Typically, it is recruited to the membrane upon growth factor stimulation and scaffolds interactions between bRaf, cRaf, MEK and ERK. While its spatial and temporal regulation have been outlined in brief, much is unknown about how—and with whom—KSR acts in health and disease. We present results of *in vivo* cross-linking experiments aimed at temporally resolving protein-protein interactions involved in KSR scaffolding.

Methods

HEK293T and constitutive Braf melanoma cell lines were transfected with epitope tagged KSR, B-RAF and C-RAF. At different time points after stimulation with EGF, cross-linker was added, quenched, and cells harvested. A variety of cross-linking reagents (DSP, DSS, formaldehyde, glutaraldehyde) and conditions (short and long incubation, varying concentrations, different methods of inoculation) were explored and optimized based on Western blots and co-Immunoprecipiation (IP) experiments. Lysates were immunoprecipitated with anti-FLAG beads and rigorously washed. Epitope associated proteins were extracted with NH4OH, digested and analysed by LTQ-Orbitrap LC-MS/MS along with control samples.

Results

Nearly every KSR associated protein identified in a previous study (1) was recapitulated in a single *in vivo* cross-linking IP experiment as well as several novel interaction candidates. We expect to achieve fine grained resolution of the assembly and regulation of KSR scaffolding in both canonical (e.g., in HEK293T) and non-canonical Raf signaling pathways (e.g., in B-Raf mutants). While our current identification approach identifies peptides unmodified by cross-linker, we are also working to establish the identities of cross-linked peptides to achieve greater spatial resolution of protein-protein interactions.

Innovative aspects

- Explore a variety of cross-linking conditions to capture protein-protein interactions.
- Study dynamic protein-protein interactions in signal transduction.

References

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Impact of tumour-derived IL-6 on the liver proteome in cancer cachexia & toxicity to chemotherapy

C256

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Introduction
Tumour-derived cytokines (eg IL-6) can disrupt lipid & carbohydrate homeostasis in the liver resulting in metabolic dysregulation & ultimately cancer cachexia - the direct cause of death in ~30% of cancer patients. Another manifestation of elevated IL-6 is repression of hepatic drug clearance[1] which causes inter-patient variability in toxicity to chemotherapy, one the major challenges of pharmacogenomics[2]. MS-based techniques were used to profile the liver proteome of tumour-bearing mice – i) iTRAQ labelled peptides with multiplexed tandem MS/MS & ii) peptide IPG-IEF with spectral counting.

Methods
Colon 26 cells were implanted s.c. and features of cachexia were assessed over 14 days. The EHS sarcoma was explanted into the quadriceps muscle. IL-6 levels were determined by ELISA. Liver lysates from cachectic C26 mice were profiled using 8-plexed iTRAQ labels with subsequent analysis by tandem MS/MS. Membrane proteins from liver lysates of EHS tumour-bearing mice were isolated by sedimentation & the crude membrane pellet was digested with trypsin in 60% methanol[2]. Tryptic peptides were separated by IPG-IEF into 24 fractions & peptides were analysed using a linear ion-trap MS[2]. The EHS liver membrane preparation was also subjected to 4-plexed iTRAQ analysis. Expression of liver genes in EHS tumour mice was analysed using microarrays (Illumina Mouse-8 v1.1 Expression BeadChip).

Results
Colon26 tumour-bearing mice lost ~15% body mass with dramatic reduction in fat and muscle accompanied by progressive increases in plasma IL-6 to 130ng/ml. 8-plexed iTRAQ analysis of whole liver proteins from cachectic mice identified & quantified 1520 protein with 165 showing differential expression. 30% of down-regulated proteins were mitochondrial, indicating that cachectic livers may be in ATP deficit. Lipid accumulation and β-oxidation were also impaired. Such changes in hepatic metabolism would compromise energy balance and thereby contribute to cachexia. RT qPCR analysis of hepatic genes involved in metabolic pathways & their regulators, especially nuclear receptors, confirmed the proteomic results.
The combination of iTRAQ, Peptide IPG-IEF with label-free MS quantitation and mRNA microarray analyses of livers from EHS tumour-bearing mice provided comprehensive profiling of all components of drug clearance pathways with strong concordance between the 2 proteomic & genomic approaches. Multiple Phase I and II enzymes were quantified (49 CYPs; 14 GSTs; 12 UGTs) with many changes observed in enzymes relevant to metabolism of anti-cancer drugs. Extensive coverage of transport proteins (24 ABC & 9 Slco family members) showed alterations in MDR/P-gp, mrps and bcrp.

Innovative aspects
- Proteomic profiling of organs distal to tumours to characterise changes in liver metabolism which impact on survival and response/toxicity to treatment.
- Combined use of iTRAQ, peptide IPG-IEF & microarray profiling to study how chronic IL-6 release by non-hepatic tumours reprograms gene expression in the liver
- Extensive coverage of liver membrane proteome with ~1600 proteins identified and quantified.

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Multi-Hybrid Strategy for Novel Lung cancer Biomarker Discovery

C257

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Introduction
Lung cancer is one of the leading causes of cancer related death world wide. However, current diagnostic methods for lung cancer, such as X-rays, MRI or PET, are showing relatively low sensitivity and fail to early diagnose in many times. It has been reported that lung cancer patients' 5-year survival rate can be increased from 15% to 80% if diagnosed IA stage in overall non-small cell lung carcinoma. For this reason, Sensitive and early diagnostic markers for lung cancer will lead to successful diagnosis and therapy.

Methods
LC-MS/MS analysis was carried out using a Thermo Finnigan's ProteomeX workstation LTQ linear ion trap MS equipped with NSI sources, as previously reported . Briefly, 12 ul of peptide sample from the in-gel digestion was injected and loaded onto a peptide trap cartridge Trapped peptides were eluted onto a 10-cm reversed-phase (RP) PicoFrit column. The mobile phases consisted of H_2O (A) and ACN (B), and both contained 0.1% v/v formic acid. The flow rate was maintained at 200 nL/min. The gradient started at 2% B, reached 60% B in 50 min, 80% B in the next 5 min, and 100% A in the final 15 min. Data-dependent acquisition (m/z 400-1800) was enabled, and each survey MS scan was followed by five MS/MS scans within 30 sec, with the dynamic exclusion option on.

Results
Sample collection- Not only for biomarker discovery but also for further verification and validation, large number of sample cohort collection is on process. For serum or plasma collection, sample collection protocols were set up for collection time, amount, and clinical information of patients etc. At present, about 1000 samples are collected and those include 288 healthy controls, 255 lung cancer samples (adenocarcinoma, squamous cell carcinoma, small cell carcinoma) and 400 samples of respiratory disease or other types of solid tumors, such as tuberculosis, asthma, idiopathic pulmonary fibrosis, breast cancer, hepatocellular carcinoma.
Strategy for glycoprotein enrichment- (i) For the enrichment of total glycoproteins, multi-lectin affinity column was used. The elute, total glycoproteins of serum, was collected by centrifugation and analyzed. (ii) To enrich specific glycan attached proteins, albumin and IgG were removed from the samples (sera) using a ProteoPrep Immunoaffinity Albumin & IgG Depletion kit prior to the lectin extraction. Briefly, lectin extraction was performed using agarose-bound AAL and SNA and absorption buffer and elution buffers Fucosylated and Sialylated glycoproteins from healthy and lung cancer patients pooled sera were extracted, and those were dissolved in loading buffer and resolved by 1DE-SDS-PAGE. Proteins were excised from the gel and tryptic digestion prior to the LC MS/MS analysis.
Described biomarker discovery analysis is carried out for lung adenocarcinoma, squamous cell carcinoma and small cell carcinoma and results were compared each other.

Figure1. Strategy to identify lung cancer glycoprotein biomarkers. using lectin extraction system.

Innovative aspects
- Glycoprotein, which plays many biological important function, targeted biomarker discovery can be successful.
- Targeting overall histological types of lung cancer biomarker discovery will reveal histological specific or lung cancer common biomarkers

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Development of MRM assays for a large panel of low-abundance proteins in ovarian cancer ascites

C258

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Introduction
Biological fluids are considered an attractive source of cancer biomarkers due to their lower complexity and elevated levels of disease-relevant proteins. Quantitation of large panels of low-abundance proteins in biological fluids can be achieved with antibody-free multiple reaction monitoring (MRM) assays. Rapid development of large sets of MRM assays requires combination of discovery data and bioinformatic approaches to provide robust quantitative assays. Here we describe a workflow for discovery of low-abundance proteins in ovarian cancer ascites, translation of discovery data into sets of MRM assays, and quantitation of a large panel of low-abundance proteins, some of which may be potential ovarian cancer biomarkers.

Methods
Sample preparation at the discovery phase included tryptic digestion of ovarian cancer ascites followed by SCX fractionation of peptides. In addition, a one-bead-one-compound combinatorial peptide library was used prior to digestion and SCX to facilitate the discovery of low-abundance proteins. Each SCX fraction was later analyzed with LS-MS/MS (Orbitrap) and searched with Mascot and GPM software. Discovery data, such as peptide MS/MS fragmentation patterns, was translated into MRM assays using Pinpoint software (Thermo Fisher Scientific). MRM assays were further optimized and run on a triple-quadrupole mass spectrometer (TSQ Quantum). High-resolution MRM mode (resolution up to 5000) was used to provide enhanced selectivity of analysis in the presence of complex matrix.

Results
Discovery phase resulted in a list of 484 proteins identified with 1% false-positive rate. Filtering criteria (1) were applied to reduce the list to 134 secreted and membrane low-abundance proteins that were later considered for MRM assay development. Forty-three peptides were pre-screened with MRM assays, and successful MRM assays were developed for 20 low-abundance proteins. To confirm the utility of MRM assays for biomarker quantitation, we measured kallikrein 6 in the range of 29 – 1912 ng/mL in ovarian cancer ascites from several patients. MRM results were in good correlation with ELISA data. Some low-abundance proteins discovered in the present work will be further assessed as potential ovarian cancer biomarkers.

Innovative aspects
- Rapid development of MRM assays for a large panel of unpurified proteins
- MRM quantitation of low-abundance proteins in ascites without extensive sample preparation

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Involvement of BAG2 and its complexes in cell senescence

C259

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Introduction
Suppression of c-Myc oncogene was reported to prompt cell senescence leading to carcinogenetic arrest in many tumors. Preliminary observations suggested that overexpression of BCL2-associated athanogene 2 (BAG2), either at mRNA and at protein level, happened along with attenuation of the c-Myc protein abundance. Is BAG2 involved in the senescent process? We initiated the project trying to address whether BAG2 induces cell senescence or which role of BAG2 plays in the process.

Methods
An inducible stable TRE293 cell line with BAG2 overexpression was generated by transfection of pCDNA4/TO-BAG2. The cell behaviors were assessed through growth curve depiction, BrdU incorporation, senescence-associated acidic β-galactosidase (SA-β-Gal) staining and Western blot. The carcinogenicity associated with BAG2 expression was evaluated by colony formation at cellular level or tumorigenicity detection in nude mice. To explore the proteins interacted with BAG2, the lysates of TRE293 were incubated with or without the recombinant BAG2 protein. The protein complexes involved in interaction of BAG2 were isolated by affinity chromatography followed by protein separation by gradient SDS-PAGE and identification by MALDI TOF/TOF MS. Several interesting interactive components were further confirmed by Western blot.

Results
The TRE293 cells with stable overexpression of BAG2 were successfully established. The growth curve depiction and BrdU incorporation assessment revealed that BAG2 overexpression caused a significantly decreased cell growth. With assay of SA-β-Gal staining, the cells with BAG2 overexpression exhibited obvious senescent signals (Figure 1) and up-regulated p21CIP1, a classically senescent marker. When the BAG2 cells were transplanted onto nude mice, the animal carcinogenicity was attenuated significantly. All the results indicated that BAG2 was closely correlated with senescence, at least for TRE293 cells. The proteomic analysis of the BAG2 complexes revealed that a total of 16 differential proteins were identified by MALDI TOF/TOF MS (Figure 2). Of these proteins, 9 were reported as the p53-associated ones, even though no p53 protein was detected by mass spectrometry. Thus we adopted another approach to examine the presence of p53 in the complexes. Using antibody against p53 in Western blot, it was indeed found in the complexes generated from the BAG2 pull-down experiments. We come to the conclusion that BAG2 interacts with several proteins to form a stable complex in TRE293, in which the p53 related may be the core elements, possibly involved in senescent process.

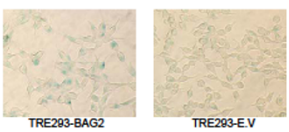

Figure 1. The overexpression of BAG2 could induce senescence of TRE293 cells

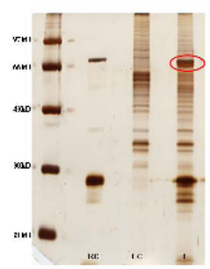

Figure 2. Separate protein complexes involved in interaction of BAG2 by gradient SDS-PAGE. The ones red-marked are some of differential bands denoting interacting proteins with BAG2. T: test sample with recombinant BAG2 and TRE293 cell lysates; RC: control sample with recombinant BAG2 only; LC: control sample with cell lysates only.

Innovative aspects
BAG2 can interact with p53 through p53/p21CIP pathway, which possibly causes cell senescence.

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C260

The cysteine modification of human AKR1C proteins and its biological significance

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Introduction

Aldo-keto reductases (AKRs) are highly conserved enzymes comprising more than 150 members. AKR1C is the biggest AKR subfamily which shares more than 60% identity and the similar three dimensional structures with other AKR members. (1)Recently a member of AKR1C in mouse, AKR1C12, was found to have a reactive cysteine residue, Cys87, which was easily modified under oxidative stress leading to decreased catalysis. According to multiple alignment and comparative protein modelling, 15 members in all 25 AKR1C subfamily proteins possess the cysteine residue at the position, and 6 others have serine substitution. In human tissues, four AKR1C members are identified, AKR1C1, AKR1C2, AKR1C3 and AKR1C4, in which AKR1C1 and AKR1C4 contain the conserved cysteine residue. Compared of AKR1B enzymes (2), there is no report yet that the human AKR1C proteins are sensitive to oxidative stress. Since Cys87 in the AKR1C12 is accessible for oxidant molecules, we inquire whether the enzyme activity of some human AKR1C proteins is modulated due to their cysteine modification under oxidative environment.

Methods

The human AKR1C proteins were generated through pET construction and purification system. The four AKR1C recombinant proteins were incubated with iodoacetic acid (IAA) followed by measurement of their enzyme activity. To identify the modification site(s), the AKR1C proteins were first incubated with 20mM IAA to react with the accessible cysteine residue(s), in turn treated with 8M urea and added 20mM N-ethylmaleimide (NEM) to modify the rest cysteine residues. The modified cysteine sites were identified by mass spectrometry. After that, the mutants containing cysteine to serine substitution at positions 87 (AKR1C1-C87S and AKR1C4-C87S) were generated and the enzyme activities for wild and mutant forms were estimated with/without IAA.

Results

The enzyme activities of AKR1C1 and AKR1C4 were significantly changed (P<0.05) after modified by IAA, in which AKR1C1 lost more than 85% activity and AKR1C4 remained about 30% activity, whereas the IAA treatment did cause insignificant changes in AKR1C2 and AKR1C3(Fig 1). With chemical modification followed by peptide identification by mass spectrometry, two peptides, lwcnshrpelvrpaler for AKR1C1 and lwctffqpqmvqpalesslk for AKR1C4, were identified to carry out the typical mass shift of 31da which was distinguished from IAA dnd NEM modification to cysteine residue. The modified cysteines in the two peptides were just Cys87 in AKR1C1 and AKR1C4, respectively. The mutant of AKR1C1C87S and AKR1C4C87S were further examined their sensitivity to alkylation reagent. The data obtained from enzyme activity demonstrated that both mutants were insensitive to IAA treatment. Taking all data above, we come to the conclusion that Cys87 residue of AKR1C1 and C4 is critical important to the enzyme catalysis, possibly playing a role in regulation of substrate binding or product releasing. On contrary, the AKR1C2 and AKR1C3 may have different regulatory mechanisms especially for substrate binding due to the absence of Cys87 in the two proteins.

Innovative aspects

- For the first time, we have provided the convincing evidence to confirm the hypothesis that cysteine residue in AKR1C family is modified under oxidative stress, which leads to loss of enzyme activity.

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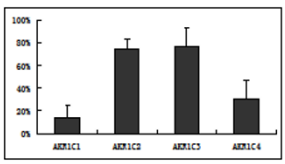

Figure 1. Relative activity of AKRs after cysteine modification treated with IAA. Suppose the enzyme activity before modification is 100%. N=3. This shows that the modification resulted in loss of enzyme activity in AKR1C1 and AKR1C4, while did not seriously affect the enzyme activity of AKR1C2 and AKR1C3.

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Quantitative Proteomic Analysis of Metastasis-Related Proteins in Lung AdC

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Introduction

In recent years, the incidence rate of human primary lung adenocarcinoma (AdC) has clearly been on the increase. And the patients of lung AdC have very low survival rate and very poor prognosis. Cancer cell metastasis is the major cause of high mortality and poor prognosis in lung AdC. A variety of positive and negative factors may be involved in this process, which play important role in promoting or inhibiting cancer cell metastasis. Therefore, directly screening metastasis-associated proteins is to provide the foundation for finding predictive metastatic biomarkers.

Methods

To screen metastasis-associated biomarkers of lung AdC, laser capture microdissection (LCM) (1) was used to purify the cancer cells from human primary lung AdC with (LNM AdC) and without lymph node metastasis (non-LNM AdC) according to clinical diagnosis of lymph node metastasis and distant metastasis. Then two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed to isolate the total proteins of the pooled microdissected cancer cells from non-LNM AdC and LNM AdC. The differential proteins between non-LNM AdC and LNM AdC were analyzed by Decyder software and further identified by mass spectrometry (MS). The partial differential proteins including were validated by Western blot and immunohistochemistry.

Results

In our study, 2D-DIGE patterns of microdissected non-LNM AdC and LNM AdC were established, and 20 differential proteins in the above two tissues were identified, 13 out of which were up-regulated and 7 were down-regulated in LNM AdC compared to non-LNM AdC. Western blot results indicated that S100A9, annexin A1, annexin A2 and annexin A3 were significantly up-regulated in LNM AdC compared to non-LNM AdC; B23 was significantly down-regulated in LNM AdC compared with non-LNM AdC. Immunohistochemical analysis further indicated S100A9, annexin A1, annexin A2 and annexin A3 were up-regulated in LNM AdC compared with non-LNM AdC; B23 was down-regulated in LNM AdC compared with non-LNM AdC. Furthermore, annexin A1, annexin A2 and annexin A3 were up-regulated in positive lymph nodes compared with primary lung adenocarcinoma. Statistical analysis indicated the increase of the three annexins expression levels in these tumors was significantly associated with lymph node metastasis and advanced clinical stage.

Innovative aspects

- The data will facilitate understanding of human lung AdC metastasis and provide some direct proof for mining markers for predicting metastasis and patients' outcome so as to improve the diagnosis and treatment of lung AdC.

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C262

Secretome based discovery of pancreatic cancer biomarkers

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Introduction

Pancreatic cancer has very low 5-year survival rate, partially because lack of early detection and diagnosis. Some markers, like CA19-9 and CA 242, are widely used in the clinic, but none of them with enough sensitivity and specificity. The secreted proteins from cancer cells or organs (cancer "secretome") have been recognized containing biomarkers related to carcinogenesis (1). Thus, identifying additional secreted biomarkers that may improve early detection of pancreatic cancer is greatly needed.

Methods

We analyzed secretome of pancreatic cancer from culture medium (CM) using comprehensive proteomic strategy. Pancreatic ductal adenocarcinoma (PDAC) and their matched adjacent normal pancreas tissues were harvested. After primary organ culture, the secretome from culture medium was analyzed by both top-down (SDS-PAGE-HDMS) and bottom-up (Shotgun-LC-MS/MS) strategies. The generated peak lists were searched the RefSeq database (20071019) and Mascot servers.

Results

Under stringent criteria, finally total 247 and 735 proteins from normal pancreas and PDAC secretome were identified, and 570 high-quality pancreatic cancer specific secreted proteins (PCSPs) were defined. Bioinformatic analysis revealed that nearly 40% of the identified proteins were found extracellular localization and 60% of them had signaling peptides. Meanwhile, overlap was found between PCSPs and the known cancerous secretome databases (32%), and PDAC pancreatic juice proteome (21%) as well. Furthermore, 81 PCSPs have been reported to be relevant with pancreatic cancer, and some proteins like cathespin B, cathespin D, PGK1, were even proved to be elevated in the sera of pancreatic cancer patients using ELISA assay. In general, the newly established massive pancreatic cancer secretome may encompass useful candidate biomarkers and meantime further insights into the mechanisms of carcinogenesis of pancreatic cancer.

Innovative aspects

- Culture medium (CM) of pancreatic cancer tissues were analyzed by two bottom-up proteomic strategies
- 570 high-quality pancreatic cancer specific secreted proteins (PCSPs) were defined
- 81 PCSPs have been reported to be relevant with pancreatic cancer

References

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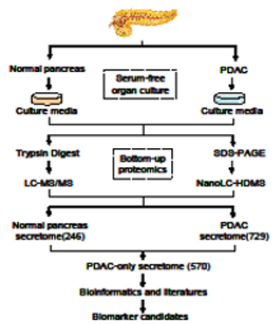

Figure 1. Workflow of the proteomic analysis.

C263

The Loss of Phenol Sulfotransferase 1 in Hepatocellular Carcinogenesis

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The discovery of biomarkers for detecting early hepatocellular carcinoma (HCC) is urgently needed. To identify biomarkers of HCC, we performed a comparative proteomics analysis, based on 2-DE of the HCC tissues and the surrounding non-tumor tissues. Interestingly, seven xenobiotics enzymes were remarkably down-regulated in HCC. Among theses, phenol sulfotransferase (SULT1A1) was validated by Western blot analysis in 105 HCC patients. SULT1A1 showed a significant decrease in 98.1% of the HCC tissues and it displayed 88.6% sensitivity and 66.7% specificity for detecting HCC. We further performed immunohistochemistry for SULT1A1 and compared it with that of glypican-3, which is a well-known marker of HCC. The down-regulation of SULT1A1 and the up-regulation of glypican-3 were observed in 63.8% and 87.2% of the HCCs and their combination significantly improved the sensitivity up to 95.7%. Moreover, SULT1A1 was useful to distinguish early HCC from benign dysplastic nodules. Clinically, the down-regulation of SULT1A1 was closely associated with an advanced UICC stage and a high level of serum alpha-fetoprotein. In conclusion, our studies demonstrate that the loss of SULT1A1 appears to be a characteristic molecular signature in hepatocellular carcinogenesis. SULT1A1 could be a useful biomarker for detecting early HCC and to predict the clinical outcome of HCC.

C264

Screening of methylation silenced genes in leukemia by Quantitative proteomics

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Introduction
DNA methylation is one of the most common epigenetic mechanisms on the regulation of gene expression, and the abnormal methylation genes not only play an important role in the pathogenesis of leukemia, but also provide a specific target for the treatment of leukemia. Therefore, screening for new methylation silenced genes in leukemia can help to reveal its pathogenesis, and provide the important theoretical and scientific basis for the prevention and cure of leukemia.

Methods
In the first step ,we used two-dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) to discover up-regulated proteins in leukemia cell line HL-60 following the treatment of 5-aza-CdR.Western blot was used to validate the results of 2D-DIGE, and Methylation specific PCR (MSP) was adopted to detect the methylation levels of gene promoters. Reverse transcription polymerase chain reaction (RT-PCR) explore whether the gene promoter methylation aused the downregulation of mRNA .In the second step,four leukemia cell lines (HL-60,Jurkat, U937, Raji),235 cases of leukemia bone marrow cells and 45 cases of control marrow cells was used for further research of the gene promoters methylation levels by MSP,RT-PCR and Western blot.

Results
We analyzed the molecular response of HL-60 cells to 5-aza-CdR treatment by using 2D-DIGE studies. Proteomic study evidenced that 53 proteins were differentiated expressed (of >1.5-fold), 47 proteins were up-regulated, 6 proteins were down-regulated after the 5-aza-2dC treatment. Forty non-redundant differential proteins were identified by MALDI-TOF-MS and ESI-Q-TOF-MS, of which 34 proteins were up-regulated and 6 proteins down-regulated. Western blot validated partial proteins: Galectin-1, HSP47, S100A8, S100A9 (S100A8,S100A9 was also up-regulated after 5-aza-2dC treatment in microarray analysis study in our team).RT-PCR confirmed that the mRNA of Galectin-1, HSP47,S100A8,S100A9 were down-regulated after the 5-aza-2dC treatment in HL-60 cell. MSP proved that promoters of Galectin-1, HSP47 genes are high frequently methylated in HL-60 cell. Additionally, we also demonstrated promoters of Galectin-1, HSP47 genes are high frequently methylated in leukemia cell lines and in clinical leukemia bone marrow cells samples by MSP, RT-PCR and Western blot analysis. In conclusion, we demonstrate that an integrated proteomic and Epigenetic approach not only can be effectively utilized to elucidate the changes in post-transcript and transcript levels following a treatment of leukemia cells with 5-aza-CdR, but also can provide a comprehensive idea to screen methylation silenced genes in leukemia cells.

Innovative aspects
- An integrated Quantitative proteomic and epigenetic approach for Screening methylation silenced genes
- Novel confident 2D-DIGE strategy for Differential protein identification
- Systematically studied different level of leukemia for confident result

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C265

Proteomics Identification of Proteins Involved in Colorectal Cancer Metastasis

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Introduction
Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer deaths in developed countries. The high mortality of CRC is largely due to extensive metastatic spread of the disease, with secondary hepatic tumours being the most common occurrence. Acquisition of metastatic phenotype by cancer cells thus represents one of the most lethal aspects of cancer progression. Patient survival in CRC will greatly benefit from earlier recognition (and intervention) of metastasis but as yet, proper markers indicating metastases are not available.

Methods
E1 is a highly metastatic cell line that had been derived from hepatic metastases following splenic injection of HCT116 primary colon cancer cell line into nude mice. To identify proteins associated with CRC metastasis, we carried out comparative proteomics of HCT116 and E1 cells. Whole cell lysates and fractions obtained from an enrichment step of heparin affinity chromatography were analyzed by two-dimensional difference gel electrophoresis (2-D DIGE) coupled with tandem mass spectrometry. Subsequently, western blots were performed to validate up-regulation of stathmin in E1. Cell viability and migration capability in stathmin knockdown E1 cells were determined using MTT and transwell assays respectively.

Results
In total, 143 protein spots were detected to be differentially expressed between the 2 cell lines and they corresponded to isoforms of 86 different proteins as identified by MS analyses. A majority of these proteins are implicated in cell growth, survival, motility, invasiveness and angiogenesis, which are indispensable steps in the metastatic cascade. They may represent candidate biomarkers for colorectal cancer metastasis as well as potential therapeutic targets for treatment of metastatic colorectal cancer.
We proceeded to functionally validate one of the proteins, stathmin, which was the most up-regulated protein in the E1 cell line as compared to its parental HCT116 cell line, with the view to establish its precise role in the metastasis cascade. RNA interference was carried out to investigate the functional effects of stathmin knockdown in E1. Our results showed that knockdown of stathmin in E1 did not affect cell viability but decreased cell migration significantly. We also showed that stathmin expression levels correlate strongly with increasing metastatic potential in other paradigms/models of colorectal metastasis.

Innovative aspects
- Generation of metastatic E1 from HCT116 using an *in vivo* liver colonisation model
- Proteomics identification of biomarkers for colorectal cancer metastasis
- Functional characterization of stathmin's role in colorectal cancer metastasis

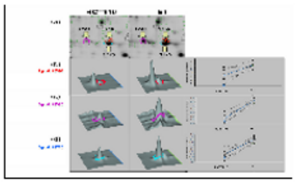

Figure 1. Stathmin was found in 3 protein spots from the 2-D DIGE analysis of heparin-bound fraction which were shown to be up-regulated by +1.83 to +3.03 fold in E1 as compared to HCT116.

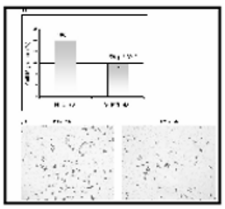

Figure 2. Cell migration assay showed that stathmin knockdown resulted in significant reduction in E1 cells migration. (a) Cells migration was found to decrease by 41% in stathmin knockdown E1 cells. (b) Representative images of migrated E1 cells in the transwell assay. Significantly lesser cells were migrated in STMN-KD cells.

C266

Clinical Proteomics of Human Saliva on Oral Squamous Cell Carcinoma

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Abstract:
Oral cancer is one of the most prevalent cancers in Asia and specially India where it share the maximum cancer related morbidity and mortality. The sole reason for this high mortality is late diagnosis, mainly due to subtle presentation in form of ulcer or plaque but also due to lack of expertise of pathologist and areas of grey between the precancerous and cancerous lesions. We found in our study, some expressible markers in saliva which are largely found in tissues. Our saliva based clinical proteomics approach may prove fruitful in providing a patient compliant and physician friendly method for detection of the disease at an earlier stage without much of expertise and hence, changing over all prognosis of the disease.

Introduction
The use of saliva for oral cancer screening is still in infancy, but it does look very promising. The use of saliva for the detection of oral cancer yet to come to fruition [1]. Overall incidence and mortality attributed to oral Squamous cell carcinoma (OSCC) is increasing, with current estimates of age-standardized incidence and mortality of 6.6/100,000 and 3.1/100,000 in men and 2.9/100,000 and 1.4/100,000 in women, respectively [2]. The most common type of carcinoma in India is Oro-pharyngeal carcinoma and it also has the sixth place in US. Recent studies confirm that oral cancer forms a large part of the cancer load in parts of India.[3]. Tobacco and alcohol are the two most important known risk factors for the development of oral cancer. With current approach based on proteomics like sub-cellular fractions from OSCC proteins like statherin, cofilin, C-reactive protein precursor, creatine kinase m-chain, fatty acid-binding protein, keratin type II, myosin light chain 2 and 3, nucleoside diphosphate kinase A, phosphoglycerate mutase 1, plakoglobulin, and retinoic acid-binding protein II were found to be differentially expressed. Our study for the first time reports the over expression of CKAP -2 in saliva of OSCC patients.

Methods
Sample Collection
5 ml of saliva was collected from normal subject not consuming tobacco in any form and patients of oro-pharyngeal carcinoma as diagnosed by clinico-histo- pathology but not having clinically documented disease of oral mucosa, diabetes, autoimmune diseases or other cancers from Head and Neck Cancer clinic, Department of Surgical oncology, Dr. B.R.A. Institute of Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi-110 029. over a period of two hours from control and case and was collected 15 minutes after light breakfast, followed by proper mouth washing by spitting into the vial in septic conditions and presence of proteolytic inhibitors and stored in – 70 deg. C till use.

2 -Dimensional Gel Electrophoresis
Samples were centrifuged at 20,000 ×g for 30 minute and the supernatant was used for precipitation. The sample was precipitated by acetone and dissolved in lysis buffer, followed by rehydration of the IPG strips (24 cm) with 1000 µg of protein sample as described previously. The rehydrated strip was equilibrated with buffer and iso-electric focusing was done on IPGphor ™ iso - electric focusing system (Amersham Biosciences) for 100,000V hours. Gels were stained with colloidal Coomassie blue (G-250). Computer analysis of 2D-PAGE images is performed using MELANIE VIEWER software.The spots on gel were digested as described before and LC-ESI-qTOF MS/MS analysis was carried out.

Results
When compared to the coomasie (G-250) stained gel of normal human whole saliva with that of Squamous cell carcinoma of buccal mucosa and was matched for the differential spots. When both the gels were compared using MELANIE VIEWER, three differentially expressed proteins were found to be present in the case of Squamous cell carcinoma, viz., Cytoskeletal associated protein -2 (CKAP – 2), Cytokeratin I, C-14 and a hypothetical protein.

Innovative aspects
- Use of saliva for the diagnosis of oral cancer will not only fruitful for the patients as it will increase compliance but also to the physicians for providing them bedside diagnosis of the disease.

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C267

The overexpression of Annexin A2 in the survival cells of CNE1 irradiated with the sublethal dose

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Introduction
Nasopharyngeal carcinoma (NPC) is one of the most common head neck malignant tumors in southern China.The radiotherapy recurrence is a major obstacle preventing effective treatment of NPC.The biology characteristic of the irradiated survival cells is relatived with the radiotherapy recurrence of NPC.Despite extensive studies,the molecular mechanisms involving in NPC cell reaction to ionizing radiation have yet to be fully understood.Greater understanding of the molecular mechanisms to ionizing radiation is likely to provide more promising treatment for NPC patients.Here the survival cells treated with the sublethal dose were analyzed for their changed proteins by two-Dimensional gel Electrophoresis and MALDI-TOF-MS.

Methods
The sublethal dose of NPC cell line CNE1 was measured by MTT assay for 6MV-X-ray,and CNE1 cells were exposed to the sublethal dose.The survival cells and the parent CNE1 cells were tested for the cell stage distribution by the flow cytometry,and their changed proteins were analyzed by two-Dimensional gel Electrophoresis and MALDI-TOF-MS.

Results
The sublethal dose of CNE1 was 15Gy by MTT assay.The survival cells were attained after CNE1 cells irradiated with the dose of 15Gy.These survival cells were cultured and collected.The survival cells compared with the parent CNE1:a higher G1/G0 and lower G2/M stage,48 different protein spots(36 spots were overexpression and 12 spots underexpression) were found in the 2-DE maps of the survival cells,and identified by MALDI-TOF-MS.This test found that the irradiated survival cells had changed their biological characteristics and the expressed proteins.There was higher Annexin A2 expressed in the survival cells than in the control parent CNE1 cells.Some studies found that overexpression of Annexin A2 can predict rapid recurrence[1].Annexin A2 is a growth-regulated gene,whose expression is significantly increased in various human cancers[2].Annexin A2 is believed to play an important role in the oxidative stress-induced cancers and also functions as a prosurvival factor[3].In our test,overexpression of the Annexin A2 may relatived with the survival of the irradiated CNE1 cells and the cell stage distribution.Annexin A2 maybe take a important role in the survival of the irradiated cells.

Innovative aspects
- These survival cells were analyzed by 2-DE and MALDI-TOF-MS after CNE1 cells treated with the sublethal dose .
- Annexin A2 is a growth-regulated gene,and first found overexpression in the survival cells of NPC cell line CNE1.
- The cell stage redistribution may relatived with the survival of the CNE1 cells .

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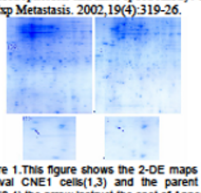

Figure 1.This figure shows the 2-DE maps for the survival CNE1 cells(1,3) and the parent CNE1 cells(2,4),the arrow instruct the spot of Annexin A2

Differential proteome analysis to detect APP as a secreted protein regulated by EGFR in NPC

C268

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Introduction Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in Southern China and Southeast Asia. Secreted proteins play an important role in cell signal conduction and are neoessary for tumor growth, invasion and metastasis. Epidermal growth factor receptor (EGFR) is usually overexpressed in NPC. The study will further reveal the signaling pathway involved in activation of APP expression regulated by EGFR in CNE2 and the potential significance of EGFR and APP in the occurrence of NPC.

Methods CNE2 was cultured in the media with the absence and presence of TGF-α respectively. The secreted proteins in the media were collected and further separated by two-dimensional gel electrophoresis (2-DE), and the differential proteins were identified with MALDI-TOF Mass Spectrometer. The results indicated that Amyloid beta A4 protein (APP) was upregulated by EGFR. In order to further clarify the regulation of APP activity involved in the precise signaling pathway, the EGFR tyrosine kinase selective small molecular inhibitors (PD153035), MAPK inhibitor (PD98059), PI3K inhibitor (wortmannin) were respectively treated with the cells. Western blotting was performed to analyze the changes of APP expression levels.

Results The 2-DE results showed that EGFR could upregulate the expression of APP. And the upregulation of APP could be hampered by special inhibitors as PD153035, wortmannin and PD98059. And the wortmannin inhibit APP expression more significantly than PD98059. Furthermore, PD153035 could inhibit APP expression in a concentration-dependent manner.

Innovative aspects

- The data provides useful information for the study of NPC secretome and new clue for revealing the mechanism of NPC regulated by EGFR
- In the NPC occurrence and development, EGFR regulates APP expression possibly mainly through the PI3 kinase pathway to display their functions.

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Establishing an "Index of Individuality" for the Chicken Plasma Proteome Using Label-free Proteomics

C269

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Introduction
The index of individuality is an important clinical metric for assessing the appropriateness of applying normal ranges to biochemical constituents (e.g., biomarkers)[1] In an effort to explore the utility of this metric in mass spectrometry-based biomarker discovery, label-free proteomics was used to globally quantify proteins in longitudinally-procured plasma samples from 242 chickens over a one-year period. The chicken has emerged as an important animal model of epithelial ovarian cancer and serves herein as a means to establish a "global proteome index of individuality" wherein candidate biomarkers can be assessed relative to a "normal range" of spectral counts.

Methods
Plasma was collected from 242 age-matched laying hens starting at 2.5 years of age (October 2007) for 1 year at 3 month intervals (5 time-points total). At the conclusion of the 1 year sample procurement period, the remaining birds were bled, euthanized, and necropsied to retain "normal" and "cancerous" tissues. Tissue sections were preserved by snap freezing in LN_2, RNAlater, and formalin for proteomic, genomic, and pathological characterization respectively. Each set of 5 longitudinal plasma samples per bird were fractionated together on separate lanes of a 1D gel, gel bands were excised, and then subjected to in-gel tryptic digestion. Tryptic peptides were analyzed using an Eksigent 2D-Ultra nLC coupled to a Thermo LTQ-Orbitrap mass spectrometer. Protein identification and quantification was accomplished using Mascot (Matrix Science) and ProteoIQ (Bioinquire) software packages, respectively.

Results
A total of 106 birds from the original 242 birds remained at the conclusion of the 1 year sample procurement (October 2008). Preliminary evaluation of the necropsy data showed that 39% of the birds showed neoplasms on the either the ovaries and/or neighboring organ tissues (e.g., oviduct, intestine, and liver) (Figure 1). The origin of the tumors is currently under investigation although EOC is suspected in many cases based on past experience with this animal model and the extent of tumor growth on the ovaries. Importantly, 7 birds (17% of neoplasms) had tumors localized solely on the ovary suggesting "Early Stage EOC". A group of longitudinal plasma samples from "healthy" (no neoplasms), cancer controls (no neoplasms on the ovary), and early and late stage EOC were selected for initial analysis. The sample preparation methods, nLC-MS/MS data, within- and between-gel analytical variation, and intra- and inter-chicken label-free results from this dataset will be presented.

Innovative aspects

- Label-free proteomics study of longitudinal plasma samples from an animal model of epithelial ovarian cancer
- Established a global proteome index of individuality to assess the value of spectral count variation (i.e., candidate biomarkers) relative to normal biological and analytical variation.

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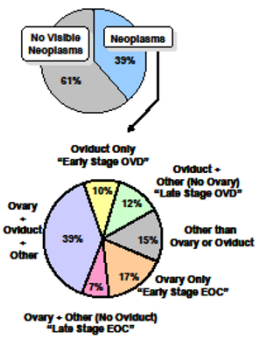

Figure 1. Gross pathology of the 106 necropsied birds (Oct. '08)

Annexin A2, a Novel Serological and Histological Candidate for Hepatocellular carcinoma

C270

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Introduction
Hepatocellular carcinoma (HCC) is a common tumor worldwide. Alpha-fetoprotein (AFP) is the only available HCC serological biomarker and has limited effect at early stages. Previous work has proven that AFP-producing and -nonproducing HCC cells have different gene/protein expression profiles (1). To identify potential markers of HCC, especially for AFP negative cases, as well as understand tumorigenesis mechanisms of HCC cases with differential AFP expression, a subcellular proteomic analysis of multiple liver cancer cell lines corresponding to different AFP expression were carried out.

Methods
Cytosol, Membrane/Organelle and nuclear subcellular fraction proteins from multiple liver cancer (HepG2, Hep3B and SK-HEP-1) and normal liver cells (HL-7702) were separated and identified using a 2-DE/MALDI-TOF-TOF approach. Annexin A2 was selected and verified in multiple liver cancer cells and 126 HCC specimens using Western blot and immunohistochemical staining. Meanwhile, its expression level in serum was evaluated in 140 sera samples using established indirect sandwich ELISA assay. The diagnostic performance of AFP and Annexin A2 was evaluated by ROC curve.

Results
In total, 50 differentially-expressed proteins corresponding to AFP characteristics were identified from different fractions including the cytosol, membrane/organelles and nuclear proteins of live cells. Among them, the tumor-related protein Annexin A2, was verified to be significantly upregulated in the cytosol, membrane/organelle and nuclear fractions of multiple AFP negative liver cancer cells. In addition, a large-scale clinical specimen validation showed that Annexin A2 expression was elevated in the HCC tissues (63.5%, 80/126) especially in AFP-normal cases. Serum levels of Annexin A2 measured by an established ELISA assay were found to be significantly elevated in HCC patients compared with healthy individuals. The serum concentration was also correlated with histological grade. Combined use of AFP and Annexin A2 could increase the diagnostic performance of HCC. A constructed interaction network also revealed a potential role of Annexin A2 in hepatocarcinogenesis in AFP-deficient HCC. These data suggest that Annexin A2 may serve as a novel serological and histological candidate involved in HCC, especially among individuals with normal levels of serum AFP.

Innovative aspects

1. Annexin A2 was verified to be expressed in the cytosol, membrane/organelle and nuclear fraction of multiple AFP-negative liver cancer cells.
2. Annexin A2 was significantly up-regulated in HCC cases (63.5%, 80/126), especially in AFP-deficient clinical tissue specimens.
3. Serum levels of Annexin A2 were significantly elevated in serum of HCC patients compared with healthy individuals. The diagnostic accuracy of AFP and Annexin A2 was comparable.

References

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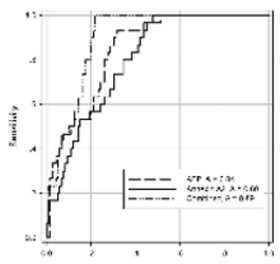

Figure 1. Receiver-operating characteristic (ROC) curves of AFP and Annexin A2. Serum concentration of AFP and Annexin A2 of 119 HCC samples and 30 healthy control samples was determined by a chemiluminescence immunoassay and a sandwich ELISA, respectively. ROC curves were derived by plotting the relationship between specificity and sensitivity at various cutoff levels.

High-throughput Study of the Effects of Rapamycin Treatment on Acute Myeloid Leukaemia Cells

C271

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Introduction
In this study we have investigated the possibility of using rapamycin (rapa) as a therapy for acute myeloid leukaemia (AML). The regulatory effects of rapa and the perturbations caused by rapa treatment on the cell as a whole are still vague. All the previous studies pertaining to the anti-proliferative effects of rapa employed the traditional approach of investigating individual pathways such as mTOR and PI3K/Akt (1). In this context, we aim to study the effect of rapa on AML at the global level, by mapping all the transcriptomic and proteomic modulations caused by the drug.

Methods
In-vitro cytotoxicity assays were conducted on 5 AML cell lines to gauge their responsiveness to rapa. Two of these cell lines- MV4-11 and THP-1 were chosen for further studies. The IC_{50} dosage for MV4-11 was 50nM and for THP-1 was 500nM. The cells were treated with their respective IC_{50} dosages of rapa and whole scale genomic and proteomic studies were conducted using Affymetrix microarray and iTRAQ™ platforms respectively. The data was analysed with the Ingenuity pathway analysis software and functional evaluation of the pathways identified was performed using FACS analysis of cell cycle and western blots.

Results
Using MTS assays, it was found that among all the AML cell lines tested, MV4-11 was the most sensitive to rapa treatment with an IC_{50} of 50nM while THP-1 was the least sensitive with an IC_{50} of 500nM. These two cell lines were hence chosen for all further studies. To map the transcriptomic and proteomic alterations induced by rapa the Affymetrix microarray and iTRAQ™ techniques were used respectively. The data provided us with lists of genes and proteins regulated by rapa in AML. Pathway analysis of these datasets showed that Cell cycle: G1/S checkpoint regulation, IGF-1 signalling, PI3K/Akt and Insulin receptor signaling were the top pathways regulated by rapa. We found that rapa caused down-regulation of IGFBP2, an important member of the IGF-1 pathway. Cell cycle analysis using FACS showed that rapa induced a time dependent G1 arrest in both the cell lines. Western blot analysis of the major cell cycle regulatory proteins indicated that rapa acted by down-regulating SKP2, which leads to accumulation of p27 and hence repression of Cdk2 and Cdk4, thus leading to G1 arrest.

Innovative aspects

- Creative strategy employed: Comprehensive study of the regulatory effects of rapa using a systems biology approach combining transcriptomic and proteomic strategies
- Identification of novel pathways regulated by rapa such as cell cycle: G1 arrest and IGF-1 signalling
- Providing a functionally validated mechanism for the G1 arrest induced by rapa in AML

References

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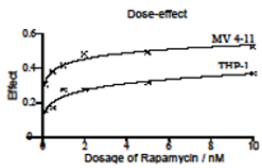
Figure 1: Dose-response curves of cell lines after 48 hours treatment with Rapa.



The inhibitory effect of rapa was studied by growing the cells with increasing dosages of the drug. The most and the least Rapa-sensitive cell lines are shown.

C272

Role of PCNA interactions in cancer: proteomics view

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Introduction. Cancer is an example of systems biology disease where many pathways are involved. Cancer researchers have been focusing their studies on the identification of molecular differences between cancer and healthy cells, often using a proteomics approach. Cancer proteome usually deals with those proteins that are differentially expressed or mutated in cancer cells. However, studying the mode/role of these "cancer proteins" is very challenging. PCNA is a cellular proliferation marker and its role in DNA replication as an essential accessory protein is well known. Additionally, we found previously that PCNA is highly expressed in cancer cells, and interacts with many different proteins.

Methods. A combination of Far-Western blotting, high resolution two-dimensional gel electrophoresis (2DE), and mass spectrometry was applied to analyze proteins isolated from normal and cancer cells.

Results. We have detected many PCNA-interacting proteins. Some of them are involved in energy producing cascade, upon which the cancer cells are heavily dependent.

Innovative aspects. A surprising role of PCNA in glycolysis has emerged from our current study. We think that PCNA may activate or inactivate certain cellular functions by simultaneously binding different protein partners that are involved in many different cellular functions including glycolysis. A certain aspect of this PCNA function may be important for cancer cell survival and proliferation.

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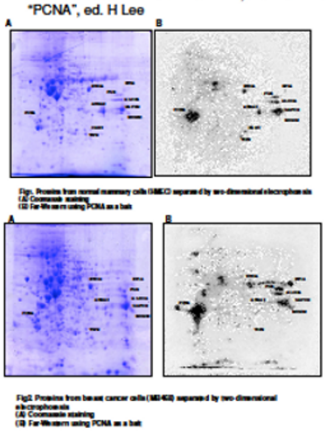

C273

Proteomic Studies on Conditioned Media Collected from Primary Culture of Non-Small Cell Lung Cancer

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Introduction
Detection of lung cancer at early stage is critical for successful clinical therapy and increased survival rate (1). Having moved from one-gene-at-a time analyses to more synthetic approaches, genomic researchers are profiling thousands of gene transcripts to generate entire patterns of information. This study is to develop a profile of proteins associated with non-small cell lung cancers (NSCLCs) and to search for the biomarkers in the circulating plasma.

Methods
Based on the novel method established in this laboratory previously, which could be used to detect cancer-related free proteins released into the blood, primary organ cultures were generated from the tumor tissues and matched adjacent normal bronchial epithelia derived from 9 cases of NSCLC. The conditioned media were collected from the primary cultures, and subsequently analyzed by LC-MS/MS (liquid chromatography-mass spectrometry/MS). Bioinformatic analysis with a label-free quantitative proteomic technique was adopted to explore the spectrum of proteins dysregulated in the lung cancer. The resulting proteins were then validated in human samples of peripheral plasma by ELISA.

Results
In total, about 1,000 unique proteins were identified with high confidence. Quantitative protein differences between the pair samples were determined by comparing the spectral counts (label-free). Gene Ontology term enrichment analysis revealed that the predominant functional classes of the proteins were related to the oxidoreductase activity, glycolysis and the subcellular location was mainly membrane-bound. According to the statistical analysis together with the gene annotation information, alpha-enolase, ALCAM (activated leukocyte-cell adhesion molecule), MMP1 (matrix metalloproteinase 1) were validated in human specimens. Examining by ELISA, the plasma level of alpha-enolase was significantly higher in lung cancer patients than that in patients with nonmalignant diseases in lung and healthy individuals, while the ALCAM plasma level was significantly lower compared to the control subjects. Moreover, the result showed that the high levels of MMP-1 in both tumor tissue and peripheral plasma were correlated with lung cancer progression and poor prognosis.

Innovative aspects
- Label-free quantification method could be a feasible and effective strategy to search potential protein biomarkers of cancer in the simplified conditional media system.
- The differentially expressed proteins reported in this study would make promise for a group of protein markers for non-small cell lung cancers.

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C274

Differential Profiling in Human Chang Liver and MHCC97-H Cells using Integrated Proteomics

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Introduction
Human hepatocellular carcinoma (HCC) is one of the most malignant tumor particularly induced by unregulated metastasis and a leading cause of deaths, and the screening for markers for detection and therapeutic targets of HCC will be seriously important. Here we applied an integrated 2-DE/MS and LC-MS/MS strategy to investigate the differential profiling of samples from human normal liver cells, Chang liver cells, and MHCC97-H cells established from the parent HCC MHCC97 cell line with highly metastatic potential, with the aim of improved understanding of the mechanism of HCC relevant to pharmaceutical development.

Methods
For 2-DE/MS analysis, proeins samples were separated by 2-DE, in-gel digested, and the protein spots differentially expressed were identified by MALDI-TOF-TOF MS. For LC-MS/MS, corresponding another homogenate of each sample was fractioned in water-soluble and membrane-riched fraction. The extraction of the fractions in the mixture was then in-solution digested, separated by *nano*-LC coupled with C_{18}PepMap precolumn, and identified by LTQ-orbitrap MS. The number of times peptides for each identified protein was used as semiquantitative measurements of relative proteins abundance in mixtures [1]. The data from 2-DE/MS and LC-MS were searched against the IPI human database using criteria as described previously. The tranmembrane properties of the identified proteins were analyzed using TMHMM Server [2], and western blot analysis was applied to verify the proteomic result for the selected proteins identified.

Results
Total 78 differentially expressed proteins were identified by the combination of the two approaches. Of those, 12 proteins were identified in both analysis while there were 32 (including protein isoforms) and 54 unique proteins were contributed from 2-DE/MS (MS/MS) and LC-MS/MS, respectively. In particular, comparison showed that LC-MS/MS detected an increased coverage for tranmembrane proteins (31 vs 11) and obtained more basic (pH>9) proteins than 2-DE analysis.
Some of the differentially expressed proteins based upon the functional classification were identified as mainly up-regulated in MHCC97- H cells and involved in cytoskeleton constituents, stress resistance, metabolism and energy production, and cell redox homeostasis. Some others associated with mitochondrial, heter-ribonucleoproteins as well as pre-mRNA splicing were down expressed. While others such as those related to transcription and translation machine, DNA repair, and those whose molecular functions remain uncertain were found to be over- or underexpressed. Of the proteins differentially expressed some were previously shown to be involved in HCC or linked with other types of cancer, while others such as heat shock protein (HSP) 10, stress induced phosphoprotein 1 (STI1), alpha skeletal muscle E, and phosphoglycerate mutase 4 etc were not previously reported, which might be apparently or potentially related with HCC and served as novel markers for diagnosis and therapy of HCC. In conclusion, Our study demonstrated the capability of integration of both the Gel-based and Gel-free proteomics for more comprehensive expression profiling analysis, which expanded significantly the known proteome coverage. Our observations by identifying the proteins with differential profiling would improve the understanding of the complex mechanism of tumorigenesis of HCC relevant to the diagnosis of the disease and subsequent pharmaceutical development.

Innovative aspects
· Combine both the 2-DE/MS and LC-MS/MS to investigate differential profiling between human Chang liver cells and MHCC97-H cells with highly metabasis
· Some tranmembrane proteins of high hydrophobia not obtained by 2-DE/MS were detected by LC-MS/MS
· Some proteins were implicated in hepatoma cells relative to normal cells for the first time, which were apparently or potentially related with HCC, and might be served as novel markers for diagnosis and therapy of HCC

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C275

Comprehensive Proteomic Profiling of Direct Expressed Prostatic Secretions

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Introduction
Clinically-obtainable fluids that are proximal to organs contain a repertoire of secreted proteins and shed cells reflective of the physiological state of that tissue, and thus represent potential sources for biomarker discovery and assay development. The prostate gland secretes many proteins into a prostatic fluid that combines with seminal vesicle fluids to promote sperm activation and function. Proximal fluids of the prostate that can be collected clinically are seminal plasma and expressed-prostatic secretion (EPS) fluids. We reason that characterization of the prostate specific protein components of EPS obtained from prostate cancer patients prior to prostatectomy should facilitate discovery of new biomarkers and development of novel diagnostic assays for prostate cancer.

Methods
Direct expressed-prostatic secretion (EPS) fluids were obtained from men under anaesthesia about to undergo prostatectomy for prostate cancer. EPS samples from 9 men diagnosed with prostate cancer [Gleason 6-7, stages pT2a-pT3b] were analyzed by on-line MudPIT using a LTQ-Orbitrap mass spectrometer. MS-data was searched with SEQUEST and stringently filtered by Peptide and Protein Prophet (>95% confidence). Only proteins identified with ≥ 2 unique peptides were accepted, resulting in the confident identification of 891 unique proteins. Integrated bioinformatics data mining was performed using the Protein Centre software suite.

Results
Analyses of 9 EPS samples with using a shot-gun proteomics approach led to the identification of 891 unique proteins the largest inventory of the prostatic secretion proteome to date. Of note, there were few apparent blood derived proteins present in these clinically collected fluids. After computational comparison to other published proteomic studies of urine, seminal plasma, and prostate cancer cell line secretions, as well as, our own (unpublished) proteomics data from ovarian cancer ascites, we short-listed ~100 candidate proteins – including some previously proposed markers and known markers like prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), underlining the feasibility of our mining scheme (**Fig. 1**). Five putative candidates have currently been quantified by ELISA in an additional panel of 40 EPS samples. Since the rapid, multiplexed quantification of putative biomarkers seems a promising and reasonable approach for future biomarker research, we are currently developing a Selected Reaction Monitoring (SRM-MS) method for selected candidates in direct EPS and EPS urine samples. An SRM-MS workflow for detection and quantitation of two of these candidates, PSA and PAP, has already been optimized. This method will allow for further scrutinizing of the occurrence of these putative biomarkers in patient samples in different contexts (i.e. stage, recurrence, chemotherapy resistance etc).

Innovative aspects
- Most comprehensive coverage of the EPS proteome
- Unique body fluid for biomarker discovery
- Integrated analysis to shortlist putative biomarkers

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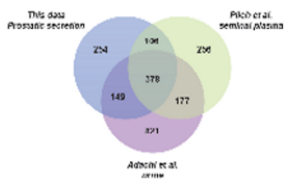

Figure 1: 891 proteins (887 protein clusters) were identified by proteomic analyses of EPS. Comparison to other proteomics studies (urine and seminal plasma) is shown.

C276

Oxythiamine specifically inhibits cellular phosphoproteome in pancreatic cancer cells

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Introduction
The non-oxidative pathway, catalyzed by transketolase (TK) and transaldolase (TA) in the pentose phosphate pathway, play an important role in regulating glucose carbon recruitment toward *de novo* nucleic acid ribose synthesis and cell proliferation *in vitro* and *in vivo* to sustain cancer growth. Inhibition of TK with oxythiamine (OT) has been shown to reduce tumor cell proliferation *in vitro* and *in vivo* leading to cell cycle arrest and apoptosis. In this study, we investigated the molecular mechanisms of the anti-proliferative action of OT at both translational and post-translational levels in MIA PaCa cancer cells using a new proteomic technique that can determine differential expression and protein synthesis.

Methods
Cell culture: Experiments were set up in three groups: group 1 MIA PaCa cells were cultured in MEM medium containing 1 mg/ml of natural amino acids; group 2 cells with 50% of ^{15}N algal amino acid mixture (98% ^{15}N); and group 3 cells with 50% of ^{15}N algal amino acid mixture (98% ^{15}N) plus 10 μM OT. After incubation for 72 hours, the cell pellets were collected and stored at –80°C.
2-DE and protein identification: 400 μg protein was separated by using 2-DE as described in our previously study1. Selected phosphoprotein spots were excised and trypsin digested before identification by MALDI-TOF/TOF MS.
Protein synthesis rate: The synthesis rates of the phosphoproteins identified were calculated according to our newly developed algorithms[1,2].
Data analysis: The synthesis rate of each protein is the average of two to four fragments. One-way ANOVA with Tukey's adjustment was used for multiple comparisons in SPSS 16.0 (SPSS Inc., Chicago, IL).

Results
Oxythiamine caused cell cycle arrest through the MAPK pathways: OT was found to increase significantly the percentage of the cancer cells in G1/G0 phase. MIA cell cycle arrest by OT treatment may be through specific deactivation of the p38MAPK cascades.
Oxythiamine suppressed expression of cellular phosphoproteome: In comparison of the phosphoproteome in OT treated group to control, we found that expression level of the cellular phosphorylated proteins was significantly reduced.
OT interrupted protein synthesis rate: Protein turnover in cells is the result of protein synthesis and degradation. The turnover rate of a protein indicates the functional significance of the protein and can explain the incongruence between transcriptome and proteome data. To understand the role of the phosphoproteins differentially expressed play in OT treated MIA cells, Using our recently developed method[1,2], we measured the synthesis rates of the phosphoproteins differentially expressed in OT treated MIA cells. The results show that the protein synthesis rate in 72 hours was roughly classified into two categories: <80% and >80%. Proteins with a turnover rate <60% include PDIA 3, while proteins with a turnover rate >60% are nuclear autoantigenic sperm protein (89%), 60S acidic ribosomal protein P0 (72%), Glutathione S-transferase P (72%), and Peroxiredoxin-6 (73%) and Hsp 27 (~70%).

Innovative aspects
- A new quantitative proteomic technique allows the simultaneous determination of protein turnover
- High protein turnover rate will be considered useful to identify signaling target in chemotherapy.
- Novel approach is offered in study of chemotherapeutic resistance.

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C277

Quantitative profiling of secreted proteins in wild-type and EGFRvIII glioblastoma cells using hydrazide chemistry and label free LC-MS

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Introduction
Glioblastomas (GBMs) are the most common and malignant brain tumors in adults. Amplification/mutation of the epithelial growth factor receptor (EGFR) gene is one of the main genetic alterations identified in GBMs. The most frequent mutation of the EGFR gene (referred as EGFRvIII) is an in-frame deletion of exons 2-7; this results in the constitutive activation of the receptor tyrosine kinase domain which dramatically enhances the tumorigenic and angiogenic properties of GBM cells. In order to understand mechanisms involved in tumorigenesis and angiogenesis in GBMs, this study aims to identify differences in secreted protein expression between wild-type and EGFRvIII human glioblastoma cells (U87MG) using glycopeptide capture coupled with label-free LC-MS based approaches.

Methods
Secreted proteins contained in the serum-free conditioned media of wild-type and EGFRvIII U87MG cells were collected and concentrated using 3 kDa MWCO ultrafiltration devices (Millipore). Secreted proteins are commonly glycosylated and since they could represent promising therapeutic targets for cancer, both CM were subjected to hydrazide capture as described previously to enrich for N-linked glycopeptides (1). The captured N-linked glycopeptides were analyzed by nanoLC-MS using nanoAcquity UPLC coupled with Q-TOF Ultima (Waters). This was followed by quantification and normalization of peptide levels between wild-type and ΔEGFR U87MG cells using MatchRx software (2). Peptides were identified using a target list-dependent nanoLC-MS/MS analysis followed by semi-automated alignment of data with the corresponding original LC-MS data using MatchRx. Mascot was used as the database search engine. Differentially expressed glycoproteins between wild-type and EGFRvIII U87MG-cells were then classified into functional categories (Panther) and associated with tumor progression using a custom literature mining software.

Results
In this proteomic profiling study, more than 1000 unique peptide ions were detected by MatchRx, 90% of which were N-linked glycopeptides. Among them, a total of 23 N-linked glycoproteins were found to be differentially expressed between wild-type and ΔEGFR U87MG-CM. Panther ontology analysis indicated that the majority of these glycoproteins (22%) are ECM proteins and involved in cell signalling (43%). Literature mining revealed that 14 out of the 23 glycoproteins were previously associated with cell invasion, for example SPARC, which was 6.5 fold up-regulated in EGFRvIII U87MG-CM compared to wild-type U87MG-CM (Fig.1). This suggests that SPARC may contribute to the enhanced malignancy of GBMs expressing EGFRvIII. In conclusion, these results demonstrate that the combination of hydrazide capture, label-free LC-MS analysis and MatchRx-based quantification represents a powerful approach to identify relative changes in glycoproteins in complex samples such as U87MG-CM. Proteins highly secreted by EGFRvIII U87MG cells, including SPARC may prove to be valuable markers of GBM progression and malignancy.

Innovative aspects
- Quantification of secreted N-linked glycopeptides in U87MG cell lines using a combination of hydrazide capture, label-free LC-MS analysis and a novel software MatchRx.
- Identification of a group of glycoproteins differentially expressed between wild-type and EGFRvIII U87MG cells that could potentially be used for identification and/or therapeutic targeting of GBM harbouring the EGFRvIII mutation.

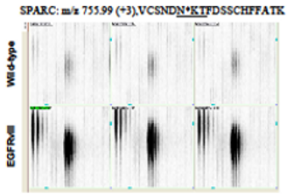

Figure 1. LC-MS Image (MSight) of a formerly N-linked glycosylated peptide of SPARC in triplicate technical repeats. SPARC is 6.5 fold up-regulated in EGFRvIII U98MG-CM compared to wild-type U87MG-CM based on MatchRx analysis after normalization. Retention time for this peptide is detected between 23.52 and 23.59 min. N*KT corresponds to the N-linked glycosylation site.

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C278

Identification of Prostate Specific Antigen Isoforms in Clinical Samples

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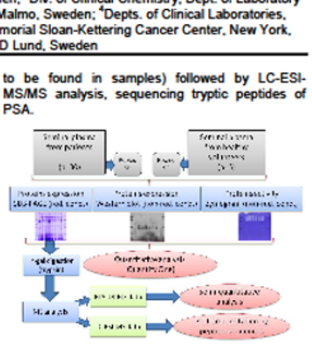
Based on its versatile diagnostic value in serum, and prior findings that the distribution of multiple molecular forms of prostate specific antigen (PSA) may further distinguish the pathological condition, we aimed to perform a comprehensive identification of PSA variants occurring in the seminal fluid.
Prostate-specific antigen (PSA) is a serine protease produced by epithelial cells of the prostate (1) that has restricted chymotrypsin-like endoproteolytic activity, which is not only limited towards to the biological substrates, semenogelin 1 and 2, but also to fibronectin, insulin-like growth factor binding protein and gelatin. The expression of PSA in cancerous tissue remains exceedingly high even though it may be reduced up to 1.5-2-fold compared to the normal epithelial. On the other hand, the levels of PSA released into the blood (normally corresponding to $\leq 10^{-6}$ of that released in the seminal fluid) become elevated in blood due to increased leakage from the prostate. Because of the sensitivity and tissue specificity, PSA in blood (median level at age 40-50 of 0.6 ng/ml) is the most valuable clinical biomarker for the diagnosis of prostate carcinomas, contributing to their early detection. However, increased levels of PSA in serum do not simultaneously allow a combination of very high sensitivity and specificity in the discrimination of prostate cancer of an unquestionable significance, "indolent" disease, or benign conditions, such as hyperplasia (BPH) or prostatitis which increase steeply in frequency after age 50. Therefore, 65-75% of men who undergo a prostate biopsy due to a moderate PSA elevation (≈3-10 ng/mL) do not have evidence of cancer on the biopsy.
For successful identification of PSA forms in seminal plasma various gel-based methods and mass spectrometric strategy was developed. Protein expression and enzymatic activity was monitored by reducing SDS-PAGE, consecutive Western blot analysis (from non-reducing gels) and zymogram gel electrophoresis, respectively.
Following quantification from gels, relevant bands were cut out and trypsinated prior mass spectrometric analysis. Identification of PSA in gel bits was performed by MALDI-MS peptide fingerprinting (at least three peptides of PSA had to be found in samples) followed by LC-ESI-MS/MS analysis, sequencing tryptic peptides of PSA.



Work flow of the multiplex analysis for identification of PSA variants in seminal plasma.

In SDS-PAGE gels, 1-7 PSA bands were clearly stained in the range of 25-36 kDa, whereas only 1-3 and 0-4 bands were detected by Western analysis (25, 28 kDa) and zymogram gels (30, 32 kDa), respectively. Quantitative analysis revealed high concordance between PSA concentration measured by a commercialized time-resolved fluorescence immunoassay (Free/Total PSA Prostatus, Perkin-Elmer, Turku, Finland) and gel bands quantification or semi-quantitative analysis of MALDI-MS results.
Despite notable advances in our understanding of the molecular and functional details of PSA, there is still lacking consensus as to which may be the best strategy to obtain optimal clinical value from PSA-testing. Consequently, we believe that our observation of various isoforms of PSA in patients reported in this communication may contribute important insight further an identification of disease-relevant heterogeneity of PSA, including transcriptional and post-translational modifications present due to various stages and causes of prostate disease. This may allow comprehensive structural characterization of this important target protein in low abundant samples such as blood in the near future.

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C279

Screening for Methylation Silenced Genes in Human Leukemia by Quantitative Proteomics

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Introduction
DNA methylation is one of the most common epigenetic mechanisms regulating gene expression, and the abnormal methylation genes not only play an important role in the pathogenesis of leukemia, but also provide a specific target for the treatment of leukemia. Therefore, screening for novel methylation silenced genes in leukemia can help to reveal its pathogenesis, and provide the important theoretical and scientific basis for the prevention and cure of leukemia.

Methods
In this study, 2D-DIGE and MS were preformed to identify up-regulated proteins in leukemia cell line HL-60 following the treatment of 5-aza-CdR, Western blot was used to validate the results of 2D-DIGE, and methylation specific PCR (MSP) and reverse transcription polymerase chain reaction (RT-PCR) were adopted to detect the methylation and mRNA levels of the encoding genes of the identified proteins, respectively, to discover the methylation silenced genes in HL-60. In addition, other 3 leukemia cell lines (Jurkat, U937, Raji), and clinical samples including 235 cases of leukemia bone marrow cells and 45 cases of control marrow cells were used for further validate the methylation silenced genes by MSP and RT-PCR.

Results
40 differential proteins were identified by 2D-DIGE and MS in HL-60 cells following 5-aza-CdR treatment, of which 34 proteins were up-regulated, and 6 proteins were down-regulated. 4 differential proteins (Galectin-1, HSP47, S100A8, S100A9) were confirmed by Western blot. RT-PCR and MSP showed that Galectin-1 and HSP47 genes were methylated in HL-60 cells. Additionally, we also demonstrated that Galectin-1 and HSP47 genes were high frequently methylated in the other leukemia cell lines and clinical leukemia bone marrow samples.

Innovative aspects
- Galectin-1 and HSP47 are methylation silenced genes in human leukemia.
- An integrated quantitative proteomic and epigenetic approach can be used to screening methylation silenced genes.
- The methylation inactivation of Galectin-1 and HSP47 genes may play an important role in the pathogenesis of human leukaemia.

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Verification of Bladder Cancer Markers through Quantitative Proteomic Analysis of Urine

C280

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Introduction:
Urinary analysis using cytology and immunodiagnostic tests has shown to aid in the detection of bladder cancer. However, there is a need to improve on the sensitivity and specificity offered by the use of these methods. Quantitative proteomic analysis, using iTRAQ and multiple reaction monitoring (MRM), of protein extracted from urine of bladder cancer patients offers the potential to identify and verify novel markers to improve the detection of bladder cancer. Here we demonstrate the discovery and verification of a multi-parametric panel of biomarkers in the urine of bladder cancer patients.

Methods:
Twenty micrograms of extracted protein from pooled urine was trypsin digested and labelled with iTRAQ. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed on a QSTAR Elite in information dependent acquisition mode. ProteinPilot was used for database searching and relative quantification. Differentially expressed candidates plus those selected from literature were subjected to MRM analyses in individual patient urine samples. MRM transitions were selected through experimental and *in silico* methods. Transitions were experimentally tested and an optimized list created. Digests were analyzed by nano-flow reversed-phase C_{18} chromatography coupled to a triple quadrupole-linear ion trap mass spectrometer (QTRAP 4000). Relative quantitation was performed by MultiQuant software.

Results:
Through relative quantitation analysis by iTRAQ, 30 candidate proteins were identified to be differentially expressed and an additional 120 candidate proteins identified in the literature were selected for targeted MRM analysis. For each protein, two peptides and two transitions for each peptide were selected for MRM. Before sample analysis our workflow was optimized through protein extraction optimization to determine recovery and LC-MRM optimization to determine optimal sample loading. From this we determined that acetone precipitation and 2µg was optimal for protein extraction and loading respectively. Reproducibility of retention time and peak area calculation was determined (range from 0.09-0.73%, 4.15-23.24% respectively) for our workflow by triplicate runs of a test urine digest for five candidate proteins. Molecular pathway analysis of the identified candidates provides insight into perturbed networks involved in bladder cancer.

Summary:
This study demonstrates for the first time the feasibility of monitoring 150 candidate proteins in urine using an optimized workflow for sample preparation and MRM quantitation.

Innovative Aspects:
- Optimized large scale monitoring of 150 proteins by MRM
- Sensitive quantitation and comparative analysis between patient groups utilizing *in silico* methods
- Molecular network analysis of differentially quantitated candidates

Identification of metastsis-associated secreted proteins in non-small cell lung cancer by comparative proteomic analysis

C281

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Introduction
Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers and the 5-year survival rate is less than 15% (1). The low survival rate is mainly due to the lack of suitable metastatic biomarkers for early detecting cancer metastasis. In metastatic processes, secreted proteins abounded in the extracellular microenvironment and increased tumorigenic metastasis by promoting cell migration and invasion (2). Therefore, the objective of this study focused on characterizing secreted proteins associated with NSCLC metastasis.

Methods
We compared the secretome profile of CL1-0 cells (non-metastatic capability) and CL1-5 cells (highly metastatic capability) using the hollow fiber culture (HFC) system, and the cell growth in the HFC system was monitored by daily glucose consumption and lactate generation. Daily conditioned medium was collected and concentrated for 2-DE analysis. Silver stating was used to detect individual protein spots. After in-gel digestion, liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify proteins. Secreted proteins were determined using Human Protein Reference Database (HPRD) and three prediction software: SignalP 3.0, SecretomeP, and TMpred.

Results
Daily glucose consumption and lactate production increased gradually in both CL1-0 cells and CL1-5 cells (Fig. 1A and 1B). Protein concentrations of condition medium also revealed a time-dependent increase in both CL1-0 cells and CL1-5 cells for 10 days (Fig. 1C). Condition media were pooled from the first six days based on the aggressive growth of CL1-0 cells and CL1-5 cells. Separation of secreted proteins in the conditioned medium was performed using 2-DE. A set of six analytical gels from CL1-0 and CL 1-5 were run simultaneously. An average of 540 protein spots in each of the CL1-0 gel and 640 spots in each of the CL1-5 gel were detected. After image analysis and identification by LC-MS/MS, 43 secreted proteins generated from 77 protein spots were identified differentially expressed between CL1-0 cells and CL1-5 cells. Among them, 23 proteins were up-regulated in CL1-5 cells while 20 were down-regulated in CL1-5 cells (Fig. 2A). Forty proteins (97%) were classified as secreted proteins based on the searching of HPRD and predictor software: SignalP 3.0, SecretomeP 2.0, and TMpred (Fig. 2B).

Innovative aspects
- Our experimental strategy was able to sufficiently enrich and identify the metastasis-associated secreted proteins for following studies.
- Approximately 97% (40 out of 43) of these secreted proteins could be associated with NSCLC metastasis.

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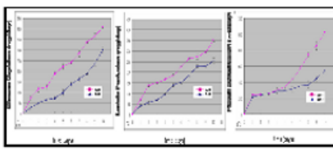

Figure 1. Monitoring of cell growth and protein secretion of CL1-0 cells and CL1-5 cells in the HFC system. (A) Daily glucose consumption, (B) Daily lactate production, and (C) Daily protein concentration of CL1-0 cells and CL1-5 cells in the HFC system for 10 days.

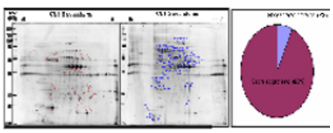

Figure 2. Representative 2-DE gels of secretome profiles of CL1-0 cells and CL1-5 cells. (A) Secretome profile of CL1-0 cells and secretome profile of CL1-5 cells were performed by 12% polyacrylamide gels, stained with silver nitrate (spot number: L: proteins were downregulated in CL1-5 cells; H: proteins were overregulated in CL1-5 cells). (B) Classification of secreted proteins.

Proteomic analysis of membrane glycoproteins of HepG2 cell by affinity enrichment and MS identification

C282

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Introduction The cell surface membrane proteins provide a physical boundary between the cell and its environment. One particularity of mammalian membrane proteins is that a major part of them is extensively glycosylated. Glycosylation of membrane proteins plays a significant role in the processes such as signal transduction, cell adhesion and cell recognition , and some membrane glycoproteins have been reported involved in cancer progression and immune responses[1]. Therefore, many membrane glycoproteins have been used as therapeutic targets and biomarkers for cancer prognosis, diagnosis, and monitoring.

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignant diseases worldwide and it is usually diagnosed at the advanced stage with a low 5-year survival rate. The extremely poor prognosis of HCC is largely the result of a high rate of recurrence and metastasis[2]. The adhesion factor and receptor factor associated with HCC metastasis are mostly membrane glycoproteins. The research on membrane glycoproteins of HCC may help shed light on understanding the mechanism of HCC carcinogenesis and metastasis, adding further support for screening of novel biomarkers. Despite their importance, the membrane glycoproteins of HCC cells are largely uncharacterized.

In this work, we firstly developed a purification approach to effectively enrich membrane glycoproteins of a standard protein mixture and HepG2 cell line, which involves biotinylation of cell surface membrane glycoproteins, affinity enrichment of these glycoproteins and identification by mass spectrometry.

Methods HepG2 cells were firstly incubated with sodium periodate to oxidize vicinal hydroxyl groups on the carbohydrates of glycoconjugates. Excess periodate was then removed by washing from the cells. Using biotin-LC-hydrazide, it was possible to link biotin residues via aldehyde groups to HepG2 cell surface glycoproteins. After lysis of the labeled cells by ice-cold hypotonic buffer with dounce homogenization and centrifugation to remove large debris, the cell surface membrane components in the cell lysate were fractionated by an ultracentrifuge step. For the affinity capture, we adopted the "protein level" and "peptide level" enrichment strategies. Finally peptides treated with PNGase F were analyzed by nanoLC/ESI-MS/MS using an LTQ-FT.

Results We demonstrated the feasibility and characterized the capture efficiency of this affinity approach, simultaneously comparing the enrichment strategies of "protein level" and "peptide level", using a protein mixture consisting of five different proteins. Close to 100% capture efficiency was obtained, and "peptide level" strategy can simplify the complexity of peptide mixture and benefit the following identification of mass sepctrometry. Then we applied our capture approach to HepG2 cell line. Totally 119 glycosylation sites on 116 glycopeptides , corresponding to 79 different protein species were identified. Among the proteins，CD proteins such as CD44 and CD147, Epidermal growth factor receptor precursor, MHC class I have been reported previously as the potential biomarkers of hepatocellular carcinoma. In contrast to already mentioned glycoproteins and glycopeptide enrichment methods, targeted affinity enrichment is direct, simple, and rapid, which increases the relative amount of glycopeptides by trapping and removing the non-glycopeptides in the sample. Furthermore, the identified membrane glycoproteins also provide a representative database of membrane glycoproteins from HCC cell lines, with the view of helping to elucidate the mechanisms of HCC carcinogenesis and metastasis and probe the novel biomarkers for the early diagnosis of the disease.

Innovative aspects
- A direct, simple, rapid and global approach to profile the genuinely cell surface membrane glycoproteins was developed.
- Totally, 119 different N-glycosites within 79 different glycoproteins were identified from HepG2 cells, which make a very useful database for digging the biomarker for early diagnosis of Hepatocellular carcinoma.

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Prognostic and Predictive Biomarkers for Colorectal Cancer

C283

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Introduction
The rising health burden associated with colorectal cancer (CRC) in Australia and the Western world demands more efficient and reliable approaches to treating these patients. After lung cancer, CRC has the highest mortality rate with over 4700 deaths in Australia per annum. Currently, patients with Stage C disease (lymph node involvement) or metastatic disease receive chemotherapy, however, only 50% of patients respond to treatment and 20% of patients experience severe chemotoxicity as a result of treatment. Prognostic biomarkers that inform on patient outcome, tumour response, and toxicity to chemotherapies would greatly assist in delivering appropriate personalised patient care.

Methods
For prognostic biomarkers we profiled paired normal mucosa and epithelial cell-rich CRC from 16 patients using 4-plex iTRAQ quantitative mass spectrometry. Differentially expressed proteins were validated by Western blotting and immunohistochemistry. Candidate biomarkers were evaluated using IHC with archival tissue on a tissue microarray using a consecutive cohort of 477 Stage C patients with 5-year survival data.
A further experiment has been conducted using 8-plex iTRAQ-MS analysis of CRC from 7 Stage C patients with poor survival outcomes compared to 7 Stage C patients with acceptable survival outcomes. To increase the number of identified proteins we conducted two LC/MS experiments, the second using an ion exclusion list generated from the first LC/MS experiment.
For predictive biomarkers a set of 38 selected reaction monitoring (SRM) assays were designed for common plasma proteins. We have screened plasma from 16 patients receiving fluoropyrimidine-based chemotherapy to identify proteins predictive of response and haematological chemotoxicity.

Results
Prognostic biomarkers (mucosa vs CRC): Over 1400 proteins were identified and quantitated, with 118 observed as differentially expressed by 1.3-fold or greater with a false discovery rate of p<0.05. Validation experiments using immunoblotting and immunohistochemistry (IHC) showed strong concordance with iTRAQ data. Gene ontology analysis indicated that proteins with increased expression levels in CRC tumours include those associated with glycolysis, calcium binding, and protease inhibition. Proteins with reduced levels in CRC tumours were associated with loss of ATP production through: (i) reduced beta-oxidation of fatty acids, (ii) reduced NADH production by tricarboxylic acid cycle and (iii) decreased oxidative phosphorylation activity. Additionally, biosynthesis of glycosaminoglycans and proteoglycans were significantly reduced in tumour samples. Tissue microarray IHC of 477 consecutive clinicopathological Stage C tumours demonstrated high expression of the actin bundling protein, fascin, was shown to be an independent predictor of diminished survival (hazard ratio 1.7, CI 1.2-2.3), while the serpin, maspin showed no correlation with survival in this cohort.
Prognostic Biomarkers (Poor vs acceptable survival): Using an exclusion list strategy with 8-plex iTRAQ we have identified and quantitated 2700 proteins in CRC from these patients. Bioinformatic analysis is underway to identify differentially expressed proteins prior to validation using tissue microarrays.
Predictive Biomarkers: Plasma samples from 16 patients have been screened by SRM for 38 assays. We have preliminary evidence that peptides from vitronectin and paraoxonase-1 predict for outcome after 15 days therapy and Apolipoprotein B predicts for chemotoxicity.

Innovative aspects
- A comprehensive quantitative analysis of protein expression in normal mucosa and CRC from 16 patients.
- Identification and large scale validation of fascin as a prognostic biomarker in CRC.
- Preliminary identification of plasma biomarkers predictive of early chemotoxicity and response for CRC chemotherapy

C284

Identification of small-molecule Inhibitors of the Sox2-DNA Interaction Using a High Throughput Fluorescence Polarization Screen

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Sox2 belongs to a family of high-mobility group (HMG) transcription factors that is expressed in pluripotent mammalian cells and plays a key role in the maintenance of stem cell self-renewal. Increase in Sox2 expression is known to trigger differentiation of ES cells into neuroectoderm, mesoderm, and trophectoderm. Over expression of Sox2 is also associated with tumorigenesis in human gastric carcinoma, stomach adenocarcinomas, malignant glioma and breast cancer and is hypothesized to play a key-role in the self-renewal of cancer stem cells. Discovering specific inhibitors of Sox2-HMG DNA interactions will be of interest to develop therapeutic leads against cancer stem cells, as chemical agents to promote targeted differentiation of stem-cells into specific cell-fates, in chemical reverse-genetic studies or as chemical tools *in-lieu* of RNAi based techniques.

Targeted disruption of specific protein-DNA interfaces remains an under-utilized target in small-molecule based drug discovery. To address this challenge, I will describe the current in-house efforts in the development and establishment of a high-throughput screen using fluorescence polarization to identify *in-vitro* small molecule inhibitors of Sox2-HMG binding to a consensus Sox binding DNA element (CCND1 cis-regulatory element). Gradually the fluorescence polarization based high-throughput screening will be expanded to include other transcription factors implicated in disease states related to aberrant transcription.

C285

Analysis of the Melanoma Proteome Associated with Poor Prognosis by DIGE and iTRAQ

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Introduction
Australia has the highest recorded incidence of skin cancer world-wide. In figures published for 2002-3, melanoma is the rarest of the three main types of skin cancer accounting for 2.5% of cases, but causes 75% of skin cancer fatalities. If melanoma is detected early it is readily treatable by surgical excision, but after metastasis the 5-year survival rate drops to 7-19%. However, there is a spectrum of responses amongst patients with a small sub-set of individuals who have a low remission rate and a long survival rate, while apparently similar individuals with an identical stage melanoma have a poor prognosis and a survival rate of less than 6 months. Investigation of the proteomic differences between these two groups of patients should provide a better understanding of the molecular mechanisms associated with poor prognosis, and identify potential prognostic markers.

Methods
This study used stage 3 melanoma secondary tumours which had been removed as a sentinel lymph node during treatment. Under expert guidance by the pathologist the tumour was dissected and snap frozen. Twenty patients were selected for the study from two groups of either extremely poor prognosis or extremely good. Approximately 200 mg of each tumour was ground under liquid nitrogen and extracted in DIGE sample buffer. A portion of the extracted proteins was pooled, creating a global standard. Samples were analysed by difference gel electrophoresis (DIGE) [1] and quantitative shotgun proteomics [2]. In the DIGE analysis, proteins were labelled with size- and charge-matched fluorescent Cyanine dyes and then focused on 17 cm pH 3-10 non-linear IPG strips, before running on Tris-glycine 8-18% SDS-PAGE gels. Gels were analysed, proteins of interest were excised, subjected to in-gel trypsin digestion and identified by peptide mass fingerprinting or de novo sequencing. For the iTRAQ analysis, proteins were digested with trypsin, labelled with one of 8 iTRAQ tags, pooled together, and the peptides were fractionated by strong cation exchange then reverse phase chromatography. Peptides were analysed by tandem mass spectrometry using a QSTAR XL mass spectrometer and these data were processed using ProteinPilot 3.0.

Results
Through careful analysis of the 30 gel images, 400 spots were matched between all gels (10 patient samples for good prognosis, 10 for poor prognosis and 10 global standards). While all the samples gave reproducible protein spot patterns, abundance of particular proteins varied considerably between individuals in each group as well as between groups. However, a small number of proteins showed a statistically significant ($p < 0.05$) differential abundance between the good and poor prognosis groups.

Preliminary analysis of the iTRAQ data lead to the identification of more than 750 proteins. While the data showed a similar heterogeneity in protein abundance within the two patient groups, there were also a significant number of proteins whose abundance correlated with good or poor prognosis. An example is human periostin. While further work needs to be done to thoroughly mine the two complementary data sets obtained, the preliminary analysis indicates a number of proteins that correlate with good or poor prognosis for stage 3 metastatic melanoma.

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C286

Proteomic analysis of lung cancer cell lines aimed to identify novel target proteins for miR-17-92

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Introduction
MicroRNAs (miRNAs) are small non-coding RNAs (20-23 nucleotides) that negatively regulate gene expression at post-transcriptional level by interacting with 3'UTRs of their target mRNAs. Aberrant expression of miRNAs in cancer indicates that miRNAs play key roles in human cancers acting as either oncogenes or tumor suppressor genes depending on their targets. The miR-17-92 cluster located on C13orf25 is well known as oncogenic miRNA, which is overexpressed in lung cancer and malignant lymphoma. However, few data have been collected about the targets of miRNAs, including miR-17-92 cluster, and their roles in tumorigenesis. To identify the target proteins of miR-17-92 cluster directly, we performed proteomic analysis in lung cancer cell lines using 2-DE and LC-MS/MS.

Methods
We profiled miRNA expression in a subset of 31 of cancer cell lines by TaqMan Real-time PCR (ABI). Two lung cancer cell lines, SBC-3 and LK-79, which overexpress miR-17-92 cluster were identified. We performed two-dimensional protein electrophoresis analysis (2-DE) of SBC-3 lung cancer cell line treated with anti-miR-cluster locked nucleic acid (LNA). Whole cell lysate of SBC-3 cell was isolated by standard method. 2-DE was performed using pH3-10 IPG DryStrip (BIO-RAD), followed by SDS-PAGE. After SDS-PAGE, the gels were treated within the SYPRO Ruby (Invitrogen) according to the manufacturer's protocol. The images of gels were obtained on image analyzer FLA-3000 (FUJIFILM) and analyzed using PDQuest software (BIO-RAD), with spot densities compared at defined coordinate positions. The number of valid spot was 1866 in a total of 2387 detected spots and 347 distinct spots were recognized as upregulated by anti-miR-LNA, using a 1.5-fold threshold for significance. Among these spots, 112 proteins were identified as candidates for miR-17-92 cluster immediate target proteins by LC-MS/MS (Agilent).

Conclusions
Our results of exhaustive analysis revealed significant differences in the proteomic profile between anti-miR-LNA treated and non-treated lung cancer cell lines. Among these upregulated proteins by anti-miR-LNA, we identified potential immediate target proteins of miR-17-92 cluster. Further studies are needed to determine direct target genes of miR-17-92 cluster. Analysis of these potential target proteins may enhance understanding of the mechanism of miRNA related tumorigenesis.

Key words ; micro-RNA, proteome, lung cancer

C287

Serological Proteome Analysis in Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma (NPC) is one of the most common cancers in Southeast Asia as well as in southern China. Because of vague clinical symptoms and metastasis at the early stages of the disease, NPC is often difficult to early diagnose, and results in the strong decrease in the possibility of cure and the survival time in NPC patients. Therefore, identification of novel NPC biomarkers would be very helpful for its diagnosis and treatment.

To discover novel NPC biomarkers, serological proteome analysis (SERPA) was used to identify proteins that commonly elicit a humoral immune response in NPC. Sera from 19 newly diagnosed NPC patients and 19 healthy individuals were analyzed for IgG autoantibodies against NPC proteins resolved by 2-DE. Protein spots that exhibited selective reactivity with sera from NPC patients were identified by MS. As a result, a total of 13 proteins that induced autoantibodies in at least 21% NPC patients but not or rarely in healthy individuals were indentified. Among 13 identified proteins, CK19, EBP1, and Rho-GDI-2 induced autoantibodies in more than 36.8% of NPC patients.

To validate the findings of SERPA, occurrence of autoantibodies against the three proteins (CK19, EBP1, and Rho-GDI-2) was detected by immunoprecipitation and Western blotting in additional 30 NPC patients, 23 other types of cancer patients and 20 healthy individuals. Results showed that frequency of autoantibodies against CK19, EBP1 and Rho-GDI-2 in NPC patients was significantly higher than that in other types of cancer patients and healthy individuals. The results not only validated the findings of SERPA, but also indicated that autoantibodies against these three proteins were specific in NPC patients.

To explore the mechanisms for autoantibody development of the three proteins (CK19, EBP1, and Rho-GDI-2) in NPC, Western blotting and immunohistochemical staining were performed to determine the expression and localization of CK19, EBP1, and Rho-GDI-2 in NPC tissues and NNET. Upregulation of CK19 and EBP1, but not Rho-GDI-2 were observed in NPC as compared with NNET, and subcellular localization of the three proteins in NPC tissue was same as that in the normal tissue. The results indicated that overexpression of CK19 and EBP1 may be one of the mechanisms for their autoantibody development in NPC, and a protein translational modification may be related to Rho-GDI-2 autoantibody development in NPC.

To explore the value of autoantibodies against the four proteins (CK19, Rho-GDI-2, HSP70 and LAP3) for diagnosing NPC, we detect the serum levels of their autoantibodies in additional 36 NPC patients, 20 other types of cancer patients and 20 healthy individuals by ELISA, and the sensitivity and specificity of individual autoantibody and four autoantibody combination for discriminating NPC patients and controls were analyzed. The results showed that the serum levels of four autoantibodies in NPC and other types of cancer patients were significantly higher than these in the healthy individuals. Using individual autoantibody as a judge marker for NPC patiens, Rho-GDI-2 autoantibody had better sensitivity, and HSP70 autoantibody had the highest specificity. Using four autoantibody combination as a judge marker for NPC patiens, the sensitivity was 41.7% (15/36), and the specificity was 95% (19/20).

We conclude that CK19, EBP1 , HSP70 ,LAP3 and Rho-GDI-2 antigens and their autoantibodies may have utility in NPC screening, diagnosis and immunotherapy.

KEYWORDS nasopharyngeal carcinoma, serological proteome analysis, tumor antigen, autoantibody, diagnosis, marker

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Differential Proteome Analysis of Ewing Family of Tumors and Neuroblastoma Cell Lines

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Introduction

Ewing Family of Tumors (EFT) and Neuroblastoma belong to the family of small, round blue cell tumors and derive both from the neural crest [1]. Differential diagnostics was improved in the past, but the development of new biological markers for prognostic and/or differentiation purposes would be valuable, especially for the correct classification of patients into their risk groups. Here we describe a proteomic approach for identification of new biological markers using Difference Gel Electrophoresis (DIGE) and Western-blotting for differential glycoproteomics.

Methods

For DIGE experiments protein extracts from six EFT- and three Neuroblastoma cell lines were separated by 2D-Gel Electrophoresis with IPG 3-12 and IPG 4-7 according to previously published protocols [2]. The preparation of the protein extracts for the DIGE-experiments with CyDyes (GE Healthcare) was according to the manufacturers protocol. A total of 27 2D-Gels per pH-Range were run and scanned. Analysis was performed with Progenesis Same Spots Software (Nonlinear Dynamics). Detected spots with an ANOVA ≤ 0.05, a power ≥ 0.80 and a consistent expression profile in both tumor types were considered as differentially expressed. For Western-blotting protein extracts were separated by IPG 3-12 and blotted onto an PVDF-membrane. Glycoprotein detection was performed with DIG Glycan Differentiation Kit (Roche) according to the manufacturers protocol.

Results

With DIGE we were able to identify 8 differentially expressed proteins between EFT- and Neuroblastoma cell lines with IPG 3-12 and seven proteins with IPG 4-7. Interestingly no „total-regulation" (i.e. present vs. absent) of proteins could be observed, only minor (1.1 to 1.9-fold) regulation. The glycoproteomic approach revealed three proteins which are either differentially glycosylated or are a „total-regulation", because none of them are detected by the algorithm in the DIGE-experiments so no conclusion of their regulation can be made. The function of the identified proteins ranges from cytoskeletal maintenance, cell division, proton-pumping, splicing to conversion of amino acids. A comparison of EFT cell lines, which characteristically express a EWS/FLI1 fusion protein, but with different fusion points [3], revealed four proteins differentially expressed with IPG 3-12 and three proteins with IPG 4-7. These differentially expressed proteins are involved in glycolysis, splicing, rearrangement of sulphur bonds, ATP synthesis, regulation of cell growth and cell survival.

Innovative aspects

- First approach for differential proteome analysis of EFT- and Neuroblastoma cell lines by DIGE and glycoproteomics
- First approach for differential proteome analysis of EFT cell lines with different types of EWS/FLI1 fusion proteins by DIGE

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C289

A Systems Approach for Discovering Biomarkers and Therapeutic Targets In Pancreatic Cancer

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Introduction

A multi-pronged approach to tackling pancreatic cancer should entail early detection as well as targeted therapy. Currently available biomarkers such as CA 19-9 are not sensitive or specific. Although the genetics of pancreatic cancer is beginning to be unveiled, there are no molecular targets for therapy. We have taken a systems approach to systematically characterize the proteomes of pancreatic cancer cell lines, cancer tissue, serum and urine. We have also established a compendium of candidate biomarkers based on an extensive literature survey. Finally, we have used affinity methods to capture kinase signaling pathways that are activated in pancreatic cancer cell lines and tissue – these are attractive targets for personalized therapy of pancreatic cancer.

Methods

Quantitative proteomic profiling using either SILAC or iTRAQ was used to identify differentially expressed proteins as candidate biomarkers from a panel of pancreatic cancer cell lines or tumor tissues (with corresponding adjacent normal tissue), serum and urine of pancreatic cancer patients. For identifying potential therapeutic targets, we enriched tyrosine-phosphorylated peptides from pancreatic cancer tissue using antibodies. The samples were analyzed on a QTOF (Agilent) or LTQ-Orbitrap mass spectrometer.

Results

The compendium of biomarkers (1) which we created includes 2,516 genes that were reported as potential biomarkers. SILAC based membrane proteomics of pancreatic cancer resulted in the identification of MYADM a promising biomarker that was differentially expressed in many pancreatic cancer samples (Fig 1). Lipocalin 2 and Deleted in malignant brain tumor 1 were some of the other promising candidates identified in our secretome analysis. In order to compare the endogenous levels of these differentially expressed proteins, we have carried out a differential proteomic analysis of serum from pancreatic cancer patients. Validation of the markers through multiple reaction monitoring are currently underway. In addition to serum, analysis of the urinary proteome is being done to detect markers that are excreted in the urine. In order to identify activated tyrosine kinases, an unbiased study was carried out using a panel of pancreatic cancer cell lines (2), which revealed activation of the EGFR signaling pathway. Since only a subset of tumors had activated EGFR, we extended our study to identify other activated tyrosine kinases. Phosphotyrosine profiling at the peptide level using pooled tumor tissues led to identification of several tyrosine kinases that were hyperphosphorylated in tumor tissues but not in normal tissues. HCK and YES were significantly hyperphosphorylated in tumor tissues – these could serve as targets for individualized therapy.

Innovative aspects

- Systems approach to identify pancreatic cancer biomarkers

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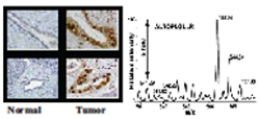

Figure 1: Overexpression of MYADM in pancreatic cancer

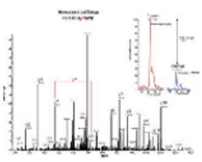

Figure 2: Hyperphosphorylation at Y209 site of HCK Kinase

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Oxidation of thiol proteins by Diethylnitrosamine in rat hepatocarcinogenesis

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Introduction

Conversion of Protein-SH into disulphides is an early event during the radical-mediated oxidation of proteins (1), this has prompted a great interest in the study of changes of these thiol proteins. The aim, was to detect the most sensitive thiol proteins to reactive oxygen species generated by metabolism of Diethylnitrosamine (DEN) in the rat liver. DEN has been widely used to induce experimental hepatocellular carcinoma (2). In our comparative analysis they were many changes in the number of oxidized thiol proteins, which correlated with the time of exposure to the carcinogen.

Methods

Twenty Male Fischer 344 (180-200 g) rats were randomized into 4 groups: 3 groups were treated with DEN at 200 mg/kg weight. Each group was sacrificed at 6h (DEN 6), 12h (DEN 12) and 24h (DEN 24) after DEN administration, respectively and the livers were dissected. The last group was used as negative control (NC). To evaluate the formation of disulphide proteins, we compared the three DEN groups against the non treated rats , for that purpose, total proteins were extracted and labeled with saturation dye according with method Redox-DIGE (3) with some modifications, this method coupled to mass spectrometry was used to identify the differential oxidation protein profile. We performed functional classification of the identified proteins using PHANTER software.

Results

The comparative analysis of the three DEN treated groups against non treated rats group,showed a tendency to increase the number of oxidized thiol proteins, through the time of DEN exposure coupled to a possible increase in degradation of proteins. The differentially expressed proteins at 6h, 12h and 24h were 407, 690, and 665 respectively; of those 6, 12 and 90 increased and 45, 12 and 51 decreased, taking as reference two folds in their oxidation status respect to NC group. Some proteins were already identified from the different groups and they are summarized in Table 1.
According to functional classification, the identified proteins in our study are categorized as chaperone, oxidoreductase, activity isomerase, hidrolase, protein binding, cytoskeleton, activity transferase, among others.
Additionally, we found an increase in the levels of low molecular weight proteins correlated with time to DEN exposure. This result likely shows protein degradation instead a increase of oxidized proteins, but this aspect has to be validated experimentally.

Innovative aspects

- DEN has an oxidative effect on thiol proteins.
- There is a tendency to increase the number of oxidized thiol proteins which depends of the time of DEN exposure and very likely related to the mechanism of carcinogenesis initiation.
- The proteins in addition to oxidation probably may be degraded by DEN effect.

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C291

Prediction of Diseasomal Proteins from Liver Disease- and Liver Cancer-Related Disease Networks

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Introduction

A series studies have reportedly identified various cellular networks, e.g. metabolic networks, genetic networks, protein networks, and disease networks (1). Proteins close to one another in a network cause similar functions and disease. In the studies for prediction of new disease-associated genes, the basic idea is that a network-neighbor of a disease related gene is to cause same or similar disease (2). We extensively employed an assumption that disease-related proteins tend to be work as an important factor for architecture of the disease network. In this study, we constructed disease networks from the liver disease- and the liver cancer-related protein-protein interaction network and then derived diseasomal proteins.

Methods

The protein-protein interaction data was obtained from the Human Protein Reference Database [HPRD, http://www.hprd.org/] and Disease data obtained from the Online Mendelian Inheritance in Man [OMIM, http://www.ncbi.nlm.nih.gov/omim/]. The protein networks can be derived the map of the relationship between different disease proteins, denoted disease interaction network. We demonstrated that the associations between diseases are directly correlated to their underlying protein-protein interaction networks. From disease-protein bipartite network, we derived diseasomal proteins (2).

Results

Both liver disease- and liver cancer-protein protein interaction networks showed a scale free network character in which a few highly connected hub proteins are considered an important factor. We derived liver disease- and liver cancer-disease networks from both related protein networks (Figure 1). From the liver disease-protein bipartite network, we obtained liver diseasomal proteins, BRCA, TP53, CTNNB1, JAK2, and PIKCA. All diseasomal proteins are hub proteins in liver disease-related protein network except BRCA2. We also elucidated liver cancer diseasomal proteins, APC, CTNNB1, PIK3CA, and TP53. All proteins are hub proteins in liver cancer-related protein network. These results demonstrate that hubs in the protein network tend to be diseasomal proteins in the disease network. Although we use the relatively small liver related disease networks, it is sufficient that the discovery of protein interaction networks assigned by diseases will provide insight into the underlying molecular mechanisms and biological processes in complex human disease system.

Innovative aspects

- Construction of disease-disease network from disease-related protein-protein interaction network
- From the liver disease-protein bipartite network, prediction of the diseasomal proteins

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(a)

(b)

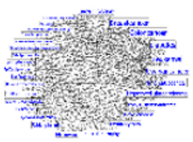

Figure1. Liver disease-related protein-protein interaction network (a) and liver disease network (b). Liver disease-related protein-protein network was constructed by using information of the liver disease-related proteins and protein-protein interactions. Liver disease network derived from the constructed disease-related protein network.

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Prostate Cancer Tissue Imaging

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Abstract: The diagnosis and treatment of prostate cancer (PCa) is largely dependent on clinical and pathologic staging based on cellular morphology of tissues using immunohistochemical methods. The recent advent of Imaging mass spectrometry (IMS) has proven it to be a powerful technique that enables us to identify and localize biological compounds directly on tissue without the need for radioactive or fluorescence labels or immunochemical reagents. Profiling and Imaging Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS) provides an effective means to measure and assess peptides and proteins present in a particular tissue. The aim of this study was to undertake experiments using imaging MALDI for the presence and localization of proteins/peptides in prostate cancer versus benign tissue.

Methods

MALDI has been utilized to provide high resolution spatial and chemical information. We obtained localized peptide and protein images from 12 µm thick human PCa and benign tissue sections. The sections were washed and allowed dry prior to coating with matrix solution alpha-cyano-4-hydroxycinnamic acid prepared in a mixture of 50% acetonitrile, 50% deionized water, and 0.2% trifluoroacetic acid which was deposited on the tissue sections by the ImagePrep (Bruker, Daltonic, Germany) vibrational coater. Using a raster of the tissue surface by a laser beam 60 microns in diameter, images of samples were produced over the m/z range 1000-100,000. Individual m/z values were then assembled from the mass spectra to produce selected m/z images.

Results

In this preliminary study we have employed MALDI imaging mass spectrometry (IMS) to investigate prostate cancer to compare proteins/peptides differentially expressed in diseased tissue with those in benign tissue. We will show multiple protein/peptide expression changes identified that correlated with the presence or absence of PCa,.

Innovative Aspects

- direct spatial analysis of peptides/proteins in intact tissues
- The importance of sample preparation conditions in order to get quality IMS data

C293

Serum Quantitative Proteomics of HCC with/without Recurrence after Liver Transplantation

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Introduction

Primary liver cancer is the third most common cause of death from cancer. The prognosis of hepatocellular carcinoma (HCC) remains poor. LTx (Liver transplantation) is considered as the best therapeutic option for cirrhosis-associated HCC. However, tumor recurrence and metastasis after LTx still remain a significant problem in patients with HCC. In order to improve the diagnosis and prognosis of HCC, it is mandatory to identify molecular tumor markers predictive of recurrence that can define a subset of HCC patients that stand to benefit from LTx.

Methods

Samples were obtained from HCC patients undergoing LTx in our hospital with complete clinicopathological and follow-up data, within UCSF criteria and sharing a similar disease background, which were categorized into two groups: recurrence and disease-free survival (DFS) groups. Samples were immunodepleted for 14 highly abundant serum proteins, and then labled with mass-balanced isobaric tags (iTRAQ), and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results

Totally 95 proteins with quantitation information were identified. Forty-eight of these proteins (50.5%) were displayed differentially in the recurrent group, compared to the DFS group. Among these 48 proteins, 11 were found to be up-regulated (>1.2-folds), 13 were down-regulated (<0.8-folds). All these proteins were classified according to their molecular function, using the tools at www.geneontology.org.Those were classified into eight groups: binding activity (32.56%), enzyme activity (25.58%), lipid metabolism (9.30%), transporter activity (6.98%), immune function (6.98%), complement function (2.33%), antioxidant activity (2.33%), and others (13.95%).

Innovative aspects

The results implied that iTRAQ combined quantitative proteomics have provided a special protein expression patterns in metastatic and recurrent states of HCC patients undergoing LTx. These differential proteins maybe serve as new prognostic biomarker and potential therapeutic targets particularly for HCC patients undergoing LTx, which warrants further investigation.

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C294

Search for a Protein Phosphatase(s) Responsible for Mst 1/2 Dephosphorylation in Cell Contact Inhibition.

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Introduction

Contact inhibition is a pause of the inhibition of cell proliferation by tight cell-cell contact and a fundamental characteristic of normal cells. But, most human cancer cells are refractory to contact inhibition. The STE20-family members purported Hpo orthologs, denoted Mst, are reported to be involved in the cell contact inhibition via phosphorylation of a down stream target protein(s) during cancer development. Here we searched for some protein phosphatases that may be implicated in the dephosphorylaion of Mst1/2 during contact inhibition. These results may indicate that the regulation of the relevant phosphatase(s) can offer a potential opportunity to interfere with cancer development through contact inhibition.

Methods

Mst and Yap were visualized by immunofluorescence and detected by SDS-PAGE and western blotting, respectively, in HeLa cells. To find proteins showing interaction with Mst1/2, we experimented using uv-cross linking method and co-immunoprecipitaion and identified the proteins by mass spectrometry.

Results

Most human cancer cells are refractory to contact inhibition possibly because of activation of phosphatase(s) which dephosphorylates Mst proteins. To search for the phosphatase(s) responsible for the Mst dephosphorylation, we used photo cross linking to covalently fix interactions between the two proteins in living cells. We observed that cross-linked dimmers were formed with photo amino acids (Fig. 1a). And to overproduce the Msts interacting with the partner phosphatase(s), we have cloned Mst1/2 genes into a polyhistidine tagging vector. Then the constructs were transfected into HeLa cells and the stable transfectants were selected through immune blot analysis (Fig. 1b). The lists of candidate phosphatases is shown in Table 1, which will be subject to confirmation studies for pin-pointing relevant one.

Innovative aspects

- A pin-pointing of a relevant phosphatase(s) with Mst may provide an important clue to interfere with cancer development via regulation of contact inhibition.

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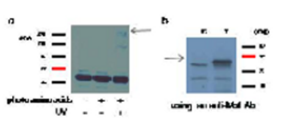

Figure 1. (a) HeLa cells were grown in limited media (without Lucine and Methionine) in the absence(-) or presence(+) of photo-Leu, photo-Met and then some of them were irradiated with UV(+). Cross-linked products formed after UV irradiation were detected by SDS-PAGE and WB. (b) Mst1/2 genes were cloned and transfected to overproduce the Msts. To purify the recombinant proteins and the partner phosphatase(s), co-immunoprecipitation was performed based on the purification on metal-chelating resins.

Table 1. Lists of the phosphatase candidates for Msts.

Accession no.	Identities	MASCOT Score	No. of unique peptides
IPI009025**	Serine/threonine-PP 0-alpha catalytic subunit	125	6
IPI000274**	PP 0, catalytic subunit, alpha isoform 3	123	3
IPI000443**	Isoform 3 of Type I 0-bisphosphate 4-P	60	3
IPI002955**	Receptor-type tyrosine-PP 0	55	5
IPI007459**	Protein tyrosine P, receptor type, 0 isoform a	53	5
IPI002987**	Serine/threonine-PP 0 regulatory subunit 10	48	7
IPI004801**	Isoform 1 of 0 P SAC2	48	8

C295

Secretome-based proteomic profiling of Ras-transformed MDCK cells reveals extracellular modulators of epithelial-mesenchymal transition

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Introduction:

Epithelial-mesenchymal transition (EMT) is a morphogenetic process involving an orchestrated series of transcriptional reprogramming and protein modification events that alter epithelial cell morphology, diminish polarity and adhesion, and enhance motility and invasiveness. Commonly described during embryonic development, fibrosis, and wound healing, EMT has been more recently implicated as a mechanism utilised by cancer cells during the early stages of metastasis. Despite significant progress made in identifying key molecular players and signalling pathways during EMT, a detailed knowledge of extracellular proteins involved in the process remains limited.

Methods:

To gain insights into proteins released from cells that modulate the EMT process, we developed a method to enrich for the secretome, a cellular sub-proteome comprising both secreted proteins/peptides, as well as membrane-derived nanoparticles termed exosomes, for proteomic analysis. Secretome samples from MDCK cells, and MDCK cells undergoing oncogenic Ras-induced EMT, were analysed by 2-D Fluorescence Difference Gel Electrophoresis (DIGE), as well as 1-D SDS-PAGE-based proteomic profiling. Subsequent LC-MS/MS was performed using Orbitrap instrumentation for protein identification. Proteomic findings were further substantiated by mRNA gene expression profiling using Affymetrix GeneChip microarrays. Functional contribution of extracellular modulators during EMT was confirmed using siRNA technology.

Results:

DIGE analysis revealed 47 secretome proteins to be differentially expressed during Ras-induced EMT. Label-free spectral counting identified 70 proteins with extracellular annotation to be significantly dysregulated following Ras-transformation. Strong concordance (~70%) was observed between proteomic analyses, as well as protein and mRNA transcript expression levels during EMT. Ras-transformed MDCK cells reduce expression of cell-cell and cell-matrix adhesion proteins, as well as structural components of the basement membrane. Concurrently, expression of proteases, motility factors, and extracellular matrix constituents that promote cell migration were increased during EMT. Enhanced cell migration of Ras-transformed MDCK cells was attenuated via RNA interference to key extracellular EMT modulators.

C296

Effect of TIMP-1 Expression Level on Cancer Cell Growth and Survival

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Introduction
Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a glycoprotein that is a natural inhibitor of the matrix metalloproteinases (MMPs), a group of peptidases involved in degradation of the extracellular matrix. TIMP-1 can regulate cell survival, proliferation and apoptosis independent of their MMP inhibitory activity. In this study, we regulated the expression level of TIMP-1 in Hep3B cells through TIMP-1 overexpression and down regulation by shRNA, in order to investigate the effects of TIMP-1 level on cell growth and apoptosis. In addition, phosphopeptides involved in TIMP-1 triggered cell survival signaling were monitored.

Methods
Effects of TIMP-1 expression level on cancer cell was induced apoptosis by staurosporine and viability was test by MTT assay, and anoikis was investigated following culture in the soft-agar plates (Figure 1). The dependence of the dynamism of phosphoproteome on TIMP-1 level was also monitored by TiO_2 column chromatography and nano-LC-FTICR/LTQ mass spectrometry (Table 1, 2).

Results
Effects of TIMP-1 level and/or TIMP-1 glycosylation mutants on cell growth and apoptosis revealed that TIMP-1 overexpression enhanced the rates of cell growth and cell survival while TIMP-1 knock-down reduced the rates in Hep3B. Search for dependence of the dynamism of phosphoproteome on TIMP-1 level indicated that a high TIMP-1 level confers the benefits for cancer cell growth and survival during cancer progression via modulation of phosphoproteome in cancer cells. However, more details of the effects of TIMP-1 in on cancer cell survival need to be scrutinized through further molecular study.

Innovative aspects
- Protein glycosylation affects to the efficacy of TIMP-1 as a growth stimulatory trigger.

References
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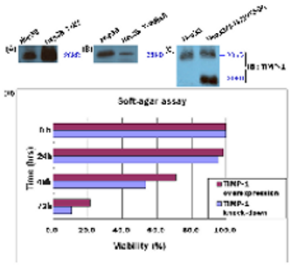

Figure 1. Establishment of stable transfectants of TIMP-1
(A) Stable cells with TIMP-1 overexpression (B) TIMP-1 knock-down and (C) a glycosylated TIMP-1 expression were confirmed by immunoblot. (D) effect of TIMP-1 expression on anoikis was investigated following culture in the soft-agar plates.

Table 1. Lists of phosphopeptides founds in TIMP-1 overexpressing Hep3B cells.

Accession no.	Identities	MASCOT Score	No. of unique peptides
IPI00018***	OOO receptor coactivator 2	64	9
IPI00293***	OOO novel protein	77	8
IPI00019***	OOO family member 1	72	6
IPI00306***	OOO receptor-related protein 4	65	8
IPI00455***	OOO of extracellular matrix protein	98	22

Table 2. Lists of phosphopeptides founds in TIMP-1 silencing Hep3B cells.

Accession no.	Identities	MASCOT Score	No. of unique peptides
IPI00738***	Isoform 4 of protein kinase OOO	91	6
IPI00296***	OOO complement component OO	78	6
IPI00001***	OOO translational activator OOO	80	10
IPI00167***	OOO isoform 7 of OOO activating death domain protein	76	12

C297

Proteomic approaches to the identification and quantification of the protein tyrosine phosphatase (PTP) "redoxome"

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Introduction

Recent studies suggest that reactive oxygen species (ROS), particularly H_2O_2, act as intracellular second messengers in many signalling pathways, including those for PDGF and EGF[1,2]. PTPs are believed to be primary targets of ROS. PTPs contain a conserved catalytic cysteine with an unusually low pKa (4.5-5.5)[1,2]. This property facilitates nucleophilic attack on substrate phosphotyrosyl residues, but also renders PTPs highly susceptible to oxidation[1,2]. We are interested in developing global proteomic approaches to identify and quantify PTP oxidation in both physiological and pathological signaling.

Methods

Oxidized PTPs are unstable and cannot withstand direct isolation; hence, an indirect approach was applied to isolate them. Ligand-induced PTP oxidation results in two pools of PTPs: oxidized (PTP-SOH; inactive) and reduced (PTP-S-; active). Active PTPs were irreversibly alkylated (PTP-S-Alkyl; resistant to any further modification), while oxidized PTPs are resistant to alkylation. Oxidized PTPs were reduced and subsequently oxidized to the sulfonic acid state (PTP-SO_3H). Oxidized PTPs were then isolated using the monoclonal oxidized PTP active site antibody (oxoPTP Ab)[3]. The relative levels of PTP oxidation were then quantified using multiple reaction monitoring (MRM). This protocol can also be adapted to profile the PTPome in any given cell type or tissue.

Results

A limitation to understanding the roles of reactive oxygen species (ROS) in cell signaling has been identifying the PTPs inactivated by ROS in specific signaling pathways. Given their potential toxicity, it seems likely that ROS generation is highly controlled within cells to restrict oxidation of those PTPs that must be inactivated for signalling to proceed. Thus, identifying ROS-inactivated PTPs may be tantamount to finding the PTPs that critically regulate a specific signaling pathway. We have developed the first global proteomic method to identify and quantify PTPs that undergo redox regulation. Since the oxoPTP approach is an indirect method to detect PTP oxidation, the method was applied to profile PTP expression in a number of cell lines and tissues, where 32 of the known 38 PTPs were monitored. The relative susceptibility of all PTPs expressed in NIH3T3 cells was monitored in response to a range of H_2O_2 concentrations. Each PTP showed a differential susceptibility to oxidation. Lastly, the basal oxidation state in a number of different cancer cell lines was determined. Not surprisingly, there was a notable difference in the oxidation state of all monitored PTPs.

Innovative aspects
- OxoPTP Ab robustly identifies PTPs in cell lines and tissue
- Successfully developed the first global proteomic method to identify and quantify PTP expression and oxidation
- Application of the method include profiling PTP expression and quantifying PTP oxidation in normal, transformed and growth factor stimulated cells

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C298

Proteomic Investigation of the Mechanism of Therapeutic Decoys for Prostate Cancer

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Introduction
Androgen receptor (AR) regulates genes involved in proliferation and survival of prostate cancer. Current therapies for prostate cancer target the AR C-terminus. AR is a phosphoprotein that interacts with 160 proteins. Transcriptional activity resides in the AR N-terminal domain (NTD) that contains most of the phosphorylation sites and is essential for protein interactions. A decoy encoding amino acids 1-558 of the AR NTD inhibits the growth of prostate cancer (1). Here, we map the regions of the NTD as therapeutic decoys and elucidate the underlying mechanisms using proteomics approaches.

Methods
Construction of the viral vectors to express smaller regions of the AR NTD (decoys) were applied to LNCaP prostate cancer cells in tissue culture (*in vitro*) and in xenografts in mice (*in vivo*) to assess their efficacy. Proteins interacting with the decoys and changes in the phosphorylation state were identified. Proteins were immunoprecipitated and immunocomplexes were analyzed by mass spectrometry. For in vivo therapeutic response, serum prostate-specific antigen (PSA), tumor volume, and protein expression were measured in male NOD-SCID mice bearing LNCaP xenografts treated with decoy. Biological activity and expression of decoy molecules were evaluated using luciferase reporter gene assays and western blot analysis.

Results
We have identified that decoys encoding 1-233 and 234-391 amino acid regions of the AR NTD have antitumor activities. Both decoys inhibited the growth of xenografts in castrated mice. Temporal changes in phosphorylation of serine 210/213 in AR NTD were shown by western blot analysis. Serine 94 in AR 1-233 was also identified in forskolin-treated cells (Fig 1). Forskolin is known to transactivate the AR NTD. Additionally, proteins that interact with decoys were identified by immunoprecipitation followed by mass spectrometry analysis. Transcription factors and coregulator proteins known to interact with and modulate AR activity were also detected. In conclusion, decoys of the AR NTD are effective in blocking activation of the full-length AR and show therapeutic response in vivo for prostate cancer. The mechanism is suggested to involve competition of the decoy for proteins required for activation of the full-length AR by preventing or altering phosphorylation and interaction with proteins. These data begin to elucidate potential novel targets for the development of new therapeutics for prostate cancer.

Innovative aspects
- Paradigm shift in prostate cancer therapy from the C-terminal ligand-binding domain to the NTD of the AR
- Mapping the region of the AR NTD required for inhibition and evaluation of phosphorylation events in the decoy molecules
- Identification of proteins that interact with the decoy molecules

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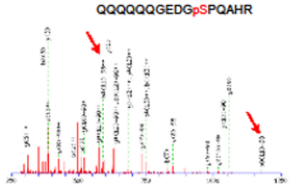

Figure 1. MS/MS spectrum of the phosphorylated peptide (QQQQQQGEDGpSPQAHR, *m/z* 634.69) of the decoy molecule identified from forskolin-treated LNCaP cells.

C299

The novel human gene UBE_2Q_2 and its protein product are up regulated in breast cancer tissues.

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Introduction
The ubiquitin-proteasome pathway facilitates the degradation of damaged proteins and regulates growth and stress response. The activation of this pathway in various cancers and malignancies including breast cancer is established. According to our previous report (1), the novel human gene, UBE_2Q_2, with a putative ubiquitin-conjugating enzyme activity, is located on chromosome 15, and over expressed in tumor mass and invasive epithelium in head and neck squamous cell carcinoma. Here, we have investigated the expression patterns of UBE_2Q_2 gene in both normal and cancerous tissues of human breast.

Methods
We have used real time PCR to investigate the expression levels of UBE_2Q_2 gene in a collection of 25 breast cancer tissues matched with normal adjacent counterparts. Immunohistochemistry was also performed on formalin-fixed paraffin-embedded tissue sections using a rabbit polyclonal antibody that we generated against an amino acid sequence predicted from the DNA sequence of UBE_2Q_2 gene.

Results
Increased expressions of UBE_2Q_2 gene (8.8±2.83 folds) were observed in 76% of tumor samples when compared with normal tissues. Consistently, mmunohistochemistry data showed a higher level of immunoreactivity for UBE_2Q_2 protein in invasive epithelium of cancerous tissues when compared with that in the normal epithelium.

Innovative aspects
- This is the first report which has demonstrated the expression of UBE_2Q_2 gene in both normal human breast and its cancerous counterparts.
- Expression pattern of UBE_2Q_2 protein has been shown in both affected and unaffected tissues of breast cancer.

References
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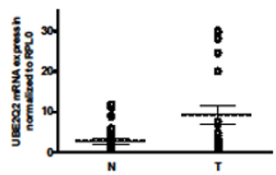

Figure 1. Relative expression of UBE_2Q_2 mRNA in cancerous tissues (T) against corresponding normal tissues (N). UBE_2Q_2 mRNA was measured using a quantitativeRT-PCR. Expression of mRNAs was quantitated as a ratio to the amount of RPL0.
* $p < 0.001$ by the Mann-Whitney test. Horizontal lines indicate mean±SEM value of each group.

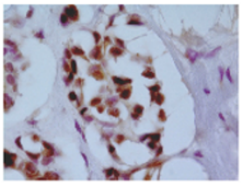

Figure 2. Cytoplasmic and nuclear immunohistochemical detection (X 700) of the protein product of UBE_2Q_2 gene in cells of human breast.

C601

Proteomic Identification of RKIP as A Metastasis Suppressor Protein in Nasopharyngeal Carcinoma

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Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in southern China. Early metastasis is one of distinctive characteristics of NPC and the main cause of death for NPC patients. Although numerous efforts have been made to reveal the molecular mechanism of NPC metastasis, it remains poorly understood. Identification of NPC metastasis-related molecules will be helpful for diagnosis and treatment of NPC, and may provide new insights into its metastasis.

Methods

2-DE was performed to separate the total proteins from 10 paired NPC and adjacent non-cancerous nasopharyngeal epithelial tissue (ANNET), image analysis was used to find the differential protein spots between the two types of tissues, and both MALDI-TOF-MS and ESI-Q-TOF MS/MS were performed to identify the differential proteins. As a result, 21 differential proteins were identified, and Raf kinase inhibitor protein (RKIP) was significantly downregulated in NPC as compared with ANNET. To explore the roles and mechanisms of RKIP in NPC metastasis, Western blotting and immunohistochemistry was respectively used to detect RKIP expression in 5-8F and 6-10B NPC cell lines with the different metastatic potentials, and in NNET, primary NPC and NPC metastasis. Furthermore, high metastatic 5-8F with low RKIP expression and non-metastatic 6-10B with high RKIP expression were stably transfected with plasmids that expressed sense and antisense RKIP cDNA, respectively, or with empty vector. The effects of RKIP expression on *in vitro* cell invasion, and the activity of Raf-1/MEK/ERK and NF-κB signaling pathway were analyzed in the transfected cells.

Results

The results showed that RKIP was significantly downregulated in 5-8F compared with 6-10B, in NPC compared with NNET, and not detectable in NPC metastasis. Overexpressed RKIP in 5-8F could decrease its *in vitro* cell invasion, whereas downregulated RKIP in 6-10B could increased its *in vitro* cell invasion. RKIP negatively regulated Raf-1/MEK/ERK and NF-κB signaling pathway in NPC cells, and activation of the two signaling pathways by RKIP downregulation increased *in vitro* invasion of NPC cells.

Innovative aspects

- Decreased RKIP expression is associated with the increased invasive capability of NPC cells possibly through the activation of Raf-1/MEK/ERK and NF-κB signaling pathway.
- It is first time report that RKIP may be a NPC metastasis suppressor.

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C602

The Functional Role of PI3K for Personalized Cancer Treatment by Label-Free Targeted Proteomics

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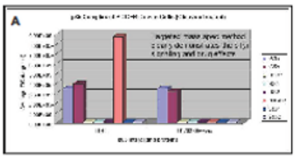
Introduction. The insulin signaling pathway in eukaryotes is central to gene translation, cell growth and proliferation. Central to this pathway is the activation of AKT by phosphoinositide-3-kinase (PI3K). We have established a simple label-free targeted proteomics method using an Orbitrap to probe the functional role of PI3K by quantifying the receptor tyrosine kinases (RTKs) and adaptor proteins that PI3K utilizes in various cell lines and tissues. This information will eventually help us choose kinase inhibitor drugs to treat patients through a personalized approach.

Methods. For the PI3K immunoprecipitations (IPs), a polyclonal antibody against the p85α regulatory subunit of PI3K was used to IP the catalytic p110 and p85 regulatory subunits of PI3K. The protein complex was run partially into a SDS-PAGE gel just to resolve IgG heavy chain. The gel piece above 55kDa was excised and digested with trypsin in order to avoid suppression from antibody contamination. A list of twenty *m/z* values representing eight known p85 binding proteins that primarily bind through phosphotyrosine residues and SH2 domains including p85 as a control were used for a one hour Targeted Ion MS/MS (TIMM) experiment. Samples were run using an EASY-nLC nano-HPLC and an Orbitrap XL-ETD mass spectrometer. The MS/MS data were searched against the reversed Swiss-Prot database using Sequest. PI3K interacting proteins were quantified by averaging the MS/MS TIC from all targeted peptide hits using in-house developed software called *NakedQuant* that was specifically developed for MS/MS based label-free quantification.

Results. The targeted and quantitative proteome surrounding the PI3K complex was established using label-free proteomics and different cancer cell lines and tumor tissues showed very different patterns representing the cell's abnormalities that drive proliferation and tumor growth. This method replaces the equivalent of performing at least eight different Western blots to study the functional role of PI3K. Figure 1a shows that H1703 cells are driven by PDGF binding directly to p85. In this case, the PDGFR inhibitor Gleevec was used to inhibit the growth of these cells or in the case of a patient tumor, Gleevec could be used to stop cancer growth. In other cell lines, other PI3K adaptors drive AKT activation such as IRS proteins or Gab proteins. In these cases, other RTK inhibitors such as Met or EGFR inhibitors may be necessary to stop cancer cell proliferation. We have also been successful in getting the strategy to work in mouse Xenograft models with cells that show an ALK translocation (Figure 1b). We show that treatment of the mouse with an ALK inhibitor shuts down binding of p85 to IRS1 and IRS2 and inhibits AKT. Additional cell lines and tissues show different PI3K profiles.

Innovative aspects

- A novel label-free and targeted proteomics approach to assessing the functional role of signaling proteins and their protein interactions
- Establishment of a proteomics based assay to study signal function that can be directly applied to treating patients via "personalized medicine"



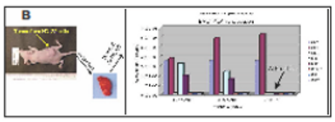


Figure 1 (A). Quantified PI3K protein complex from H1703 cells via a label-free Orbitrap MS/MS functional assay. (B). Mouse Xenograft model of PI3K complex with and without kinase inhibition.

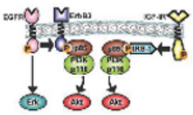


Figure 2. The PI3K-AKT pathway surrounding the p85 bait protein.

References

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C603

Early-Stage Breast Cancer Patients Have Active Degradome and Rich Blood Peptidome

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Introduction

Proteases are well-established components of cancer progression and invasiveness. Human blood peptidome contains many endogenous proteolytic peptides resulted from various exoprotease activities, which may provide tumor-specific information for clinical applications. We developed a novel strategy for comprehensive top-down characterization of the blood plasma peptidome, and discovered that early-stage breast cancer patients have a much richer peptidome compared to healthy women. The endogenous proteolytic peptide "signature" can serve as a potential surrogate for breast cancer biomarkers.

Methods

One pool of plasma samples from 15 early-stage breast cancer patients and another from 15 age-, race- and menopausal status-matched healthy women were used. The blood plasma peptidome was isolated through application of immunoaffinity depletion followed by size exclusion chromatography (species <20 kDa were collected), which provides an enrichment factor of >300. The intact peptidome components were then separated by ultra-high pressure LC with a peak capacity of ~300 and analyzed by LTQ-Orbitrap using high resolution MS/MS (Fig. 1). Both database searching- and de novo sequencing-based unique sequence tags (UStags) methods (1) were used for the identification of the peptidome components and their modifications. Random false hit rate was evaluated using the decoy database approach.

Results

A total of >1000 unique peptides (modified and unmodified) originated from ~75 proteins were identified from single analyses of the cancer and normal pools. This is significant in that only 35 proteins have been previously reported from a total of 25 published top-down proteomic studies of serum and plasma samples. Additionally, error-free identification for every single plasma peptide was achieved by combining the specificity for generating UStags with additional stringent mass restrictions. Strikingly, breast cancer plasma peptidome contained 2X more peptides than that in its normal counterpart, although most of the proteins from which the peptides originated were observed in both samples. The "extra" peptides observed in breast cancer blood were mainly contributed by a handful of specific blood plasma proteins. The degradation of these proteins by tumor-specific protease activities, as well as the content and characteristics of the resulting peptidome products hold great promise for surrogate biomarker discovery towards early detection of breast cancer and for gaining valuable insights on cancer pathogenesis and progression.

Innovative aspects

- Novel strategy for effective enrichment and unambiguous identification of plasma peptidome components
- Unparalleled coverage for blood plasma peptidome using top-down approach
- Many novel PTMs and point mutations identified for plasma peptidome components

References

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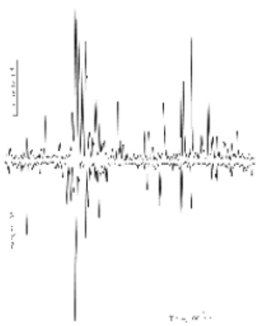

Figure 1. LC-orbitrap MS analysis of peptidome samples enriched from pooled plasma samples of early stage breast cancer patients (upper trace) and age-, race- and menopausal status-matched healthy controls (lower inversed trace).

C604

Analysis of Serum Peptidome in Cancers from Digestive System

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Introduction

The amount and repertoire of serum peptides probably reflect the physiological or pathological state of an individual. Tumor-specific pattern of serum peptides have been reported to serve as powerful biomarkers to distinguish different cancers that are different anatomical system-derived. However, how does the serum peptidome express in the cancers that develop in the same anatomical system remains unknown. In this study, we are aiming at evaluate the serum peptidome as a cancer biomarker in gastric cancer (GC) and colorectal cancer (CRC) that both come from the same digestive system.

Methods

Serum from 59 normal males and females, 57 GC patients and 82 CRC patients were collected and stored according to a standardized procedure. The peptides fraction and profiling were done by combination of hydrophobic bead and high throughput MALDI-TOF/TOF mass spectrometer. Peaks detection was done by ClinProt™ bioinformatic software. Mann-Whitney U test was performed between GC and CRC group and among some subsets defined by gender, age, clinical stage, and pathological differentiation. Two optimal subsets of peaks was selected by using the newly proposed CARS method and pattern recognition equations were established through partial least squares-discriminant analysis (PLS-DA).

Results

Using this approach, 161 normalized peptide peaks were detected totally .The number of peaks with *p* value less than 0.01 in gender, age, clinical stage, and pathological differentiation subsets were all <=2.5% of the total number. More than 40 peaks are significantly different ($p<0.00001$) between normal and cancer group (GC or CRC). Conversely, no peptide is obviously different between GC and CRC group ($p>0.01$). Comparing the 161 peaks of CRC group and normal group, an optimal subset of 5 peptide peaks is selected by CARS. A discriminant equation using these 5 peaks (Z_1=0.5733X_{70}+0.4129X_{80}+0.5388X_{84}-0.4420X_{106}-0.4899X_{120}) is established to distinguish CRC from normal with a 10-fold cross validation accuracy of 95.1%(Figure 1). Another discriminant equation (Z_2=0.4835X_{44}+0.5390X_{93}+0.5189X_{119}-0.4614X_{127}-0.4317X_{137}) is established in the same way and obtains a 10-fold cross validation accuracy of 96.6%. in distinguishing GC patient from normal person.

Innovative aspects

- Exclude the biases caused by gender, age, clinical stage and pathological differentiation;
- Cancers from the same anatomical system may be with the same or similar serum peptidome.
- Establishment of two discriminant equations for CRC and GC with dramatic diagnosis efficiency.

References

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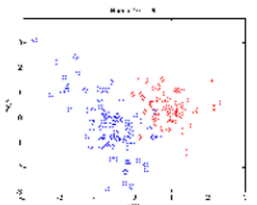

Figure 1. The score plot of partial least squares-discriminant analysis of the normal group (red, n=59) and CRC group (blue, n=82) using the 5 peaks (X_{70},X_{80},X_{84},X_{106},X_{120}) selected by CARS.

The Sea Around: Can the Secretome of Tumor Cells be a Mimetic Microenvironment? The Core Metastatic Signature Initiative

C605

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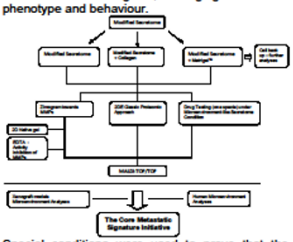
Introduction

On a multi-step cancer development, invasion and metastasis acquaintance are the darkest sides of the molecular events that orchestrate malignancy. As metastatic events still an unknown frontier on cancer progression, all efforts to elucidate the very early steps and development of this stage, are extremely relevant. As all cancer biologists and physicians agree, to set hallmarks along metastatic development, specially on the it very beginning, is to guarantee a better prognosis and an efficient tailor-made treatment for those patients that have higher chances on *ex situ* occurrences. Within an experimental progressive point of view, we develop The Core Metastatic Signature Initiative that aims to comprehend the very early stages that coordinate invasiveness and metastasis, evaluating from simple models (*in vitro*) to very complex ones (*in humans*), under an integrative approach, in order to find markers on these differential events, within different conditions.

Methods

The first part of this Initiative is to evaluate *in vitro*, the immortalized tumor cell's secretome, under the comprehension that, it truly acts as a mimetic microenvironment, enfolding the tumor cells as a sea of exocited signals, changing the cell´s phenotype and behaviour.



Special conditions were used to prove that the secretome is an information bridge, similar to tumoral microenvironment. We experimentally incorporated in the cell culture, typical elements of the stroma - collagen and Matrigel™ – using the original secretome, as the negative control. We also performed a novel data assessment to test a single drug in the presence of added stromal elements. To evaluate the proteins profile under each condition, Proteomic tools, such as in-gel activity assays, 2D gels and MALDI TOF/TOF spectrometry were used.

Results

As we compare the different situations, differential profiles were analysed and the results enforced the idea of a secretome as a microenvironment-like. Deriving, with the incorporation of typical stromal elements, the mimetic secretome may be far more suitable for *in vitro* analyses. Potentially, the outcomes may open a broader scope regarding to *in vitro* tumoral drug test screenings, as the pro-drug could acts not in the tumor *per si*, but in some element of the secretome, operating in upper cascades, preventing downstream pathways. This point of view, widespread the target´s range for a drug´s candidate, enriching the knowledge about the molecular mechanisms of metastatic events, from its early beginnings, as well providing some light and accuracy on the possible mechanisms of *in vitro* drug tests.

Innovative aspects

- The Core Metastatic Signature Initiative as a progressive approach to establish a core signature to invasion and metastasis;
- A novel data assessment of *in vitro* drug tests;
- An attempt to elucidate some of the molecular mechanisms involved on metastatic events, from simple to complex models.

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Systematic -omics Analysis of HBV-associated Hepatocellular Carcinoma

C606

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Hepatocellular carcinoma (HCC) is a kind of malignant tumor with high morbidity and mortality. In China the chronic infection with hepatitis B virus (HBV) is one of the major risk factors. To clarify the carcinogenesis of HBV-associated HCC, and to diagnose HCC at an early stage, we initiated the systematic-omics research of HBV-associated hepatocellular carcinoma at the levels of genomics, transcriptomics and proteomics.

Genomics studies have been made to find out comprehensively susceptibility genes in the process from HBV infection to HCC onset. The T29C polymorphism in ESR1 gene, together with other two polymorphisms ((TA)n repeat in promoter region, and PvuII RFLP in intron 1) were associated with susceptibility to HBV-related HCC and that the haplotypes derived from the polymorphisms in strong LD in the ESR1 gene were also shown to be associated with the risk of HBV-related HCC. And our unpublished data showed that EGFR 521R allele might be another genetic marker for the development of HBV-related HCC. However, functional polymorphisms in the promoters of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12 and MMP-13 are not associated with HCC risk. Replication in independent populations and genetic susceptibility studies in genome-wide association studies of the polygenes involved in HBV-related HCC have been on the way in our lab.

Proteomics researches on cell lines, tissues and sera were carried on to elucidate the dysregulated proteins in HBV-associated HCC, which could be used as candidate protein markers for diagnosis and therapy. Secreted proteins from HBV genome-integrated hepatoma cell line HepG2.2.15 were subtracted with those of its parent cell line HepG2, and 69 unique proteins highlighted by chaperone machinery proteins were identified in HepG2.2.15. The expression level of three unique proteins was compared in sera from healthy control, chronic hepatitis B and HCC to show their diagnosis potential in chronic hepatitis B or HCC. HCC tumor tissues and adjacent non-tumor tissues were compared using DIGE (fluorescent difference gel electrophoresis), and the over-expression of two up-regulated proteins (HOP and hnRNP C1/C2), was further confirmed by immunohistological analysis in 70 HCC patients. Sera from AFP negative HCC patients were analyzed by ClinProt and a peak pattern with more than 90% sensitivity and specificity was discovered. The validation of those dysregulated proteins in larger serum samples was going on.

To understand systematically the earlier events of HCC metastasis, 4 HBV induced hepatocellular carcinoma cell lines (MHCC97L, MHCC97H, HCCLM3 and HCCLM6) with the same genetic background and progressive metastatic abilities were selected for transcriptome and proteome investigation synchronously. Data sets of other non-metastatic samples were also integrated, including transcriptome of Hep3B (an HBV-induced hepatocellular carcinoma cell line without metastasis ability), transcriptome and proteome of normal human liver tissue and LCM-collected hepatocyte. Transcripts and proteins associated with metastasis were extracted and analyzed using bioinformatics (mathematics) artifices and systems biology strategies. Some of them were found with good agreement with previous findings and others have never been reported before. Patterns in transcriptome and proteome showed a general tendency with a few explainable modes. Follow-up experiments that based on -omics screening results had been carried out in both biopsy sample validations and cell line perturbations.

Cardiovascular Proteome Biology and Medicine

(C301 – C324)

C301

Targeted Proteomic Analysis of Potential Biomarkers of Hypertrophic Cardiomyopathy

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Introduction
Hypertrophic cardiomyopathy (HCM) is a complicated disease of the myocardium and is associated with high risk of sudden death, particularly in young people (1). Currently there is not a rapid and reliable method for simple detection of HCM from human plasma in clinical practice. However, several protein candidates are suggested as potential plasma markers of HCM (2). We have tested whether these proteins can be validated by Multiple Reaction Monitoring (MRM).

Methods
Plasma samples from patients and control group were collected and processed for subsequent analysis. FASTA format of candidate proteins was used for creating MRM transitions in TIQAM digestor. Data acquired on 4000 QTRAP (Applied Biosystems) were processed using TIQAM viewer. The correct assignment of peptide sequence was confirmed by MIDAS™ (MRM Initiated Detection And Sequencing) and final MRM method for analysis of HCM related peptide was created.

Results
Using TIQAM software, we created a MRM method for detection of proteins of interest and we verified this method using depleted and non-depleted plasma samples obtained from patients and control group.

Conclusion
We have used targeted proteomic approach as a suitable tool for validation of large number of potential biomarkers of various diseases and subsequent quantitative studies for analysis of proteins possibly involved in HCM.

Innovative aspects
- MRM method for validation of potential biomarkers of hypertrophic cardiomyopathy

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Acknowledgements
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C302

A Proteomic Study of Hypertension using Quantitative Assays on a Congenic Rat Model

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Introduction
When a disease has many contributing and exacerbating environmental factors entwined with underlying genetic traits, such as in hypertension, then any single proteomic assay will struggle to identify useful biomarkers. Through the collection and multi-level analysis of biological fluids and tissues a fuller understanding of the disease could be obtained. Plasma, urine, kidneys, hearts and brains were collected from 21 week old rats that were either wild type, disease prone or a congenic strain. For induction of hypertension a salt treatment was started at 18 weeks for half the cohort.

Methods
Body fluids were enzymatically digested before being separated and analysed by reverse phase LC-ESI-MS (3000 Dionex; HCT & microTOF, Bruker; QSTAR, AB), with label-free quantitation (Decyder MS, GE Healthcare) and subsequent targeted MS-MS analysis.
Tissues were section by cryostatic microtome and thaw mounted onto MALDI targets for mass spectrometry imaging (MSI) or standard glass slides for macro dissection followed by minimal labelling 2-D gel electrophoresis (DiGE). MSI performed at 15-150µm resolution using a 10µm diameter laser (Ultraflex III, Bruker).
A total 6 cohorts were analysed, salt treated vs non-treated for wild type (WKY), Spontaneous Hypertensive Rat Stroke Prone (SHRSP) and a Congenic strain with SP background (SP.WKYGLA2K).

Results
MS based label-free analysis of plasma detected >12,000 features which were classified by both pairwise T-test and by Rank Product (adapted from the method applied to microarray experiments) (1), both with a false discovery rates of 5%. Comparisons were made within the salt/no-salt cohorts, between pairs of strains (Fig. 1). The Rank Product approach characterised a similar number of features to be targeted for MS-MS identification as T-test, but importantly there was little overlap between the selected datasets.
MALDI MSI and subsequent off-tissue MS-MS identification of proteins aided in the targeting of tissue regions for macro dissection of material for the DiGE analysis, as well as providing a simple molecular histological comparison between the strains and treatment cohorts. Additionally, the tissue bathing solutions used in MSI preparation provided a further fluid that was analysed by MS label-free quantitation
DiGE protein intensity profiles aided in the identification of proteins exhibiting intermediate expression in the congenic strains.

Innovative aspects
- Application of statistical methods to MS label-free data that are normally applied to microarray experiments
- Near cellular resolution (15µm) MALDI mass spectrometry imaging used to target tissue regions for DiGE

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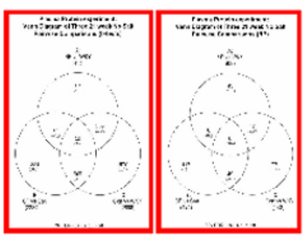

Figure 1. Examples of Venn diagrams grouping peptides of significant variance (as classified by different statistical tests) between treatment cohorts, as well as between strains.

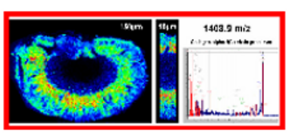

Figure 2. Example of increased spatial resolution obtained when kidney imaged at 15µm raster and example of off-tissue MS-MS identification.

C303

Differential Proteomic Analysis of the Plasma of Swines with Chronic Myocardial Ischemia

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Title: Differential Proteomic Analysis of the Plasma of Swines with Chronic Myocardial Ischemia

Introduction To identify and explore the specific protein markers for chronic myocardial ischemia .We used an established swine model with myocardial ischemia to explore the differential expressed proteins by 2D-DIGE.

Methods Three pigs were instrumented with a size-matched Ameroid constrictor on the anterior descending branch under general anesthesia in sterile conditions, and a sham group was also included (n=3). The chronic myocardial ischemia was evaluated by coronary arteriography and echocardiogram. It was proved that the time window from 30-day to 60-day after operation is the stable state for myocardial ischemia. Blood was obtained from the model group and control group at 0d, 30d and 60d after operation. Plasma samples were centrifuged and then stored at -80℃. All plasma samples were depurated with 2D-Cleanup Kit, and were quantitated with 2D-Quant Kit. The plasma samples were examined by the fluorescence differential in-gel electrophoresis (2D-DIGE). Analysis with Decyder software revealed signicant differential proteins spots which were identified by MAIDI-TOF/TOF.

Results
There were 25 proteins having significant differences between the groups. MAIDI-TOF/TOF successfully identified 15 proteins. The proteins up-regulated at the point of 30-day were haptoglobin, Phosphoribosyl pyrophosphate synthetase-associated protein 1(PRPPs1), and the protein down-regulated at the point of 30-day was Nicotinic acid receptor.The protein was up-regulated at the point of 60d were O-fucosylpeptide-3-beta-N-acetylglucosaminyltransferas, and the protein down-regulated at the point of 60d was: S-adenosylhomocysteine hydrolase (SAHH). Some protein were up-regulated at the points of 30d and 60d: Ch4 and secrete domains of swine IgM, myosin heavy polypeptide 2, Ppfibp1 protein, and some protein were down-regulated at the points of 30-day and 60day: hemopexin , albumin.

Innovative aspects
The detected differential expressed plasma proteins of chronic myocardial were associated with inflammatory reactions and energy metabolism. The differential proteins at 30d point were associated with inflammatory reaction principally, and the differential proteins at 60d point were associated with the formation and metabolism of adenosine.

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IEF-LC-MALDI Analysis of Rat Model of Chronic Heart Failure Using iTRAQ Labels

C304

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Introduction:
Chronic heart failure (CHF) is one of the most common, deadly and costly diseases, affecting between 1-2% of adult European population. Despite applications of modern therapeutic approaches, 50% of CHF patients die within 4 years from the initial diagnosis. To get a deeper insight into virtually unknown molecular pathology of CHF, we performed proteomic analysis of failing cardiac tissue obtained from rats with advanced CHF, induced by chronic volume overload due to arterio-venous fistula (AVF).

Methods:
Arterio-venous fistula was performed in 10 weeks-old Wistar male rats using needle technique (1). In this model, animals develop asymptomatic cardiac hypertrophy soon after induction of AVF, that gradually progress into heart failure by 20-26 weeks after AVF induction. We analyzed cardiac samples from control (sham-operated) animals and AVF and animals in decompensated phase of CHF. Protein samples from left ventricles of 6 AVF and 6 sham-operated animals were digested with trypsin and labeled with iTRAQ reagents. Labeled peptides were separated first by peptide isoelectric focusing on immobilized pH gradient strips and then on nano-RP-LC. Eluting peptides were robotically collected, mixed with matrix and spotted on MALDI plates.
In parallel, we performed gene expression profiling of the same myocardial samples using RatRef-12 BeadChip (Illumina).

Results:
At 24th week, all AVF animals showed clinical signs of heart failure (dyspnoea, lethargy, ascites). AVF rats had 2.4x elevated heart mass/body weight and pulmonary congestion as evidenced by 1.7x increase of lung weight.
In myocardial samples, twice repeated LC-MALDI analyses (ABI 4800 MALDI/MS) of total 64 IEF fractions obtained from 2 IPG strips resulted in collection of ~28 000 MALDI spots. Approximately 78 000 MS/MS collected spectra provided high-confidence identification and relative iTRAQ-based quantitation for 1410 proteins using Protein Pilot software. Thirty nine proteins were considered to be differentially expressed in AVF hearts compared to sham-operated controls.
The list of differentially expressed proteins was correlated with the mRNA data from our microarray experiment performed with samples from identical animals.
Most proteins identified as differentially expressed in our proteomic analysis were also differentially expressed (in the respective "up" or "down" direction) at the mRNA level. Among the differentially expressed proteins clearly dominate enzymes involved in fatty acid oxidation and several structural, stress and regulatory proteins.

Innovative aspects:

- Employment of advanced model of chronic heart failure
- Application of a shot-gun method so far unused in heart proteomics
- Correlation with mRNA data

References:

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Indepth Proteomic Analysis of Coronary Vasculature

C305

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Introduction
Atherosclerosis results in plaque development which can lead to occlusion of the coronary vessel. Previously we have reported decreased expression of a cytoskeletal protein, Hsp27 within the vasculature of patients with transplant atherosclerosis. Here, we wish to compare and analyse diseased and control vessels to determine a possible cardioprotective role for proteins in native atherosclerosis.

Methods
Coronary arteries were isolated from both control individuals (unused donors for heart transplant) and patients with Ischaemic Heart Disease (IHD). Vessels were subjected to 2D-DIGE and differentially expressed proteins analysed and identified by LC-MS/MS. As membrane proteins are poorly represented by 2-DE, enrichment of membrane proteins using Triton X114 was carried out on both the control and diseased vessels according to a previously described method (1). Pooled control and diseased vessels were labelled with one of eight iTRAQ 8plex labelling reagents, fractionated by OFFGel Electrophoresis and identified by MS/MS.

Results
Hsp27 was shown to be significantly decreased in ischaemic vessels (35.4%) when compared to control vessels. Further validation by western blotting demonstrated a specific decrease in all three Hsp27 phosphorylated forms in IHD only Phosphorylation of Hsp27 at serine residues 82, 78 and 15 was decreased by 37.2%, 49.9% and 43% respectively. Other cytoskeletal proteins including vimentin, tropomyosin and cofilin showed decreased expression while transgelin was shown to be upregulated. Cytoskeletal proteins were primarily identified in this part of the study. Since few membrane proteins were identified using 2-DE, membrane proteins were successfully enriched for using Triton X114. Western blot analysis showed enrichment of the membrane protein, CD31 and similarly a depletion of the cytoskeletal protein, GAPDH in the detergent fraction. Thus Triton X114 was subsequently used to successfully enrich for membrane proteins.

Innovative aspects

- This is the first DIGE investigation to examine differential protein expression in coronary vessels.
- Cytoskeletal coronary re-organisation may protect the vasculature against atherosclerosis.
- Triton X114 is a novel means of enriching for membrane proteins in the coronary vasculature.

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Fig. 1

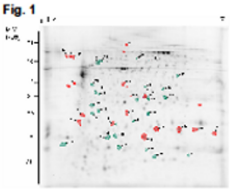

Figure 1. Differential protein expression in vessels with IHD. A total of 49 spots were differentially expressed (P<0.05) In coronary arteries (red=↑, green =↓).

Fig. 2

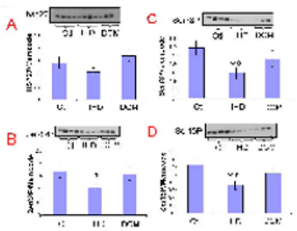

Figure 2. 1D Immunodetection of Hsp27 and phosphorylated Hsp27 in coronary vessels. Vessels from IHD show a significant decrease in phosphorylated Hsp27 at serines 82, 78 and 15 (p=0.02, 0.008 and 0.004 respectively) Fig 1B, 1C and 1D.

Detection and Site Identification of Nitroxyl Induced Thiol Modifications Using a Modified Biotin Switch Assay.

C306

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Identification of the cysteine residues involved disulfide bonds is challenging and limited success has been achieved with global screening by MS. We modified the traditional biotin switch assay in order to target and characterize nitroxyl (HNO) induced thiol modifications in heart. HNO is the one electron reduced and protonated form of nitric oxide (NO^-) which reacts with free thiol groups on proteins to form either sulfinamide or disulphide bonds. HNO donors are now considered promising pharmacological tool to improve cardiac function in subjects with heart failure. Two HNO donors, Angeli's Salt (AS) and 1-Nitrosocyclohexacetate (NCA), increase maximum force of contraction while only NCA treatment has been found to increase Ca^{2+} sensitization.

Methods: The contractile myofilament proteins, isolated from rat cardiac tissue, were exposed to 25 µM NCA, 500 µM AS or their decomposed equivalents. The remaining free thiols were blocked with NEM in the presence of 2.5% (w/v) SDS. After the excess NEM had been removed, HNO- modified cysteine residues were reduced with 5 mM DTT and subsequently labeled with biotin-HPDP. Samples were digested with either trypsin or chymotrypsin and then labeled peptides were captured with streptavidin and identified by LC/MS/MS (LTQ).

Results: A total of 12 HNO sites on 8 different myofilament proteins were identified between the two treatments (n=3). Of those, 3 proteins (tropomyosin C190, actin C257 and myosin heavy chain C947, 1750) were found to be specifically modified by NCA. Western blots for actin, tropomyosin, myosin light chain 1 and myosin heavy chain displayed additional higher molecular weight species with HNO treatment which were reversed with DTT indicating the formation of dimers by disulphide bonds, validating the site mapping studies.

Conclusion: We have shown that this modified biotin switch method is able to identify the disulfide bonds that are likely involved in the changes in cardiac muscle response found after treatment with HNO donors.

Development of a sequential immunoassay for characterizing circulating cardiac troponin

C307

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Introduction
The detection of cardiac muscle specific troponins in blood is the current gold standard for the diagnosis of patients with myocardial infarction (MI). The cardiac troponin (cTn) complex comprises 3 tightly interacting subunits cTnI, cTnT, and cTnC. With MI, cTn is released from the heart into the circulation where it can be detected using a variety of immunoassays that independently quantify cTnI or cTnT. Here we describe the development of a novel sequential immunoassay to directly probe the primary and ternary structure of the circulating cTnI using diffractive optics technology (dot®).

Methods
dot® uses a diffraction grating formed from affinity reagents such as antibodies. This grating is comprised of repetitive lines and generates a specific diffraction pattern when interrogated with a laser. As molecules exhibit affinity for the capture molecules making up the grating, the diffractive intensity (DI) is increased. An anti-cTnI antibody to the constant region was immobilized on a sensor to capture cTnI, which was sequentially probed with multiple antibodies to assess cTnI integrity, phosphorylation status, and association with cTnC and/or cTnT. The binding in each step was recorded as an increase in real-time DI and the differences were utilized to characterize the circulating form of cTnI. Each immunodetection was independent of one another but was done in a single assay.

Results
cTnI is a complex analyte. Its circulating forms may include different ternary structures as well as disease-induced post-translational modifications such as the specific and selective degradation of the N- and/or C-terminus. It is accepted dogma that cTnI circulates in the blood as a cTnI-cTnC complex, yet this is based primarily on indirect evidence. There has been no clinically applicable assay that has directly characterized the circulating ternary form of these biomarkers, in part, due to a limitation in technology.

A novel diffraction-based immunoassay using the dotLab® System was developed to characterize the primary and ternary structure of the circulating form of cardiac troponin. The new assay was able to directly probe the integrity and modification of cTnI and determine if the protein is degraded or phosphorylated. The detection of circulating cTnI bound to cTnC and cTnT from serum obtained from an AMI patient indicated the dimer or intact cTn complex (cTnI-cTnT-cTnC) is present. Further development of this assay in a new multiplex format with other cardiac markers is currently underway.

Innovative aspects

- We have developed the first clinically-adaptable immunoassay to address the direct interactions of the subunits within the cTn complex.
- Direct measurement of these protein interactions in complex samples may provide a better characterization of the disease state using existing cardiac markers.
- Real-time binding data may allow a more thorough understanding of these clinical biomarkers and has the potential to improve their clinical utility.

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An optimal protocol for the proteomic analysis of stenotic aortic valves

C308

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Introduction: Degenerative aortic valve stenosis (AS) is the most common of the valvular diseases. AS pathogenesis shares a number of features with atherosclerosis, such as endothelial dysfunction, leukocyte adhesion/infiltration and calcification. Both pathologies are related to the same clinical risk factors.
Despite the high prevalence and increasing mortality of this condition, little is known regarding the cellular and molecular factors involved in the development of AS and their possible relation with coronary artery disease.
A proteomic study of AS might help to improve our knowledge of this pathology.

Methods : AS valves obtained from necropsies (control samples) or by surgery patients were homogenized in extraction protein buffer. Both samples were analyzed using 2DE, 2D-DIGE and LC-MS/MS.
Furthermore, in order to confirm the proteomics results, 10 AS and control leaflets were studied by immunohystochemical (IH) and Western blot (WB) analysis, using a panel of specific monoclonal antibodies for inflammatory and cytoskeletal/ contractile proteins.

Results: Here we present an effective protocol to extract proteins from AS valves for their subsequent proteomic analysis. This protocol efficiently extracts most of the proteome of calcified valves and it is compatible with 2-DE and 2D-DIGE.
The result of 2D-DIGE analysis of the proteome of AS valves compared with control valves reveals the expression protein alteration in several proteins such as 27 Heat shock protein, osteopontin, vimentin. Western blot and IH techniques were performed to corroborate the 2D-DIGE results.
Furthermore, we have characterized the cellular composition of degenerative aortic stenotic valves by IH. The fibrosa layer of AVL had a higher cellularity than spongiosa and ventricularis (elastic) and it was the principal layer which was damaged in the lesion.
Our results show that a proteomic approach to study the pathogenesis of AS might provide useful information to understand the development of AS and help define its possible relation with coronary atherosclerosis.

Innovative aspects:
-Analysis of human stenotic aortic valves with different proteomics tools.
-Sequential protein extraction protocol in human aortic valves.
-Differential analysis expression (DIGE) between healthy and stenotic valves subjects.

Proteomic profiling of the cardiomyocyte plasma membrane

C309

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Introduction: Membrane proteins make up 30% of all known proteins and 50% of current drug targets. However, membrane proteins are relatively low in abundance and tend to be understudied because they are difficult to isolate and solubilise. Here we have used cationic silica beads to enrich for membrane proteins in cardiomyocytes. Multidimensional protein identification technology identified 3420 proteins with high confidence in cardiomyocytes whereby 379 of these were significantly enriched in the cell-surface membrane.

Methods: Mouse neonatal cardiomyocytes were harvested and cultured for 2 weeks. Membrane proteins were cross-linked to cationic silica beads (1, 2). Samples were centrifuged to isolate the beads resulting in a homogenate containing cytosolic proteins and a membrane fraction attached to the beads. Membranes were eluted using detergent 1% Triton-X100 or 8M urea buffer to aid solubility of membrane proteins. Samples were analyzed by triplicate LC-MS runs using an LTQ Ion Trap. High confidence membrane proteins were obtained by stringent filtering and removal of proteins with gene ontology terms of nuclear, mitochondria and cytosol. Further filtering kept only those proteins with a two-fold enrichment in the membrane fraction.

Results: A total of 3420 unique proteins were identified with high confidence in cardiomyocytes. These proteins clustered uniquely to either the membrane or cytosolic fraction (Fig 1). Enrichment of membrane protein and depletion of cytosolic proteins was confirmed by western blotting analysis of both membrane and cytosolic fraction. Stringent filtering allowed us to refine further a list of 379 known and novel proteins that mapped with high confidence to the cardiomyocyte membrane. Comparison of our proteome dataset with two published mouse heart proteome maps showed more than two-thirds of the proteins identified in this study overlapped with the existing data sets. However, we identified 120 unique proteins in the current study. A comparison of this data to a dilated cardiomyopathic mouse model, the phospholamban R9C mutant mouse, showed 87 proteins in our cell-surface associated fraction were up-regulated in the diseased heart.

Innovative aspects:
- First comprehensive study of cell surface-associated proteins in the heart
- Generation of a cardiomyocyte membrane protein database

References:

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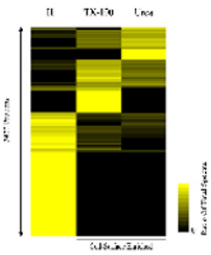

Figure 1: Hierarchical clustering of 3,420 high-confidence protein identifications in homogenate and TX-100 and Urea buffer eluted membrane fraction as a fraction of their total spectral counts.

The Proteomic Elucidation of Calcineurin Mediated Cyto-Protection.

C310

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Introduction
Here we performed a proteomic analysis of a mouse model of hypertrophic cardiomyopathy using Calcineurin transgenic mice (CNA), which demonstrate a hypertrophic phenotype beginning as early as 2 weeks of age. Interestingly, Calcineurin activation has been shown to be necessary for survival of cells undergoing endoplasmic reticulum (ER) stress (1). Calcineurin activity was previously demonstrated to be protective against apoptosis in ischemia-reperfusion models (2). Therefore this mouse model offers an attractive opportunity to employ comprehensive proteomic profiling to identify potential factors mitigating cardiac apoptosis.

Methods
We performed a large scale proteomic profile of mice with cardiac specific overexpression of Calcineurin (Chugh et al. unpublished). High confidence proteins (>99% confidence score) from CNA and WT mice were compared using two different statistical models including a global linear model previously described (3), as well as a local model to identify changes between subcellular fractions. We assayed the degree of ER stress and basal apoptosis in CNA and WT mouse hearts. We also assayed apoptosis in cultured cardiomyocytes derived from CNA and WT neonatal mouse hearts.

Results
We found that basal apoptosis in CNA hearts was comparable to WT hearts, yet markedly reduced compared to another mouse model previously described as having increased apoptosis (3, Figure). In addition, we showed that Calcineurin over-expression protects cultured cardiomyocytes from apoptosis. The comprehensive proteomic dataset derived from these animals was analysed to confidently identify significantly altered proteins via two different statistical methods with global and local approaches, respectively. This stringent analysis identified 59 proteins which were significant under both statistical models. Several of the latter factors were validated with immunoblotting. We also found that ER stress was elevated in CNA mice as evidenced by activation of both IRE-1 and PERK, two integral signalling pathways triggered by ER stress. Within the group of 59 significantly altered proteins we found that several cyto-protective chaperones, including Crystallin, which has repeatedly been demonstrated to be anti-apoptotic, were significantly elevated. In contrast, we found cytochrome c, an integral component of the apoptosome was significantly reduced in CNA hearts.

Innovative aspects
1. Derivation of a biologically testable hypothesis from stringent statistical analysis of a cardiac proteomic dataset.
2. Demonstration of reduced apoptosis despite elevated ER stress.
3. The utilization of a broad scale proteomics approach to identify several proteins as potential factors of Calcineurin mediated cyto-protection.

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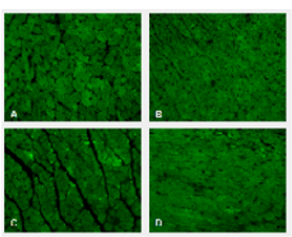

Photomicrographs demonstrating negligible fluorescent TUNEL labelling in the left ventricle of a WT heart (panel A), and a CNA heart (panel B), but high degree of TUNEL labelling in the positive control heart (bright spots, panel C). Panel D, the negative control, in which the labelling enzyme was omitted.

Proteomic Characterization of the Murine Heart

C311

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Introduction
Cardiomyopathies are complex multi-factorial diseases with varying etiologies. Thus identifying disease fingerprints through proteomics may facilitate discovery of novel disease targets and increase specificity of treatments. Development of standard proteomes describing all of the protein involved in normal physiology will facilitate the delineation of disease mechanisms. Here we characterize the wild-type (WT) cardiac proteome using data obtained from a sub-cellular fractionation protocol in combination with a multidimensional protein identification proteomics approach.

Methods
We recently performed a comprehensive proteomic survey of cardiac ventricles from a mouse model of dilated cardiomyopathy and their WT littermates (1). This gel-free shotgun sequencing was carried out using an HPLC pump linked to an LTQ linear ion trap mass spectrometer. High confidence proteins (>99% confidence score) from WT mice only were allocated to organellar compartments in which they were most abundantly identified. The dataset was subjected to several validation protocols in addition to comparison to several previously published cardiac and organellar proteomes.

Results
We identified 4906 proteins which were allocated to either cytosolic, microsomal, mitochondrial matrix or mitochondrial membrane fractions with relative abundance values in each fraction. We subjected these proteins to hierarchical clustering, gene ontology terms analysis, immunoblotting, comparison to publicly available protein databases, comparison to 4 distinct cardiac transcriptomes, and finally, to 6 other related proteomic datasets, including other cardiac proteomes (2, 3). This study provides an exhaustive analysis of the cardiac proteome and is the first large scale investigation of the subcellular location for over 2000 unannotated proteins. We also employed the newly developed and publicly available confocal subcellular atlas of the human proteome to validate the unannotated protein locations (Figure). Furthermore, Using a subtractive transcriptomics approach we have also extended our analysis to identify 'cardiac selective' proteins in our proteome. Finally, using specific filtering criteria we identified proteotypic peptides for subsequent use in targeted studies of both mouse and human.

Innovative aspects
- Most comprehensive cardiac proteome to-date.
- First large scale sub-cellular location and relative protein abundance description for 2019 unannotated proteins in cardiac muscle, with validation using the publicly available human proteome database.
- Computational identification of 1875 proteotypic peptides mapping to 927 unique orthologous mouse/human proteins.

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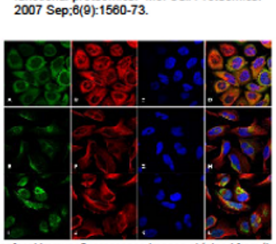

Confocal immunofluorescence images obtained from the Human protein Atlas. Immunofluorescence (A, E, I), cytoskeletal-phalloidin staining (B, F, J), nuclear Dapi staining (C, G, K), and the composite images showing integrated fluorescence (D, H, L). Panel A-D, A-431 cells were stained to detect endogenous levels of Adenylosuccinate lyase with cytoplasmic staining. Panel E-H corresponds to proteasome subunit beta type 4 showing microsomal staining. Panel I-L shows Isocitrate Dehydrogenase with mitochondrial staining.

C312

Identification and Characterization of Cell-Surface Associated Proteins of the Human Heart

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Introduction
Cardiovascular disease (CVD) is a leading cause of death in North America (1) and the development of new therapeutic strategies depends on a greater understanding of cardiac cell function. Many clinical drugs that treat CVD, target cardiac membrane proteins (1), however these proteins are difficult to isolate. Recently, we used cationic silica beads to enrich for cell-surface proteins in murine cardiac cells followed by extensive mass spectrometry (MudPIT)-based proteomics. Here, we have followed up this proof-of-concept study to enrich for cell-surface proteins in human cardiomyocytes.

Methods
Primary human cardiomyocytes were acquired commercially from PromoCell (Germany) and were harvested and cultured. Plasma membrane proteins were cross-linked to cationic silica beads (2), lysed and centrifuged. The resulting supernatant containing the cytosolic fraction was separated from the plasma membrane pellet attached to the beads. Membranes were eluted using either 1% Triton-X-100 or 8M urea. Samples were analyzed by liquid chromatography and mass spectrometry runs using an LTQ Orbitrap mass spectrometer. High confidence membrane proteins were obtained by stringent filtering and removal of proteins with Gene Ontology terms of "nuclear" and "mitochondria". Further filtering kept proteins with a three-fold enrichment in the membrane fraction.

Results
In total, 1685 unique proteins were identified with high confidence in the human cardiomyocyte. These proteins clustered uniquely to either the membrane or cytosolic fraction. Membrane protein enrichment and depletion of cytosolic proteins was confirmed by western blot analysis. Following extensive filtering we refined further a set of 333 known and novel cell-surface associated proteins, in which majority of the proteins contained transmembrane domains (Figure 1). Comparison of our proteome dataset with the mouse heart membrane proteome showed an approximate one-third overlap. Ongoing investigations focus on identifying cardiac enriched proteins through performing similar studies using endothelial cells, smooth muscle cells and *in vivo* cardiomyocytes obtained from adult myectomies; and characterizing these novel membrane proteins.

Innovative aspects
- Identification of novel human cardiac plasma membrane proteins.
- Development of a human cardiomyocyte cell-surface associated protein database.

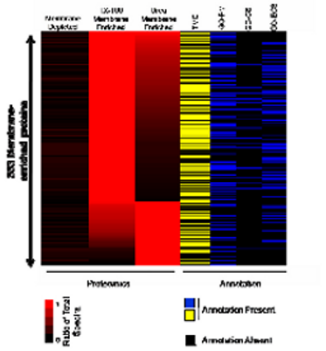

Figure 1. Heatmap representation of the 333 cell-surface associated proteins. Approximately 54% of proteins had a predicated transmembrane domain (TMD), 34% had a GO-term of "plasma membrane" (GO-PM) and together approximately 65% of the proteins identified can be classified as cell-surface associated proteins.

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C313

Proteomic Survey of a Mouse Model of Dilated Cardiomyopathy Reveals microRNA Modulation

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Introduction
Cardiac related disease is the premier cause of mortality in the developed world[1]. To understand the molecular events of heart failure, our laboratory has performed a proteomic survey of a mouse model of human genetic dilated cardiomyopathy (DCM). Our model overexpresses phopholamban (PLN) with the Arg[9] to Cys mutation (R9C) found in humans, from this proteomics study we identified 287 proteins with significantly altered expression[2]. MicroRNAs (miRs) have recently emerged as regulators of protein expression; therefore we are interested in delineating miR mediated protein expression in DCM

Methods
To gain insight into protein regulation, we profiled miR expression at the 8 weeks time-point utilizing two different microRNA microarray platforms, Exiqon and Agilent. Expression signals were normalized, and then two differentiality scores were calculated: the simple difference, and t-test p-value. Results were integrated by combining two different platforms. miRs displaying consistent and significant expression patterns were selected as hits. Using the target prediction algorithm TargetscanS, the predicted targets of the altered miRs were mapped against R9C proteomics data. We later showed correlated miR expression levels using Real-Time PCR, and concordant expression of its protein targets through immunoblotting

Results
Combining the microRNA expression profiles from two different microarray platforms; a portion of these miRs have been previously linked to heart pathologies or myopathies, either by profiling studies or genetic perturbation experiments, confirming the reliability of our results **Figure 1**. Utilizing publicly available sequence-based microRNA-target prediction algorithms, we identified proteins consistently related to heart disease (e.g oxidative metabolism, cytoskeleton remodelling, growth factor pathways, ER Stress). We are in the process of validating the expression of miRs and their protein targets based on framework provided by our miR-1 pilot study which has yielded encouraging results. We are able to confirm our microarray data through Real-Time PCR showing downregulation of miR-1 in the R9C disease model, and increased expression of predicted miR-1 targets through both the proteomics and immmunoblotting in R9C.

Innovative aspects
- High throughput proteomics guided delineation of microRNA mediated protein expression in heart failure
- Identification and Investigation of novel microRNAs previously unassociated with cardiac disease

References

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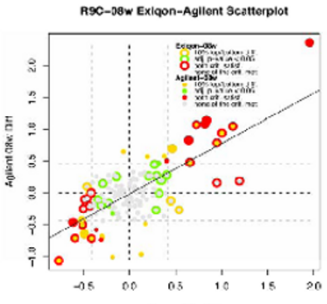

Figure 1 Using quantile normalization followed by log transformation, the miRs analyzed showed an $R^2 \sim 0.7$. miRs among the top 10% that were upregulated and top 10% downregulated and had a P<0.05 from a KS test. Were chosen for investigation

C314

Creation of a transgenic mouse line that over-expressing TXND4 protein

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Introduction
Heart failure is a major source of global morbidity and mortality in the world wide. Recently we have reported that a mutation in the human phospholamban (*PLN*) gene at Arg-9 to a Cys residue (PLN-R9C) resulted in an inherited human dilated cardiomyopathy (1). A large scale comparative proteomic study using wild type and transgenic PLN-R9C mouse model was performed previously and identified several elevated proteins. Included among these proteins were many proteins involved in ER stress including TXND4 (2). Our aim was to gain more information on the molecular machines controlling oxidative folding in human cells. Here, we designed and characterize a novel ER resident folding assistant protein Thioredoxin domain-containing protein 4 (TXND4).

Methods
A double affinity streptavidin and calmodulin binding peptide tags were used to generate a TXND4 construct. Subcellular localization of TXND4 protein was observed in mammalian HEK293T cells and validated by western blot analysis. To gain more information of the molecular machines controlling oxidative folding in human heart disease an inducible transgenic construct was generated. *TXND4* transgenic construct was created by multiple subcloning steps. The entire construct is driven by an EF1-alpha promoter and neomycin cassette flanked with two loxp sites. Neomycin cassette was excised in the presence of cre-recombinase. Induction of the transgenic construct was monitored on HEK293T cells in the presence of cre-recombinase using CBP antibody.

Results
Sub-cellular localization of TXND4 double affinity tagged construct was detected by immuno-fluorescence assay and validated by western blot analysis. A 55 kDa single protein band was detected in microsomal fraction of HEK cell lysates. Confocal imaging showed TXND4 expressions in the lumen of endoplasmic reticulum. We are currently developing a transgenic expression mouse model of TXND4 with the double affinity tags to investigate TXND4 biology in heart disease *in vivo*. The expression of TXND4 transgenic construct was studied in HEK cells with and without cre-recombinase. In the presence of cre-recombinase the TXND4 is expressed in the lumen of ER. No signals were detected in the absence of cre-recombinase. In parallel studies the role of TXND4 transgenic construct will be further studied in a conditional transgenic mouse using gene trapping. Thus, this transgenic mouse model will provide a suitable *in vivo* system to analyze the expression of the TXND4 under physiologic conditions.

Innovative aspects
- To gain more information of the molecular pathways controlling oxidative folding in human cardiac muscle.
- Utilization and extension of the expression of TXND4 under physiologic conditions.

References

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C315

Candidate Biomarkers for Thoracic Aortic Aneurysms: A Novel Platform for Biofluids

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Introduction. Thoracic aortic aneurisms (TAA) are the fifteenth leading cause of death in the United States. TAAs are asymptomatic and may be undiagnosed until crisis occurs due to rupture and dissection. Aneurysms undergoing rupture or dissection are associated with a high degree of morbidity and mortality, and early diagnosis or identification of individuals predisposed to disease may be life saving, permitting monitoring and early surgical intervention. Biomarkers that may provide early diagnosis or predisposition to the disease are therefore of great utility, but because of the nature of the disease, acute and stable phase biomarker discovery requires the study of explanted tissues.

With this in mind, we cultured perioperative aortic tissue from control (C), stable (SA), and acute aneurytic dissections (AD). The conditioned media were analyzed for cytokine, protein, and peptide levels to identify candidate biomarkers. Biofluids pose unique challenges for protein biomarker discovery: electrolytes and metabolites may interfere with separations; high-abundance proteins limit the assayable dynamic range and adsorb low-abundance proteins/peptides; and the presence of numerous endogenous proteases are but a few confounding factors. We have devised an efficient and reproducible platform for fractionation and quantification of both proteins and peptides from biofluids that achieves differential analysis from the same low-volume samples (BAP-Figure 1).

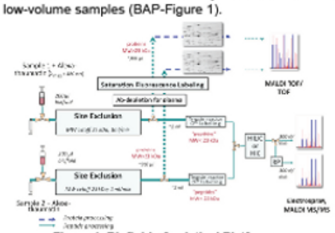

Figure 1. Biofluids Analytical Platform

Methods. The platform utilizes a fluoresecent internal size standard that defines a protein/peptide molecular size cutoff by which biofluid proteins are pooled separately from peptides. Denatured samples (to disrupt molecular interactions) are mixed with a fluorescently labeled protein standard and fractionated by size-exclusion chromatography. Protein and peptide pools are created automatically according to the elution profile of the standard. The protein pool undergoes antibody depletion, followed by saturation labeling with an uncharged dye [1, 2]. 2D gel electrophoresis (2DGE), and identification of differentially abundant proteins by MS. The peptide pool is labeled *via* $^{16/18}O$ exchange ensuring double-isotopic substitution at the C-terminus, with relative quantification and identification by LC-MS/MS.

Results. *Cytokine Analysis.* Conditioned media from 9 Cs, 19 SAs, and 6 ADs were analyzed for 25 human cytokines using bead-based immunoassays. Four cytokines were found to be statistically significant ($p < 0.05$)—IL-6, RANTES, IL-8 and MCP-1. Of these, IL-6, RANTES, and IL-8 were increased in the acute dissections. Unsupervised cluster and decision-tree analyses were performed to establish cytokine relationships to acute dissections.

Protein/peptide Discovery Analysis. Media from a subset of the cytokine-analyzed samples (3 Cs, 10 SAs, and 4 ADs) were fractionated and analyzed. Sixty-two proteins were selected ($p<0.05$) and relative abundance and grouped. Preliminary cluster analysis identified 20 proteins uniquely elevated in dissections, with stable aneurysms demonstrating levels intermediate between controls and dissections (Figure 2). Continuing studies will yield protein/peptide identifications.

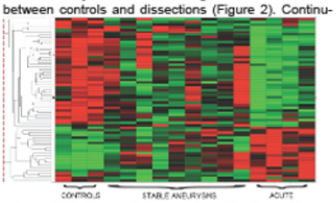

Figure 2. Heat map of expressed proteins in conditioned media from controls, stable aneurysms, and acute, dissected aneurysms.

Innovative aspects.
- BAP minimizes adventitious loss of target (low-abundance) proteins upon Ab-depletion;
- BAP reproducibly pools proteins and peptides from a single biofluid sample
- Combined with saturation fluorescence labeling with uncharged dyes, candidate biomarkers for stable and acute TAAs can reliably be identified.

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C316

Targeted Analysis of Transgenic Mice asssociated with Familial Hypertrophic Cardiomyopathy

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Introduction

Mutations in the cardiac troponin T gene occur in 10-15% of families with familial hypertrophic cardiomyopathy (FHC). The clinical manifestations of cardiac troponin T mutations that cause FHC are diverse, ranging from asymptomatic with high life expectancy to severe heart failure and sudden cardiac death. Transgenic mice containing wild-type and I79N mutation in human troponin T have been generated and the I79N mice show significant cardiac dysfunction (1). 2D gel elctrophoresis and label free MS expression profiling were utilized to determine the signaling pathways that are affected in these mice.

Methods

To dissect the proteins that are differentially expressed in diseased (I79N) hearts label-free expression profiling was carried out on a Thermo LTQ mass spectrometer. Samples were prepared from whole hearts and digested with trypsin as previously described (2). Samples from three wild-type and three 179N transgenic mice were investigated and the MS data analyzed using Scaffold. Two-dimensional difference in-gel electrophoresis (DIGE) was also utilized to compare the expression of proteins in 179N and wild-type hearts.

Results

Using label free quantification of total spectral counts from MS/MS fractions it was found that transgenic mice expressing I79N showed significant increases in proteins associated with metabolism when compared to mice expressing wild-type troponin T. Many mitochondrial proteins involved in metabolism were found to be increased in I79N mice including beta-enolase, pyruvate dehydrogenase, lactate dehydrogenase, cytochrome c and citrate synthase. Heat shock proteins, such as HSP60, were also found to be upregulated in I79N mice using label free quantification. Using DIGE, hearts from transgenic mice containing the I79N mutation also showed changes in several proteins when compared to wild-type transgenic mice (Fig. 1). One such protein is the HSP60 (arrow points to the heat shock protein which is >2 fold increased in I79N mice when compared to wild-type mice). Interestingly, although some proteins were found to be significantly altered between I79N and wild-type hearts using both proteomic methods employed, label free and DIGE both yielded some unique results. It seems that these differences were due to some proteins being better resolved by one technique over the other.

Innovative aspects

- Comparison of DIGE versus label free quantification of heart samples.
- The I79N mutation studied is atypical in that it causes sudden cardiac death without cardiac hypertrophy unlike other FHC mutations.

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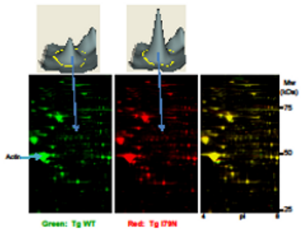

Figure 1. Two dimensional Fluorescence Difference in-Gel Electrophoresis (DIGE) of hearts from wild-type and 179N transgenic mice. The left and middle figures represent the wild-type and I79N transgenic mice hearts respectively. The right figure is an overlay of the left and middle figures. The inset above the gels shows DeCyder analysis of the intensity of the heat shock protein 60 (HSP60).

C317

Quantitative Signatures of Cardiac Hypertrophy and Failure using Mass Spectrometry and Bioinformatics

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Introduction

Cardiac hypertrophy is a compensatory response which is initially beneficial to heart function but can ultimately lead to de-compensation and failure.(1) In response to increased afterload to the heart, individual myocytes grow as a means of increasing cardiac pump function and decreasing ventricular wall tension. Over time, however, this increased demand leads to chamber dilation, wall thinning and reduced ejection fraction. Although the physiological manifestations of this phenotype are well understood (2) the systems-level reprogramming responsible for this shift from compensation to failure is unknown. Specifically, a major limitation of the current treatment for heart failure is the inability to determine when cardiac function will deteriorate before this process has become irreversible. To address this issue we are developing systems-level signatures for the progression of cardiac failure following mechanical overload using proteomics and bioinformatics in a mouse model.

Methods

Pressure overload hypertrophy was induced in adult balb/c mice by ligating the transverse aorta (TAC) over a 26-gauge needle. The mice were sacrificed at 1, 2 and 4 weeks after the aortic ligation or from SHAM-operated animals as a control. Hearts were then homogenized in a sucrose buffer containing both protease and phosphatase inhibitors and the homogenate centrifuged at 1000g for 10 min at 4°C. The pellet from the initial centrifugation step was overlaid on a 1M sucrose cushion and centrifuged at 1600g for 5 min at 4°C in order to separate the nuclear fraction. Enrichment of cardiac nuclei was confirmed by electron microscopy and immunoblotting for organelle specific proteins. Nuclear proteins were separated by SDS-PAGE and analyzed on a Thermo Orbitrap mass spectrometer. Mass spec data was searched against the mouse IPI database version 3.46 using both Mascot and Sequest search algorithmns. Proteins were identified on the basis of ≥ 2 unique peptides per protein using the following criteria: *for SEQUEST,* Xcorr values of >2.0 (+1), >3.0 (+2), >4.0 (+3), and >5 (+4); and Delta CN>0.1; *for MASCOT,* protein score >50; 10ppm parent ion mass tolerance; 0.5 dalton product ion mass tolerance. False positives were ferreted out with a reversed database search. The Elucidator software (Rosetta) was utilized to identify quantitative changes in protein expression and modifications at various time points after TAC.

Results

We have characterized the basal and diseased nuclear sub-proteome within the myocardium. Bioinformatic analysis of this sub-proteome has allowed characterization of quantitative signatures of protein abundance and post-translational modification changes in the nucleus that accompany the remodeling of the heart following pressure overload. This signature has unique components that differentiate the compensatory growth phase from the de-compensation to failure. We reason that this proteomic signature could ultimately be used to diagnose underlying pathologies of the heart and to suggest new therapies.

Innovative aspects

- Characterization of basal murine nuclear proteome in heart
- Identified quantitative protein abundance and post-translational modification changes in this subproteome during disease
- Developed systems-level signatures for the progression of cardiac failure

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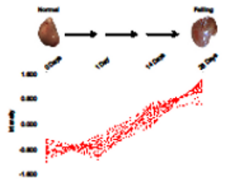

Figure 1. Quantitative Analysis of Nuclear Proteome to Reveal Cardiac Signatures. Top Panel. Cardiac hypertrophy is a progressive disease that leads to gross changes in heart morphology and function, the systems-level basis for which is unknown. Bottom Panel. Nuclear proteins were isolated and identified from mouse hearts following TAC or SHAM surgery to induce pressure overload hypertrophy. Quantitative changes in the abundance of these proteins at various time points after TAC were determined using the Elucidator software. Relative intensities of identified proteins were converted to z-score values (y-axis) to compare these changes across several orders of magnitude. A subset of these proteins was found to be up-regulated following pressure overload and to peak in intensity at 28 days after surgery when the compensatory response has lead to heart failure. These proteomic changes can be used to identify disease signatures for the progression of cardiac failure following mechanical overload in the mouse.

C318

Role of profilin-1 in endothelial cell dysfunction in ouabain-treated endothelial cells revealed by proteomics study

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Introduction

The cardiac steroid ouabain has been shown to involve in a variety of endothelial cell variability and function, so as to affect the pathogenesis of hypertension, but the precious mechanisms of its actions are not fully understood. To understand the underlying mechanisms, we investigated the protein expression profiles by 2-DE in endothelial cells (ECs) treated with ouabain and we found that the actin-binding protein profilin-1 was regulated in ouabain-treated ECs by proteomic study. We suppressed profilin-1 expression in ECs by RNA-interference to address the role of profilin-1 in ouabain actions.

Methods

We employed human umbilical vein endothelial cells as our model system. cell migration, nitric oxide production and endothelin-1 release were detected. Furthermore, two-dimensional electrophoresis(2-DE) in conjunction with mass spectrometry were used to reveal the different expressed proteins. Cell variability, migration, nitric oxide production and endothelin-1 release were tested when profilin-1 expression was suppressed.

Results

Nitric oxide production and endothelin-1 release revealed that ouabain played dual effects on cell function. Proteomics analysis revealed that the ouabain-induced nitric oxide production was accompanied by regulated expression of cytochrome C1, endothelin converting enzyme, claudin-1, reticulon-4, galectin-1, calnexin, profilin-1 and heat shock protein 70. Data from both cell viability and transwell experiments showed inhibition of cell migration when profilin-1 expression was suppressed. Gene silencing of profilin-1 led to significant reduction in the nitric oxide production induced by ouabain. Loss of profilin-1 expression was also associated with increased dynamics of endothelin-1 secretion simulated by ouabain.

Innovative aspects

1. Ouabain effect on ECs function with different pathways.
2. Profilin-1 may act as the important target protein of ouabain on ECs and play important role in endothelial cell dysfunction.

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C319

Characterization of Nitrated Tyrosine Residues in Succinyl-CoA: 3-oxoacid CoA transferase (SCOT) of Heart Mitochondria

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Introduction

Over the past several years, substantial evidence has been accumulated that the tyrosine nitration of mitochondrial proteins was widely found in several tissues of diabetes due to the presence of reactive nitrogen species. The studies in Murad lab have demonstrated that the decrease of catalytic activity of succinyl-CoA: 3-oxoacid CoA-transferase (SCOT), a mitochondrial matrix protein, was accompanied by an accumulation of nitrotyrosine of this protein in the hearts of diabetic rats. These early observation, however, did not answer a fundamental question, what was the correlation between SCOT nitration and the enzyme activity of nitrated SCOT. We thus initiate this project to specifically localize the modified tyrosine and to accurately determine the SCOT enzyme activity.

Methods

An E.Coli expression vector, pET30a-SCOT, was constructed by inserting mouse SCOT into a reading frame and the recombinant protein was purified through a modified protocol using the buffer containing 0.5% sarkosyl. The catalytic activity, either for recombinant SCOT or for the isolated mouse heart mitochondria, was examined using spectrophotometric approach. Nitration was induced by addition of peroxynitrite and monitored by Western blot using 3NT antibody. The modified residues of nitrated SCOT were employed by LC-ESI-MS/MS and further confirmed the catalysis dependent residues by site-directed. The results revealed that SPITC treatment approach seems to present relatively high identification rate (68.8%) as compared with the untreated samples (45.8%). In addition, the conclusion elicited from one approach would be strengthened by the evidence obtained from the other one. A combined strategy was therefore profitable to avoid missing interpretation caused by a single de novo approach. Together all, the identification rate increased to 73.6%, which reached the same level of efficiency and reliability as conventional database-search strategy.
By using topological analysis and reference search to predict the cellular locations of the identified proteins, we found about 86.4% of the membrane identifications were deemed part of membrane, which indicated the successful protein extraction. Function analysis of differential proteins revealed that some membrane proteins participated in iron transport were differentially expressed as external pH elevated and most of differential proteins with mutagenesis. Furthermore, a transfection vector to induce iNOS in mitochondria was constructed. With checking Western blot and mass spectrometry, the nitration status of SCOT in mitochondria in H9c2 cells was analyzed.

Results

The recombinant SCOT was examined that it was capable to catalyze the conversion of Succinyl-CoA to acetoacetyl-CoA and its activity was comparable with the native station, in mouse heart mitochondria. The SCOT modified by peroxynitrite through Western blot, revealing that the nitration of SCOT was steadily enhanced responding to the increased concentrations of peroxynitrite. Thus, we first provided the evidence that the tyrosine residue (s) in the purified SCOT was indeed modified by NO, which led to loss of its catalysis. To define the nitrated tyrosine site, the peroxynitrite-modified SCOT was completely digested by trypsin and the digested products were delivered to ESI MS/MS. The spectra of MS/MS exhibited a +45 mass unit ion shift from [M +H]+ m/z in Tyr4 and Tyr76. Through generating the site-directed mutated SCOT, the tyrosine nitration and catalytic activity of mutants were analyzed with and without ONOO- treated. Mutation of tyrosine residues of SCOT at Tyr4 and Tyr76 was not only significantly protecting modification of ONOO-, but also dramatically preventing loss of the SCOT enzyme activity. Furthermore, a transfection vector to induce iNOS in mitochondria was constructed. With *checking Western blot and mass spectrometry, the nitration status of SCOT in mitochondria in H9c2 cells was analyzed.*

Innovative aspects

- The nitrated tyrosine sites of SCOT were identified at Tyr4 and Tyr76, which are critical important for regulating the enzyme activity of SCOT.

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C320

Proteomic analysis of homocysteine induced proliferation of cultured neonatal rat vascular smooth muscle cells

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Introduction
Atherosclerosis is the leading cause of death and disability. Hyperhomocysteinemia has been recognized as a common independent risk factor for atherosclerosis. we examined the protein expression alterations in proliferative VSMCs induced by Hcy using 2-DE. The expression of 11 proteins was found altered in proliferative VSMCs and eight of which were identified by mass spectrometry. These results provide some clues for comprehensively understanding the mechanism of VSMC proliferation and pathogenesis of atherosclerosis induced by Hcy.

Methods
VSMCs were prepared from thoracic aorta adult of Wistar rats and treated with homocysteine. Cell viability was measured by MTT and cell cycle analysis was performed with FACSCalibur flow cytometer by DNA content analysis. Protein expression alterations in proliferative VSMCs induced by Hcy were examined using two-dimensional gel electrophoresis (2-DE) and were identified by MALDI- TOF MS. The function of some differential expressed proteins were conformed by western blot and assay of enzyme's activitives.

Results
Hcy induces the proliferation of VSMC obviously in a manner of dose- time dependent. After comparing the protein expression profiles of VSMCs between the Hcy-treated and non-treated groups, 11 protein spots were found altered markedly in proliferative VSMCs with expression of eight protein spots increased and three protein spots decreased. In the differentially expressed proteins, eight protein spots were identified successfully including glycolytic metabolism proteins: pyruvate kinase M2 (PKM2), triosephosphate isomerase (TPI) and aldose reductase (AR); cytoskeletal proteins: lamin C and vimentin; three other proteins: calreticulin; similar to WDR1 protein and LIM and SH3 protein 1. The differentially expressed proteins were further validated by Western blot and confirmed by assay of enzymes' activities and ATP content. These results may provide some clues for comprehensively understanding the mechanism of VSMC proliferation and pathogenesis of atherosclerosis induced by Hcy.

Innovative aspects

- Globally analyze the protein changes in proliferative VSMCs using proteomic techniques.
- Some novel altered proteins were found in Hcy treated VSMCs.

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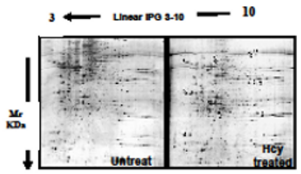

Figure 1. Representative 2D map of rat VSMCs from untreated (left) and Hcy treated (right) samples. Spot differentially expressed with p<0.05 calculated with Student's t test, are indicated with circle.

C321

Proteomic Analysis of Human LDL Reveals the Presence of PCL1, a H_2O_2-Generating Enzyme

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Introduction
Lipoprotein particles are stabilized in the bloodstream by association with highly evolved proteins called apolipoproteins, which through lipid-binding regions containing both hydrophilic and hydrophobic surfaces facilitate binding between protein and phospholipids (1).The molecular mechanisms underlying the relationship between low-density lipoprotein (LDL) and the risk of atherosclerosis are not clear. Therefore, detailed information on the protein composition of LDL may help to reveal its role in atherogenesis and coronary artery disease in humans.

Methods
In this study liquid-phase IEF has been used to separate human LDL proteins isolated from healthy subjects, into well-defined pools with individual pI values, which improves the subsequent detection and resolution of low abundance proteins (2). The fractionated samples were collected, desalted, concentrated by ProteoExtract Protein Precipitation Kit (Calbiochem) according to the manufacturer's instructions, separated on 7–17% polyacrylamide gradient gels, and stained with the Colloidal Blue method. All detectable gel bands were excised and digested with trypsin for MS analysis with a Q-TOF spectrometer (Micromass) connected to a Micromass CapLC capillary chromatograph.

Results
Besides known LDL-associated proteins this approach revealed the presence of several proteins not previously described to reside in LDL, including prenylcysteine lyase, PCL1 (an enzyme crucial to the lysosomal degradation of prenylated proteins), orosomucoid, retinol-binding protein, apoL-1, apoH, and paroxonase. PCL1 is a flavin adenine dinucleotide-dependent thioether oxidase which generates free cysteine, isoprenoid aldehyde and hydrogen peroxide. Addition of the substrate farnesylcysteine to lipoprotein resulted in a time-dependent generation of H_2O_2. This reaction was stronger in VLDL than in LDL or HDL, reflecting the greater content of PCL1 in VLDL. Farnesol, a dead end inhibitor of the PCL1 reaction, reduced H_2O_2 generation by VLDL. PCL1 is generated along with nascent lipoprotein, as shown by its presence in the lipoprotein secreted by HepG2 cells in culture, a popular model used for studies on lipoprotein metabolism. The finding that an enzyme associated with atherogenic lipoproteins (VLDL and LDL) can itself generate an oxidant suggests that PCL1 may play a significant role in atherogenesis. Acknowledgement of support from EC contract n° LSHM-CT-2007-037273-PROCARDIS.

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Acknowledgement of support from EC contract LSHM-CT-2007-037273-PROCARDIS

C322

Plasma Proteomic Biomarkers of Acute Heart Transplant Rejection

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Introduction
Heart transplantation is the primary therapy for patients with end-stage heart failure. However, acute and chronic rejection remains major risk factors for early and late graft loss, respectively. Currently, heart transplant patients are typically monitored in serial fashion for acute rejection by endomyocardial biopsy, an invasive, risky and expensive procedure. The aim of this work is to discover reliable plasma protein diagnostic markers of acute heart allograft rejection for use in effectively guiding clinical care.

Methods
Plasma protein markers of acute heart transplant rejection were identified using iTRAQ-MALDI-TOF/TOF and advanced statistics. Plasma samples were depleted of the 14 most abundant proteins by immunochromatography to enhance the detection sensitivity in plasma. A longitudinal time course quantitative analysis on 79 samples from 26 patients was performed to compare acute rejection (AR, ISHLT Grade ≥ 2R) samples at the time of rejection *versus* non-rejection (0R, ISHLT Grade=0R) samples from similar timepoints.

Results
A panel of five plasma proteins was identified exhibiting statistical significant differential relative concentrations between acute rejection and non rejection ($p<0.01$ and fold-change>1.2), of which 3 were increased and two were reduced in acute rejection. A score based on these protein relative levels clearly discriminates acute rejection from non rejection, and characterizes two groups of mild rejection. Together, the identified markers achieved a satisfactory classification (100% sensitivity and >91% specificity).Several of the identified plasma proteins were validated using ELISA and new patient samples. Rejection-regulated proteins belong to cellular and humoral immune responses, acute phase inflammatory pathways, and lipid metabolism biological processes. A parallel strategy using genome microarray analyses of peripheral blood cell RNA identified a panel of genomic biomarkers for both acute and chronic of heart rejection. Proteomic and genomic combinatorial analyses resulted in 100% sensitivity and specificity for heart rejection.

Innovative aspects

- Plasma protein biomarkers for heart transplantation were identified using quantitative proteomics.
- The panel of 5 plasma proteomic biomarkers of acute heart rejection clearly discriminate acute rejection from non rejection with 100% sensitivity and >91% specificity
- The results hold promise for transit from proteomic discovery to clinical utility.

C323

Vimentin Interacts with hnRNPs and Dengue NS1 Protein and Its Role in Viral Replication and Release

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Introduction
Dengue virus (DENV) is classified as a member of flavivirus genus in *Flaviviridae* family. There is no effective vaccine currently available. Better understanding of the virus-host interplay is therefore crucial for vaccine development. Our previous study (1) using expression proteomics demonstrated that many proteins, particular five forms of heterogeneous nuclear ribonucleoproteins (hnRNPs) were up-regulated in human endothelial cells upon DENV infection. To address functional significance of these proteins in response to DENV infection, we performed a functional proteomics study to identify hnRNPs-interacting proteins in the DENV-infected endothelial cells.

Methods
Human endothelial cell line, EA.hy926, was infected with DENV serotype 2 (DENV-2) at a multiplicity of infection (MOI) of 10 and subjected to immunoprecipitation (IP) using antibody specific to hnRNP C1/C2 and K. Interacting partners were resolved by 2-D gel electrophoresis and then identified by Q-TOF MS and/or MS/MS analyses. Functional characterization of vimentin, a common partner of both hnRNPs was performed. The interaction between hnRNPs and vimentin, and between vimentin and dengue NS1 protein was confirmed by reciprocal IP and by double immunofluorescence staining under vimentin network disruption by 4 mM acrylamide. Moreover, cell-associated virus and extracellular virus were determined by focus forming assay to examine role of vimentin in viral replication and release.

Results
IP followed by 2-D gel electrophoresis and mass spectrometric analyses revealed 18 and 13 interacting partners of hnRNP C1/C2 and hnRNP K, respectively. Interestingly, vimentin was a common partner for both hnRNP C1/C2 and K. The interaction between vimentin and these hnRNPs was confirmed by reciprocal IP followed by Western blot analysis and also by double immunofluorescence staining. Disruption of vimentin intermediate filament by acrylamide not only dissociated these complexes but also reduced nuclear hnRNPs expression, whereas cytosolic hnRNPs expression was unchanged. We also demonstrated that vimentin was strongly associated with dengue NS1 protein. Disruption of vimentin intermediate filament not only dissociated this complex but also reduced dengue NS1 expression, as well as viral replication and release.

Innovative aspects

- Novel interaction of host (hnRNP C1/C2, K and vimentin) and viral proteins (dengue NS1)
- Vimentin plays a crucial role in replication and release of dengue virus

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C324

How Protein Networks Impinge on Genomic Networks, Causing Heart Cells to Fail

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Stress to the heart during disease leads to cardiac failure, a condition characterized by irreversible morphological abnormalities and impaired pump function. Although the physiological manifestations of this phenotype are well understood, the systems-level reprogramming responsible for impaired function at the cellular level is unknown. Conserved genetic networks have been implicated in heart failure, with specific modules of genes activated during the development of disease. How protein networks influence these modules through direct regulation of the genome is unknown. To address this issue we are studying genomic and proteomic networks responsible for the progression of cardiac failure following mechanical overload in a mouse model. Nuclear proteins were analyzed on an Orbitrap mass spec and identified by searching the mouse IPI database using both Mascot and Sequest. The Elucidator software (Rosetta) was utilized to identify quantitative changes in protein expression and modifications at various time points during the development of disease and in different compartments of the nucleus. In-house algorithms were used to reveal properties governing interaction between modules of proteins and modules of genes that establish the cardiac phenotype and whose regulation goes awry in heart disease. The aim of these studies is to establish a basis for understanding proteome-genome dialog in the heart cell and to find new systems-level targets for therapy.

Chemical Proteomics

(C401 – C417)

C401

Development of Novel Fluorescent Reagents for Easy and Highly Sensitive Detection of Proteins

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Introduction

Proteomic studies are integral to understanding disease states and normal metabolic pathways. Biochemists involved in proteomic research are therefore investigating various protein detection methods that offer the advantages of easy handling, rapid monitoring, high sensitivity, and good binding linearity to facilitate both qualitative and quantitative analyses of proteins.

In this study, novel fluorescent molecular probe based on the cyanopyranyl moiety (Compound 1) was designed and synthesized in order to detect proteins via non-covalent bonding.

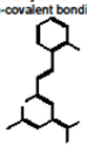

Figure 1 Chemical structure of Compound 1

Results

Compound 1 did not produce any fluorescence emission in the absence of a protein. On the other hand, strong fluorescence emission was observed after the addition of BSA. These fluorescence spectral changes upon binding proteins were caused by intramolecular charge transfer. The fluorescence intensities were plotted as a function of protein concentrations. A good linear relationship was observed up to 325 μg/mL of protein and the detection limit was found to be 50 ng/mL under the given assay conditions. Similar results were observed for the measurements of not only BSA but also other proteins (IgG, etc.).

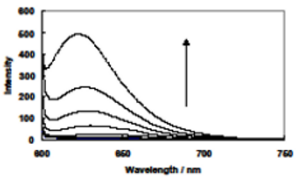

Figure 2 Fluorescence spectra of compound 1 before and after the addition of 0 to 325 μg/mL BSA. HEPES, 20.0 mM (pH 7.2). Excitation occurs at 550 nm in the fluorescence measurements.

To demonstrate the application of compound 1, proteins after electrophoresis using SDS-PAGE minigels were stained by 1 and were scanned using the image analysis systems. Figure 3 shows the gel image of BSA and sheep IgG samples after 1 staining. The association of 1 with these proteins and a visual examination of the staining response were successful. Moreover, simplification of the in-gel protein staining protocol was successful in order to achieve the high-throughput SDS-PAGE analysis of proteins.

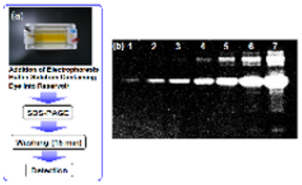

Figure 3 Rapid protein staining protocol using compound 1 after SDS-PAGE (a), and typical gel images of BSA (b). The amount of protein in each well is as follows: (1) 0.02 μg; (2) 0.05 μg; (3) 0.1 μg; (4) 0.4 μg; (5) 1.1 μg; (6) 3.3 μg; and (7) 10.0 μg.

Innovative aspects

The experimental results clearly showed that these probes are good protein indicators for the easy and highly-sensitive detection. Moreover, our group collaborated with KANTO CHEMICAL CO., INC. in Japan and excellent fluorescent protein reagent for SDS-PAGE, Rapid FluoroStain KANTO, has been developed based on above knowledge, which is commercially available now. Recently, proteomic analysis has become an important field, and it is becoming increasingly important to refine the techniques used to generate proteomic data. The highly sensitive, rapid, and easy-to-handle staining method using compound 1 should prove to be convenient and widely applicable to several scientific disciplines, including biochemistry, medicine, and pharmacology.

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C402

Mode of Action Analysis of Sorafenib by Integrating Chemical Proteomics and Phosphoproteomics

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Introduction

Multi-targeted kinase inhibitors such as sorafenib (Nexavar®, Bayer HealthCare AG) have emerged as promising anti-cancer drugs. However, due to their broad selectivity, it is particularly challenging to understand their modes of action in a cellular context. Therefore, systems-wide approaches that integrate comprehensive drug target identification and global phosphoproteome analysis are essential to gain valuable insights into the inhibitor's impact on signal transduction pathways. Such information may predict potential side effects or point to additional therapeutic applications.

Methods

The cellular target profile of sorafenib was analyzed applying a quantitative chemical proteomics workflow. SILAC-labeled PC3 cell lysates were incubated with immobilized sorafenib and competed with free compound. The bound proteins were analyzed by high resolution quantitative LC-MS/MS allowing identification and quantification of the cellular target proteins.

For global phosphoproteome analysis triply SILAC-labeled PC3 cells were incubated with sorafenib for 0, 30, and 90 min. Proteins were digested and phosphopeptides were enriched using SCX-IMAC/TiO_2. High resolution LC-MS/MS data were processed using the MaxQuant software (1). Identified phosphorylation sites were further statistically analyzed and mapped to signal transduction pathways to decipher the drug's cellular modes of action.

Results

We integrated advanced chemical proteomics and global phosphoproteomics approaches to reveal new modes of action of sorafenib. We confirmed previously known kinase targets such as B-Raf and p38α. In addition, previously unknown targets Map3k1, Taok3, and Mylk were identified. Mltk was found to have the highest affinity to sorafenib (K_D=30nM). In parallel, quantitative phospho-proteomics upon sorafenib treatment was conducted in four biological replicate experiments. More than 20,000 phosphorylation sites were identified. About 60% of these were found in at least three of the four replicates. About 700 phosphorylation sites were significantly regulated at a false discovery rate of 1%. Mapping of the regulated phosphosites to mitogen-activated protein kinase pathways revealed the known down-regulation of the Raf-Mek-Erk pathway and thus confirmed the expected cellular inhibition of B-Raf activity. In addition, several other pathways were down-regulated. In particular, the JNK and NF-κB pathways were found to be down–regulated, most likely due to inhibition of the new sorafenib target Map3k1.

Innovative aspects

- Application of quantitative chemical proteomics to identify new cellular targets of sorafenib
- Application of global quantitative phospho-proteomics to analyze the effect of sorafenib on cellular signal transduction pathways
- Integration of quantitative chemical proteomics and phosphoproteomics to gain valuable insights into a targeted drug's modes of action

References

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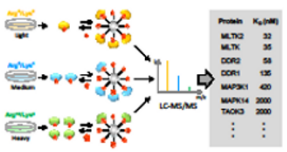

Figure 1: Chemical Proteomics workflow. SILAC encoded cell lysates are incubated with immobilized inhibitor and bound proteins are competed with free compound (red diamonds) revealing target proteins and their affinities to the free compound by quantitative mass spectrometry.

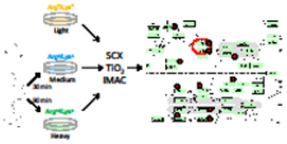

Figure 2: Phosphoproteomics workflow. Phospho-peptides from SILAC-labeled, sorafenib-treated cells were enriched and analyzed by LC-MS/MS. Significantly regulated phosphorylation sites (red circles) were mapped on signal transduction pathways.

C403

Substantial gain in reproducibility/precision in quantitative proteomics assays by targeted analysis

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Introduction

We described a chemical proteomics approach using immobilized non-specific kinase inhibitors (Kinobeads™) that capture a large fraction of the kinome[1]. In a competition binding assay binding potencies of ATP-competitive inhibitor compounds are determined by measuring relative amounts of affinity precipitated kinases as function of inhibitor concentration using MS (iTRAQ). Affinities of inhibitor compounds against >100 kinases are determined in one LC-MS/MS run. The shotgun approach has limited reproducibility in kinase identification in studies containing larger panels of inhibitor molecules.

Methods

Experiments were performed on a LTQ-Orbitrap mass spectrometer operated in PQD mode and coupled to an Eksigent NanoLC HPLC. Gradient elution was performed over 4 hours at a flow rate of 200 nl/min. Shotgun runs were acquired using a repeat count of 1 and dynamic exclusion for 120 s. Protein identification was performed using mascot and all data were imported into a Oracle data base. Inclusion lists were generated based on statistical analysis of several hundred such analyses and contained approx. 1500 include masses (up to 10 per kinase). Eluting peptides matching to charge state retention time and precursor ion mass (+– 7.5 ppm) were selected for up to 4 consecutive MS/MS scans using PQD.

Results

Reproducibility of the proteomics assay and precision of protein quantifications is substantially improved by targeted acquisition. Based on a historic data set of hundreds of experiments from an Orbitrap MS we developed mechanisms for selecting best peptides for proteins of interest. Key parameters include reproducibility, ion score, retention time, reporter ion intensity, uniqueness etc. By selecting up to 10 include masses per kinase, we saw a 25% increase in kinase coverage. Due to the high mass accuracy of the instrument isobaric peptides not included in the list were identified only to a minor extent (< 10%). By allowing multiple MS/MS events per peptide species fragmentation occurred across eluting peaks resulting in an improved precision of reporter ion based quantification (>2-fold). The number of reliably quantified kinases increased by >50% and, the number of kinases quantified in 7 out of 7 repeat experiment increased by 90%.

The targeted approach has proven valuable for kinase drug discovery projects. Increased reproducibility and precision of the proteomics assay enables a more comprehensive comparison of binding profiles of candidate inhibitor molecules.

Innovative aspects

Targeted data acquisition approach for improved reproducibility and precision of quantification kinase affinity profiling experiments.

References

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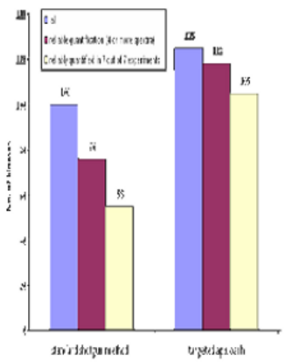

Figure 1: Comparison between identified and quantified kinases for the standard shotgun approach and the targeted approach.

C404

Small Molecule Capture Compounds - Towards a Targeted Reduction of Proteome Complexity

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Introduction: The isolation and identification of proteins from complex biological samples is a challenging task in proteomics. Therefore, a targeted isolation of enzyme families would be highly desirable. This requires the functional separation of sub proteomes from the whole biological sample in a form which allows the identification by state-of-the-art bioanalytics and bioinformatics. Separation by physical properties like molecular weight and charge can reduce the complexity of protein mixtures but lacks any selectivity concerning protein function. Affinity chromatography and chip based technologies achieve functional separations by immobilized selectivity functions but struggle from surface effects and steric hindrance. Here we present a novel technology, called Capture Compound Mass Spectrometry (CCMS) (1), which allows the isolation of sub proteomes based on their interaction with synthetic small molecules, called Capture Compounds (CC).

Methods Capture Compounds combine three different functionalities within one individual molecule: 1) a selectivity function for the specific affinity driven interaction with proteins, 2) a reactivity function for the photo cross-linking of Capture Compound and protein, and 3) a sorting function, biotin, for the isolation of captured proteins.
The data and experiments presented here, demonstrate the application of this novel technology for the analysis of three different interactomes: 1) the SAH interactome, 2) the cAMP interactome and 3) the staurosporine interactome. The CCMS technology is compared to pull down experiments. In contrast to pull down assays the data demonstrate a higher yield of identified proteins out of complex samples analyzed by gel electrophoresis and mass spectrometry.

Results We conclude that CCMS is a promising, novel technology for the functionality directed reduction of complexity and a higher enrichment of low abundant proteins compared to other proteomic approaches. The gel-free LC-MS analysis of captured protein complexes is unique for the CCMS technology. The versatility of the method allows a straightforward synthesis of novel Capture Compounds for many different applications and areas of life sciences and drug discovery. Capture Compounds can be used for the isolation, identification and profiling of novel and known proteins within complex biological samples.

Innovative aspects

- Significant and functional reduction of proteome complexity
- Covalent binding of proteins to CC allows the isolation of even weakly interacting target proteins
- CCMS can be customized for any sub-proteome by exchanging the selectivity function

References

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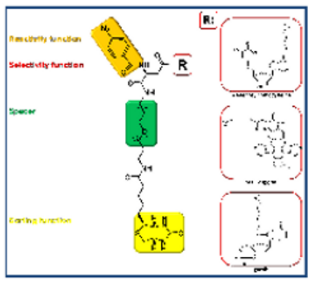

Figure 1. The core of Capture Compound Mass Spectrometry is a small, tri-functional molecule, the Capture Compound which consists of a sorting function, reactivity function and a variable selectivity function.

C405

Poly (methyl methacrylate) microchip gel isotachophoresis for simultaneously separation and concentration of bovine serum albumin and its immunocomplex with monoclonal antibody

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Introduction
Microchip electrophoresis (MCE) systems are of major importance in clinical diagnostics. However, for the trace analysis of biological samples, it is absolutely necessary to find a way for the concentration of sample to be higher than the detection limit of MCE system. In this regard, our group introduced a fully automated microchip gel isotachophoresis (ITP) for simultaneously concentration and separation of bovine serum protein and its immunocomplex in a standard cross-channel microchip made of poly(methyl methacrylate) (PMMA).

Methods
Combination of PMMA Microchip gel electrophoresis and ITP was applied for the separation and concentration of protein immunoassay sample. Sample injection, concentration, and separation were done continuously and controlled solely by a sequential voltage switching program. In this study, we also investigated the ITP concentration effect using combination of leading electrolytes (LEs), terminating electrolytes (TEs) and other factors.

Results
We concentrated the bovine serum protein and its immunocomplex by a fully automated online ITP-based concentration MGE which does not require time-consuming steps and complicated chip design. Because this method combined ITP with MGE, we can simultaneously concentrate and separate the sample which cannot be separated using the common ITP method. By combination of LEs and TEs, we can optimized the concentration enhancement factor and decrease separation time. Fig. 1 shows the electropherograms of BSA and its immunocomplex with monoclonal antibody obtained by on-line ITP concentration (Fig. 1 a, b) compared to normal MCE (Fig. 1c). The peak intensity for the immunocomplex was enhanced more than 2,000-fold by the use of Tris-H_3PO_4 as LE and Tris-γ-amino butyric acid (GABA) as a TE compared to the peak intensity in normal MCE. We also found that TE ions exert strong influence on concentration enhancement factors and that lower mobility of terminating ions result in higher concentration effect (1). In addition, we also realized concentration of BSA sample in water which was more than 20,000-fold and was the result of the concentration effect from combining ITP and the sample stacking techniques.

Innovative aspects

- Fully automated direct ITP-based concentration microchip gel electrophoresis which does not require time-consuming steps and complicated chip design.
- By combination of leading electrolytes and terminating electrolytes, we can increase concentration enhancement factor and decrease separation time.
- Exceeding 20,000 fold concentration enhancement factors with combination of this method and sample stacking techniques

References

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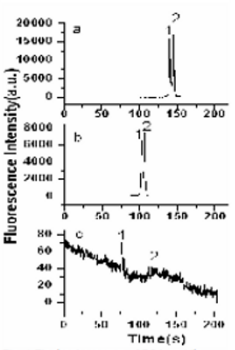

Fig. 1 Electropherogram of BSA and its immunocomplex, (a) was obtained by the ITP–based concentration using Tris-H3PO4 as a LE and Tris-GABA as a TE;
(b) was obtained by the ITP–based concentration using Tris-HCl as a LE and Tris-glycine as a TE;
(c) Normal MGE without ITP concentration.
Peak 1: BSA-FITC; peak 2: BSA immunocomplex.

C406

Quantitative Chemical Proteomics Investigation of the Bisindolylmaleimide binding proteome in a phorbol ester stimulated breast cancer cell line

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Introduction
Chemical proteomics utilizes synthetic chemical probes to selectively capture and enrich particular classes of proteins from complex cellular samples, enabling their characterization by proteomic techniques. In our study we have engineered a generic probe that harbor's a potent Protein kinase inhibitor, bisindolylmaleimide (Bis) as the reactive group and developed a robust chemical proteomic method to characterize cellular targets in a breast cancer cell line (MDA-MB-231). We also adapted a SILAC strategy to quantitate the differentially captured proteins following stimulation by phorbol 12- myristate 13-acetate (PMA).

Methods
MDA-MB-231 breast cancer cells were activated with 100nM PMA for 30min. The cells were lysed and 100µg of protein samples were incubated with 5µM Bis-probe for 1h at 37°C with intermittent mixing, prior to capture with avidin agarose beads. The eluate was gel purified, reduced, alkylated and trypsin digested prior to nano LC-MS/MS using an LTQ-MS. For SILAC experiments the cells were grown in light (L-lysine,L-arginine) and heavy ($^{13}C_6$ L-Lysine and $^{13}C_6$ $^{15}N_4$ L-Arginine) isotope containing media. Cells grown in the heavy media were stimulated with 100nM PMA for 30min and a second plate was stimulated for 60min.The light and heavy lysates were then mixed 1:1 and a combined extract of 100µg was used for pull down by the Bis-Probe. The SILAC ratios of the differentially captured sub-proteome at 0min to 30min and 30min to 60min stimulation were quantitated using Protein Pilot software.

Results
We have developed a robust chemical proteomics method to capture, identify and quantify proteins in the MDA-MB-231 breast cancer cell line. Using immunoblotting for detection we demonstrated the use of the Bis-probe to capture PKC-α, one of the known target of bisindolylmaleimeide small molecule inhibitors, in MDA-MB-231 cells.
Using a shotgun proteomics method we identified 210 proteins bound to the Bis-probe following PMA stimulation in MDA-MB-231 cells. The captured proteins included previously identified protein kinases PKCα, GSKα, GSKβ, MARK2, SRPK1 and PKR and non protein kinase targets, like NQO2. Apart from these, other enzymes identified had ATP or nucleotide binding sites and could be broadly categorized as hydrolases, transferases and synthtases. Approximately 50% of the bis-bound protein that were identified in our study had ATP binding sites (42%) and GTP binding sites (6%). On the basis of cellular activity 21% of the identified targets had nucleoside triphosphate activity and 6% had protein kinase activity (Fig 1).
We quantitated the differentially captured sub-proteome at 0-30min and 30-60min PMA stimulation. 7 proteins were significantly differentially expressed in both time points, which included proteins such as Vimentin, Pyruvate kinase and Glyceraldehyde-3-phosphate dehydrogenase. In addition, 16 proteins were up-regulated and 6 proteins were down-regulated following 30 min PMA stimulation. 9 proteins were up-regulated and 7 proteins were down regulated between 30 to 60 min stimulation.

Innovative aspects

- Engineered a potent Bis-probe for capturing purine binding proteins
- Developed a robust quantitative chemical proteomics method to study the purine binding proteome of breast cancer cells.

References

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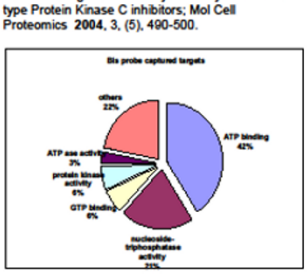

Figure 1. Cellular targets of Bis-Peg-Biotin probe captured in PMA stimulated MDA-MB-231 cell

C407

Protein Complex Discovery Using Chemical Proteomics

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Introduction
Chemical proteomics is widely used in drug discovery such as target hunting, mode of action(MOA) analysis, and toxicological study. We previously developed bioinformatics tools for analysing interaction between compounds and proteins last year. Here we have developed an improved bioinformatics tool to analyze the protein complexes and pathways for binding proteins and compounds.

Methods
One hundred different drug compounds were immobilized to beads and prepared as affinity columns. HCT116 cell line and mouse whole brain lysates were applied to each column and binding proteins were purified. As the results of mass spectrometry analysis, approximately 12,000 unique binding proteins were identified and the results were installed to our new tool with their relative binding strength, enrichment factor(EF). This database may include candidates of primary binding targets, secondary binding targets and their binding partner proteins(Figure 1(a)). In another word, we may have chance to find target pathway via direct binders and complex information in our datasets. Figure 1 shows the principle of the protein complex discovery, a protein B, which has the similar binding patterns as protein A that always binds to the compound group X and not binds to the compound group Y, so the protein B might be considered as one of the complexes of protein A. Based on this concept, we developed a relational database and a computer algorithm for discovering protein complex by searching the similar proteins against the database.

Results
To show the performance of our complex discovery strategy, we first analyzed the complex of 14-3-3 gamma as an example. After searching with our new software, we found that 14-3-3 gamma binds to 57 and not binds to 43 out of total 100 compounds and detected 14 candidates as the 14-3-3 gamma complex proteins from the similarity of binding protein patterns. After adding reference knowledge, we confirmed that those 7 proteins were reported as the known complex of 14-3-3 gamma in the literatures. Motif analysis for remained 7 proteins also suggested that those proteins would be the same complex members.
The same approach could be utilized for discovering complexes of interesting proteins.

We will further present the detail information of this tool and algorithms.

Innovative aspects

- Novel method for protein complex discovery
- Database and computer algorithm for detection of the protein complex from huge amount of binding protein lists

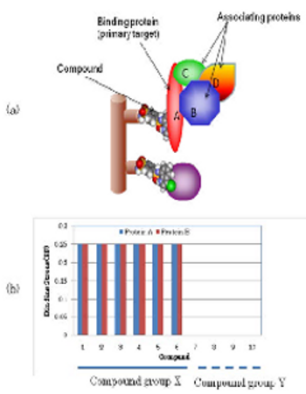

Figure 1. A schematic illustration on binding proteins to the compound, it may contains the primary, secondary targets and associating proteins(a), and the principle of protein complex discovery(b).

Proteomic Analysis of the European Clone *Staphylococcus aureus* Exoproteins C408

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Introduction
Community acquired meticillin resistant *Staphylococcus aureus* (CA-MRSA) was first described in Europe at the beginning of this decade. It belongs to clonal complex 80, which is known as the European clone. It is a threat to human health worldwide, accounting for 25% of bloodstream and 40% of skin and soft tissue infections (1). It is known that exoproteins are virulence factors involved in its pathogenesis. To gain a complementary understanding and to produce a reference map of the exoproteins produced by European CA-MRSA, we identified proteins in their culture media.

Methods
Representative strains of European clone *Staphylococcus aureus*, ER13 and ER21, isolated from sputum and skin abscess, respectively (2) were cultured in tryptic soy broth to stationary phase of growth. Exoproteins were precipitated from culture supernatant using trichloroacetic acid (10% final concentration), washed with ice cold acetone, and dissolved in SDS sample buffer. The proteins were separated on a SDS-PAGE gel and cut into 13 slices. Each slice was reduced, alkylated, and digested with trypsin. These tryptic digests were analyzed by nanoflow LC-linear iontrap-TOF MS. Data processing and databank searching were performed with Mascot software against NCBInr database.

Results
One hundred and twenty eight and 222 proteins were identified from ER13 and ER21 culture media, respectively, at the stationary phase of growth. Of the identified exoproteins, 64 from cultures matched between the two strains.These unique proteins of the European CA-MRSA culture media were separated into categories based on functional annotation. Fifteen exoproteins (23.4%) are associated with virulence such as the two components of PVL toxin (LukSPV and LukFPV), alpha and delta hemolysins, enterotoxins (seb and seu), and fibrinogen binding proteins. Twenty proteins (31.25%) are involved in carbohydrate utilization like pyruvate dehydrogenase, pyruvate carboxylase, pyruvate kinase, enolase, acetoin reductase, and transketolase. Culture supernatant contained 22 (34.37%) proteins associated with stress response such as alkaline shock protein, chaperone protein HchA, and alkyl hydroperoxide reductase subunit C. The strains also produce 7 (10.93%) exoproteins of unknown function.

Innovative aspects
- To our knowledge, this study is the first proteomic analysis of exoproteins produced from the European clone CA-MRSA.
- These data could be used as a reference map of exoproteins produced from European clone CA-MRSA for comparing different strains selected for their pathogenicity.

References:
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CCMS Technology – A Unique Tool for the Investigation of the Human Kinome C409

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Introduction
Their enormous diversity and role in signaling has made kinases attractive biomarkers and targets for drug discovery. Capture Compound Mass Spectrometry (CCMS) (1) addresses the need for a tool to stably and reliably profile the kinome of different biological samples. Capture Compounds™ (CC) are multifunctional probes (Fig. 1) with a selectivity, a reactivity and a sorting function allowing the captured proteins to be isolated for mass spectrometric analysis. In this study staurosporine, a broadband ATP-competitive kinase inhibitor, was used as selectivity function.

Methods
CCMS allows the isolation and identification of functional sub-proteomes in four consecutive steps. Captured protein targets are subsequently identified unambiguously by gel-free LC-MS/MS analysis. The CCMS process includes the following steps:

(a) Upon incubation with the biological sample, the variable selectivity function of the CC reversibly interacts affinity-driven with the target proteins.

(b) The reactivity function forms a covalent bond with interacting proteins by UV photo crosslinking thereby 'freezing' the equilibrium between selectivity function and interacting partners.

(c) Captured proteins are directly isolated via the biotin sorting function by means of streptavidin-coated magnetic beads.

(d) Protein digestion followed by LC-MS/MS analysis.

Results
Here we demonstrate the capability of CCMS to analyze the human kinome. The covalent attachment of the staurosporine-CC to the targets in HepG2 lysate allowed the profiling even of low-abundant kinases that bind with low affinity (e.g. tyrosine-protein kinase CSK). Among the identified kinases are e.g. the proto-oncogene kinases Fyn, Src and Yes, the serine/threonine-protein kinases PKN1, 2 and TBK1, the calcium/calmodulin-dependent protein kinase KCC2A, B, D, G and KCC1D. In one single capture experiment 41 kinases could be unambiguously identified by LC-MS/MS (Fig. 2b). In total, so far 54 distinct kinases could be assigned.
A direct comparison with the shotgun proteomic approach revealed the capability of CCMS to functionally enrich target proteins. This was reflected by a significant increase of specific kinase identifications (Fig. 2).

The superiority of CCMS was demonstrated in experiments where photo-crosslinking was omitted (pull-down). Here, significantly less specifically bound kinases were identified (Fig. 2b).
The results presented here demonstrate the power of CCMS for the comprehensive profiling of functional subsets of complex biological samples of limited abundance.

Innovative aspects
- Significant and functional reduction of proteome complexity
- Covalent binding of proteins to CC allows the isolation of even weakly interacting target proteins
- CCMS can be customized for any sub-proteome by exchanging the selectivity function

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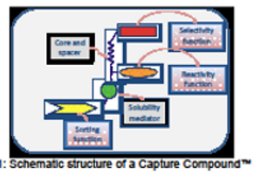

Fig. 1: Schematic structure of a Capture Compound™

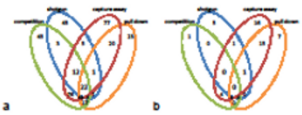

Fig. 2: Venn Diagrams of capture assay, competition, pull-down, and shotgun
a) Total numbers of identified **proteins**
b) Total numbers of identified **kinases**

A label- and immobilization-free proteomic approach for identifying the targets of drugs C410

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Introduction
The majority of drugs (i.e. small molecules) interact physically with one (or more) specific protein targets, causing inhibition or activation of function. However, while many drugs are used in therapeutics and increasingly in chemical biology, the relevant targets are not always known or are difficult to validate. Understanding drug mechanism-of-action, including off-target effects, therefore involves investigation of which proteins are specifically bound by drugs. While many experimental methods have been devised to address this, most have important shortcomings. Here, we report a novel proteomic approach to identify a drug's targets in an unbiased manner.

Methods
The core concept behind this technique for identifying drug targets is based on co-elution of bound drug with its interacting protein(s) during non-denaturing high performance liquid chromatography (HPLC). The presence of drug and protein(s) in each fraction is then determined using tandem mass spectrometry. For example, protein extract is prepared either from drug-treated cells or cell lysates are spiked with drug; free drug in buffer alone is also fractionated to establish a reference baseline. Target-bound drug containing fractions are identified using multiple reaction monitoring (MRM), while the co-eluting protein targets are identified by shotgun sequencing (LC/MS).

Results
We demonstrate the utility of this assay to monitor the binding of the protein targets of well-established drugs in a series of proof-of-principle experiments. For example, in **Figure 1**, we monitor co-elution of the anti-cancer agent methotrexate (MTX) with a recombinant form of its target dihydrofolate reducatase (DHFR). Similar results were obtained for the anti-proliferative agent radicicol and its target, the chaperone Hsp90. Further experiments were performed in which drug-target binding was monitored using complex biological mixtures consisting of soluble protein extracts and endogenous targets to demonstrate interaction specificity. Using our method, we have identified a putative new target for the synthetic anti-fungal agent, compound 4513-0042.

Innovative aspects
- We present a flexible, quantitative method to identify the direct protein target(s) of drugs
- Neither the drug or nor the protein needs to be derivatized or labelled to monitor binding
- Potentially applicable to most drugs and virtually any cell, tissue or organism

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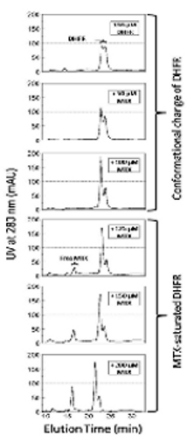

Figure 1. Proof-of-Concept: Bound MTX co-elutes with DHFR. Free MTX is evident after target saturation, with the signal proportional to the amount of excess MTX. A conformational change in DHFR is also evident upon binding.

Insights into the Progressing of Formaldehyde Cross-linking by Time-Course Studies C411

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Introduction
Formaldehyde cross-linking can be used to study native protein complexes (1). Its mechanism consists of: formaldehyde-induced amino acid modification is followed by covalent bond formation between two proteins. The specificity of the second step has yet to be determined. Enzymatic digestion converts cross-linked proteins into a complex mixture of unmodified and modified peptides, with a small subset of cross-linked dipeptides which are difficult to detect. Understanding the progression of both reaction steps would aid the identification of dipeptides and clarification reaction specificity.

Methods
Paraformaldehyde (PFA, Fisher) was dissolved in PBS at 4% (w/v) and added to a PBS buffered myoglobin (Sigma-Aldrich) solution with a final concentration of 100μM. The reaction was performed at 37°C, with the reaction progression monitored by quenching aliquots of the reaction solution at 8 time points between 0-6 hours. The reaction was quenched by addition of 1M Tris buffer. Samples were cleaned via a 3kD molecular weight cutoff filter, then either spotted onto a MALDI plate with CHCA (Sigma-Aldrich) matrix, or enzymatically (Trypsin, Promega or Glu-C, Roche) digested prior to spotting. MALDI-MS was performed on an Applied Biosystems 4700 Proteomics Analyzer. Offline LC separation was done on a GE Biosciences Ettan MDLC using a 75μm x 150mm C18 Zorbax column (Agilent).

Results
Using myoglobin as a model, a time course study showed modified amino acid residues and cross-linked intact myoglobin at longer time points (>1hr). Upon digestion, peptides displayed modification on amino acids, primarily lysine, although local reactivity seems to play a role (2). For example, at all reaction times, a peptide near the C-terminus had a higher average level of modification than a peptide in the central part of the sequence, despite the latter having more reactive residues. Longer-time-point samples produced new, higher-mass species. These newly formed species correlate with the production of cross-linked intact myoglobin, suggesting that they correspond to cross-linked dipeptides which are indicative of the actual sites of cross-linking. Sequencing of these species proved impossible due to low abundance. The simplicity of the formaldehyde-induced cross-link still allows confirmation of their origin. As cross-linking is not predicted to induce further mass shifts in the modified peptide forms, we are currently correlating the masses and levels of modification of the known peptides with the production of the new species to characterize protein cross-links.

Innovative aspects
- Formaldehyde cross-linking of proteins was observed directly by mass spectrometry
- Time-course studies revealed a link between protein modification and cross-linking

References
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C412

Development of A Quantitative Method to Study the Nitroxyl-induced Modifications

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Introduction
The effects of nitroxyl (HNO) on proteins have begun drawing attention due to its important effects on many biological functions including inhibition of platelet aggregation. Protein thiols are considered to be the major molecular target of HNO, therefore the chemical changes that free thiols undergo when in contact with HNO have been studied (1, 2). However, no method currently exists for determining the selectivity and extent of HNO-induced modification on proteins. We aim to develop a method to quantitate these modifications, and study their effect on platelet proteins ex-vivo.

Methods
Angeli's Salt (AS) was used as the HNO donor and a cysteine-containing tryptic peptide (EKPLQNFTLCFR-NH_2) and bovine serum albumin (BSA) were used as models. AS-treated peptide was reduced with dithiothreitol (DTT, 0, 0.1mM, 1mM and 10mM) for 1h at 56°C, and analyzed on MALDI 4700-Proteomics Analyzer. AS-treated and untreated BSA were reacted with thiol-modifying reagents including iodoacetamide (IAA), DTT and isotope-coded affinity tags (ICAT), trypsin-digested and analyzed by hybrid quadrupole/linear ion trap instrument (Q-Trap). For general analysis of the effects of HNO, HNO-treated platelet lysates were separated by 1D SDS-PAGE, digested, analyzed by Orbitrap and searched for oxidative modifications using the Global Proteome Machine (www.thegpm.org).

Results
Reduction of AS-treated EKPLQNFRLCFR-NH_2 with DTT showed that 0.1mM DTT fully reduced disulfide bonds, while full reduction of sulfinamide required up to 10mM. These features were used to devise an assay using differential thiol-modifying reagents to distinguish HNO-induced effects. AS-treatment is followed by addition of IAA to block unmodified free thiols. Then, a high amount of DTT is added, producing an AS-treated sample where endogenous disulfide bonds and HNO-modified thiols are reduced and a control sample where free thiols are IAA-modified while endogenous disulfide bonds are reduced. This method is currently being employed for the identification and relative quantitative analysis of the modification by comparing ratios of ICAT-labeled peptides between AS-treated and untreated samples. Finally, general effects of HNO were also investigated. Using the Global Proteome Machine (www.thegpm.org) we have seen an increase (~2x) in the number of oxidation sites on amino acid residues in HNO-treated platelets, such as tryptophan, tyrosine and lysine.

Innovative aspects
- Development of a novel assay to determine the selectivity and the extent of nitroxyl-induced protein modification
- Characterization of these modifications on platelet proteins

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C413

Phosphoproteomics of *Klebsiella pneumoniae* NTUH-K2044 reveals a tight link between tyrosine phosphorylation and virulence

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Introduction
Protein phosphorylation is one of the most biological relevant and ubiquitous post-translational modifications in both eukaryotic and prokaryotic organisms. It is generally considered nowadays that bacteria harbor different phosphorylation systems, namely phosphoenol pyruvate : carbohydrate phosphotransferase (PTS) system, two-component system, and ATP/GTP-dependent system. Recently, more and more accumulated evidence suggests that Ser/Thr/Tyr phosphorylations play versatile roles in regulating a diverse range of cellular responses and physiological significances in prokaryotes. As for tyrosine phosphorylation, it has been reported with surprise to be the key of exopolysaccharide biosynthesis in several bacteria.
Capsular polysaccharide of *K. pneumoniae* has been determined as the major factor of virulence. Encapsulated *Klebsiella pneumoniae* is the predominant causative agent of the emerging infectious disease, pyogenic liver abscess, which usually complicated with metastatic meningitis or endophthalmitis in the world. The regulation of capsular polysaccharide biosynthesis is still unclear except that protein tyrosine kinase and phosphatase are involved. The identification and characterization of these signaling regulators would significantly advance our understanding of the underlying mechanism that controls the formation of capsule and would become the therapeutic targets.

Methods
The bacteria are grown to mid-log phase at 37℃ incubation and then harvested, meanwhile, tyrosine phosphorylated proteins were enriched by immune-precipitation against phosphotyrosine so as to catalog more pTyr sites. The antibody enriched portion was then digested with Lys-C and trypsin endopeptidase. Apart from protein level enrichment, peptide level enrichment was also carried out by TiO2 following SCX chromatography fractionation. Subsequently, these captured peptides were analyzed by nanoscale LC coupled to the LTQ-Orbitrap. To increase the coverage and confidence of phosphopeptide identification, samples were analyzed for multiple times and then searched against MASCOT algorithm. Finally, site-directed mutants were generated and then their virulence thereof was assessed in capsule quantification, serum killing assay and mouse lethality model.

Results
117 phosphopeptides were identified from 81 *K. pneumoniae* proteins with the false positive rate of less than 1%. 93 distinct phosphorylation sites were assigned confidently, including 29 serines (31.2%), 14 threonines (15.1%), 24 tyrosines (25.8%), 12 histidines (12.9%) and 14 aspartates (15.1%). In GO enrichment analysis, most of phosphoproteins involves in translation as well as protein and carbohydrate metabolism in the functional annotation. In the view of cellular component, most of the over-represented phosphoproteins located in cytoplasm or constituted ribosome. However, these identified pTyr proteins only sorted in the biopolymer metabolic process were statistically over-represented. Notably, three identified pTyr proteins, protein tyrosine kinase (Wzc), phosphomannomutase (ManB) and undecaprenolphosphate glyolytransferase (WbaP), located within *cps locus*, were presumed to be the candidates for converging the signal transduction. Since Wzc has been well characterized, we focused on ManB and WbaP. Two site-directed phenylalanine substituted mutants, therefore, were generated and the virulence was assessed by capsule quantification, serum killing assay and mouse lethality assay. Comparison with those of the wild-type strain, the capsular polysaccharides of WbaP mutant (WbaY5F) was dramatically reduced quantitatively and the LD50 increased by 200 fold in mouse peritonitis model. However, the capsular poly-saccharides of ManB mutant (ManBY26F) showed no difference in quantity and the LD50 slightly increased by only 6 fold in mice test. Thus, tyrosine phosphorylation of WbaP is clearly involved in the regulation of the biosynthesis of capsular polysaccharide that correlates closely with virulence.

Innovative aspects
- Most pTyr proteins are sorted in biopolymer biosynthesis.
- The phosphorylation on Tyr^5 residue of WbaP is demonstrated to influence CPS production leading to virulence for the first time.
- Several phosphorylated residues except for tyrosine were identified either in Walker A motif or in the periplasmic loop region of Wzc.

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C414

Identification of a new biomarker against genotoxic carcinogens

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Introduction
Toxicoproteomics is an emerging field aiming to identify critical proteins and pathways in biological systems that are affected by adverse chemicals and environmental exposures based on systematic analysis of global protein expression. To that extend, the potentials of genotoxic carcinogens towards genetic mutation or cancer have been emphasized to be monitored at the early stage of their exposure to the biological systems. In this study, we identify and validate a new biomarker protein as an indicator of the exposure to genotoxic carcinogens towards mouse lymphocyte model cells.

Methods
Mouse lymphocyte cells, L5178Y, were treated with genotoxic carcinogens for 2 h to prepare the proteome samples of 2-DE and western blot analysis. Proteome samples were separated by 2-DE and image analysis was conducted using Image Master Platinum 5. As the result, spots which showed more than 1.5-fold increased or decreased expression pattern compared with solvent control were selected. Peptide mass finger printing was analyzed by using 4800 MALDI-TOF/TOF mass spectrophotometer. Monoisotopic peptide mass was obtained by using Data Explorer 3.5. MASCOT search engine was applied to identify the protein and western blot analysis was conducted to validate this result.

Results
To discover the specific biomarker against well known genotoxic carcinogens, 1,2-dibromoethane and glycidol, 2-DE and MALDI-TOF/TOF MS analysis were conducted using the cellular proteome of L5178Y (mouse lymphocyte cells) treated with the compounds. As the result, 50 spots which showed more than 1.5-fold increased or decreased expression were selected and identified. Among these proteins, we focused on a candidate biomarker GCB1 (Genotoxic Carcinogen Biomarker1) that showed increased expression pattern against compounds treatment (Fig 1). Western blot analysis using monoclonal GCB1 antibody validated the protein and the increased expression of GCB1 upon exposure to the compounds. Furthermore, GCB1 was also increased in the cells treated with additional 7 genotoxic carcinogens, indicating that GCB1 could be a reliable biomarker toward genotoxic carcinogens (Fig 2). Collectively, the new biomarker GCB1 was identified and validated for monitoring genotoxic carcinogens and this study demonstrated proteomics is a powerful tool to identify new biomarkers for the biological safety of chemicals and environmental exposures.

Innovative aspects
- Proteomics-based biomarker discovery
- Application of proteomics to evaluation and diagnosis of the biological safety of chemicals.

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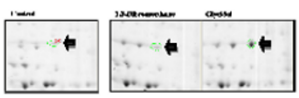

Figure 1. Spot selection of GCB1. Proteome samples were separated by 2-DE and spots which showed more than 1.5-fold increased or decreased expression pattern compared with solvent control were selected. Arrows indicate that GCB1 spot of each gel.

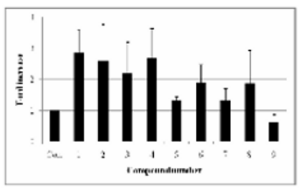

Figure 2. The expression level of the new biomarker GCB1 treated with solvent control and 9 genotoxic carcinogens. Among 9 test samples, 6 presented more than 1.5-fold increase, suggesting that GCB1 could be used as a biomarker protein to monitor genotoxic carcinogens exposure towards mouse lymphocyte cells.

C415

Functional Proteomics Identifies EEF1D as a Biomarker for CK2 Inhibition

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Introduction
Protein kinases have emerged as promising targets for cancer therapy based on their role in transformation and tumorigenesis. Protein kinase CK2, representing a small family of serine/threonine protein kinases, exhibits oncogenic activity in transgenic mice and is frequently elevated in tumours and leukemic cells. Accordingly, CK2 has emerged as a promising candidate for molecular-targeted therapy. In order to enable the validation of CK2-targeted therapies, we undertook an unbiased functional proteomics approach to identify biomarkers for CK2 inhibition.

Methods
Human cervical carcinoma HeLa cells, were biosynthetically labelled with 32P in the presence or absence of commercially available CK2 inhibitors (TBB, TBBz, DMAT). Phosphorylation profiles were analyzed by 2D gel electrophoresis and proteins exhibiting significant decreases in 32P incorporation were identified by mass spectrometry. For validation of EEF1D as a bonafide substrate for CK2, constructs encoding EEF1D with N- or C-terminal FLAG tags were expressed in HeLa cells. To overcome decreased phosphorylation by CK2 inhibitors, rescue experiments were also performed using constructs encoding inhibitor-resistant mutants of CK2.

Results
A systematic, unbiased functional proteomics strategy employing 2D electrophoresis and mass spectrometry led to the identification of eukaryotic elongation factor 1 delta (EEF1D) as a protein with decreased 32P labelling in the presence of CK2 inhibitors. Since EEF1D is phosphorylated in cells at a site conforming to the consensus for CK2 phosphorylation, it was selected for further investigation as a candidate CK2 substate and potential biomarker for CK2 inhibition. To validate EEF1D as a bonafide physiological substrate for CK2, it was expressed in HeLa cells treated with or without CK2 inhibitors. Analysis by 2D electrophoresis confirmed significant changes in EEF1D-FLAG phophorylation profiles indicating the inhibitory effect of the CK2 inhibitors. Kinase assays performed using immuno-precipitated EE1D-FLAG as substrate for wild-type and inhibitor-resistant forms of CK2 reinforced the conclusions that EEF1D is a direct substrate for CK2. Overall, these studies demonstrate a systematic approach for the identification of physiological substrates for protein kinases and for the identification of biomarkers for kinase inhibition.

Innovative aspects
- systematic functional proteomics approach to characterize protein kinase inhibitors
- identification of a novel substrate for protein kinase CK2
- translation elongation factor EEF1D is a potential biomarker for CK2 inhibition

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Two Novel Strategies for a Targeted Proteomic Analysis of Protein Citrullination

C416

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Introduction

Protein citrullination results from enzymatic deimination of peptidylarginine. Despite its important role both in health and disease, the identity and function of citrullinated proteins *in vivo* remain widely unknown. Proteomic studies of protein citrullination will require tools for a targeted analysis of citrullinated polypeptides which are currently not available. Here we describe two novel strategies for such analyses which are based on chemical modification of citrulline: (i) signature ion based identification of citrullinated peptides; (ii) specific enrichment of citrullinated peptides.

Methods

Signature ion based analysis: Digests of citrullinated proteins were incubated with 2,3-butanedione and antipyrine at low pH. After work-up by SCX and RP chromatography, samples were analyzed on an HCTultra ion trap instrument using alternating collision induced dissociation (CID) and electron transfer dissociation (ETD).
Enrichment of citrullinated peptides: Glyoxal derivatives were covalently attached to beads via a base labile linker. These citrulline reactive beads (CRB) were incubated with digests of citrullinated proteins at low pH resulting in immobilization of citrullinated peptides. After extensive washing, bound peptides were cleaved off at high pH and analyzed by MALDI-TOF/TOF. For data evaluation, variable modifications of arginine were implemented in the search engine.

Results

Citrulline residues can be specifically derivatized by either 2,3-butanedione/antipyrine or by glyoxal derivatives (Fig. 1). No other amino acid side chain is modified.
CID of butanedione/antipyrine-modified citrulline generates an intense fragment ion at m/z 201.1028 which serves as a signature ion for monitoring of citrullinated peptides in LC-MS/MS. In ETD, the modification remains intact and MS/MS spectra show nearly complete sequence coverage. Therefore, LC-MS/MS with alternating CID and ETD allows targeted identification of protein citrullination sites, as shown by the analysis of myelin basic protein (MBP) which was deiminated *in vitro* (1).
Two variants of CRB were prepared, carrying either in-house synthesized 4-glyoxalbenzoic acid or commercial 4-hydroxyphenylglyoxal. Both CRB allowed a highly specific enrichment of synthetic citrullinated peptides added to protein digests (Fig. 2). CRB were applied to enrich and identify citrullinated peptides from digests of enzymatically deiminated proteins (e.g. MBP) (2). Further, a soluble and tagged glyoxal derivative was synthesized and applied to enrich citrullinated peptides from synovial fluid of rheumatoid arthritis patients.

Innovative aspects

- Specific chemical modification of citrulline
- Signature ion based detection and identification of citrullinated peptides
- Specific enrichment of citrullinated peptides by beads functionalized with glyoxal derivatives

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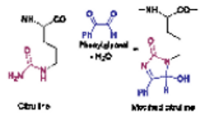

Figure 1. Modification of citrulline by phenylglyoxal

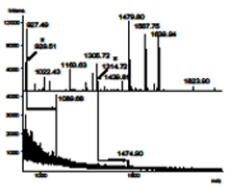

Figure 2. Enrichment of two synthetic citrulline containing peptides added to a tryptic BSA digest by CRB. MALDI-TOF spectra before (top) and after enrichment (bottom).

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cAMP Capture Compounds: Fishing Rod for the Identification of cAMP Signaling Pathway Proteins

C417

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Introduction

cAMP is an important second messenger which regulates multiple targets including different cAMP/cGMP-dependent protein kinases (PKA/PKGs), phosphodiesterases (PDEs), guanine nucleotide exchange factors directly activated by cAMP (Epacs) and cyclic nucleotide gated ion channels (CNGs). To elucidate the molecular basis of these pathways, identification and profiling of the cAMP-binding proteins is an essential step. Unique and robust tools, cAMP-Capture Compounds™ (cAMP-CCs, **C1-C3**, Fig.1), were developed to specifically target the cAMP-binding proteins. The cAMP-CCs are trifunctional small molecule probes that carry a modified cAMP as selectivity function, a photoactivatable diazirine moiety as reactivity function and biotin as sorting function. Here, we demonstrated the capability of the cAMP-CCs to profile the proteome subset of cAMP-binding proteins.

Methods

In the Capture Compound Mass Spectrometry (CCMS) workflow, the selectivity group (modified cAMP derivatives) first provides equilibrium binding of the cAMP-CCs to the target proteins. Second, upon irradiation, the reactivity function freezes this interaction through covalent cross-link to the target proteins. Third, using streptavidin magnetic beads, the CC-protein conjugates can be easily isolated from the complex protein mixture *via* the biotin sorting function. The captured proteins are then directly subjected to proteolytic cleavage and automated LC-MS/MS based protein identification. The DTA files were analyzed using SEQUEST™, and Scaffold™ was used to compile and assign probability scores.

Results

We applied the cAMP-CCs approach to human derived HepG2 cell lysate and solubilized rat brain synaptosomes. The generally low abundant cAMP binding proteins and some of their interactors were efficiently enriched. All known protein kinase A regulatory subunit isoforms (PKA-R) could be identified in HepG2 (500µg protein input). In addition, we have identified for the first time in mass spec-based proteomics the transmembrane hyperpolarization-activated cyclic nucleotide-gated channels (HCN1, HCN2) at the level of the endogenous proteins from rat brain synaptosomes (80µg protein input). Notably, distinct selectivity profiles were observed with different cAMP-CCs, in which N6 or C2 modified cAMP-CCs (**C2**, **C3**, Fig. 2) exclusively bound to PDEs while C8 modified cAMP-CC (**C1**) has an excellent binding capacity to Epac4.
Furthermore, several potential and *bona fide* interaction partners of cyclic nucleotide-binding proteins, such as ryanodine receptor, GABA receptor, protein kinase C, and A-kinase anchoring proteins were identified with the cAMP-CCs.
The results presented here demonstrate that cAMP-CCs are unique tools to specifically target cAMP binding proteins.

Innovative aspects

- Novel fish rod for profiling the cAMP signaling pathway proteins
- Suitable for biopsies considering minute amounts of complex protein mixtures
- Uniquely sensitive tool to address low-abundant transmembrane ion channels due to the covalent CC-protein interactions

References

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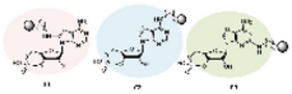

Fig. 1: Structure of cAMP-CCs (**C1**, **C2** and **C3**). Grey ball refers to a scaffold with diazarine and biotin as reactivity and sorting functions respectively.

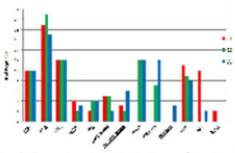

Fig. 2: Examples of cAMP-binding proteins identified by cAMP-CCs (**C1**, **C2** and **C3**) capturing in rat brain synaptosomes (triplicate experiments, Scaffold™ protein probability >99% and peptide probability >95%).

Computational Approaches and Proteomic Datasets

(C501 – C552)

MacroSEQUEST: an analysis of candidate-centric searching for proteomics

C501

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Introduction
Here we formally describe a general strategy for improving the performance of computer-assisted spectral matching algorithms that we call "candidate-centric searching", and demonstrate its utility and advantage through its implementation in a modified version of SEQUEST that we call MacroSEQUEST. This optimized version of SEQUEST is able to analyze large and complex sets of spectra (10,000 spectra matched to a database of 150,000 proteins) on a single CPU computer in several minutes, whereas legacy SEQUEST takes days.

Methods
MacroSEQUEST was written in ANSI C and compiled on a Unix-based system. Both the preliminary (Sp) and XCorr scoring systems were replaced with the Fast XCorr algorithm as described in Eng etal 2008 (1). The correlation identity results between MacroSEQUEST and legacy SEQUEST are identical. Additionally, an E-value as described in Eng et al 2008 is implemented.

Results
SEQUEST, a thoroughly established program, correlates peptides contained in a translated genomic sequence database with experimental tandem mass spectra from a proteomics dataset (2). The most-likely identity for each spectrum is matched by first digesting and scoring every protein in a given database *in silico*, then matching these theoretical spectra to experimentally generated spectra. Published in 1994 when datasets were relatively small, SEQUEST is run once for each spectrum, resulting in a tremendous duplication of effort for analyses of contemporary proteomics runs. In particular, we identified two areas of redundancy: database parsing and theoretical spectra scoring. Our 'candidate-centric' method reduces this wasted work by initially collecting and organizing all the spectra in a dataset before searching the database (Fig. 1). We have generated three datasets with which to examine the effect of candidate-centric searching: a large-scale Human sample, a small-scale Yeast sample and a large-scale Human sample enriched for phosphorylation by TiO2, each with approximately 10,000 acquired spectra. MacroSEQUEST is able to score each data set with a reduced set of steps, resulting in a dramatic improvement in productivity, defined as the number of spectra analyzed per unit time (Fig 2).

Innovative aspects
- Able to search through a FASTA database more quickly than a traditional indexed search
- E-value calculated from Fast XCorr distribution
- Reduced computational resources required to run SEQUEST

References
(1) Eng et al, A Fast SEQUEST Cross Correlation Algorithm; J Proteome Res (2008) vol. 7 (10) pp. 4598-602
(2) Eng et al, An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database; J. Am. Soc. Mass Spectrometry (1994)

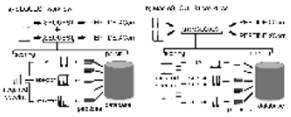

Figure 1. Comparison of SEQUEST and MacroSEQUEST workflows. a) SEQUEST considers each individual experimental spectra to be a discrete search event and therefore must work through and entire search cycle for each acquired spectra. B) MacroSEQUEST considers all of the acquired spectra to be a single set and thus is able to doing all requiring database parsing and spectra scoring in one search cycle.

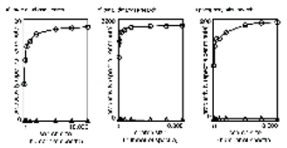

Figure 2. Comparison of SEQUEST and MacroSEQUEST productivity a) large-scale human sample with 20,208 average matched peptides scored from 150,324 proteins. b) small-scale yeast sample with 2,153 average matched peptides scored from 12,854 proteins. c) large-scale human TiO2 sample with 98,163 average matched peptides from 150,324 proteins.

Mesothelial cell stress response to peritoneal dialysis fluid – potential role of glucose

C502

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Introduction
In this proteomic study the stress response of MeT-5A cells, representing mesothelial cells (MCs), to therapeutic stress by peritoneal dialysis fluid (PDF) is elucidated. Biocompatibility research almost exclusively focuses on toxic physicochemical properties such as acidosis, high lactate concentrations and presence of glucose degradation products. In this 2DGE based work we focus on the role of glucose as a stressor to MCs.

Methods
In a Two-Dimensional Gel Electrophoresis (2DGE) experiment, samples of a control *vs.* a stress group were compared. In these experiments 100 proteins significantly different in abundance were identified by MS and MS/MS.
A KEGG database search was performed with the whole dataset of identified proteins in order to find the pathways they are assigned to. A chi square test allowed us to extract those pathways which were significantly "enriched" after PDF stress. As this work particularly focuses on the glucose associated stress response proteins associated to these pathways were in the centre of our interest.

Results
Bioinformatic treatment of the identified significantly over/under- abundant proteins revealed several *„biological pathways"* being over-represented within this protein set. Out of 12 *„biological pathways"* being glucose-metabolism associated, 4 were found to be significantly over-represented ($p<0.05$).
These pathways were *glycolysis* (5 proteins), *reductive carboxylate cycle* (2), *pentose phosphate pathway* (3) and *pyruvate metabolism* (3). 2 proteins were assigned to two pathways. These 11 proteins (present in 15 spots) populating the 4 mentioned pathways were further investigated.
Key enzymes of the *"glycolysis pathway"* were found over-expressed. This result seems to indicate that cells enhance glycolysis, producing energy equivalents (ATP) needed for stress response.
The over-expression of two enzymes of the *"pentose phosphate pathway"*, a turnoff from the glycolysis pathway, is supposed to stay in the context of an increased need for the reduction equivalent NAPDH. NADPH, a cofactor for the regeneration of the antioxidant glutathione, might be needed for the detoxification of reactive oxygen species (ROS) discussed as end-products of glycose metabolism in the diabetes model (1).

Innovative aspects
- Analysis of the role of glucose in peritoneal dialysis
- Discussion of pathways related to glucose-associated stress response

References
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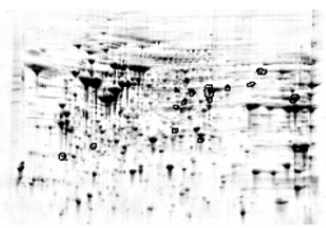

Figure 1. Coomassie brilliant blue stained 2D Gel exhibiting proteins from the MeT-5A cell lysate. Proteins significantly altered in abundance after PDF exposure and assigned to significantly over-represented glucose-associated pathways are encircled.

Gene-centric knowledge-base of human proteins coded in 18th chromosome

C503

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Introduction
Within the Human Proteome Project (HPP) the problem of collection, storage, usage and re-usage of the information about the proteins seems to be very important. Russian part of HPP is aimed on the inventory of proteins coded by 18th chromosome. It assumes a new, gene-centric approach in proteomics in contrast to disease-centric paradigm now dominant in the field. HPP is going to characterize the protein linkage to the genome in terms of evidence on proteome level, e.g. evidence in mass-spectrometric experiment. So, LC-mass-spectra of the protein and its fragments are going to be the main attributes of protein relation to a specific gene.

Methods
In present report we introduce a concept of gene-centric knowledge-base of human proteins coded by 18th chromosome.
Knowledge-base has to accumulate a mass-spectrometric data currently available and newly acquired within HPP project. mzData – open XML-based data format was chosen for mass-spectra storage. Considering gene-centric paradigm all newly uploaded mass-spectra would be identified within the knowledge-base using unified procedure. It allows us to achieve the best linking of mass-spectra to the knowledge-base proteins derived from the DNA coding sequences. Unified format of mass-spectrometric data and identification data provides large field for bioinformatics analysis.

Results
The presented knowledge-base based on database which encompasses entities like protein, peptide, mass-spectrum linked to the genes of 18th chromosome. Current realization is built upon the links between UniProt, PeptideAtlas/PRIDE and ProteinAtlas systems. Today KB encompasses information for 45 proteins which comprise ~17% of all protein coding genes of 18th chromosome. Each of these proteins has at least one proteotypic peptide mass-spectra. Six of these proteins were mapped by antibodies in course of ProteinAtlas project.

Innovative aspects
- Concept of gene-centric proteomic knowledge-base
- Integration into Human proteome project

C504

CPFP – The Oxford Central Proteomics Facility Pipeline

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Introduction
Core proteomics facilities frequently lack the manpower necessary to carry out state-of-the-art analysis of mass spectrometry (MS) proteomics data, and typically use a single search engine such as Mascot (Matrix Science). We present the Central Proteomics Facility Pipeline (CPFP), which uses multiple search engines, modelling tools, and target-decoy validation, to provide confident peptide and protein identifications with minimal user intervention. The software is accessed via an intuitive web interface, and is aimed at core facilities rather than specialist laboratories.

Methods
Raw data in mzXML format or peak list data in .mgf or .pkl format is automatically submitted to the Mascot, X!Tandem, and OMSSA search engines with parameters specified on a single web form. Translation of parameters is handled by the software. Results are combined using the Trans-Proteomic Pipeline (TPP) PeptideProphet, iProphet, and ProteinProphet tools [1]. The concatenated target/decoy protocol is used to filter results according to a given empirical false discovery rate (FDR). The software is written in Perl using the Catalyst framework. Search and analysis jobs are submitted to a Sun Grid Engine cluster. We are now adding automatic submission of results to a local installation of the PRIDE proteomics repository [2], and integrating PTM prediction and identification tools.

Results
The Institute of Systems Biology (ISB) standard Mix 7 (Orbitrap) and Mix 2 (QTOF1) datasets [3] consisting of 10 MS/MS runs each were searched against SwissProt v57.1 using Mascot v2.2 and the CPFP. Identical parameters were used for each method. CPFP results were viewed at the default 1% target/decoy FDR. Mascot results were examined at 1% FDR using both the identity and homology thresholds. Submission of the 20 data files required 8 minutes of user time for Mascot vs 11 minutes for the CPFP. Total processing time was 49 minutes for Mascot on a 16 core cluster and 53 minutes for the CPFP on a 40 core cluster.

The CPFP identifies a greater mean number of peptides than Mascot for an equivalent FDR (Figure 1). Within the pipeline the combined number of peptides identified exceeds those of the individual search engines. Results can be easily shared with facility clients via the web interface.

Innovative Aspects
- Automated data analysis with multiple search engines and state-of-the art tools.
- Simple interface and automated processing aimed at core proteomics facilities, rather than specialist bioinformatics staff.
- Scalable clustered execution of search and analysis jobs.

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[3] J Kilmek et al. The Standard Protein Mix Database: A Diverse Data Set To Assist in the Production of Improved Peptide and Protein Identification Software Tools, J Proteome Res. 7, pp 96-103, 2008.

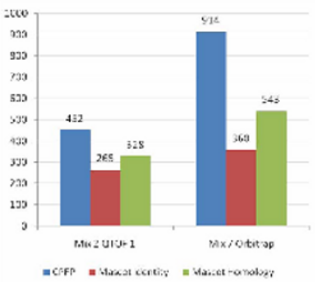

Figure 1: Mean peptide identifications at 1% FDR, CPFP vs Mascot, for ISB QTOF1 (mix 2) and Orbitrap (mix 7) datasets.

C505

Improving transition selection using Synthetic Peptide Database for TPM/MRM pipeline

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Introduction
With the ever increasing abundance of tandem mass spectrometry data from shotgun proteomics, MS/MS-spectral repositories will be useful to build targeted mass spectrometry assays using TPM (Targeted Peptide Monitoring) and MRM (Multiple Reaction Monitoring) approaches. Detection of multiple low abundance proteins depends on optimized instrument settings. However, there is still no clear target selection and method optimization pipeline for building assays to simultaneously monitor multiple targets in complex mixtures. Manual construction of multiplex assays is cumbersome, time consuming and may result in suboptimal detection. Hence we have developed a principled, automated strategy based on the generation of reference MS/MS-spectral information using synthetic reference peptides to devise assays with optimal experimental parameters and minimal overlap in mass or retention time. Our method allows for the building of assays showing a considerable improvement in sampling efficiency and detection reliability.

Methods
Our method consists of a reference spectral database and web interface to allow relevant data to be added and extracted to build suitable instrument methods. The web interface allows for the query of target-specific transitions for protein targets of interest and exports CSV-files which can be imported into the instrument software (e.g. Xcalibur). Additionally, assays can be devised and visualized using fuzzy-logic-based retention time maps to inspect the optimal assay construction.

Results
MS/MS-spectra and retention time information recorded for hundreds of reference synthetic peptides mapping to low abundance protein components of mammalian signaling pathways were stored in an indexed database (1,2). Experimental product ion intensities and retention time information were accessed to build multiplex LC-MS/MS assays. A web-interface allows custom design of high-density assays optimized for monitoring multiple targets. Functionalities such as most intense product ions, least overlap of predicted m/z-values and optimal retention time windows were calculated using fuzzy-logic. With using the present web tool, we found the optimal retention time and transitions for high density (>100 peptides) detection assays. We generated and successfully applied pathway-level assays to monitor and quantify low abundance transcription factors and protein kinases in self-renewing and differentiation mouse embryonic stem cells (2).

Innovative aspects
- Construction of a high-quality MS/MS-spectral reference library based on synthetic peptides to optimize multiplex target assay design.
- Functional web-interface for choosing suitable transitions/peptides for selected proteins.
- Optimization and visualization of the retention time overlaps for multiple targets/transitions.

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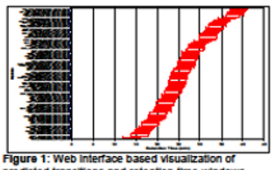

Figure 1: Web interface based visualization of predicted transitions and retention time windows for multiple target assays.

Figure 2: Web interface for querying the reference database, for example either by Protein Name, Sequence, Precursor or Product mass range, Retention Time, Product Ion Intensity, and/or Charge state.

C506

Variations of Peptide Ion Intensities in ESI LC-MS Experiments

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Introduction: Variations in ESI signal strength of peptides in LC-MS experiments is the source of much of the variability in mass-spectrometry based proteomics. The present studies apply new data analysis tools for comparing MS1 signal strengths of peptide ions in protein digests. These enable the measurement and examination of various contributors to this variability. Full scan MS/MS serves to identify components, while MS1 peak maxima derived from the raw data provide relative intensities.

Methods: Data analysis methods were developed to extract information from raw data files and combine it with results of peptide identification results (from traditional sequence library and spectrum library searches). Ion trap, orbitrap and qtof classes of instruments were examined. 'Clusters' of m/z peaks corresponding to a individual ions were perceived, their chromatographic maxima found and when possible they were connected to peptide identifications derived from MS2 spectra. Data originated from multiple analyses of various 'standard' mixtures employed for interlaboratory NIH.NCI CPTAC studies as well as from egg yolk and pure protein digests.

Results: Relative intensities of individual peptide ions across multiple LC-MS runs of both simple and complex mixtures were examined for various series of runs: 1) successive analysis; 2) replicate runs, but with intervening runs of other mixtures; 3) replicate runs done weeks apart, 4) different loadings; 5) different LC gradients; 6) major tuning differences, especially 'in-source' voltages. Variability is analyzed as a function of various factors, including retention time (hydrophobicity), peak intensity, peptide class and others factors. Systematic trends are found for early and late eluters, probably a result of solubility. Loading and gradient generally also had significant effects on variability. Irregularities in electrospray could lead to regions of elution showing large deviations from average behaviour, though short term ESI variations were generally not significant. Observed variations are expressed as median deviations, providing values and metrics for describing 'intra-sample' variability. This, in turn, should enable the reliable determination of 'inter-sample' variations in future studies.

Innovative Aspects:
1) Signal intensity variations were measured for peptides in 'standard' tryptic digests with controlled variations in analytical conditions
2) Metrics were developed for describing these 'intra-sample' variations.
3) Provides basis for future 'inter-sample' studies.

C507

Spectral counting - An easy to use and cost effective method for label free proteomics.

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Abstract content: "Label free" approaches are the upcoming methods for quantitative proteomics. Spectral counting is a very easy to use and cost effective approach for this.

Title: Spectral counting - An easy to use and cost effective method for label free proteomics.

Introduction
Biomarker discovery has become even more and more important in proteomics. Most of the biomarker discovering techniques today uses labeling techniques for peptides or proteins. Problems of chemical or isotopic labeling are the additional variations by using the labeling steps and the labeling efficiency. "Label free" Proteomics approaches by quantifying on LC-MS runs will have a high impact in the near future, but the workflow is very complex and today's software tools have many disadvantageous. However we will show a very simple and easy to use label free approach by using spectral counting.
Methods
An amount of 5/50/100/250 and 500 ng of a mixture covering eight proteins was used together with an amount of 500 ng protein sample of a lung carcinoma cell line (A549). Each sample was measured five times with a Bruker MicroTof-Q by using 3h LC-MS/MS-runs. The resulting mgf files were merged into one mgf or by using ProteinScape (Bruker Daltonik GmbH, Bremen, Germany) to process them with Mascot in one search. The results of peptides and proteins together with the spectrum identification and therefore with the run, were exported to Excel and analyzed for spectral counting by only using the Pivot Table function of Microsoft EXCEL 2007.
Results (maximum 1200 characters, including spaces)
We have identified some of the proteins from the spiked mixture. They can be quantified as differential in the sample, but not in the same way as expected theoretically from the desired dilution series. There is some bias between the expected ratios and the measured ratios. However only the spiked proteins could be found as differentially expressed (ratio larger than 1.3 and lower than 0.76 together with a t-test smaller than 0.05). The proteins of the background (500 ng A549) were not identified as differential because they are in all runs the same. However a quantification of some spiked proteins in totally different concentrations is possible. This simple approach of spectral counting is useable to quantify proteins down to a low amount of several fmol. The quantification is not dependent on the amount of the protein, but more on the possibility to ionization of the peptides. The measured ratios did not represent the real ratio in the sample, but the method is suitable to detect differentially expressed proteins in complex mixtures for biomarker discovery.

C508

Quantitative toponome analysis of KRAB ZNF data sets present in the Proteinatlas database

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Introduction: To obtain spatial information on cell-type specific and disease-pathway related protein expressions related to KRAB ZNF protein functions, information on protein expressions presented by the Proteinatlas database were downloaded and computationally analyzed, partly guided by www.toponostics.de. Informative antibody repertoires were selected to determine protein expressions that are inversely correlated to the expression of KRAB ZNF genes since Krüppel-associated box (KRAB) zinc finger (ZNF) genes encode the largest mammalian family of proteins with strong transcriptional repressor activities (Margolin et al., PNAS 1994)

Methods: The Swedish Protein Atlas (Release 2008-12-03) content (www.proteinatlas.org) currently displaying immunostainings of 6103 antibodies relating to 5683196 images including microarray tissue samples from 48 different normal tissue types, 20 different types of cancer and various cell lines were analyzed by in-depth in-silico analysis in order to determine the most informative toponome profiles and antibody sets in respect to KRAB ZNF gene functions. Pearson correlation distances were calculated of immunostainings of i. normal tissue, ii. cancer tissue and iii. cell types singly and in combination to evaluate how expressions profiles diverge from each other and to determine tumour expression profiles that are more closely or more distantly correlated with each other as well as in respect to KRAB ZNF protein expressions. A minimum spanning tree - a concept from graph theory- was employed. Pearson correlation coefficients were measured between every pair of samples using R software and the correlation coefficients translated into correlation distances. The minimum spanning tree of a full graph was computed using Kruskal's algorithm. To obtain images of computed networks the open source software GraphViz was used. Immunostainings on cell lines and cells were quantitated by making use of the Definiens software.

Results: Immunostainings of 54 KRAB ZNF antibodies were used to visualize the relateness of toponome profiles of Proteinatlas immunostainings displaying KRAB ZNF protein expressions in respect to the expression of putative candidate proteins. Hereto, antibody sets showing the most divergent expression patterns within 48 tissue types, 20 cancer types and numerous cell lines in respect to KRAB ZNF expressions were assessed. Minimum spanning trees thereof were visualized based on Pearson correlation distances to determine tissues and proteins thereof that display most divergent protein expression profiles. To validate the quality of the monovalent KRAB ZNF antibodies used, the epitope signatures of these antisera were assessed as well against all corresponding PrEST sequences, see Lorenz et al, 2009. In general, the protein expression analysis of tumour tissues in conjunction to normal tissues revealed that the extracted data sets describing tumour types are highly diverse within individual cancer types revealing the presence of great inherent heterogeneities. KRAB ZNF toponome signatures within these tissues and cell types thereof were correlated with each other as well as studied in respect to the phylogenetic relateness of KRAB ZNF genes under study by making use of the SysZNF database (see Ding et al., 2009: URL http://epgd.biosino.org/SysZNF). Our in-silico analysis demonstrates that the Proteinatlas database encompasses a unique reservoir of pathway information waiting to be revealed. The workflow described leads to the most informative antibody sets for in depth analysis of putative KRAB ZNF target gene functions in complex tissue specimen.

Innovative aspects: I. Immunostainings should be systematically quantified by imaging software. II. Each polyclonal serum should be characterized by their epitope pattern on PrESt peptide sequences. III. KRAB ZNF genes have to be grouped in phylogenetic clusters before analyzing them on the toponome level.

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C509

Antibodypedia – a portal for sharing antibody and antigen validation data

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Antibodies are useful tools to characterize the components of the human proteome and to validate potential protein biomarkers discovered through various clinical proteomics efforts. The lack of validation results across various applications for most antibodies often makes it necessary to perform cumbersome investigations to ensure specificity of a particular antibody in a certain application. A need therefore exists for a standardized system for sharing validation data about publicly available antibodies and to allow antibody providers as well as users to contribute and edit experimental evidence data, including data also on the antigen. Here, we describe a new publicly available portal called Antibodypedia (http://www.antibodypedia.org), which has been developed to allow sharing of information regarding validation of antibodies in which providers can submit their own validation results and reliability scores. We report standardized validation criteria and submission rules for applications such as Western blots, protein arrays, immunohistochemistry and immunofluorescence. The contributor is expected to provide experimental evidence and a validation score for each antibody and the users can subsequently provide feedback and comments on the use of the antibody. The database thus provides a virtual resource of publicly available antibodies towards human proteins with accompanying experimental evidence supporting an individual validation score for each antibody in an application-specific manner.

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C510

Modeling of Protein Networks in Complex Systems for the Optimization and Control of Bioprocesses

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Introduction

The use of technologies and methods of the "white biotechnology" plays an essential role for several industrial production processes, e.g. in producing drugs or antibodies. Especially in fields like biotransformation, fermentation, biocatalysis and metabolic engineering the innovative technologies of proteomics (e.g. quantification via spectral counting) are of growing importance. For the development and optimization of the process in the lab and the monitoring of the actual production process the exact knowledge of the complex regulation mechanisms is of great interest.

Methods

For identification of relevant process markers it is necessary to determine the state of the system as a whole and to understand its dependence on external parameters (temperature, pH). Therefore spectra and results are analyzed with multivariate statistical analyses (e.g. unsupervised like clustering and PCA or supervised like classification via neural networks or support vector machines) to find relationships between process parameters (e.g. stability or drug yield) and mass, peptide or protein signals.
Next to experimental findings and empirical discoveries simulation-driven approaches are used to predict the reaction of the host organism to external stimulations. Therefore several experimental parameters and internal measurements (proteomics results) will be collected in a first step. These will be converted into standardized formats (e.g. formats of the Proteomics Standards Initiative PSI – http://www.psidev.info) (annotated with controlled vocabulary terms) and stored a standards-compliant database.

Results

Until experiments have been performed and data sets are generated, a review of existing proteomic data sets in literature or public repositories (e.g. PRIDE – http://www.ebi.ac.uk/pride) will be done. These data will be extended with genomic data sets (gene expression data or metabolomic data) and similarities and differences between gene and protein quantities will be characterized.
Another aspect in the direction of systems biology of the host biology is to utilize existing knowledge about protein interactions or to reveal new interactions specific for the host system used.
A last possibility for the understanding of the process influencing parameters is the inspection of pathways, especially whether they are strengthened, disturbed or blocked. The field the experiments take place is well suited for a utilization of interaction and pathway knowledge, as the host organisms are usually well described and the focus lies on metabolic instead of interaction pathways.

All the described data will then be assembled and used to generate a model of the process and to be able to predict its behaviour.

Innovative aspects

- Utilization of systems biology approaches for biotechnology applications (drug or antibody production)
- Relating data from public repositories with own data to improve models

C511

The Contaminant Repository for Affinity Purification (CRAP) project

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Introduction

Affinity purification followed by mass spectrometry (AP/MS) has become the method of choice for the identification of protein interactions and complexes [1]. One of the main challenges of this approach is co-purification of contaminant proteins, leading to false positive protein interactions. To address this problem, we have developed a new resource - the Contaminant Repository for Affinity Purification (CRAP). Our main objective is to create a central repository to store, annotate, statistically analyze and disseminate lists of protein contaminants likely to be observed in AP/MS studies.

Methods

Raw MS data (RAW or mgf format) for negative controls (e.g. tag alone) are collected from recent or ongoing AP/MS studies. All datasets are processed using a uniform data analysis pipeline using X! Tandem search engine, RefSeq database, and the TPP suite of data validation tools [2]. The lists of protein identifications and the semi-quantitative protein abundance information (spectral counts) are stored in the relational database ProHits developed at SLRI. The CRAP database contains annotation of the most relevant experimental conditions, including tag type and cell line, cell growth, lysis, purification, and LC-MS/MS analysis. Search and display options are being built in to help with the annotation of the data, data extraction, and subsequent statistical and data mining applications.

Results

The pilot version of the repository already contains a substantial number of AP/MS experiments, including data from several large-scale studies (e.g., 200 gel-based control experiments [3]). Among more than 1000 human proteins in the database, close to half were identified in multiple experiments and with high spectral counts. The top examples include such proteins as PRMT5, WDR77, and STK38.

The repository will be substantially expanded in the near future. We are continuing to gather data on different cell types, organisms, and purification strategies. An open letter to the proteomics community inviting data submission received an overwhelmingly positive response.

The contaminant repository will significantly improve the ability of individual researchers discriminate between spurious and real interactors and to decide which proteins they are interested in following up on, based on the propensity of the protein to be identified as a contaminant across multiple analyses. The database can also be used for statistical analysis to compare lists of contaminants observed under different conditions. Preliminary analysis has already uncovered groups of contaminants specific to a certain tag type, or overrepresented in gel-based vs. gel-free studies.

Innovative aspects

- New resource for detection of likely false protein interactions in AP/MS-derived datasets
- Data mining of contaminant protein profiles across different conditions
- Data contribution from multiple research groups representing diverse AP/MS conditions

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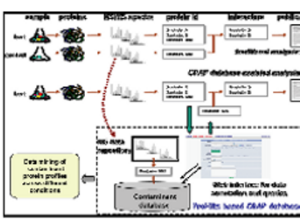

Figure 1. Outline of CRAP project for optimal use of negative control runs in AP/MS based interactome studies.

C512

Identification of peptide PTMs and allelic changes using an unbiased search algorithm

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Introduction
MS database searches are constrained to the data in the database used for the search. Probability scoring schemes for MS database searches are efficient at detecting random peptide-spectrum matches due to poor quality spectra but do not perform well in the case of high quality spectra that correspond to a peptide sequence that is not in the database due to allelic variation or modifications not included in the search. This often leads to the misidentifications of variant forms of peptides from high abundance proteins as unmodified peptides from other rare proteins.

Methods
Rat skeletal muscle endothelial cells were grown in RPMI ± stable isotope labelled arg (+10) and lys (+6) under high and normal glucose conditions. Proteins were extracted from high and low glucose samples, mixed and digested with trypsin. Peptides were separated by C18 chromatography and analyzed using a Thermo LTQ mass spectrometer. Resulting data files were searched against the rodent (taxon: 9989) portion of the UniProt database with Sequest including M (+16) and C (+57) variable modifications.

Results
We have developed an algorithm that uses the results of the initial search of the sample with Sequest to identify additional variant peptides from abundant proteins in the sample. After the initial search, the list of abundant proteins in the sample is used to identify alternative matches to unidentified spectra or spectra assigned to proteins with single peptide matches. To do this, the mass difference between the experimental spectrum and the calculated mass of peptides in the new database is considered as a potential modification at each site in the database peptide. For spectra that match better to the variant peptide, the mass difference is compared to known post-translational modifications or allelic changes. Even with limits imposed on the potential size differences, such a computationally intensive search is only tractable with a very small database of potential proteins. Since the list of abundant proteins found in the first pass in most samples is usually less than 100 proteins, this can be accomplished using the two-pass approach.
To test this approach, we used a sample that is composed of both SILAC labelled and unlabeled peptides. This sample mimics the situation in which abundant peptides have increased mass due to the SILAC label are present but not included in the Sequest search. Validation of the method is achieved if the mass shift caused by the SILAC label can be found in an unbiased search. As shown below (Figure 1), the unbiased search is able to accurately identify peptides containing SILAC label by the mass shift and location.
The second pass used to identify variant peptides is independent of the initial search engine or parameters used. We are currently using this method to survey biological samples to determine the extent and nature of the modifications detected as well as optimizing the algorithm and adapting it for using in a cluster or cloud-computing environment.

Innovative aspects
- Identifies variant peptides without needing to specify the mass shift and amino acid.
- Identifies both PTMs and allelic differences
- Reduces incorrect peptide assignments

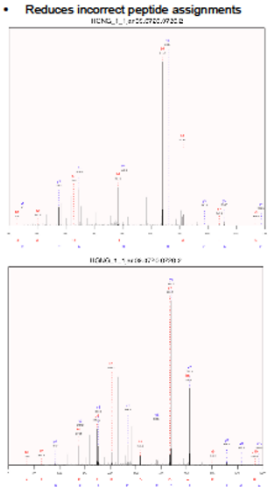

Figure 1. Spectral Assignments before (top) and after (bottom) correction showing correct identification of the mass shift due to the SILAC label.

C513

ProHits: an Open Source flexible integrated LIMS and analysis platform for interaction proteomics

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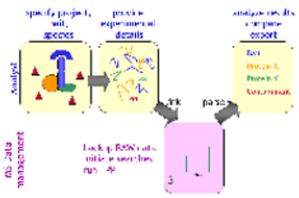
Introduction
Data management remains a major bottleneck in large proteomics studies. For protein interaction studies, an additional challenge is the interpretation of the data resulting from AP/MS studies in a biologically meaningful manner. Here we present ProHits, a modular Open Source system designed for interaction proteomics. Data management, database searching, statistical and quantitative analysis can be controlled across many projects, multiple instruments and several security layers. Integration with other Open Source software (X!Tandem, TransProteomics Pipeline, Cytoscape) allows for a seamless analysis of the AP/MS data. Detailed tracking of experimental details and bait to prey relationships, filtering options for contaminants and visualization/export functions enable cross-platform protein interaction studies.

Methods
The program is a client based HTML program written in PHP with data stored in a MySQL database. ProHits consists of two main modules: a Data Management module and an Analyst module. A ProHits "light" version consisting of only the Analyst module with an import function for search results is also available. Raw data from all instruments in a facility is copied to a secure storage location in a scheduled manner and converted to appropriate format for database searching which is initiated either manually or automatically (currently Mascot and X! Tandem are fully supported). Full integration of the ISB TransProteomics pipeline allows comparison of data searched with different engines. Search results are parsed to the Analyst module, in which a bait and experimental description are annotated. Within the Analyst modules, search results can be viewed, filtered and exported. Multiple links to external databases (e.g. NCBI, BioGrid) enhance the biological information content. Multiple AP/MS analyses can be viewed as side-by-side comparisons, and search functions further enhance the usefulness of the system. Flexible export functions allow for further analysis of the data, including direct visualization in Cytoscape, with MS data for the interactions (spectral counts, unique peptides, scores from the search engines) encoded and visualized as edge attributes.

Results
ProHits forms since 2002 the backbone of the MS analysis at the Samuel Lunenfeld Research Institute and supports gel-based and gel-free interaction proteomics. As such, it handles data from 10 laboratories, 9 instruments, ~90 users and thousands of experiments. ProHits is also undergoing beta testing in 6 other research groups in Canada and elsewhere. ProHits is also used as the backbone for community-supported projects, e.g. the Contaminant Repository for Affinity Purification (poster by A Nesvizhskii) and a benchmarking set for cross platform validation of AP/MS (poster by AC Gingras). ProHits is also undergoing further development, e.g. the integration of the SAINT algorithm (Significance Analysis of Interactome, posters by H Choi, M Tyers). While it was initially designed as a tool to handle protein interaction data, the modular design of Prohits allows for easy modification of the workflows supported.

Innovative aspects
- ProHits Analyst enables biologists to explore functional relationships between bait and prey in a biologically meaningful manner, filter contaminants, and visualize results
- ProHits facilitates detailed experimental annotations and traceable links to the RAW files and search results
- ProHits provides complete data management across instruments and projects including pre-scheduled analysis workflows



C514

Possible reasons of ambiguity in expression study by microarray and ion trap proteomics

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Introduction
Possible reasons of ambiguity were analyzed in the high-throughput study of the RNA and protein expression in the same biological specimen. Expression data on HepG2 cell line were compared to establish, at its best, the minimal threshold for the signals from the RNA expression microarray and mass-spectrometry-based identification to be considered biologically relevant.

Methods
HepG2 cells were not synchronized and used for genome-wide microarray RNA-analysis and proteome analysis.
Briefly, for RNA data the genome-wide DNA microarray system was used according the vendor's protocol (Agilent Technologies, CA). The microarray signal from each probe located on the chip was analyzed by GeneSpring software (Agilent). Primarily, the home Agilent identifiers were used for each probe.
Protein expression was estimated by 2D-LC-MS/MS procedure using XCT Ultra Ion Trap equipped by ChipCube interface (Agilent). Spectra were analyzed by SpectrumMill software and proteins were identified to assign the Uniprot identifiers. Minimal SpectrumMill overall score to be accounted was as low as 7.
The home Perl script was used to get information on the correspondence of the Agilent DNA probes and protein identifiers using the Ensembl resource (www.ensembl.org). Some probes were excluded due to the absence of corresponding protein identifier.

Results
Matching the protein and RNA tables it was shown that not all human proteins had their corresponding probes on the Agilent genome-wide array. These proteins were further not considered. It was also found that the rest of proteins have 1 to 13 corresponding probes, the expression level for different probes of the same protein being different, sometimes, by several logs. Of these probes, the probe having maximum intensity was taken for further calculations. No significant correlation was shown between protein spectral parameters (total score, number of spectra, sequence coverage etc) and probe hybridization intensity. The estimation of the minimal threshold for the signals from the RNA expression microarray and mass-spectrometry-based identification remains challenging.

Innovative aspects
- Simultaneous use of the Agilent microarray and mass-spectrometry proteomics platform to study the same cell culture
- Analysis of the reasons of ambiguity observed in such a multiplatform study

C515

InterPro: Deciphering the Human Proteome

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Introduction
The human proteome currently stands at 25,635 proteins. Some of these proteins have been experimentally characterized, but the majority has not and there is a pressing need to elucidate what functional role they play. InterPro is a powerful diagnostic tool for protein classification and function prediction. It uses a vast array of protein signatures to associate proteins of unknown function with those that are well characterized, providing comprehensive annotation on function, structure, splice variation, taxonomic distribution and hierarchical classification of proteins from all organisms.

Methods
InterPro, a free downloadable resource, is accessible at: http://www.ebi.ac.uk/interpro/. Ten major databases - PROSITE, PRINTS, PFAM, PRODOM, SMART, TIGRFAM, PIRSF, PANTHER, GENE3D, SUPERFAMILY – together contribute over 60,000 protein signatures to InterPro. These signatures model the conservation of amino acids found within protein families, domains, repeats and conserved sites using a variety of computational means: patterns, fingerprints, clustering and Hidden Markov Models. The models are run over the UniParc database to predict the classification/features of uncharacterized proteins. Novel sequences not yet submitted to UniProt can be queried through InterProScan, a downloadable tool that runs the complete set of InterPro signatures against the query protein to find a match.

Results
InterPro signatures match ~80% (almost 6M) of the protein sequences in UniProtKB. Of these, 18,861 are of human origin.
InterPro enables in-depth analysis of the human proteome, providing information on protein classification, domain occurrence, and the distribution of functional roles. The most common human protein families in InterPro are GPCRs, olfactory receptors, and the major facilitator superfamily, while the most common domains are those with an immunoglobulin fold, C2H2 zinc fingers and kinase-like domains.
InterPro capitalizes on the specialization of each member database by linking different but related signatures into hierarchical classification schemes that reflect their underlying evolutionary biology. For example, InterPro divides the kinase superfamily into over 40 different families and subfamilies based on sequence identity. InterPro provides 14,000 PDB structures, 1.65M ModBase and SwissModel homology models and CATH/SCOP classifications that can be directly compared with signature matches, as well as GO mapping and links to databases such as MEROPS and IUPHAR. With its multi-layered approach, InterPro is a valuable resource for analyzing the human proteome.

Innovative aspects
- InterPro provides the widest and deepest coverage of functional annotation of any database, covering 74% of the human proteome (18,861 proteins).
- InterPro provides direct structure-sequence comparisons in both 2D and 3D formats.
- InterPro enables a comparative analysis of human proteins with over 550,000 species.

References:
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Using the Global Proteome Machine Database to Define Protein Interaction Environments

C516

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Introduction (maximum 600 characters, including spaces)
The Global Proteome Machine database (GPMDB) is currently the largest curated and publicly available data repository for proteomics information derived from tandem mass spectrometry (1). We hypothesized that the GPMDB contains information on protein interaction environments, i.e. proteins interacting with a protein of interest, or sharing its cellular localization and having the potential to form such interactions. We tested this hypothesis by parsing the GPMDB for proteins that are commonly coincident and have highly correlated occurrences and intensities.

Methods (maximum 800 characters, including spaces)
All models in which the small GTPase Rap1b was identified were extracted from the GPMDB (gpmdb.thegpm.org/) with the experiment type unknown. Models not showing protein identification scores of log (e)<-10, and at least two matching peptides were removed. Of the remaining models, those with high protein confidences and small model size (number of identified proteins less than 30) were selected and extracted as a proxy for the type of results generally obtained from immunoprecipitation experiments. Conversely, models with ratios of high confidence proteins vs total number of proteins of less than 20% were considered global proteomics experiments and thus excluded. The protein information from the remaining models was merged and used to predict a "global protein interaction environment".

Results (maximum 1200 characters, including spaces)
Two isoforms of Rap1b (accession number ENSP00000250559 and ENSP00000377085) appeared in the GPMDB. 53 different proteins were identified for both transcripts across the models that fell within the criteria mentioned in the methods section, 167 additional proteins were found for isoform 1. Of the shared proteins, abundant cytoskeletal proteins and platelet-related proteins dominate the Rap1b interaction environment, presumably reflecting the elevated expression level of Rap1b in this tissue. The presence of talin, integrin alphaIIb and beta-3, and several cytoskeletal proteins is truthful reflection of the functional role of activated Rap1b in integrin alphaIIbbeta3 activation via talin recruitment and cytoskeletal organization (2). Other small GTPases such as Rap1a, Rap2b, Cdc42p2, and several Rab proteins have relatively high appearance numbers, coincident with independent GTPase immunoprecipitation experiments performed in our laboratory. We are currently analyzing data from different Rap1 isoforms and other small GTPases for comparison, which will help further distinguish contaminations from information on protein organization and interactions.

Innovative aspects (maximum of 3 bullet points)
- Studying protein interaction environments in databases is a novel way to observe protein organization and interactions.
- GPMDB supports the observation of proteins that commonly coincident and have correlated occurrences and intensities.

References (maximum 3 references)
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A new versatile file translator for proteomics standards

C517

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Introduction
Information derived by proteomics experiments such as sample preparation and sample separation, mass spectrometry, protein identification etc..., is represented using different and sometimes incomprehensible file formats, resulting not very suitable for daily life

To deal with this problem, several initiatives have been developed: HUPO-PSI MIAPE and XML-based formats [1], XML-based repository: PRIDE [2], etc... All of them have contributed effectively, but currently the translation between some of the previous schemas is not clear.

Our contribution is a new tool to overcome this situation.

Methods
This tool is a Sun Java application and it is free accessible through the Internet (http://proteo.cnb.csic.es/). The application has been designed using common graphics libraries and latest XML parsers offering a friendly and easy interface avoiding complex configuration dialogs.

Currently, the application is able to manage the following file formats (proteomics standards): MIAPE Gel, MS and MSI documents (MS Excel and XML format), GelML, MzML and PRIDE.

Results
To illustrate this innovative work we realized two tests, both of them based on the translation between GelML and MIAPE Gel schemas. Input files were creating from 1) HUPO PSI Microsoft Excel template and 2) ProteoRed MIAPE web site (www.proteored.org)

The combination of both schemas (MIAPE Gel and GelML) is quite interesting: The first one presents a lot of information according to protocols, methods and images and GelML could exchange this information with other bioinformatics tools, but some problems appeared during the transformation:

MIAPE presents a lineal schema (well defined pipeline) whereas GelML is a FUGE based schema, dividing the information along several linked sections. Furthermore, any assumption can be done about the cardinality of the relationships between both schemas. Other issues are related with the relationships between elements within GelML schema.

After created a set of mapping files, the results were a perfect correspondence between both formats, allowing a complete translation between MIAPE Gel and GelML schemas. Of course, some configuration files had to been defined to drive the transformation but finally any instance of GelML could be generated.

Innovative aspects
- Novel user-friendly interface for standards schemas.
- Any comprehensible schema can be added
- Free tool to improve the automation of the daily pipeline

References
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Images

Figure1: Translation between proteomics standards will be easier.

Computational Refinement of Post-translational Modifications Predicted from Tandem MS Data

C518

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Introduction
A post-translational modification (PTM) is a chemical modification that occurs on proteins. Many of these modifications are known to play a pivotal role in the regulation of protein function and are linked to diverse diseases like Parkinson's, Alzheimer's, diabetes and cancer. There is currently a surge of interest in analyzing tandem mass spectrometry data to discover PTMs on a genome-wide scale, and several innovative search algorithms have been reported including one recent method from our group termed SIMS (1). However, this approach is subject to noise in terms of the mass measurements and uncertainty in the actual modification sites, which can result in false PTM assignments. We have devised a machine learning algorithm that accounts for the noise in the data and clusters the data points into more probable PTM groups. To our knowledge, this algorithm is the first principled technique that systematically and objectively addresses outstanding problems in PTM discovery.

Methods
We introduce a novel probability model and learning algorithm that addresses the noise (spurious variance) and uncertainty encountered when using unrestricted PTM search algorithms. It accomplishes this by modelling the hidden relationships between the composition of amino acids in the peptide sequence, the modification mass, the modification site, and the identity of the amino acid the PTM likely occurs on (**Fig. 1**). The inputs to the algorithm are a list of peptide predictions and for each prediction the modification site and modification mass. We used the expectation-maximization (EM) algorithm to perform inference and learning of our model, and devised a recursive merge model selection method that will effectively evaluate and identify the optimal setting for the number of clusters K, to achieve a desired false detection rate. A background cluster is added to allow the model to handle spurious data.

Results
We used our technique to process and refine a set of ~20,000 putative yeast modified peptides consisting of real and decoy peptides, predicted by SIMS, and have identified both known and putative novel PTMs. Weighting the trade-off between the number of bogus 'decoy' peptides allowed and the number of real peptide candidates detected, we settled on a model selection that resulted in ~58% of the peptides grouped into one of 112 clusters with an improvement in the False Discovery Rate from ~8% to ~2%. [The rest of the peptides were placed into the background cluster.] In addition, our algorithm was able to map PTMs seemingly present at protein and peptide termini, a feature that is not found in other popular unrestricted PTM search algorithms.

To validate the biologically relevance of our model, we have compared our predictions with the results from two recently published datasets aimed specifically at studying phosphorylation in yeast (2, 3). The union of the reference datasets consists of 75 non-redundant PTMs on 103 unique proteins, 15 of these were initially mapped to a different site by SIMS, but were subsequently corrected by our new algorithm. In addition, there were 0 instances where our algorithm incorrectly moved a properly matched modification site to the wrong position.

Innovative aspects
- Characterization of experimental PTM data generated from unrestricted PTM search algorithms such as SIMS (1) into more accurate (both mass and site) and biologically significant PTM groupings.
- Ability to simultaneously consider PTMs in the middle and at the terminal ends of peptides and proteins

References
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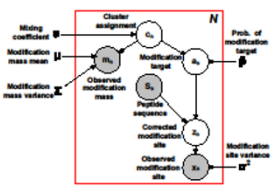

Figure 1. A Bayesian network describing our model. The modification targets (amino acid) and the PTM groupings are inferred from the input peptide sequences, observed modification sites and modification masses

Analysis of multiple protein marker panels: Optimizing sensitivity and selectivity

C519

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Introduction
Proteomic discovery experiments are rapidly generating lists of putative biomarkers for diseases and pathologies. Verification of these markers in multiplexed assays poses a statistical challenge as traditional ROC (Receiver Operation Characteristic) curves used to calculate the sensitivity and specificity of a diagnostic or predictive assay are based on single markers. The ability to combine quantitative information from several markers could potentially improve the diagnostic accuracy of existing tests and facilitate the development of new tests. However, standardized approaches to representing panels of markers remains controversial.
In the present study, we used an existing data set of quantitative SRM-based assays of 18 individual (putative) plasma protein markers for Trisomy 21 derived from an analysis of maternal plasma in previous discovery experiments (1).

Methods
Clinical samples:
Maternal serum samples from Trisomy 21 and normal first trimester pregnancies were provided by the Fetal Medicine Foundation, London, UK.
SRM Assays:
Specific transitions were selected for representative peptides from the 18 putative marker set and were measured for each patient using LC-MS/MS SRM assays (1).
Bioinformatic analysis:
The quantitative data from the SRM assays were used in the multiple marker statistical analysis.

Results
Targeted peptide expression levels for the 18 individual putative markers were measured by SRM assay. All possible double and triple candidate markers panels were selected from the pool of 18 candidate single markers. Marker thresholds were optimized by exploring true positive and false negative rates for all possible threshold / marker combinations. From this, we visualized the candidate panel as a sensitivity *vs* 100-specificity scatter plot. This unique visualization explored the threshold space for all sets of markers and designated a boundary for optimal panel efficacy. Various combinations of markers as opposed to single markers resulted in enhanced selectivity for the tested data set.

Innovative aspects
- Novel statistical procedure for depicting optimal specificity and sensitivity in a biomarker panel consisting of multiple markers.

References
(1) D. Sarracino, *et. al.*, "Efficient biomarker discovery coupled with SRM assay development in maternal serum from normal and Trisomy 21 first trimester pregnancies", HUPO 2009 Poster

Metabolic labeling validation of peptide MS/MS spectral library for spectral matching Strategy

C520

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Introduction

The current sequence database searching methods contain some notable drawbacks, such as unnatural use of peak intensities and little use of recurring irregular fragmentation patterns. Those drawbacks are prone to render false peptide identifications. An effective alternative is to construct spectral libraries for peptides and compare candidate MS/MS spectra to reference spectra. In this work we present an experimental approach to verify peptide matches by ^{15}N metabolic labeling. A new spectral matching algorithm using retention information to confine the searching range was also developed.

Methods

Two types of *E. coli* K12 cells were cultured individually in normal and ^{15}N-enriched media. The cells were harvested, lysed and subjected to acetone precipitation, reduction, alkylation, trypsin digestion, strong cation exchange fractionation, RPLC QTOF MS/MS analysis and mascot database search. An in-house program was developed to consolidate all the replicate identifications and compare the identified peak patterns of the same peptide identification between labeled and unlabeled peptide spectra. The verified consensus spectra were then compiled to form the annotated *E. coli* tryptic peptide MS/MS spectral library. A new spectral matching algorithm using retention information to confine the searching range was implemented to demonstrate the sensitivity and specificity of our strategy.

Results

With 2D-LC separation and QTOF MS/MS analysis, ~27000 identifiable spectra were collected and 11000 unique peptides were identified in each unlabeled or ^{15}N labeled sample. By measuring the similarity of the identified peak patterns of the same identification between unlabeled and labeled spectra and applying similarity filters, we verified 70% of peptide identification using the predicted mass shifts of the b and y ions (i.e., ^{14}N vs. ^{15}N) in corresponding MS/MS spectra. In the spectral library, verified peaks, non-verified but identifiable peaks and unidentifiable peaks were stored and annotated, along with all the retention information of each identification. In addition, an in-house spectral matching program was developed and optimized by properly processing spectra and assigning different weighing factors to different types of peaks in the reference spectrum. By calculating the dot product between the candidate and reference spectra, our strategy clearly showed good discriminatory power (<0.5% FDR). These results demonstrate that our strategy allows the construct of an experimentally verified spectral library and provides a good alternative to sequence database search methods.

Innovative Aspect

An experimentally validated spectral library was constructed and a new spectral matching algorithm using retention information was developed.

Analysis and interpretation of multiple proteomic datasets – biologically relevant information obtained in less than 3 hours

C521

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Introduction

A growing issue in proteomics is data interpretation, in particular the time-consuming steps of analyzing proteins and peptides identified by database search engines, extracting biologically meaningful information, and sharing results with co-workers and collaborators. This poster shows the application of a bioinformatics tool that was used to manage protein and peptide lists, put them into a biological context and enable the results to be shared directly. Within 3 hours, output generated by protein database search engines sourced from multiple proteomic data sets, was translated into biological information and shared with collaborators.

Methods

The bioinformatics tool, ProteinCenter (Proxeon) uses biological annotations from multiple resources to produce a biologically-relevant overview in large-scale proteomics studies. ProteinCenter contains public sequence databases to form a comprehensive and consistent superset of 12 million protein sequences derived from over 50 million protein records from GenBank, Refseq, EMBL, UniProt, Swiss-Prot, Trembl, PIR, IPI, PDB, Ensembl etc., including more than 5 million outdated accession numbers. The ProteinCenter database is built using Sun Java technology and Microsoft mySQL database technology for optimal performance.

Result

Here we present the bioinformatic analysis and comparison of proteomics datasets derived from the PRIDE database, including an organ-specific proteome map for *Arabidopsis thaliana* and HUPO projects. Protein identifications were clustered using ProteinCenter algorithms based on indistinguishable proteins or sequence homology. The results of ProteinCenter data processing will be presented including statistical analysis of over- and under-represented features such as gene ontology categories, PFAM annotated proteins, signal peptide proteins, trans-membrane annotated proteins, enzymes, involvement in KEGG pathways and others.

Innovative aspects

A bioinformatics tool that gives access to all major protein repositories enabled analysis and interpretation of multiple, large scale proteomics datasets, sourced directly from search engines, to be performed within 2-3 hours.

A novel Protein Scoring Algorithm for shotgun proteomics

C522

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Introduction

We present a novel annotation algorithm for the reliable identification of proteins in complex mixtures using shotgun proteomics. The proposed algorithm not only considers the matching quality of the peptides, as most other approaches do, but also takes into account important statistical parameters such as overall size and redundancy of both search databases and sets of MSMS spectra, protein size, counts and redundancy of the identified peptides. The superiority of this annotation approach was demonstrated by analyzing two non-related complex proteomes spiked in each other at various ratios.

Methods

Lysates of a human HELA cell line and of *B. subtilis* were mixed at various ratios, digested with trypsin, and 15 ng protein digest were analyzed in triplicate by nanoLC ESI-MS/MS using a ThermoFisher LTQ-Orbitrap. Parent masses were acquired either in the Orbitrap analyzer (at 30,000 resolution) or in the LTQ analyzer operated in enhanced mode while MS/MS spectra (5 most abundant ions of a full mass spectrum, dynamic exclusion set for 30 s) were acquired in the LTQ analyzer. Raw files were searched using the Sequest algorithm against a composite human-*B. subtilis* database (36700 proteins) concatenated with its corresponding decoy allowing either 10 ppm (Orbitrap) or 1.5 Da (LTQ) tolerance for the parent mass and 1.0 Da error for the fragment masses.

Results

For each detected protein, the algorithm:

- groups all its identified peptides and generates a preliminary score using the peptide probability scoring calculated by established algorithms (such as Mascot or PeptideProphet);
- calculates the exact probability that at least m peptides (m were assigned to the protein) are found in n (the submitted number of spectra) peptides randomly selected from a set of N (the non-redundant count of theoretical peptides in the search database);
- calculates the final protein score as a function of the two above results.

Identified proteins are clustered in independent groups, defined as single proteins, or sets of several proteins sharing exactly all group's identified peptides, that can be experimentally distinguished from other groups by at least one peptide. The best member's protein score becomes the group score.
The False Discovery Rate (FDR) for each detected protein is the fraction of decoy groups (determined from the decoy database) in the set of all better-scoring independent groups.
An increase of up to 20% identified proteins (with FDR<0.01) was obtained independently of instrument resolution relative to a standard annotation workflow.

Innovative aspects

- Additional experimental parameters are taken into account in an unbiased manner.
- Realistic FDR estimation at the protein identification level.
- Protein grouping provides a solution on how to account for sequence database redundancies and greatly simplifies the visualization of results.

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Automatic Alignment, Statistical Restoration and Quantification of Raw LC/MS and 2-DE Data

C523

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Introduction

Recent HUPO initiatives have highlighted the need for an automated and sensitive informatics workflow to facilitate large scale high-throughput analysis and objective cross-validation between laboratories. Towards this goal, we advocate integrated statistical modeling of raw data rather than the conventional sequence of lossy processing steps. Combined with our established image alignment method, we present the groundwork for novel automatic statistical deblurring of LC/MS maps and 2-DE images, which for the first time provides confidence levels for detected spots and their quantification.

Methods

LC/MS data is first converted to a virtual gel. Our RAIN method (1) then automatically aligns the images. A Variational Bayes approach to Blind Image Deconvolution (2) is then employed to statistically remove blur caused by imperfect focusing, electrophoretic and chromatographic migration, under the sole assumption that under ideal conditions protein appears in homogeneous shapes. All parameters are estimated from the data, including the weight given to this assumption and the nature of the blur (rather than typical application of a 2D Gaussian). Consequently, the quantity of protein in each deblurred pixel has associated with it a statistical distribution to be harnessed by proceeding differential analysis. To segment co-migrating proteins, an ellipse-covering algorithm can be used (3).

Results

Fig. 1(a,b) shows the result of automatic image-based alignment with RAIN (7.5 mins). Furthermore, ref. 4 in (1) demonstrates the effectiveness of the technique on large datasets from the HBPP Pilot Studies. Statistical restoration with Blind Image Deconvolution (5 mins) is shown in Fig. 1(c,d). A number of weakly expressed and co-migrating spots are now uncovered, and spot boundaries are clearly determined. Since the error range of each pixel's expression level has also been computed, the probability of false positive detection is more accurately deduced.
This study demonstrates the use of statistical restoration techniques in proteome imaging. Further modelling will reveal additional power. For example, blur varies spatially over 2-D gels and in LC. Modelling this change will improve sensitivity and provide more constraints than conventional fitting of parametric spot models to proteins in isolation. The related technique of Blind Source Separation will be used to integrate background subtraction statistically into the restoration process. Also, concepts from Multiframe Deconvolution will be harnessed to integrate replicate experiments at this early analysis stage.

Innovative aspects

- Novel problem formulation of restoration in proteome imaging, with all parameters estimated from the data (*i.e.* no assumed parametric spot model)
- Fully statistical treatment at the pixel level computes uncertainty levels in the resulting protein detection and quantification
- New analysis paradigm opens up further statistical modeling opportunities *e.g.* spatially varying blur, background and artifact subtraction, fusion of replicate experiments

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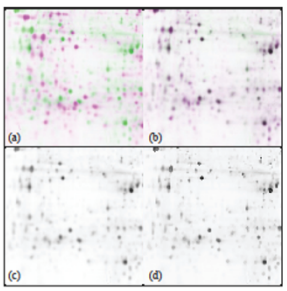

Fig. 1. Automatic image-based alignment and statistical restoration. (a) Before and (b) after RAIN alignment. (c) Before and (d) after Blind Image Deconvolution.

C524

Pathway Analysis of Heart Disease Proteomic Profiles using Enrichment Maps

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Introduction
Profiling global protein abundance for different forms of cardiac disease can highlight biological processes contributing to pathogenesis. However, the interpretation of large-scale proteomic expression patterns is difficult as the raw data is often noisy, no individual protein may meet the threshold for statistical significance and the sheer quantity of results makes it difficult to identify important players. We have addressed these problems by looking for functional 'gene-sets' significantly enriched in altered proteins, and visualizing the results as a network of enriched gene-sets, an 'Enrichment Map'.

Methods
Proteins from two distinct mouse models of heart disease, dilated cardiomyopathy and hypertrophic heart failure, were quantified using the spectral counting technique and scored to assess differential protein expression. We adapted the Gene Set Enrichment Analysis (GSEA) technique(1) in an effort to uncover aberrant processes and pathways. Typically, GSEA results are organized as lists of up and down-regulated gene-sets; the large number of gene-sets and their intrinsic redundancy can make the interpretation of GSEA results elusive. In order to better visualize and explore the output from GSEA, we transformed its results into an Enrichment Map, a network of significant gene-sets represented by nodes connected by edges representing the overlap in membership between gene-sets. Highly-related gene-sets form compact clusters in the map (2).

Results
Enrichment Maps generated for the two models of heart disease highlighted key areas of dysregulation during the progression of the disease. These included, for example, actin remodelling, cell cycle control, metabolism, transcription, translation and apoptosis. Furthermore, using terms derived from the Gene Ontology a few striking differences in differential expression were evident from the maps of the two models. Dilated cardiomyopathy had unique perturbations in RNA processing, heart processes and ATP activity (Figure 1) whereas hypertrophic had a large cluster relating to the electron transport chain providing mechanistic insights that map to using curated pathways. Moreover, while the two diseases shared enrichment in actin and microtubule processing, cellular respiration, sugar catabolism, the degree of up and down regulation was different in informative ways.

Innovative Aspects
- Biological patterns, functional similarities and mechanistic differences in complex proteomic profiles are readily highlighted using Enrichment Maps
- Mining of the individual models and comparison of the two cardiac models using Enrichment Maps indicated perturbed processes and pathways and suggested differences in the etiologies of each condition.

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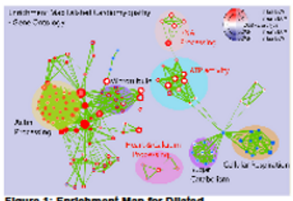

Figure 1: Enrichment Map for Dilated Cardiomyopathy with GO terms.
The colour of the inner node represents the significance of the geneset at early stage disease. The colour of the outer node represents the significance of the geneset at mid-stage disease. Clusters indicated in red are not found in the hypertrophic cardiomyopathy map.

C525

Peptide Detectability Prediction using Support Vector Machines with Novel Kernels

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Introduction
Accurate protein detection and quantification is very important in systems biology. Current tools for proteomics mass spectrometry are dependent on peptide centric approaches. A very important concept in this context is the concept of proteotypic peptides (PTP) (1). In contrast to experimental PTPs, the algorithmic prediction of PTPs does not require a laborious prior discovery stage of the proteome.
Here we present a support vector machine (SVM) based approach using novel peptide kernels for predicting the detectability of peptides in a mass spectrometer. In contrast to previous approaches, which focus on extraction of properties of peptides such as length, amino acid frequencies, etc., we focus on properties of theoretical spectra of the peptides, which capture better the way the peptides are detected in the mass spectrometer.

Methods
We use SVMs for classification and the novel kernels are implemented as part of http://www.csie.ntu.edu.tw/~cjlin/libsvm/. As kernel functions for SVMs are defined for pairs of objects, in our case peptides, the novel SVM kernel we propose is a string based kernel that captures the similarity of sets of suffixes and prefixes of the amino acid sequence of the peptides. This kernel is based on the idea that if a peptide whose detectability needs to be predicted, generates similar theoretical spectra with peptides that we already know they fly well and are detectable, then quite probably our peptide of interest has a high detectability value too.
The training data we use are part of the Model Organism Proteomes (MOP) initiative, and are mainly *Drosophila* (2) peptides, although *Arabidopsis* data is also used for evaluation.

Results
We carried out a preliminary empirical analysis of our predictor, comparing the achieved results with established PTP predictors (3) as well as the spectral data for *Drosophila*, *Arabidopsis* as well as other model organisms. For this purpose, we extracted the 100 most abundant proteins of the respective organism for all MOP experiments based on spectral counts and all the peptides resulting from their theoretical *in-silico* digestion proteis were labelled as seen or not seen. Our approach achieves an accuracy of 75% of predicting the visibility of *Drosophila*, whereas existing approaches (3) result in up to 51% accuracy.
The higher accuracy comes from the fact that the new peptide kernels we use, that work on the level of theoretical spectra of peptides can discern better the features affecting the detectability of peptides. For example, the two peptides AAEDELGVIPK and AGDPVILEEAK, coming respectively from the proteins CG12340-PA and CG6084-PA of *D. melanogaster* are anagrams with each other: they are composed of the same set of amino acids with the same frequencies. As such, every tool based only on amino acid frequencies, length and mass, will predict the same detectability for these peptides (e.g. PeptideSieve (3)). However, the peptide AGDPVILEEAK has been observed 83 times in (2) and AAEDELGVIPK has not been observed at all although we have seen the respective proteins with different other peptides. Our tool predicts correctly AGDPVILEEAK as detectable (score 0.5) and AAEDELGVIPK as not detectable (score 0.04).

Innovative aspects
- Novel method for prediction of peptide detectability by capturing properties of theoretical spectra of peptides
- Higher accuracy than existing methods in predicting the detectability of peptides as indicated by empirical results and comparisons with experimental data
- Possibility to fine tune the predictor to specific organisms when experimental proteomics data is available

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C526

Integrated Time Alignment Application for Complex LC-MS Datasets

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Introduction
The retention time shifts between multiple LC-MS chromatograms is unavoidable in comparative quantitative label-free profiling of complex biological samples. In order to compare the same peaks in the statistical analysis of multiple chromatograms, it is necessary to correct these shifts. We present a new strategy to adapt two dimensional time alignment algorithms to take account the three-dimensional structure of LC-MS data using Components Detected Algorithm (CODA) [1]. We have successfully improved three algorithms using Correlation Optimized Warping (COW) [2], Dynamic Time Warping (DTW) and Parametric Time Warping (PTW) [3].

Methods
High-quality mass traces were selected prior to the alignments and various trace selection based on CODA methods were tailored specifically to the time alignment algorithms. The benefit functions of each algorithm were then modified to combine the alignment of selected mass traces into one warping function. The global quality of the different algorithms was compared based on the overlapping area of chromatograms pairs after time alignment. The performance of the algorithms was assessed on LC-MS data obtained with depleted human serum and acid precipitated urine samples from ordinary biomarker discovery studies. In addition, the sensitivity of these new algorithms for the selection of the reference chromatogram was observed.

Results
All modified algorithms were able to align the chromatograms in all the three studied datasets with relatively high precision, in contrast to the performance of the original algorithms, which produced high number of misalignments particularly in the urine dataset. Amongst the three modified algorithms, COW-CODA averagely performed the best, but the use of the algorithm was accompanied by longer running time. DTW-CODA required the least running time and mostly resulted in more precise time alignment than COW-CODA and PTW-CODA. In addition, DTW-CODA improved the peak shape distortion problem that was found in the original algorithm. However, DTW-CODA was more sensitive with respect to the choice of the reference chromatogram. PTW-CODA provided an acceptable average performance, but the number of misalignment observed was higher than the other modified algorithms. As an example, Figure 1 shows the local performance of the time alignment by the modified and original algorithms on one omnipresent standard peptide in urine dataset. The figure shows the large extent of peak shape distortion of the original DTW algorithm (DTW-TIC), which is solved on the aligned chromatograms obtained by the modified DTW algorithm (DTW-CODA).

Innovative aspects
- Two-dimensional benefit function of COW, DTW and PTW algorithms in combination with CODA to take local information into account for time alignment.
- Global and local assessment and comparison of algorithms using three different type of highly complex LC-MS datasets different concentration variability.
- Strategy to select the best reference for time alignment.

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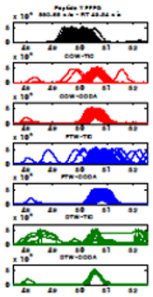

Figure 1. Peak alignment of one standard peptide in urine dataset with 50 chromatograms by different algorithms with one-dimensional (COW, DTW, PTW-TIC) and two-dimensional (COW, DTW, PTW-CODA) benefit function.

C527

Towards a Better Understanding of Matrix Interferences in SRM

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Introduction

Peptide quantitation by selective reaction monitoring (SRM) is plagued with co-eluting matrix interferences [1,2]. These interferences often cause problems with both the identification and quantification of the peptide of interest. In order to better understand matrix interferences in complex mixtures, a yeast digest was monitored with a range of SRM transitions on a triple quadrupole mass spectrometer. The resulting data were then analyzed in an effort to understand and potentially make predictions about the effects of co-eluting peptide interferences on sensitivity and selectivity of SRM assays.

Methods

Yeast cell lysate samples were collected and enzymatically digested, then analyzed by LC-MS/MS. SRM assays were developed on a TSQ Quantum Ultra triple quadrupole mass spectrometer, Surveyor MS pump, Surveyor Autosampler and an IonMax ESI Source (ThermoFisher Scientific). Reverse phase separations were carried out on a 1mm X 50mm Hypersil Gold 1.9µm C18 particle. Solvent A was LC-MS grade water with 0.2% (v/v) formic acid, and solvent B was LC-MS grade 30% (v/v) acetonitrile with 0.2% (v/v) formic acid (Optima grade reagents, Fisher Scientific). In order to minimize the complexity of the data, a short segment of the gradient and a range of SRM transitions were used for data analysis with Pinpoint software. For each SRM transition, matrix interferences were characterized.

Results

A complete data set of matrix interferences for a range of SRM transitions in a yeast digest sample was produced. The data provide a foundation for further studies which will attempt to link biological matrices with SRM transitions and predict their interferences. Specifically, it will allow for better understanding of the SRM transitions that have the highest chance of co-eluting interferences in a particular LC segment of a biological matrix separation. Further, data generated from the experiments were used to refine predictive computational models.

Once matrix interferences are well characterized in an LC segment, it allows for targeted method development which can improve the selectivity of problematic transitions. In particular, HSRM (Q1 FWHM 0.2) and FAIMS were shown to provide significant improvement in resolving matrix interferences. Adjusting the LC gradient to chromatographically resolve co-eluting interferences from peaks of interest in regions and for transitions with high interference, was another strategy that was successfully applied.

Innovative aspects

- A complete analysis of SRM matrix interferences in yeast digest

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Chromatographic peak centred approach improves phospho-peptide identification C528

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Introduction
In recent years the analysis of protein phosphorylation by mass spectrometric techniques has taken a giant leap forward. An important part of the development has been the introduction of stringent approaches for handling false positive hits present in any mass spectrometric data set e.g. by using the reversed data base approach. Here we report that for samples of moderate complexity false positive estimation can be significantly improved using a chromatographic peak centered approach. The method was evaluated on data sets generated by four different fragmentation techniques on an Orbitrap.

Methods
Kinases were affinity-purified from a K562 cell lysate using immobilized broad spectrum kinase inhibitors. [1] The kinases were in-gel trypsin digested and phosphopeptides were enriched with IMAC. Eight identical aliquots were generated of which two were analyzed with each fragmentation method. The LTQ-Orbitrap was set up with equal trap filling time (250ms), isolation width (2.5 Da) and target value for MS1 (1e6 ions) and MS2 (1e4 ions) to allow a fair comparison between the methods. Samples were analysed in duplicate using either CID, MSA, PQD or MS3 as fragmentation technique. For MSA and MS3 neutral loss masses were set to 24.5, 32.7, 49 and 98 Da below the precursor mass. The gradient time was 180 minutes for all experiments.

Results
Different ways of enhancing the reliability of phospho peptide identification are studied. We found that for samples of moderate complexity a false positive estimation based on the spectra will lead to acceptance of a larger number of false positives than expected. The reason is that a large number of species are sequenced several times per chromatographic peak. We developed a method for selecting the best spectrum associated to a chromatographic peak based on experimental conditions. Further, we have refined a neutral loss criterion for flagging phospho peptides and finally a feedback mass accuracy filter was developed that takes into account the systematic shift of identified peptides and the quantity of false positives in a particular experiment. The new methods were tested on kinase phosphopeptide enriched samples using four different fragmentation techniques: CID, PQD, MSA and MS3. For MS3 the precisely determined m/z of the MS2 precursor is also used for searching the MS3 spectrum. When compared to peptide centric false positive estimation an improvement of 7-31% is observed, depending on the fragmentation method used and the desired false positive rate.

Innovative aspects (maximum of 3 bullet points)
- Chromatographic peak centred data processing approach for phospho-peptide identification.
- Feed-back mass accuracy filter improves high confidence identifications.

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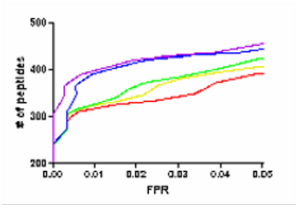

Figure 1: Pseudo ROC plots indicating the impact of individual method improvements on sensitivity for phospho-peptide identification. Red: peptide centric false positive estimation Yellow: chromatographic peak centric, Green: + Neutral loss filtering, Blue: + feed-back mass accuracy filtering, Purple: peak centred matching across duplicates.

Human Genome Re-annotation by Proteogenomic Mapping C529

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Introduction
The flood of genome sequences being produced far outpaces their annotation. Presently, human genome annotation relies primarily upon cDNA/EST libraries and gene prediction algorithms, which identify gene transcripts. Recent findings from the ENCyclopedia of DNA Elements (ENCODE) project revealed pervasive gene transcription and unexpected alternative splice forms [1]. This raised unanswered questions, such as: which of these transcripts are translated into protein(s), and when? Proteome-based genome annotation may answer these questions, however, technical challenges remain. In this work, we used a well-studied MS/MS data set to determine whether whole human genome-based search provides additional data about translation of un-annotated transcripts or alternative splice forms compared to the standard protein database search.

Methods
We used a reference data set from the Human Plasma Proteome Project, consisting of 5,806 MS/MS spectra. Its content was well characterized by a previous study of several MS/MS identification methods using standard protein database search, with 677 previously identified and manually verified matches [2]. We compared those previous results with a whole human genome search of sequence draft 18, using our Genome Fingerprint Scanning (GFS) software running on a cluster [3]. To perform searches, we partitioned the genome into 5 megabase segment (MB) with 2 kilobase (KB) overlap. We then combined the results from the resulting 600 searches, calculating an E-value for each MS/MS match using a combination of Python and Perl scripts. We examined the resulting matches, comparing them against gene, cDNA, and gene prediction annotations, as well as comparing them against the protein database search.

Results
Among the 677 spectra previously reported and manually verified as matching the protein databases, we found that 26 had better-scoring, significant matches elsewhere in the genome than the location of the originally reported protein match. We investigated each of them using UCSC Genome Browser, revealing that 22 of them matched putative transcribed and/or coding regions. Ten of the spectra matched regions supported by predicted genes or EST evidence, while 12 spectra correspond to potential alternative spliced variants of known genes. Only four of the matches did not have any other supporting evidence. It was surprising that nearly 4% of the identification results had better matches than the ones previously assigned, and were due to limitations in the available protein databases.

We searched the 5,180 previously unidentified spectra from the data set, and found 115 additional hits within controlled 1% false positive rate (FP) rate, and 356 hits within controlled 5% FP rate. Examining some of these in more detail revealed cases of peptides that match within annotated introns, and others that match cDNA or EST evidence. An example is a match to the Human Metabotropic Glutamate Receptor 8 (GRM8) that had several alternative splice forms already annotated. Our data suggests an additional alternative splice variant that falls within a region annotated as an intron.

From this analysis, it appears that as many as 50% additional MS/MS peptides might be identified by whole genome search at the 5% false positive rate. However, limitations to both the speed and the specificity of the whole genome search remain the greatest challenge to its widespread use.

Innovative aspects
- Direct comparison of protein database search to whole genome search using a well-known sample
- Identification of novel peptides
- Identification of novel alternative spliced forms of known genes.

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ITM Probe: analyzing information flow in protein networks C530

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Introduction
Many current approaches apply the concept of a random walk to extract biologically relevant information from protein interaction networks. These approaches, however, do not model information loss/leakage that naturally occurs in all networks. For example, proteases constantly degrade proteins, diminishing the strength of information propagation. We have recently developed a mathematical framework to model information flow in networks with a novel ingredient, damping/aging of information (1). Here we present a web application *ITM Probe*, which implements our theory.

Methods
ITM Probe models information flow in a protein interaction network through discrete random walks. Unlike classical random walks, our model allows the walker a certain probability to dissipate or damp (that is, to leave the network) at each step. Each walk, simulating a possible information path, terminates either by dissipation or by reaching a boundary node. We distinguish two types of boundary nodes: sources (emitting information) and sinks (absorbing information). *ITM Probe* offers three models: absorbing, emitting and channel, containing respectively sinks, sources and both sources and sinks as boundary nodes. Each selection of boundary nodes and dissipation rates provides the biological context for the information transmission modeled.

Results
Both the absorbing and emitting models navigate neighborhoods of selected nodes and illuminate the protein complexes associated with them. The channel model is suited for discovery of potential pathways linking proteins of interest or biological functions associated with them. Using multiple sources may reveal the potential points of crosstalk between information channels, while a solution of multiple sinks chosen according to a set of competing hypotheses may suggest the most biologically plausible pathways among many possible ones. To assist *in silico* investigations on the impact of knocking out certain genes and to remove the effect of proteins with a large number of non-specific interaction partners, *ITM Probe* allows users to specify nodes to exclude from the network. *ITM Probe* returns the set of most relevant proteins with weights and a graphical representation of the corresponding sub-network (Fig 1). With a click, the user may send the resulting protein list for Gene Ontology (2) term enrichment analysis to facilitate hypothesis formation or confirmation. The software can be found at http://www.ncbi.nlm.nih.gov/CBBresearch/qmbp/mn/itm_probe.

Innovative aspects
- Aging/damping of random walks models leakage of information from networks.
- Proteins relevant to the context are retrieved from the entire network without prior restriction to the sub-network of interest.
- Novel concept and implementation of information channels through networks, connecting origins and destinations.

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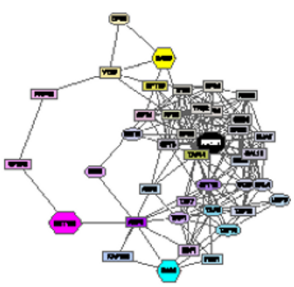

Figure 1. An image of the sub-network resulting from running the *ITM Probe* channel model on the yeast protein-protein interaction network. The sources (associated with basic colors) are the histone acetyltransferases Sas2p (cyan), Rtt109p (magenta) and Sas3p (yellow), while Rpo21p, the catalytic subunit of RNA Polymerase II, is a sink. At each node, the intensity of each basic color represents the number of visits by a random walk from the corresponding source to that node. The basic colors are mixed to show the relative influence of each source.

Bioinformatics for Systems Biology: Directed Shotgun Proteomics & Deterministic Protein Inference C531

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Introduction
We have previously suggested a two-step strategy to enable global quantitative proteome analysis reminiscent of microarray-based gene expression analysis [1]. In a first step, the discovery phase, the proteome observable by mass spectrometry is mapped out to saturation. A key limitation for extensive proteome coverage is the large redundancy of identification for the most abundant proteins. Novel strategies that allow to reach deep proteome coverage would help to saturate the open-access proteome catalogs that result from the discovery phase. These can be used to select peptides specific to only one protein, so-called proteotypic peptides (PTPs) [2]. These PTPs form the basis to move towards quantitative targeted proteomics using mass reaction monitoring (MRM).

Methods
We have devised a novel, directed shotgun proteomics strategy that allows to reach very high proteome coverages at reduced levels of redundancy. Central element of this generally applicable strategy is a feedback loop that iteratively cycles between experimentation and bioinfomatic and statistical data analysis, which we term ADE (Analysis Driven Experimentation). Various physico-chemical and functional protein parameters are assessed and allow to adapt the experimental approach in order to target under-represented areas of the proteome.
Furthermore, a novel peptide classification scheme based on the information content of each peptide was developed, which enables to report conservative lists of protein identifications and to seamlessly integrate proteomics and transcriptomics data.

Results
We have applied the ADE feedback loop strategy on a large shotgun proteomics dataset of *D. melanogaster* and identified close to 70% of the protein-coding gene models, an unprecedented coverage of the proteome of a complex eukaryote. Focusing a series of targeted experiments on the class of under-represented short proteins helped to identify many previously unidentified short proteins at reduced levels of redundancy and established the validity of our targeted approach.
Our novel deterministic peptide classification and protein inference scheme differs from the existing approaches in three ways: i) it is the only approach that considers the gene model – protein sequence – protein accession relationships; ii) in contrast to probabilistic approaches, it only considers peptides above a certain confidence threshold after the peptide spectrum matching process, not peptides of lower score. Thus, a conservative cumulative protein list can be generated, allowing researchers to draw firm conclusions from the final dataset; iii) it enables the seamless integration with transcriptomics datasets.
In silico analysis of model organism and human protein databases indicates that our classification represents a generally applicable strategy. We will present data highlighting its benefits for the analysis of shotgun proteomics datasets.

Innovative aspects
- directed shotgun proteomics strategy minimizes redundancy of protein identifications and enables deep proteome coverage
- protein inference based on novel peptide classification scheme allows to generate conservative protein lists with minimal error and a seamless integration with transcriptomics data

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Variability in Sample Preparation Protocols and LC-MS/MS Analysis

C532

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Introduction
Metrics currently used to compare differences between samples or methods in LC-MS/MS experiments include counting the number of peptides and proteins along with protein sequence coverage. A more rigorous analysis of datasets can be performed using our software, NISTMSQC (1). This software makes reliable peptide identifications using tandem mass spectral reference libraries and calculates metrics for quality control. In this study, the variability in data originating from the LC-MS/MS platform and sample preparation will be identified for tryptic digestion of a standard protein.

Methods
Human serum albumin (HSA) was added to Tris buffer alone or with the addition of either 0.2% RapiGest (Waters), 6 M urea, or 20% acetonitrile. Samples were alkylated with iodoacetamide and diluted to an appropriate concentration for tryptic digestion. Following tryptic digestion at 37°C, equivalent amounts of peptides were loaded onto an Atlantis dC18 trap column (Waters). Peptides were eluted at 450 nL/min onto an Integrafrit C18 column (New Objective) coupled to a linear ion trap mass spectrometer (Thermo Fischer Scientific). Samples were prepared and analyzed by LC-MS/MS in triplicate. Peptides were identified with NISTMSQC using a peptide tandem mass spectral reference library containing tryptic and semitryptic peptides (NIST).

Results
NISTMSQC was used to identify peptides and their abundance for samples of HSA prepared with different protocols. Typical values for percent coefficient of variation (% CV) for most metrics such as number of peptide identifications and chromatographic peak widths were well below 10% for each sample and between replicate samples prepared with the same method. One significant source of variation was found to be related to the peptide intensity measured during the MS1 scans. For samples prepared using different protocols, differences in peptide intensity were also found and were assigned to specific classes of peptides (i.e. tryptic, tryptic with missed cleavages, and semitryptic peptides). Changes in intensity were also mapped to individual HSA peptides (Fig 1); therefore, tracking these changes for different sample/instrument protocols is important when performing LC-MS/MS experiments targeted for specific peptides. This study shows that NISTMSQC can be used to generate metrics to identify differences in datasets along with metrics for instrument performance. Ranges of values and % CV determined from this data can be used as a benchmark for quality control in future experiments.

Innovative aspects
- Bioinformatic tools for quality control of MS/MS data
- Identification of changes in peptide abundance for different digestion protocols

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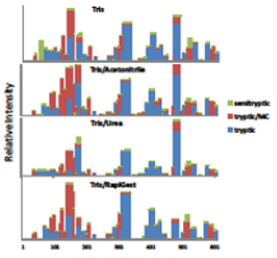

Figure 1. Histogram showing relative average intensities of HSA peptides from MS1 scans according to amino acid position in the protein. Intensities are shown for 3 classes of peptides (tryptic, tryptic with missed cleavages, and semitryptic peptides) for samples digested in different buffers (Tris buffer alone or with acetonitrile, urea, or RapiGest added).

Searching Peptide Mass Spectral Libraries Using a "Target-Decoy" Strategy

C533

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Introduction

The increasing coverage of peptide mass spectral libraries advocates a practical examination of search strategies. We focus on adapting the popular "target-decoy" method as described by Elias and Gygi (1). In this method, decoy databases are used to estimate false discovery rates (FDRs). Here, we present evidence that non-overlapping spectral libraries can be used as decoy by applying a simple correction to account for bias towards decoy spectra seen for incorrect identifications. This bias is similar to what is observed when using decoy sequence databases created by stochastic methods.

Methods

In this work we test a new search program developed at NIST, called MSPepSearch. This program is an implementation of the NIST MSSearch dll, both of which are available at http://peptide.nist.gov. This engine processes input spectra in MGF format and searches the binary version of the NIST peptide spectral libraries. The scoring is based on a modified dot-product (2,3) but with minor adjustments which increase the specificity of the algorithm for peptide spectra (e.g., peak annotations). The discriminate score used for these studies is labelled 'Score' in the MSPepSearch output. Spectral libraries from a different species with overlapping spectra removed were used as decoy. Test data include 1D RPLC, LTQ-Orbitrap replicates of the SigmaUPS1 or a yeast reference lysate.

Results

According to (1), in order for estimates from the 'target-decoy' strategy to be valid, the following two assumptions must be true: 1) the target and decoy libraries must not overlap and 2) false positives must be equally distributed between target and decoy databases. To accomplish 1), we simply removed identical or "homologous" spectra from the decoy library. Then we examined the distribution of the target masses as a function of precursor mass accuracy (Fig. 1). These estimates indicate differences only in the low (300-400) and high m/z regions (1,800-2,000). Precursors in these ranges were excluded from the analysis. Next, the likelihood of a false positive match occurring to decoy vs. target was estimated from low-ranking matches. Results indicated that a false positive identification was approximately 20% more likely to occur in to the *Drosophila* library used as a yeast decoy. Finally, FDR can be calculated according to the following:

FDR = (2 * D * b * r) / (D * b * r) + T

where D and T equal the sum of decoy or target matches at a given score or better, respectively. b and r equal the pre-determined ratios of the decoy/target bias or library size, respectively.

Innovative aspects

- Provides users of spectral library searching a simple method to perform 'target-decoy' searches.

References

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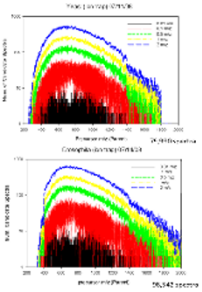

Fig. 1 Distribution of precursor *m/z* values for yeast (target) and *Drosophila* (decoy) spectral libraries at different precursor tolerances.

A Bayesian Approach to Distinguish Signal and Noise Peaks in Peptide Tandem Mass Spectra

C534

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Introduction

The inevitable presence of noise peaks in peptide tandem mass spectra complicates, and sometimes prevents their correct identification, a key step in proteomics data analysis. Strategies to de-noise spectra vary, but rarely go beyond using the peak intensity or rank as a cutoff criterion. This simple approach often fails for noisy spectra where the signals are weak, as in the case of low-abundance peptides. In this work, we adopted a Bayesian approach to train a discriminator to distinguish signal and noise peaks, incorporating peak characteristics that are overlooked by simple denoising methods.

Methods

The training sets of signal and noise peaks were established during consensus spectral library building from 121,357 SEQUEST-identified spectra [1]. Specifically, spectra with the same identifications (replicates) are combined, whereby only peaks found in over 60% of all replicates are considered "signal" and enter into the consensus. All other peaks are considered "noise."

We proceeded to extract peak characteristics that are good discriminator between the signal and noise sets. Among the characteristics considered so far are m/z, the peak rank, the presence of complementary fragments and sister peaks a fixed distance away. A Bayesian model is built, and the prior and conditional probabilities learned from the training set are used to calculate the probability of any given peak being a signal.

Results

We found that the peak rank is, unsurprisingly, the strongest discriminator: the top-ranked peak has a 98% of being a signal whereas the 50th-ranked peak has about an even chance of being signal or noise. A signal peak has a 67% chance of having a sister peak at +/- 1 Da, presumably due to isotopes, whereas for noise peaks it is only 30%. In all, signal peaks show a distinct enrichment of sister peaks (Figure) at where common neutral loss fragments are expected, whereas noise peaks exhibit no such pattern.

For a set of lower-quality spectra (distinct from our training set) defined as having an Xrea[2] score of below 0.5, we found that for the same number of peaks retained (50), filtering by the probability calculated from our model retains about 10% more signal peaks than a simple intensity-based filtering.

We also observe significant differences between the Bayesian models we obtained for spectra from +2 and +3 precursors. For example, the chance of having a -9 sister peak is higher for +3 spectra than +2 spectra (40% to 29%), and the opposite is true for -18 sister peaks (43% to 58%). These results may enable us to guess the precursor charge state based on spectral pattern.

Innovative aspects
- An independent way to divide peaks into signals and noise based on consensus spectrum creation
- A Bayesian approach to distinguish signal and noise peaks based on several peak characteristics
- Improved quantitative understanding of the spectral patterns of peptide CID fragments

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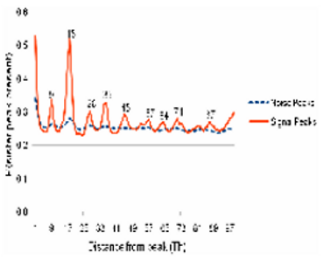
Figure



Mass Spectrometry-Based Gene Annotation – a study of Cell-Wall Surface Proteins from *C. difficile*

C535

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Introduction

Although the bioinformatics techniques for homology searching and transferring information from genome to genome have seen a great deal of progress, weaknesses still remain with respect to the annotation of primary gene sequences. Mass spectrometry-based protein identification has become an attractive accessory method to confirm gene expression and verify the localization of proteins predicted to be present in subcellular compartments. For instance, the identification of proteins present in the cell wall of pathogenic strains of bacteria might provide insight on virulence factors and hence speed up the prioritization of proteins that could potentially be used for diagnosis or treatment. This strategy has been successfully applied for the identification of cell surface proteins from *Clostridium difficile*, a gram positive anaerobic bacterium that causes enteric diseases in humans.

Method

C. difficile isolate QCD-66c26 was grown in brain-heart infusion broth under anaerobic. Cell pellets were washed and resuspended in lysis buffer containing 1 mg of lysozyme, desalted using 3 KDa Amicon ultrafiltration units and proteins precipitated with TCA/acetone. Thirty micrograms of protein was resolved on a 3 cm 12% SDS-PAGE gel. The entire lane was subjected to automated band excision to generate 18 bands. Proteins were in-gel digested and peptide extracts analyzed by liquid chromatography-tandem mass spectrometry (MS). MS data was searched aagainst a database containing all virtual tryptic peptides from the 3745 open reading frame (ORF) proteins predicted to be expressed in isolate QCD-66c26.

Results

The QCD-66c26 isolate used in the study is one of eight epidemic and non-epidemic isolates whose genomes have been sequenced with the Roche GS-FLX on the Sequencing Platform of the McGill University and Genome Quebec Innovation Centre (MUGQIC), as part of a Genome Canada/Genome Quebec funded project. Sequenced genomes were annotated using an in-house developed browser. An initial mass spectrometry analysis of a sample enriched in cell surface proteins readily confirmed the expression of 24 out of 32 predicted cell-wall associated proteins in strain QCD-66c26.

Additional data-mining efforts were carried out so as to maximize the number of MS-detectable cell-wall proteins by accounting for variations between gene prediction methods and database search engine peptide identifications and unaccounted post-translational modifications. As a part of our study we developed a procedure to import proteomics data onto a genome browser for rapid visualization of protein expression and localization.

Innovative aspects

- Isolation and characterization of C. *difficile* cell-wall proteins.
- Development of a gene annotation browser.
- Development of an integrated proteogenomic browser

C536

DomPep-a method for predicting modular domain-linear peptide motif recognition

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Introduction
The formation of macromolecular complexes in cells is often mediated by small modular protein domains (i.e. SH2 domain family) binding to short peptide ligands (<10 amino acids) in partner proteins. Recently, Proteomic methods were developed to investigate binding specificities of a few domain families. Meanwhile, using proteomic data, a few computational approaches were developed for predicting ligands for the domains at the level of domain family [1-3]. Here we describe a prediction method called DomPep that can cover different domain families.

Methods
DomPep was based on the data from peptide/domain-array experiments, whereby domains are compared using the same set of peptides. We defined a simple measure called ligand binding identity (LBI) to quantify specificity similarity between two domains by comparing their peptide ligands. Since LBI is calculated by comparing binding peptides of a domain pair in the same peptide pool, LBI is able to directly and accurately measure the specificity similarity of the two domains. We used LBI to calibrate two widely used parameters, i.e. sequence identity (SI) and Position-Weight-Matrix (PWM) distance, for finding domains with similar specificity, because both parameters can measure specificity similarity using all available interaction data rather than array interaction data only.

Results
We demonstrated DomPep by investigating two domain families (PDZ and SH2) as study cases, based on their domain/peptide-array data [1,3]. BLI for PDZ domains decreases with an increase in PWM distance and BLI seems independent of SI. In contrast, the SH2 data suggests that BLI decreases with SI and with the increase in PWM distance.
We tentatively set LBI=0.7 as the cut-off to differentiate two domains as similar. This cutoff was used to determine the cutoffs of SI and PWM distance for searching for similar domains. The ligands of the similar domains were combined to build predictors.
Using the same training sets and independent test sets, DomPep showed better performance than Netphorest [3] for SH2 domains and MDSM [1] for PDZ domains, but similar to Structure-based Position-Specific Scoring Matrix (SPSSM) [2] for PDZ domains. We further found the performance of both DomPep and SPSSM increases with the number of training data. Therefore, the final DomPep built using all interaction data should be better than SPSSM built using array data. Additionally, DomPep can predict ligands for a domain without a DomPep predictor only if it is similar to a domain trained in DomPep (≥SI cutoff).

Innovative aspects
- DomPep has better performance than available prediction approaches for domain families and can be used for any domain families.
- Unlike most prediction methods that are limited to specific domains trained in the methods, DomPep can be used for domains without trained in DomPep.

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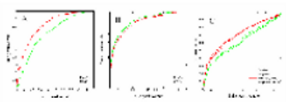

Figure 1. Comparison of DomPep to other prediction methods by ROC curve. (A) DomPep vs MSMD. (B) DomPep vs SPSSM. (C) DomPep vs Netphorest using a test set of 9 SH2 domains. The 9 SH2 domains relate to 9 Netphorest predictors composed of both PSSM and ANN models. Since the ANN models were built using the same set of training data for building DomPep models, we further compared the performance of these ANN models with DomPep. DomPep is significantly better than MDSM and Netphorest (p-value <0.05) and similar to SPSSM (p-value=0.14).

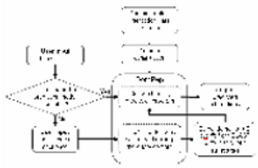

Figure 2. The framework of DomPep program.

C537

Significant improvements to the HUPO-PSI mass spectrometer data file standard mzML

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Introduction
Nearly every mass spectrometer vendor has developed its own proprietary data format for storing data acquired in mass spectrometers. It is impractical for software tools developed for general use to support all these different formats. Thus the approach in proteomics has been to employ open, XML-based formats, and develop the software to work with these formats. Two different formats have emerged, mzXML and mzData. However, the availability of two different formats has caused confusion about which format to use in the community and software developers must expend extra effort supporting two formats. Therefore in order to address this problem, the Institute for Systems Biology (ISB) and the Proteomics Standards Initiative (PSI) have collaboratively designed and implemented a new single format, mzML, taking from the best aspects and requirements of both original formats.mzML Version 1.0 was release at ASMS in June 2008.

Methods
The format is implemented in XML using schema definitions in conjunction with a controlled vocabulary implemented within the Open Biological Ontologies framework. Semantic validation, in addition to standard XML validation, is achieved via a special semantic validator tool. A reference implementation (ProteoWizard) and a number of other tools were contributing to test writers and readers.

Preliminary results
Version 1.0 of mzML has shown some data ambiguities left in the standard, so the format has undergone more internal and external review and we are ready to release a significant update to the schema: version 1.1. Several software tools are available to utilize the new format, including writers, readers, and validators (e.g. ProteoWizard, TPP, TOPP, jmzML, Phenyx, Pride, etc.). Details can be obtained on the PSI website (http://psidev.info)

Innovative aspects
- Unified open standard format for storing mass spectrometer output data
- Several open source (and non-GPL) tools already implemented to ensure fast adoption
- Supports new data types and coupled with improved controlled vocabulary
- Online or offline semantic validation (MIAPE-MS compliant validation is also available)

Schema improvements in 1.1
- Added support for precursor ion and neutral ion loss spectra
- Added support for Selected Reaction Monitoring aquisitions
- Schema-level enforcement of instrument components
- Removed spectrumDescription abstraction
- Removed xsd:IDREF spectrum/chromatogram id (id is replaced by nativeID)
- Moved "softwareRef" from dataProcessing to processingMethod to allow multiple software to act on a single spectrum/chromatogram
- Added "defaultDataProcessingRef" to spectrumList/chromatogramList
- Merged "acquisition list" and "scan" concepts into scanList
- Removed obsolete "number" from scan (née acquisition)

More info
All information about the status of the format, including specification documents, example documents, implementations can be obtained at http://psidev.info/index.php?q=node/257

C538

Fingerprinting Bacterial Strains Utilizing LC/MS-based Profiling of Intact Proteins

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Introduction
Combining retention time with accurate mass from LC/MS data is a powerful approach for profiling compounds present in complex biological matrices. This untargeted workflow has been applied to both metabolomic and proteomic samples, but proteomics has been limited to peptide digests as the feature extraction algorithm relied on resolved isotopes. A new algorithm for extracting protein features now allows this profiling approach to be applied to intact proteins. In this study, we explore fingerprinting of bacterial strains by differential analysis of the protein features in cell lysates.

Methods
Three strains of *E. coli* were grown to the same optical density, and then cells were harvested, washed and lysed. Proteins were extracted from the supernatant and analyzed by nano-LC/MS on an Agilent HPLC-Chip interfaced to a 6420 Q-TOF mass spectrometer. Data was collected in MS-only mode and protein features were extracted from the data using the large molecule feature extraction algorithm (LMFE). LMFE first finds peaks in the LC/MS analysis, aligns peaks with the same retention time and elution profile, and groups the different charge states of the same proteins together by algebraic charge state deconvolution. This results in a list of features which are then compared between the different strains using dedicated mass spectrometry based chemometrics software.

Results
Preliminary results on a standard strain of *E. coli* demonstrated that the LMFE algorithm was effective in extracting many protein features in a time-efficient manner. For a 150 minute separation, 597 protein features were extracted in 15 minutes (Figure 1). In contrast, integrating and extracting spectra from the chromatographic peaks with subsequent maximum entropy deconvolution produced only 192 compounds in 90 minutes. To verify that the masses produced by LMFE were valid, some of the measured masses were compared to intact proteins in the EcoProDB database. This resulted in 42 intact protein masses matching (within 20 ppm) to proteins known to be in standard *E coli* samples, including proteins such as superoxide dismutase, adenylate kinase, enolase, malate dehydrogenase, cysteine synthase A, aspartate carbamoyltransferase, serine hydroxymethyltransferase,, enolase, and 30S ribosomal protein S2. A pilot study of bacterial lysates showed that feature extraction was reproducible within sample replicates. Further, the strains were well differentiated by both hierarchical clustering and principal components analysis (Figure 2).

Innovative aspects
- Algorithm for extracting protein features (defined by mass and retention time) from LC/MS data
- Nano-LC/MS based differential profiling of intact protein for fingerprinting bacterial strains
- Dedicated mass spectrometry based chemometrics software

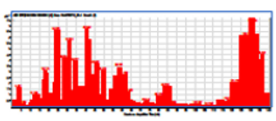

Figure 1: LC/MS analysis of proteins from an E. Coli lysate. The LMFE algorithm extracted 597 features from this data.

Figure 2: Hierarchical clustering of replicate LC/MS from three bacterial isolates clearly differentiates the strains.

C539

An Integrated Data Management Platform for Proteomics and Biomarker Discovery

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Introduction:
Proteomic technologies have progressed significantly to provide powerful tools for biomarker discovery and validation. These advances bring significant challenges in standardizing protocols, and integrating large and complex datasets. We have deployed a data management platform that combines clinical, biospecimen, analytical, and protein expression data to support advanced querying and data analysis. Capabilities are demonstrated by bladder cancer and prostate cancer progression studies involving analysis of patient urine and blood plasma by label-free, iTRAQ, and MRM quantification.

Methods:
Three distinct database systems were integrated: the TissueMetrix biospecimen tracking system (AIM Inc., Toronto), the Synapseus LIMS, and the Integrated Proteomic Analysis Database (IPAD) (Figure 1). Plasma samples were labelled and logged into TissueMetrix. Patient clinical annotations (e.g. age, disease stage, recurrence status, urine creatinine) were imported into Synapseus. Label-free, iTRAQ and MRM workflows and their experimental annotations were recorded. Raw data, protein database searches, and result parsing were also managed by Synapseus. Protein identification and quantification results were imported into IPAD and database query tools were applied to extract data reports for statistical and pathway analysis using R and Ingenuity Pathway Analysis software.

Results:
The technical design of the database platform is provided, including data types and data flow spanning sample processing, MS analysis, and bioinformatics processes (Figure 2). Standard operating procedures implemented included urine/plasma depletion, protein extraction, enzymatic digestion, isobaric labelling, fractionation, MRM transition selection, and LC-MS/MS data acquisition. Mapping of quantification data to database structures is defined, including direct relationships between discovery and MRM data elements. Example queries and results are given, including: a) peptides identified with a given average fold-change between patient groups (e.g. low-grade/invasive, BPH/metatstatic); b) proteins uniquely identified within a patient group; d) correlation of peptide relative intensity between label-free/iTRAQ and MRM datasets. User interfaces are shown to illustrate how results and reports were accessed by researchers from multiple facilities during the course of the studies.

Summary:
We describe an integrated data management platform used for managing large-scale quantitative proteomics studies, enabling standardization, quality control, and complex data analysis.

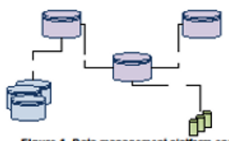

Figure 1. Data management platform enabling Integrated querying and analysis of clinical, analytical, and protein expression data

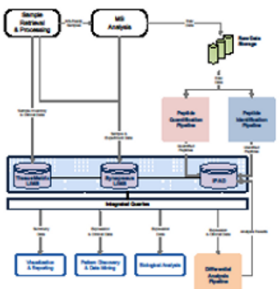

Figure 2. Analytical data flow from sample processing MS analysis, and bioinformatics processes into the integrated database platform.

Innovative Aspect:
- Integration of clinical, biospecimen, analytical, and protein expression data to support complex querying and data analysis.
- Integration of quantification results from discovery and verification methods.

C540

New Functionality for the Trans-Proteomic Pipeline: Tools for the Analysis of Proteomics Data

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Introduction
The Trans-Proteomic Pipeline (TPP) is an open-source software suite with well-established community acceptance(1). The TPP provides a free proteomics analysis solution, spanning: conversion of raw MS/MS data to open formats; support for searching MS/MS spectra with various search engines, including X!Tandem, Sequest, Mascot, Phenyx, and others; statistical validation of peptide and protein ids with PeptideProphet and ProteinProphet; quantitative proteomics with XPRESS, ASAPRatio, and Libra; and tools for visualization of and interaction with results. Here we present recent updates to the TPP.

Methods
We have recently introduced several new features in the TPP. We provide a new tool (iProphet) which allows one to statistically combine and analyze peptide identifications from multiple experiments, searches, runs, etc, to provide more accurate probabilities; it reduces false discovery rates, especially on large datasets. We have added preliminary support for results from the OMSSA, MyriMatch, and InsPecT search engines. We have added many features to the GUI, including: spectral library download and management, generation of Libra condition files for any number of channels; support for compressed (gzip) files. Recent speed optimizations to ASAPRatio have cut down analysis times by a factor of 10 to 300. Several bug fixes and improvements to the user interface have also been made.

Results
The usage of the TPP tools has been steadily increasing since their original release a few years ago, as evidenced by the number of downloads from the Sourceforge website, and the number of posts and members in our email discussion list.

Over the past 12 months, there have been over 8,000 downloads of the various versions of the TPP source code (which can be compiled on Windows and Linux/Unix operating systems) and the pre-compiled Windows distribution of the TPP tools from our Sourceforge site (2). Furthermore, several hundred researchers from around the world have attended the week-long in-depth course on these tools, presented semi-annually at the Institute for Systems Biology.

An easily searchable email distribution list, hosted at Google Groups (3), was established as a means to provide free support and build a user community around these tools. This list has been quite active and useful to the end users; there are currently 770 members. Nearly 7,000 messages have been posted, in topics spanning 1,600 threads, such as installation problems, usage queries, bug reports, and feature requests, among others.

Innovative aspects
- Improved peptide identification discrimination with additional models and integration of multiple search strategies
- Performance and installation enhancements
- Additional search engine support

References
(1) http://tools.proteomecenter.org/
(2) http://sourceforge.net/project/showfiles.php?group_id=69281&package_id=120912
(3) http://groups.google.com/group/spctools-discuss

C541

Efficient and Confident Identification of Protein Phosphorylation by Combining Dephosphorylation Reaction and High Mass Accuracy LC-MS Data

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Introduction
Several mass spectrometry-based methods have been developed for charactering phosphoproteins. We have demonstrated the capability of a computational algorithm in mining phosphopeptide signals in large LC-MS data sets by measuring the mass shift due to phosphatase treatment (Wu et al., 2007). Mass accuracy seems to play an important role in efficiently selecting out phosphopeptide signals. Here, we developed a process to incorporate LC-MS data that was generated from an LTQ/Orbitrap mass spectrometer into our strategy for taking advantage of the accurate mass measurement.

Methods
The analytical strategy was described in Figure 1. LTQ/Orbitrap raw files were converted to the open file format mzXML via ReAdW.exe program. To find peaks that were contained in each mzXML file, an open-source computer program, msInspect, was utilized to locate isotopes and assemble those isotopes into peptides. An in-house program, LcmsFormatConverter, was utilized for signal filtering and format transformation. A proposed in-house program, DeltaFinder, was modified and used for defining signals with an exact mass shift due to the dephosphorylation reaction, which generated a table that listed potential phosphopeptide signals. The retention times and m/z values of these selected LC-MS signals were used to program subsequent LC-MS/MS experiments to determine phosphorylation sites.

Results
Compared to our previous work finished by using a quadrupole/time-of-flight mass spectrometer, a larger number of phosphopeptides in the casein mixture were identified by using LTQ/Orbitrap data, demonstrating the merit of high mass accuracy in our strategy. Besides, the characterization of the lung cancer cell tyrosine phosphoproteome revealed 67 tyrosine phosphorylated peptides, corresponding to 64 unique peptides/proteins. For 10 samples, our strategy is relatively reproducible while comparing to the conventional data-dependent LC-MS/MS analysis. Overall repeatability in the direct LC-MS/MS analysis was 16-16.7% and the repeatability rose to 69.8~72.5% (Fig 2) while applying our strategy. The characterization of the lung cancer cell tyrosine phosphoproteome revealed that the use of alkaline phosphatase treatment combined with accurate mass measurement in this strategy increased data repeatability and confidence.

Innovative aspect
- The combination of alkaline phosphatase treatment, high mass accuracy measurement, and computational methods increased repeatability and confidence in phosphopeptide identification.

Reference
(1) HY Wu et al., Mining phosphopeptide signals in liquid chromatography–mass spectrometry data for protein phosphorylation analysis. J Proteome Res. 2007, 6, 1812–1821.

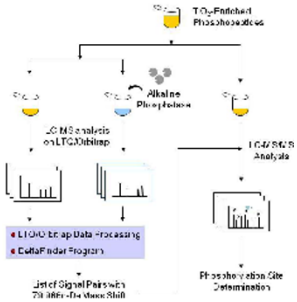

Figure 1. Analytical strategy. The TiO_2-enriched peptide mixture, treated or un-treated with alkaline phosphatase, were analyzed by an LTQ/Orbitrap. Signals with ~79.966n-Da mass shift were exported by DeltaFinder. MS/MS experiments were conducted on those potential phosphopeptide signals for the determination of phosphorylation sites.

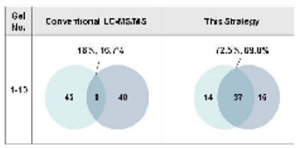

Figure 2. Comparison of overall repeatability between conventional LC-MS/MS analysis and this strategy.

C542

ICPL meets ESI platforms: ICPL-*ESIQuant* for analyzing stable isotope labeled proteomic data

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Introduction
One big bottleneck in quantitative proteomics remains the availability for powerful software to evaluate mass spectral raw data automatically. For the isotope coded protein label technology (ICPL™, Serva) (1) in combination with MALDI-TOF/TOF mass spectrometry systems, ICPL*Quant* was developed in our lab to quantify ICPL multiplexed peptide data on the MS level (2).
The lack of a powerful tool to handle ICPL labeled MS data generated from ESI instruments is becoming critical since many users have started using the ICPL technology followed by ESI mass spectrometry.

Methods
The efficiency of ICPL-*ESIQuant* is demonstrated by the comparative analysis of four mixtures with different ratios of three proteins (Ovalbumin, Horseradish Peroxidase and Rabbit Albumin) spiked into the complex protein background of the DGPF Proteome Marker. All peptide mixtures were double-digested with trypsin and Glu-C, separated by on-line nanoLC and analyzed by electrospray tandem mass spectrometry. The experiments were performed on an Agilent 1200 nanoflow system connected to an LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany). Data analysis with ICPL-*ESIQuant* was supported by Mascot as the database search engine for peptide identifications.

Results
Here we present a software tool, ICPL-*ESIQuant*, which comprises a set of modules to process ICPL-multiplexed MS data acquired on high resolution Orbitrap mass spectrometers. ICPL-*ESIQuant* works with deisotoped and deconvoluted mzXML data files as input and is able to quantify ICPL doublet, triplet or quadruplet peptide pattern with a user defined sensitivity and selectivity. The quantification of an ICPL quadruplex labeled peptide is done by integrating the four XICs of each ICPL peptide state (Fig. 1).
The Mascot MS/MS results, which were acquired automatically during an ESI-MS run, can easily be merged with the quantitative information.
Additionally, a precursor list can be created for a targeted identification of all regulated ICPL peptides.

Peptide and protein summary tables are provided in Excel format and can be visualized by several external programs.
The data sets, including quantified multiplets (doublets, triplets, quadruplets), position in the separation space, peptide and protein information etc. can be stored in a database, which can provide a very valuable basis for further statistical analysis and data mining steps, especially in larger proteomic projects.

Innovative aspects
- Accurate detection and quantification of ICPL multiplets measured by ESI instruments
- Generation of peptide and protein summary result tables which can be imported into databases
- Generation of precursor lists for a targeted MS/MS search

References
(1) A Schmidt, J Kellermann, F Lottspeich, A novel strategy for quantitative proteomics using isotope-coded protein labels; Proteomics 2005 (5)1, 4-15 (in press).

(2) Brunner A, Keidel E, Dosch D, Kellermann J, Lottspeich F. ICPL*Quant* – a software for non-isobaric isotope labeling proteomics (submitted for publication).

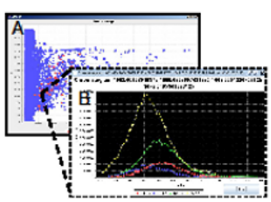

Figure 1. ICPL-*ESIQuant* result views: (A) visualizes a 2D peak map (mass vs. scan number) which shows detected ICPL quadruplex labeled peptides (in red); B shows a XIC of one detected ICPL quadruplet candidate.

C543

Effective mapping of disulfide bridges in proteins combining informatics and $^{16}O/^{18}O$ labeling

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Introduction
It remains a great challenge to locate all the likely disulfides in a protein containing multiple disulfide bridges. There are some reports where oxygen eighteen (^{18}O) water is used to facilitate the identification of disulfide-containing peptides. However, this approach has been marred by poor labeling efficiency. Recently, we have optimized the labeling procedure to ensure efficient ^{18}O incorporation (1). Based on this labeling technique, we have integrated computer programming to develop a high-through approach to effective identification of disulfide-containing peptides.

Methods
This approach consists of four steps: (A) Peptide labeling: The protein is treated with trypsin and the product is divided in two parts. After completely dried, one part is treated with trypsin in regular buffer, and the other is incubated with trypsin in the buffer made of ^{18}O water. (B) Mass spectrometric analysis: The above two samples are subjected to one LC-MS analysis together. (C) Informatics analysis: The MS data is examined using in-house computer programs, which can annotate isotope clusters in the MS data, define the masses of candidate disulfide-containing peptide conjugates and find the likely compositions of these conjugates. (D) Peptide verification: The MS/MS of the above conjugates are collected to verify their identities and to map the exact locations of the disulfides.

Results
Using albumin as a prime example, we analyzed this protein first with $^{16}O/^{18}O$ labeling and Orbitrap MS. There were mainly three type of isotope clusters observed in the MS data (Fig 1). Our proprietary computer programs could indeed pick up disulfide-linked peptides based on the criterion that there is an 8- or 12-dalton difference between the isotope clusters with or without ^{18}O labeling. With another set of programs, we have successfully verified the peptide conjugates with the disulfide bridges previously reported (2). Most strikingly, we have identified a group of novel disulfide bridges that can be detected in albumin preparation for clinical use as well as in plasma albumin from normal individuals. This approach will lead us to better understand how protein structures and functions can be diversified through modulation of disulfide linkages.

Innovative aspects
- Optimization of a procedure for efficient $^{16}O/^{18}O$ peptide labeling
- Development of computer programs for comprehensive identification of interchain disulfide-containing peptides
- Mapping of the disulfides in proteins containing multiple disulfide bridges
- Prediction of multiple conformations for a protein with a defined amino acid sequence

References
(1) Y. Chen et al. Identification of proteomic changes using an optimized $^{16}O/^{18}O$ labelling procedure, *Manuscript in preparation*
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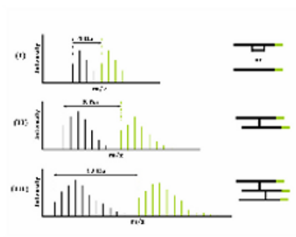

Figure 1. The MS patterns of isotope clusters using our approach. Pattern (I) is observed for a single peptide chain without any disulfide or only with intrachain disulfide; pattern (II) is for two peptide chains linked with at least one interchain disulfide; and pattern (III) is for three peptide chains linked with at least two interchain disulfides

C544

Bioinformatics analysis of quantitative proteomics datasets using soft clustering algorithms.

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Introduction:
The success of mass spectrometry-based proteomics techniques has created a number of challenges when analyzing large numbers of complex samples. However, these challenges have been met by the emergence of new approaches and techniques for sophisticated bioinformatics analysis. Historically, clustering algorithms are frequently used for the analysis of microarray data. With the advent of SILAC, iTraq and label-free quantitative methods there is a growing requirement to transfer existing bioinformatic solutions from genomics to proteomics. In our current study we present protein profiling of proteomics quantitative datasets based on soft clustering algorithms.

Methods:
To evaluate the newly developed software tool, we used the training dataset and proteomics data derived from quantitative studies available from scientific publications. The experimental data, including accession numbers, peptide sequences, search engines scores, and quantitative information, was exported into ProteinCenter (Proxeon). Proteins were clustered according to an "indistinguishable proteins" algorithm. Protein profiling based on soft clustering was applied to generate a set of clusters sharing the same tendency during kinetic studies. The proteins extracted for each cluster were further processed for Gene Ontology (GO) and pathway analysis.

Results:
ProteinCenter enabled several alternative partitions of protein data (data sets, filtering, clustering), allowing the user different views for directed data analysis. Profiling represents another way of partitioning proteins based on their quantitative data characteristics across a data set such as iTRAQ or SILAC assays. The primary action of profiling involves a soft clustering of the proteins, based on their selected quantitative data values. In conventional clustering each protein has a single and exclusive membership to one particular cluster. Soft clustering, however, embodies a more generalized partitioning approach, where each protein belongs to every cluster to a certain degree. Here we demonstrate the application of protein profiling based on soft clustering using examples of proteomics kinetic studies.

Innovative aspects:
Application of soft clustering algorithms for quantitative proteomics.

C545

Protein List Comparator (ProLiC): a Framework for Comparison of Protein Lists

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Introduction
The rise of Proteomics yields huge amounts of data. However, lists of identified proteins are frequently not easily comparable. A major problem is that list entries often include arbitrary chosen representatives of a group of related proteins. Furthermore, results are sometimes given as accession numbers of different databases and these numbers may change. Comparison of results from different surveys leads to the definition problem of identity between certain entries. Here, we present a software solution (ProLiC) for comparison of protein lists based on sequence and/or peptide information.

Methods
ProLiC is an open source software library written in the Java programming language (JDK 6, Sun Microsystems, Inc., Santa Clara, CA, United States).
Therefore, user-written applications can be developed that utilize the ProLiC framework.
Using JavaBeans ProLiC supports application of builder tools for graphical user interfaces (GUI) or an easy development of web applications using the JavaServer pages (JSP) technology.
The huge amount of Proteomics data, which need to be stored and the complex data interrelationship normally takes advantage of database usage. Therefore, ProLiC provides interfaces for utilization of the open source database management system MySQL. Furthermore, ProLiC supports retrieving of sequence information by using accession numbers.

Results
Three different ideas were considered for comparison of protein lists:
1. Grouping. Based on sequence or peptide information obtained from the protein lists, which are given as input, protein groups are constructed. These groups contain proteins that are regarded as identical. As a consequence, only protein groups are reported as intersections of different protein lists.
2. Calculation of a similarity ratio by alignment algorithms or BLAST on two sequences. A threshold is given by user input in order to determine a sequence similarity criteria for compared proteins.
3. Comparison with consideration of peptide information. This approach is probably the most suitable for proteomics data. Proteins with no "original" evidence (i.e. with a unique peptide pattern) can be left out from the groups (**Occam's razor** principle, fig. 1, a) or enclosed into the group structure (fig. 2, b). In the former case each protein occurs in only one group. In the latter case proteins can be member of several groups.
Based on the given input (i.e. with or without peptide information) ProLiC uses the adequate algorithm for protein group definition.

Innovative aspects
- Protein list comparison on the basis of protein groups and different identity criterias (based on global alignment and/or peptide information)
- Automated choice of the adequate comparison algorithm with respect of the given input information

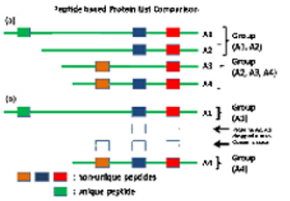

Figure 1. Two examples for peptide-based protein list comparison. a: The group structure when Occam's razor is *not* applied => all possible proteins appear (probably in more than one protein group).
b: The group structure when the general design principle "Occam's razor" is applied: proteins without "original" peptide evidence (i.e. with a unique peptide pattern) are left out (proteins A2 and A3).

C546

Public Proteomics Data - of Carrots and Sticks

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Proteomics is generating a vast amount of data from a broad array of technologies. However, the experimental data supporting published conclusions is still often not available, incomplete, or related datasets are not comparable due to the lack of standards, or their implementation. Still too often, data is effectively hidden in PDF tables and not accessible to third party analysis. Examples from other domains, in particular microarrays, show significant improvements to data analysis by third parties who did not originally generate the data. Such methodology improvement, as well as independent validation of published conclusions, is only possible if experimental datasets are accessible for further analysis, and if they are accessible in a re-usable form. The HUPO Proteomics Standards Initiative (PSI) develops standards for data representation in proteomics, and promotes their implementation by experimentalists, instrumentation and software providers, databases such as PRIDE, and journals. Since the conception of the PSI in 2002, significant progress has been made in the availability and structure of public proteomics data, but obstacles still remain. We will discuss the current status of systematic proteomics data collection, tools and motivations ("carrots") to encourage deposition of proteomics data in public repositories, as well as "sticks", requests by journals and funders for increased public availability of proteomics data supporting scientific publications. After all, if the Human Genome Project had not followed an open data release policy, what would we be searching our mass spectra against today?

C547

Deconvolution of multiply charged states of Intact Proteins

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Introduction
Analysis and identification of intact proteins and peptides through LC/MS is widely used in biochemical laboratories. Even with high resolution instruments, high molecular weight intact proteins are not always isotopically resolved. There is great interest in the identification of different protein modifications, such as glycosylation or oxidation. Deconvolution based on graph theory and using appropriate scoring will allow identification of zero charge masses and relative quantification of their modification with high accuracy and reliability.

Methods
Typical individual spectra from LC-MS do not produce enough charge states to perform deconvolution. The first step is to average enough spectra to provide good statistics for different charge states. On a formal graph model, all unknown charge states are presented as possible states. The relation between different states is formalized as the probability of belonging to the same mass. Then all charge states belonging to the same mass present a Charge State Chain. The problem of deconvolution can be formalized as the problem of search of all optimal paths in the graph. The scoring function assigned to the optimal charge state reflects the reliability of identification. From all possible charge state chains the chain with best score is assigned to a mass

Results
Several methods are used for deconvolution of intact proteins and peptides such as Max Entropy. Maximum Entropy method demands the evaluation of peak width. It is not always possible to identify peak width with enough accuracy. An additional problem is that peak width is changes along m/z region, even for the same compound. An alternative method based on graph search of all optimal paths is free from this problem. It also works well for co-eluting charge states. It is demonstrated that a deconvolved spectrum, after appropriate averaging to get enough different charge states and improve charge state envelope, reveals multiple glycosylation sites. Other antibody variants are revealed as well. Analysis of separate charge states with clear variant patterns confirms the validity of modifications in the deconvolved signal.
Deconvolution provides not only correct measurement of intact protein molecular weights but also allows accurate quantification.
The method works well for high resolution spectra and for low resolution spectra. It can also be applied to isotopically resolved data also. The deconvolution algorithm was applied for to data acquired using both orbitrap and ion traps.

Innovative aspects
- Presentation of an approach to accurate deconvolution of multiply charged states of intact proteins and peptide based on graph algorithm
- Characterization of different modifications of intact proteins

References
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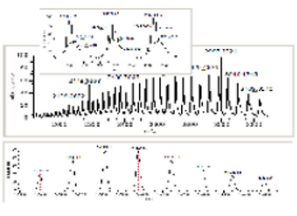

Figure 1. Deconvolved Spectrum of Sigma IgG (IT spectrum on an Orbitrap Instrument). Deconvolved spectrum reveals the same modification pattern as individual modification clusters.

C548

GeneMANIA: Gene Function Prediction using a Multiple Association Network Integration Algorithm

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GD Bader,
QD Morris

INTRODUCTION

GeneMANIA (http://beta.genemania.org) simplifies the task of navigating through the maze of large-scale biological datasets. It is an interactive network browser that also predicts gene function using many large publicly available datasets including proteomic, protein-protein and genetic interaction networks, gene expression data, protein domain information, and subcellular localization. GeneMANIA uses these data to analyze gene lists to predict function and identify high-throughput data sources in which your query genes are highly associated.

METHODS

We have collected high throughput data and gene identifier information from several web accessible proteomic and genomic databases. Based on a query list of proteins, GeneMANIA generates a query-specific functional association network upon which it applies the guilt-by-association principle to identify genes and proteins that share function with the query list. GeneMANIA's database is continually updated, so gene function predictions will be based on the most recent information. GeneMANIA supports six model organisms (arabidopsis, worm, fly, mouse, human, yeast), with plans to expand this list in the future. GeneMANIA uses Cytoscape-Lite, a lightweight interactive network visualizer to show how the genes in the query list relate to one another.

RESULTS

GeneMANIA consists of a web interface, the GeneMANIA algorithm (1), and our data warehouse. When a user enters a list of functionally related query genes, data sources are identified that are most relevant to the query genes. The GeneMANIA algorithm was entered in the MouseFUNC competition (2) and was found to be faster, and at least as accurate as the other algorithms in the competition. The GeneMANIA algorithm is fast enough to be used in an interactive web-based interface, providing the ability for users to enter arbitrary query gene lists and receive predictions in seconds. GeneMANIA-Beta (http://beta.genemania.org) is free, web-based, open source and still a work in progress. We welcome any comments, suggestions and collaborations.

INNOVATIVE ASPECTS

- Fast, accurate gene function prediction using a web-based interface
- A large data warehouse containing more than 100,000,000 interactions
- An interactive network visualization tool to view composite networks

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C549

Protein Ontology (PRO) for Proteomics Data Integration

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Introduction

Protein diversity is mainly achieved by alternative splicing and/or post-translational modifications. Many of these protein forms are key components of biological pathways, and they may be functional distinct. However, the current functional annotation of a protein may represent composite annotation of several forms, which may lead to misinterpretation of data mining results. PRO provides the means to represent the multiple forms of a protein as well as their relationship, and therefore assign an annotation to the correct protein entity, instead of the canonical protein [1].

Methods

The ontology and the annotation can be searched and retrieved from the PRO website (http://pir.georgetown.edu/pro/). The TGF-beta signaling pathway components from Reactome [2] and Pathway Interaction Database (PID) [3] were mapped to the corresponding PRO IDs and the integrated pathway was derived. The PRO-Reactome mapping is available from PRO FTP site. The PRO-PID mapping is forthcoming.

Results

Both PID and Reactome contain manually curated data for human pathways. In many cases there is no complete overlapping. Therefore, the integration of the information from these resources can provide a comprehensive insight into a given pathway. Figure 1 shows the integrated view for the TGF-beta signaling pathway in Reactome and PID. The common components are shown within the pale orange area. The boundaries for the pathway are significantly different in both databases. PID also includes pathways that cross-talk and regulate the TGF-beta signaling, whereas the Reactome pathway provides information on the TGF-beta intracellular processing. PRO allows the integration of these two datasets providing a unique ID for each protein object, and also the relevant annotation (distributed in the PRO association file PAF). In addition, the PRO-pathway mapping supports modeling of the specific objects involved in a given disease in the pathway context. For example, it is possible to model the cardiac myocyte apoptosis, a process in which activated caspase-3 generates the cleaved form of rho-associated protein kinase 1 (PRO:000000563). This form is constitutively active and promotes apoptotic signals, as has been observed both in the mouse myopathy model and in human heart failure patients. This allows a better representation of the scientific knowledge available to the research community, thereby facilitating the understanding of human health and disease.

Innovative aspects

- PRO assigns a unique ID for each protein object
- PRO mappings facilitate data integration and interpretation
- PRO allows precise definition of objects needed for pathway analysis and/or in disease modeling.

References

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(2) Reactome: http://www.reactome.org/
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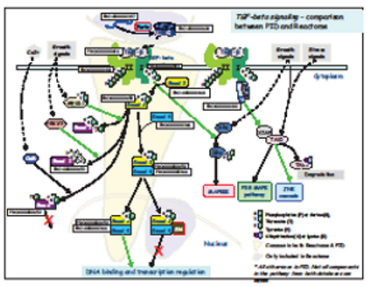

Figure 1. TGF-beta pathway data integration.

C550

APCF: a large proteomics computational resource and APCF UNITE for launching and viewing data

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Undoubtedly, the Achilles' heel of current proteomic analyses is the processing and validation of the vast volumes of MS/MS spectra obtained from current mass spectrometry instrumentation. Our approach for addressing these needs has been met by the construction of the Australian Proteomics Computational Facility (APCF) in 2007. The APCF is both a hardware and software solution for rapid, high volume proteomics mass spectrometry data analysis at a single online internet site. This shared national resource strategy addresses the needs of large scale automated mass spectrometry data analysis allowing access to multiple industry-standard algorithms installed on an advanced high-performance multi-processor computing cluster. To date, the facility has met with wide acceptance from the proteomics community with over 60,000 individual data searches being performed by many groups both in Australia and around the world, independent of distance from the facility based in Melbourne.

The APCF's large computational power of over 1000 CPU cores is managed by multiple interactive systems that direct resource allocation and act as gateway systems to the computational nodes. The APCF has constructed a sophisticated queuing system, which allows multiple concurrent MS/MS databases searches to be performed, without competition, using best utilization of computational resources combined with fault-tolerance to ensure the integrity of each submitted search. Specially designed web based software provides a unified interface to receive jobs from a range of tools including the APCF designed automated interface "UNITE", Mascot Daemon and standard web pages in a highly secure manner through 128 bit SSL encryption.

The APCF constructed interface "UNITE" is a world's-first tool that allows users to seamlessly access multiple industry-standard MS/MS databases searching algorithms (Mascot, X!!Tandem and OMSSA) remotely and simultaneously at the APCF. In addition, a unique result file download utility *Mass Extraction Download Information Centre* (MEDIC) allows any number of result files to be downloaded to the user's PC for subsequent downstream analysis.

UNITE is a complete automation utility where raw instrument files are automatically converted from many different instrument manufacturers before database searches are performed by single algorithm searches or simultaneously submitting them to multiple algorithm searches at the click of a button. APCF also provides access to many regularly updated sequence databases, including SwissProt, IPI and the LudwigNR, a non-identical database of over 7.5 million entries.

This unique world's first integrated approach to sharing of proteomics computing and databases has placed Australia at the forefront of efforts to identify the proteins associated with the early detection of major human diseases as well as many other research programs including plants, animals and microbes requiring proteomic analysis. In addition, the APCF gathers together expertise to provide leadership for proteomic data interpretation on locally generated data. This data can also be used in contributions to other world-wide large-scale proteomic efforts. The APCF can be accessed by a secure user account which can be obtained from the APCF at www.apcf.edu.au. The APCF is free and open to all researchers in other countries.

C551

ProteomeCommons.org Annotations and Project Management Resource

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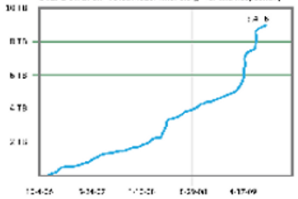
Introduction

Accurate and complete annotation has been a difficult problem for the field of proteomics – with compliance, ease-of-use, and the evolving nature of research being some of the challenges encountered. In an effort to respond to these challenges, ProteomeCommons.org has implemented a system of annotation and project management that applies the concepts of Web 2.0 social networking to the annotation of data sets that are stored in the ProteomeCommons.org Tranche Repository.

Methods

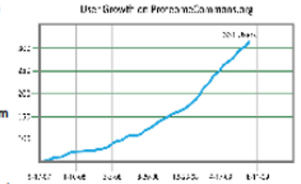
Underlying the updated ProteomeCommons.org is the new Tranche Annotations Database software. Developed in Java and coupled to a MySQL database, Tranche Annotations stores descriptions of data sets in an accompanying Tranche repository. There is not a built-in information model, so the annotations are able to evolve along with the field.

The new system enables researchers to sign up and form groups to share, manage, and annotate data sets. Once public, the data sets are accessible through proteomecommons.org and are also disseminated by other resources like PRIDE (Proteomics Identifications Database) and GPMdb (Global Proteome Machine Database.) The annotations have also been made more accessible by making some of the annotations available to caBIG (cancer Biomedical Informatics Grid.)



Results

Since its release, the annotation and project management system has already had an impact on repository usage, and several large proteomics initiatives, including the NCI CPTAC (National Cancer Institute Clinical Proteomic Technology Assessment for Cancer) centers and the ACS (American Cancer Society) Multiple Myeloma Research Foundation, are using it to enhance their proteomics collaborations. Usage statistics represent one measurement of usefulness to the proteomics community. The Tranche repository began collecting proteomics data in October, 2006 and has experienced exponential growth in the amount of data submitted and made available online. The modified system was deployed on February 2, 2009, with 178 registered users and 4.8 terabytes of data stored in the Tranche repository. As of July 15, 2009, the system now has 331 registered users and 9.4 terabytes of data. A registered used is a user that is submitting content. Registration is not required to browse content or download from the Tranche repository. The pace of growth in these metrics are shown in the figures below.



This new community resource is available immediately and can be accessed at https://proteomecommons.org.

Innovative Aspects

- Modelling annotation standards on a dynamic information model allows them to evolve with proteomics technologies.
- Applying concepts of Web 2.0 social networking to the annotation of data sets and to project management accurately models important aspects of collaborative research.
- Exposing the information stored in the ProteomeCommons.org Tranche Annotations Database to caBIG data grid – making the annotations and the data sets stored on the accompanying Tranche repository more accessible.

Recent and Upcoming Development in the Tranche Distributed Data Repository

C552

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Introduction

Tranche is designed to meet the data sharing and public dissemination requirements of proteomics research, and is currently available at ProteomeCommons.org. Ongoing development of the Tranche Distributed Repository (www.trancheproject.org) deals with issues of scalability, reliability, performance and security. Tranche combines some of the strengths of a centralized, client-server model with a distributed architecture, but likewise must accommodate the challenges associated with both. Here we discuss these developments in the context of the related challenges.

Methods

Tranche is a highly-configurable, Java-based application currently used by ProteomeCommons.org (tranche.proteomecommons.org). Continuous development is directed by user feedback and well as testing (unit, stress and user), while major revisions are developed to address issues we can identify.

Challenges

- Scaling to petabyte capacities while minimizing required connections between servers as well as between client-servers
- Compensating for inevitable hardware failures
- Detecting and repairing corrupt data
- Offline servers, either temporarily (e.g., power outage) or indefinitely
- Managing security risks, even if compromised
- Network changes, such as new servers or adjusted server configurations

Results

Current Statistics

- 16 geographically-distributed servers
- 330 registered users
- 7,569 data sets
- 9.4 TB of data (uncompressed)
- 12,573,412 files

Recent developments

- Servers devote their maintenance time to downloading new data and deleting old data (actively balancing), as well as checking for (and repairing) corrupted data and balancing local database b-tree
- Data is verified on clients and servers when uploaded and downloaded to check for integrity
- 'Shadow' Tranche network (with different trusted X.509 certificates) actively backup ProteomeCommons.org Tranche network
- Recent performance benefits from batching multiple requests as well as having servers 'compete' for a request

Upcoming developments

- Multiple servers be managed by single 'routing' server
- Clients will connect to fewer servers
- Servers will have degrees of trust so can add data to each other
- Network will 'lazily' balance on demand by user requests
- Actions on network are logged and queried by servers coming online
- Server maintenance schedule more intelligent based on what was most recently profitable

Innovative aspects

- Redundancy re-introduced (lazily and actively)
- Servers assist each other in repairing corrupted data
- Demonstrates data pedigree and integrity

Figure 1. (1) A data set is simply a collection of files. (2) When stored on a Tranche network, a ProjectFile is created to describe the files and how to retrieve them. (3) Each file (as well as the ProjectFile) is made up of a meta data chunk and one or more data chunks.)

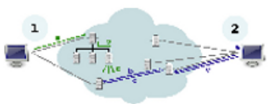

Figure 2. New server model. Note that each client connects to a limited number of servers (grey lines). Client 1 uploads chunk to routing server (a), which passes it to another server (b), which replicates it to network (c). Client 2 downloads a chunk (a-d).

Environmental Proteomics

(E101 – E110)

E101

Proteome Analysis of Sulfur-reducing Hyperthermophilic Archaeon *Thermococcus onnurineus* NA1

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Introduction

Thermococcus onnurineus NA1, isolated from a deep-sea hydrothermal vent area, is sulfur-reducing hyperthermophilic archaeon. The strain requires elemental sulfur as a terminal electron and organic compounds such as peptides, amino acids and sugars as carbon source. This strain shows the optimum growth at 80℃ and pH 8.5. Recently, the genome sequence of *T. onnurineus* NA1 was completed. In the present study, we analyzed the proteome of *T. onnurineus* NA1 at heterotrophic and lithotrophic culture condition in order to understand its metabolic versatility.

Methods

Harvested *T. onnurineus* NA1 cells were suspended in sample buffer (100mM Tris-HCl (pH6.8), 4% (w/v) SDS, and 4mM EDTA) and boiled for cell lysis. The soluble proteins were prepared by centrifugation at 15,000 rpm for 20 min and separated on 12% SDS-PAGE. The gel was fractionated according to molecular size and in-gel digestion was performed with trypsin (Promega, USA). The resulting peptides were pooled and lyophilized in a vacuum concentrator. Tryptic peptides were dissolved with 0.5% (v/v) TFA solution and used for LTQ-FTICR - MS/MS analysis (Thermo Fisher Scientific, Bremen, Germany). The MASCOT v2.2 was used for protein identification and emPAI calculation. The metabolic pathway analysis was performed with the Excel 2007 and DAnTE v1.0, R-based bioinformatics tool.

Results

More than 1400 proteins were identified by 1-DE/MS-MS analysis, which are the above of 69% of genome of *T. onnurineus* NA1. By the comparative analysis of metabolic pathways, glyoxylate and dicarboxylate metabolism were primarily increased in lithotrophic growth condition while reductive carboxylate cycle and pyruvate metabolism were decreased (Figure 1). In particular, hydrogenase gene clusters (1) responsible for biohydrogen production were prevalently increased in the lithotrophic condition than heterotrophic one (Figure 2). The altered metabolism is probably due to the feasible proteomes of *T. onnurineus* NA1 for high biohydrogen production. This result suggests that the switching culture of *T. onnurineus* NA1 can give a possibility of the industrial application to produce the biohydrogen.

Innovative aspects

- Proteome analysis of novel hyperthermophilic archaeon
- Analysis of lithotrophic pathway in *T. onnurineus* NA1

References

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Figure 1. Comparison of metabolic pathways between heterotrophic and lithotrophic culture condition. The glyoxylate and dicarboxylate metabolism were primarily increased in lithotrophic growth condition while reductive carboxylate cycle and pyruvate metabolism were decreased.

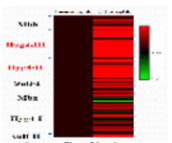

Figure 2. Expression profile of hydrogenase gene clusters between heterotrophic and lithotrophic condition. The hydrogenase gene clusters were prevalently increased in the lithotrophic condition than heterotrophic one.

E102

Proteomics of Bronchoalveolar Lavage Fluid: Adjuvant Effects of Ambient Particulate Matter

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Introduction

Vehicular traffic and combustion result in increased cardiorespiratory morbidity and mortality. Size-fractionated ambient particulate matter (PM) was used to induce Th2 allergic inflammatory responses (1). The enhanced *in vivo* adjuvant effect of ultrafine particles (UFP; <2.5 μm diameter) correlates with a higher *in vitro* oxidant potential and a higher content of redox-cycling organic chemicals. The mechanistic link between the adjuvant effect of PM and the proteome can be used to develop biomarkers for allergic inflammatory responses caused by air pollutants.

Methods

Aliquots of bronchoalveolar lavage fluid (BALF) supernatants corresponding to 6 mice in each group (control and UFP-dosed) were pooled and subjected to 2D gel electrophoresis. Protein spots were excised from the Sypro Ruby-stained gels and subjected to trypsin digestion. The resulting proteins were identified by using an LC-quadrupole time-of-flight (QTOF) system (Dionex/LC Packings nano-LC and Applied Biosystems/Sciex QSTAR Pulsar XL mass spectrometer). Mascot search program (Matrix Science, London, UK) was used to search the MS/MS spectra against the Swiss-Prot protein sequence database. The highest up-regulated 7 proteins were confirmed by western blotting and RT-PCR.

Results

We investigated alterations in global protein expressions in bronchoalveolar lavage fluid in allergic airway inflammation using a proteomics approach. A total of 30 spots from 16 proteins were significantly altered. 7 of these proteins were further confirmed by western blotting and RT-PCR. These include surfactant protein-D, polymeric immunoglobulin receptor (PIGR), complement C3, neutrophil gelatinase-associated lipocalin (NGAL) and a family of chitinases, including chitinase-3-like protein 3 (Ym1), chitinase-3-like protein 4 (Ym2), and acidic mammalian chitinase (AMCase). Among them, Ym1/Ym2 and AMCase showed a significant surge in their expression (at least>18 fold on mRNA level) by UFP introduction. It was known that AMCase and Ym1/Ym2 is involved in allergic inflammation via a Th2-specific, IL-13 and IL-4/STAT 6 signal pathway respectively (2). It is still not clear how they modulate the inflammation processes, especially the link between the adjuvant effect and the hierarchical oxidative stress response. The proteins identified in this study may be useful for identifying the mechanism of the adjuvant effect of ambient PM on allergic sensitization related to air pollutants.

Innovative respects

- Global protein profiles measured in bronchoalveolar lavage fluid show for the first time differences from allergic airway inflammation induced by size-fractionated ambient PM challenge.
- Ym1, Ym2, AMCase, PIGR, complement C3, and NGAL demonstrated significantly enhanced up-regulation by UFP with a polycyclic aromatic hydrocarbon content and a higher oxidant potential.
- These proteins may be the important specific elements targeted by PM through the ability to generate reactive oxygen species in the immune system.

References

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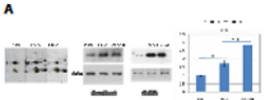
A



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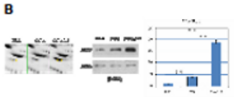

Figure 1. Differential expression of neutrophil gelatinase-associated lipocalin (NGAL) (Panel A), and acidic mammalian chitinase (AMCase) (Panel B). After 2D gel electrophoresis and LC-MS/MS analysis, western blotting and real-time PCR were performed for these proteins. These proteins showed significant increased expression in the presence of OVA plus UFP compared to the OVA alone.

E103

Proteome analysis of the different stages of the Biofilm formation in *Rhizobium etli*.

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Introduction.

The nitrogen-fixing α-proteobacterium *Rhizobium etli* is a model for symbiotic plant–microbe interactions. Most microbes as *R.etli* grow in the rhizosphere as organised biofilm communities on surfaces, sesile bacteria (biofilm), constitute a major component of the bacterial biomass in many environments. Bacterial cells attached to, and growing on, surfaces in mature biofilms are physiologically distinct from their planktonic counterparts. We are analysing with proteomics methodology the phenotypic changes that take place when planktonic cells of *R. etli* CE3 make the transition to the biofilm mode of growth.

Methods.

High-resolution two-dimensional gel electrophoresis (2DE) was used to demonstrate phenotypic differences between plancktonic cells grown in Peptone Yeast medium (PY) in agitation (6 hours) versus 24, 72, 120 and 240 hours-old biofilm cells grown in glass-pearls. Approximately 1000 distinct protein spots were resolved in the pH range from 3 to 10 after Blue Coomassie staining for each experimental condition. Comparison of the 2DE protein patterns obtained indicated specific protein spots expressed in the biofilm mode of growth. This finding supports several recent observations that a number of functions are required de novo in each biofilm stages of development. Three hundred protein spots were selected and subjected to identification by Mass Spectrometry (MALDI-TOF) to define their role of the biofilm metabolism in *R. etli*.

Results.

Our preliminary results indicate that the biofilm proteome differed from the planktonic (sessile) proteome. The sessile proteome, at early stages (24 and 72 h), differed from the more developed biofilm (10 days) proteome, in addition using confocal microscopy we observed than the biofilm at different stages of development shown structural differences, mainly between early stages (24 and 72 h) against late stages (120 and 240 h) we conclude than the observed difference was not due to a single factor; rather, it was due to a multitude of up- and down-regulated proteins, and posttranslational modification of proteins may also have been involved.

Part from this work is supported by CONACYT grant 60641 and DGAPA IN-222707 grant .

E104

Development of Biomarkers for Pesticide Induced Carcinogenicity in Rodents : A Proteomic Approach

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Introduction

Pesticides are known to pose serious health hazards to non-target species including humans. Various pesticides have been shown to possess genotoxic and carcinogenic properties in mammals. As long-term bioassay for carcinogenicity is expensive, time consuming and involves a large number of animals, biomarker development for short term exposure are needed. We are attempting to develop proteomic signature of known carcinogens and pesticides already proven as carcinogens with an aim to develop primary screening of other newly registered pesticides. Glyphosate is one such pesticide, and its tumorigenic potential is unknown.

Methods

Tumorigenic potential of glyphosate (25 mg/kg b.wt, topically,3 times/week) has been evaluated using 2-stage mouse skin initiation-promotion protocol. Further a proteomic approach, based upon 2-dimensional gel electrophoresis (2-DE) coupled to matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF/TOF) and liquid chromatography mass spectrometry (LC-MS) was used for the comparison of differentially expressed proteins which were modulated both by the topical exposure of glyphosate and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA).

Results

The *in vivo* bioassay results revealed that glyphosate has tumor promoting potential in mouse skin. However glyphosate exposure failed to provoke tumorigenesis when tested for tumor initiating and complete carcinogenic activity. Further proteomic analysis, showed differential expression (>2 fold) of total 23 spots among treated groups as compared with untreated group. Among them, proteins (translation elongation factor eEF-1 alpha chain, carbonic anhydrase III, annexin II, calcyclin, fab fragment anti-VEGF antibody, peroxiredoxin -2, superoxide dismutase [Cu-Zn], stefin A3, calgranulin –B) were common between glyphosate and TPA treated skin tissues. These proteins were mainly involved in apoptosis and growth-inhibition, anti-oxidation, energy metabolism, angiogenesis, calcium binding and protein biosynthesis. From the proteomics results it is concluded that glyphosate application on mice skin generates similar response in terms of differential protein expression as shown by TPA, a known promoter of carcinogenesis. Taken together, the combination of 2-stage mouse skin carcinogenesis model and proteomic analysis suggests that glyphosate has tumor promoting potential in skin carcinogenesis.

Innovative aspects

- Development of novel biomarker against glyphosate induced skin carcinogenesis.
- Toxicoproteomic approaches can lead to a better understanding of the mechanism underlying pesticide carcinogenesis at an operational level.

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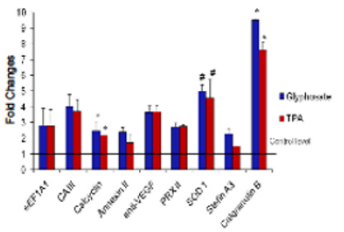

E105

Differential Expression of Proteins by LPA and ACI between Young and Senescent HDFs

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[1]. Korea Basic Science Institute, [2].School of Biological Sciences and Biotechnology Graduate School Chonnam National University

Introduction

Senescent human diploid fibroblasts (HDFs) were reported to be mostly insensitive to mitogenic stimuli. However, we found that proliferative potential of senescent HDFs was increased by lysophosphatidic acid (LPA) and adenylyl cyclase Inhibitor SQ22536 (ACI).

Methods

We have performed two-dimensional electrophoresis and protein identification by mass spectrometry of young and senescent HDFs after LPA and ACI treatment for 30min. We fully identified 22 proteins, which changed more than two-fold up regulation between young and senescent HDFs treated with LPA and ACI (Fig 1).

Results

In young HDFs PKM2, GAPDH, CFL1 and CALR were increased by LPA, and PKM2, GAPDH, PPIB, CFL1 and CALR were increased by ACI. In senescent HDFs SHROOM3, YA61P, PPIB and CFL1 were increased by LPA, and YA61P were increased by ACI. These increased proteins in senescent involved in regulation of transcription, cell growth, cell cycle, meiosis, signal transduction, DNA binding and cell polarity. Our findings suggest that the differential protein expression profile may provide a clue for the molecular mechanism of altered responsiveness of senescent cells to LPA and ACI. And based on these proteomic findings, more refined physiological, biochemical and cell biological studies can be designed to further our understanding of the mechanical mitogenic response in senescent HDFs.

Innovative aspects

- Selective increased proteins by LPA and ACI in senescent HDFs involved in cell proliferation
- These findings provide a clue for the molecular mechanism of altered responsiveness of senescent HDFs

References

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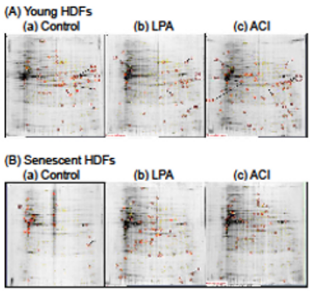

Figure 1. 2-DE patterns of young HDFs (A) and senescent HDFs (B) after LPA and ACI treatment. And indicate number of each protein spot changed expression compared to each other.

Table1. List of proteins identified on the 2-DE map of young and senescent HDFs after 2-DE followed by MALDI-TOF analysis.

E106

Assessment of Chemical Effects in Neural Stem Cell using Proteomics and Cyclic Voltammetry

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Introduction

A developmental exposure to environmental neurotoxicants produces alterations in the dopaminergic system and has been linked to neurodegenerative disorders, including Parkinson's disease. The present study was undertaken to elucidate polychlorinated biphenyls (PCBs)-responsive gene expression profiles in neural stem cells (F3 cell).

Methods

The cells were seed in the RGD (Arg–Gly–Asp) coating gold substrate and treated with different concentration of PCBs for 24h in culture, which induced the change of the cyclic voltammetry (CV) current peak. F3 cells were exposed to 100 nM PCBs for 0, 24, 48 and 72h in culture, after which gene expression profiles were analyzed by the proteomics analysis and bio-cell chip.

Results

Treatment PCB caused the loss of cell viability and accelerated apoptosis both in a concentration- and time- dependent manner. In proteomic analysis of human neural cells, 25 different proteins showed significant quantitative changes during the experiment, which were associated with PCBs time -dependent manner. The results involved the characteristics of proteins, which can be categorized into skeleton, signal transduction, cytokine, apoptosis, and reactive oxygen species metabolism. The apoptosis induced PCB increased the expression level of caspase-3 and bax. In conclusion, results suggest that the identified proteins provide crucial information to understanding possible mechanisms of toxicity of PCBs-induced endocrine disruption.

Innovative aspects

- Electrochemical signal detection in neural stem cell chip using cyclic voltammetry
- Monitoring of neurotixidant effect in neural stem cell chip
- Proteomics biomarker for polychlorinated biphenyls exposure in stem cell

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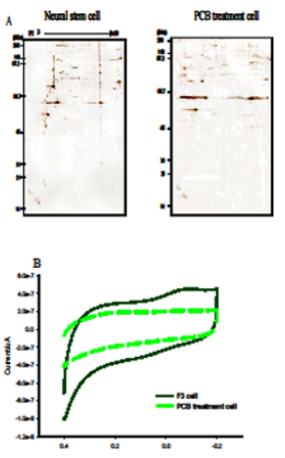

Figure 1. 2D-Gel Electrophoresis of human stem cell proteins (A) and cyclic votammograms of stem cell at 100m V/s (B). (A) The proteins (1mg) from the whole stem cells were extracted and separated on pI 3 to 10 nonlinear immobilized pI-gradient strips, followed by 9% to 18% polyacrylamide gel. The gel was stained with Coomassie's brilliant blue G250.

E107

Identification of PerR-Regulated Secreted Virulence Factors of Environmental Pathogen *Streptococcus Pyogenes* Using Comparative Proteomics Analysis

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Introduction

Streptococcus pyogenes is an important Gram-positive human pathogenic bacterium in the environment that causes various diseases. Previous studies were reported that *S. pyogenes* can secrete many proteins to influence host-pathogen interactions. One of *S. pyogenes* strains demonstrated that *perR* gene deficient was highly attenuated the virulence in animal model. PerR protein was recently reported to be involved in the resistance response to peroxides but little is known about the regulation of secretome involved in virulence (1,2). Therefore, the objective of this study is to investigate how the *perR* regulates the expression of *S. pyogenes* secretome involved in virulence.

Methods

A *perR* deficient mutant (*perR* -) was constructed to be used for comparative proteomics analysis with wild type *S. pyogenes*. The *perR* gene product expression level was measured by RT-PCR and Western blotting. The virulence in wild type strain and *perR* deficient mutant was determined by murine air sac model. The supernatants containing secreted proteins of a wild-type *S. pyogenes* and a *perR* deficient mutant (*perR* -) at stationary phase were collected for differential proteomics analysis. Because proteases derived from *S. pyogenes* may degrade secreted proteins in the supernatants, resulting difficulty in interpreting two-dimensional gel electrophoresis (2DGE) data, we added protease inhibitor cocktail into culture medium. Differential protein spots were identified by liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS).

Results

The expression level of *perR* gene products in the deficient mutant was verified and found to be less than 5% of that in the wild type pathogen. The *perR* deficient mutant was attenuated in virulence by 67% within two days using an animal model.(Fig. 1) More than 330 protein spots were detected on 2D gels. We identified 23 unique secreted proteins that showed decreased abundance, relative to the wild type, in *perR* deficient mutant. Another 15 secreted proteins were increased in abundance in the deficient mutant. (Fig. 2) Among these identified secreted proteins, mitogen factor 3 (MF3) is found to be a novel virulence factor regulated by *perR* gene. The analysis of PerR-regulated secretome provided information for the elucidation of the disease mechanisms and the development of chemotherapeutic strategies to prevent or treat severe streptococcal diseases.

Innovative aspects

- This is the first report on protein secretion regulated by *perR* gene.
- Mitogen factor 3 (MF3) is found to be a novel secreted virulence factor regulated by *perR* gene and could be a potential therapeutic target.

References

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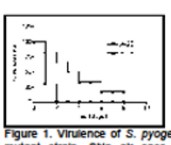

Figure 1. Virulence of *S. pyogenes* wild-type and *perR* mutant strain. Skin air sacs of BALB/c mice were injected with 10^8 CFU of strain A20 (wild type) or *perR* deficient mutant (perR'). The results are expressed as percentages of survival rate over time.

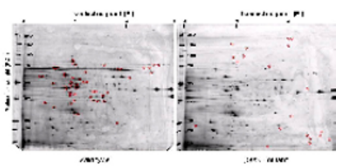

Figure 2. 2DGE silver-stained gel of secretome is isolated from wild type strain (left) and *perR* deficient mutant (right) of *S. pyogenes*. The protein spots with differential abundance (indicated by red circles) were cut out and subjected to LC-MS/MS analysis.

E108

Mass Spectrometric Analysis of Protein Fatty Acyl Modifications in Membrane Signal Transduction

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Introduction

Protein posttranslational modifications play important role in the complex biological processes. Covalent fatty acyl modifications anchor signaling proteins to the cytoplasmic face of plasma membrane in response to environmental stimuli and affect protein's subcellular localization and interactions with other proteins. While other modifications such as phosphorylation is widely studied [1], fatty acyl modifications are currently under-represented due to the lack of suitable methods. Here we describe an IFAT strategy (Isotope-Coded Fatty Acid Transmethylation) for qualitative identification and quantification of fatty acyl modifications [2].

Methods

S. cerevisiae cells were grown in either YPD media or DDT containing (24ng/μl) YPD media. Logarithmically growing cells were harvested and lysed by a buffer containing 1% SDS. Cell lysates or SDS-resistant pellets were dried under nitrogen gas and then directly mixed with 100 μl regular methanol or d3-methanol solution of 0.5M NaOH. Then add n-hexane solution on the top and the final volume of hexane is 200 μl. Each vial was treated by 1 minute of ultrasonication (100W, 40KHz). Then let the vials stand for 5 minutes before the supernatant hexane parts were transferred. Combine equal amounts of hexane layers of d0-methanol derived fatty acid methyl esters with those derived by d3-methanol. The combined n-hexane layers were then washed with pure water and anhydrous Na_2SO_4 was used to absorb trace water remaining in the hexane solution. Finally, n-hexane layers were analyzed by GC-MS (QP2010PLUS, Shimadzu, Japan).

Results

As shown in Figure 1, both soluble cell lysates and SDS-resistant pellets were analyzed. Light and heavy methanol derived fatty acid methyl esters were separated by a 30 m long capillary column before they were analyzed by the mass spectrometer. Due to the diverse structures of fatty acids, the identification was achieved by both database searching and comparison of the chromatographic retention time with that of standard fatty acid methyl esters. It was apparent that cells of *S. cerevisiae* contained an increased level of cellular C12:0, C14:0, C16:0, C18:0 and C18:1 but no significant changes in C16:1 when grown in DDT containing media. Observed mean peak ratios (control: DDT treated) were 0.65, 0.79, 0.89, 0.88, 0.90 and 0.98 respectively. Standard deviations ranged from 0.01 to 0.07. Similarly, in-pellet delipidation can be accomplished by directly mixing pellets with 0.5M NaOH solution of methanol and n-hexane. After 1 minute of sonication, the top n-hexane layer was used for analysis of fatty acid methyl esters. It was found that there was no significant changes of C14:0, C16:1, C16:0, C18:1 and C18:0 in the SDS-resistant pellets when cells were treated with DDT. Observed mean peak ratios (control: DDT treated) were 1.12, 1.08, 1.14, 1.01 and 1.09 respectively. Standard deviations ranged from 0.12 to 0.25.

Innovative aspects

- One-step transmethylation and isotope labeling
- Novel strategy for quantification of fatty acyl modifications
- Analysis of SDS-resistant pellets

References

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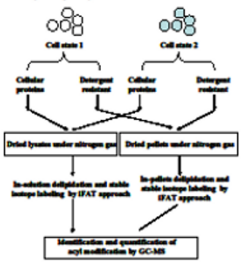

Figure 1. The IFAT strategy for quantitative analysis of fatty acid composition in different samples. Two samples have been derived with isotopically light and heavy methanol respectively and the resultant fatty acid methyl esters were simultaneously transferred into n-hexane layer with the aid of ultrasonication. The ratio of the original amounts of samples have been recovered in

E109

Environmental proteomics, tool to search for biological effects of emerging pollutants

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Complex mixtures of pollutants are often found in our environment. The biological impact on human health remains largely unknown. Traditional attempts to predict effects on organisms and to assess the quality of specific ecosystems based on chemical analysis have proven inefficient. Difficulties arise not only from the wide variety of chemicals but also because the biological effects can be caused by additivity or synergism. However, the application of proteomic could advance knowledge in the safety, environmental and human health implications of exposure to emerging pollutants.

Therefore, it was necessary to develop assessment tools that can handle the complex biological effects that environmental stress cause. Our research group has been working in the development of proteomics based methods for marine pollution or chemical assessment that could provide protein expression signatures or peptide profiles[1-3]. The analysis of an organism's proteome allows the detection of changes in the level of individual proteins in response to environmental stressors and provides a more robust approach for environmental assessment. Quantitative proteomics could lead to the discovery of biomarkers of exposure, to define the biological effects and to provide new insights into the mechanisms of toxicity. Examples from laboratory and field experiments applying peroxisomal proteomics, liquid chromatography couple with 2-DE to obtain protein expression signatures will be discussed. Finally, a new strategy using peptide profiling and MALDI-TOP MS for the assessment of human pharmaceuticals will be presented.

Innovative aspects:

1. Increasing the robustness against biotics and abiotic factors
2. Scalable to automation
3. Requirement of an affordable instrumentation.

References:

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E110

Phosphoproteome analysis of pathogenic and non-pathogenic *Pseudomonas* species

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Introduction

With the recent whole-genome sequencing projects reporting the presence of Ser/Thr kinases and two-component proteins both in prokaryotes and eukaryotes, the importance of protein phosphorylation in bacteria is gaining acceptance. While conventional biochemical methods failed to obtain a snapshot of bacterial phosphoproteomes, advances in mass spectrometry methods have led to in-depth mapping of phosphorylation sites. Here, we present phosphoproteomes of two ecologically diverse non-enteric Gram-negative bacteria captured by a nanoLC-MS based approach combined with a novel phosphoenrichment method.

Methods

Two batches each of *P. aeruginosa* PAO1 wildtype, *P. putida* PNL-MK25 wildtype (WT), *morA* mutant[1] and *morA* overexpression[1] strains were grown in Luria-Bertani (LB) medium until the late-log phase (OD 1.7). After tryptic digestion, phosphopeptide enrichment was done using TiO_2- and ZrO_2-packed minicolumns according to hydroxy acid-modified metal oxide chromatography (HAMMOC)[2,3]. A Thermo LTQ-Orbitrap coupled with a Dionex Ultimate3000 pump and a CTC HTC-PAL autosampler was used for nanoLC-MS/MS analyses. Proteins were identified by automated database searching using Mascot v2.2 against NC_007492 (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Pseudomonas_fluorescens_Pf0-1/) for *P. putida* samples and Pseudomonas_aeruginosa_PAO1_2007-06-19 (http://www.pseudomonas.com/downloads/sequences) for *P. aeruginosa*.

Results

In order to collect a large-scale data set of *Pseudomonas* Ser/Thr/Tyr phosphorylation sites, we employed two phosphopeptide enrichment methods i.e., lactic acid-modified titania HAMMOC and 3-hydroxypropanoic acid-modified zirconia HAMMOC. This is a single-step enrichment method without fractionation, hence eliminating the risk of sample loss confirmed by the similar number of phosphopeptides captured compared to published bacterial phosphoproteomes($P < 0.01$). *P. putida* had 53 phosphorylation sites (Ser-52.8%,Thr-39.6%,Tyr-7.5%) while *P. aeruginosa* had 55 sites (Ser-52.7%,Thr-32.7%,Tyr-14.5%). 10-30% of identified phosphopeptides matched uncharacterized proteins while the rest were from proteins with either predicted or experimentally proven function(s). Since c-di-GMP signalling is well established in Gram-negative bacteria, we were interested in understanding its mechanism of action in *Pseudomonas*. Phosphoproteome analysis of *P. putida* that either lacked or overexpressed MorA, the GGDEF/EAL domain-containing protein resulted in changes in phosphorylation patterns of 41 phosphopeptides from 24 phosphoproteins while only 14 show absolute dependency on either presence or absence of MorA.

Innovative aspects

- First study on *Pseudomonas* phosphoproteome
- First on bacterial c-di-GMP signalling by using HAMMOC and LC-MS approaches
- Protein phosphorylation seems a likely mechanism for large-scale posttranscriptional affects of c-di-GMP signalling and may involve novel Ser/Thr/Tyr protein kinases in *P. putida*.

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Glycoproteomics

(G101 – G124)

The Plasma Glycome: An Approach to the Early Detection of Cancer

G101

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The early detection of cancer Mechanisms underlying disease pathogenesis are not well understood in the context of common etiological factors such as microbial infection, inflammation, malignancy or tissue breakdown. Such processes may be elucidated by identifying disease-related molecular markers, such as acute phase proteins, cytokines, cytoskeletal fragments and autoantigens.

In an attempt to identify such markers, we have developed a novel platform, namely multiple lectin affinity chromatography (M-LAC) to study the plasma glycoproteome in patients and healthy donors in a series of studies ranging from cancer to autoimmune disease. The M-LAC platform allowed the evaluation of changes in concentration of glycoproteins, and to comprehensively survey the plasma proteome. We then used a second method, intact peptidomics, to assess changes in endogenous proteolytic activity by analyzing the low molecular weight (LMW) component of blood. The integrated proteomic and peptidomic analysis of plasma samples identified a number of cytoskeletal and Ca^{2+}-binding proteins and their proteolytic fragments in the disease samples.

The measurements were compared to healthy donors and several of the observed differential quantitations were independently verified by ELISA. The identified changes in plasma proteome and peptidome, and the underlying changes in glycosylation together with altered endogenous protease activity may result in the generation of novel autoantigens. We and others have confirmed this hypothesis by the observation of autoantibodies in patients and upon extension of these studies to larger populations of patient; we may gain additional understanding of the role of etiological factors in different disease pathways.

Fucosylated glycoproteins as biomarker candidates for Heptocellular Carcinoma

G102

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Introduction Hepatocellular carcinoma is the most common primary malignant tumor of the liver. However, accurate diagnosis can be difficult as most of the patients who develop this tumor have no symptoms varied from those related to their longstanding liver disease. Here we applied lectin-array methods to investigate the serum glycoprotein profiling difference between the cirrhosis and hepatocellular carcinoma (1). Then the differentially expressed fucosylated proteins were identified by using Exactag labeling, AAL enrichment and LTQ-MS/MS.

Methods The twelve of the high-abundance proteins were removed by IgY-12 column. Then all the thiol groups of proteins in the depleted serum were labeled with Iodoacetyl -PEG2-Biotin. Each serum was diluted 100 times with 1% BSA in PBST for the lectin-array. One hundred μg proteins from each serum were labeled with one of the ExacTag (PerkinElmer) reagents which generate different isobaric tags, followed by mixing the ten labeled samples (5 of HCC group and 5 of cirrhosis group). Then the fucosylated proteins were enriched by Agaorse-bound AAL. The variation of fucosylated protein expression was calculated based on the identified peptides and relative peak intensity of featured isobaric tags using LC-MS/MS.

Results Sixteen lectins were used, in which AAL and LCA showed significantly higher signals in the HCC group than the Cirrhosis group, indicating the expression of fucosylated proteins was increased in HCC. Ninty-nine fucosylated proteins were identified by LC-MS/MS. The fucosylation of 14 proteins were up-regulated in the HCC group including hepatocyte growth factor activator, vitamin D-binding protein, angiotensinogen, ceruloplasmin, complement C3, histidine-rich glycoprotein and cholinesterase precursor. Together, these proteins discovered by lectin-array and comparative proteomics can provide useful clues to develop fucosylated glycoproteins as biomarkers of hepatocellular carcinoma.

Innovative aspects
First application of lectin-array to depleted serum sample.
Identification of novel fucosylated biomarkers for hepatocellular carcinoma using isobaric tags labeling and lectin enrichment.

References
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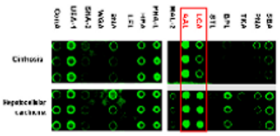

Figure 1 Lectin-array of serum from cirrhosis and hepatocellular carcinoma

Quantitative analysis of N-glycoproteins in search of the disease biomarker in HCC

G103

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Introduction
The change of N-llinked glycan structures is significantly related to the tumorigenesis and progression of hepatocellular carcinoma, one of the most common fatal cancers worldwide. In this study, we described a simple and an efficient strategy for selective identification and quantitation of N-linked glycoproteins without extensive enrichment steps. With our approach, the change of N-linked glycoproteins between healthy and HCC patient plasma were selectively quantified after iTRAQ labeling.

Methods
Human plasma proteins were deglycosylated with treatment of PNGase-F. N-linked glycopeptides of human plasma were selectively identified using pre-peak exclusion (PPE) method (Scheme 1). The change of N-linked glycoproteins between healthy and HCC patient plasma were selectively quantified after iTRAQ labeling using PPE method. The characterization of glycan structure of candidate glycol-biomarker for HCC was performed with combination of Q-STAR and LTQ. All peaks were manually assigned.

Results
Here, we identified a total of 74 differentially changed N-linked glycopeptides in HCC. Additionally, the glycan structure of intact glycopeptides for vitronectin (Asn-169, 242)(Figure 1) and antithrombin III (Asn-225) was changed differentially in HCC plasma was also characterized. Moreover, we found that the intact glycopeptide was also present with increased level in HCC tumor tissue. Take together, it is suggested that our approach with high throughput potential will be useful for the global profiling and quantification of the N-linked glycopeptides and the identification of disease biomarkers.

Innovative aspects
- Selective detection of N-linked glycopeptides without extensive enrichment steps
- Selective quantitation of N-linked glycopeptides between normal and HCC patient plasma using iTRAQ
- Characterization of glycan structure for candidate glycosylated biomarker of HCC

References
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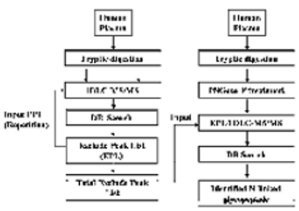

Scheme 1. The overall strategy for selective detection of N-glycopeptides from human plasma

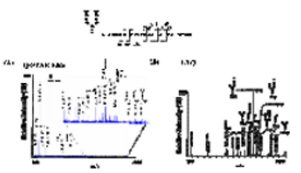

Figure 1. Manually assigned MS/MS spectra of intact N-linked glycopeptide.
N'ISDGFDGIPDNVDAALALPAHSYSGR sequence was acquired by Q-STAR (A) and LTQ (B). ■: mannose (Hex) ●: N-acetylglucosamine (HexNAc) ◆ : a-N-Acetylneuraminic acid (NeuAc)

(This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea [A030003 to YKP])

Characterization of Glycopeptides in Complex Systems Matrices Using LC-MS^E G104

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Introduction
The post-translational glycosylation of proteins can have a profound effect on their biological function and structure. Hence the ability to identify and characterize glycosylated peptides in systems samples (protein digests) with minimal manipulation of the starting material is advantageous in systems biology. Here we describe a strategy to target glycopeptides in a discovery experiment by observing characteristic fragment ions in the elevated energy channel time-aligned to eluting glycopeptides. Ions are measured at high mass accuracy from all precursor charge states to assure specificity.

Methods
Proteins and sera were reduced, alkylated (Iodoacetamide) and digested with trypsin following standard procedures. Data were acquired on a nano UPLC (nanoACQUITY, Waters Corporation) system using gradient elution of peptides on a C_{18} column coupled to the nano electrospray source of a hybrid quadrupole/time of flight mass spectrometer (Synapt HDMS, Waters Corporation) operated in alternate scan (high/low collision energy) mode (MS^E). Data were processed in ProteinLynx Global Server version 2.4 with glycopeptide detection enabled. Glycopeptide identification is based on the observation of target oxonium ions in the elevated energy channel, the mass difference between the observed precursor and the candidate peptide corresponding to a reasonable combination of carbohydrates, the observation of the glycopeptide Y1 ion (peptide + HexNAc) in elevated energy, and, for N-glycans, the presence of the NxS/T sequence motif.

Results
Prior efforts to identify glycopeptides in complex mixtures have relied on data dependent schemes (1). The present work uses the same data independent scheme used to identify ordinary tryptic peptides in digest mixtures (2) to identify glycopeptides as well.
A digest of bovine fetuin was used for initial proof of concept, where numerous N- and O-linked glycopeptides were identified. Extending the investigation to digests of human plasma, again numerous glycopeptides previously reported in the literature were identified, including the example from alpha-2-macroglobulin (3) illustrated in Figure 1. The elevated energy spectrum is characterized by oxonium ions at low m/z (eg. 204.0871, 292.1005, 366.1391, 657.2341), the Y1 ion at m/z 2366.2773, and many ions at higher m/z reflecting fragmentation of the glycan.
Results of the application of this scheme to investigate the role of glycosylation in differentiating lysosomal storage patient plasmas and controls will also be presented.
In conclusion, our method allows simultaneous identifications of proteins in a systems sample and identification of glycosylated peptides in those proteins.

Innovative aspects
- Data independent identification of glycopeptides in complex mixtures
- Accurate mass data for precursors and fragments obtained from all precursor charge states simulatneously
- Glycosylation information obtained simultaneously with protein identification

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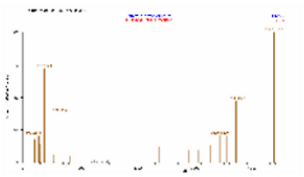

Figure 1. Elevated energy (MS^E) spectrum of glycopeptide VSNQTLSLFFTVLQDVPVR from human alpha-2-macroglobulin detected in a tryptic digest of plasma. This displays the signal oxonium ions at low m/z, Y1 at 2366.2773, and fragmentation of the glycan at higher m/z.

Analysis of Sulfated Glycans by MALDI-Orbitrap and DHB/N,N-Dimethylaniline Matrix G105

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Introduction
Sulfation is a key structural determinant regulating numerous biological functions, such as lymphocyte homing through L-selectin, thus, structural assignment is key to understanding these mechanisms. Here we demonstrate the use of 2,5-dihydroxybenzoic acid/N,N-dimethylaniline (DHB/DMA) matrix[1] for the analysis of sulfated glycans by MALDI-Obitrap. This matrix offers significant improvements over the conventional DHB matrix. Furthermore, the use of MALDI-Orbitrap provides accurate and reliable MS and MS/MS information which combined with high mass resolution brings definiteness to analysis.

Methods
High-molecular weight mucins were isolated by size-exclusion and O-linked glycans released by β-elimination. Sulfated oligosaccharides were eluted from porous graphitized carbon solid phase extraction cartridges using 10% MeCN. Samples were deposited onto a MALDI target by mixing the analyte and matrix solutions (DHB, DHB in 70% etOH, and DHB/DMA respectively) (0.5 µL each) on-target and allowing the mixture to dry at room temperature. All MALDI experiments were performed on a Thermo Fisher Scientific MALDI LTQ Orbitrap XL mass spectrometer. MS analysis was conducted in the Orbitrap mass analyzer region at a resolution of 60000 and 100000 as specified and MS/MS analysis was conducted in the LTQ ion trap for ions of sufficient abundance.

Results
Analysis of sulfated glycans by mass spectrometry has been a challenge for many years due in part to the limited capacity of these compounds to ionize. A further challenge in mass spectrometry strategies is the ability to distinguish between sulfation and phosphorylation, since these groups are isobaric. A factor more complicated by the identical data generated by MS/MS data of these species.
O-linked glycans from mucin were separated into three equimolar proportions (calculated volumetrically), and spotted onto the MADI target using three different matrices, DHB, DHB (in 70% ethanol) and DHB/DMA. Mass spectra were acquired in the negative mode to evaluate ionization efficiency of all three matrices. In contrast to these two matrices, substantial improvements in sensitivity were observed using DHB/DMA as well as the number of sulfated glycans detected far exceeded those using the conventional DHB matrix. Molecular weights of the glycans were measured within 0.5 ppm of calculated monoisotopic values allowing for differentiation between sulfation and phosphorylation without MS^n analysis. However, ions were of sufficient abundance to allow MS/MS for further structural confirmation.

Innovative aspects
- The use of DHB/N,N-Dimethylaniline matrix for enhanced sensitivity in the analysis of sulfated glycans by MALDI-Orbitrap mass spectrometer
- Ability to distinguish Sulfated glycans from phosphorylated glycans during MS analysis

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Improved Analytical Strategy for Glycoproteome by Combined Enzymatic Deglycosylation G106

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Introduction
Traditional methods to identify N-linked glycosites are removal of oligosaccharides by PNGase digest, which converts asparagines to aspartic acid. But the conversion between the two residues is common in nature or during experiments. Besides, accurate determination of 0.98 Da by MS/MS is not easy. Recently, a partial deglycosylation by Endo digest with a remaining GlcNAc is introduced (1), though only a few glycosites had been found by Endo. Here, we modified method by using lectin to capture more glycopeptides before addition of Endo, and proved the advantage of more reliable lectin + Endo.

Methods
Concanavalin A (Con A) affinity column was used to enrich glycopeptides from tryptic peptides of rat liver tissue. The captured glycopeptides were respectively treated by peptide: N-glycosidase F (PNGase F) and endoglycosidase H (Endo H). Con A mainly captures glycopeptides of high mannose type, which is also the main substrate type of Endo H (2). Then the deglycosylated peptides were separated by nanoflow RP-HPLC and analyzed by online ESI-LTQ-Orbitrap tandem mass spectrometry. Endo cleaves N-glycans with the last GlcNAc or fucosylated GlcNAc still attaching to the peptide backbones. So modification (0.98 or 203.19, 349.33 Da) in motif (NXS/T, X≠P) was used to filter the spectra searched by SEQUEST.

Results
846 and 492 Con A binding glycosylation sites were identified by PNGase F and Endo H digest, corresponding to 471 and 317 glycoproteins, respectively (figure 1). Endo H got less identifications probably because the remaining GlcNAc reduced the fragmentation of peptide backbones. As a result, a total of 911 different glycosites within 498 unique glycoproteins were successfully determined just in 1D LC by the two complementary methods. The data is larger than previous studies. Besides, the results can be further improved by J replacing of modificated N in motif to search (data not show).
Moreover, by PNGase F digest with 0.98 Da mass shift, more than 6% of all peptides were identified with modification contain no motif. While by Endo H digest with higher mass increase, the percentage was only about 2% (figure 2). It is indicated that, although more results can be obtained by PNGase F, the results of Endo H are more reliable. Anyway, we improved the strategy for N-glycosylation sites analysis using lectin capture with complementary enzymatic deglycosylation, and proved the advantage of Endo by experiments.

Innovative aspects
- Maximum the results of N-glycosites identification by lectin capture and combining PNGase F and Endo H deglycosylation
- Proved the advantage of Endo H: more reliable though less identifications

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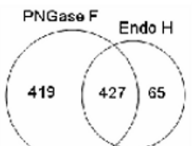

Figure 1. Venn diagram showing the overlay of glycosylation sites detected by PNGase F and Endo H.

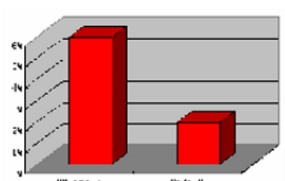

Figure 2. Percentage of peptides detected with modification but no motif with PNGase F or Endo H.

Glycopeptides Analysis Using Cellulose-based Separation Cartridges and LTQ Orbitrap ETD. G107

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Introduction
Glycosylation of proteins currently constitute an active area of research and ETD MS/MS fragmentation[1] is method of choice to characterize this type of PTM. In this work, we describe a novel workflow, which combines a new method for efficient extraction of glycopeptides from a complex digest mixture with nearly negligible loss of glycopeptide material or carryover of non-glycosylated peptides into the isolated glycopeptide fraction and subsequent ETD analysis

Methods
Digest of reduced and alkylated 10 or 12 standard protein mixture containing 3 glycoproteins (human serotransferrin, chicken ovalabumin and bovine alpha-lactoalbumin) or bovine fetuin from Sigma were used in this study. The mixture was divided into 6 aliquots and labeled according to manufacturer provided protocol with 126, 127, 128, 129,130 and 131 TMT tags[2] respectively. Upon labeling, samples were mixed one to one and dried.
The labeled samples were then used in method development for separating glycopeptides from peptides. We used cellulose powder, packed into a small plastic solid phase extraction reservoir tube as our glycopeptides extraction medium. Purified glycopeptides were analyzed by LTQ Orbitrap XL ETD.

Results
Aqueous protein digest mixtures were loaded onto a custom-made cellulose column, equilibrated with solutions containing a high proportion of organic solvents (ACN and isopropanol). Bound peptides were removed from the column by a step-gradient elution with an incremental increase in water content. The water concentration at each step, as well as the volumes applied at each concentration were optimized according to the volume of the cellulose material used. Bound glycopeptides left on the column during peptide extraction were then eluted with a mobile phase containing a significantly higher proportion of water. LC-MS/MS analysis found minimal carryover of the non-glycosylated peptides into the isolated glycopeptides fraction. Importantly, majority of the glycopeptide material was recovered from our sample mixtures, as indicated by subsequently seeking for glycans released with PNGase F endoglycosidase in both peptide and glycopeptide fractions. The described glycopeptide enrichment strategy significantly improved rate of success of ETD analysis and simplified the overall glycoproteomics workflow. Furthermore, it provided comprehensive site-specific glycan heterogeneity analysis.

Innovative aspects
- Selective enrichment of TMT labelled and intact N- and O-glycopeptides by cellulose based chromatography
- Novel workflow for comprehensive site-specific glycan/glycopeptides analysis

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Glyco Chain Explorer: a comprehensive platform to interpret glycopeptides

G108

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Introduction
Elucidation of glycopeptides fragmentation from tandam mass spectra remains the bottleneck of glycoproteomics study. We've developed Glyco Chain Explorer (GCE), a software to interpret glycopeptides spectra quickly and accurately. Relational network constructed from peaks of glycopeptide fragments, and unique features of glycopeptide fragmentation in different MS instruments are integrated into the program(Figure 1).

Methods
Horseradish peroxidise (HRP) was analysed on LTQ-Orbitarp and QIT mass spectrometer. Spectra with relatively good quality were interpreted by GCE, a program developed in MATLAB. GCE build relational network of the spectra by exhaustly comparing the peaks. After that, sub network and relational trees are extracted for further analysis. High-quality relational trees will be searched in the database for possible compositions. In order to improve the applicability and flexibility of the program, various kinds of features, such as "feature mass" and "precursor recalibration" are also included.

Results
In LTQ data, all 8 glycosylation sites of HRP are successfully revealed by GCE in very short time. Several sites are also indentified in QIT data. For a certain glycopeptide, GCE built different relational network for spectra from different mass spectrometry, and both revealed the correct answer. These results shows that GCE serves as a solid platform in glycoprotein research.

Innovative aspects
- Construction of relational network to select correct peaks and reduce false positive results.
- Focus on constant breakage of glycans on glycopeptides.
- Suitable for different MS instruments.(e.g MALDI & ESI source)

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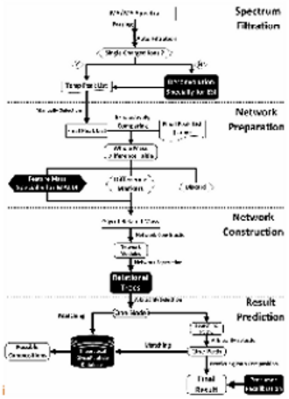

Figure 1. Overall workflow of GCE.

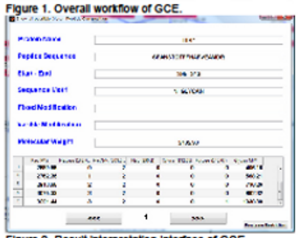
Figure 2. Result interpretation interface of GCE.

Membrane glycoproteomics of liver inflammation in the presence of a distal benign tumour

G109

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Introduction
It has been shown that the presence of a distal non-metasizing tumour (EHS sarcoma) can ultimately induce liver inflammation and cause reduced metabolism and clearance of anticancer drugs in mice[1]. Label-free quantitative proteomics using peptide IPG-IEF was applied to determine whether liver inflammation affected the abundance of membrane proteins. Triton X-114 phase partitioning was used to enrich membrane proteins for global glycan profiling to determine if glycosylation of the liver membrane proteins was affected by the distal tumour.

Methods
EHS sarcoma cells were injected into the hindlimb of transgenic FvB male mice. Membrane proteins from homogenized liver lysates were isolated by sedimentation and the crude membrane pellet was either digested with trypsin in 60% methanol[2] or, subjected to Triton X-114 phase partitioning[3] for further membrane enrichment. Tryptic peptides were separated by IPG-IEF into 24 fractions and peptides were analysed using a linear ion-trap MS[2]. MS/MS spectra were searched against the *M musculus* database for protein identification. Triton X-114 partitioned membrane proteins were dot blotted onto PVDF and subjected to PNGase F and β-elimination to release *N* and *O*-glycans respectively. Glycans were separated by graphitized carbon LC and analysed using an ion-trap MS in negative ion mode.

Results
Peptide IPG-IEF and label-free quantitation identified 236 differentially expressed integral and membrane-associated proteins. Most of these proteins are involved in biological pathways such as fatty acid metabolism, protein detoxification and protein glycosylation. Enzymes responsible for protein glycosylation were found to be upregulated in the livers of tumour-bearing mice, particularly sialyltransferases.
Global glycan profiling of Triton X-114 phase partitioned proteins identified glycans that were expressed uniquely to hepatic membrane preparations from tumour-bearing mice. In addition, glycan isomers expressed in different ratios were identified in diseased samples but not in controls. Interestingly, the glycans which were identified in only the liver membranes of tumour-bearing mice showed increased *N*-acetyl and *N*-glycolyl sialylation. Cancer is well-known to be associated with alterations in membrane protein glycosylation and the identification of these glycan differences, together with the associated glycosyltransferases which are differentially expressed, gives a greater insight into the cell-cell interactions that are changed in organs besides the tumour itself.

Innovative aspects
- Peptide IPG-IEF identified differentially expressed membrane proteins
- Glycosyltransferases were found to be upregulated in livers distal to the tumour
- Glycan profiling showed increased sialylation on liver membranes from tumour-bearing mice, which reflect the increased expression of the sialyltransferases.

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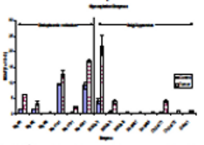
Figure 1 Relative abundance of enzymes involved in the glycosylation machinery. P<0.05

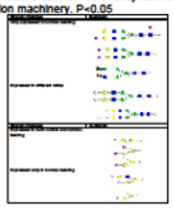
Figure 2 Glycan differences between control and tumour-bearing liver membranes

Automated N-Glycan Composition Analysis with LC-MS/MS

G110

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Introduction
Compared to proteomic profiling and differentiation the glycan analysis still employs a lot of manual work and can be a burden with increasing number of spectra. The aim of this study is to ease the task by using in house developed glycomic software methods in combination with existing proteomic tools (Fig 1). The resulting workflow is targeted especially to glycan LC-MS/MSMS analytics and can be run with a minimal amount of human intervention. The final goal is to apply the developed methods to profile and differentiate stem cell surface glycans.

Methods
The sample consists of permetylated glycans, using a method described earlier (Ref. 2).
The sample is analysed with an LC-MS/MS run of the LTQ Orbitrap XL mass spectrometer. The values of potential glycans (features) are extracted with Progenesis LC-MS expression analysis software (Nonlinear Dynamics Ltd) and the MS^2 spectra is extracted with Mascot Distiller (Matrix Science Ltd).
The next steps at the glycan analysis workflow are built on the in house developed R function library. The workflow includes spectrum filtering, search of potential glycan compositions based on feature masses, matching composition fragments with MS^2 spectra, combining MS and MS^2 results and deconvolution. The MS^2 matching includes a novel probability based scoring scheme (developed from the scheme at Ref 1.).

Results
The methods were tested with a mixture of commercially available N-glycan structures. The workflow was shown to be able to decrease the analysis time considerably.
The focus of the method is to identify glycan compositions. If detailed structure or linkage analysis is needed, the workflow can be used as a pre process prior the other analysis software, for example the GlycoWorkbench (Ref 3).

Innovative aspects
- LC-MS/MS glycan analysis
- Novel probabilistic MS^2 scoring scheme

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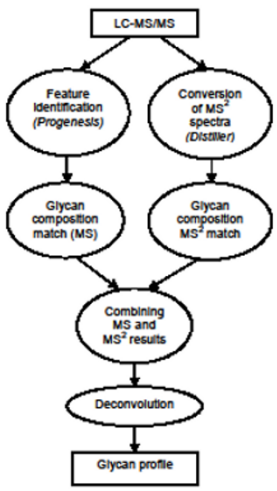

Figure 1. Glycan profiling workflow.

Glycoprotein profiling using Tandem Mass Tags and Staudinger ligation of azido sugars

G111

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Introduction
Protein glycosylation is the most abundant and diverse family of post-translational modifications found in mammalian cells. Identification of the proteins of the glycoproteome is essential to understanding basic cellular biology as well as elucidating variations associated with disease states. Here we used a metabolic labeling approach to incorporate azido sugar derivatives into cells for subsequent detection and capture with phosphine probes utilizing the Staudinger ligation (1). Glycoproteins were identified and profiled by mass spectroscopy facilitated by isobaric Tandem Mass Tag Reagents (2).

Methods
Cells were incubated with tetra-acylated azide-modified sugar derivatives of glucose (GlcNAz), galactose (GalNAz) or mannose (ManNAz) for 72 hours before harvesting and lysis. Cell lysates were labeled with phosphine-derivatized fluorescent dyes for verification of azido sugar incorporation. For mass spectrometry analysis, azido-labeled glycoproteins were reacted with a phosphine-derivatized biotin and isolated using streptavidin agarose. Captured proteins from control and azido-labeled cell lysates were deglycosylated, reduced, alkylated and enzymatically digested before labeling with Thermo Scientific Isobaric Tandem Mass Tags (TMT®) Reagent (Figure 1). Peptides were analyzed using the Thermo Scientific LTQ Orbitrap mass spectrometer and identified using the Thermo Scientific Proteome Discoverer software and the IPI database.

Results
We used a bioorthogonal approach to metabolically label and enrich specific glycoprotein subfamilies for MS identification. Our results show highly sensitive and selective reactivity of azido sugars with phosphine derviatized fluoresoent dyes in cell lysates compared to mock-treated samples. Phosphine-based Staudinger ligation chemistry was also used to biotinylate azido-labeled glycoproteins for isolation and enrichment using streptavidin agarose. A sixplex of TMT isobaric tags were employed to label mock-treated and azido sugar-labeled peptides (GlcNAz, GalNAz and ManNAz). The addition of isobaric tags not only allowed for increased sample throughput, but also improved identification of novel glycoproteins. Overall, more than 200 glycoproteins encompassing sialic acid-containing cell surface proteins and O-linked GalNAc and GlcNAc intracellular proteins were identified using this approach.

Innovative aspects
- Glycoprotein profiling using isobaric mass tags
- Bioorthogonal labeling and capture of glycoprotein family subclasses using Staudinger chemistry
- Identification of novel glycoproteins

References
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Figure

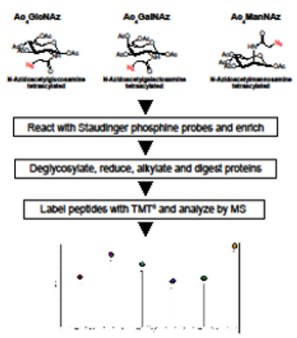

Figure 1. Schematic of azido sugar metabolic labeling, capture and glycoprotein MS identification workflow.

Quantitative Proteomic Approach to Analyze Glycoprotein Regulation In Response To Hypoxia

G112

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Introduction

Under low oxygen level conditions (hypoxia), malignant cells undergo a variety of biological responses such as activation of signaling pathways that regulate proliferation, angiogenesis, cell migration, and apoptosis (1). Cancer cells have adapted to hypoxia, facilitating an aggressive tumor behavior. Because many of the oncogenic pathways overlap with hypoxia pathways, we are interested in analyzing and comparing the regulation of the *N*-glycosylated proteins in response to hypoxia in breast cancer cell lines presenting different stages of invasiveness.

Methods

We used a label-free quantitative proteomic technique to quantify the protein glycosylation changes in response to hypoxia treatment. Low invasive (MCF7 and T47D) and high invasive (Hs578T and MDAMB231) cancer cell lines were grown at 80% confluency and treated at different time points (6 and 24 hrs) in normoxic and hypoxic conditions. Protein extraction was performed on crude extracts. For each cell line, equivalent amount of proteins was digested prior N-linked glycosylated peptide enrichment (2). The glycopeptides were injected on a LTQ-Orbitrap Hybrid mass spectrometer and quantitative data analysis was performed using the MSBID and Corra softwares (3). MS/MS spectra were searched against the IPI human database (version 3.18) using the X!Tandem algorithm.

Results

Analysis of MS1 data revealed a distinct clustering of features of the different cell lines. We also observed a difference in the hypoxic response between the low and the high invasive cancer cell lines. Features from the low invasive cell lines cluster distinctly at 6 hrs of treatment whereas longer treatment was necessary for the high invasive cell lines. The comparison of the protein expression profile of the low and high invasive cell lines during normoxia allowed us to identify glycoprotein signatures related to the degree of invasiveness of the cell lines. We found a difference in the regulation of the energy metabolism between the different cell lines and many proteins involved in cell migration, focal adhesion, cell proliferation were up-regulated in the high invasive cell lines. We could confirm some of the identified candidates by western blot analysis.
Under hypoxic conditions, we observed the up-regulation of proteins classified as "high invasive protein signature". We also identified proteins that are known to be hypoxia-related (e.g. GLUT1, LOXL2) and their regulation was depending of the cell line category.
Altogether, these results suggest that the cell-specific regulation of glycoproteins upon hypoxia could potentially be used to identify novel cancer biomarkers.

Innovative aspects

- N-linked glycopeptide enrichment of mammalian cells treated by hypoxia
- Label-free quantitative proteomics approach to analyze cancer cell lines
- Identification of hypoxia regulated proteins using a proteomic approach

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A novel human *in vitro* translation system for glycoprotein expression.

G113

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Introduction

One of the key features of the human proteome is the abundance of post-translational modification, with more than 50% being glycosylated (1). Current expression systems available for producing glycoproteins *in vitro* are limited. *E.coli*- and wheat germ-based in vitro expression systems cannot glycosylate proteins and protocols involving rabbit reticulocyte lysates in combination with canine microsomal membranes only inefficiently glycosylate proteins. In collaboration with RIKEN, we have developed a novel human cell-free expression system that can sustain expression of proteins for over 6 hours and in addition produce biologically active glycoproteins (2).

Methods

Human cell lines grown in shaker flasks were lysed to produce lysates that are capable of supporting *in vitro* translation of exogenously added mRNA or DNA containing either encephalomyocarditis virus-internal ribosome entry site (EMCV IRES) or beta-globing leader sequences. Protein expression was monitored using GFP mRNA. Glycosylation of *in vitro* -expressed glycoproteins was confirmed using deglycosylating enzymes. The alpha and beta subunits of human choriogonadotropin (hCG) glycoprotein were co-expressed in the human *in vitro* expression system and the resulting hCG complexes were purified. Functionality of the *in vitro*-expressed hCG was compared to native hCG using a testosterone induction assay in rat testes.

Results

Lysates prepared from human cell lines were able to sustain *in vitro* expression of proteins for over 6 hours with exogenous addition of mRNA at 2-3 hour intervals (Fig.1). Commercially available rabbit reticulocyte-based mammalian systems failed to produce protein beyond 1.5 hours, limiting the resulting expression level. We examined the glycosylation capacity of our cell-free extracts using the glycoprotein complex, hCG. We observed that co-expressed hCG subunits did form functional complexes *in vitro* and when purified and added to rat testes in petri dishes produced testosterone similar to the levels produced by native hCG. In addition, the glycosylation pattern of the HIVgp120 protein produced *in vivo* and *in vitro* using the human cell-free expression was found to be identical (Fig. 2). These results suggest that using our human cell-free expression system we can produce functionally active glycoproteins.

Additionally, we will also report on construction and expression of large scale libraries consisting of clones that can be directly used in our cell-free protein expression systems.

Innovative aspects

- A novel human cell-free expression system for efficient and higher level expression of proteins.
- A novel cell-free expression system that produces functionally active post-translationally modified glycoproteins at higher levels.

References

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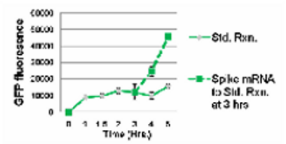

Figure 1. Expression of GFP in human cell based in vitro translation system continues over 6 hours. The solid and the dashed lines represent expression of GFP without and with exogenous addition of GFP mRNA, respectively.

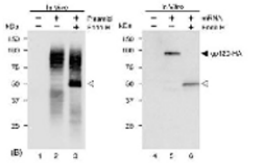

Figure. 2. HIV gp120 expressed in vivo in Hela cells or expressed in vitro in our human cell-free extracts was found to be identical. The expression pattern was compared with and without the deglycosylating enzyme endoglycosydase H (Endo H).

Analysis of glycoproteins from cancer stem-like cells by a lectin affinity approach

G114

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Introduction

Recent findings support the belief that cancer stem cells are responsible for tumor formation and ongoing growth. The identification of cancer stem cell surface antigens, especially their glycosylation sites, is crucial to the therapy of cancers. Therefore, it is necessary to uncover the membrane glycoproteins of cancer stem cells and their glycosylation sites. In the present work, the cell surface glycan expression patterns were first analyzed using lectin microarrays, and then the glycoproteins were enriched by lectin affinity and identified by LC-MS/MS.

Methods

Sixteen lectins were dissolved in 10% PBS to a concentration of 1 mg/ml and printed on SuperAmine slides. U373 glioblastoma cells and 20913 glioma-derived stem-like cells were labeled with CFSE dye and incubated with lectin microarrays [1] at room temperature for 15 min. After washed with PBS for 5 min, the slides were air-dried and scanned with a microarray scanner. Genepix 6.0 was used to analyze the images. Glycoproteins were enriched by lectin affinity [2], followed by digestion of these proteins with trypsin and PNGase F. The resulting peptides were analyzed by LC-MS/MS, and the glycans were analyzed by MALDI hybrid ion trap and time of flight.

Results

PNA and TKA lectins showed large differences in fluorescent intensity. This implies that these lectins may be used to distinguish the glioma cell subpopulations. Both of the two lectins have binding specificity to galactose. Agarose-bound PNA was employed to enrich glycoproteins from the cancer stem-like cells by affinity chromatography, and 333 proteins were identified from this fraction by LC-MS/MS. About a half of these proteins were membrane proteins, including several cell surface antigens such as CD36, CD71, CD90, CD138, CD147 and CD280. The elucidation of these glycoproteins may provide useful clues for the diagnosis and treatment of glioma.

Innovative aspects

Analysis of cell surface glycan expression patterns by lectin microarray and identification of glycoproteins from cancer stem cell by lectin affinity coupled LC-MS/MS.

References

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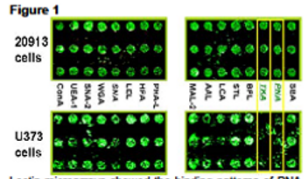
Figure 1



Lectin microarrays showed the binding patterns of PNA and TKA lectins were significantly different between 20913 glioma-derived stem-like cells and U373 glioblastoma cells

Identification of urinary glycoprotein biomarkers for human bladder cancer by multi-lectin affinity chromatography and LC-MS/MS

G115

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Introduction

Early clinical diagnosis of bladder cancer remains a major challenge and the development of noninvasive urinalysis is desirable for both patients and health care providers. Glycoproteins have been recognized as one rich source for diagnostic biomarkers due to the critical roles they play in biological functions and disease progression. To discover potential biomarkers for bladder cancer, novel methodologies for urinary proteome profiling by our group were further optimized and applied to the large-scale identification of human urinary glycoproteins.

Methods

Urinary glycoproteins were extracted from naturally micturated human urine using multi-lectin affinity chromatography, Wheat Germ Agglutinin (WGA) and Concanavalin A (Con A). The captured glycoproteins were subjected to trypsin digestion and followed by deglycosylation with peptide-N-glycosidase F (PNGase F). Then PNGase F digests were identified using µHPLC-ESI-MS/MS, and further characterized by Trans-Proteomics Pipeline (TPP) software. Differences in glycoprotein abundance between normal and cancer were quantified by the label-free spectral counting method. Functional and pathway analyses of the glycoproteins were carried out by Ingenuity Pathway Analysis (IPA) software. The glycan was also separated and analyzed using MALDI hybrid ion trap and time of flight.

Results

In total, 42 urine samples obtained from 20 healthy individuals and 22 bladder cancer patients were analyzed by LC/MS-MS. A list of 265 distinct glycoproteins was produced from the analysis with high confidence (ProteinProphet probability scores >= 0.9). According to the t-test results of normalized spectral counting for each glycoprotein, 169 glycoproteins were produced with P value < 0.05 and 40 glycoproteins were further identified with P value < 0.01. 13 cancer-associated glycoproteins have been identified as promising biomarker candidates based on the criteria that a protein must have a more than 2 fold difference in concentration and must be present in at least half of the cancer samples to qualify as a potential bladder cancer marker.

Innovative aspects

13 cancer-associated glycoproteins have been identified as promising biomarker candidates in urinary glycoproteomics for bladder cancer.

References

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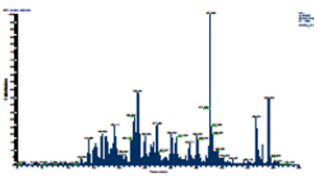

Figure 1. Base peak chromatogram of the urinary glycoproteins from bladder cancer patient (0481). The initial protein amount: 200 µg.

Figure 2. Network of : 11 out of 13 identified glycoproteins with p < 0.01 and based on the criterion that a protein must be present in at least half of cancer samples.

G116

Tandem ^{18}O Stable Isotope Labeling for Quantification of N-glycoproteome

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Introduction
It is believed that glycoproteins could play important roles in many life processes of physiology and pathology. Changes of occupancy of N-glycosylation at specific sites have been shown to be closely related to aggressive cancer cell behavior such as tumor cell invasion, metastasis and other diseases. Thus, methods for the quantitative profiling of glycoproteins especially for the N-glycosylation sites are very important. Here, we described a tandem ^{18}O stable isotope labeling (TOSIL) method which can accurately and effectively quantify the changes in N-glycosylation levels.

Methods
The strategy of tandem ^{18}O stable isotope labeling (TOSIL) in quantitative glycoproteomics is shown in Fig. 1. The two peptide mixtures to be compared are firstly tryptic digested in either $H_2{}^{16}O$ or $H_2{}^{18}O$, and then glycopeptides are captured by sepharose method(1) and exposed to PNGase F for deglycosylation of the N-linked glycopeptides in corresponding $H_2{}^{16}O$ or $H_2{}^{18}O$ used for trypsin. Thus, after treated by trypsin and PNGase F in $H_2{}^{18}O$, the glycopeptides with single N-glycosylation site are labeled with three atoms of ^{18}O (two are located at the C-termini of the peptides, the rest one is in the N-glycosylation site). The two samples labeled with ^{16}O or ^{18}O are then mixed and analyzed by Nano-LC-ESI-MS/MS. Peptides are quantified by measuring the relative signal intensities of peptide ion pairs in the MS mode(2).

Results
First, the TOSIL method was tested using four glycopeptides from two standard glycoproteins. Consequently, the linearity was good within the tenfold dynamic range with the correlation coefficient $r^2 > 0.99$, and the SD varied from 0.06 to 0.21.
Furthermore, the TOSIL method was applied to quantify the N-glycosylation changes between two biological samples for the first time, human normal serum and ovarian cancer serum as exemplified in this study. In this result, a total of 86 N-glycosylation sites occupancies were quantified from the comparative analyses, of which N-glycosylation levels of 56 N-glycosites were significantly changed. Most changes of occupancy of N-glycosylation sites were in accordance with the changes of protein expression levels, and three glycosylation sites occupancy were inconsistent with protein expression levels attracting most of our attentions. Probably, these glycopeptides would be potential clinically significant in terms of disease etiology and biomarkers. Western blot was performed for five important proteins and the results were consistent with the quantitative information obtained by the TOSIL method.

Innovative aspects
- After treated by trypsin and PNGase F in $H_2{}^{18}O$, three ^{18}O atoms labeling were introduced into glycopeptides. So high throughput and accurately quantitative analysis of N-glycosylation site occupancy was achieved.
- A single process can be carried out not only for identifying the N-glycosylation sites but also for quantifying the change both in glycosylation level of a certain glycosylation site and in protein expression level. between two samples, e.g. normal and diseased.

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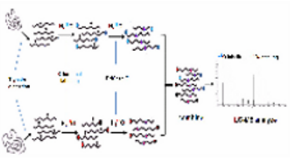

Figure 1. Analytical strategy of Tandem ^{18}O Stable Isotope Labeling (TOSIL) in quantitative glycoproteomics.

G117

A Novel Two-step Protease Digestion Approach for More Confirmed Glycoprotein Identification

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Introduction
Glycopeptide capture by hydrazide chemistry is a popular approach with high specificity in glycoproteomic researches. However, due to its high specificity, usually glycoprotein identification can only depend on a limited number of captured peptides containing N-glycosites. Herein, a modified solid-phase extraction of N-linked glycopeptides strategy was developed: two different proteases were novelly applied into this approach, introducing more confirmed glycoprotein and glycosite identification while making use of the high specificity of hydrazide chemistry.

Methods
As shown in Fig. 1, before glycopeptide oxidization and coupling, Lys-C (cutting at K) was used as the first protease to digest proteins into relatively larger peptides. The large peptides containing oxidized sugar groups were captured by hydrazide resin then to simplify the peptide mixture. After glycopeptide coupling and several washing steps, trypsin (cutting at K, R) as the second protease was used to cut off small peptides (LT-peptides, in the red dashed circle) from Lys-C produced glycopeptides captured by the solid phase. The LT-peptide supernatant was collected and analyzed by 1D LC-MS/MS, the way as the later PNGase F released glycopeptides (TF-peptides, in the red dashed rectangle).

Results
Combinative use of the newly introduced LT-peptides (not the TF-peptides only as traditionally) for the final glycoprotein and glycosite identification, not only improved glycoprotein sequence coverage, but also provided more confirmed N-glycosite information. Because LT-peptides indicated the existences of glycan coupling happened beside them, even if some glycosites were insensitive to PNGase F.
We applied this approach to 293T cell lysate. Finally, 668 unique peptides with a 0.98 Da mass increase on Asn in NXS/T sequon were identified from 280 glycoproteins, and 543 N-glycosites were recognized. Combining with the LT-peptides identification result, nearly 40% of the identified glycoproteins obtained improved sequence coverage (an average increase of 88%). Meanwhile, 141 of the identified N-glycosites were further confirmed by identified LT-peptides beside them.

Innovative aspects
Novelly applying two-step protease digestion to hydrazide chemistry capture approach and combinatively making use of newly introduced LT-peptides to improve traditional TF-peptide result for glycoprotein and glycosite identification.

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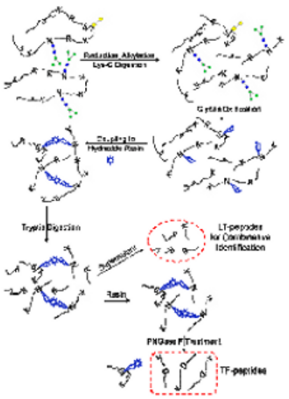

Figure1. Workflow of the Two-step Protease Digestion Glycopeptide Capture Approach

G118

Altered Glycoproteins in Distal Renal Tubular Cells upon Calcium Oxalate Dihydrate Crystal Adhesion

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Introduction
Calcium oxalate dihydrate (COD) crystals can adhere onto apical surface of renal tubular epithelial cells. This process is followed by crystal growth and aggregation, resulting in kidney stone formation. Glycoproteins have been thought to play roles in response to crystal adhesion. However, components of the glycoproteome that are involved in this cellular response remain largely unknown. Our present study was therefore aimed to identify altered glycoproteins upon adhesion of COD crystals onto tubular epithelial cells representing distal nephron, the initiating site of kidney stone formation.

Methods
Madin-Darby Canine Kidney (MDCK) cells were maintained in culture medium with or without COD crystals (100 μg/mL) for 48 h (n=5 for each set). Thereafter, the cells were harvested into the tube containing 0.5 M EDTA in PBS to dissolve the adherent COD crystals and were washed with PBS. Cellular proteins were then extracted and resolved by 2-D gel electrophoresis. The separated protein spots were visualized by SYPRO Ruby total protein stain, whereas glycoproteins were visualized by Pro-Q Emerald 300 glycoprotein stain (Invitrogen – Molecular Probes). These spots were matched and analyzed by Image Master 2D Platinum software (GE Healthcare). Significantly differed glycoprotein spots, whose corresponding total protein levels did not change, were subjected to identification by Q-TOF MS and/or MS/MS analyses.

Results
There were 5 gels derived from 5 individual culture flasks in each condition (totally 20 gels were analyzed). Approximately 850 total protein spots were detected by SYPRO Ruby stain, whereas about 240 glycoprotein spots were detected by Pro-Q Emerald dye. Quantitative intensity analysis revealed significantly altered levels of 16 glycoprotein spots, whose corresponding total protein levels did not change. These altered glycoproteins were then identified by Q-TOF MS and/or MS/MS analyses.

Innovative aspects
- This is the first study that defines altered glycoproteome in distal renal tubular epithelial cells upon COD crystal adhesion.
- These findings may provide some novel insights into the pathogenic mechanisms of kidney stone disease.

G119

Quantifying N-Glycosylation Distribution in Therapeutic Recombinant IgG using MRM strategies and triple quadrupole linear ion trap MS technology

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Introduction
Glycosylation patterns determine the stability and biodisposition of recombinant protein drugs *in vivo*. Analysis of therapeutic antibodies is focused on complete characterization and quantification of glycosylation, since heterogeinty of glycosylation patterns obtained from cell culture influences efficacy, folding, binding affinity, specificity and pharmacokinetic properties. Specific, quantitative methods for monitoring glycoprotein isoforms in biological samples are crucial.

Previously, we developed a simple workflow on a hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Analytical Technologies) to quantify glycopeptides. Here, we extended this MRM based strategy to increase specificity and enable quantification of glycopeptides from specific glycoforms of a therapeutic antibody in biological samples, and profile their pharmacokinetic differences.

Methods
NanoLCMS was performed on a triple quadrupole linear ion trap MS system (Applied Biosystems/MDS AnalyticalTechnologies) using multiple acquisition strategies. After tryptic digestion of the glycoprotein, glycopeptides are selectively detected by Precursor Ion Scanning for *m/z* 204.1/366.1/Y1 –ion and their molecular weights are confirmed by high resolution LIT-MS. The major glycopeptide species are characterized in detail using MS2/MS3 analysis and SimGlycan™ data processing. Using the resulting experimental information and *in silico* prediction of all possible glycan structures, a comprehensive list of MRM transitions to all expected/possible glycopeptide species is created. These MRM methods are used to specifically detect and distinguish all glycopeptide species and used for quantitative monitoring. Overlays of specific MRM sets per glycopeptide increase specificity and confidence in quantitation.

Results
The approach has been first optimized using commercially available immunoglobulin G from human serum. This IgG sample contains both different protein sequence isoforms (IgG1-3) and nine different glycan isoforms. Due to the high sensitivity of MRM methods, quality MRM and MS/MS data was achieved on only 100 fmol of glycopeptides on column. First, the glycan structure of the glycopeptide was characterized from MS2 and MS3 from the precursor ion scanning and MRM experiments using SimGlycan software, a predictive carbohydrate and glycopeptide analysis tool for MS/MS data. Most glycoforms were detected with high confidence and confirmed with MS/MS. Using known and predicted glycan structures, we designed expanded quantitative MRM assays with more specificity and information to distinguish glycoforms in complex biological matrices. The resulting specific MRM data for sugar marker ions (HexNAc, HexHexNac, Fucose, Hexose and Sialic acid) as well as glycopeptide Y1 marker ion run on the digested IgG sample provided more specificity because of multiple co-eluting transitions.Further work will be done to increase specificity through use of additional glycopeptide ions and MS3 quantitation.

We extended this approach to test on therapeutically interesting antibodies. Production of these IgG molecules in cell culture results in varied glycoprotein structure due to incomplete processing of the galactose and fucose residues from the biantennary glycans, therefore creation of *in silico* list of possible glycan variations and therefore MRM detection methods is possible. Next we will apply this strategy to profile their pharmacokinetic differences in animal samples.

Applying this strategy, specific quantitative assays can be developed to study antibody clearance in biological samples.

Innovative aspects
- Quantitative profiling of specific glycan pattern variability at the glycopeptide level using MRM and MS3 strategies
- Data processing using Multiquant software and SimGlycan software

G120

Beyond Proteomics: everywhere we look, the sugars they are a-changin'

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Introduction.
The primarily extracellular location of glycans makes them intrinsic to cell–cell interactions e.g. pathogenic infection, fertility, immunity, and cancer. Glycans thus are an alternative, under-exploited, molecular class to discover disease biomarkers and drug targets. Glycomics, the study of all glycans from a cell, tissue or system, is facilitated by recent advances in mass spectrometry. Bioinformatics tools for the analysis of this data are lagging behind, but it is relatively easy now to compare the glycosylation profile of the total cell membrane proteins in different biological systems.

Methods
Mass spectrometric data was obtained on released glycans and glycopeptides from glycoproteins derived from crude membrane fractions of a range of biological systems (e.g. milk, saliva, cell lines, tissues) by Triton X-114 phase partitioning[1]. These fractions were trypsin digested for glycopeptide analysis or were dot blotted onto PVDF and subjected to PNGase F and β-elimination to release *N* and *O*-glycans respectively. Glycopeptides were separated by C18 reversed phase (ProteoCol, SGE) chromatography, glycans were separated by graphitized carbon LC and both were analysed using an ion-trap MS (Bruker HCT) in positive and negative ion mode respectively.
Glycoprotein-enriched (>100kDa) fractions were bound to HA crystals and incubated with bacteria stained with Hoechst 33342 before imaging.

Results
Glycosylation data on a diverse range of cells and secretions will be presented as a function of time and biological state. Bioinformatic tools were developed to facilitate some of the analysis of the mass spectrometric data; such as tools for locating glycopeptide masses in a complex mass spectrum and for comparing large datasets of glycan structures. Global glycan profiling of milk fat globule membranes during lactation of different women showed differential expression of Lewis antigens but did not express blood group antigens. In contrast, salivary mucin glycoproteins showed expression of different blood group epitopes and interacted differently with microbial pathogens. The differential binding of the pathogenic species was quantitated using a fluorescent binding assay(Fig. 1). Marsupial milk glycosylation was found to be more similar to human milk in the glycoprofile of its milk than cow's milk. In addition, different glycans were expressed on the membranes of colon cancer cells cultured under different conditions compared to tissues, on liver tissues distal to a tumour and on leukaemia cells selected for resistance to cancer drugs.

Innovative aspects
- Rapid, easy comparative glycoprofiling of membrane glycoproteins isolated from different biological systems.
- Development of bioinformatic tools for facilitating mass spectrometric glycoanalysis
- Glycan profiling shows that different expression of glycan epitopes occurs in cells and secretions depending on source, time and seemingly whenever there is a biological perturbation.

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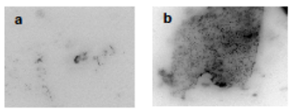

Figure.1 Salivary glycoproteins attached to crystals (HA) bind differently to fluorescently labelled bacteria a) *E.coli* b) *S.gordonii*

G121

Profiling the *N*-linked Glycome from Chicken Plasma to Identify Epithelial Ovarian Cancer Biomarkers

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Introduction
The chicken has emerged as an important animal model for epithelial ovarian cancer (EOC) research given the high prevalence of ovarian tumors (>15%) in birds 2-4 years of age. A one-year effort to procure longitudinal plasma samples from 242 chickens (2.5 years of age) recently concluded resulting in a substantial biospecimen repository. A recently developed method[1] in this lab for profiling *N*-linked glycans from human plasma proteins was applied to a subset of these longitudinal samples in an effort to identify candidate biomarkers for the onset of EOC and is reported herein.

Methods
Plasma was collected from 242 age-matched laying hens starting at 2.5 years of age (October 2007) for 1 year at 3 month intervals (5 time-points total). Longitudinal plasma samples from 4 "healthy" (no neoplasms present) and 4 "Early Stage EOC" (neoplasms localized on the ovaries) were denatured and 10 mU of peptide N-glycosidase (PNGase F) were used to release glycans from glycoproteins found in plasma. Samples were then enriched and purified using porous graphitized solid phase extraction cartridges. Purified *N*-linked glycans from the longitudinal plasma samples were quantified (relative) and identified using an Eksigent 2D nLC system (HILIC stationary phase) coupled to a Thermo LTQ-FT MS.

Results
Confident identification using accurate mass and MS/MS data revealed the glycan structure shown which has a fucosylated core. Analysis of the peak area of this glycan normalized to the internal standard peak area as a function of time revealed the following. First, at *t*=1 (Oct '07), the Control Chicken (red squares) had a statistically significant (t-test) higher abundance of this glycan than did the EOC Chicken (blue diamond's). Second, at *t*=2 (Jan '08) and *t*=3 (Apr '08), the data analysis indicated that there was not a statistically significant difference between the Control and EOC chicken. Third, at *t*=4 (Jul '08) the data support that the Control again had a higher concentration of this glycan. Fourth, at *t*=5 (Oct '08), the abundance of this glycan was higher in the EOC than in the Control. Importantly, very different conclusions would be reached if only a single-time point collection was used. Data will be presented which clearly demonstrates that **rational biomarker discovery** must include human samples but also longitudinal sampling in a model organism where the genetic background and environment can also be controlled.

Innovative aspects
- *N*-linked glycome profile of chickens with and without epithelial ovarian cancer as a function of time and compared to results obtained from humans.
- Defining "normal ranges" for N-linked glycans in chicken plasma.
- Assessment of each glycan in the context of this normal range within and between birds to define a "glycome index of individuality".[2,3]

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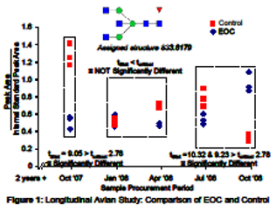

Figure 1: Longitudinal Avian Study: Comparison of EOC and Control

G122

Comprehensive analysis of core-fucosylated glycoproteins from mouse embryonic fibroblast (MEF)

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Introduction
α1,6 Fucosyltransferase (Fut8) catalyzes the transfer of fucose from GDP-Fuc to the innermost GlcNAc residues of N-linked complex and hybrid type glycans on the glycoproteins[(1)].
The level of core fucose is altered under pathological conditions such as hepatocellular carcinoma[(2)]. Therefore, α1,6 fucosylated protein(s) could be used as candidates to discover potential prognostic cancer biomarker.
We will present our comprehensive proteomic analysis of core fucosylated glycoproteins in the MEF cells prepared from wild type mice. We also used the MEFs from Fut8 KO mice as a negative control.

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Methods & results
Fut8$^{+/+}$ and Fut8$^{-/-}$ MEF cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cultured Fut8$^{+/+}$ and Fut8$^{-/-}$ MEFs were harvested in T-PER lysis buffer. The cell lysates (~4.5x10^7) were prepared by removing the debris by centrifugation. To analyze the soluble secreted glycoproteins from the cells, the MEF cells were also cultured in a serum free condition. The media and cell lysates (<1 mg/ml of proteins) were respectively applied to *Lens culinaris* agglutinin (LCA) affinity columns. The columns were then washed with 20mM TBS (pH7.6), 1mM calcium chloride, 1mM magnesium chloride, and 1mM manganese chloride. After that, the bound fractions were eluted with 0.4M α-methyl-D-mannnopyranoside.
The bound fractions were concentrated using an Amicon ultra filtration membrane, and their protein contents were determined, using a BCA protein assay kit. The samples were then subjected to sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and subsequently analyzed by both silver staining and LCA lectin blotting analysis. The LCA-positive bands specifically observed in the sample from WT mice were manually cut out from the gel and digested with trypsin. Matrix Assisted Laser Desorption/Ionization quadrupole ion trap mass spectrometer (MALDI-QIT-MS) analyses were performed using an AXIMA-QIT-MS (Shimadzu).
These data were searched by protein database of the Mascot MS/MS ion search engine. We identified extracellular matrix proteins and lysosomal enzymes as possible core-fucosylated proteins.
Innovative aspects
We identified some core-fucosylated glycoproteins obtained from MEF lysates and media using a lectin affinity column.

G123

Drug resistance inhibits expression of α2-6 sialylated glycans on cell membrane proteins

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Introduction
Drug-resistance is one of the problems of cancer chemotherapy and knowledge of the molecular changes associated with the development of resistance enables an understanding of the cellular mechanisms that are involved. We focused on studying the changes in glycan structures on membrane proteins of drug resistant cancer cells, as the glycans are involved in cell recognition, adhesion, migration, lymphocyte homing and apoptosis (1). We analyzed the glycan structures of human leukaemia cells that have acquired microtubule targeted antitumor drug-resistance.

Methods
CCRF-CEM cells, a T-cell acute lymphoblastic leukaemia cell line, were used. These CCRF-CEM cells were selected for acquired drug-resistance against desoxyepothilone B (dEpoB), vincristine (VCR) and paclitaxel (TAX), all of which are microtubule-targeted antitumor drugs. These cells were homogenized, the cell membrane fraction was obtained using Triton X114 detergent phase partitioning and ultracentrifugation. The fractions were dissolved in 8M Urea followed by dot blotting onto PVDF membrane. The glycomic profile of the cell membrane glycoproteins was obtained by sequential release of *N*- and *O*-glycans from the dotblot with PNGase F and β-elimination respectively (2). Both of the released *N*-glycan and *O*-glycan alditols were then separated by porous graphitized carbon LC-ESI MS/MS.

Results
The major *N*-glycan on CCRF-CEM membrane glycoproteins is the disialylated (α2-3 and α2-6), core fucosylated biantennary structure. CCRF-CEM cells resistant to TAX (5 nM) did not alter their membrane glycosylation. Resistance to dEpoB (30, 60 and 140 nM) did not change, however, resistance to dEpoB (300 nM) resulted in loss of α2-6 sialic acid on all *N*-glycans. Moreover, resistance to VCR (3nM) caused the loss of both α2-6 sialic acid and α2-3 sialic acid, resulting in an abundance of asialo-*N*-glycans and de-sialylated *O*-glycans. It is clear that the membrane glycosylation of leukaemia cells changes during acquired resistance to chemotherapeutic drugs and that this change is related to a reduced sialylation of the glycans, presumably caused by a decrease/ increase of expression or activity of the cell's sialyltransferases/sialidases. We tested the expression of these enzymes in homogenized cells by western blot using sialyltransferase (ST6Gal-I) and sialidase (NEU1) antibodies. Activity of these enzymes was also measured by HPLC analysis using the substrates asialo-biantennary *N*-glycan and disialo-biantennary *N*-glycan labeled with 2-amino pyridine.

Innovative aspects
- Sequential analysis of *N*-glycan and *O*-glycan structures from cell membranes of 10^7 cells
- Glycan structures change on cancer cells that have acquired microtubule targeted antitumor drug-resistance
- Inhibition of expression of α2-6 sialylated glycans on membrane proteins by acquired drug-resistance

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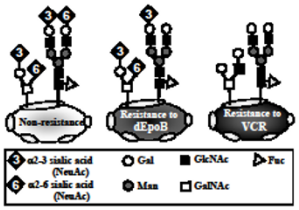

Figure 1. Major *N*-glycan and *O*-glycan structures on cell membrane proteins of CCRF-CEM cells and the cells with acquired drug-resistance against dEpoB (300 nM) and VCR (3 nM).

G124

Protein O-GlcNAc site assignments by electron transfer dissociation(ETD) mass spectrometry

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O-GlcNAc is a dynamic covalent modification of nuclear and cytosolic proteins in all metazoans studied, and is implicated in intra-cellular signaling, protein expression, activity, trafficking and degradation as well as human diseases, e.g. diabetes, Alzheimer, and cancer. Deciphering its role in modulating protein function has lacked rigorous methodology to assign the sites of modification easily[1,2]. We have recently shown that both ECD and ETD energy deposition techniques retain O-GlcNAc in the GlcNAcylated peptide sequence ion series required for unambiguous site assignment[3].

Methods: As described elsewhere(3), murine postsynaptic density pseudoorganelle was isolated from murine brains of 8 week old LCI animals[3] with PUGNAc present during the protocol. Two mg aliquots were digested with trypsin and subjected to wheat germ agglutinin affinity enrichment of O-GlcNAcylated peptides. Fractions from the WGA lectin column were analyzed using a 75 micron C18 column in a Nanoacuity(Waters)/LTQ-Orbitrap ETD(Thermo) platform and processed using tools in ProteinProspector. In most experiments both CID and ETD spectra were acquired, but the CID spectra are virtually devoid of sequence ions containing the O-GlcNAc moiety.

Results: We have established that both ECD and ETD energy deposition preferentially forms peptide sequence ion series that retain the O-GlcNAc moiety and thus permit the assignment of modification sites. We have recently reported the assignment of 58 sites corresponding to 15 proteins present in the murine postsynaptic density preparation(3). In addition, since ETD spectra contain direct information and identification of the peptide residue bearing the modification, we were able to established the presence of 8 peptides that were modified at sites of N-linked glycosylation as well. In subsequent work, we have established the presence of some ten additional O-GlcNAc modification sites in PSD and have been able to obtain ETD spectra that permit the assignment of a site of phosphorylation and two sites of O-GlcNAcylation on the same peptide from Disks large-associated protein 1. Both O-GlcNAc moieties were assigned in our previous report, but the exact phosphorylated residue of among N-terminal SSSP… could not be assigned then. This result establishes the presence of both a phospho- and two O-GlcNac moieties separated by four and five residues respectively, SSpSPPPR[O-GlcNac]T[O-GlcNAc]TTTVR.

Innovative aspects:

• We have established that WGA affinity chromatography enriches O-GlcNAcylated peptides

• ETD provides a robust technique to preserve O-GlcNAcylation in electrospray peptide sequence ion series that reveals readily sites of modification

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Human Proteome Projects

(H101 – H122)

Russia on 18th chromosome: strategy and end-point H101

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Introduction The main goal of the Russian part of Human Proteome Project (RPHPP) is the identification of human proteins coded by 18th chromosome.

Methods and results The backbone of the RPHPP consists of the following items:
(a) Data-mining for chromosome selection. Gene-centric principle of HPP assumes the selection of one fixed chromosome for each of the participating countries. The selection of chromosome is made accounting for the total number of genes and for its linkage with the disease. We selected 18th-chr because it contains minimal number of genes (462) and relatively high disease-related nsSNP coverage (over 0,97 per gene).
(b) *Selection of the liver and plasma as biological material for the analysis.*
(c) *Computational mining for transcripts and expressed proteins of 18th-chr.* For these purposes we should use data-mining strategy for the analysis of global genomic and proteomic databases (including UniProt, EntrezGene and ProteinAtlas) and text-mining approach.
(d) *Experimental transcriptome analysis* of 18th-chr transcripts of HepG2 cells and hepatocytes and *trivial experimental proteomic analysis* of high-(10^{-3} -10^{-7} M) and medium-copied (10^{-7}-10^{-12} M) proteins and peptides of liver and plasma using MS/MS and quantitative analysis (MRM/AQUA approaches).
(e) *Creation of transcriptome 18th-chr microchip.*
(f) *Creation of AFM/MS combination technology for the analysis of low- and extra-low copied proteins* and identification of low- and extra-low copied proteins expressed by 18th-chr at the range of 10^{-12}-10^{-18} M in plasma and dozens of protein molecules per liver cell.
(g) *Proteotyping of proteins expressed by 18th-chr in plasma and liver.*
(h) *Creation of gene-centric knowledgebase of 18th-chr proteins.*

Evaluating the impact of salts on IEF protein separation H102

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Introduction
2-DE offers an outstanding resolving power to analyze complex protein samples. To work in conjunction with protein identification by mass spectrometry and bioinformatics, this technique has been widely applied in proteomic research. Although 2-DE has evolved into a robust method, one of the major technical challenges in the first dimension of separation is the salt content in the sample. In this study, the interferences of salts in IEF protein separation were investigated. Samples contained a variety of salts with various concentrations were analyzed and the effects were evaluated.

Methods
Protein samples were prepared by extracting from swine liver tissue and reconstituted for 2-DE. Salt to be investigated was added to the assigned final concentration as indicated prior to IEF. Proteins were focused on strips by IPGphor II system and the second dimension was performed by 12.5% SDS-PAGE (GE Healthcare BioSciences). Imidazole-zinc reverse staining methods was performed by using a commercial VisPro 5 min protein stain kit (Visual Protein, Taipei, Taiwan). For gel image analysis, ImageMaster 2D Platinum software version 5.0 (GE Healthcare BioSciences) were applied to detect the protein spots.

Results
To look into the interference effects due to the presence of salts, various salts were exogenously added to the desalted 2-DE samples respectively. Distinctive patterns of interference were observed with different salts added. Since this effect is due to the presence of salt, the vertical streak is referred as the salt front in this study. To understand the association between the degree of interference and the concentration of salt in IEF experiment, samples with exogenously added salt were investigated. As the applied Vh increased, the salt fronts moved toward the electrodes gradually. When sufficient Vh was provided to the sample, the salt interference disappeared completely and all proteins can be identified just like the original sample without exogenous salt. However this method cannot be expanded unlimitedly. When extreme high salt content was presented in the sample, electrodesalting cannot completely eliminate the residual ions in the strip. Base on the salt concentration, applied Vh and IEF result, here we proposed a mechanism to illustrate how ions behave during IEF. As the ion concentration increased, the travel distance of ions diminished and the range of separation decreased.

The cutback of the migration mobility is not only due to the increased number of ions but mostly because of the effect of internal build-in electric field. This force repels the ions that are traveling toward the electrodes and slow down the migration of ions. Unlike simple salts, week ionized salts are not dissociated into ions completely, the charge and species of molecules change according to the environmental pH. It was quite different from what observed with the simple salt. A vertical streak was observed in the central region of the gel. The positions of the vertical streak move closer to the pKa value of the buffer salt as the Vh increased.

Innovative aspects
Our result displays the correlation between salt interfering behaviors and these factors. Mechanisms for interfering behaviors were proposed for simple salt and buffer salt respectively. In addition, this work provides valuable information for the selection of salt composition during sample preparation for 2-DE.

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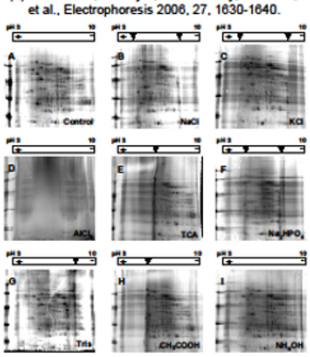

Figure 1 Evaluation of the interference of various salts on IEF.

A Human Plasma Proteome in the PeptideAtlas with Estimated Protein Concentrations H103

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Introduction The proteome of human blood plasma is of great interest as a source of potential disease biomarkers. We have recently announced a tandem MS derived human plasma proteome comprising about 2400 proteins with a false discovery rate (FDR) of 1%, the largest high-quality proteome compiled to date (1). Here, we apply spectral counting techniques to the human plasma proteome in order to estimate protein concentration using the number of peptide-spectrum matches (PSMs) for each protein.

Methods Spectra from 73 conventional MS/MS experiments were searched against a human spectral library, including decoys, using SpectraST, a spectrum library search engine developed at ISB. Spectra from another 22 glycocapture experiments were searched against a target-decoy sequence database using X!Tandem. All PSMs were assigned probabilities using the Trans Proteomic Pipeline (TPP) statistical validation tools. Spectra above a PSM FDR of 0.0002 were compiled into a Human Plasma PeptideAtlas. The smallest protein set necessary to explain all identified peptides was compiled. PSMs for each protein were counted, and these counts were normalized to the number of predicted highly observable peptides. A formula for predicting concentration was then calibrated against experimentally-derived concentrations (Figure 1). The resulting formula was used to predict concentrations for all proteins in the Human Plasma PeptideAtlas.

Results The spectra included in this new Human Plasma PeptideAtlas represent about 2400 distinct proteins with a protein-level FDR of 1% as estimated by decoys. For each of these proteins there is now an estimate of the average abundance in plasma. These estimates will be useful for experiment planning, especially for targeted experiments with spiked-in in reference peptides, whose injected abundance should be similar to the abundance of the endogenous peptide.

Innovative aspects
- Largest high-quality (FDR=1%) human plasma proteome to date, enhancing the HUPO Human Plasma Proteome (3)
- Protein concentrations estimated using spectral counting

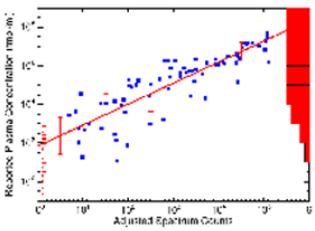

Figure 1. Spectral counting is calibrated for the Human Plasma PeptideAtlas using about 100 known plasma concentrations from Polanski & Anderson (P&A) (3). Blue dots represent PeptideAtlas spectrum counts plotted against P&A concentrations. Red arrows to the left indicate P&A proteins that we do not see in the atlas. Red bars on the right show how well PeptideAtlas covers the P&A proteins at different concentration levels.

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How much is enough? An algorithm for optimizing IEF condition in 2-DE experiments

H104

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Introduction
2-DE is a widely applied technique for proteomics researches. This technique sorts proteins in two consecutive steps: IEF and SDS-polyacrylamide gel electrophoresis. IEF separates proteins according to their isoelectric points by applying certain Vh to the sample. Salt and buffer ions in the sample can request an increase of the total Vh requirement and sometimes impede the quality of protein focusing. In this study, the influences of salts in IEF were investigated thoroughly and an algorithm for optimizing the total Vh was developed to ensure the quality of IEF.

Methods
Using conductivity as the parameter to monitor the ion contents in the sample, appointed Vh can be customized according to the variations of sample compositions. Other factors including the length of the IPG strips, the pH gradient, and the quantity of protein were also taken considered to determine the total Vh for the sample.

Results
In this study, the portable conductivity meter has been demonstrated to be a simple and accurate apparatus to estimate the salt contents in 2-DE samples. Prior to IEF procedure, knowing the level of salts in 2-DE samples helps the optimization of IEF program. We have proposed an algorithm using the conductivity and the amount of protein in the 2-DE sample, as well as the length and pH range of IPG strip to predict the required total Vh in the IEF experiment. This algorithm enables scientists to design a customized IEF program to reach the optimum focusing condition according to the nature of samples. This algorithm has been examined persistently with a number of different samples with adequate focusing results revealed, and hence was proven to be a reliable guide for performing IEF experiments. It is of great consequence to address that a quick and simple measurement in conductivity can significantly improve the quality of 2-DE experiments by tailoring to optimum.

Innovative aspects
The algorithm developed in this work provides a convenient guide to set up an IEF program when performing 2-DE.

- $Vh_{total} = Vh_{protein} + Vh_{salt}$
- $Vh_{protein\_exp} = Vh_{protein\_ref} \times \frac{M_{exp}}{M_{ref}} \times \frac{\text{strip length}_{exp}}{\text{strip length}_{ref}} \times \frac{\text{pH range}_{ref}}{\text{pH range}_{exp}}$
- $Vh_{salt\_exp} = Vh_{salt\_ref} \times \frac{S_{exp} - S_{blank}}{S_{ref} - S_{blank}} \times \frac{\text{strip length}_{exp}}{\text{strip length}_{ref}} \times \frac{\text{pH range}_{ref}}{\text{pH range}_{exp}}$

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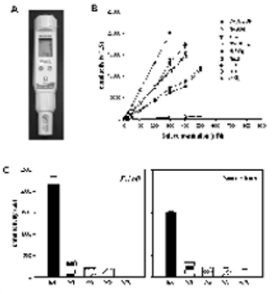

Figure 1 Utilization of the conductivity to monitor the salt contents in 2-DE samples. (A) A portable conductivity meter. (B) The correlation between the conductivity (μS) and salt concentration (mM) for various salts dissolved in the 2-DE rehydration buffer. (C) The conductivity (μS) of protein samples underwent different cold acetone washes after TCA precipitation. Samples were extracted from *E. coli* and swine liver respectively. Ext. indicates the crude extracted samples. W1, W3, W5, and W10 indicated one, three, five and ten acetone washes respectively.

Acknowledgement
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Phosphoproteomic study of insulin resistance in Type 2 Diabetes

H105

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Introduction
Type 2 diabetes (T2D) is a complex disorder characterized by impaired insulin secretion from beta-cells and insulin resistance (IR) in peripheral target tissues. Skeletal muscle is a key tissue site of the IR, with evidence of decreased insulin-stimulated glucose uptake and storage as glycogen. Although a number of abnormalities have been identified in skeletal muscle from type 2 diabetic subjects, the exact molecular mechanisms for IR has not been established. Several studies indicate that IR might be a result of impairment in the regulation of protein kinases and phosphatases.

Methods
The phosphorylation profiles of diabetic and control myotubes are characterized and compared using quantitative phosphoproteomics. The myotubes are quantitative labeled using iTRAQ 8plex as this form of labeling can be directly implemented on subsequent *in vivo* studies. Combining efficient pre-fractionation (Strong Cation Exchange) and highly sensitive phosphopeptide enrichment (SIMAC, Sequential elution from IMAC) gives the best characterization of the protein phosphorylation occurring in the myotubes. The phosphopeptides are analyzed using Multi-Stage Activation (MSA) in combination with higher-energy C-trap Dissociation (HCD) on a LC-ESI coupled to LTQ-orbitrap XL.

Results
Four comprehensive experiments were performed in order to compare the phosphorylation profiles of myotubes from obese, lean and obese T2D patients during both short-term and long-term insulin stimulation. The phosphorylation profiles were characterized and compared using quantitative phosphoproteomics.
We have identified approximately 2000 proteins from low amounts of starting material using quantitative phosphoproteomics and tandem mass spectrometry. Through bioinformatics analysis, we found that proteins involved in glucose metabolism were over-represented in these datasets. Furthermore, a large part of the proteins identified have been characterized as having muscle-related functions, positively validating our strategy. We have identified several interesting phosphorylation sites, some which confirms recently findings in diabetes studies.

Innovative aspects Insulin resistance, Type 2 Diabetes, Phosphoproteomics

Acetyl-Proteomics for the Investigation of Pathological Molecules In Rheumatoid Arthritis

H106

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Introduction
Rheumatoid arthritis (RA) is an intractable systemic autoimmune disease with severe inflammation. Pathogenesis of RA remains to be solved. Recent studies have demonstrated that development of autoantibodies to citrullinated proteins is a specific and sensitive marker for the diagnosis of RA (1). Thereby, investigation of citrullination, one of post-translational modification (PTM), in the pathogenesis of RA have been started recently. However, roles of other PTM in RA have rarely investigated until now. Thus, we here investigated whether acetylation, another PTM, was involved in pathogenesis of RA. Specifically, we detected and identified proteins which were predominantly acetylated in RA by proteomic analysis.

Methods
Peripheral blood mononuclear cells (PBMCs) were obtained from patients with RA and healthy donors. Proteins extracted from PBMCs were separated by 2-dimensional electrophoresis. The separated proteins, stained with Sypro Ruby, were transferred onto a PVDF membrane. The membrane was subjected to western blot with anti-acetylated lysine antibodies. Acetylation levels of protein spots were compared between the RA and healthy groups. By matching the western blot image and the Sypro Ruby image, we identified location of the highly acetylated protein spots on the 2D gel. Then the proteins, digested with trypsin in the gel, were identified by MALDI-TOF/TOF mass spectrometry.

Results
In PBMCs from the RA patients, 22 acetylated protein spots were detected by the western blot. By the comparison of the western blot between the RA and healthy group, multiple protein spots were found to be acetylated at significantly higher levels in RA than in the healthy. Two of such acetylated proteins were identified to be α-enolase (ENO1) and isocitrate dehydrogense 1 (ICDH1) by MALDI-TOF/TOF mass spectrometry. In order to clarify how acetylation of ENO1 and ICDH1 is related to the pathogenesis of RA, we are now trying to investigate whether acetylation affect the enzyme activity of them.
In conclusion, ENO1 and ICDH1 were found to be acetylated at significantly higher levels in RA than in the healthy. Acetylation might be another PTM involved in the pathogenesis of RA. Also, acetylation should be investigated from the aspects of diagnosis and monitoring of RA.

Innovative aspects
- Relation of Acetylation, one of PTM , to RA
- Acetylation of ENO1 and ICDH1 is related to the pathogenesis of RA or might be marker for the diagnosis and monitoring of RA

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Proteomics Analysis of Hepatocellular Carcinoma Caused by Hepatitis C Virus

H107

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Abstract content:

Introduction: Hepatocellular carcinoma (HCC) is highly fatal cancers for Japanese people. One of the major causes of HCC is the infection with hepatitis C virus (HCV). The pathogenesis of HCV-related HCC in its incipient stage from the infection to the onset of cancer is now being elucidated. Until now, a number of proteins have been reported to date as candidates for biomarkers in HCC from proteomics of HCC tissues.
Here we performed proteomic differential display analysis of cancerous tissues and corresponding non-cancerous tissues to identify new expectable diagnostic or therapeutic biomarkers for this cancer.

Methods: The investigations were performed by using SDS-polyacrylamide gels (SDS-PAGE), 2-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [Figure 1]. The proteins of cancer tissues and corresponding non-cancerous tissues were well resolved by pH 4-7 strip Gel (IPG) 11cm, and SDS-PAGE. After electrophoresis, the gels were stained with Flamingo™(BioRad). The position of the protein spots on cancerous and non-cancerous tissues were recorded by using ProExpress 2-D Proteomic Imaging System (Perkin-Elmer, Waltham, MA). The position and intensities of the spots were analyzed individually with PG240 software (Perkin Elmer). We picked up the protein spots of cancerous tissue whose expression level significantly increased or decreased by more than 1.5-fold ($p<0.05$) compared with that of non-cancerous tissue. After statistical analysis, the gels were stained with See Pico™ (Benebiosis, Seoul, Korea). The protein spots of high expression level were selected to pick up for MS analysis. The totally differential unique 22 proteins were identified by LC-MS/MS.

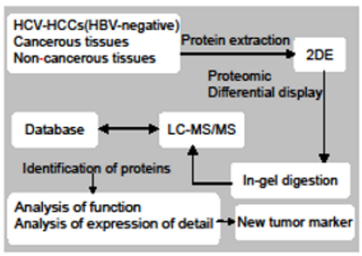

Figure 1. The concept of proteomic analysis study for HCV-HCC. 2DE:2D electrophoresis, HCB:Hepatitis B virus, HCC:Hepatocellular carcinoma, HCV:Hepatitis C virus, LC-MS:, MS:Mass spectrometry,
The proteomic differential display method is popular method to analyze protein expression profiling in carcerous tissues and noncancerous tissues. 2D electrophoresis (2DE)and mass spectrometry (MS) have been used for this differential display method.

Results: A total of 22 proteins were differentially expressed in liver tissue infected with HCV. Compared with the non-cancerous tissues group, the protein in cancer tissue group exhibited 10 spots increased, and 12 spots are decreased.

Innovative aspects: These proteins could be candidates as new diagnostic or therapeutic biomarkers for HCC.

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H108

Atlas of Korean Plasma Proteome

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Introduction
Plasma is the most obtainable and clinically valuable specimen of human [1]. Reference data of healthy Korean plasma would be useful information to find serological disease biomarkers. To make a reliable protein catalog for plasma proteome, we used various experimental methods to obtain mass spectra and identified by two separate search engines (Mascot and Sequest). PeptideProphet/ProteinProphet were used to estimate the FDR for any minimum probability as a cut-off of search results [2]. We provide high-quality protein catalog which can be searched by two different search engines with its FDR of less than or equal 1%. These results are publicly available at http://proteomix.org/koreanproteome.

Methods
Plasma samples were treated by MARC™ to remove abundance proteins. ProteoMiner™ was used to reduce the complexity of proteins. Proteins were separated by 1D-LC, SEC, PF2D, or 2DE. Separated proteins were analyzed by LTQ or MALDI-TOF/TOF. Peptide identification was subject to an analysis of Sequest and Mascot. PeptideProphet was used to estimate the FDR for any minimum probability as a cut-off of peptide identification. For protein identification, more than two peptides with less than 1% FDR were required but one unique peptide was also used. UniprotKB release 14.8 was used for peptide identification [3]. GO, tissue expression, protein domain, biological pathway and disease association of proteins (biomarker) were examined.

Results
At present, total 190 proteins have been identified by either SDS-PAGE separation with LTQ analysis or size exclusion column separation with LTQ. In addition, plasma proteins which had been separated by narrow pH range of IEF and SDS-PAGE separation, from pH 3.5 to 11, protein spots are being analyzed by MALDI-TOF/TOF and compared to those already known plasma proteome from other ethnic groups (see Table 1).

Innovative aspects
- Useful reference proteome of Korean plasma for identification of disease biomarker.
- High quality of protein identification
- With various methods and good sample size, we could lower experimental bias and cover wide range of protein characteristics.

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Table 1. Partial list of Korean plasma protein.

H109

Global Detection of Human Protein Complexes by Fractionomic Profiling and Tandem Mass Spectrometry

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Introduction
Protein complexes are critical to virtually every biological process. The human "interactome" is of particular interest, but no comprehensive study of human multi-protein complexes has been reported to date. By combining non-denaturing fractionation (HPLC) with exhaustive gel-free liquid chromatography–tandem mass spectrometry (LC-MS/MS) and consecutive bioinformatics data analyses, we have initiated a study to systematically identify endogenous soluble protein complexes from representative human cell lines. Here, we present proof-of-principle experiments confirming the suitability of our method to monitor stable protein complexes using HeLa cells as a model.

Methods
We introduce a novel profiling technology based on extensive fractionation of soluble protein extracts using Ion Exchange (IEX) HPLC with protein identification and quantitation using label-free shotgun sequencing as an alternative method to existing affinity purification (e.g. TAP tagging) or immunoprecipitation for the detection of protein complexes in mammalian cells and tissues. Our technology termed "Fractionomic Profiling" predicts membership in protein complexes based on the identification and correlation of co-eluting subunits during IEX-HPLC fractionation.

Results
HeLa cytoplasmic extract was prepared under non-denaturing conditions (1). Stable complexes in each extract were separated and fractions collected using an optimized high resolution tandem IEX-HPLC system (2). Co-eluting protein fractions were precipitated, and the proteins identified by LC-MS/MS using a linear ion-trap instrument. More than 2,000 high confidence proteins were identified in the 100 collected fractions. 35% of the identified proteins were previously reported to be part of known complexes in MIPS reference database (3), which we used as a reference benchmark. **Figure 1** shows some examples of co-purification of subunits of annotated complexes present in our dataset. These data establish the potential of our approach to monitor human protein complexes in a comprehensive manner. We are currently developing a sophisticated data mining strategy to accurately predict novel components and new multi-protein complexes.

Innovative aspects
- Simple, generic approach that can be applied to examine the global interactome of virtually any cell line or tissue using standard equipment.
- The method can potentially be used to compare different cellular states (e.g. changes in interaction networks under different growth conditions).
- No requirement for epitope tagging, antibodies or isotope labelling.

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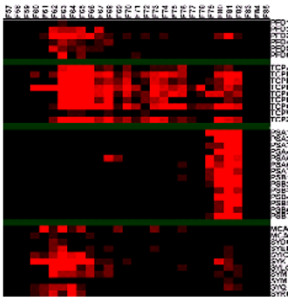

Figure 1. Examples of co-elution of known components of protein complexes (e.g. from the top to the bottom: **PFD, CCT, 20S Proteasome, and Multisynthetase**). Indicate on the actual image curated in the MIPS reference database (3). Increasing red color intensity indicates higher spectral counts.

H110

Novel Proteins Associated with Uterine Receptivity In Fertile Women

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Introduction
Molecular mechanisms which operate in spatio-temporal manner to endow the endometrium with receptivity are not yet elucidated in humans. This may be attributed to our incomplete knowledge of the key players, which are of crucial relevance in endometrial receptivity. In the present study a proteomics based approach was adopted to generate a catalogue of the major secreted and cellular proteins expressed during the proliferative and mid secretory phase/receptive in fertile women.

Methods
Women of reproductive age with a history of regular menses were enrolled in the study. Uterine fluid samples collected during the mid secretory phase (n=6) and proliferative phase (n=5) were resolved by two dimensional gel electrophoresis in the linear range of pI 4-7. Fifteen protein spots found differentially expressed were excised from the 2D gel of mid secretory phase uterine fluid and identified by MALDI-TOF. On comparing the mid secretory phase endometrial tissue with that of the proliferative phase 8 proteins were found differentially expressed between the two phases. Immunohistochemical analysis was performed to confirm the inferences drawn from the densitometric analysis of the 2D gels.

Results and discussion
Mass spectrometric analysis of the spots in the mid secretory phase uterine fluid revealed the identity of several proteins including alpha-1 anti-trypsin precursor, anti-chymotrypsin precursor, and β actin fragment. It is for the first time that the presence of HSP 27 was demonstrated in human uterine fluid. Our study is the also the first to report cycle dependent variations in the expression of endometrial calreticulin. The present study offers valuable clues regarding the differential expression of novel as well as some already known proteins expressed during the mid secretory/receptive phase of the endometrium. Some of these identified proteins could be analyzed further to be proposed as biomarkers to distinguish the receptive and non receptive state of the endometrium.

H111

Novel Putative CRP and sFLT1 Sensors for the Point-of-Care Diagnostic

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Introduction:
C-reactive protein (CRP) as an acute phase-protein and soluble fms-like tyrosin kinase-1 (sFLT1) as an antiangiogenic protein are important clinical biomarkers reflecting high-degree inflammation and endothelial dysfunction, hypertension, and proteinurea in preeclampsia. Here we report about novel putative CRP and sFLT1 sensors for the point-of-care diagnostic identified using a high-throughput screening technique, the yeast two-hybrid (Y2H) system.

Methods:
CRP and sFLT1 full length protein as well as six extracellular sFLT1-domain (D1 to D6) encoding sequences were cloned into a LexA DNA-binding domain encoding bait vector and screened for interaction using a cDNA-library derived from an *Arabidopsis thaliana* root cell culture (courtesy of C. Koncz, MPI Cologne, Germany). This library was already cloned into a Gal4 activation domain encoding prey vector (Clontech, Mountain View, CA). Based on lithium acetate method, yeast transformations were performed as previously described (1, 2). Self-activation tests of the bait hybrid proteins LexA-CRP, LexA-sFLT1 and LexA-sFLT1-D1/-D2/-D3/-D4/-D5/-D6 were performed using the empty prey vector and of the prey hybrid proteins using the empty bait vector. All yeast clones were incubated for 7 days at 30°C.

Results:
CRP and sFLT1 interacting candidates were identified in Y2H experiments using CRP, sFLT1 and six sFLT1 protein domains (Fig 1) as bait hybrid proteins. More than 10 screens were performed resulting in >1×10^6 transformants/bait hybrid protein and yielding in >1000 primary positives of which a maximum of 96/screen were further investigated. In total 125 interactions (secondary positives) were identified (55xCRP, 6xsFLT1-D1, 5xsFLT1-D2, 19xsFLT1-D3, 30xsFLT1-D4, 0xsFLT1-D5 and 10xsFLT1-D6). Sequence analysis of CRP secondary positives resulted in 12 different prey encoding genes and of sFLT1-D1/-D2/-D5/-D6 secondary positives resulted in histone encoding genes only. Histones are proved to be unspecific interactors, thus excluded from subsequent analysis. However, interactions with 10 and 16 different prey proteins were identified for sFLT1-D3 and sFLT1-D4. Affinity copurification experiments are in progress in order to confirm found interactions *in vitro*. For this we developed a protein expression platform containing almost all expression systems (www.unipep.de) and expressed CRP, sFLT1-D3/-D4 and interacting prey proteins as GST or 6xHis fusion proteins (data not shown).

Innovative aspects:
- Development of a protein expression platform for quick and parallel expression of proteins in multiple organisms
- Development of cheap sensors for clinical biomarkers for the point-of-care diagnostic

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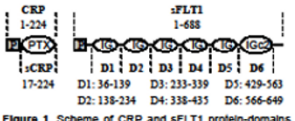

Figure 1. Scheme of CRP and sFLT1 protein-domains used for interaction analysis in yeast two-hybrid experiments. The soluble part of the c reactive protein (CRP) designated sCRP, the soluble part of soluble fms-like tyrosin kinase-1 (sFLT1) and six domains of sFLT1 (D1/D2/D3/D4/D5/D6) were used for the identification of interacting candidates using the yeast two-hybrid system. Numbers indicate amino acid positions in the full-length protein. P, signal peptide not present in the native protein; PTX, pentaxin domain; IG, immunoglobulin-like domain; IGc2, immunoglobulin-like domain.

Acknowledgements:
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H112

Sequential Proteome Profiles of Joint Inflammation in Arthritis Patients.

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Introduction
Juvenile idiopathic arthritis (JIA) comprises a poorly understood group of chronic, childhood onset, autoimmune diseases with variable clinical presentations, outcomes and therapeutic responses. In a prior study, the synovial fluid (SF) proteome of an oligoarticular patient subgroup was compared to patients who show a spread of disease in the first year to involve new joints, the extended oligoarticular subgroup. Current laboratory tests are unable to flag those patients at a higher risk of disease spread to multiple joints, who could benefit from earlier therapy to prevent joint damage.

Methods
This study investigated how SF proteome profiles which discriminate JIA subgroups, fluctuate in individual patients over time as disease progresses. A difference gel electrophoresis (DIGE) approach was used to separate and quantify approximately 1000 synovial proteins. SF samples from 22 JIA patients were analysed: 10 oligo-, 5 extended oligo- and 7 polyarticular disease. Samples from each patient were labeled with Cy dyes and subjected to protein separation by 2-DE. The strength of correlation between levels of a select panel of SF proteins and inflammatory activity was tested over a two year period. Protein spots of interest which were over expressed beyond a two-fold threshold between patient subgroups were identified by MALDI-TOF mass spectrometry.

Results
Samespots software analysis of SF gel scans was used to highlight joint-specific proteins which were differentially expressed across disease classifications. Expression levels of a previously selected set of 40 proteins matched across the three clinical subgroups appear to follow a trend mimicking joint inflammation. The levels of a number of proteins rise commensurate with the numbers of joints inflamed in individual patients, as shown in **Figure 1**. Proteolytic fragments of apolipoproteins (33), complement component C3c (30), and haptoglobin (900) were identified ($p<0.05$) amongst the discriminatory proteins.

Innovative aspects
- Synovial fluid proteome profiles have been used to classify patients based on clinical subgroup definitions.
- Time resolved protein expression patterns have also been observed as disease spreads to multiple joints within individuals.
- The panel of identified proteins may play a role in spread of joint inflammation. With further validation, at functional levels, these putative prognostic biomarkers could improve the clinical management of patients.

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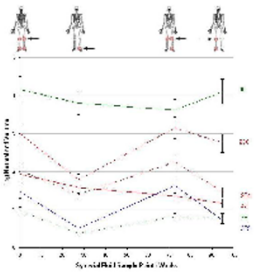

Figure 1. The protein spot volumes (LNV) from DIGE images of samples from a representative patient are plotted against time in weeks. The body above each data point indicates the number of swollen joints at the time of aspiration (encircled red) and the joint on which DIGE proteome analysis was conducted (black arrow).

H113

Semen Proteomics: Novel Technique to Identify Biomarkers of Reproductive Tract Disorders

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Introduction
Advances in mass spectrometry permit characterization of proteins in tissues or tissue cultures, serum or other bodily fluids. Such proteomic profiling studies provide means of searching for markers of various diseases either for the purpose of diagnosis, prognosis or monitoring response to treatment. Seminal plasma is a fluid that provides a safe medium for survival of spermatozoa. It contains secretions from the testes, epididymis, vas deferens, seminal vesicles and prostate; hence proteomic studies may identify potential biomarkers of infertility as well as prostate and testicular cancer.

Methods
Pooled seminal plasma samples (n=5) from fertile individuals (controls) and patients with obstructive-azoospermia (OA) were reduced, alkylated and digested in triplicate. Protein digests were then fractionated using strong-cation exchange (SCX) chromatography. SCX fractions were further fractionated using reverse-phase chromatography coupled in-line with LTQ-Orbitrap XL mass spectrometer. Resulting spectra was searched against the Human-IPI database using Mascot and X!Tandem and uploaded into Scaffold.

Results
We were able to identify over 1300 proteins in control and OA pools, and over 1500 proteins in control and OA pools combined. Compared to previous work by Pilch *et al* (1) where over 900 proteins were identified using an LTQ-FT instrument, over 600 are in common. Upon comparison of proteomes from control and OA pools, 15 proteins were found to be absent from OA pools, while 13 were absent from control pools. Additionally, spectral counting showed 39 proteins to be underexpressed in OA. Of these 67 proteins, 14 are of testicular or epididymal origin. These proteins are candidate markers of infertility. In addition, 259 proteins that are in common with proteins identified in prostasomes by Poliakov *et al* (2) potentially may be used as markers for prostate disease.

Innovative aspects
- Use of seminal plasma for identification of biomarkers of infertility, prostate and testicular cancer

References

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H114

CrkRS/CDK12 interacts with a novel isoform of Cyclin K & phosphorylates the C-terminal domain of RNA Pol2

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Introduction:
CrkRS (Cdc2-related kinase, Arg/Ser), or CDK12 (Cyclin Dependent Kinase 12), is a ser/thr kinase believed to coordinate transcription and RNA splicing. While CrkRS complexes were known to phosphorylate the C-Terminal Domain (CTD) of RNA Pol2, the responsible cyclin was not known. However, others showed that co-expression of CrkRS and Cyclin L could affect the alternative splicing of a model substrate[1]. We have isolated CrkRS protein complexes and identified Cyclin K as the primary cyclin partner of CrkRS.

Methods:
CDK/cyclin protein complexes were isolated by immunoprecipitation (IP) using polyclonal antibodies to the endogenous protein or by IP with an anti-FLAG antibody to isolate protein complexes containing an ectopically expressed protein fused to the FLAG epitope tag. Protein complexes were separated by SDS-PAGE, entire lanes were excised and digested in gel with trypsin. Proteins were identified by electrospray LC-MS/MS and spectra were assigned to proteins using the Mascot and X!Tandem algorithms. False positives were excluded by comparison to a mock IP experiment performed in parallel. The presence or absence of a protein in a complex was also analyzed by MRM. MRM transitions were developed using LC-MS/MS spectra obtained from ectopic FLAG-tagged fusion proteins isolated by IP.

Results:
We identified a novel 65 kDa isoform of Cyclin K in endogenous CrkRS protein complexes, whereas the known isoform of Cyclin K is 43 kDa[2]. The MS/MS data contained spectra which were assigned to tryptic peptides in an alternative Cyclin K open reading frame that could be generated by alternative pre-mRNA processing. We did not observe Cyclin L in CrkRS complexes by LC-MS/MS or by MRM. Previously, Cyclin K complexes were known to phosphorylate the CTD, yet the responsible kinase was not identified. Also, yeast-two hybrid and co-expression experiments identified CDK9 as a Cyclin K partner and that CDK9/Cyclin K could regulate transcription[3]. We show that Cyclin K complexes isolated from mammalian cells contain CrkRS but do not contain CDK9, and that CDK9 complexes contain Cyclin T1 and not Cyclin K, within detection limits. Co-expression of CrkRS with Cyclin K showed increased CTD kinase activity compared to controls. Our data suggests the primary cyclin partner for CrkRS is Cyclin K and that Cyclin K is sufficient to activate CrkRS to phosphorylate the CTD. The results indicate that our IP and mass spectrometry methodology can be used to clarify ambiguous CDK/cyclin interactions.

Innovative aspects:
- We identified a novel splice isoform of Cyclin K in CrkRS/CDK12 protein complexes by immunoprecipitation and mass spectrometry.
- We used Multiple Reaction Monitoring analysis to detect the presence of specific CDK/Cyclin sub-complexes within a CDK IP.

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H115

Understanding Innate and Adaptive Immunity: A Large Scale Proteomic Evaluation

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Introduction
The objective of this study is to address immunological mechanisms involved in defense against infectious disease, with an emphasis on immune response gene polymorphisms and their effect on protein expression that contribute to inter-individual variations in immune response. We are correlating these genetic polymorphisms with response to phenotypes, such as severity of infection, or with vaccination outcome. Here we present the results to date from this study obtained from a variety of proteomic studies including Luminex bead assays, defensin analysis, 2-D DIGE, and shotgun proteomics.

Methods
One thousand subjects in Kolkata India were vaccinated against typhoid using a Vi-polysaccharide antigen. On day 0, 3, and 28, saliva, serum and plasma samples were collected for DNA resequencing, SNP analysis (Illumina platform), 2-D gel electrophoresis (DIGE), IPG-IEF based shotgun proteomics, MS based defensin assays, and cytokine panels. Response to vaccination (responders and nonresponders) was measured using a newly developed Luminex bead assay. Samples were classified and analyzed according to response to vaccination, gender, age and, ethnicity. A large scale statistical analysis was applied to the 2-D gel data based in part on treatment of mRNA expression data. Significant research on proteomic sample prep and analysis for shotgun and targeted analysis was also performed[1,2].

Results
All proteomic and genotyping studies as part of this project are being compared against a new Luminex based assay to measure response to vaccination. This method produces substantially less background than standard ELISA based assays and is much more sensitive.

All proteomic assays are being compared against this response to vaccination standard, and are also being analyzed demographically. For example, trends have been observed in defensin levels as a function of age (HNP-2 & -3) and gender (HNP-1 & -2). Current studies are also focusing on gene copy polymorphisms and the effect on expression levels for these peptides involved in innate immunity.

Global proteomic studies performed by 2-D DIGE (700+ samples) were analyzed statistically using modified versions of ad hoc shrinkage (SAM) and empirical Bayes (LIMMA) shrinkage methods. Similar demographic trends were observed involving age, gender, and ethnicity. Studies involving response to vaccination are currently being interpreted (several cytokines have also shown strong correlations with response to vaccination as well).

Here we present a summary of these large scale study results and their correlation with response to vaccination, demographic trends, and gene polymorphisms observed in this population. Their effect on innate and adaptive immunity will also be presented.

Innovative aspects
- Large scale study examining the effects of polymorphisms on innate and adaptive immunity
- Large scale biostatistical treatment of over 700+ DIGE gels adapted from the microarray community
- Development of various saliva based proteomics assays for shotgun and targeted (defensin) studies

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HT-Yeast Two-hybrid for generating antibodies against the human proteome

H116

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Introduction

Affomix Corporation is developing a pipeline for the HT-generation of recombinant antibodies. *We are particularly interested in developing high-throughput methods (HT-methods) that can yield information about the affinity-binding of the antibodies during the initial screening process.* In the Y2H method (see Figure 1), it has been shown that the beta galactosidase (βGal) activity can be correlated with binding affinity of the protein-protein interaction. Likewise, we are developing a second method (Phage ESCape) that likewise can yield information about the binding kinetics of the antibody to labeled antigen.

Methods

The yeast two-hybrid system (Y2H) has been utilized extensively for identifying protein-protein interactions due to its ease of screening large numbers of potential interactors. We have constructed a high-titer scFv library from human cDNA in yeast which is used to screen against any protein epitope. Each well in a Master Library plate contained approximately 10k-100k different cDNAs. Each cDNA was represented within a well by about 1000 different yeast cells. For automated identification of interactors in the arrayed library, each pooled subset of the master library was aliquot to daughter plates, into which were seeded a bait-expressing strain of the opposite mating type. After mating, diploids are subjected to prototrophic selection and assayed for βGal activity. Each well of the three 96- well microtiter plates represents βGal assay on prototrophic selected diploids arising from a bait plasmid-expressing yeast strain mated to ca. 1000 yeast clones of a scFv library.

Results

In Figure 1, the right column in each plate represents both positive and negative controls. CPRG was used as the chromogenic substrate. Dark red wells are indicative of the presence of an interaction occurring within the well [see Buckholz, 1999]. (bottom, right) Test of *in vitro* specificity. For the ELISA, ELISA plates were coated with yeast lysates from cells expressing the target bait or a BSA control. The wells were blocked, incubated with phage for 2 hours at room temperature, and then incubated with anti-M13-HRP antibody. The ELISA was developed with Ultra TMB reagent, and reactions were stopped with 2M sulfuric acid. Absorbance was measured at 450 nm. Background represents bait lysates incubated without phage.

Innovative aspects

We describe a novel system of making the discovery of immunoaffinity reagents faster and cheaper. The option for expressing and purifying antibodies directly from *E. coli* or yeast is another potential application of the system.

The major advantages of the Y2H system include the elimination of the time and expense needed for purifying antigen (with the trade-off of being able to sample a smaller scFv sequence space in yeast and lack of an ability to look at most post-translational modifications). High value antigens can be screened in a second round by reshuffling first-round antibodies either by Y2H or phage screening.

References

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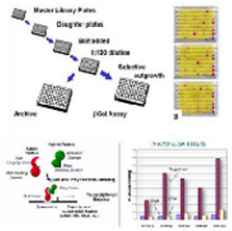

Figure 1. Schematic representation of the automated Y2H screen for random human scFv cDNAs. (top) Automated assay. **(bottom, left)** The yeast two-hybrid system (Y2H) has been utilized extensively for identifying protein-protein interactions. **(bottom, right)** Test of *in vitro* specificity.

Identification of Hypermethylated Genes in Lung Cancer Cell Line by Proteomics

H117

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Introduction

Lung cancer remains the leading cause of cancer death in many countries including China, and in recent years, lung adenocarcinoma (AdC) has an increasing frequency(1). DNA methylation is one of the epigenetics mechanisms of gene expression regulation, and promoter methylation of tumor suppression genes plays a critical role in carcinogenesis (2). Therefore, Identificating for methylation silenced genes may help to reveal the pathogenesis of AdC, and provide the important theoretical and scientific basis for the prevention and cure of it.

Methods

Demethylation agent 5-aza-2dC was used to treat A549 cell line, MTT assay was used to detect the cell growth, and flow cytometry was adopted to detect the cell cycle distribution and apoptosis. Two-dimensional gel electrophoresis (2-DE) was performed to separate the proteins of treated and untreated A549 cells with 5-aza-2-dC, PD-Quest software was used to analyze 2-DE images, and Q-TOF-MS was used to identify the differentially expressed proteins between the treated and untreated A549 cells. Then RT-PCR and Western blotting were performed to examine the expression levels of nm23-H1 mRNA and protein, one of the differential expression proteins in the treated and untreated A549 cells. Methylation-specific PCR (MS-PCR) was performed to detect the methylated level of nm23-H1 gene in the treated and untreated A549 cells.

Results

2-DE maps of the treated and untreated A549 cells with 5-aza-2-dC were established and a total of 56 differential protein spots were detected in treated and untreated A549 cells. 39 non-redundant differential proteins were identified by MS, of which 21 proteins were up-regulated and 18 proteins were down-regulated after 5-aza-2-dC treatment. The results of Western blotting, RT-PCR and MS-PCR showed that nm23-H1 is a methylation silenced gene in A549 cell line.

Innovative aspects

- High-throughput proteomics technology offer the potential ability to find methylation silenced genes in AdC.
- 5-aza-2dC could decrease the methylation level of nm23-H1 gene promoters, upregulate its expressions, inhibit cell proliferation, and promote cell apoptosis in AdC.

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Identification of Novel Biomarkers for Diabetic Retinopathy in Human Tears

H118

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Introduction

Diabetic retinopathy is a sight-threatening, chronic ocular disorder that affects nearly all patients with diabetes. This affects the retinal aspect of the eye, with abnormal blood vessels establishing and maturing on the surface of the retina. Without intervention, fibrosis of blood vessels, clinical macula odema and ultimately visual loss will eventuate. Tear proteins are important in the pathogenesis and characterization of various diseases of the eye. Using 2D-LC and in solution electrophoretic partitioning (MF10, NuSep), we investigated the differential tear profiles of diabetic and diabetic retinopathy patients against a healthy population for potential biomarkers to prognosticate the onset of diabetic retinopathy in human tears.

Methods

Open eye reflex tears were collected from patients with diabetes type 2 without retinopathy (N=??) and diabetes type 2 with retinopathy (N=??) and healthy controls (N=??) that were age and sex-matched. Samples were tryptically digested and analysed using 2D-LC. Additionally, samples were fractionated into multiple size fractions using an MF10 apparatus. The resulting fractionated samples were digested and proteins were identified using LC-MS/MSProteins were identified using MASCOT, searching a non-redundant database (NCBI; feb 2007). Data from these identifications were analysed using spectral counting (Scaffold v2.0) to determine quantitative changes of proteins between the three biological conditions.

Results

From the MF10 fractionation and 2D-LC analysis, several proteins were found to have differential abundance levels between the three biological states in human tears. A considerable number of newly identified proteins in tears presents us with new information which may be used to elucidate previously unidentified pathways in tear biology.

Innovative aspects

- Use of 2D-LC shotgun proteomics with human tears.
- MF10 fractionation to enrich tear peptidome.
- Characterization of proteins in tears with onset of diabetic retinopathy.
- Novel proteins identified which have previously not been detected.

The Protein Production Pipeline in the HPR-project

H119

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Introduction

The activity in the field of proteomics has increased rapidly during the last decade as a result of the large amount of sequence information available. A common problem when performing high-throughput experimental studies is often the availability of protein. To be able to supply the need for protein samples, groups around the world have used different approaches to set up high-throughput protein production pipelines. Here we will present how this was done in the HPR-project, an antibody-based proteomics effort with focus on protein profiling in human tissues ((1), www.proteinatlas.org).

Methods

In the Protein Factory module of the HPR-project, milligram amounts of high-quality protein, used for production of affinity purified polyclonal antibodies, are needed. To reach the monthly production goal of presently 600 pure proteins, the production setup needs to have a high capacity. For this purpose we have chosen to produce the proteins in shake flasks and use *E. coli* as host organism. To be able to purify all samples, a fully automated protein purification has been set up (2). The method is based on IMAC which is possible since all proteins are produced with an N-terminal His_6-tag. The protein concentration is determined using the BCA protein assay and the purity using SDS-PAGE. As a quality control MS is finally used to determine the molecular weight of all pure proteins.

Results

From the start of this project, six years ago, optimizations have been performed in parallel with the production to improve the success rate and increase the capacity. Almost every step in the workflow has been evaluated during this process and other options tested to find the optimal setup for the majority of all proteins (3).
To improve the protein expression culture volume, culture media, cultivation conditions and *E. coli* strain are some of the parameters that have been evaluated. The resulting changes have increased the success rate as well as the capacity in the protein expression.
Protein purification was initially performed on gravity columns on a lab bench, but as the number of samples increased this was no longer possible. For that reason the protein purification was fully automated.

Until today, the Protein Factory has cultivated over 48 000 cultures, representing more than 31 000 recombinant proteins. The optimized protocol used today has not only increased the capacity, it has also increased the overall success rate from 64% to 81%. Regarding the capacity, all optimizations have increased the throughput from just a few proteins to four batches of 72 proteins per week.

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The Effect of transforming tobacco leaves with a gene expressing the Heavy and Light Chain of rabies antibody on the proteome

H120

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Introduction

According to information obtained from the World Health Organization, more than 50 000 cases of human rabies are reported annually in the world and most of them are from developing countries. Once the signs and symptoms of rabies start to appear, there is no treatment and the disease is almost always fatal. Since the early 20th century, immune sera has been used as an essential therapeutic agent for the treatment of rabies but lately, plantibodies are being created from genetically derived plants and are used for the treatment of rabies. Amongst the advantages of producing recombinant proteins using plants are: the low risk of product contamination by mammalian viruses and pathogens, production for large scale biomass and the ability to introduce multiple trangenes. Using proteomics, we examined the changes to tobacco as a result of introduction of MABE559 polypeptides with a view to generate information that could facilitate optimisation of tobacco as a host expression plant-based system for the large-scale production of rabies antibodies.

Methods

Constructs of the heavy chain (HC), light chain (LC) and the cross (C) antibody were transformed into *Agrobacterium tumefaciens (A. tumefaciens)* and the clones were used to transform *Nicotinum tabacum cv. Petit havana (N. tabacum)*. For western blot analysis HC and LC were detected with goat anti-human antibodies conjugated to horseradish peroxidase. Human- derived mAb was used as a positive control. Expression of both chains was confirmed using Elisa to determine the effect of expressing the antibodies in *N. tabacum*; protein extraction, 2D SDS PAGE was carried out in replicates. Images were digitalized and analyzed with PD Quest software (BioRad Laboratories). Normalized protein spot volume was calculated for each gel. Spots were excised and analyzed by MALDI-TOF-MS (Bruker Daltonics).

Results

When comparing protein patterns on the 2-DE gels, we observed that the number of protein spots that were common between gels from HC, LC, C, and wild type ranged from 33-60. It was also discovered that gels expressing the LC of the rabies antibody had less protein spots than those found in gels expressing the HC of the rabies antibody (**Figure 1 and Table 1**). Proteins which were differentially expressed were analyzed and identified using Mascot. The preliminary run resulted in 21 protein spots being successfully identified, and other analyses are ongoing. From these preliminary results, it can be concluded that the presence of the various antibody chains resulted in the differential expression of endogenous tobacco genes.

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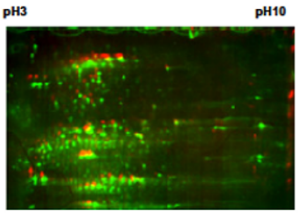

Figure1. An overlay of two dimensional gel electrophoresis protein profile of silver stained HC (green spots) and LC (Red spots). 22ug of protein was separated on first dimensional pH 3 -10 linear IPG gels and second 12% vertical gels. The isoelectric point is given at the top. Spots of interest were identified using MALDI-TOF MS. The preliminary number of protein spots that were matched are shown on table 1.

Gel Name	Number of Protein Spots	Matched
Non transgenic tobacco leaves	172	33
Heavy Chain tobacco leaves	236	34
Light Chain tobacco leaves	60	60

Table 1. Comparison of protein spots between the non-transgenic tobacco, tobacco expressing the light chain, and tobacco expressing the heavy chain of the rabies antibody.

A Human Protein Atlas for Profiling Cells, Tissues and Organs

H121

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The new version 5.0 of the Human Protein Atlas (www.proteinatlas.org) contains 8,000 validated antibodies targeting 6,800 genes corresponding to approximately one third of the protein-encoded genes in humans. The portal contains more than 6 million high-resolution images generated by immunohistochemistry and confocal microscopy. The antibodies have been generated to regions of low homology (1) and the long-term objective is to generate paired antibodies towards the protein targets with separate and non-overlapping epitopes (2). Pilot projects have been initiated to also generate recombinant affinity reagents (3) and a community-based portal for sharing antibody and antigen validation data has recently been launched (4) as well as a pilot version of a Rodent Brain Protein Atlas (5). We have used the human protein atlas as a discovery tool to find potential biomarkers for cancer diagnostics.

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Mapping and Measuring Proteomes

H122

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The human genome project has taught us that a complete map -in the case of the genome project the complete genomic sequence - along with computational tools to navigate the map, represent invaluable resources for experimental and theoretical biologists. A main consequence of such a complete map is that all the biological processes have to be explainable with the components that constitute the map.

For technical and conceptual reasons the generation of complete maps has been more challenging in the field of proteomics. One of the main difficulties has been the definition of a suitable end point, e.g. definitive proof that all the proteins in a sample have been identified. To overcome this limitation, we have chemically synthesized multiple proteotypic peptides for each ORF in the genome of the yeast S. cerevisiae and generated reference fragment ion spectra for these peptides, thus generating for the first time a complete map of a mass spectrometry observable proteome.

In this presentation we will discuss experimental and computational challenges related to the generation of complete proteomic maps and demonstrate the value of the generated reference spectra libraries for shotgun as well as selected reaction monitoring (SRM) based targeted proteomics strategies that will transform proteomics from perpetual proteome re discovery to accurate proteome measurement.

Innovation in Peptide & Protein Separation Technologies

(I101 – I133)

I101

A New protocol for High-Yield Purification of Recombinant Human CXCL8$_{(3-72)}$K11R/G31P Expressed in *Escherichia coli*

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Introduction
The ELR-CXC chemokines are important to neutrophil inflammation in many acute and chronic diseases. Among them, CXCL8 (IL-8), binds to both the CXCR1 and CXCR2 receptors with high affinity and the expression levels of CXCL8 are elevated in many inflammatory diseases. Recently, an analogue of human CXCL8 with mutations at both K11 and G31, CXCL8$_{(3-72)}$K11R/G31P (hG31P) has been developed. hG31P is a high affinity antagonist for both CXCR1 and CXCR2. We have developed a new protocol for high-yield purification of hG31P and for the removal of lipopolysaccharide (endotoxin) associated with hG31P due to the expression in *Escherichia coli*. The purified hG31P was tested by various biological assays.

Methods
Salek-Ardakani et al. reported a two-column chromatography method to remove both the LPS and Triton X-114 from the recombinant protein. We have modified the two-column chromatography method to a single SP Sepharose column method for hG31P. Because of its high PI value, hG31P can bind tightly onto the SP Sepharose resin under a low salt buffer condition. A citrate buffer with 180 mM sodium chloride and 1% Triton X-114 was used to wash and remove LPS from hG31P. The column was washed by citrate buffer to clear the residual Triton X-114.

Results
By using this method, the final LPS level of purified hG31P can be reduced from 13,500 EU per mg protein to 54 EU per mg protein which is less than the commercial standard of 100 EU per mg protein. The yield was found to be more than 92% with the Triton X-114 treatment. The purity of the hG31P protein was checked by SDS PAGE and mass spectroscopy to be > 95%. The final yield is 9.7 mg hG31P per gram of cell paste.

Innovative aspects
- High yield single column purification of high PI value proteins
- Novel protocol to remove lipopolysaccharide (endotoxin) associated with *E. coli* expression
- Can be applied to purify various proteins with high PI value and LPS association

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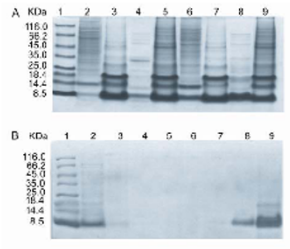

Figure 1. Analysis SDS-PAGE (A) difference procedure for cell lysis and pellets: lane 1, marker; lane 2, the cell lysate supernatant with 100 mM sodium chloride and not heating; lane 3, dissolving the pellet which was treated with 100mM sodium chloride and not heating; lane 4, the cell lysate supernatant with 100mM sodium chloride and heating for 10 min; lane 5, dissolving the pellet which was treated with 100mM sodium chloride and heating for 10 min; lane 6, the cell lysate supernatant with 700 mM sodium chloride and not heating; lane 7, dissolving the pellet which was treated with 700mM sodium chloride and not heating; lane 8, the cell lysate supernatant with 700mM sodium chloride and heating for 10 min; lane 9, dissolving the pellet which was treated with 700mM sodium chloride and heating for 10 min; (B) lane 1, marker; lane 2, the supernatant before loading into column; lane 3, the supernatant after loading into column; lane 4-7, washing with 60, 120,180+1% Triton X-114, 240 mM sodium chloride; lane 8, eluted with 600 mM sodium chloride; lane 9, purified (K11R/G31P)IL-8 and concentrated to 1 mM

I102

QUANTIFICATION OF PREDICTIVE TOXICOPROTEOMIC BIOMARKERS OF INFLAMMATORY DISEASES BY IMMUNOAFFINITY CAPILLARY ELECTROPHORESIS

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Many diseases caused by inflammatory processes, in particular chronic diseases, can become devastating illnesses with unbearable pain, deterioration of quality of life, and an overall poor prognosis for long-term survival. To address inflammatory diseases effectively, early detection and novel therapeutics are required. However, early detection of inflammatory diseases is challenging, in part because of the lack of adequate predictive biomarkers and the limited availability of powerful multi-dimensional technologies and methodologies, which are essential for the characterization and quantification of key predictive biomarkers present in biological fluids, cells and/or tissues.

Modern screening technologies in the field of genomics, proteomics and metabolomics have created new opportunities for the development of sensitive and specific diagnostic tools. Furthermore, the availability of biological fluids, cells and tissues from biorepositories or human specimen banks are facilitating our ability to monitor inflammatory diseases from the very early onset all the way to an advanced and terminal stage.

While this and other studies highlight the ongoing need for state-of-the art technologies and methodologies, with high-sensitivity and high-throughput capabilities, a dedicated, portable, miniaturized immunoanalytical analyzer, for determination of multiple biomarkers, is not yet commercially available. Despite the fact that many new biomarkers have been discovered in the last five years, no approach has yet been made to bring this advancement in diagnostic technology to the patient's bedside. The aim of this study is to present an overview of the various advances available today to further the discovery of predictive biomarkers of diseases related to inflammatory processes; in particular this paper discusses the technology of immunoaffinity capillary electrophoresis, which combines the use of antibodies as capture agents with the high resolving power of capillary electrophoresis. This two-dimensional technology permits the quantification and characterization of several biomarkers simultaneously, including structural changes of the separated proteins and peptides, such as variants, isoforms, peptide fragments, and post-translational modifications. Furthermore, the results are rapid and can be performed at a relatively low cost. The analyte-concentrator cartridge or device containing the immobilized antibodies may be reused many times. The design of the device permits integrated sample preparation and processing, and sample volumes range from a few microliters to milliliters improving significantly the sensitivity of the tests and permitting efficient sample analysis over a broad dynamic range of concentrations. The portable instrument covers applications of relevance to military, medical, pharmaceutical, industrial and research fields.

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I103

3D-Gel Electrophoresis, a New Approach to Protein Analysis

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Introduction
2-DE separation has not been considered suitable for large-scale comparative protein expression studies due to its limited throughput. We have developed a high-throughput 2-DE analysis method and instrument based on 3D geometry gel electrophoresis. With the 3D-gel instrument in its last phase of development and testing, we are investigating and initiating applications in molecular discovery, clinical diagnosis, pharmacology and toxicology, like protein monitoring during disease development and screening of drug candidates for their effect on protein expression.

Methods
Following conventional isoelectric focusing (IEF), up to 36 IPG-strips are arrayed on the top surface of a 3D-gel body and samples are transferred electrokinetically to the gel. SDS-PAGE occurs in vertical direction perpendicular to the loading surface. For optimum resolution and sensitivity, proteins are labeled according to differential in-gel electrophoresis (DIGE) protocols and detected online using laser-induced fluorescence (LIF). As the proteins pass a laser-illuminated detection plane in the 3D-gel, images are acquired by a digital camera and recorded as a 3D image stack. Image processing software draws vertical sections out of the 3D image stack, representing a series of 36 conventional 2-DE slab gels. Image matching and statistical analysis is performed using DECODON Delta2D software.

Results
To our knowledge, the 3D-gel is the only method for high-throughput 2-DE analysis with online LIF detection, providing results directly in electronic format without any further gel processing steps. A specific thermal management ensures that SDS-PAGE occurs under identical conditions for all samples, thereby permitting the immediate comparison of the separation patterns without elaborate software corrections. We tested the instrument for protein expression analysis of *Haemophilus influenzae* treated with antibiotics. Image matching revealed an unprecedented congruence of the separation patterns, leading to precise and highly reproducible results. A unique way to apply the instrument is to introduce the sample index (n = 1...36) as a third parameter to the analysis, *i.e.*, denoting the time, concentration, or dose of the sample treatment. The 3D-gel quantitatively measures the protein abundance as a function of that parameter, making it a valuable tool for large-scale comparative protein analysis.

Innovative aspects
- All samples of a series are analyzed simultaneously in the same 3D-gel: Direct comparability of separation patterns, no gel-to-gel variations, significant improvement in the detection of small differences in protein expression.
- Online data collection by photodetection: Results are immediately accessible without time-consuming gel incubation and documentation (no fixing, staining, destaining, scanning).
- A single 3D-gel accommodates up to 36 IEF-prefractionated samples: High throughput, easy handling, cost saving.

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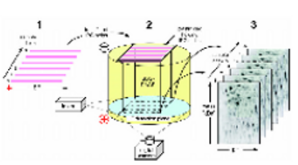

3D-gel electrophoresis workflow. *Step 1:* A series of 36 fluorescently labeled protein samples is separated by conventional IEF. *Step 2:* All 36 IPG-strips are arrayed in a slotted plastic grid and placed on the 3D-gel. Electrophoresis is activated and samples are transferred to the 3D-gel. During SDS-PAGE, laser-induced fluorescence is detected online by a digital camera and recorded as a 3D image stack. *Step 3:* Software generates vertical sections of the 3D image stack, representing a series of 36 conventional 2-DE gels.

RPLC Proteome Fractionation for Shotgun Proteome Mapping of MCF-7 Cell Membranes

I104

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Introduction

Comprehensive proteome profiling of membrane fraction of a cell extract is challenging, as it contains many hydrophobic proteins with varying concentrations. Protein level separation is needed to reduce its complexity. In this work, we report an optimized reversed-phase liquid chromatography (RPLC) method for membrane protein separation, followed by shotgun analysis of individual protein fractions using LC electrospray ionization (ESI) quadrupole time-of-flight (QTOF) MS/MS. Using this method, a relatively large proteome profile of breast cancer MCF-7 cell membranes has been generated.

Methods

MCF-7 membrane proteins were solubilized in urea, and separated by RPLC using a macroporous reversed-phase C18 column. Individual protein fraction was subjected to tryptic digestion, followed by strong cation exchange (SCX) chromatography. The peptides in the collected SCX fractions were quantified by LC-UV and selectively combined, dependent on sample amounts, or further fractionated by high-pH reversed-phase chromatography according to the peptide complexity gauged by LC-ESI MS/MS. All peptide fractions were then subjected to reversed-phase capillary LC ESI-QTOF MS/MS. Peptides were identified using the MASCOT search engine and Homo sapiens in the Swiss-Prot database. False positive matching rate was determined by searching the forward and reverse proteome sequences.

Results

In our initial work, we used a sequential solubilization method by NH_4HCO_3, MeOH and SDS to dissolve acetone-precipitated proteins from MCF-7 membrane protein. The sample complexity was simplified to a large extent. 5011 proteins were identified with a false positive rate of less than 1%. Although this method is extremely simple, it suffers from low separation efficiency, i.e., protein fractions have large overlaps. For NH_4HCO_3, MeOH and SDS dissolved fractions, 45%, 3%, 27% of identified proteins were unique to each fraction, respectively. To improve separation efficiency, RPLC was employed with optimized protein solubilisation and gradient elution conditions. In a preliminary test, MCF-7 proteins were solubilized in urea and separated by RPLC into three fractions. Each fraction was subjected to shotgun analysis and 306, 469, 358 proteins were identified from these fractions, respectively. Among them, 57%, 60%, 64% of identified proteins were unique to each fraction, respectively. This result illustrates a significant improvement over the sequential solubilization in terms of resolution. At present, the MCF-7 membrane proteins are being analyzed and results will be reported.

Innovative aspects

- RPLC fractionation of membrane proteins combined with LC-ESI QTOF MS/MS for improving protein identification efficiency
- Comprehensive profile of MCF-7 membrane proteome

Identification and Absolute Quantitation of *Clostridium acetobutylicum* proteins using 2D RP/RP LC MS

I105

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Introduction

Single-dimension and multi-dimensional reversed-phase nanoUPLC chromatography combined with the use of an LC-MSE acquisition mode was used to conclusively and stringently identify proteins from *Clostridium acetobutylicum*. Samples employed in the analysis were tryptic digests of whole cell lysates from different growth conditions. The application of the 'Ion Accounting' database search algorithm ranks preliminary peptide identifications against researched physiochemical characteristics of fragmented tryptic peptides, which provides a further level of confidence in protein identification. Simultaneous absolute quantification of the identified proteins showed detection of proteins present over 3 to 4 orders of magnitude using the 1D and 2D approaches respectively. Furthermore, the numbers of proteins identified showed a two fold increase on moving from 1D to 2D RPRP chromatography

Methods

Complex mixtures of proteins were extracted from three different growth conditions of *Clostridium acetobutylicum*; solventogenesis, acidogenesis and alcohologenesis. Individual samples were subjected to tryptic digestion, including reduction and alkylation, prior to analysis. Complex tryptic peptide mixtures were separated using a RP nanoUPLC system. In 1D this consisted of a trapping and analytical column with a gradient which rose to 40% ACN over 90 mins. In the 2D experiment an additional initial RP step was employed at high pH producing 6 fractions of the original sample, which were then processed on-line by the same trapping/analytical column configuration. The data-independent analysis mode (MS^E) was employed on the SYNAPT MS mass spectrometer enabling precursor and fragment ions from digests to be analyzed simultaneously.

Results

In all experiments numbers of identified proteins essentially doubled when moving from a one dimensional to a two dimensional RP chromatographic strategy. In the case of the ZBU315 sample 323 and 652 were identified in the 1D and 2D approaches respectively. In comparing the numbers of proteins identified in the replicate 1D and 2D RPRP experiments we find that many of the protein identifications are common to both analyses, with many additional identifications being made using the 2D workflow. In ZBU315 686 total replicated proteins were identified across both chromatographic methods. Of those 34 were found uniquely in the 1D experiment, 362 were found uniquely in the 2D experiment and 290 were identified as common to both experiments. These protein identifications were replicates over multiple analyses. The inclusion of a spike of phosphorylase B tryptic digest of a known concentration to the samples allowed the software not only to identify the components of the complex mixtures but also to calculate the absolute amounts of identified proteins1. In all the growth conditions of *Clostridium acetobutylicum* tryptic digests analysed, 3 orders of magnitude of protein concentration were detected in the 1D RP analysis and 4 orders in the 2D RPRP analyses. For example, the column loading for a single injection of the ZB313 sample analysed with 2DRPRP ranged from proteins detected between 0.02ng and 284.7 ng on column. The total column loading can also be calculated using this methodology – a total column loading of 333µg allowed the confident identification of 384 ZBU321 proteins under stringent conditions using 1D RP chromatography. A false positive rate of 0.26% was calculated for this experiment.

Innovative aspects

- Increased number of proteins identified by 2 D RP/RP separation
- Comparison of the *Clostridium acetobutylicum* proteome under different growth conditions
- Absolute quantitation of the identified proteins over 3 orders of magnitude

Sample Name	Number of replicated proteins identified by 1D RP	Number of replicated proteins identified by 2D RPRP
ZBU313	324	631
ZBU315	350	652
ZBU321	384	659

Figure 1. Numbers of replicated protein identifications in 1D and 2D RPRP analyses

Simultaneous Detection of Posttranslational Modifications of Proteins with Quantum Dot Technology

I106

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Introduction

The number of protein isoforms in the human proteome has known to be much higher than the number of genes in the human genome. This is in large part due to posttranslational modifications (PTMs) of proteins. With quantum dot technology, we developed a new proteomic method for simultaneous and multicolor detection of various PTMs of proteins on a single Western or immunoblotting analysis. The purpose of the study is to find new diabetic markers with disease-specific PTMs.

Methods

For detecting protein carbonyls, we used biotin-hydrazide (BHZ) as a derivatization reagent (1). After the derivatization with BHZ, proteins were separated by agarose 2-DE method (2, 3) and transferred to a PVDF membrane. Specific binding of biotin to Quantum dot (QD) 565-streptavidin conjugates was utilized for visualizing the biotin-derivatized protein carbonyls. Using a pair of primary antibodies to different PTMs on a single protein where one of the pair is a mouse antibody and the other is a rabbit antibody, a variety of PTM detection was possible. For detecting oxidatively induced methylglyoxal, we used mouse anti-methylglyoxal primary antibody and QD 585-anti-mouse IgG conjugates. For detecting polyubiquitination as a protein degradation signal, rabbit anti-ubiquitin antibody and QD 605-anti-rabbit IgG conjugates.

Results

We applied this method to examine the changes of the PTMs of proteins from skeletal muscle in a diabetes model Otsuka Long-Evans Tokushima Fatty (OLETF) rat and its control Long-Evans Tokushima Otsuka (LETO) rats. Protein carbonyls, polyubiquitination and methylglyoxal were detected simultaneously on a single blot. Both actin and creatine kinase were found carbonylated in the OLETF rats. However, only actin was barely ubiquitinated, indicating carbonylated actin was accumulated in the OLETF muscle.

Innovative aspects:

- A sensitive and quantitative method for multiplexed proteomics
- A means toward clarifying a comprehensive view of abnormal PTMs of proteins on disease proteomics
- Accelerating detection of marker proteins with disease-specific PTMs

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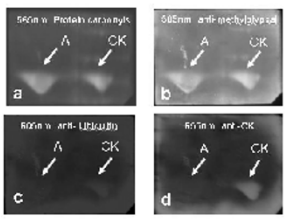

Figure 1. Western blot of skeletal muscle (gastrocnemius) proteins from a diabetic model OLETF rat. A: actin; CK: creatine kinase. (a) Biotinhydrazide derivatized protein carbonyls detected by QD 565-streptavidin conjugates; (b) Methylglyoxal detected by mouse anti-methylglyoxal antibody and QD 585-anti-mouse IgG; (c) Polyubiquitination detected by rabbit anti-ubiquitin antibody and QD 605-anti-rabbit IgG; (d) Creatine kinase detected by goat anti-muscle creatine kinase antibody and QD 655-anti-goat IgG. Each panel was obtained with respective narrow bandpass filters.

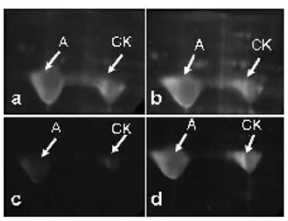

Figure 2. Chronological analysis of protein carbonylation of actin (A) and creatine kinase (CK). (a), (b) diabetic OLETF rats; (c), (d) control LETO rats. (a), (c) 12-week-old rats; (b), (d) 30-week-old rats.

Investigating Reproducibility of LC-MS Analysis of Complex Peptide Mixtures using FFPE Tissue

I107

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Introduction

In this work, we apply multiple LC-MS strategies to the analysis of extracted Formalin-fixed paraffin-embedded (FFPE) tissue archives, which potentially provide an alternative resource to fresh tissue samples. Five FFPE tissue samples have been independently extracted, digested, and analyzed using LC-MS to assess technical reproducibility and variability in sample preparation. 1D LC-MS was subsequently compared to 2D RP-RP LC-MS (1) approaches to assess depth of coverage and analytical reproducibility.

Methods

Proteins extracted from five FFPE tissue slices were digested with trypsin and diluted to 1µg/µL using aqueous 0.1%formic acid plus ADH internal standard. Samples were analysed by 1D, 2D-5step and 2D-10step LCMS at 400ng, 2.5µg and 4.5µg respectively. 2DLC was performed using a 150µmx5cm XBridgeC18(5µm) column with a discontinuous step gradient at pH=10 in the first dimension, followed by a pH=2 separation on a 75µmx20cm BEHC18(1.7µm) column in the second dimension. In each case triplicate injections were performed. Column eluent was coupled to the nanoelectrospray source of a Q-ToF MS, using the alternating low energy / elevated energy MSE analysis technique. Data was processed, database searched and compared with PLGS2.3 and a non-redundant Human database.

Results

Analysis of the different FFPE tissue extracts showed that the number of proteins identified was highly consistent across the replicates, using 1D LC-MS. For the 2D analyses, merging of individual fractions and searching shows that an increasing number of proteins are identified when the number of first dimension steps is increased. Approximately 450 proteins are identified in each of the 2D 5 step experiments and >600 proteins in each of the 2D 10 step experiments. This increase in protein identifications can be correlated to the loads which increase with the number of steps. When assessing the data for replication between the experiments using the same number of steps, high reproducibility between repeat injections of the 2D RP/RP technique is observed at both the peptide and protein level for each individual step, **figure 1**, and for each of the steps combined. Absolute quantitation (2), utilising the intensity of the top three peptides from ADH internal standard, shows that with increased numbers of first dimension steps, there is an increase in the number of low abundant species that are sampled, **figure 2**.

Innovative aspects

Reproducibility of 2DLC analyses of FFPE tissue.

References:

(1) M.Gilar, *et. al., J. Sep. Sci 2005, 28, 1694-1703.*
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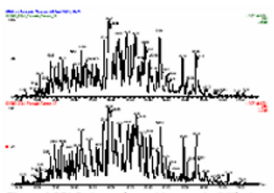

Figure 1 2DLC chromatographic reproducibility. Low energy MS^E chromatograms from the 17.4% first dimension acetonitrile step from repeated 2DLC analyses of formalin fixed paraffin embedded tissue tryptic digest. These traces were acquired approximately 14hrs apart.

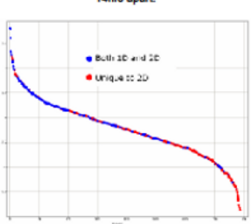

Figure 2 Absolute quantitation for observed proteins in 1D and 2D analyses. The graph shows that the 2D strategy samples the less abundant suggesting a much greater dynamic range for 2DLC.

I108

SRM to Identify Low Abundant Olfactory Receptors from Human Spermatozoa

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Introduction
The human spermatozoon is a highly specialized and glycosylated cell type which alters during capacitation process. Its plasma membrane (PM) includes important proteins for chemotaxis, signaling and fertilization.
Selected reaction monitoring (SRM)-experiments are especially suited for unambiguous identification of low abundant membrane proteins (e.g. olfactory receptors).
In the present study we could identify several low abundant olfactory receptors (ORs; part of the GPCR-family) in the plasma membrane after enrichment by an aqueous biphasic system and especially generated SRM-transitions.

Methods
Membrane proteins isolated from human spermatozoa were enriched by an aqueous biphasic system containing polyethylene glycol (PEG) and dextran (Dx; with or without coupled wheat germ agglutinin (WGA)) according to Persson and Jergil [1].
Pelletized membrane fractions were digested by a combination of trypsin and chymotrypsin overnight and obtained peptides were separated by multidimensional protein identification technology (MudPIT).
For ongoing SRM-experiments (TSQ Vantage; ThermoFisherScientific) 223 unique transitions have been chosen from 33 identified ORs [2].

Results
The complex proteome of human spermatozoa contains various high and low abundant signaling proteins. More than 4500 proteins could be identified after plasma membrane protein enrichment by an aqueous biphasic system with and without dextran coupled WGA. 347 plasma membrane proteins (PMPs) are part of the membrane fraction (2362). Interestingly, 36% (125) of the identified PMPs are G-protein coupled receptors (GPCRs).
Based on these results several unique peptides of 33 ORs (GPCRs) have been selected for SRM-approaches. Generally, a single peptide would be sufficient to clearly identify the OR due to its unique transitions. For data validation, up to three different peptides for a single receptor have been chosen.
Improvement of low abundant protein enrichment and fractionation techniques yields qualitative data for 19 receptors from 20 peptides by such SRM-experiments. Interestingly, the absence of coupled WGA is leading to an increasing number of 29 receptors from 34 different peptides.

Innovative aspects
- Identification of low abundant proteins due to specific transitions in SRM-experiments
- Method validation for low abundant ORs based on selected unique transitions for specific peptides
- Beside identification of low abundant proteins by SRM-approaches, simultaneous quantification of them will be enabled

References
[1] A. Persson and B. Jergil, Purification of Plasma Membranes by Aqueous Two-Phase Affinity Partitioning; Analytical Biochemistry 1992
[2] B. Han and R. E. Higgs, Proteomics: from hypothesis to quantitative assay on a single platform. Guidelines for developing MRM assays using ion trap mass spectrometers; Briefings in Functional Genomics and Proteomics 2008

I109

Analysis of proteins secreted by cultured cells in serum-containing media

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Introduction
The most common approach for secretome characterization is the analysis of conditioned media (CMs) obtained by cells cultured *in vitro*. Most secretome studies use serum-free conditions to eliminate the background due to serum proteins. Serum deprivation, however, often reduces cell viability and can affect secretome composition. We present a strategy - based on metabolic labelling and protein concentration linearization - to analyze CMs obtained by cells grown in standard media containing 10% bovine serum. The strategy was used to compare CMs obtained from three different breast cells lines.

Methods
Two breast cancer cell lines (MDA-MB-231 and MCF-7) and a non-tumorigenic immortalized epithelial breast cell line (MCF-10A) were cultivated in standard media containing 10% bovine serum. Cellular proteins were metabolically labelled by adding an excess of heavy amino acids (Val, Lys or Arg) to the culture media. After 3-days incubation, the CMs were collected and treated with the Proteominer hexapeptide resin to reduce the dynamic range of protein concentration. After protein identification by geLC-MS, isotope labelling was used to confirm the cellular origin of the identified peptides. The proteins reliably considered as human were relatively quantified in the three CMs using spectral counting or SILAC quantification.

Results
The treatment of the CMs with the hexapeptide resin yielded a significant reduction of the dynamic range of protein concentrations, as observed by SDS-PAGE (Figure 1) and confirmed by geLC-MS analyses. The total number of proteins identified in the mammalian database was at least 3-fold higher following the linearization, compared to untreated samples. Accordingly, the numbers of spectra matching to the most abundant proteins (i.e. serum proteins) significantly decreased after linearization. The presence of at least two labelled peptides was used to confirm the cellular origin of the identified proteins. By applying this constraint, for each linearized CM it was possible to prove as actually cellular about the half of the proteins matched to the human database. Metabolic labelling was thus essential to distinguish the cellular proteins from the serum ones, avoiding misleading identifications due to the high homology between bovine and human sequences.
To focus on the proteins differentially present in the three CMs, the relative amount of the genuine human proteins in the different samples was estimated using spectral counts and SILAC quantification.

Innovative aspects
- The CMs were obtained from cells cultivated in presence of serum (i.e. "standard" conditions)
- The Proteominer hexapeptide resin was use to "linearize" CMs in a comparative study
- Metabolic labelling was used to rule out false positive bovine proteins

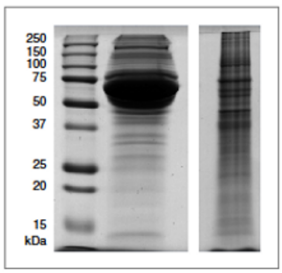

Figure 1. Coomassie stained SDS-PAGE of medium conditioned by MDA-231 cells cultivated in presence of 10% bovine serum. Left: raw CM. Right: CM treated with the Proteominer hexapeptide resin. The two lanes were loaded with 80 and 20 µgr of proteins, respectively.

I110

The Interaction Network of the Phosphatase PP1 through optimized LCMS for gel-free AP-MS samples

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Introduction

One of the most abundant serine/threonine phosphatases in mammalian cells is PP1. Through various regulatory subunits it targets hundreds of substrates with high specificity. Affinity purification mass spectrometry (AP-MS) is a technique used to identify protein interactions, and infer protein function and pathway connections. Gel-free AP-MS samples offer a challenging mixture that differs significantly in complexity and dynamic range from both gel bands and from cell lysates. Here we present the PP1 interaction network resulting from optimizing various LCMS conditions for gel free analysis.

Methods

FLAG-tagged PP1 protein was stably expressed to near endogenous levels in HEK293 cells and purified along with interacting proteins using anti-FLAG conjugated agarose beads (Sigma). After base elution, proteins were trypsin digested in solution. Resulting peptides were analyzed by LCMS using an Agilent 1100 HPLC and a ThermoFisher LTQ. Nano-LC was achieved with a passive split-flow and 75um x 10cm C18 packed tips. C18-C18 LC pre was done with Waters Xbridge and Symmetry media at pH 10 and 2.5. An Agilent 3100 OFFGEL system was used for PI based fractionation of peptides.

Results

The PP1 interactions detected include many known interactions as well as several new protein interactions that have not been previously reported. The gradient length of LCMS analysis significantly affected peptide and protein detection. Optimal gradients for 1D LCMS plateaued at 2h. While the impact of 2D-LCMS on a lysate sample was significant with respect to the total number of peptides and proteins identified, it had less impact than expected on APMS samples. While more peptides were detected using 2D-LCMS, the additional LCMS time required was less efficient, and there was not a substantial increase in protein identification. Similarly, the impact of pre-fractionating peptides based on PI was more pronounced with higher amounts of material loaded. At scales normally used for LCMS, the additional fractionation effort did not result in a measurable improvement.

Innovative aspects

Novel PP1 protein interactions
LCMS optimization for gel-free APMS samples

I111

Highly reproducible data from a microfluidic separation platform for proteomics

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The cHiPLC nano platform enables the acquisition of high quality, highly reproducible data by establishing a robust chromatographic platform for nanoliter separations. The reduction in retention time reproducibility and the ability to compare data across runs and columns opens avenues of study in discovery proteomics.

Introduction
For the analysis of complex proteomic mixtures the variability in the observed MS data and protein identification is a result of a combination of diverse factors. The enormous complexity of many proteomic samples can have thousands of peptides in abundances over many orders of magnitude. Online reverse phase separations have peak capacities in the hundreds, and the finite dynamic range of the mass spectrometer (both in terms of electrospray performance and detector considerations) often makes it difficult to achieve reproducible results. To compound this issue, inter-column reproducibility is difficult to achieve for capillary columns that operate at nanoliter flow rates. This is because the making of the connections for these nanoliter/min separations is highly skill dependent, and method robustness very difficult to achieve.
Microfluidic devices have inherent appeal as a chromatographic medium as the microchannels are defined lithographically and are highly reproducible. However, the difficulty in implementing microfluidic devices is in achieving a chip-to-world interface which does not sacrifice the performance gains from the microdevices themselves. We reported recently on a microconnector which facilitates reproducible microfluidic connections at nanoliter flow rates with a very high degree of consistency. In this poster, we will be reporting on our progress on a new microfluidic platform based on this microconnector for nanoliter separations on microchips and demonstrate that robust, high quality separations can enhance the reproducibility of proteomic experiments.

Methods
Nano2D-Ultra
Thermo LTQ
cHiPLC nano in direct injection format (15cm column, Bischoff C18 3micron phase)
New Objectives Integral Frit column (15 cm column, ProIFC-75-PP2-15, ProteoPepII)

BSA digest, Michrom standard digest
Protein mixture, HUPO/Invitrogen protein complement, reduced/alkylated/trypsin digest

Results
cHiPLC nano performance:
- BSA digest peptide retention times within 2%, intra- and inter-column
- >85% overlap in identified BSA peptides inter-run and inter-column
- >85% overlap in identified HUPO standard proteins inter-run and inter-column

Innovative aspects

Protease Substrates Discovery Using Terminal Amine Isotope Labeling of Substrates (TAILS)

I112

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Introduction. Proteases are essential to cell homeostasis and proteolysis constitutes an important, irreversible protein post-translational modification. Proteolytic activity ranges from limited processing (eg: propeptide removal) to complete degradation (protein turnover), modulating protein structure and functions. To better understand the biological activity of protease, we need to identify their biological substrates. Here we present Terminal Amine Isotope Labeling of Substrates (TAILS), a method allowing the identification of protease substrates from biological samples.

Methods. TAILS allows comparison of peptides from 2 proteomes, subjected to proteolysis or uncleaved, using quantitative MS (see Fig). Proteolysis generates new N-terminus. In TAILS, protein N-termini and lysines are labeled and blocked by reductive dimethylation with heavy and light formaldehyde for protease-treated and control samples, respectively. Proteins are mixed and trypsinized, generating internal tryptic peptides with free N-termini. Tryptic peptides are depleted using a newly developed, soluble, high molecular weight, amine reactive polymer, enriching the blocked peptides. These are analyzed by LC-MS/MS and quantified. Protease-generated N-termini peptides are found only in the protease-treated sample and are represented by N-terminally labeled singletons in the MS1 spectrum.

Results. Characterization of the high molecular weight, soluble, hyperbranched polyglycerol polymers developed especially for TAILS reveals very low non-specific interactions and binding capacity of up to 2.5 mg peptide/mg of polymer. To demonstrate that TAILS can identify protease substrates we digested the secreted proteome of cultured fibroblasts with GluC, an enzyme with strict cleavage specificity. Before enrichment of the blocked peptides, 49 GluC-generated peptides were identified, while 180 were found after the enrichment step. The proportion of tryptic peptides was reduced to 5% after the negative selection. We then sought to identify new matrix metalloproteinase-2 (MMP-2) substrates using TAILS. Following treatment of a secretome of MMP-2$^{-/-}$ embryonic fibroblast with exogenous MMP-2, TAILS identified 21 known MMP-2 substrates, 113 cleavage sites from reported, but previously non-validated substrates of MMP-2 or other MMPs, and 48 novel fibroblast secretome substrates. Four new MMP-2 substrates were validated *in vitro*, so proving the utility of TAILS for substrate degradomics.

Innovative aspects.

- The HPG polymers present the highest peptide binding capacity reported so far and demonstrate very low non-specific interactions with peptides.
- TAILS allows for the enrichment and identification of natural and protease-generated N-terminal peptides by quantitative mass spectrometry in a single experiment.
- Four new, biologically relevant, MMP-2 substrates were identified using TAILS.

Ultra-high-performance nanoLC-MS/MS Analysis of Complex Proteomic Samples

I113

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Introduction
Determination of the proteome and identification of biomarkers is required to monitor dynamic changes in living organisms and predict the onset of an illness. One popular method to tackle contemporary proteomic samples is called shotgun proteomics, in which proteins are digested, the resulting peptides are separated by high-performance liquid chromatography (HPLC), and identification is performed with tandem mass-spectrometry. Digestion of proteins typically leads to a very large number of peptides. For example digestion of a cell lysate easily generates 500,000 peptides. The separation of these highly complex peptide samples is one of the major challenges in analytical chemistry.

Methods
The main strategy to improve the efficiency of packed columns is either to increase column length or by decreasing the size of the stationary phase particles. However, to operate these columns effectively the LC conditions need to be adjusted accordingly. Naturally, the on-line coupling to MS systems has to be taken into account in the optimization process.

Results
Here, we report on the performance of nanoLC columns operating at ultra-high pressure. The effects of column parameters (particle size and column length) and LC conditions (gradient time, flow rate, column temperature) were investigated with reversed-phase (RP) gradient nanoLC. High-resolution LC-MS separations of complex proteomic peptide samples are demonstrated by combining long columns with 2 µm particles and long gradients. The effects of LC parameters on performance and the influence on peptide identification are discussed.

Innovative aspects

- Ultra-high performance nanoLC
- Combined optimization of stationary phase, column length and LC conditions.

Design of Monolithic nanoLC Columns for Ultra-High-Efficiency LC-MS Peptide Mapping

I114

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Introduction
Peptide sequencing by LC-MS/MS applied in proteomics research has made great advances over the past years in terms of sensitivity and scan speeds. However, in order to truly tackle present-day proteomic samples, separation performance is still insufficient. Different strategies have to be followed to increase separation power and this includes the development of novel column technologies.
Polymer monolithic columns have become an attractive alternative for packed columns. Especially for the nanoLC-MS analyses of complex samples they offer high-efficiency separations due to the absence of inter-particle mass transfer.

Methods
The porosity of monolithic materials can be influenced to tune the permeability and to reach maximum separation efficiency. In this study we optimized the morphology and column dimensions of monolithic columns to maximize the LC performance for peptide separations with nanoLC-MS. The porous properties of the monolith were systematically altered and investigated with scanning electron microscopy. The performance and the permeability of nanoLC columns upto 1 m in length was tested by the gradient-elution of complex tryptic digests. Experiments were conducted at low and high pH mobile phases with different ion-pairing agents. The nanoLC columns were coupled via electrospray to an ion-trap mass spectrometer operated in positive and negative ion mode.

Results
With the optimized monolithic structure, high-efficiency LC-MS peptide separations were obtained on long high porosity monolithic columns yielding peak widths smaller than 2 s. The maximum peak capacity on long poly(styrene-co-divinyl benzene) monolithic nanoLC columns was obtained at much longer (shallower) gradients than typically applied for conventional (5 cm long) monolithic columns. Peak capacities exceeding 1000 were achieved when using 1 m long monolithic columns with optimized morphology and applying 1 -5 h gradients.

Innovative aspects

- Application of 1 meter long polymer monolithic columns
- Very high efficiency LC-MS separations of complex peptide samples.

Protein Equalizer Based on scFv Displaying M13 Phage Library for Serum Proteome Analysis

I115

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Introduction
The decrease of proteome complexity by removing high abundance proteins is essential to discover low abundance ones with important biological functions. Although antibody columns are proven effective to deplete several kinds of high abundance proteins (1), it is impossible to remove unknown high abundance proteins. Righetti proposed the technique of protein equalizer by immobilizing combinatorial peptide ligands on silica, by which proteins were bound to beads according to the specific interaction with ligands instead of the amount (2). In our recent work, a novel protein equalizer based on scFv displaying M13 phage library was developed with monolithic cryogel as the matrix, by which the identified protein number from human serum was nearly increased by a factor of 1.85.

Methods
Macroporous monolithic acryogel was prepared at low temperature with acrylamide, N, N'-methylenebisacrylamide and allylglycidyl-ether as monomer, crosslinker and porogen. scFv displaying M13 phage library was covalently bonded to the matrix to achieve a novel protein equalizer. 2 mL of human serum proteins diluted by PBS to the final concentration of 0.2mg/mL was loaded onto 5mL cryogel matrix immobilized with scFv displaying M13 phage library, and kept at room temperature for 30 min. Then the collected eluants were loaded repeatedly for 5 times, followed by in sequence washing with 50mL PBS (pH7.4), 2M NaCl, 50mM Gly-HCl buffer (pH2.5) and 5mL thrombin solution (0.01U/µL). All collected fractions were neutralized, desalted, concentrated and frozen before analysis by µRPLC-ESI-MS/MS with a serially coupled long microcolumn.

Results
In our recent study, a novel protein equalizer was developed with scFv displaying M13 phage library covalently bonded on monolithic cryogel. The monolithic matrix with 10–100 µm wide interconnected pores was found suitable for the immobilization of M13 phage with decreased risk of pore blocking and improved hydrophilicity to avoid non-specific adsorption of proteins. By measuring phage library titer before and after immobilization, up to 5.2×10^{12} M13 phages were successfully immobilized on 5 mL cryogel, corresponding to 5×10^{6} copy of each kind of displayed scFv fragment, which meant that almost all phages with scFv fragments displayed were immobilized, ensuring high specific binding capacity between scFv fragments and target proteins in plasma.
For eluting bounded proteins, 2M NaCl was used to decrease ion-ion dominating interaction; 50mM Gly-HCl (pH2.5) was applied to disrupt strong conformation related interaction; thrombin solution was added to cleave the specific external fragmentation on phages, ensuring proteins with extremely strong binding capacity to be released completely.
All proteins or peptides collected from each elution step were further analyzed by µRPLC-ESI-MS/MS with a serially coupled long microcolumn. Compared to the untreated samples, the identified protein number was increased from 151 to 430 after treated by protein equalizer technique, as shown in Table 1. All these results demonstrate that the novel protein equalizer with scFv displaying M13 phage library immobilized on cyrogel could effectively reduce protein concentration difference in proteome samples, enabling more deep proteins to be found.

Innovative aspects

- Protein equalizer to decrease dynamic range of proteomes
- Monolithic acryogel as matrix with decreased non-specific adsorption of proteins
- scFv displaying M13 phage library as ligands to improve specific binding capacity with proteins

References

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Table 1. Identified protein number from human serum without and with the treatment by protein equalizer

Crude serum	Serum treated by protein equalizer				
	direct sample loading eluants	NaCl eluants	Gly-HCl eluants	Thrombin eluants	Total protein
151	186	89	64	151	430

I116

Optimization of the Sample Preparation in Urinary Proteomics

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Introduction
The workflow in a urinary proteomic study is usually complex and time consuming. All steps are possible sources of protein loss and could also contribute to variability. This work describes method development and optimization of the workflow in a urinary proteomic study, with special emphasize on keeping the protein recovery high and also making the steps as compatible as possible with each other. After optimization, a sample of pooled patient urine was analyzed to test the suitability of the final method in a complete bottom-up proteomic experiment. The repeatability was evaluated using both qualitative and quantitative parameters.

Methods
Sample preparation of urine was done according to figure 1. All steps have been evaluated and optimized. The patient sample was pooled urine from 3 kidney transplanted patients which after sample preparation were analyzed on an ion trap MS detector. Identification of proteins was done in MASCOT. Total protein for recovery data was measured using Bradford assay. For quantification purpose in biomarker search, an approach using ^{18}O-labeling was evaluated and implemented.

Results
Sample enrichment and desalting was done using centrifugal filtration (5 kDa cut-off). Although 8 M urea yielded highest protein recovery, TrisHCl/NaCl was chosen to redissolve the residual over the filter due to its better compatibility with the next step in the sample preparation. Separation of the trypsin generated peptides in an off-line two-dimensional Hydrophilic Interaction Chromatography (HILIC) – reversed phase chromatography (RP) system showed both good orthogonality and a high peak capacity. Repeatability of peak intensity in the second dimension (a measure for the entire methods variability) was evaluated using kidney transplanted patients urine (n = 6). Relative standard deviations between 11 % and 30 % were observed. We identified a total of 1668 peptides and 438 proteins from a single urine sample despite the use of low-resolution MS/MS equipment. Quantification using $^{18}O/^{16}O$ labeling in this workflow (figure 1) appears to be satisfactory for complex samples like urine (figure 2).

Innovative aspects
- Increasing downstream compatibility for less sample handling and reduced variability
- Identifying extent of total variability in a bottom-up urinary proteomic experiment
- Accelerated ^{18}O labeling in bottom-up urinary proteomics

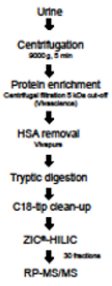

Figure 1 Overview of the sample preparation.

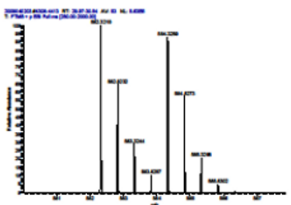

Figure 2 MS-analysis of 1:1 mixture of identical peptides: LVNELTEFAK labeled with $H_2{}^{16}O$ and $H_2{}^{18}O$ respectively.

I117

Specific Enrichment of Multiply Phosphorylated Peptides in Aliphatic Hydroxy Acid-Modified Metal Oxide Chromatography (HAMMOC)

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Introduction
Previously, we reported a novel phosphopeptide enrichment method using aliphatic hydroxy acid-modified metal oxide chromatography (HAMMOC). This approach made it possible to enrich thousands of phosphopeptides directly from cell lysates[1)]. We further developed a method for the successive and selective release of phosphopeptides captured by HAMMOC to enlarge the phosphoproteome coverage[2)].
However we found that identification efficiency for multiply phosphorylated peptides was not sufficient compared to that for singly phosphorylated peptides. To improve the identification efficiency of multiply phosphorylated peptides, specific isolation methods targeting multiply phosphorylated peptides are required. In this study, we examined the selective elution of singly and multiply phosphopeptides by optimizing elution conditions for HAMMOC to enlarge the coverage of phosphoproteome.
Methods
Commercial titania and zirconia beads were obtained from GL Sciences and ZirChrom Separations, respectively. A mixture solution of tryptic digest of α-casein, fetuin and phosvitin and the cytoplasmic fraction of HeLa cells digested by trypsin were prepared. These tryptic peptides were loaded onto self-packed titania/C2 or zirconia/C2-StageTips. Lactic acid and 3-hydroxypropionic acid (HPA) were used as chelating enhancers for titania and zirconia, respectively. Ammonium hydroxide, pyrrolidine, sodium methylphosphonate and triethylammonium phosphate (TEAP) were used for the phosphopeptide elution. The eluted fractions were desalted by C18-StageTips and then analyzed by nano-LC/MS/MS equipped with Thermo Fisher Scientific LTQ-Orbitrap or ABI/MDS-Sciex QSTAR XL. Database searches were performed with Mascot (Matrix Science) against SwissProt protein database.
Results
Firstly, to examine the properties of phosphopeptides enriched by HAMMOC and IMAC, HAMMOC-enriched phosphopeptides from 100 μg of HeLa cell lysates were loaded onto IMAC columns. As a result, most of the multiply phosphorylated peptides were captured and eluted in IMAC whereas singly phosphorylated peptides were dominantly observed in the flow-through fraction of IMAC. Note that the number of the HAMMOC-enriched multiply phosphorylated peptides captured by IMAC was larger than that by single IMAC enrichment and ca.60% of the multiply phosphopeptides by IMAC was covered by this HAMMOC-IMAC method. These results suggested that a lot of multiply phosphopeptides lost the chances for identification by interference of abundant singly phosphopeptides even though multiply phosphorylated peptides were present in the sample solutions for LC-MS in HAMMOC approach. We also examined the recovery in IMAC for HAMMOC-enriched phosphopeptides, and found that only 40% of phosphopeptides were recovered during this process. Based on this, we decided to develop new approaches for HAMMOC-enriched multiply phosphorylated peptides without using IMAC.
Recently Alpert reported electrostatic repulsion hydrophilic interaction chromatography (ERLIC) for selective isolation of phosphopeptides using WAX column[3)]. Since metal oxides such as titania and zirconia show anion exchange properties under the acidic conditions, the ERLIC-like elution, i.e., elution according to the phosphate number per phosphopeptide, would be expected. Therefore we followed the WAX-ERLIC elution conditions using titania HAMMOC. Consequently, it was found that the methylphosphonate buffer was effective to reduce the non- and singly phosphorylated peptides. Based on this, we combined the acidic elution buffers with the basic elution conditions which we have already developed previously as the successive and selective release (SSR) approach[2)] to maximize the phosphopeptide coverage including multiply phosphopeptides. The preliminary results indicated that this novel strategy increased twice the number of identified multiply phosphorylated peptide without combining other phosphopeptide enrichment approaches such as IMAC.
Innovative aspects
Phosphoproteome coverage was extended by optimizing conditions to elute singly and multiply phosphopeptides selectively in HAMMOC.
References
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(3)A. J. Alpert, *Anal. Chem.*, ***80***, *62 (2008)*

I118

Mapping the trypsin-resistant proteome by targeted multi-enzyme digestion of large tryptic peptides

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Introduction
Shotgun analysis after trypsin digestion of proteins to peptides is the most used workflow in proteomics. However, the proteome theoretically contains about 20-30% proteins or protein domains which are largely resistant to trypsin digestion because they contain very few or no basic residues. In the typical shotgun experiment this results in polypeptides too large to be easily detected and identified by tandem mass spectrometry. We have developed a strategy which targets specifically such trypsin-inaccessible polypeptides to obtain an improved coverage of the proteome.

Method
Our approach is complementary to the classical workflow. After tryptic digestion of the proteome, large tryptic peptides and non-digested proteins (Mw ≥ 3 kDa) were separated from smaller peptides by Size Exclusion Chromatography (SEC) and then subjected to specific enzymatic (Glu-C, chymotrypsin) or chemical (formic acid) hydrolysis. Both the entire tryptic digest and the large peptide fractions after the subsequent digestion were analyzed by RP-LC-MS/MS. Mascot database searches were performed with data from entire tryptic digest and separately with data from the large peptide fractions after secondary digestion. To quantify the gains achieved with this procedure, stretches of sequence identified uniquely in the large peptide fraction were mapped with a custom-made script.

Results
The method was applied to the soluble protein fraction of the yeast *Saccharomyces pombe*. 321 proteins were identified after trypsin digestion and a simple SEC/RP-LC-MS/MS analysis. For 160 of these proteins (average sequence coverage 23%) the analysis of the large peptide fraction after secondary digestion identified an average 4-5% unique sequence (a 20% relative increase) which was not detected in the trypsin dataset (Figure 1). The tryptic peptides found in large peptide fractions were mostly acidic (average pI of 4.5) and had an average mass of 4.2 kDa (max. 9.8 kDa). Thus the second digestion step allowed identification of amino acid stretches which were undetectable when the same fraction was analyzed after trypsin digestion only. By specifically targeting large fragments this method does not significantly increase the workload and allows the use of several cleavage methods to map difficult proteins or regions. Another advantage of this method over others in which the whole proteome is cleaved by two or more enzymes is that the small tryptic peptides retained their MS-suitable size. Hence, sequence loss caused by "over-digestion" of these peptides does not occur.

Innovative aspects
- Size exclusion separation of proteolytic peptides in the range 0.5-10 kDa
- Specific targeting of large, trypsin-resistant polypeptides by alternative enzymatic digestion
- Potential applications for the comprehensive study of post-translational modifications

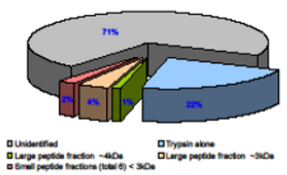

Figure 1 An illustration of average sequence of *Saccharomyces pombe* proteome obtained with employment of proteases Trypsin and Glu-C.

I119

Simple Immunoprecipitation of Phosphorylated Proteins From CHO Cells Using Magnetic Beads

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Introduction
Cellular responses to stimulus such as hormones and growth factors are commonly mediated by the activation of tyrosine kinases. Phosphorylation of tyrosine residues in proteins are due to the activity of both tyrosine kinases and phosphotyrosine phosphatases. Specific phosphatase inhibitors such as vanadate and pervanadate are useful tools in the study of tyrosine phosphorylation. Vanadium salts have been shown to have insulin-like effects in cell cultures.
We have studied the effect of protein tyrosine phosphorylation in CHO cells after treatment with pervanadate, (generated by vanadate peroxidation in the presence of H_2O_2). We have developed a preparation method using immunoprecipitation with anti-phosphotyrosine (anti-pTyr) antibodies, cross-linked to Protein G Mag Sepharose™ magnetic beads (GEHC, Sweden) followed by nanoLC MS/MS mass spectrometry analysis. Tyrosine phosphorylation is in very low abundance. By using a small amount of magnetic beads with highly selective antibodies and a large sample volume, both enrichment and concentration of phosphorylated peptides is achieved, resulting in increased sensitivity and efficiency during MS analysis.

Methods
CHO-cells were grown in rich medium and three hours before cell harvest, pervanadate, to a final concentration of 100μM, was added to the medium. The cells were centrifuged and a cell pellet corresponding to about $75x10^6$ cells was lysed with Mammalian Protein Extraction Buffer (GEHC, Sweden) supplemented with protease and phosphatase inhibitors. Non-soluble material was removed by centrifugation. The anti-pTyr antibodies (clone PY20, Sigma, USA) were bound to and cross-linked to Protein G Mag Sepharose, and finally the beads were washed with TBS. The beads were mixed with the cell extract followed by incubation for 1 hour at room temperature. After three washes with TBS and 1M urea, bound proteins were eluted with 100 mM phenylphosphate. The eluted proteins were reduced by adding TCEP to final concentration of 10 mM and heating the samples for 3 minutes at 95°C. The samples were allowed to cool to room temperature followed by cysteinyl alkylation with an excess of iodoacetamide for 30 minutes at 37°C. Trypsin was added and the proteins were cleaved over night at room temperature. 2.5 μl of the peptide sample was injected onto a C-18 RPC column (pepmap C-18, LC Packings, USA) and peptides were eluted with a gradient of increasing acetonitrile concentration. Finally, the peptides were analysed by MS/MS using an ion trap instrument (LTQ, Thermo Fischer, USA) followed by a database search against a rodent database.

Results
About 75 proteins were detected and from these about 50 proteins were unique in the pervanadate treated cells. Some of these proteins such as Caveolin-1, FAK and SHC are known to be involved in focal adhesion. These proteins may be involved in the regulation of the actin cytoskeleton. Additionally we also report other proteins that were identified and possible actions of these.

Innovative aspects
- Enrichment and concentration of phosphorylated proteins from extremely low levels using anti-pTyr antibodies immobilized on magnetic beads.
- This preparation method enables further studies of tyrosine phosphorylated proteins, that are known to be of very low abundance.

Comparative Peptide Separations by on-line MudPIT and IPG-IEF in an Off-gel Set-up

I120

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Introduction
High resolution peptide separation is crucial for successful shot-gun proteomics, thus propelling invention and improvement of ever more sophisticated approaches. Here we present the first comprehensive comparison of the established Multiple Dimension Protein Identification Technology (MudPIT)[1] to the newly emerging OFFGEL fractionation[2] followed by RP-LC-MS/MS. Using a membrane-enriched sample, we show that both methods perform comparably in terms of peptide/ protein identifications and reproducibility, but OFFGEL-RP-LC-MS/MS proves superior in peptide focusing.

Methods
Membrane-enriched fractions were isolated by cross-linking cationic silica beads to membrane proteins of murine C2C12 myoblasts[3]. Samples were centrifuged to isolate the beads resulting in a homogenate containing cytosolic proteins and a membrane fraction attached to the beads. Membranes were eluted using detergent 1% Triton-X100 to aid solubility of membrane proteins. After trypsin digest, peptide mixtures were subjected to either OFFGEL fractionation (i.e. IEF in an off-gel set-up) into 12 fractions over a pH range of 3 – 10 and recovered fractions analyzed by 1D-RP-LC-MS/MS, or directly introduced into an on-line MudPIT comprised of 12 steps to allow for accurate method comparison. All analyses were performed in quadruplicate.

Results
Similar numbers of proteins were detected with both platforms in total (MudPIT 1,428, OFFGEL 1,378) and on average (MudPIT 937, OFFGEL 893 per sample) with the majority of identifications (~ 64%) being identical (fig. 1). Significant overlaps of protein identifications were observed in-between replicates for both techniques, demonstrating good and highly comparable reproducibility. After OFFGEL fractionation, peptides are distributed over the whole pH range, yielding a trimodal repartition, whereas in MudPIT analyses, peptides were found to elute mostly in the early steps. OFFGEL demonstrates higher focusing quality, binning analytes into only one fraction for 88 % of all identified peptides on average. On the contrary, for MudPIT analyses, on average 61 % of the identified peptides are confined to one step and other peptides can be found in up to seven steps with a higher percentage, as mirrored in fig. 2. From GO-analysis and further investigation of proteins annotated as transmembrane proteins, we conclude that both techniques are equally capable of the challenging analyses of membrane-enriched samples.

Innovative aspects
- First comprehensive comparison of two state-of-the-art peptide separation techniques

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2. Horth, P.; Miller, C. A.; Preckel, T.; Wenz, C., Mol Cell Proteomics 2006, 5, (10): 1968-74

3. Durr, E.; Yu, J.; Krasinska, K. M.; Carver, L. A.; Yates, J. R.; Testa, J. E.; Oh, P.; Schnitzer, J. E., Nat Biotechnol 2004, 22, (8): 985-92

Figure 1: Protein detections are comparable with both approaches and the majority of identifications are identical

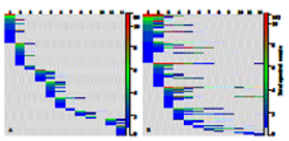

Figure 2: OFFGEL separation (A) confines a higher percentage of peptides to a single fraction than MudPIT (B)

Automated High Sensitivity Platform for Multidimensional LC/MS Analysis of Phosphopeptides

I121

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Introduction
Proteomics-based analysis of protein phosphorylation has the potential to elucidate signaling networks; however phosphopeptides are difficult to work with, and large scale analysis often requires milligram quantities of biological material. We have developed an automated high sensitivity mDLC-MS platform allowing for the identification of thousands of phosphopeptides from as little as 100 micrograms of cellular extract. Importantly, this methodology is compatible with multiplex quantitation schemes like iTRAQ, facilitating exploration of multiple biological conditions in a single experiment.

Methods
Cells were lysed in the presence of phosphatase inhibitors, proteins digested with trypsin, and when applicable, peptides labelled with iTRAQ reagent. After desalting, phosphopeptides were enriched using Fe^{3+}-NTA IMAC. Phosphopeptides were analyzed using a novel mDLC fractionation scheme which employed two Waters NanoAcquity LC pumps, autosampler, and 25 um columns with integrated emitter tips for ultra-high ESI-MS sensitivity. The platform was interfaced to a hybrid ion-trap/orbitrap mass spectrometer, or a QTOF mass spectrometer. A Digital Picoview Source (New Objective, Woburn, MA) was used to automatically position the tip at a predefined wash station during sample loading periods to help reduce build-up on the emitter.

Results
We have developed a platform (Figure 1) allowing for the identification of thousands of phosphopeptides from as little as 100 ug of cellular extract. Importantly, phosphopeptides are enriched in a single highly selective isolation step, and remain in-line during the entire mDLC fractionation scheme, avoiding losses of sample to plastic surfaces. In addition, the platform employs recently described ultra low flow LC/MS columns for high sensitivity nanoflow ESI-MS.[1]

To demonstrate the sensitivity of the platform, we analyzed 100 ug of extract from K562 cells, and detected >10,000 unique phosphopeptides. This represents a 100 fold improvement in efficiency as compared to other methods.[2] In addition, nearly 70% of phosphopeptides were unique to each fraction, indicating the high peak capacity of the separation scheme. We next performed quantitative profiling of phosphorylation events in a model system of human myeloid leukemia. Using this method, we identified >4000 phosphopeptides using 100 ug of extract from each biological condition. Work is ongoing to place regulated phosphorylation events in a functional context.

Innovative aspects
- Novel high peak capacity mDLC separation of phosphopeptides
- Inline separation of phosphopeptides to avoid sample loss
- 25 um columns and low nL/min flow rates for high ESI-MS sensitivity

(1) Ficarro et al, Anal. Chem. 2009 (EPub Ahead of Print).
(2) Dephoure et al, PNAS, 2008, 105, 10762-10767.

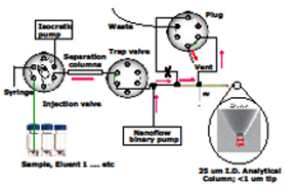

Figure 1. Valve and column scheme for automated mDLC/MS analysis of phosphopeptides (trapping configuration shown)

Quantitative Study of the Yield and Recovery of Proteomics Fractionation Methods

I122

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Introduction
Mass spectrometry analysis is a concentration-based assay, so low abundant yet important proteins such as signaling proteins and biomarkers are often not detected in complex samples like cell lysates and serum. It has become increasingly common to reduce the sample complexity via fractionation either at the protein level or at the peptide level. With quantitative approaches, we have compared both the yield and the recovery in four different fractionation systems: 1-D gel, SCX, OFF-GEL from Agilent and MicroRotofor from BioRad.

Methods
In this project, we used proteins extracted from honeybee head tissues and all mass spectrometry analysis was done in a LTQ-FT coupled with a nano-LC system.
To compare the yield from each condition, we mimicked the real experiment by fractionating the proteins or tryptic peptides into 10 or 12 fractions and then analyzed each fraction in mass spectrometry. To quantify the recovery rates, we used triplexed, reductive dimethylation to label peptides from different conditions [1]. Proteins and peptides were then fractionated, combined, mixed and a small fraction of the triplex mixture was analyzed in mass spectrometry. Data was searched against the Honey Bee Official Gene Set 1 [2] via Mascot, with a cut-off score corresponding to $p<0.05$. Data quantitation was done by MSQuant.

Results
With 10-12 fractions, 6,000-15,000 total peptides and 3,000-5,000 unique peptides were identified in one experiment and consistent differences were observed across the different methods. The fractionation step with each method has increased the number of unique peptide identified in comparison with the control sample in which every 1/10 of the sample was analyzed repeatedly. Protein fractionation had higher yields than peptide fractionation and the 1-D protein gel approach was most effective. (In peptide fractionation experiments, SCX and the two IEF systems (OFF-GEL and MicroRotofor) worked similarly well in improving the identification of non-redundant peptides.)

To determine the recovery rates, fractionation was coupled with chemical labelling to quantify the loss associated with each method. We have observed consistent recovery rates across three replicates with each of the two gel-based separation systems (1-D protein gel and OFF-GEL). Another interesting observation is the higher recovery from OFF-GEL peptide separation than from OFF-GEL protein separation. These findings allow us to tailor the fractionation procedures based on the goals of a given experiment.

Innovative aspects
- Rigorous, quantitative assessment of the yield and recovery in four fractionation systems
- Compare the overall effectiveness between protein and peptide fractionations
- Optimize the separation conditions in experimental settings

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Characterization of a Novel Rapid Fluorescent Gel Stain

I123

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Introduction
Fluorescent stains are commonly used in gel-based proteomics due to their high sensitivity and wide dynamic range. A novel fluorescent metal complex has been developed that can be used in solution to stain SDS-PAGE gels and the performance of this stain has been characterized with respect to its spectral characteristics, sensitivity, protein-to-protein variability and compatibility with mass spectrometry. Performance relative to commercially available gel stains was also evaluated.

Methods
Dilutions of a standard protein mixture were run on SDS-PAGE. The gels were stained with the novel metal complex and imaged using UV-transillumination. The resulting gel images were evaluated for appearance, limit of sensitivity, linearity and protein-to-protein variability. Excised gel plugs were subjected to in-gel digestion and MS analysis. Two-dimensional gels of complex samples were also stained and evaluated. All experiments were run in parallel using SYPRO™ Ruby staining for comparison.

Results
The stain absorbs maximally in the UV region of the spectrum and exhibits little visible light absorbance. Emission is broad and centered around 600 nm. This broad Stokes shift renders the stain ideal for use with UV-transilluminator-based imaging systems.
Optimal staining results were obtained using a simple protocol that required only immersion of the gel in the stain solution for 90 minutes without fixing or destaining.
Staining sensitivity, linearity and protein-to-protein variability were all equivalent to, or better than SYPRO Ruby. The stain was fully compatible with 2-D electrophoresis and MS-based protein identification.

Innovative aspects
- A new fluorescent gel stain has been developed that compares favourably with SYPRO Ruby in terms of all tested criteria.
- The new stain is uniquely rapid and simple to use, requiring only one ninety minute staining step.

I124

GELFREE: Molecular Weight Based Fractionation with Liquid Phase Recovery

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Introduction
Proteomics holds special promise for the development of improved disease diagnostics. Key to unlocking this potential is the development of technologies for the comprehensive discovery and validation of low abundance proteins and peptides in complex biological samples. Here we present a novel technology termed Gel Elution Liquid Fraction Entrapment Electrophoresis (GELFREE), which permits molecular weight-based fractionation with liquid phase recovery. The technology is capable of high sample loading, high protein recovery, excellent reproducibility, and high throughput.

Methods
A prototype eight channel GELFREE system was developed and assembled in house. Liver homogenate was prepared by lysis in T-Per lysis buffer (Pierce, Rockford, IL). The final protein concentration was adjusted to 10 mg/mL. 100 µL was prepared for electrophoretic separation by reduction with dithiothreitol (DTT) and heating in the presence of sodium dodecyl sulfate (SDS) to denature the sample. The sample was loaded and constant voltage of 120V applied for ca. 90 minutes. Upon the completion of each entrapment period, the fraction was removed from the collection chamber. SDS was optionally removed using precipitation and each fraction analyzed either using one-dimensional gel electrophoresis or liquid chromatography mass spectrometry using an Agilent 6340 ion trap.

Results
Fractionation conditions were optimized. Voltage was applied for an initial 14 minutes. Collection of subsequent fractions was as follows: fractions 1-5, 1 minute per fraction collection interval; fractions 6-10, 2 minutes per fraction collection interval, fractions 11-13, 5 minutes per fraction collection interval; fraction 14-16, 15 minutes per fraction collection interval. Figure 2 shows the 1D gel analysis of each of the fractions. LC/MS/MS data further confirmed the fractionation. The system was critically evaluated on the basis of reproducibility, recovery, and loading capacity. Reproducibility was found to be highly dependent on the consistency of the buffers as well as the casting of the gels. As a model protein, cytochrome C was simultaneously run and collected on eight independent GELFREE runs. The 1D gel data of the fractions confirm highly reproducible separation/collection. For recovery, 500ng of BSA was applied to the system and collected and run side by side on a 1D gel with a control of 500ng. No detectible difference in relative concentration was observed, suggesting >90% recovery. Loading capacity was determined to be ~1mg total protein.

Innovative Aspects
- Novel system for MW-based fractionation with liquid phase recovery
- >90% protein recovery
- Loading capacity of ~1mg total protein

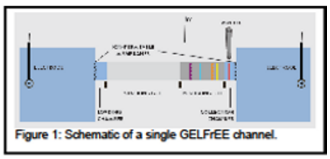
Figure 1: Schematic of a single GELFrEE channel.

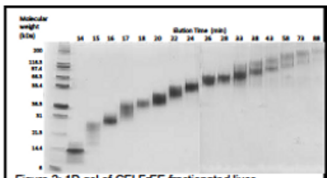
Figure 2: 1D gel of GELFrEE fractionated liver homogenate.

I125

Enrichment of Phosphorpeptides by a Ceramic Hydroxyapatide Micro Spin Column With Stepped Elution for Mass Spectrometric Analysis

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Introduction A major goal of current proteomics efforts is to identify phosphoproteins and understand their functional role. Quantitative phosphoproteome analysis requires isolation or fractionation of either phophoproteins or phosphopeptides. Currently, titanium dioxide or immobilized metal affinity chelates with bound Fe^{3+} or Ga^{3+} ions is the material of choice for phosphopeptide enrichment prior to MS analysis. CHT® ceramic hydroxyapatite is a sintered matrix composed of calcium and phosphate. Chromatography on hydroxyapatite is a well established technique for selective fractionation of phosphorylated analytes. In this study, we examined the specificity of a CHT micro spin column to enrich for phosphopeptides.

Methods We have developed a simple protocol which employs micro spin columns packed with CHT particles. Peptides (0.2 – 0.5 ml) were introduced onto the column and incubated for 5 min. After a series of washes, singly-phosphorylated peptides could be eluted with a low stringency buffer whereas multiply-phosphorylated peptides were eluted with a higher stringency buffer. The peptides were buffer-exchanged into a mass spectrometry-compatible solution prior to MALDI analysis.

Results To characterize the CHT protocol for phosphopeptide enrichment, we compared it with a Pierce phosphopeptide isolation kit in a series of tests. We first demonstrated that both methods could separate phosphorylated peptides from the non-phosphorylated ones using two commercial peptide standards. Both methods also isolated the phosphopeptides from the tryptic digest of a protein mixture containing α-casein, β-casein and ovalbumin. However, the step-elution in the CHT method provided a better detection of the multiply-phosphorylated peptides by MALDI-TOF-MS compared to the Pierce protocol. To further determine the selectivity of these methods, we applied 10 µg of the non-phosphorylated protein bovine serum albumin (BSA) digest to the columns. A significant binding of BSA peptides was observed with the Pierce column but not with the CHT column. The CHT method, but not the Pierce method, was able to enrich the multiply-phosphorylated peptides from a protein mixture with 1 µg of α-casein and 100 µg of BSA for MALDI-TOF-MS detection.

Innovative aspects Our study demonstrates that the CHT micro spin column offers an alternative to existing techniques for phosphopeptide enrichment. The CHT procedure provides rapid fractionation and high selectivity, especially for the multiply-phosphorylated peptides.

I126

High efficient proteome analysis with gel free protein prefractionation and peptide separation

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Introduction
Analysis of whole proteomes is an extraordinary challenge because of the complexity and wide dynamic range of proteins. Therefore, protein pre-fractionation is pre-requisite to achieve high efficient proteome analysis. In this study, gel-free protein pre-fractionation by preparative microscale solution isoelectric focusing (PMSIEF), off-line protein digestion, peptide separation by 2D-SCX-RPLC with serially coupled long microcolumn, and protein identification by ESI-MS/MS were combined, by which 1862 proteins were identified from rat brain.

Methods
1.95 µg of rat brain tissue lysate was separated into five fractions by a commercial PMSIEF device from Invitrogen within 4 hours (1). Proteins from each fraction were precipitated by acetone, and then redissolved in 50 mM Tris-HCl buffer (pH 8.0), followed by in solution digestion at 37 ℃ overnight, which were further analyzed by microscale 2D-SCX-RPLC-ESI-MS/MS (Fig. 1). For SCX, a total of 5 salt steps were performed with salt concentration as 160 mM，300 mM，500 mM，1000 mM and 2000 mM, and µRPLC was performed with a 30 cm long serially coupled microcolumn by connecting three columns with different lengths (5 cm, 10 cm and 15 cm of 300 µm I.D.) via two-port unions.

Results
In PMSIEF, 5 chambers with well-defined pH range, respectively as 3.0-4.6, 4.6-5.4, 5.4-6.2, 6.2-7.0 and 7.0-10.0, were divided by isoelectric membranes, and used for gel-free protein fractionation according to their isoelectric points (*pIs*). Protein recovery of 85% was achieved, much higher than that performed in gel. Furthermore, proteins focused in each chamber were digested, and further separated and identified by microscale 2D-SCX-RPLC-ESI-MS/MS. To improve the separation capacity of µRPLC, serially coupled microcolumn with length up to 30 cm was applied (2). As shown in Table 1, with 100 µg protein digests injected, over 200 proteins were identified for each fractionated proteins, and in total 1862 proteins were identified from rat brain. In addition, the fraction overlapping ratios of two adjacent fractions were respectively as 55.6%, 35.6%, 33.6% and 47.8% for fractions between 1 and 2, 2 and 3, 3 and 4, and 4 and 5, which showed the good pre-fractionation capacity of gel free PMSIEF. All these results demonstrate that by such a combination strategy, high efficient proteome analysis could be achieved, not only due to the in solution protein pre-fractionation and enrichment by gel free IEF with improved protein recovery, but also owed to the increased separation capacity of serially coupled long microcolumns applied in on-line microscale 2-D SCX-RPLC-MS/MS system.

Innovative aspects
- Effective method for protein pre-fractionation by gel free IEF with high protein recovery and low protein overlapping ratio
- A simple way to improve peptide separation capacity by on-line 2-D SCX-RPLC-MS/MS with a serially coupled long column for µRPLC.

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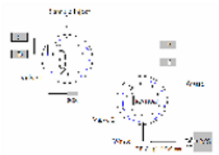
Figure 1. Diagram of on-line microscale 2-D SCX-RPLC-MS/MS system with serially coupled long microcolumn for the second dimensional separation.

Table 1 Identified proteins number for each fraction separated by PMSIEF followed by 2-D µHPLC-MS/MS analysis.

Salt steps	1	2	3	4	5	Total
MCE01	154	91	47	14	24	260
MCE02	292	351	336	39	9	734
MCE03	353	321	184	90	35	693
MCE04	96	67	99	69	14	205
MCE05	299	332	277	153	30	742
Total	1862					

I127

Parallel Electrophoretic Peptide Enrichment System for High Throughput Quantitative MRM Analysis

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Introduction
Peptides are important in drug development, as they represented nearly 10% of the total pharma market (US $31B) in 2001 (1). The analysis of analogues in the discovery phase demands generic high-throughput LC/MS procedures. Quantification from complex sample matrices requires efficient sample preparation to remove interferences that cause instrument pollution and ion suppression. Existing methods, such as protein precipitation or SPE, do not provide the desired precision or sensitivity. We present a novel electrophoretic technology for the enrichment of peptides for high throughput LC/MS/MS.

Methods
A custom multiwell plate was constructed that comprises 96 independent electrophoretic circuits, each of which contains a sample introduction chamber, a gel separation channel, and a peptide collection chamber defined by a 500 Da dialysis membrane. A 96 channel electrophoretic controller was constructed to provide independent voltage/current to each electrophoretic channel. A mixture of 10 test peptides (substance P, Neurotensin, met enkephalin, leu enkephalin, fibronectin binding protein peptide D3, and ACTH fragments 18-39, 5-10, 1-14, and 1-10) were spiked into human plasma at various concentrations. All data were generated using an Agilent 1200 LC system and an ABI/Sciex 4000 triple quadrapole mass spectrometer.

Results
The prototype is shown in figure 1. The sample well frame contained 96 sample wells, with corresponding tubes that were filled with both stacking and resolving gels (3% stacking, 8% resolving) using a Tris Glycine buffer system. A sample collection tray interfaced the columns with dialysis membrane. The base reservoir contained return buffer. Optimized operating parameters consisted of constant 30V (2mA) for ca 30 minutes for all wells simultaneously. Acid-cleavable anionic surfactant (Protea Biosciences) was used as a replacement for SDS. All samples were directly loaded onto the LC column and analyzed at a flow rate of 1mL/minute, permitting high throughput. Recovery was determined to be >80% for each of the 10 model peptides. CVs <10% were calculated for all 10 test peptides. No substantial retention time drift was detected with >300 injections, suggesting complete removal of matrix interferences. The LLOQ was found to be <1ng/mL for all peptides. The novel electrophoretic system was shown to provide the reproducibility, recovery, precision, and sensitivity required for broad peptide quantification experiments necessary to support pharmaceutical drug discovery efforts.

Innovative Aspects
- Novel system for high throughput peptide quantification
- Removal of matrix interferences
- CVs <10%; sensitivity <1ng/mL

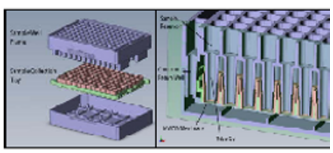
Figure 1: 96-channel electrophoretic cartridge.

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I128

Comparative proteome study on midbrains of normal and PD rats using 2D-HPLC with fluorescence detection

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Introduction
Since about 3% of people beyond 65 years old are suffered from Parkinson's disease (PD) [1], the proteome study is important for both diagnosis and treatment, which were performed by 2D-PAGE and 2-D DIGE with various cellular and animal models. In our study, after pre-column derivatization with our synthesized cyanine dye, b-sb-cy3-NHS, proteins extracted from midbrains of normal and PD rats were separated by off-line 2D-SEC-RPLC with fluorescence detection, and from 9 different fractions, a total of 20 proteins were identified, among which 15 proteins were not reported in previous study.

Methods
Proteins extracted from midbrains of normal and PD rats were firstly denatured in 0.95% SDS under 40 °C for 30 min. Then 8 mM b-sb-cy3-NHS was added and further stirred at 50 °C for 60 min in the dark to achieve protein derivatization. Finally, the derivatized proteins were separated by off-line 2D-SEC-RPLC with fluorescence detection with excitation and emission wavelengths respectively at 551 and 565 nm. The obviously differential peaks were manually collected according to retention time, which were further digested with trypsin, following by separation and identification by μRPLC-MS/MS.

Results
The water-soluble fluorescent reagent, b-sb-cy3-NHS, was developed for protein derivatization, as shown in Fig 1. After derivatization, samples were loaded on SEC column for pre-separation and totally 11 fractions were obtained. Each fraction was further separated by RPLC column. In our experiments, chromatogram peaks showing at least 3 folds altered in area were defined as the discrepancy expression peaks. One of RPLC chromatograms (from fraction 7 of SEC) was shown in Fig 2. It could be seen that the response of proteins after derivatization were obviously increased (Fig 2a1and b1) compared with that with UV detection (Fig 2a2 and b2) so that discrepancy proteins with low concentrations could be recognized.
By comparative analysis, total 20 proteins from 9 altered peaks were identified. Of these, 2 proteins, tubulin polymerization promoting protein, and superoxide dismutase, has been documented in recent studies annotating as "documented". Three proteins (α-internexin, ribosomal protein S27A, and isoform 1 of camp-dependent protein kinase catalytic subunit alpha) were annotated as "probable", because these proteins have been reported in the model of PD or AD determined by proteomics methods but no further evidence validating. The other 15 proteins were annotated as "potential" because none of them have been reported in PD research. Further work on the validation of such proteins is undergoing.

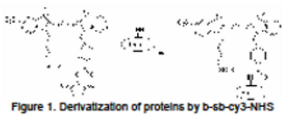

Figure 1. Derivatization of proteins by b-sb-cy3-NHS

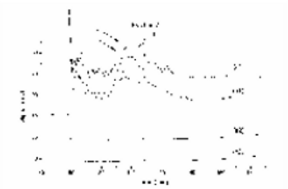

Figure 2. RPLC chromatograms of proteins from midbrains in Fraction 7 of SEC separation. a1 and b1 representing PD and the control with fluorescence detection, while a2 and b2 with UV detection.

Innovative aspects
- Proteins derivatization with water-soluble cyanine b-sb-cy3-NHS to improve detection sensitivity.
- Comparative proteome analysis combined with pre-column derivatization and off-line protein separation by 2D-SEC-RPLC with fluorescence detection for PD study.

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I129

A Global Isolation and Identification Method for Blocked N-Terminal Peptides by DITC-MNPs and MS

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INTRODUCTION N-terminal peptides play an important role in the determination of the half-life of a protein *in vivo*, and the terminal sequences of proteins can be used as ideal sequence tags for rapid protein identifications and confirmation of N-terminal processing [1]. However, N-terminal modifications result in blocked N-termini, and then hinder the analyses of the N-termini of proteins by conventional Edman degradation sequencing [2]. Therefore, selective isolation and identification of N-blocked peptides from the tryptic digest of the protein mixture remains a challenging work . ,In this study, a novel magnetic nanoparticle (MNPs) modified with isothiocyanate (DITC) groups was synthesized and then applied to the selective isolation and identification of N-blocked peptides by MS. The experimental results with the standard peptide and then the peptide mixture from protein extract from HepG2 cell showed that the newly synthesized isothiocyanate-coupled magnetic nanoparticle is very effective to separate and enrich the N-blocked peptides and can be used in N-termini determination of proteins in large scale.

MATERIALS Ferroso-ferric oxide was used as the core of particles, and coated with DITC to generate functional DITC-MNPs. A model peptide, Kemptide (LRRASLG), was used to test the performance of the functional magnetic particles. After treatment with DITC-MNPs, the left peptide solution was analyzed by MS. For further verification of novel functional materials by real application, the tryptic digest of proteins extract from HepG2 cells was first separated by SCX and then the fractionated with DITC-MNPs and analyzed by MS.

RESULTS AND DISCUSSION The structure of the nanoparticles was characterized by transmission electron microscope (TEM, JEOL 2000FX, Japan) and the average diameter of the prepared nano-scale particles was ~15 nm. Infrared spectrometry was used to further characterize the material which indicated that DITC groups were successfully bound to the nanoparticles.
Kemptide, was first used to validate the performance of new materials. The result showed that the free amino group of the kemptide could effectively react with DITC-MNPs as described as the reaction mechanism in Edman degradation method. But the acetylated kemptide was still left in the solution. So the N-blocked peptides could be isolated from the N-free ones, and then can be analyzed by MS. The binding capacity of DITC-MNPs to N-free kemptide was determined to be 2.67 nmol/mg by HPLC, and the recovery of N-blocked kemptide determined by iTRAQ method was 88%.
The effectiveness of the approach for the enrichment and identification of blocked N-terminal peptides in a complex biological sample was further verified by using the SCX fractions of tryptic digest of protein extract from HepG2 cells. In total, 215 different N-terminal peptides were identified, including 129 acetylated N-terminal peptides, 52 cyclized-peptides and 34 N-free peptides. The successful application suggests that the newly developed functional magnetic nanoparticles can be used to selectively enrich blocked N-terminal peptides for their easy and reliable identification by MS in large scale..

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I130

Novel separation and identification techniques for proteome study

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Introduction
With the in-depth research in proteomics, new techniques for proteome study have been paid more and more attention. In the past five years, a Chinese research team has been devoting into the development of novel materials, methods and techniques to achieve high resolution separation and high sensitive identification of proteomes, focusing on the depletion of high abundance proteins, enrichment of low abundance ones, multidimensional separation and high sensitive identification of proteins by MS/MS.

Methods
To decrease the dynamic range of proteomes, multidimensional array chromatography was proposed to remove various high abundance proteins simultaneously according to UV absorbance. To achieve the selective enrichment of low abundance phosphopeptides, several kinds of novel enrichment materials, such as Ti^{4+}-IMAC, Zr^{4+}-IMAC, magnetic particles coated by metal oxides (Fe_3O_4@M_xO_y) were synthesized. To establish an integrated platform for proteome study, on-line protein separation by ion exchange chromatography, digestion by microenzymatic reactor, peptide separation by μRPLC, and protein identification by ESI-MS/MS were combined. Furthermore, various ionization techniques and data mining methods were developed to improve the identification capacity of MS/MS.

Results
A platform of multidimensional array chromatography was established with SCX HPLC as the first dimension, and 8 parallel columns of RPLC as the second dimension. With UV absorbance unit over 0.1 V at 214 nm as the threshold, after the removal of 58 high abundance proteins from human liver tissue, the identified protein number was increased from 451 to 1213 (1).
A series of magnetic micro-particles coated by metal oxides (Fe_3O_4@M_xO_y), including Fe_3O_4@TiO_2, Fe_3O_4@ZrO_2 and Fe_3O_4@Ga_2O_3 were synthesized and showed high selectivity for phosphopeptides. In addition, Ti^{4+}-IMAC and Zr^{4+}-IMAC polymer particles were prepared as well, and compared to Fe^{3+}-IMAC material, 4.6 and 3.9 times of phosphopeptides were identified from mouse liver.
An integrated platform was established with proteins in sequence separated by a column packed with the mixture of weak anion and weak cation exchange particles (WAX/WCX) under a series of salt steps, on-line digested by an immobilized trypsin microreactor, trapped and desalted by two parallel C8 precolumns, separated by μRPLC with linear gradient of organic modifier concentration, and finally identified by ESI-MS/MS (Fig. 1), which was applied into the analysis of proteins extracted from human lung cancer cells. Compared to the results obtained by shotgun approach, 30% more proteins were identified with high confidence (FPR<0.05), and the total analysis time was shortened from 44 hr to 24 hr.
In addition, a method to calculate posterior probability of individual peptide identification from the "local false discovery rate" was proposed, and the sensitivity for peptide and protein identification was obviously improved.

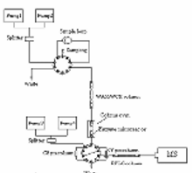

Figure 1. Schematics of the integrated platform for proteome analysis with the combination of on-line protein separation, digestion, peptide separation and identification

Innovative aspects
- Depletion of various high abundance proteins by multidimensional array chromatography
- Selective enrichment of phosphopeptides by novel magnetic particles coated by metal oxides and IMAC materials
- Integrated platform for proteome study with improved identification capacity and throughput

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I131

Novel mass spectrometry approaches to characterize ubiquitin-like protein function

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Introduction. The ubiquitin-like proteins (Ubls) are polypeptides that are covalently conjugated to proteins and other biomolecules to modulate their turnover rate, localization or activity. The full range of Ubl functions is only beginning to be understood, and the wide variety of Ubl conjugates is only beginning to be identified. Moreover, how Ubl conjugation is regulated, and how Ubl conjugate populations change e.g. in response to stress or in various disease states remains enigmatic. We are applying mass spectrometry and high throughput screening methods to better understand Ubl function.

Methods. While mass spectrometry represents a powerful tool for the characterization of many types of PTMs, standard sample preparation and data search methods are not amenable to the identification of many types of Ubl conjugates. Considering the importance of Ubls in normal cellular physiology, and their roles in disease etiology and progression, it is critical to develop improved high-throughput MS methods for the study of Ubls. To this end, we developed SUMmOn (1), an automated pattern recognition tool designed to detect diagnostic PTM fragment ion series within complex MS/MS spectra. To better understand Ubl function, we have also combined our mass spectrometric studies with large-scale functional screening (synthetic genetic array, or SGA analysis, and high throughput microscopy).

Results. Systematic functional genomics approaches were used to map networks centered on the *S. cerevisiae* SUMO (2) and Rub1/NEDD8 Ubl systems. Over 250 physical interactions were identified using the SUMO protein as bait in affinity purification-mass spectrometry (AP-MS). More than 500 genes were mapped by synthetic genetic array (SGA) analysis. The resultant global SUMO network highlights its role in 15 major biological processes. Using this resource, we identified specific roles for the SUMO system in the function of Cdc48p, lipid metabolism, Dna2p, and recovery from the DNA damage checkpoint.
We also mapped a network of 110 synthetic genetic interactions for Rub1, and over 320 Rub1 physical interactions were identified via AP-MS. The global Rub1 functional network highlights a role for this PTM in invasive growth, mitosis, chromatin remodeling and transcription.
Our SUMO and Rub1 functional maps represent valuable resources that can be mined to identify the roles of these regulatory systems in the control of individual proteins, protein modules, and signaling pathways.

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I132

Novel Proteomic Approaches for Biomarker Discovery in Clinical Samples

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Introduction: Prognosis and outcome of patients with astrocytoma is quite dependent upon stage of disease. While grade II and III tend to have lower mortality with treatment response, grade IV – more commonly known as glioblastoma multiforme (GBM) – is commonly lethal, demonstrating a median survival of just over one year. The identification of new therapeutics, companion diagnostics, and biomarkers represents a major, unmet medical need. Previous discovery efforts were hindered by the complexity, dynamic range, and quality of proteomes in clinical specimens.

Methods: Gemini™ integrates powerful laser-capture microdissection (LCM) based targeted sample capturing technology with a proprietary capillary isotachophresis/capillary zone electrophoresis (CITP/CZE)-based first dimensional separation coupled with nano-LC/MS/MS. In this study, we analyzed 7 grade II and III astrocytomas, 5 GBM, and 2 normal brain tissues using Gemini™ technology. Following LCM, 10 g of protein was used for tryptic digestion and peptides were fractionated using CITP/CZE coupled to nano-LC/MS/MS. The OpenMass Spectrometry Search Algorithm (OMSSA) was used to search the peak list files against a decoyed UniProt human database. A false positive rate based on an E-value threshold corresponding to a 1% was used as the cutoff in this analysis.

Results: By enriching as few as 10,000 tumor cells from each tissue specimen, a single proteome analysis produced over 15,000 unique proteins with 2 or more high-confidence peptide identifications. Proteins found exclusively in all 7 grade II and III astrocytomas, all 5 GBM, and both normal brain tissues were defined as "group steady proteins". Comparisons of "group steady proteins" from normal and tumor identified tumor-specific and normal-specific proteins. In addition, comparison of "group steady proteins" from different tumor stages revealed proteins related to disease progression.

Conclusions: In essence, our technology offers effective global proteome profiling from homogeneous cell populations isolated from clinical tissue specimens and a variety of body fluids, thereby ensuring the generation of biologically-relevant data and providing a direct view of the pathways involved in GBM. Because of the quality of the resulting data and the large numbers of proteins which may be quantified, it is possible to explore unique insights into the underlying molecular phenotypes that characterize GBM

I133

Exploring the complexity of post-translational modifications on salivary basic proline-rich proteins

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Introduction: Salivary peptides are usually classified in 5 different main groups: proline-rich proteins (PRP), histatins, cystatins, statherin and defensins. Basic and glycosylated (basic) PRP are the most complex group of salivary peptides, expressed by four different genes named PRB1-PRB4 clustered on chromosome 12p13.2. PTMs including phosphorylation and glycosylation could raise the complexity of forms belonging to this group as well as the several alleles for each PRB gene (1). Despite instrumental advances, up to date there is no experimental evidence of these modifications.

Methods: Unstimulated whole saliva was collected at 10:00 a.m. by direct draining into a saliva collection tube from 10 subjects, who had refrained from eating and drinking for at least 2 hours. Samples were acidified with trifluoroacetic acid (TFA 0.2%) in the proportion of 1:1, centrifuged at 12000g for 30 minutes (4ºC) and peptides were enriched by passing the supernatant through 50kDa filter cut-off. Peptide fraction was injected onto a LC system (Ultimate 3000, Dionex) using a linear gradient 0-55% ACN in 60 minutes. Fractions of 30nl were collected using Probot and all the fractions were analysed by MALDI-TOF/TOF (4800, Applied Biosystems). Data was processed using Mascot installed in a GPS (Applied Biosystems).

Results: The study of salivary proteins/peptides was achieved using peptidomics. We present the identification and PTMs characterisation of endogenous peptides resulting from proteolysis and of naturally occurring salivary peptides by nano-HPLC-MALDI-TOF/TOF. Using this methodology it was possible to identify for the first time i) several *N*-glycosylation sites: 1 in BPRP1, 3 in BPRP2, 2 on BPRP3 and 2 in BPRP4; ii) other modifications such as N-terminal pyro-Glu (4 modifications found in BPRP2) and phosphorylation in serine, iii) the 3 variants of BPRP1 (S, M and L) and 2 variants (from 3) of BPRP4 and 1 dbSNP of BPRP4.

Innovative aspects:

- Characterization of PTMs in endogenous salivary peptides;
- Determination of O- and N- glycosylation sites in salivary proteins/peptides;
- Using a peptidomic approach it was possible to demystify a part of the BPRP complexity.

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Integration and Storage of Proteomics Data

(I201 – I207)

I201

OmicsHub, a new generation of Data Standards Oriented LIMS integrating Analysis workflows

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Introduction
The diversity of instruments and workflows in Proteomics research can result in large amounts of data that requires versatile and robust software to handle such information efficiently. OmicsHub has been designed as a central data management system to collect, analyze and annotate proteomics experimental data enabling users to automate tasks yet remains flexible enough to allow manual control of final validation. It allows laboratories to effortlessly comply with standard initiatives, such as PRIDE[(1)], for standardized data management and to enable large collaborative and multicenter projects.

Methods
Based on a flexible and robust architecture, OmicsHub easily adapts to different laboratory environments providing seamless integration with the most common MS instruments, protein search engines and annotation tools. OmicsHub's back-end, based on FuGE[(2)] and PRIDE[(1)] object models and their corresponding XML data exchange formats, provides the required flexibility ensuring compatibility with future standard versions and extensibility to other 'omics' domains. In addition, OmicsHub is able to handle high-throughput proteomics data efficiently. Its user interface has been created using Web 2.0 technologies following basic usability rules to minimize the learning curve and improve user experience.

Results
OmicsHub supports both LC–MSMS and 2D-gel workflows and can be easily extended to meet custom laboratory needs with minimal effort. Its configuration allows users to process experiments in both parallel and concurrent modes simultaneously through customized pipelines. Identified proteins obtained as a result of PMF and/or PFF in the commonly used search engines are processed by OmicsHub to remove any redundancies automatically, providing a quick and clear idea of how many different protein families have been obtained. Furthermore, biological information extracted from a dynamic list of sources, including pathways information, can be used to annotate the resulting proteins, helping to better understand their role according to a specific experiment and/or project. Proteomics data can be exported in PRIDE format enabling lab users to easily share and publish their experimental results (Fig. 1). When providing services externally, custom HTML reports including descriptive images, can also be generated from OmicsHub, thus reducing one of the most time consuming tasks of a proteomics facility laboratory.

Innovative aspects
- OmicsHub enables users to manage proteomics experiments following PRIDE and HUPO-PSI initiatives such as FUGE.
- OmicsHub provides automatic integration of proteomics data and pathways information.
- OmicsHub for Proteomics is the first module of a wider platform which will allow users to integrate results from different "omics" techniques.

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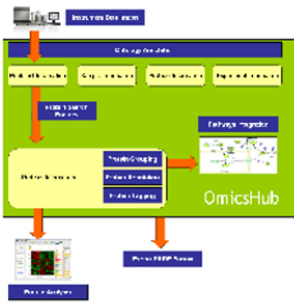

Figure 1. Overview of OmicsHub Entities & Pipelines

I202

OpenFreezer LARISA: An Enterprise Application for Reagent Tracking and Workflow Automation

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Introduction
With the creation of large-scale reagent collections and high-throughput approaches that utilize them, there is a strong demand among biological researchers for a central content management system to ensure consistent data storage, easy retrieval, and user-friendly output. We present OpenFreezer LARISA, a web-based LIMS that provides mechanisms for uniform organization of reagents and experimental results, as well as data analysis and workflow automation tools. OpenFreezer enables large-scale studies while being versatile enough to cater to smaller projects.

Methods
OpenFreezer is constructed as three-layer client-server architecture. The data layer is provided by a 3NF database running on a MySQL server. The domain logic layer consists of CGI modules written in Python employing OO programming principles; BioPython is utilized for implementing computational modules. The presentation layer is a graphical user interface written in PHP and DHTML. The number of physical tiers on the server side may range from one to two or more (database and server code situated on separate machines). OpenFreezer is platform- and browser-independent; functionality is preserved across UNIX, Windows and OSX, while the web interface is handled by Mozilla, MSIE, and Safari browsers. This architecture enhances software scalability, portability and robustness.

Results
At the core of OpenFreezer is a repository of reagents available within the laboratory and an interface for viewing virtual representations of these reagents. Users may interactively add new reagents and reagent types to the repository by defining sets of properties associated with these reagents, such as sequence, features, nomenclature, or cross-database identifiers. OpenFreezer implements a set of computational tools for experiment planning: primer design module; automated vector sequence constitution and feature mapping (using information stored in the system from vector parents); DNA to protein translation; modules for analysis and presentation of experimental data, where results are linked to reagents used in the experiments. Administrative modules enable data sharing in a multi-user environment. A rich, user-friendly graphical interface facilitates system use. We anticipate that OpenFreezer will become an essential resource and benefit the biological community.

Innovative aspects
- Versatile, expandable Laboratory Information Management System to track and organize reagents
- Development of customized features such as Primer Design, Automated Vector Constitution and Feature Mapping.
- OpenFreezer is to be open-sourced under GNU license and made available to the scientific community

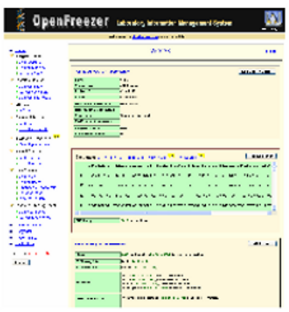

Figure 1. View of the Detailed Vector Page from OpenFreezer illustrating the information recorded for the clone V50123 including vector sequences and features.

Figure 2. Plasmid map of V50123 accessed via the "View Map" link on the Vector Details page; the map is generated using Python ReportLab graphic library each time the page loads.

I203

Accurate mass tag retention time data base for urine proteome.

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Introduction
There is a crucial need in clinical biology for new technologies allowing high-throughput identification and quantification of thousands of proteins in very short time frame (hours) to match the amount of available mRNA data. The Accurate Mass Tag –Retention Time methodology[1] is a possible answer to this need. The generation of databases of proteins involved in an organism's cellular functions is a necessary step to achieving these goals.

Methods
Sample preparation involved ultra filtration, drying, resuspention and cystein alkylation. Proteins were precipitated in acetone redissolved in lysis buffer and digested overnight with trypsin. LC MS analyses was performed using Thermo LTQ FT with homemade ESI emitter and Agilent 1100 with homemade LC column-Reprosil-Pur C18 (3um, 100 Å). All data were processed using the Mascot Distiller and the Mascot search engine software suites. Retention times for all peptides in every LC MS run were normalized using retention times of selected peptides common for all runs as internal standards. Concerning the AMT tag database format, we opted for the PNNL format database used by the VIPER software, which is freely available. MySQL v 5.0.45 with open code was used as database bases.

Results
The study group was intended to exclusively include healthy patients. They have been selected by analysis of archive information from medical records, current control for urogenital and other pathology including kidney pathology, prostatitis, arterial hypertension, and diabetes have been performed to include healthy persons. Treatments with diuretics and excessive consumption of fluids have been controlled as well. Most of the selected persons were permanent donors of blood and were included in the database. Information on harmful habits including smoking was recorded together with the time and date of urine collection. Overall, samples from 130 persons (81 males and 49 females) have been collected. Their age varied from 19 to 67 years for the whole study group (average value (±SE) was 37±1. years).Testing with Combi-Screen was performed for every sample. 39 new gene products were found in urine, from which 23 have been identified in more than 1 experiment. The final database contains over 2,900 peptides (or AMT tags) pointing to 464 protein groups. Individual proteins have been grouped by coverage with the same set or a sub-set of peptides in order to minimize redundancy.

Innovative aspects
The new accurate mass tag –retention time of urine proteome database has been created
39 new proteins were found in healthy person urine

Reference
(1) Lipton MS et al, Global analysis of the Deinococcus radiodurans proteome by using accurate mass tags, *Proc Natl Acad Sci USA*, 2002, 99, 11049–11054

I204

Salivary Proteome Wiki: A Collaborative Environment to Share and Annotate Proteomics Data

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Introduction
With the objective of enhancing our understanding of oral health and disease pathogenesis, researchers have recently completed a catalog of 1,116 proteins in human saliva using the shotgun proteomics approach (1).
The catalog that has been put together is an important first step towards the discovery and characterization of saliva biomarkers for various diseases. However, much effort is still required to enrich and refine the catalog, especially those proteins with relatively weak experimental evidence. To realize this goal, active participation from the research community at large is crucial.

Methods
The Salivary Proteome Wiki (SPW) is developed to as a central repository for researchers to store peptide and protein identification results. Contents are organized around the proteins that have been identified, integrating data from different research groups to ensure accurate characterization of the proteins. The system is built upon the MediaWiki software utilized in Wikipedia, with additional semantic plugins to help organize and evaluate the data. A text mining tool, AKS[2], has also been incorporated. Unlike other databases, the wiki encourages maximum level of interactivity and collaboration, where users have the ability to propose a wide series of changes on any data, ranging from adding new annotations to creating new data types to changing the way that information are displayed.

Results
Many proteome projects, such as the Human Salivary Proteome Project, involve researchers from across geographical locations. In addition to issues with data integration, working together on a day-to-day basis is not easy. SPW provides a collaborative model for exchanging ideas, sharing experimental results, and discovering new knowledge within a single platform.
Currently, there are more than 170 experiments, 2300 protein identifications, and 175,000 peptide hits stored in SPW as wiki pages. Pages are linked together through semantic annotations. These annotations are also used to create summary charts that offer a high level view of the data. In addition, users have access to a number of Web services (2), such as BLAST, to carry out further analysis. They can annotate the data using controlled vocabulary terms or free-text. Contributions can also be made by participating in discussions on associated talk pages.
As increasing volume of data are generated from high-throughput experiments, the ability to exploit all available resources within the community becomes more appealing than ever. Using Web 2.0 and Semantic Web technologies, this vision is one step closer to reality.

Innovative aspects
- Collaborative content development model with discussion, annotation, data import/export, and curation features
- Semantic markups to support complex *ad hoc* querying and machine-based reasoning
- Built-in tools to facilitate storage and analysis of proteomics data

References
(1) P Denny *et al*, The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions; J. Proteome Res. 2008, 7:1994-2006.
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Figure 1. Examples of figures generated in the Salivary Proteome Wiki using semantic annotations on the data to provide a real-time summary of the information stored in the system.

I205

MS Repo: Organizing, Analyzing and Sharing Proteomics Data

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We present MS Repo, a software system for the management and higher level analysis of data generated by proteomics experiments. Traditional ad-hoc data management techniques are no longer adequate for research projects that span multiple laboratories and last several years. Furthermore, publication guidelines put forth by leading journals mandate the reporting of enough data and context to assess and reproduce the results of an experiment.

The system we describe is completely web-based and manages data from multiple instruments and vendors. It has the explicit goal to support day-to-day activities in the lab with minimal overhead. The system's repository can archive data from years of experiments and make them accessible. As in any such system, it is crucial to keep metadata (the context of the experiment, sample information etc.) associated with primary data.

Much of the value of an information system for proteomics is realized at its periphery, where it interfaces with other systems such as protein identification tools, protein databases, instrument control databases etc. In place of an all-encompassing lab information management system (LIMS) we propose a federated architecture that allows independent software systems to cooperate. Pervasive use of meaningful URLs and a resource-oriented architecture make data easily accessible from external tools (Excel, ad-hoc scripts) as well as other information systems.

We close with an outlook of how the information processing landscape might look like in the context of local lab-based systems, inter-lab collaboration, and web-based resources.

I206

Improving untargeted differential analysis of mass spectrometry data by recursive feature extraction

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Introduction (maximum 600 characters, including spaces)
Processing mass spectrometric data for chemometric analysis typically involves feature extraction, followed by feature alignment across multiple sample data sets. Extraction algorithms often process data files one at a time, due to complexity and memory requirements of the raw data. Optimizing on speed over sensitivity and quality can introduce both false positives and negatives. Alignment of data containing false positives produces a sparse matrix of mass/retention time/abundance values, causing unreliable post-processing statistics and introducing noise into abundance profiling experiments.

Methods (maximum 800 characters, including spaces)
To improve the quality of feature extracted data and alignment matrix fill-in, we have developed a recursive feature extraction workflow to address the current shortcomings of mass spectrometric feature extraction on large data sets. This approach results in an iterative improvement in the quality and accuracy of mass, retention time and abundance values for each candidate feature, as well as validates the initial feature finding results, reducing false positives and false negatives.

Results (maximum 1200 characters, including spaces)
Eight high resolution Q-TOF LC/MS data files representing four technical replicates of two sample conditions were individually extracted using a feature finding and ion clustering algorithm. Between 1478 and 2389 features were found for each data file. Due to the presence of many false positives, the aligned feature list contained 4175 features. This candidate feature list was recursively re-extracted to obtain more accurate RT and signal abundance values. Following re-extraction, between 2321 and 2494 features were found per sample file, showing marked improvements in matrix fill-in (false negative reduction) and a two-fold loss of false positive noise.

Innovative aspects (maximum of 3 bullet points)
- Novel recursive feature extraction reduces false positive and false negative noise in chemometric analyses

I207

Comparison of Human Glomerulus Proteome using Antibody-based and Mass Spectrometry-based Proteomics

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Introduction
The glomerulus is structure for ultrafiltration of plasma solutes in the kidney and is also site, which most of kidney diseases originated from. Therefore, nephrologists have been focused on elucidation of molecular mechanisms for regulation of ultrafiltration in a physiological condition and for induction or progression of glomerular lesions in a pathological condition. We started proteomics analysis of normal human glomerulus to understand protein profiles of the glomerulus and to predict molecular interactions to regulate the ultrafiltration function.

Methods
Human glomeruli were isolated from kidneys, which were removed because of renal cancers, and the protein was separated into 5 fractions by liquid-phase electrophoresis and each fraction was run by SDS-PAGE. Each lane was divided into 15 gel slices for in-gel trypsinization and peptides eluted were analyzed by LC-MS/MS. Proteins derived from 2965 genes were identified by using Spectrum Mill (Agilent) and were listed in the Human Glomerulus Proteome database (http://www.hkupp.org). The mass spectrometry (MS) based-analysis was further compared with the antibody-based Human Protein Atlas (HPA) (www.proteinatlas.org) effort using immunohistochemistry (IHC) images of human kidney tissues showing the presence or absence of proteins in the glomeruli.

Results
Altogether 5,065 proteins (genes) have been analyzed by antibodies in Human Protein Atlas (HPA version 4.0) corresponding to approximately 25% of the human protein-encoded genes (approximately 21,000) and these genes were used for a comparative analysis. The analysis show that 13 % (n=660) of the total 5,065 proteins (genes) identified by HPA IHC are observed in the glomerulus proteome by both MS and antibody-based methods and 36 % (n=1800) are not observed by either assay. Thus, approximately half of the HPA proteins show concordant results (13% positive and 37% negative) based on these two completely independent analysis methods. Approximately 7 % (n=368) of the HPA proteins are identified only by MS and 44% (n=2237) are observed only by the IHC. These proteins need to be further validated preferably (i) on the immunological side, by using paired antibodies recognizing a separate and non-overlapping epitope and (ii) on the mass spectrometry side, by more sensitive assays to find low abundant proteins and procedures to rule out non-specific peptide identities.

Innovative aspects
- This study is the first comprehensive comparison of antibody- and mass spectrometry-based proteomics analysis demonstrating the power for profiling the human kidney glomerulus proteome, using two independent proteomics engines.
- Altogether 660 proteins (genes) have so far been validated by both assays as glomerular protreins and proteins uniquely identifed in the glomerulus are interesting starting points for more in-depth functional studies complemented with network analysis to explore the molecular interactions of these proteins.

References (maximum 3 references)
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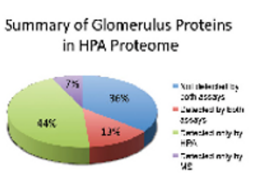

Figure Venn diagram illustration of the proteome of Human Protein Atlas detected by MS and IHC

Isotope-dilution Mass Spectrometry

(I301 – I303)

I301

Identification of the Target of an Innate Defense Regulator (IDR) using SILAC

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Introduction
Innate defense regulator peptides (IDR) are protective in mouse models of infection with important Gram-positive and Gram-negative pathogens (1). IDRs are not directly antimicrobial but instead have immunomodulatory and anti-inflammatory properties. The mechanism of action of IDRs is partially understood. They enhance the levels of selected monocyte chemokines while reducing pro-inflammatory cytokine responses. We investigated the molecular targets of IDR using Stable Isotope Labeling with Amino Acids in Cell culture (SILAC).

Methods
For the identification of IDR molecular targets, SILAC pull-down experiments were done with RAW264.7 cells grown in isotopically labeled and unlabelled media as described by Rogers and Foster (2). For pull downs, Streptavidin beads coated with desthiobiotinylated IDR or desthiobiotin alone were incubated with lysate from SILAC-labelled and unlabelled RAW264.7 cells, followed by LC-MS/MS analysis of the combined and digested bead eluate in an LTQ-Orbitrap mass spectrometer. Quantitative ratios were extracted from the raw data using MSQuant.

Results
We used SILAC to identify the main molecular target of IDR as Sequestosome 1/ p62, a highly conserved scaffold protein that has been reported to be involved in the regulation of ubiquitin modification processes in macrophages and promotes attenuation of cytokine transcription postactivation (3). Confirmatory experiments showed that IDR interacts with both the mouse and human homologues of p62.

Innovative aspects
- Discovery of Sequestosome 1/ p62 as the molecular target of Innate Defense Regulartors (IDR) using SILAC
- Confirmatory experiments of IDR's primary and secondary molecular mediators

References
(1) M Scott et al, (2007) An anti-infective peptide that selectively modulates the innate immune response. Nat Biotechnol. 25: 465-72
(2) LD Rogers and LJ Foster (2007) The dynamic phagosomal proteome and the contribution of the endoplasmic reticulum. Proc Natl Acad Sci U S A. 104: 18520-5.
(3) JY Kim and K Ozato (2009) The sequestosome 1/ p62 attenuates cytokine gene expression in activated macrophages by inhibiting IFN regulatory factor 8 and TNF receptor-associated factor 6/NF-kappaB activity. J Immunol. 182: 2131-40.

I302

Proteomic Analysis of Programmed Genome Rearrangements in *Tetrahymena thermophila* using iTRAQ and LC-MS/MS

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Introduction

Tetrahymena thermophila is a ciliated protozoan that contains two distinct nuclei: the somatic macronucleus (MAC) and germline micronucleus (MIC). During conjugation, large scale DNA rearrangements take place in the new developing macronucleus (anlagen) such as excision and elimination of internal eliminated sequences (IESs), chromosome breakage, amplification of MAC chromosomes. This unique feature makes *T. thermophila* a useful model system for the investigation of the complex processes involved during genome development. In an effort to understand these processes, we studied the nuclear protein expression from different developmental stages using the iTRAQ approach.

Methods

T. thermophila nuclei from different stages of growth and development were purified by differential centrifugation. The four types of nuclei analyzed were: MIC and MAC from vegetative cells, MAC from starved cells, and the newly developing macronucleus (anlagen) at 10 h of conjugation. Proteins from each sample are trypsinized and labeled with the differential tags, iTRAQ. The four labeled samples were pooled and separated, first by strong-cation exchange chromatography (SCX), and then by reverse-phase on a nano-LC column. Resulting MS/MS analyses of these fractions produced both sequence information and relative abundances. The MS/MS data files were searched against *Tetrahymena* genome databases using Proteinpilot (1) software for identification and quantification.

Results

A total of 322 unique proteins were identified in two replicate runs: 128 and 290 proteins were identified in run 1 and run 2, respectively. We chose a 2-fold change in expression as the threshold for significance. 18 and 30 proteins were overexpressed in the anlagen in run 1 and run 2 with 11 being overexpressed in both runs. A number of these proteins such as Pdd1p, Pdd2p, Twi1p and heterochromatin protein 1 are known to be expressed specifically during nuclear development, while overexpression of the other proteins appears to be a novel finding (2). The iTRAQ data were also correlated with expression microarray analyses of *Tetrahymena thermophila* during different stages of the life cycle (3). All the 11 proteins (overexpressed in both runs) were also significantly overexpressed by microarray data. The consistent results from both mRNA and protein level suggest that in those instances, differential expression is a result of regulation at the transcriptional level. They also indicate that these proteins might play important roles during genome rearrangement.

Innovative aspects

Proteomic analysis of genome rearrangement in *Tetrahymena*

References:
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Abstract category:
Organelle Proteomics
Quantification
Isotope-dilution Mass Spectrometry

I303

Multiplex quantification of heart failure biomarkers using Protein Standard Absolute Quantification

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Introduction

Improvement of biological diagnosis is closely related to technological developments that will provide multiplex quantification of biomarkers in biological fluids. MS-based approaches are currently attracting considerable interest due to their multiplexing capabilities. We have developed an innovative strategy, PSAQ (Protein Standard Absolute Quantification), which uses full-length isotope-labeled proteins as internal standards for MS-based quantification of proteins (1). Here, we present the potential of PSAQ for multiplex quantification of heart failure biomarkers in serum.

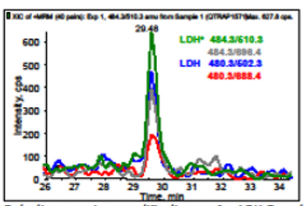
Methods

LDH-B, CKM, CKB and myoglobin were chosen as validated biomarkers of heart failure. Full-length isotope-labeled LDH-B, CKM, CKB and myoglobin (PSAQ standards) were synthesized using cell-free extracts purified and quantified. Serum samples (1mL aliquots) were spiked with defined amounts of each PSAQ standard and enriched by immunocapture. Eluates were submitted to SDS-PAGE (stacking format), proteins were digested in-gel and proteolytic peptides were extracted. Then, specific proteolytic peptides (proteotypic petides) were analyzed using Selected Reaction Monitoring (SRM) on a 4000-QTrap mass spectrometer (Applied Biosystems).

Results

To develop the assay, a titration experiment was performed using healthy serum samples artificially spiked with defined amounts of purified biomarkers and PSAQ standards. We determined from this experiment the analytical performances of the assay (accuracy, precision)

In healthy serum, all biomarkers could be detected at their physiological level (650 ng/mL for LDH-B and few ng/mL for CKM, CKB and myoglobin).



Detection and quantification of LDH-B at physiological concentrations in healthy serum using immunocapture, PSAQ strategy and SRM analysis. Transition signals from a proteotypic peptide in its two versions (unlabeled/labeled) are shown.

To validate our method, we analyzed clinical samples collected from patients with heart failure and compared our results with data obtained with reference immunological assays.

Innovative aspects
- In contrast with peptide standard-based quantification strategies such as AQUA (2), PSAQ is in essence compatible with sample prefractionation. PSAQ is thus the exquisite strategy for absolute quantification of protein biomarkers in immunodepleted or immunoenriched samples.
- We demonstrate that the combination of PSAQ, immunocapture and SRM is powerful to quantify simultaneously low-abundant biomarkers in a complex matrix such as serum. The additional use of scheduled SRM should further improve the multiplexing capabilities of this methodology.
- Coupled with SRM, PSAQ will undoubtedly advance biomarker candidate evaluation in biological fluids.

References
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Kinase-Protein Substrate Networks

(K101 – K112)

Integrated proteomic analysis uncovers the activity state of the intracellular signaling

K101

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Introduction
Although recent advance of phosphoproteomic analysis enables to identify thousands of phosphopeptides, it is still unsatisfactory for understanding the activity state of the intracellular signaling due to low coverage of the known phosphorylation sites of the key signaling molecules identified as well as lack of information on their expression level. Here, we describe a novel strategy for obtaining the activity map of the major signaling pathways by integrated proteomic analysis including phosphoproteomics and cell surface proteomics.

Methods
Whole cell extract of HCT-116 cells were reduced, alkylated, and digested with trypsin. Phosphopeptides were affinity-purified using MassPrep (Waters, MA) as described previously (1). Phosphotyrosine containing peptides were purified with anti-phosphotyrosine antibody (2). Cell surface proteins were labeled with cell-impermeable biotin and affinity-purified with avidin (3). Whole cell extract were separated with off-gel fractionators (Agilent, CA). Purified peptides were then analyzed by Paradigm MS4 nano-scale liquid chromatography system (Microm BioResources, CA) connected to the Finnigan LTQ Orbi-trap MS system (Thermo Fisher Scientific, CA). The database search was performed by Mascot Ver.2.2.02 (Matrix Science, London) against Refseq human database (NCBI). Search results were processed using in house software STEM.

Results
Independent LC-MS analyses of phosphopeptides enriched with metal affinity chromatography and anti-phosphotyrosine antibody led to the complimentary identification of phosphopeptides from HCT-116 cells, and the coverage of the intracellular signaling molecules increased significantly. This analysis also revealed that phosphorylation and activation of the non-receptor tyrosine kinases, particularly Hck, Fyn, and FAK, were striking, and adaptor proteins p130Cas and Shc appeared to be hubs of the protein complexes connecting activated receptor and non-receptor tyrosine kinases in HCT-116 cells. LC-MS analyses of whole cell extract pre-fractionated with off-gel fractionators and cell surface proteins labeled with biotin identified intracellular signaling molecules and transmembrane receptors, respectively, giving the information on their expression level. Combining the multiple proteomic analyses enables to observe whether the augmented phosphorylation of signaling molecule was stemmed from the pathway activation or up-regulation of the expression level. Thus, our integrated proteomic approach uncovers the activity states of the intracellular signaling ranging from plasma membrane to nuclear proteins.

Innovative aspects
- Coverage of the known phosphorylation sites of the major signalling molecules were enhanced by combining proteomic analyses of phosphopeptides purified with metal affinity chromatography and anti-phosphotyrosine antibody.
- Expression level of the signalling molecules including cell surface receptors were estimated by proteomic analyses of whole cell extract and cell surface proteomics.
- Combining data of phosphorylation and expression level, activity map of the intracellular signalling in HCT-116 cells could be obtained.

Reference
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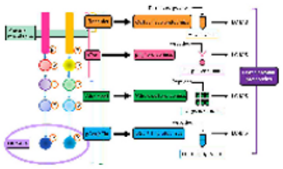

Fig.1. Strategy for obtaining the activity map of the intracellular signaling by integrated proteomic analysis. Cell surface, pTyr, phosphopeptides, and whole cell extract were isolated and subjected to LC-MS analysis independently. The obtained data were then integrated to generate activity map of the intracellular signaling.

PtpA, a *Mycobacterium tuberculosis* Protein, Blocks Macrophage's Signaling Pathways.

K102

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Introduction Since the 1990s, tuberculosis (TB) infection has strongly reemerged causing an estimated two million deaths per year[1]. TB is regarded as a growing health concern and a global health emergency. Understanding the mechanism of the disease in cells is a crucial requirement in achieving control of this deadly disease.

Macrophages are dedicated to combat infections. They normally engulf invading pathogens in organelles forming phagosomes which fuse with lysosomes – the vesicles containing digestive enzymes. Pathogens in the phagolysosome are usually degraded into peptides which are brought to the surface of macrophages to be recognized by the immune system. *Mycobacterium tuberculosis* (Mtb) blocks the fusion between the phagosome and the lysosome. This allows pathogens to "hide" in the host, multiply and infect more macrophages.

We have previously shown that Mtb interferes with the host's signaling pathway by secreting a protein tyrosine phosphatase, PtpA. PtpA was shown to block the phagosome-lysosome fusion required for the pathogen's elimination[2]. PtpA targets VPS33B (human vacuolar protein sorting 33B), a host cytosolic protein involved in the regulation of vacuolar protein sorting and membrane fusion[2]. We showed that PtpA dephosphorylates VPS33B thereby inhibiting the fusion of the phagosome with the lysosome required for pathogen digestion.

We hypothesized that in phagosome maturation arrest, PtpA dephosphorylates proteins downstream of VPS33B.

Methods Proteomic approach using PtpA incubation with cellular extracts followed by 2D electrophoresis revealed PtpA dependent dephosphorylation of 16 Host proteins. To verify if these proteins are bona fide of PtpA or VPS33B, the corresponding genes of interest were cloned and expressed for biochemical assays.

PtpA's ability to modulate proteins downstream of VPS33B activity was tested to determine if phagosome maturation arrest is solely due to the dephosphorylation of VPS33B or if it is due to direct dephosphorylation of these downstream proteins by PtpA.

Results Our studies show that all three Rab5 isoforms (Rab5a, Rab5b and Rab5c) as well as Rab7 are affected by PtpA. Rab5 isoforms and Rab7 are small GTPases involved in phagosomal maturation and facilitate fusion events between early and late organelles.

The mechanism of PtpA dephosphorylation and kinetics of dephosphorylation on its target proteins was determined. Through this approach, we hope to have a better understanding of the way PtpA succeeds in blocking phagosome and lysosome fusion and how it eludes the attention of the immune system.

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Exploring Signaling Pathways by Combining APMS and Phosphorylation Enrichment

K103

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Introduction

Understanding the protein network that impacts the specificity of kinases and phosphatases is critical to a better understanding of cellular signaling regulation. Phosphorylation and dephosphorylation events are mediated through regulatory subunits, adapter proteins, complexes, and direct enzyme/substrate interaction. In this study we have revisited the optimization of affinity purification and phosphoryation enrichment methods in unison to increase kinase and phosphatase network data. Expanded networks of protein interactions and phosphorylation sites for the yeast kinase MPS1 and mammalian phosphatase PP1 are shown.

Methods

FLAG-tagged proteins were purified from yeast or mammalian cell lysate using FLAG antibody passively coupled to protein G coupled magnetic beads. Samples were digested directly on beads, and the tryptic peptides were enriched for phosphorylated peptides. Samples were analyzed by nano-LCMS using an Eksigent Ultra HPLC and a ThermoFisher Orbitrap. LCMS data was analyzed with Mascot and X!Tandem, and phosphoryation sites were manually validated.

Results

The use of magnetic beads for affinity purification allowed for highly reproducible purifications in a shorter timeframe. The combination of efficient lysis, fast purification, efficient digestion and sensitivity gel-free analysis resulted in many new protein interactions and phosphorylation sites for both MPS1 and PP1 networks. More than 30 specific protein interactions for MPS1 were identified, along with more than 30 phosphorylation sites on interacting proteins and more than 35 phosphorylation sites on MPS1. Several novel protein interactions and phosphorylation sites within the PP1 network were also identified.

Innovative aspects (maximum of 3 bullet points)

- Novel kinase and phosphatase protein interactions
- Novel kinase and phosphatase network phosphorylation sites
- Comparison of phosphorylation enrichment techniques for APMS samples

K104

The Human SH2 Project: T-Cell Receptor mediated Protein-protein interaction Signaling network via SH2 domains

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Introduction

Selective protein-protein interactions, frequently mediated by interaction modules such as SH2 domains, are important in organizing and regulating cellular processes. The SH2 domain, is the largest family of protein modules that specifically recognize phosphotyrosine sites in signaling proteins and plays a pivotal role in relaying and modulating signals initiated by PTKs (1). Here we describe a targeted analysis of T-cell receptor signaling pathway using SH2 domain mediated affinity purification followed by LC-MS/MS (AP-MS/MS), peptide array and binding affinity studies.

Methods

Total cell lysate from CD3&CD28 treated Jurkat E6.1 T cells were IP purified using different GST-SH2 domains and separated using polyacrylamide gel-electrophoresis. In-gel digested protein bands were analyzed using nano LC-MS/MS system with reverse phase-LC Q-TOF. Protein identities were searched through both Mascot (Ver. 2.2.02) and Agilent Spectrum Mill servers. In-order to search possible phosphorylated sites identified proteins were filtered through Phosphosite & SMALI. Peptide arrays generated using those phosphopeptides were probed with relevant SH2 domains and in-solution binding assays were conducted to confirm direct interactors. Various data sources such as Ingenuity, Ambient pathway software, expression database etc were used to map physiological pathways of those identities.

Results

Protein- protein interactions mediated by 9 SH2 domains drawn from different subgroups of SH2 specificity-wheel led to identify 745 interactions involving 317 unique proteins from the AP-MS/MS approach (Figure 1) (2). Significant number of peptides on peptide array exhibited binding to the intended SH2 domain with some proteins harbouring more than one binding site. Combination of AP-MS/MS, peptide array and in-solution binding methods helped us to arrive at a high resolution signaling network for NCK1_HUMAN, which contains direct interactors (1st layer) and indirect interactors (2nd and 3rd layers) (Figure 2). The first layer interactors include proteins that contain at least one pTyr residue that displayed binding to the SH2 domain of NCK1. The remaining proteins were assigned by connection them through existing protein-protein interaction databases such as STRING, BIND etc.

Innovative aspects

- GST-SH2 pull down method is capable of recapitulating direct –phosphoprotein interactions
- Pathway mapping based on total protein identities through existing databases allow us to understand the underlying phosphotyrosine signaling pathways.
- Physiologically relevant protein-protein interaction identities will proceed on researching potential kinase/network inhibitors in cell based assays

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Figure 1 A free scale interaction network depicted from proteins identified from 9 SH2 domains following AP-MS/MS approach.

Figure 2 A high resolution TCR signalling network derived from AP-MS/MS, peptide array screening and in-solution binding studies for direct interactors for NCK1_HUMAN

K105

Selective reaction monitoring (SRM) reveals coordinated phosphorylations and protein interactions associated with EGFR endocytosis

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Introduction

Aberrant expression, activation, and downregulation of the epidermal growth factor receptor (EGFR) have causal roles in many human cancers, and post-translational modifications including phosphorylation and ubiquitylation, and protein-protein interactions directly modulate EGFR function. In an earlier study (1) we reported previously uncharacterized sites of EGFR phosphorylation at positions Y998 and S991, and showed them to be modulated by EGFR-directed inhibitors in xenograft tumors. Site Y998 (equivalent to Y974 without signal sequence) is part of an adaptin binding site and important in EGFR endocytosis (2). To address the function of these modifications their roles in EGFR signaling, trafficking, and protein-protein interactions were assessed by using MS and complementary methods.

Methods

MS analyses were applied to immunoprecipitated EGFR complexes from stably transfected human epithelial kidney (HEK293) cells. Proteins and modifications were identified and semi-quantified (spectral counting) with an LTQ-Orbitrap. Label-free relative quantification was achieved by selected/multiple reaction monitoring (SRM) with a triple quadrupole instrument (TSQ Quantum Ultra). Identical nano scale HPLCs and ion sources (Proxeon) were used with both MS platforms to facilitate data comparisons and SRM method development. Phosphorylation-defective proteins were made by site-directed mutagensis. Protein interactions and ERK kinase activation were confirmed by western immunoblotting. The stress-activated MAP kinase p38 was modulated by using the inhibitor SB 202190. EGFR endocytosis was monitored by immunofluorescence and quantified by FACS (Rhodamine-EGF internalization) and by loss of surface labelled (biotinylated) proteins.

Results

In response to EGF stimulation of cells, phosphorylations at EGFR S991 and Y998, measured by SRM analysis, accumulated more slowly than at receptor sites involved in RAS-ERK signaling. Phosphorylation-deficient mutant receptors activated ERK in response to EGF similar to wild type receptors, but were impaired for receptor endocytosis. The mutant receptors retained a network of interacting proteins, but with decreased association with certain proteins associated with receptor endocytosis including CBL, and ubiquitin. The endocytosis-defective mutant receptors were found to have elevated p38 MAP kinase-dependent phosphorylation at residues that mapped to a serine/threonine-rich region of the receptor previously implicated stress/cytokine-induced EGFR recycling (3). This suggests that receptor recycling and endocytosis are coordinated in part through discrete receptor phosphorylations.

Innovative aspects

These findings reinforce the notion that the function of the EGFR drug target is manifest through spatially and temporally regulated protein-protein interactions and phosphorylations, and demonstrate that these functional, pharmacodynamic parameters are measurable by quantitative SRM-based mass spectrometry.

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K106

Characterizing NPM-ALK Phosphorylation using Tandem Affinity Purification-Mass Spectrometry

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Introduction

The oncoprotein NPM-ALK promotes tumorigenesis by stimulating its auto-phosphorylation within the activation loop of its tyrosine kinase domain to induce the phosphorylation of other tyrosine residues within NPM-ALK, followed by activating several oncogenic signaling pathways in anaplastic large cell lymphoma patients. To characterize the NPM-ALK auto-phosphorylation mechanisms, we systematically mutated three tyrosine residues within the activation loop to produce seven Histidine-Biotin-tagged NPM-ALK mutants and used tandem affinity purification to purify these mutants to identify the phosphorylated residues of each mutant by LC MS/MS.

Methods

Mutations in the NPM-ALK activation-loop were generated by mutating Tyr-338, Tyr-342, Tyr-343 to phenylalanine to generate seven mutants: three single mutants-Y338F (FYY), Y342F (YFY), and Y343F (YYF), three double mutants-(FFY), (FYF), and (YFF), and one triple mutant-(FFF) using site-directed mutagenesis. The wild type and seven mutant NPM-ALK genes were constructed into a HB vector containing RGS-6x-Histidine (H) and biotin (B) tags. The constructed plasmids were separately transfected into GP293 cells. The core NPM-ALK fusion protein and seven mutants were purified with a fully denaturing TAP method, followed by LC-ESI QTOF MS/MS analysis. The phosphorylated residues of each mutant were identified using MASCOT search and manual spectral interpretation.

Results

To increase the likelihood of identifying phosphorylation sites of the 80 kDa NPM-ALK protein, we developed an efficient TAP purification process, followed by on-bead trypsin digestion and sensitive LC QTOF MS/MS analysis with precursor ion exclusion. Besides three tyrosine phosphorylation sites in NPM-ALK which have been previously identified: pY156, pY567 and pY664, we identified eight additional tyrosine phosphorylation sites in the wild type NPM-ALK transfected sample. Taken together, a total of eleven tyrosine phosphorylation sites were identified in the wild type NPM-ALK sample. The phosphorylated tyrosine residues in each of the seven NPM-ALK mutants were identified by analyzing both tryptic and chymotryptic digests.
Based on these data, we clarified how NPM-ALK regulates the phosphorylation of each tyrosine residues within or outside its activation loop. We believe that these new data have clarified the roles of certain residues in NPM-ALK phosphorylation and provided the potential for the development of the specific small molecule tyrosine kinase inhibitors for the treatment of ALCL patients.

Innovative aspects:

(1) Generating high sequence coverage (95%) of the core oncoprotein by using optimized tandem affinity purification under the denature condition.
(2) Characterization of the NPM-ALK auto-phosphorylation mechanisms using systematic site mutation and TAP coupled with LC-ESI-MS/MS.

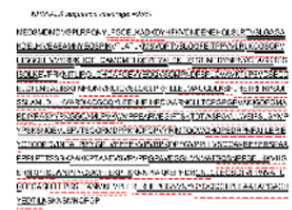

Figure 1: 95% NPM-ALK sequence coverage from both trysine (Black underline) and chymotrypsine (Red underline) digested samples.

Figure 2: Schematic depiction of NPM-ALK showing the location of the tyrosine residues, with phophorylated resides denoted.

K107

Easy Identification and quantitation of phosphorylation sites: An example using MEF2 transcription factors

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(Make certain you underline the presenting author's name)

Introduction

Identifying phosphorylation sites by mass spectrometry is difficult compared with traditional protein identification. Methods that improve the ease of identifying these sites can help in answering difficult biological questions. In this study, we used GSK-3 and p38 MAPK phosphorylation. These kinases play a central role in the development of muscle cells, by altering the activity of key proteins via phosphorylation. MEF2 is a family of transcription factors that are important in the control of muscle development. Several phosphorylation sites have been identified on members of the MEF2 family, including sites potentially controlled by GSK-3 and p38 MAPK. Understanding the quantitative aspect of this phosphorylation is important for gaining insight into the control mechanisms of this pathway.

Method

MEF2A samples treated with either GSK3 or p38 MAPK were subjected to trypsin digestion. This digest was analysed by a variety of mass spectrometry instrumentation to identify phosphorylation sites. MRM assays for these sites were designed, and these methods were run on a triple quadrupole instrument (AB Sciex). The MRM assays were designed by first identifying phosphopeptide using a precursor ion scan for the -79 phosphate ion. Prototype software was used to convert this result into a positive mode MRM method. The results of this run were searched for phosphopeptide identifications.

Results

An *in-vitro* kinase assay, using purified GST-MEF2A, identified GSK-3beta kinase has having a potential role in myogensis by targeted phosphorylation of MEF2. A sequence consensus analysis of the MEF2 family identified several potential GSK phosphorylation sites. Subsequent reporter activity assays confirmed that alteration of the activity of GSK-3beta causes slight changes in the ability of MEF2A to activate muscle specific protein expression. A cold kinase assay was performed using purified GST-MEF2A and GSK-3beta. The results of this reaction were analysed by QqTOF and LIT technology.
A separate TAP purified MEF2A sample that was expressed with p38 MPAK was also analysed by QqTOF and LIT technology. The p38 MPAK is known to phosphorylate MEF2A and demonstrates a significant change in the activity of this protein.

Potential phosphopeptides were first identified by precursor ion scanning for the -79 phosphate ion. The results of this run were used to create MRM-IDA methods to confirm and identify these phosphopeptides. Prototype software was used to automatically create these MRM methods. The results of these IDA runs were searched against protein databases. This two injection workflow resulted in almost exclusively phosphopeptide identifications, and was easy to perform. In addition, these MRM methods easily showed differences in MEF2 phosphorylation between various *in-vitro* kinase assays.

Innovative Aspect

- Quantitative analysis of the phosphorylation of a myogenic transcription factor.
- Easy identification of phosphorylation sites with no prior knowledge.

Identification of ATP binding proteins and its application to insulin signaling

K108

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Introduction
Many proteins have ATP binding pocket to use energy from ATP to play important role in many signal networks. Kinase, active membrane transporters, metabolic enzymes, microtubules, and proteolytic enzymes belong to ATP-binding protein families. Our goal is to enrich ATP binding proteins using ATP-affinity column from cell culture system and show protein profiles through proteomics technology. Furthermore, we applied this method to identify important proteins in insulin signal networks and novel target proteins to treat insulin resistance in diabetes patients.

Methods
Rat1 cells over-expressing human insulin receptors were cultured in DMEM with 10% FBS. The cells were grown till 80% confluent, starved for 16h and stimulated with 100nM insulin for 0, 5, and 30min. To confirm the insulin treatment, immuno blots were performed with phosphotyrosine antibody. For ATP-binding protein purification, ATP-affinity kit (JENA bioscience) was used. Before purification, free endogenous ATPs were removed by dialysis and then bound to the ATP resin. The eluant was dialyzed again before loaded on the SDS-PAGE for separation. In-gel digestion for each slice was processed using trypsin. Samples were analyzed by high resolution nanoUPLC Q-TOF premier MS/MS.

Results
ATP binding proteins were efficiently purified and analyzed. We identified approximately more than 300 of proteins from the Rat1 cells. As expected, most proteins identified have ATP binding motif. In addition, many proteins related to insulin signaling pathway were differently expressed over insulin treatment time. We showed for the first time the in-depth profiling of ATP binding proteins and new proteins related to insulin signaling. Finally, we analyzed this proteomic data with systems biology tools and showed several new pathways of insulin signaling.

Innovative aspects
- Establishment of a new targeted proteomic named as ATP binding proteomics.
- Identification of ATP-binding proteins in global level
- Identification of ATP- binding proteins related to insulin signal networks

References
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Evaluation of Kinase Inhibitors by Phosphoproteomics-based Phosphorylation Profiling

K109

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Introduction
Tyrosine kinase inhibitors such as gefitinib are useful as molecular-targeted drugs for cancer. Identification of the *in vivo* target proteins of these drugs is essential to develop molecular-targeted drugs because they often exhibit side-effects or no effect on selected patients. Here, we developed a method to evaluate protein kinase inhibitors by phosphoproteomics using a highly selective phosphopeptide enrichment method based on aliphatic hydroxy acid-modified metal oxide chromatography (HAMMOC) coupled with nano-LC-MS/MS.

Methods
Human cancer cells were cultured in SILAC medium and collected after stimulation with EGF in the presence/absence of various concentrations of protein kinase inhibitors. The cytoplasmic fraction from the cells was digested with trypsin. The digest was loaded onto HAMMOC tip packed with titania in the presence of lactic acid (1). After two washing steps, the bound peptides were eluted with 0.5% piperidine (2). The collected fractions were applied to nanoLC-MS/MS with LTQ-Orbitrap (Thermofisher) and Mascot (Matrix Science) search against SwissProt database was performed for phosphopeptide identification. Phosphopeptide quantitation was done based on the ratio of MS signal intensities using MassNavigator 1.2 (Mitsui knowledge Industry).

Results
First, we performed phosphoproteome analysis for 8 human cell lines under various conditions. The HAMMOC method enabled us to identify over 2000 phosphorylated sites with single LC-MS/MS run. In this study, totally 36,896 unique phosphorylation sites on 8,708 proteins containing 334 protein kinases and the related signaling molecules were identified from these cell lines. This result covers 83% of phosphoproteins registered in SwissProt database.
Our method was applied to quantitative phosphorylation profiling induced by the treatment of protein kinase inhibitors. Phosphorylation of the target kinases and their down-stream molecules was reduced by kinase inhibitor treatment (Figure 1). For gefitinib treatment, we observed the down-regulation in autophosphorylation sites of receptor tyrosine kinases other than EGFR. By clustering phosphorylation profiling data for the kinase inhibitor treatment, six kinase inhibitors were classified based that profiling the kinase inhibitors is possible without mapping the observed phosphorylation sites to signalling pathways.

Innovative aspects
- Phosphoproteome analysis with the HAMMOC method enables us to monitor inhibition of target kinase and downstream signaling.
- Protein kinase inhibitors were classified by cluster analysis for quantitative phosphoproteome data without pathway mapping.

References
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Figure 1. Inhibition of autophosphorylation of EGFR by gefitinib. Panc-1 cells were incubated with 0.01-100 nM gefitinib and EGF for 10 min at 37°C.

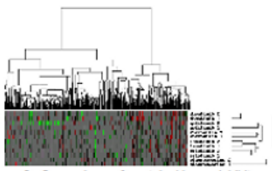

Figure 2. Comparison of protein kinase inhibitors with cluster analysis for phosphoproteome data. Panc-1 cell were treated with 6 kinase inhibitors (n = 2). Red and greed lines indicate decrease and increase by treatment with kinase inhibitor, respectively.

Building a Targeted Selected Reaction Monitoring (SRM) assay of Phosphopeptides by utilizing Discovery Information

K110

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Introduction
Targeted SRM assays are increasingly being used for the quantification and monitoring of specific disease biomarkers (1). Unfortunately, many biomarkers, especially phosphopeptides involved in cell signaling, are typically present at very low levels in a cell lysate. In the stimulated human cell line A431, epidermal growth factor receptor (EGFR) contains multiple phosphorylation modifications that directly modulate the EGFR function. An efficient, automated and robust enrichment pre-analytical step in the sample preparation workflow can facilitate the sensitive detection and routine clinical assay of these lower abundance species. We describe here an integrated, automated high-throughput workflow for the selective enrichment and identification of phosphopeptides from cell lysates using high resolution LC-MS/MS. This workflow includes the development of a quantitative biomarker assay on a triple quadrupole mass spectrometer, employing phosphopeptides identified in the discovery experiments.

Methods
Lymphocyte and monocyte cells (peripheral blood mononuclear cells, PBMC) and the stimulated A431 human cell line were collected, digested, enriched for phosphopeptides and analyzed on the LTQ-Orbitrap. This was followed by identification analysis using Proteome Discoverer to generate a list of phosphopeptides. The selected phosphopeptides were screened to identify proteotypic peptides and transitions for the targeted SRM assays using a novel software package, Pinpoint. Pinpoint utilizes the results from Discoverer to generate the targeted SRM assay. Both the PBMC and A431 lysates were then reanalyzed on the triple quadrupole platform using this assay to perform the quantification.

Results
From the discovery experiment, several hundred phosphopeptides and their sites of phosphorylation were identified. The discovery information (peptide sequences, peptide retention times, product ions, precursor intensity and sites of phosphorylation) were transferred to the SRM software Pinpoint to generate SRM assays[2]. By utilizing all the discovery information, we are able to build a highly intelligent and targeted SRM assay, which significantly increases our throughput, thereby enabling us to quantify several hundred phosphopeptides in a high-throughput manner on the triple quadrupole mass spectrometer. The various steps involved in this our, to map the liquid chromatographic elution time between the discovery and targeted platforms, choosing peptides based on their precursor intensity, and choosing transitions based on the discovery MSMS spectra. In addition to the high abundant discovery product ions, we also monitor the neutral loss ion as the signature transition. All this information helps in building an SRM assay that is selective, sensitive and high throughput for phosphor-peptide analysis.

Innovative aspects
- The described workflow enables the discovery and identification of phosphopeptides and the subsequent development of targeted SRM-based assays that may be useful for clinical application.

References
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The FGFR3 Network in Multiple Myeloma: A Phospho-Proteomic Profile

K111

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Introduction:
Signaling by activated growth factor receptor tyrosine kinases is manifest through networks of proteins that are phosphorylated by, and/or bind the autophosphorylated receptors. FGF Receptor-3 (FGFR3) is a drug target in a subset of human multiple myeloma tumors, and is mutationally activated in some cervical and colon and many bladder cancers, and certain skeletal dysplasias.

Methods:
In order to determine the FGFR3 network in multiple myeloma, mass spectrometry was used to identify and quantify protein phosphotyrosine (pY) sites associated with FGFR3 activation and inhibition. Myeloma cell and tumor proteins were subjected to label-free quantification of Orbitrap MS ion currents, and by using a triple quadrupole instrument for multiple reaction monitoring (MRM) with tandem MS.

Results:
This indicated modulation of FGFR3 kinase activity by phosphorylation of tandem tyrosine residues in the FGFR3 kinase domain activation loop (Fig.1). A set of 45 protein pY sites associated with FGFR3 activation by FGF were sensitive to inhibition by the FGFR3-directed inhibitor PD173074, and constitute an FGFR3 signaling network. The network features transactivation of additional tyrosine kinases including JAK1 and IGF1R, and phosphorylation of the cytoplasmic tail of Syndecan-1 (CD138), which is a biomarker and target in myeloma that modulates tumor-bone marrow interactions. Therefore, tumor analysis by phospho-proteomics enabled the identification and pharmaco-dynamic monitoring of drug target and target network activities. This may be a generally applicable approach to facilitate drug development and stratify tumors.

Innovative aspects:
- Identification of >275 pY peptides in Multiple Myeloma
- Ion current-based label-free quantification of pY peptides with a high-resolution LTQ-Orbitrap instrument used as a discovery step and results validated with targeted label-free MRM mass spectrometry
- Establishment of the FGFR3 network in Multiple Myeloma

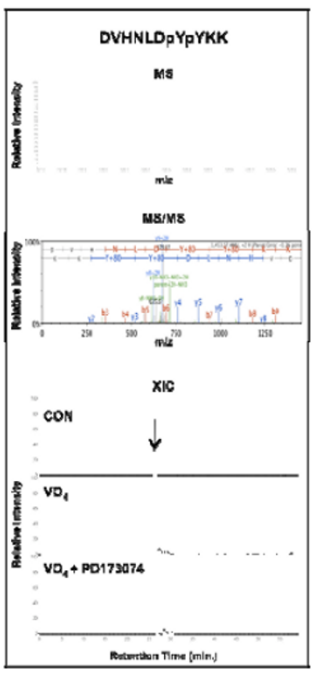

Figure 1. Label-free LTQ-Orbitrap quantification of FGFR3 activation loop phosphorylation in myeloma cells. Depicted are the MS (upper panel) and MS/MS (middle panel) spectra and the extracted ion current (XIC, lower panel) of the doubly phosphorylated DVHNLKpYpYKK FGFR3 activation loop peptide from KMS11 cells untreated or treated with pervanadate (VO4), or pervanadate and PD173074 as indicated.

K112

Network analysis of cell-specific Eph/ephrin bidirectional phospho-tyrosine signaling in co-culture

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Contact-initiated signaling between cells is an important mechanism for the development and maintenance of tissue structure. Signaling between the transmembrane Eph receptors and ephrins results in a bi-directional tyrosine kinase-dependent signal, which regulates cell migration and adhesion to control cell sorting. Until now it has been problematic to systematically study cell-specific signaling networks in distinct populations of interacting cells, primarily because the unique properties of each cell type are lost once co-cultured cells are processed for biochemical analysis.

Here, we present an analysis of bidirectional Eph/ephrin signaling in the context of direct cell-cell interaction. We have developed a novel proteomic strategy, quantitative analysis of Bidirectional Signaling (qBidS), based on lineage-specific labeling with stable isotopes of arginine and lysine, combined with mass spectrometric identification and relative quantification of tyrosine-phosphorylated peptides. In combination with siRNA analysis of Eph/ephrin controlled cell sorting and algorithm and network analysis we performed an integrative and data-driven network analysis of cell specific signaling in Eph-ephrin controlled cell sorting.

Cell lineages expressing the receptor tyrosine kinase EphB2 or its cognate ligand, ephrin-B1, were separately grown in the presence of 'light', 'medium' or 'heavy' arginine and lysine. Following, EphB2 and ephrin-B1 expressing cells were mixed to initiate bidirectional phospho-tyrosine dependent signaling, which subsequently was analyzed in a lineage specific and relative quantitative manner. We identified and quantified tyrosine phosphorylation events common to both EphB2 and ephrin-B1 expressing cells. Notably, 72% of the tyrosine phosphorylations identified were modulated in a cell line specific manner, showing that asymmetric signaling is prevalent. In addition, we performed a siRNA screen for signaling molecules involved in EphB2/ephrin-B1 controlled cell sorting. Through integrative and data-driven network modeling we identified cell specific utilization of signaling networks and tyrosine-kinases, which subsequently was identified to be regulated in a different manner between cells expressing mutant versions of EphB2 and ephrin-B1. In summary, we have identified novel and cell specific regulatory mechanisms essential for cell sorting.

Mass Spectrometry Innovations

(M101 – M126)

M101

A New Rapid and Comprehensive Peptidome Analysis by One-Step Direct Transfer Technology for 1-D Electrophoresis/MALDI Mass Spectrometry

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Introduction
A 2-DE/MS system is considered one of the major techniques for proteome analysis. However it was intrinsically difficult for mass spectrometry to directly analyze the proteins in gel separated by electrophoresis. To resolve it we sought to establish a new 1-DE/MS system. We devised a new target plate for MALDI-MS. Consequently, after electrophoresis all peptides could be transferred to the target plate in one step, and analyzed directly using MALDI-MS. The novel 1-DE/MS system that we propose can be expected to support the discovery of many new biomarkers that have not been detected by conventional peptidome analysis, due to its technical limitations.

Methods
A new target plate. To improve the transfer to and retention of peptides on the MALDI-MS target plate for 1-DE/MS, we managed to coat the surface of the conductive metal plate with a polyvinylidene difluoride (PVDF) film.
Electrophoresis and electro transferring. SDS-PAGE was carried out using NuPAGE® Gel (Invitrogen). After electrophoresis, the slab gel was sliced into ten gel strips. The strips were then placed on the target plate. Following electroblotting, α-cyano-4-hydroxy cinnamic acid (CHCA) matrix solution was applied to the target plate.
Mass spectrometry. Mass spectra were acquired on an ultra-flex II MALDI-TOF/TOF (Bruker Daltonics) interfaced with flexAnalysis Version 2.4 software (Bruker Daltonics).
Patient serum. We analyzed the serum peptidome in 3 stages (early stage, pre-DIC and DIC stages) of one patient with POEMS (polyneuropathy, organomegaly, endocrinopathy, M protein and skin changes) syndrome.

Results
A new 1-DE/MS system. After mixing five peptides (1: substance P, 2: neurotensin, 3: ACTH, 4: VIP, 5: calcitonin) with serum, SDS-PAGE was performed and the gel was stained with Coomassie blue (Fig. 1A-1). In the other part of the experiment, after electrophoresis an unstained gel was electrically transferred to a new target plate and the plate was analyzed by MALDI-MS with irradiation to 41 measuring steps on the surface of the plate, resulting in 41 single MS spectra (Fig. 1A-2). Since peptide peaks with the same *m/z* were detected in several single MS spectra, the intensities of these peaks were summed to estimate the total peak intensity of each peptide (Fig. 1A-3).
High throughput analysis of a large number of samples. This system provides a high-speed peptidome analysis owing to the one-step direct transfer of all peptides from gel to target plate. The entire process is completed within 4 hours, leading to analysis of 80 samples/day at maximum.
Differential analysis of disease period of POEMS. The patient developed cachexia and subsequent DIC, and died 178 days after serum sampling in the early stage. The peptidome patterns of 3 periods were different remarkably. In addition, we identified two fragments derived from transthyretin an α1-antitrypsin that significantly increased in DIC stage compared to those in the pre-DIC.

Innovative aspects
- High throughput peptidome analysis system, max 80 samples per a day, was completed.
- De novo identification can be done by the system.

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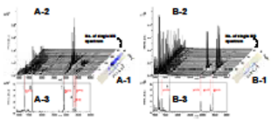

Fig. 1. Integration of MS spectrum. Five peptides (1: Substance P, 2: Neurotensin, 3: ACTH, 4: VIP, 5: Calcitonin) mixed in serum were migrated by SDS-PAGE and stained as shown in A-1. In the other part of the experiment, after electric transfer of an unstained gel, the peptides on the target plate were analyzed by MALDI-MS, yielding a total of 41 single MS spectra (A-2). Total peak intensities at the same m/z value of 41 single MS spectra were combined to generate one integrated MS spectrum (A-3). The results for peptides alone are shown in B-1, 2 and 3.

M102

Increased quantitative throughput and selectivity for triple quadrupole mass spectrometer based assays using intelligent SRM (iSRM)

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Introduction
The commonly used SRM technique provides sensitive and precise quantitative results by monitoring one or several primary SRM transitions per targeted compound. Recently this technique was extended to simultaneously confirm the identity and quantify multiple compounds in one HPLC/MS run by monitoring eight or more SRM transitions per compound. The bottleneck of this approach is that only a limited number of compounds can be targeted in one run because of the time required to monitor each transition. The newly developed instrument control software can use the specificity of a small subset of SRM transitions to simultaneously quantify and intelligently trigger the full list for confirmation, thereby allowing the analysis of up to 1000 compounds in a single LC-MS run.

Methods
A triple quadrupole mass spectrometer equipped with a nanoLC pump and a nanospray source was used. The intelligent SRM method utilizes SRM specificity in two ways. The first is compound specific quantification using a time based SRM acquisition, which monitors several primary transitions for each compound. The second is a data dependent SRM acquisition, which monitors both those primary and additional secondary transitions and is triggered only when the intensities of all primary SRM transitions simultaneously exceed the defined intensity threshold. For large scale screening experiments, the dynamic exclusion was used to trigger secondary acquisition only once for each peak for providing sufficient structural information to confirm the compound's identity without perturbing the quantification obtained with the primary SRM list.

Results
The technique was applied to quantify precisely and confirm identity of peptides in complex biological samples, including yeast cell lysates. By monitoring multiple transitions it is possible to use very low intensity thresholds to accurately trigger the data dependent SRM scan. The trigger is the leading edge of the LC peak and relatively independent of the LC peak intensity. Using known LC peak widths, the strongest intensity point to obtain the data dependant scan is easily anticipated. Instead of a full product ion scan, high quality SRM spectra monitor eight or more transitions at that point. The resulting eight spectra are assembled into a composite full scan MS/MS spectra that can be used for confirming the identity of the peptides by matching them to reference spectra stored in a library. Using a constant cycle time allows enough points across the peaks from the primary SRM scans for precise quantification. With this approach, the instrument is able to confirm and quantify most targeted peptides in the low attomol range.

This method was further employed to large scale screening experiments of peptides. 780 yeast peptides were targeted in one single LC/MS run. The yeast peptide list and the iSRM method (Two primary transitions and six secondary transitions) were generated automatically by using SRM work flow software based on MS/MS data from the previous proteomics discovery experiments. The targeted peptides were successfully identified and quantitated in a single HPLC MS run by using the iSRM software.

Innovative Aspect
- Newly developed iSRM instrument control software.
- Greatly increased throughput and selectivity for triple quadrupole mass spectrometer based SRM assays.
- Provides tremendous potential in the proteomics, environmental and food safety fields for large scale screening experiments.

M103

Relieving Experimental Artifacts in Proteomic Analysis of J774 Cells for Relative Potency of PMs

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Proteomic analyses can be valuable in obtaining information on protein profile changes specific to pollutant exposure-induced effects. Here, we have used a proteomic approach to compare the effects of a wide range of particulate matter (PM) types that can constitute a range of air pollutants in the J774 murine macrophage cell line: TiO_2, SiO_2, single-walled and multi-walled carbon nanotubes (CNT), carbon black (CB), diesel exhaust particles SRM-1650 (DEP), and urban particulate matter EHC-6802 (EHC). Cells were exposed to the particles at 0-100 μg/cm^2 (96-well plates) in serum-free medium for 24h. Shotgun proteomic analyses of cell lysates were then performed by direct MALDI-TOF-TOF-MS. Saturated alpha-cyano-4-hydroxycinnamic acid served as the matrix. The mass spectral profiles were interrogated in the region <6kDa using k-nearest neighbors clustering algorithm. Our results clearly indicated that J774 cells exposed to nanoparticles exhibited systematically more mass spectral peaks compared to that of larger particle-exposed cell lysates suggesting the assistance of nanoparticles in the MALDI process. Such mass spectral interference would impact on the relative potency estimation for these different sized particulate pollutants. We therefore assessed the clarification of cell lysates by molecular weight cut-off filtration process (30, 50, 100 kDa) to optimize the removal of nanomaterials engulfed by the cells and alleviated the interferences caused by the CNTs. Even though all particle exposures exhibited signature MS profiles, common candidate biomarkers were observed between specific particle exposures, especially with DEP and urban particle exposures. Furthermore, the MS data revealed an elevation of cellular endothelin-1 peptide in J774 cells in relation to the doses of particles. This peptide is implicated in the inflammatory status of macrophage. Characterization of proteomic biomarkers and their association with the physicochemical characteristics of particles can provide new insights into mechanisms of toxicity.

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M104

Identification and Quantification of Protein Pathways using MS/MS-Assays built with Synthetic Peptide Arrays

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Introduction
Targeted proteomic screens hold the promise of being more sensitive (1, 2) as common shotgun-based tandem mass spectrometry approaches. However, it is mostly unclear how to develop targeted LC-MS/MS assays effectively, especially when low abundant proteins must be detected. To this end, we have developed a novel approach for constructing highly effective multiplex detection assays based on the generation of synthetic peptides covering entire pathways or protein networks of interest. Moreover we use novel protein identification showing significant improvement to existing database search algorithms (3).

Methods
We first select peptides suitable for synthesis and detection by tandem mass spectrometry. These targets are synthesized in high density mode by microscale peptide synthesis. Upon solubilization, each peptide is characterized extensively by LC-MS/MS and experimental parameters were stored in a reference database. Scheduled target-directed LC-MS/MS-assays were built, either for the highly sensitive and selective detection of multiple targets using either a linear ion trap instrument or a triple quadrupole (i.e. MRM). Retention time scheduling across complex mixtures is supported by a novel alignment algorithm which corrects for experimental deviation in experimental retention time values in different samples and columns. Accurate quantification is achieved with the use of individual peptide standard curves.

Results
We show the successful construction of highly sensitive, specific and reproducible LC-MS/MS-assays of >100 peptide targets/45 min gradient. This high density is suitable for monitoring entire protein pathways and networks across multiple samples. We show further the application of developed highly-specific assays to detect entire sub-networks linked to critical low abundance transcriptional factors, such as Sox2, Pou5f1 and Stat3, implicated in stem cell fate determination from mouse embryonic stem cells We further show that our strategy can be used for other pathways or cells. Since sample amount is often limited, we further show the application of affinity enrichment techniques to increase the limits of detection to exceptional levels.

Innovative aspects
- Novel, quality controlled, targeted LC-MS/MS assay pipeline that allows for the detection of low abundant pathway components.
- Generation of a high-quality peptide reference library.
- Unique automated platform for routine proteomic screening.

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M105

Intact Protein Sequencing Using ETD-PTR in Linear Ion Trap

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Introduction
Electron transfer dissociation (ETD) is an advantageous tool for intact protein analysis because it is relatively insensible to the size, amino acid composition and post-translational modifications of proteins. ETD of intact proteins performs with high efficiency, generating very informative, yet extremely complex spectra containing highly charged product ions that are difficult, or even impossible to resolve at unit resolution. Proton Transfer Reaction (PTR) removes protons from multiply charged product ions, generating a simplified spectrum containing product ions of resolvable charge states at unit resolution. PTR has recently implemented in LTQ XL and a new dual pressure ion trap under instrument control software. Here, the performance of PTR for ETD spectra simplification and the utility of ETD-PTR for intact protein analysis in unit-resolution linear trap were investigated.
Methods
Standard peptides were purchased from Anaspec. Intact proteins were purchased from Sigma. ETD with PTR was performed using LTQ XL ETD with PTR capability under LTQ 2.6 instrument control software with developer's kit. Benzoic acid anions which are generated in the chemical ionization source at the rear of the instrument were used as PTR reagent. The anion target was adjusted from 1e5 to 2e5. PTR reaction time was from 10 -100 msec. Data analysis was performed using Proteome Discoverer 1.0 for peptide, or manually for intact protein identification. To compare the extended ETD with ETD-PTR, c/z type product ions which contain extra hydrogen from extended ETD reaction were included for identification.
Results
Our results indicated that when intact protein ETD was performed in unit-resolution instrument, the resulting spectra are information rich, yet contain multiply charged product ions which can not be resolved adequately. Thus, reducing product ion charge state to produce simplified spectra is necessary for data interpretation. Charge reduction can be achieved either by extended ETD reaction, or by PTR. Our results using standard peptides indicated that c/z type ions generated by extended ETD reaction contain one or more extra hydrogen than expected, due to multi-step charge reduction through electron transfers pathway. These ions are excluded from identification by database search software due to the unexpected mass shift. Furthermore, extended ETD reaction generated secondary fragmentation products which interfere with data analysis. PTR, which reduces product ion charge state by subtracting protons, generates c/z type ions of expected mass for data analysis software.

To investigate the utility of PTR following ETD in facilitating intact protein sequencing using unit resolution linear trap, proteins ranging in size from 8 kDa to 30 kDa were used for the study. For each ETD spectra obtained, charge reduction was performed either through extended ETD reaction, or through PTR. The reaction time for ETD, or PTR was optimized based on optimized sequence coverage. Our results indicated that ETD-PTR significantly improves sequence coverage when compared to extended ETD reaction alone. For proteins larger than 30 kDa, the benefit of charge reduction for sequence coverage becomes less significant due to the extreme complexity of spectra, the diluted signal intensity over huge number of product ions as well as the limited resolution of instrument. A combined CID, PTR and ETD approach will be discussed for larger protein top down analysis where PTR is served as a mean to determine charge state of large product ions which are selected for MS3 analysis.
Innovative aspects
Intact protein analysis using ETD-PTR

M106

qPAcIFIC: how to quantify deeper into the proteomics ocean

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Introduction
The vast majority of shotgun proteomics experiments are conducted using data-dependent precursor ion selection; i.e. ions are selected for MS2 fragmentation based on: 1) presence of a signal, and 2) intensity. While very popular and successful, this approach suffers from limited dynamic range. Recently [1], we presented a data-independent acquisition method showing superior performance on serum analysis (eight orders of magnitude dynamic range) that we named Peptide Acquisition Independent From Ion Count (PAcIFIC). Here, we present a novel quantitative, multiplexed PAcIFIC workflow.

Methods
Protein samples (standard mixture, bacterial cell lysate and plasma) are labelled using isobaric tagging approaches, e.g. TMT (ThermoFisher). A collision induced dissociation (CID) spectrum is acquired every 1.5 m/z value whether a precursor ion is observed or not followed by a pulse-Q dissociation (PQD) spectrum in the low m/z range where TMT reporter ions are expected. Thus, in a single LC-MS/MS experiment 30 such scans are acquired (i.e. 15 pairs of CID/PQD) across a range of 22.5 m/z. Repeated injections are preformed in an identical fashion until the desired m/z mass range is achieved. CID and PQD spectra are merged into a single composite spectrum using in-house software, searched with Sequest, validated using FDR < 1% and quantified using Libra (TPP, ISB, Seattle).

Results
The strategy was first applied to a plasma sample depleted of the 7 most abundant proteins. Unique dynamic range performance was achieved (eight orders of magnitude at FDR ≤ 0.5%), without any fractionation other than immuno-depletion. For quantification, first, two reference sets were designed with 14 protein standards. Theoretical ratios spanned from 0.1 to 10 (reporter 126 over 127). All 14 proteins were identified in average with >80% sequence coverage and the expected ratios for the 14 proteins matched to less than 20% deviation. A similar experiment was conducted on a whole cell lysate of Pseudomonas aeruginosa. Twice the same amount of digest was tagged to mimic a 1:1 ratio distribution. A total of 1400 proteins were identified at FDR<0.5% of which 800 were quantified. The average/median ratio distribution was 1.01/1.00 with a standard deviation of 0.15 (average/median nb of peptides/protein was 14/5). Finally, we will present results showing how this new method may be applied to more quickly and thoroughly compare differences in protein expression between normal human plasma and any altered state(s).

Innovative aspects
- Data-independent precursor ion selection
- High dynamic range achieved
- Multiplexed, quantitative workflow using isobaric tagging and data-independent selection to monitor every single peptide channel

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M107

MALDI-Immuno Screen (MiSCREEN)™
A new method for selection of high affinity anti-peptide monoclonal antibodies

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Introduction
Several new affinity proteomics applications such as iMALDI and SISCAPA require high affinity antibodies for peptide enrichment prior to identification and quantitation of target proteins by mass spectrometry. To identify such high affinity monoclonal antibodies (MAbs), thousands of hybridoma supernatants must be screened. This is often performed by various ELISA methods, or more effectively, by surface plasmon resonance (SPR) for determination of the kinetics of peptide binding and release. Although SPR is effective, it is a time-consuming and expensive approach (1). Here we describe a new technique, MiSCREEN™, for high-throughput screening of hybridoma supernatants that allows identification and selection of anti-peptide MAbs of high affinity (2).

Methods
Known amounts of selected peptide analytes were spiked into 50 µL of hybridoma supernatants containing peptide-specific rabbit monoclonal antibodies (RabMAbs) in 96 well microplates. After overnight incubation, 10 µL of sheep anti-rabbit Dynabeads® (Invitrogen) were added to each well. The plates were placed into a Kingfisher robot and incubated (with shaking) at room temperature for 2 hours. After multiple washes, the antibody-saturated Dynabeads were removed by the Kingfisher magnetic arm and the peptides eluted from the antibody by a 5 minute incubation with 50 mM TCEP, an acidified reducing agent. The eluted peptides were desalted and concentrated using ZipTips (C18) before spotting onto plates for analysis by Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry. Under the conditions used, some antibodies were clearly able to bind more peptide from solution, which was reflected in the MALDI spectra.

Results
MALDI-immuno Screen (MiSCREEN™) has been used to screen supernatants containing both rabbit and mouse mAbs, independent of the antibody concentrations, thus is useful for first-pass selection of monoclonal antibodies in hybridoma supernatants. Using MiSCREEN™ we have successfully selected high affinity (specifically low-off-rate) MAbs for a variety of peptide surrogates of protein targets including thyroglobulin (a biomarker for thyroid cancer), CA-125 (a biomarker for ovarian carcinoma), Her-2 (an over-expressed receptor tyrosine kinase in breast cancer patients), and BMAP-18 (bovine myeloid anti-microbial peptide-18, a truncated host defense peptide that is an effective inhibitor of African trypanosomes). That MiSCREEN™ was successful in identifying high affinity MAbs was confirmed by SPR analysis. In all cases, nanomolar affinities were obtained. In this way, in a relatively high throughput fashion, RabMAbs with low dissociation constants could be discriminated from those with undesirable binding kinetics.
Further automation of the MiSCREEN™ technique will allow us to process a large number of anti-peptide antibodies in a time and cost effective manner, and hence introduce an alternative method to ELISA and SPR antibody screening assays for selection of mAbs for use in affinity proteomics.

Innovative aspects
- Novel method for rapid, cost effective identification of high affinity anti-peptide MAbs
- Identification of high affinity MAbs in a first-pass screen regardless of the antibody concentration in hybridoma supernatants
- Identification of monoclonal antibodies in an assay similar to the assays of ultimate use (iMALDI and SISCAPA), i.e. binding and enrichment of peptides from solution.

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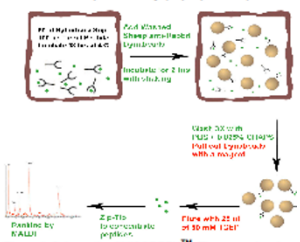

Figure 1. Schematic of MiSCREEN™. The monoclonal antibody of highest affinity will bind the most amount of peptide, which is reflected in the MALDI peak intensity.

M108

Comprehensive Proteomic Studies Leveraging a Novel SRM Technique

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Introduction

Targeted proteomics based on selected reaction monitoring (SRM) is expanding very rapidly. The technique has exquisite sensitivity, high selectivity, and a wide dynamic range was primarily applied to quantitative studies. We present here an extension of this technique enabling large-scale quantitative and qualitative experiments. A new data acquisition software enables analysis of up to thousand peptides in one single LC-MS run, by monitoring a few transitions to precise quantify, and punctually (in a data dependent manner), additional transitions to unambiguously identify each peptide.

Methods

Measurements were performed on a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific) equipped with a nano-HPLC system. Separations were performed on 75 micrometers ID reversed phase column. During one acquisition cycle (2 sec), the instrument control software switches between two modes: a) two or three (primary) transitions specific to each peptide are monitored in given time intervals for quantification; b) in a data dependent SRM manner, additional (secondary) transitions are measured when the intensities of the primary transitions exceed a defined intensity threshold. It provides sufficient structural information to confirm unambiguously the identity of the compounds without significantly perturbing the quantification obtained with the primary SRM traces.

Results

A novel instrument control and data acquisition software enabled high density SRM measurements. Scheduling and control of the elution times increased drastically the number of peptides analyzed. The SRM measurements were acquired in a data dependent mode, punctually monitoring as many as ten transitions per peptide. It allowed reconstructing composite tandem mass spectra, used to confirm the identity.

Proof-of-principle was demonstrated by analyzing isotopically labeled tryptic peptides added into a yeast cell lysate. Two primary, and six secondary transitions for each peptide of interest were monitored.

The resulting composite spectra confirmed the identity of the peptides by matching with a reference library (down to the amol range sensitivity).

A large-scale experiment was performed, including thousand peptides in one single LC-MS run. The data acquisition was carried out measuring for each peptide two primary transitions for quantification and occasionally an additional six secondary transitions to corroborate the identity. The technique was applied to study metabolic pathways in yeast, like TCA or glycolysis by monitoring large sets of peptides in full cell lysates.

Innovative aspects

- Novel SRM data acquisition technique for high-throughput analyses.
- Concomitant quantitative and qualitative analysis of peptides by SRM.
- Robust and consistent screening method for comprehensive systems biology studies

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M109

Overcoming Undersampling in Proteomic Experiments using a New Linear Trap-Orbitrap Mass Spectrometer

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Introduction

Enormous improvements in MS technology over the last few years have allowed for ever deepening analysis of complex proteomes. However, many low abundance proteins remain undetected due to the limited number of MS2 spectra acquired. To solve this problem, we developed a new linear trap-orbitrap mass spectrometer that provides faster MS2 scanning in the ion trap (up to 10 peptide sequences /s), thereby increasing the depth of analysis of complex protein mixtures. Here we evaluated the ability of the new instrument to identify more proteins, with increased sequence coverage and confidence.

Methods

Several improvements in technology have been implemented: a) a progressive stacked-ring ion guide (SRIG) with 5x increased ion transmission; b) a dual-cell differentially pumped ion trap with a higher pressure region for improved ion trapping, isolation, and CID efficiencies, and a lower pressure region for improved resolution and/or scan speed; c) predictive AGC for increased scan speed; d) an HCD-collision cell with axial field for improved performance; e) improved vacuum in the Orbitrap chamber for improved intact protein analysis. To assess improvements in proteome coverage, nanoLC-MS/MS analyses of C. Elegans and E. Coli protein digests were performed using various LC gradients. The raw data files from triplicate analyses were searched with Mascot using Proteome Discoverer software.

Results

To assess the extent of under-sampling for a complex sample, nanoLC MS/MS analysis of 1µg C. elegans protein digest was carried out using two different LC gradients (2-40% acetonitrile in 75 minutes, and 2-40% acetonitrile in 150 minutes). Extensive data analysis allowed us to estimate the extent of under-sampling for this proteome. Various contributors to under-sampling are reviewed. Using the new instrument, a two-fold increase in the number of MS/MS spectra acquired per unit time was observed. This provided identification of a higher number of proteins and peptides in the 75' run than in the 150' run using the LTQ Orbitrap XL. With the novel hybrid linear ion trap-orbitrap mass spectrometer, we were able to identify 41.5% more proteins and 63% more peptides (75' run, at 0.25% FDR) or 34% more proteins and 50.5% more peptides (150' run, 0.25% FDR) than in the corresponding runs with the LTQ Orbitrap XL. Similar results were obtained analyzing very complex E. Coli protein digest. These results indicate that the newly developed instrument can identify more proteins with increased sequence coverage.

Innovative aspects

- The problem of under-sampling in proteomics is effectively addressed by a new linear ion trap-Orbitrap mass spectrometer.

M110

Proteomics based Diagnostic and Prognostic Biomarkers for Head-and-Neck Cancer: Challenges and Opportunities

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Introduction

Over 50% of head and neck/oral squamous-cell carcinomas (HNOSCCs) have advanced disease at the time of diagnosis; at least some of this dismal statistics is a reflection of a lack of clinically established biomarkers for early cancer detection. Proteomics combined with mass spectrometry (MS) is a powerful paradigm for unprecedented large-scale determination of peptides and identification of proteins in clinical samples for biomarker discovery.

Methods

Multidimensional LC-MS/MS was used for the analysis of isobaric mass tags (iTRAQ) labeled biological samples to identify and quantify differentially expressed proteins in HNOSCCs and oral premalignant lesions (OPLs) versus non-cancerous head-and-neck tissues (control) and tumor-surrounding mucosa (field cancerization) (1,2). A panel of identified potential cancer markers (PCMs) was verified using an independent and larger set of clinical samples by immunohistochemistry (IHC) and correlated with clinical follow up of 51 patients to develop molecular predictors of clinical outcome (3).

Results

A panel of three PCMs showed 92% sensitivity and 91% specificity in discriminating HNOSCC from non-malignant head and neck tissue in iTRAQ analysis and 92% sensitivity and 87% specificity in IHC analysis. Interestingly, YWHAZ (14-3-3 zeta) and stratifin (14-3-3sigma) were also differentially expressed in OPLs, albeit at a lower level than in HNOSCCs. Kaplan-Meier survival analysis revealed reduced disease-free survival for HNOSCC patients showing overexpressions of YWHAZ and stratifin (median disease-free survival of 13 months, p = 0.02) relative to patients showing no overexpression of these two proteins (median = 38 months), underscoring the utility of these proteins as adverse prognosticators for HNOSCCs. Increased heterogeneous ribonucleoprotein K (hnRNP K) expression was found to be an early event in head and neck tumorigenesis, suggesting its utility in identifying oral leukoplakic lesions in early stages, prior to the onset of dysplasia. Follow-up study showed that cytoplasmic hnRNP K was an independent predictor of disease recurrence in HNOSCC patients (77cases) (4).

Innovative aspects

- Novel panel of 3 PCMs has been identified for HNOSCC
- A combination of two PCMs may provide better survival predictions for HNOSCC prognosis
- Nuclear hnRNP K may serve as a potential marker for early diagnosis of OPLs, while its cytoplasmic accumulation can help to identify a subgroup of HNOSCC patients with poor prognosis, suggesting its putative utility in clinical management of HNOSCC.
- Pathway analysis unraveled the perturbed biological networks and identified molecular targets for designing new therapeutic strategies

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M111

Liquid Chromatography - Electron Transfer Dissociation and Ion Mobility Studies on a QTOF Mass Spectrometer

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Introduction

Electron Transfer Dissociation (ETD) is now established as a complementary fragmentation technique to Collision Induced Dissociation (CID) for peptides. This presentation describes novel experiments that have been enabled on a quadrupole TOF mass spectrometer. The instrument is equipped with the ability to separate ions according to their ion mobilities through a combination of 3 travelling wave (TWAVE) ion guides. Ion mobility analysis of ETD product ions including the additional functionality of ETD-IMS-CID will be described. In order to acquire ETD data on the chromatographic time scale a highly sensitive glow discharge ETD reagent anion source has been developed and this will also be presented.

Methods

Azobenzene reagent anions are generated using the new glow discharge source, they are selected by the quadrupole and accumulate in the first TWAVE ion guide. Analyte ions are then admitted either via infusion for peptide and protein standards or via UPLC for peptide digest mixtures into the nanoflow electrospray ion source. Precursor ions are then selected by the quadrupole and admitted into the first TWAVE ion guide. ETD (or optionally CID) occurs within the first ion guide, product ions are then separated according to mobility in the IMS TWAVE guide and optionally subjected to further CID in the third ion guide prior to TOF mass analysis.

Results

For anion reagent generation, the glow discharge source is approximately 100x more sensitive than an APCI source (reported at ASMS 2008 - WPA_021). As such a negligible proportion of the experimental cycle time (~10%) is required to accumulate sufficient anions for ETD. This enables ETD on the UPLC (Waters nanoAcquity) timescales. To demonstrate this, peptides from a trypsin digest of 50fm Bovine Serum Albumin (BSA) were separated by UPLC producing LC peak widths of ~4secs. Triply charged precursors were automatically software selected for ETD and BSA was sucessfully identified using the Mascot database search algorithm. Other results will be presented from ETD-Ion Mobility-TOF and ETD-Ion Mobility-CID-TOF experiments of infused peptide standards and protein digests.

Innovative aspects

- Ion mobility of ETD product ions
- ETD-IMS-CID on QTOF
- Glow discharge anion source enables ETD on LCMS timescales

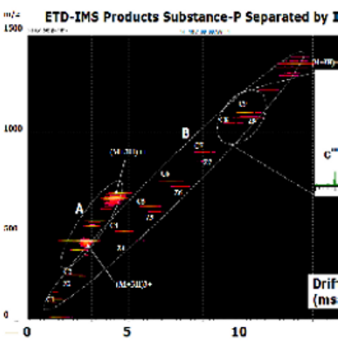

Figure 1.ETD – IMS, product ions separated by ion mobility and mass analysed by TOF. Group A are identified as doubly charged product ions and group B are singly charged products labeled c and z.

M112

12-Plexed Quantitation: Combining Tandem Mass Tags with SILAC

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Introduction
Phosphoprotein expression profiling by MS is limited by many factors, including low stoichiometry and technical reproducibility of the sample preparation workflow. We have addressed these issues with a combination of phosphoprotein and phosphopeptide enrichment with TiO2, and replicate 12-plex multiplexed quantitative analysis using SILAC and TMT with novel MS acquisition methods. The quantitative reproducibility and identification of differential phosphorylation cascades affected by activators of cell proliferation or cell death is demonstrated using this multiplexed quantitative strategy.

Methods
293T cells were grow in DMEM media containing dialyzed fetal bovine serum and supplemented with either 13C8-Lys 13C615N4-Arg or normal isotopic abundance lysine and arginine. Serum-starved cells were treated with LY294002 (25uM, 1 hour), rapamycin (100nM, 1 hour), or left untreated. Following drug treatment, 293T cells were stimulated with insulin or left unstimulated. Phosphoproteins were enriched followed by enzymatic digestion and labeling with TMTsixplex Tandem Mass Tags. Labeled peptides were combined, fractionated by strong cation exchange, and phosphopeptides were enriched with TiO2 coated magnetic beads. Mass spectrometric analysis of enriched was performed on an LTQ Orbitrap with HCD fragmentation. Data analysis was performed with Thermo Scientific Proteome Discoverer software.

Results
We employed this phospho-proteomics enrichment strategy to trace specific phosphorylation events in the PI3K-Akt signaling pathway in response to chemical inhibitors of PI3K (LY294002) and mTOR (rapamycin). Enrichment of phosphoproteins using an IMAC-based phosphoprotein enrichment kit and of phosphopeptides with TiO2 coated magnetic beads, in combination with quantitative mass spectrometry using SILAC reagents and TMT isobaric mass tags, provided high resolution replicate data of the action of these drugs in insulin responsive 293T cells. Of specific interest were the technical aspects of targeting fragmentation of isotopic paired peaks for high resolution, highly multiplexed quantitation of isobaric tags ("isotopobaric quantitation"). This strategy resulted in important biological insights such as the quantitative reduction in phosphorylation of the retinoblastoma protein, Rb, on residue T373 in response to rapamycin. Reduction in Rb (T373) phosphorylation coincides with altered phosphorylation and kinase activity of PKA (Ser99), Akt (Ser473), GSK3a (Ser9), and GSK3ß (Ser21), ultimately regulating cyclin D stability, cyclin-dependent kinase 4/6 activity, and cell proliferation.

Innovative aspects
phosphoprotein and phosphopeptide enrichment, multiplexing with SILAC and isobaric tags

M113

Novel Software Approach to Building, Processing, and Scoring Large Targeted Peptide Assays using Intelligent SRM Transitions

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Abstract content: Introduction
The role of SRM assays for peptide and protein verification and quantitation has expanded to monitor large number of candidates (500-1000 peptides) during initial screening phases. This expansion has created additional challenges to the biomarker verification workflows to effectively build intelligent methods, process data, and automatically classify proteins, peptides, and individual transitions. To address these concerns, we present a novel software package to build large SRM assays, acquire data, process and score the resulting processed data and enabling user-defined metrics to refine the SRM assay to include the optimal transitions for a final method. The software package is integrated into discovery data acquire in-house as well as public database repositories, to intelligently build large SRM assays based on experimental data. In addition, the software enables user-defined criteria to score resulting processed data to automatically parse the best peptides and transitions per protein into a refined method.

Methods
Yeast cell lysate and depleted human plasma were digested and analyzed in triplicate on an LTQ Orbitrap and processed using SIEVE to obtain a protein and peptide list. The resulting discovery data imported into Pinpoint and used to build targeted SRM assays for 500, 750, and 1000 peptides in a single experiment for verification and scoring. Each data set was acquired with four technical replicates for statistical purposes. Pinpoint was used to process each data set, scores each transition, peptide, and proteins according to user-defined filters to evaluate the success rate for each experiment in terms to SRM results (%CV, p-values, signal intensities, etc.) as well as the overlap with the LTQ Orbitrap experiments.

Results
From the discovery experiments, several thousand peptide candidates were identified from the SIEVE output with high confidence. The SIEVE spectral libraries are read into Pinpoint to create the protein and peptide lists targeted in each of the above mentioned methods. The selected peptides used in each of the methods above were chosen based on protein coverage in an effort to correlate prioritization functions in predicting proteotypic peptides. Each of the targeted peptides was monitored using a unique scan feature enabling simultaneous qualitative and quantitative detection. All SRM experimental data files were processed using Pinpoint to determine %CV, AIC, probability values against library spectra, and relative retention times as compared against the Orbitrap discovery files. The initial results show over 90% success rates for the 500 peptide method, 85% and 71% success rates for the 750 and 1000 peptide assays. The poor hits observed in each SRM assay (500, 750, and 1000) were extracted into a second assay using new experimental parameters (retention times, Q1 resolution, dwell times, and transitions) increased detection efficiencies bringing up the success rate over 90% for each of the different targeted peptide lists.

Innovative aspects
- Automated building of SRM assays containing over 8000 transitions based on spectral libraries.
- Novel processing strategies to score and classify targeted peptide in an automated method.
- Automated method refinement strategies used to build new methods based on processed data.

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M114

Differential protein expression throughout the life cycle of parasitic African trypanosomes

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Introduction
Trypanosomes are protozoan parasites responsible for the socio-economically important diseases collectively called the trypanosomiases, in sub-Saharan Africa. The World Health Organization considers these diseases to be a major impediment to the economic development of Africa. The trypanosomiases leave approximately 9 million km^2 of sub-Saharan African unsuitable for human settlement and livestock production, a 3rd of the African continent. The economic impact can be measured in billions of dollars per annum (1).

One species of African trypanosome, *Trypanosoma congolense*, has four major life cycle stages that can be grown entirely *in vitro*. One stage lives in the blood and/or cerebrospinal fluid of a mammalian host and three in the midgut and mouth parts of the tsetse fly vector. The goal of my research is to, 1) Identify which proteins are being expressed in each life cycle stage and 2) monitor the change in protein expression from one life cycle stage to the next.

Methods
Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) in conjunction with liquid chromatography tandem mass spectrometry analysis were used to measure differential expression of proteins throughout the *T. congolense* life cycle. Three complete biological replicates of all four life cycle stages were studied. The data were analyzed by comparison to the recently completed *T. congolense* genome sequence (2).

Results
A total of 9214 tryptic peptides were identified and localized to 2237 open reading frames representing about 27% of the genes predicted to be in the *T. congolense* genome. Our data are being used as an aid to annotation of the parasite genome.

All protein sequences were used in a BLAST search of the non-redundant database. Many of the sequences drew strong hits with a related African trypanosome (*T. b. brucei*) or other kinetoplastids (*T. cruzi, Leishmania sp.*). There were eleven sequences that were identified with high confidence in all three replicates that drew no strong sequence similarity to any other sequences in the database. There were an additional eleven sequences that were present in two of the three replicates that also had no strong database hits. These sequences represent new proteins that have not been described in any organism and may be unique to *T. congolense*.

Innovative aspects
- *T. congolense* is the only African trypanosome for which all life cycle stages can be cultured in vitro.
- Although iTRAQ analysis has been performed on a related kinetoplastid parasite (*Leishmania donovani)* (3) the inability to isolate enough sample for proteomics studies has prevented similar studies from being performed on African trypanosomes. By using T. congolense we solved this problem since all four life cycle stages can be grown in the lab in sufficient quantities.
- This is the first instance of a large scale proteomics study of a parasitic African trypanosome.

References
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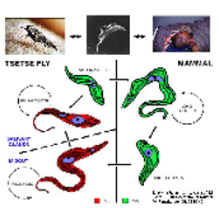

Figure 1. Diagram of the African trypanosome life cycle

M115

PTM Finder Based on PEAKS De Novo Sequencing Result

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Introduction
Identification of post-translational modification (PTM) by tandem mass spectrometry is still as major challenge in proteomics, especially if the PTMs are unknown. In typical existing software, tandem mass spectra are searched against an enlarged-database that includes all possible combinations of modified peptides. Because the search time grows exponentially with the combination of all possible modifications, only a small number of known variable modifications can effectively be included in the each search. In this abstract, we describe a new approach based on *de novo* sequencing results to identify unknown variable PTMs from an MS/MS dataset

Methods
For a peptide with variable PTM, usually only a fraction of many copies of the same peptide are modified. Thus spectra of both modified and unmodified versions of the peptide may be seen in the dataset.

PEAKS software is used to do *de novo* sequencing on all spectra. Since we do not know the mass change of the PTM, PEAKS can only provide partially correct sequence tags to the modified spectrum. Pairs of the modified and unmodified spectra are identified by recognizing pairs of similar *de novo* sequencing results of PEAKS. The local confidence provided by PEAKS is used to define the similarity score. The precursor mass difference of a spectrum pair provides information to the identity of the PTM.

Results
Two samples were prepared to test the validity and efficiency of the method. The first sample contains a known protein, and was analyzed with a LTQ-Orbitrap (Thermo) instrument. 1645 MS/MS spectra were obtained and therefore there are 1645*1644/2= 1352190 spectrum pairs. By using our PTM finder program, 1242 pairs are judged as pairs caused by variable deamidation PTM. By visually examining these spectrum pairs we are convinced that the PTM is real. The program also finds some other possible PTMs with much lower score.

The second sample is a complex mixture containing several hundred proteins. By other means, we know 7 types of variable PTMs that are in the sample. The sample was analyzed with a Q-TOF instrument and more than 30,000 MS/MS spectra were obtained. Without using the prior knowledge about the 7 PTMs, our program successfully reported all of them in the list of top-10 possible PTMs.

Novel Aspects
Software to identify unknown PTM from LC-MS/MS data by using local confidence information from PEAKS *de novo* sequencing results

M116

High Throughput Global Proteome Profiling of Mammalian Tissue Using Hybrid Triple Quadrupole / LIT Technology

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Introduction
Whole cell proteomic profiling of mammalian tissue derived homogenates can be analytically challenging due to limited supplies and laborious extractions in comparison to profiling cultured cell line preparations. Faster acquisitions and more sensitive peptide MS/MS data from very low sample amounts is needed for mid-to-high-throughput cellular profiling of mammalian tissues. We demonstrate a robust and highly sensitive technique for identifying proteins in highly complex tissue preparation by LC-MS/MS using new hybrid triple quadrupole / linear ion trap system.

Methods
AB SCIEX QTRAP® 5500 system was equipped with a prototype NanoSpray® source and heated interface for sample analysis. Nanoflow LC experiments were carried out at a flow rate of 300 nL/min with 75um nano column with Tempo™ nanoLC system. High flow experiments were operated at a flow rate of 200ul/min with a BetaBasic C18 column, 150 x 2.1mm with a standard LC system. Inner medullary collecting duct (IMCD) cells freshly isolated from rat kidney, digested with a standard trypsin protocol.

Results
Over 250 proteins were identified in a single analysis of 200 ng of total protein digested rat kidney IMCD cells using ProteinPilot™ Software for database searching at a FDR < 5%. High quality MS/MS spectra obtained reported confidence score of > 95% from sequencing fragments identified from the 100-1000 m/z mass range. Repeat injections (triplicates) demonstrated further gains in total protein ID. Experiments designed to evaluate the effect of shortening chromatography methods on the total number of protein identified demonstrated that fast and sensitive full scan MSMS acquisitions enable complete analysis times under 30 minutes.

Innovative aspects
To develop fast and sensitive protein profiling MS experiments on whole cell mammalian tissue lysates of very limited amounts.

References
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M117

Modeling ETD Fragmentation with Bayesian Network for Peptide Identification

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Introduction
In mass spectrometry–based protein identification and characterization, sequence assignment requires a successful MS/MS dissociation event, that is, production of a sufficient number of informative fragment ions. The recently introduced electron transfer dissociation (ETD) method has proven to be complementary to collision induced dissociation (CID) since it is better suited for sequencing larger and more basic peptides and are now becoming more established technologies. ETD spectra differ from CID spectra in the magnitude and the complexity of fragment ion signals. Current software tools for peptide identification are better suited for CID data and give poorer results with ETD data. We propose a Bayesian network model for peptide identification with ETD data. Preliminary data showed promising results of the model.

Methods
The peak intensity at position (m/z) was normalized using four features: global rank, local rank, log ratio of the maximum intensity and peak intensity, and log ratio of the maximum local intensity and peak intensity. In the probabilistic network, eight common ions (z', c, y, b, a, z'-H2O, c- H2O, and z'+2) were considered. We modeled the correlations between intensities of fragment ions and the existence of a real cleavage. For each edge, two fitted probability intensity curves were utilized for real cleavages and random positions. The score function is the log odds of the probabilities of real cleavages and random positions.

Preliminary results
The Bayesian network model was implemented and tested with a ETD dataset from a complex C. elegans protein mixture digested with trypsin followed by alkylation with iodoacetamide. The spectra were acquired on a LTQ Orbitrap XL ETD. The peptide mixtures were separated with Surveyor LC equipped with MicroAS autosampler using a reversed phase peptide trap and a reversed phase analytical column at a flow rate of 250 nl/min. A gradient of 5~30% acetonitrile in 90 minutes was employed. PEAKS software is used to identify peptides of the spectra from Swissprot database. 264 spectra with high identification confidence were selected. Among them, 132 of the spectra were used for training and the remaining 132 for testing. A linear combination of the four selected features was used as the normalized intensity of each peak. The normalized intensity is better than each of the four features in distinguishing real cleavages from random positions. The comparison of the ROC curves of the four features and the normalized intensity is shown in Fig. 1.

For each fragment ion, we used the training data to acquire the distribution of the normalized intensities of its peaks. Randomly generated positions were used to acquire the background distribution. Then the fitted curves of the distributions were acquired using standard fitting method. The z' ion distribution and background distribution and their fitted curves are shown in Fig. 2.

For each test spectrum S, we mutated its peptide by replacing three consecutive residues with three other residues with the same total mass. If our model is good, then it should give the mutated peptide a lower score than the real peptide. We generated the probabilistic tables using the training data. Then, we used the scoring function to compute the scores of real peptides and peptides with small mutations described above. The experimental result shows that, for the peptides with small mutations, 88% peptides have lower score than the real peptide, which showed that the scoring function has good performance on ETD.

Innovative aspects
New statistical model for ETD peptide fragmentation.

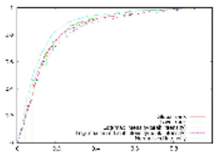

Fig. 1 Comparison of ROC curves of the normalized intensity.

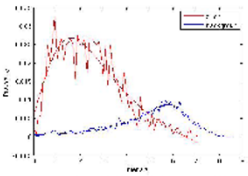

Fig. 2: Comparison of z' ion distribution and background distribution.

M118

Exploring proteome responses of *Alkalimonas amylolytica* N10 to different external pHs

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Introduction
It is very important to understand why alkaliphiles could survive from alkaline environment, which is still poorly understood yet. *Alkalimonas amylolytica* N10 is such a kind of alkaliphilic bacteria found in Lake Chahannor, which could survive from pH 7.5 to 11.0. Till now, only limited numbers of proteins were found in alkaliphiles, which are involved in regulation of pH homeostasis and play import roles in the adaptive mechanism. More proteomic surveys are highly expected to explore the pH-dependent proteins in alkaliphilic bacteria. Being absent of genome data of N10, a combined strategy, including derivative with 4-sulfophenyl isothiocyanate (SPITC) and non-derivative *de novo* peptide sequencing were used to interpret proteome data. The protein identification rate was highly improved, and approximately 40 pH-dependent proteins were found, which would give insight for survival mechanism of alkaliphiles.

Methods
Bacteria strain N10 were cultured in three pH media, 8.4, 9.4 and 10.4, respectively, and the membrane fraction and cytoplasm fraction were obtained by ultracentrifugation. Proteins in the membrane fraction and cytoplasm fraction were then extracted by acetone precipitation and solubilized in lysis buffer containing 2% ASB or 4% CHAPS. After 2DE separation and image analysis, the differential spots were excised and digested. The digestions were divided into two parts, one for SPITC treatment and then MALDI-TOF/TOF MS detection, the other one for direct MALD-TOF/TOF MS detection. All the MS/MS signals were analyzed manually or by PEAKS *de novo* software. At last, all the identified proteins were analyzed by bioinformatics software to classify their function.

Results
Approximate 400 spots for membrane fraction and 600 spots for cytoplasm fraction were averagely appeared on each 2-DE gel stained by silver nitrate. On the basis of image analysis, we totally defined 26 differential 2-DE spots in membrane protein and 46 differential spots in cytoplasm protein responding to the elevated medium pH from 8.4 to 10.4.
With traditional search of Mascot, only 18.1% of spots were identified, which is clear that protein identification with conventional database-search strategy could not achieve satisfactory rates for identifying the strain N10 proteins. By using *de novo* approach, another 40 spots were identified, including 18 spots by SPITC derivative approach, 7 spots by non-derivative approach and 15 spots by both ways. The results revealed that SPITC treatment approach seems to present relatively high identification rate (68.8%) as compared with the untreated samples (45.8%). In addition, the conclusion elicited from one approach would be strengthened by the evidence obtained from the other one. A combined strategy was therefore profitable to avoid missing interpretation caused by a single *de novo* approach. Together all, the identification rate increased to 73.6%, which reached the same level of efficiency and reliability as conventional database-search strategy.
By using topological analysis and reference search to predict the cellular locations of the identified proteins, we found about 86.4% of the membrane identifications were deemed part of membrane, which indicated the successful protein extraction. Function analysis of differential proteins revealed that some membrane proteins participated in iron transport were differentially expressed as external pH elevated and most of differential proteins with increased or bell-shape mode of pH-dependence were involved in bioenergetic process and metabolism pathways. Our data thus provide a functional profile of the pH-responsive proteins in alkaliphiles, leading to elucidation of alkaliphilic-adaptive mechanism.

Innovative aspects
- For the first time we have systematically studied the pH-dependent proteomes in membrane and cytoplasm of alkaliphilic bacteria.
- We have successfully combined non-derivative *de novo* and derivative *de novo* sequencing strategy to investigate the bacterial proteome whose genome is not available yet.

References
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M119

Combining Chip-based Enrichment of Phosphopeptides with Specific Analysis using ETD Mass Spectrometry

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Introduction
Comprehensive identification of protein phosphorylation is challenging due to the complexity and low abundance of phosphorylation events. Enrichment of the phosphopeptides improves sensitivity and can be achieved with a new microfluidic chip with an enrichment column consisting of titanium dioxide particles sandwiched between reverse-phase. Unambiguous assignment of phosphorylation sites by LC/MS/MS is best achieved with ETD. Thus in this work the Phosphochip has been combined with ion trap based ETD mass spectrometry for the precise characterization of the phosphopeptides from Erk1 protein.

Method
Active recombinant human mitogen-activated kinase Erk1 (Millipore) was denatured, reduced, alkylated and digested with trypsin. LC/MS analyses were performed on an Agilent HPLC-Chip/MS interfaced to a 6340 ion trap mass spectrometer configured with ETD. An aliquot of Erk1 tryptic digest was injected on the Phosphochip and the phosphopeptides bound to the TiO_2 were eluted to the C-18 trapping column with an aliquot of injection buffer. Then 10 µL of 10% formic acid was injected and the eluted peptides were analyzed. The Phosphochip design included: a multilayer polyimide laminate, a TiO_2 bead packed enrichment column flanked by C-18 reverse-phase enrichment columns, an integrated C-18 reverse-phase separation column and a laser-ablated metalized nano-ESI emitter.

Results
For the preliminary study, the Erk1 digest was analyzed using the Phosphochip in both flow-through and enriched modes. Protein database searching of the LC/MS analysis from the flow-through fraction, i.e., those peptides not bound to the titanium dioxide fraction, confirmed that these peptides were not phosphorylated. In contrast, the analysis of the eluted bound peptides yielded a much less complex chromatogram (Figure 1) which contained seven phosphorylated peptides. Further data will show the ETD performance and confirmation of the sites of phosphorylation. The combination of linking the Phosphochip system to a mass spectrometer with ETD capability allows very specific selection, detection and identification of phosphopeptides from simple biological systems to complex ones.

Innovative aspects
- On-line enrichment of phosphopeptides in a microfluidic chip
- Combining phosphopeptide enrichment and separation with ETD characterization

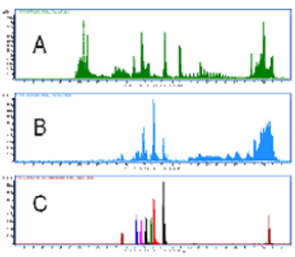

Figure 1. Results for LC/MS analysis of Erk1 digest showing A) TIC of flow-through peptides (non-phosphorylated); B) TIC of bound peptides (phosphorylated) and C) Selected EICs for phosphopeptides from the bound fraction.

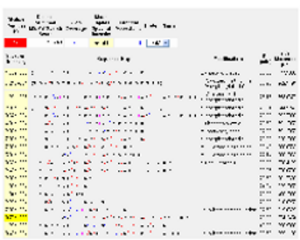

Figure 2. Results for protein database search of the Erk1 bound fraction (Figure 1B) showing 7 phosphopeptides and 3 potential phosphorylation sites.

M120

On the usage of the number of carbon atoms for peptide mass fingerprinting

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Introduction
Many mass spectrometric instruments (like the major part of TOF instruments) don't have the ability to perform ion fragmentation, for measuring masses of fragments. In proteomic applications users have to rely on the masses of peptides to get identifications. This approach works for simple protein mixes, but fails when you move to the full human proteome as any mass measurement accuracy (mma) under 0.1 ppm is not enough to uniquely identify a peptide. Additional information obtained during the mass spectra deconvolution process allows to lower mma requirements significantly.

Method
Peptide mass fingerprinting is a well known technique for peptide identification. The exact masses are compared against computed masses in a dataset, consisting of theoretically digested proteins from the proteome of interest. We enhance this method, by calculating not only the masses of peptides in the reference database, but also the numbers of carbon atoms. We have developed a new algorithm for deconvolution of isotopic clusters, which, as a byproduct, calculates the number of carbons alongside with monoisotopic masses. Comparing both against the reference database using predefined accuracy comprises the whole method. Additionally, having the database at our hands we could simulate real isotopic distributions of all peptides and search for those in non-tandem MS spectra.

Results
So far we have only checked theoretical capabilities of the method described above. Taking one of the latest ipi.HUMAN databases and theoretically digesting (with Trypsin) all the proteins gave over 3 billion peptides, removing ones, that were shorter than 5 amino acid residues left us with about 400000 peptides. For each peptide mass and the number of carbon atoms were calculated. Then the amount of peptides that can be distinguished by exact mass alone and by mass with the number of carbons was evaluated at different mma levels. Results have shown that the best effect is observed at mma of 1 to 10 ppm, where without the number of C atoms we could identify from 20 to 2 percent of all the peptides, while using the number of carbons enables identification of additional 50 to 28 percent of peptides. At the often routinely achievable level of 5 ppm the corresponding percentages are 5 and 44 – which is half of all the peptides from the currently annotated human proteome. Real life data comes when the deconvolution/deisotoping program is finished and well tested.

Innovative Aspect
Ability to use peptide mass fingerprinting to identify the major part of peptides from human proteome.

M121

Increased Proteome Definition Exploiting a New Linear Ion Trap Mass Spectrometer

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Thermo Fisher Scientific, 355 River Oaks Parkway, San Jose, CA

Introduction
Qualitative definition of a proteome is a prerequisite for investigations of proteome dynamics. Means of characterization should be exhaustive and reliable, and is currently addressed by tandem mass spectrometry for peptide/protein identification. To satisfy increasingly demanding requirements for throughput and thorough characterization, the chosen mass spectrometer is a new linear ion trap with improved scan rate, sensitivity, and dynamic range for MS/MS peptide identification. The intent is to benchmark performance of the new instrument for characterization of a complex proteome.

Methods
The new instrument features a dual pressure linear ion trap and a stacked ring ion guide source to deliver better trapping/fragmentation efficiency, greater resolving power, and superior ion flux. A novel implementation for controlling the ion population also produces a dramatic increase in scan rate for data-dependent tandem MS/MS. A *C. elegans* digest was subjected to reverse phase separation prior to introduction into the mass spectrometer run in the data dependant fashion. Peptide identifications were determined from a database search with MASCOT, conducted through Proteome Discoverer 1.0 software. Only identifications exceeding a threshold that produced a false discovery rate of 1% were considered.

Results
The new ion trap instrument demonstrates practical scan rates more than twice that of an LTQ XL while maintaining equivalent spectral quality for MS/MS fragmentation spectra of peptides, resulting in a significant increase over existing platforms in the detection and identification of both proteins and unique peptides (protein coverage) from a complex proteome. There is considerable overlap in the complement of proteins identified between the two instruments (>80%), with additional proteins seen only on the new instrument. The greatly increased ion flux into the mass spectrometer afforded by the new source design also contributes to increased detection of peptides at lower abundance. Enhanced resolution from analysis in the lower pressure trap demonstrates resolution >20,000, sufficient for isotopic resolution of small proteins, such as myoglobin and cytochrome C; a resolving power beyond that of any commercial quadrupole ion trap yet demonstrated. High practical acquisition rates for a tandem mass experiment on the new instrument also permits rapid throughput for identification of proteins in simple mixtures with short gradients and short run times.

Innovative aspects
- Significant technology and performance enhancements of linear ion trap-based instrument result in dramatically increased penetration into complex proteome

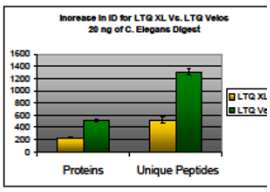

Figure 1: Increase in identification of unique peptides and proteins for 20 ng injection of C. elegans digest (lower quantity injection), demonstrating increased sensitivity.

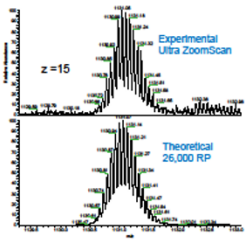

Figure 2: Resolution of intact myoglobin (~16 kDa) with Ultra ZoomScan, demonstrating >25,000 resolving power FWHM.

M122

Generating Products of All ions with an Experimental Trap-TOF.

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Introduction
The goal of a profiling experiment is to fragment as many precursors as possible extracting the maximum information in a single analysis. Previous attempts to use precursor ion scans where, a mass resolving quadrupole is stepped every amu and the transmitted ions fragmented recording all daughter ions failed as instrument sensitivity requires a dwell time in the order of 100ms per step mass resulting in very long cycle times.
Data is presented using very short dwell times providing cycle times which are compatible with nano-LC gradient profiles from an experimental Trap-TOF.

Method

Experiments were performed on a test bed instrument which consisted of a QJet, a skimmer pulsing equipped quadrupole ion guide, a mass resolving quadrupole and a LINAC II collision cell. Ions were pulsed from an ion trap post collision cell into the time of flight accelerator to achieve best duty cycle.
A sample of C2C12 myoblast was injected onto a nano-lc prior to the mass spectrometer. The resolving quadrupole was scanned in a range to cover the maximum of expected peptide m/z. Ions from each step were fragmented and all product ions recorded. The data was processed such that peptide identifications could be determined and compared to operating the instrument in a normal dependant scan mode.

Results

Using a precursor scan mode generated scan times which were between 5 and 12 seconds dependant upon the mass range scanned and dwell time which was set for each mass.
Determination of the optimal mass range to scan was performed in silico and showed that for the sample analysed the mass range of 500-700 amu would allow maximal coverage of the peptides defining 90% of the proteins expected in the sample.
Scanning 2 different mass ranges did not show any significant increase in the number of proteins identified, in a single LC-MSMS analysis scanning 500-700amu identified 1015 vs 1009 when scanning from 400-900amu at 5%FDR. At the peptide level 2105 spectra were identified from a commercial QqTOF whilst 16120 were identified from the POA analysis, a factor of 8 increase.
These results indicate that the use of a narrow mass range scan and complete fragmentation of all ions can result in sample coverage which is better than with traditional automated parent ion selection. This type of scan has been non functional until the development of technology to provide the improved sensitivity and scanning speed such as demonstrated in this presentation.

Innovative Aspects

- A novel method for extracting maximum peptide information from a single analysis
- Fragmentation of all parent ions in a single LC-MSMS analysis
- Novel Hardware allowing implementation of rapid precursor ion scans with high sensitivity

M123

INNOVATIVE MASS SPECTROMETRY TECHNOLOGY FOR THE IDENTIFICATION OF CANCER IMMUNOTHERAPEUTICS

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At present, the most effective treatment for metastatic melanoma is adoptive T-cell therapy (ACT). In this approach, tumor-infiltrating lymphocytes (TIL) are isolated from resected tumor and expanded ex vivo. After the patient's immune system is ablated by a combination of chemotherapy and total body irradiation, the TIL plus cytokine (IL-2) are re-infused. Objective (tumor skrinkage) and complete responses for this therapy in a recent clinical trial of 25 late stage patients with metastatic melanoma were 72% and 16%, respectively. Tumors the size of grapefruit disappear in a matter of months. Reported here are efforts to identify class I and class II MHC phosphopeptide antigens that are presented (1-10 copies/cell) as a result of dysregulated signal transduction pathways in cancer cells and that will make it possible to employ adoptive T-cell therapy for many additional human cancers.

M124

Of bears and birds
Genome free de novo sequencing by a combination of LysN protein digestion and electron transfer dissociation

A.F. Maarten Altelaar[1,2]; Nadia Taouatas[1,2]; Shabaz Mohammed[1,2]; Albert J.R. Heck[1,2]
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In a typical MS experiment peptides are identified by searching fragmentation spectra against large protein sequence databases and scored based on the overlap between the experimental and theoretical fragment spectrum. An alternative strategy, for species with un-sequenced or poorly characterized genomes, utilizes de novo sequencing of the fragmentation spectra. However, de novo sequencing is challenging due to the complexity of common CID fragmentation spectra. Recently, we have shown that Lys-N enzymatic cleavage in combination with ETD analysis results in fragmentation spectra almost exclusively containing N-terminal fragment ions. These, easy to interpret, ladder sequences open up a completely new window for de novo sequencing and markedly reduce the dependence on sequenced genomes. As a proof of concept we analyzed the proteomes of ostrich muscle and hibernating bear heart.

We performed a proteomics study of Ostrich muscle or Bear heart tissue through Lys-N proteolytic cleavage followed by low-pH SCX fractionation, RP-nanoLC separation and ETD dissociation. The SCX fractionation is used for isolation of the 'de novo sequence-able' peptides. These peptides produce fragmentation spectra, after ETD, dominated by c-type ions, which are relatively easy to interpret. De novo analyses of the ETD spectra is performed by an in-house developed algorithm, called LysNDeNovo, which utilizes the presence of a single fragment ion series to assign the peptide sequence. The resulting peptide sequences are, in turn, subjected to a BLAST search to find similarities between the ostrich and related species. Here we BLAST searched against a Chicken, Human and 'Bird' database, with the last one being comprised of several bird species which are in the same phylogenetic tree branch as the Ostrich. Our de novo sequencing approach results in a significant higher number of peptides identified than searching the ETD Ostrich proteomics dataset using the Mascot search engine. Moreover, the de novo results allow the determination of point mutations as well as conserved regions between proteins of different species. Furthermore, the experimental design, the de novo algorithm and the idea of consequent genome free proteomics will be discussed.

Furthermore, we are in the process of analyzing and quantifying the differential proteome of bear heart tissue from a hibernating and summer-active bear. Therefore, the LysN generated peptides are first isotope labeled using a protocol based on reductive dimetlylation of Lysines and free amines. Hundreds of proteins could be identified de novo, and several of these show significant changes in between the hibernating and summer active bear.

M125

Lys-N and Trypsin cover complementary parts of the phosphoproteome in a refined SCX-based phosphoproteomics approach

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Introduction:
In proteomics multi-dimensional fractionation techniques are widely used to reduce the complexity of peptide mixtures subjected to mass spectrometric analysis. In particular, low pH SCX separation has been widely exploited for extensive phosphoproteome screens. The combination of this technique with an alternative protein digestion strategy using a Lys-N metallo-endopeptidase has recently emerged as a promising tool for phosphopeptide enrichment. Furthermore, Lys-N combined with ETD allows the generation of simple spectra that can allow facile de novo sequencing. In this presentation we evaluate this approach against more classical approaches that use trypsin and Lys-C as the enzyme of choice in a large scale experiment.

Method:
Equal amounts of HEK293T cell lysate were digested by Lys-N, Lys-C and Trypsin. Resulting peptides were first separated by SCX chromatography on an Agilent 1100 HPLC system with a C18 Opti-Lynx guard column and a Polysulfoethyl A SCX column. Enriched fractions were analyzed by LC-MS using either an LTQ XL Linear Ion Trap Mass Spectrometer with an ETD source or an LTQ-Orbitrap.

Results:
We explored a method for global protein analysis of whole cell lysates using a combination of Lys-N proteolytic cleavage followed by low-pH SCX fractionation and RP-nanoLC-MS/MS analysis using both CID and ETD. Digestion with Lys-N yields proteolytic peptides with the lysine at their N-terminus, resulting in an increased basic entity caused by the lysine and N-terminal amines. In the subsequent low-pH SCX separation, distinct fractionation profiles could be observed in which peptides from different functional categories were extremely well separated. The four categories we were able to separate well are I) acetylated N-terminal peptides, II) singly phosphorylated peptides containing a single basic (Lys) residue, III) peptides containing a single basic (Lys) residue and IV) peptides containing more than one basic residue. A large advantage of this approach is the enrichment and targeted analysis of the phosphoproteome. ETD analysis of the purified phosphopeptides (category II) results in clear c'-ion ladder sequences of the peptides, and allows a simple read out of the location of the phosphorylation site. Subsequently, we evaluated the relative efficacy of three proteolytic enzymes (namely Lys-N, Lys-C and trypsin) in conjunction with the de facto standard for phosphoproteome screening which is based on low pH SCX. Lys-N alone allowed the identification of over 2300 phosphopeptides using approximately 300 ug of material. Combination of all 3 enzymes and a total of 1 mg of material allowed identification of over 5000 phosphopeptides. In this presentation we will show that Lys-N is a highly viable as a starting point for phosphopeptide enrichment by low-pH SCX. Furthermore, Lys-N provides a certain level of complementarity to Trypsin.

Innovative aspects:
- Lys-N in combination with SCX and RP-separation is allows a facile analysis of phosphorproteome.
- Lys-N, Lys-C and Trypsin generate complementary pools of phosphopeptides.

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M126

Optical Fibres as Multi-channel Electrospray Emitters

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Introduction
Electrospray ionization mass spectrometry (ESI-MS) has become a dominant analytical tool in proteomics research. There has been a trend toward lower flow rates to maximize ionization efficiency, sensitivity and sample utilization, but current tapered emitters make it difficult in practice to achieve consistent, robust electrospray in this regime. Here we present a novel multi-channel electrospray emitter based on optical microstructured fibre (MSF) capable of highly sensitive ESI-MS without the worry of clogging.

Methods
MSF's containing 30, 54, 84 or 168 separate channels were purchased and used for the ESI-MS of a variety of test analytes such as leucine enkaphalin, bradykinin and insulin down to 1 μM. Channels are ~5 μm in diameter, spaced ~7 μm apart in a silica fibre ~230 μm in diameter (Fig. 1). Electrospray was demonstrated at flow rates from 1000 to 50 nL/min and with highly organic solvents (methanol or acetonitrile) or highly aqueous solvents. The tips were treated with a hydrophobic silylation reagent to aid in the electrospray of highly aqueous solutions. An optical microscope was used to image the electrospray plume offline. Resistance to clogging was demonstrated by electrospraying a concentrated salt solution.

Results
The emitters are capable of stable electrospray at flow rates in the microspray regime (e.g. 1 μL/min) down to the nanospray regime (e.g. 50 nL/min) with high sensitivity. Using the hydrophobically modified tips, aqueous solutions (99.9% H_2O with 0.1% formic acid) were successfully sprayed over the entire range of flow rates (50-1000 nL/min).
The presence of multiple channels allows the formation of multiple Taylor cones, theoretically improving ion current relative to a single emitter at the same flow rate by a factor of the square root of the number of emitters in the array (1). To visualize the multispray, electrospray imaging showed that at higher flow rates (e.g. 300 nL/min) a single Taylor cone is observed covering the tip, but at low flow rates (e.g. 25 nL/min) a mist is observed.
The robustness of the emitters was demonstrated by continuous stable electrospray (<11% rsd) for >6 hours. Further, Hanks' solution (a salt solution known to clog emitters) was sprayed from the multi-channel emitter and a commercial tapered emitter. The tapered emitter clogged within seconds, but the multi-channel emitter actually sprayed the solution for 25 minutes then recovered.

Innovative aspects
- Multi-channel electrospray from a single-fibre, non-chip-based device
- Excellent robustness relative to most commercial emitters
- Good stability and sensitivity over a wide range of flow rates and solvent compositions

References
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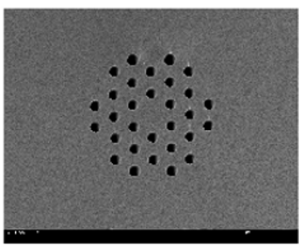

Figure 1. Emitting surface at the tip of a multi-channel electrospray emitter (30-hole). The scale bar is 50 μm.

Mass Spectrometry Based Molecular Imaging

(M201 – M206)

M201

Determination of Oxaliplatin distribution in rat kidney during HIPEC: a mass spectrometry profiling

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Determination of Oxaliplatin Distribution in rat kidney in HIPEC conditions: an IMS study.

Introduction: MALDI imaging allows the study of spatial distribution of proteins and peptides directly on a tissue sample. This technique is now coming closer to clinical applications with the discovery of numerous pathological biomarkers.
A novel tissue sample preparation tool associated with MALDI MS and imaging software, has been used in this study. This imaging chain has lead to successfully identified and localised oxaliplatin, a chemotherapeutic drug used in the treatment of peritoneal carcinomatosis, on a thin section of rat kidney.
Methods: Rat kidneys were incubated with Oxaliplatin (Eloxatin®) at 42°C during 35 min as decribed in human hyperthermic intraperitoneal chemotherapy (HIPEC) treatment. The tissue sample preparation has been achieved with a new spotting robot (Siliflow®). The integrated microscope was used to visualize the area of interests onto the rat kidney tissue slices (15µm). Then, the few nanoliters matrix droplets were deposited homogeneously on the selected zones at a predefined humidity rate for the sake of experiments reproducibility. The arrangement of the droplets forms a regular pattern of matrix spots and the spots coordinates are then exported to the 4800 Plus MALDI TOF/TOF™ Analyzer. MS imaging was performed at a laser pixel resolution of 100µm x 100µm using an MS Reflector Positive method scanning the mass range 300 - 500 amu, firing 50 laser shots per pixel. Imaging data was processed using TissueView™ software.
Results: Our IMS study allowed the identification of multi ionic forms of Oxaliplatin (Oxa + NA^+, K^+...) mass spectra signature. Interestingly, Oxaliplatin forms are localised only on the periphery of the tissue, showing an apparent penetration default of the chemotherapeutic drug in rat kidney.

Innovative aspects: The combine utilization of the new robot spotter and MALDI imaging allows for the first time the determination of the spatial distribution of the Oxaliplatin from tissue section.

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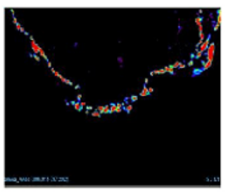

Localisation of Oxaliplatin (397 m/z) in a thin section of rat kidney using Maldi Imaging

M202

Tracking the Distribution of Tiotropium in Rat Lung Using MALDI Imaging Mass Spectrometry

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Introduction
Precise readouts defining the cellular distributions of inhaled drugs has remained a difficult but much desired task. Inhaled therapies enable rapid onset and provide higher local concentrations of compounds to specific target sites in comparison to drugs administered perorally. In the present study we have tracked the distribution of tiotropium in rat lung at different time points following inhalation and additionally we demonstrate that compounds administered by inhalation can be quantitatively detected in lung tissue sections by MALDI IMS with accuracy and precision.

Methods
Tiotropium was administered as an aerosol of a nebulized solution to rats. The animals were sacrificed at different time points after administration. The lungs were rapidly dissected out and the frozen tissues were cut into 14 µm thick sections with a cryomicrotome. The sections were transferred to and thaw mounted on conductive MALDI glass slide dried and the lung tissue sections were coated with CHCA matrix solution (5 mg/ml) in 50:50:0.1 ACN/H_2O/TFA using automatic spraying/spotting devices. MS and MS/MS experiments were carried out using the UltraFlexII MALDI-TOF/TOF MS (Bruker Daltonics, Bremen, Germany). Quantitative measurements of tiotropium on dosed tissue were performed by correlating the levels of tiotropium to tiotropium standard solutions applied to control lung tissue.

Results
The spatial distribution of tiotropium in lung tissue was determined in both MS and MS/MS mode. The MALDI IMS images showed that although the highest concentration of tiotropium was observed in the central lung compartment, tiotropium was rapidly transported from the central conducting airways to alveolar beds in the upper and lower parenchyma.
To estimate the concentration range of tiotropium present in lung tissue sections from rats administered the drug by inhalation, we compared the signals from different concentrations of drug standard manually applied to control lung tissue section to those obtained directly from tissue isolated from dosed animals. After analysis, the average intensity values from each tiotropium deposit were calculated and a linear regression curve of tiotropium concentration was constructed for the MS and MS/MS experiment, respectively (Figure 1). In MS mode there was linearity between 80 fmol to 2.6 pmol (r=0.983) and in MS/MS mode linearity was obtained in the range from 20 fmol to 5 pmol (r=0.995).
The levels obtained by MALDI Imaging MS measurements correlated very well with the ones obtained by conventional LC-MS/MS measurements.

Innovative aspects
- Drug distribution analysis following inhalation using MALDI imaging MS
- Absolute quantitative drug measurements directly on tissue sections

M203

Neuropeptidome Alterations in an Experimental Model of Parkinson Disease

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Introduction
Parkinson disease (PD) is characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta projecting to the striatum in the brain. The information regarding the expression of neuropeptides in parkinsonism is very limited. Here we have elucidated brain neuropeptide mechanisms in experimental parkinsonism using the unilateral 6-hydroxy-dopamine (6-OHDA) model to degenerate dopamine neurons. A thoroughly controlled sample preparation technique (1) together with a peptidomics approach (imaging MALDI mass spectrometry (IMS) and nanoLC ESI MS (2) and targeted neuropeptide sequence collections (3) enabled sensitive detection, identification, and relative quantitation of a great number of endogenous neuropeptides.

Methods
Mice were administered the neurotoxin 6-OHDA unilaterally to induce a selective degeneration of the nigro-striatal dopaminergic pathway of the brain. Four weeks after surgery, mice were treated with saline (n=6) or L-DOPA/benserazide (n=6; 50/12.5 mg/kg, ip) once daily for 14 days. All mice were sacrificed 15 min after the 15th L-DOPA injection. The tissue were cut by a cryomicrotome and studied by IMS (UltraFlexII, Bruker) but also analyzed by nanoLC-ESI MSMS (Q-Tof2, Waters and LTQ, Thermo) after homogenization of specific brain regions. The animal experiments were approved by the local ethics committee at Karolinska Institute. To optimize the identification process for endogenous peptides we used our in-house constructed SwePep database (3) (www.swepep.org).

Results
The analysis demonstrated several differentially expressed peptides and small proteins in the experimental models of PD. Different normalization strategies were evaluated using various settings for improved quantification of endogenous neuropeptides. The 6-OHDA treated animals displayed an increased relative level of secretogranin I, but was normalized after L-Dopa administration, the conventionally treatment of PD symptoms. Dynorphin B peptides were increased after the L-Dopa treatment in the dopamine-depleted side of the brain. In addition, some of the novel peptides identified was significantly altered in the experimental model of PD. Novel peptide families affected by neurodegeneration were identified. In the present study we have used improved approaches to unravel unidentified neuropeptides from complex biological matrices, such as the brain.

Innovative aspects
- Novel alterations in neuropeptide levels were identified in the unilateral lesioned mice with or without subchronic L-DOPA
- Novel peptide families involved in experimental PD were identified
- Targeted neuropeptide sequence collections enabled sensitive detection, identification, and relative quantitation of a great number of endogenous neuropeptides

References
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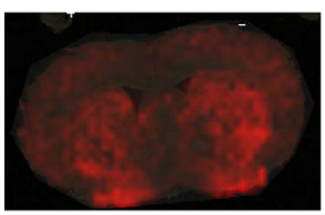

Figure 1. MALDI IMS of a brain tissue section following unilateral 6-OHDA administration. The molecular image shows the expression of PEP-19 which was predominantly defined to the striatum. After lesioning with 6-OHDA, there was a significant decrease in the striatal expression (6-OHDA lesion in left hemisphere).

M204

A Novel Statistical Approach to Interpret Complete MALDI Mass Spectrometry Datasets

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Introduction
Mass spectrometry imaging enables biomolecules to be analyzed directly from tissue providing spatial information. The data collected is complex and within one tissue section, a large number of signals are detected and thousands of pixels can be defined. In a non-targeted experiment, signals over a large mass range metabolites, lipids, peptides and proteins may be observed and identifying relevant signals from molecules of interest proves challenging. In order to present a series of images, one has to scan all masses to observe the differences, and similarities, between molecule localizations.

Methods
An unsupervised statistical approach examines the entire dataset. Unlike the traditional approach of dividing the dataset into different regions and comparing the abundance of molecules in each region. Here we compare the distributions of molecules throughout the entire tissue section by extracting scan distributions of each m/z present. A scan distribution is a plot where the intensity of an ion is extracted for every pixel present. Comparing all scan distributions enables a statistical algorithm to group molecules which have similar localization by comparing their scan distributions.

Results
MALDI Imaging data obtained from frozen mouse uterus tissue sections demonstrate the statistical approach. Data was acquired with ion-mobility to provide an extra dimension of separation for very complex sample datasets and therefore to have a true distribution of molecules without any interference.

The data interpretation workflow starts with the extraction of the scan distribution for each m/z value. For each m/z value, the scan distribution represents its pixilated image without taking into account their spatial coordinate. After processing which may include; smoothing, background subtraction, removal of noise and/or normalization, the dataset can be interrogated using different multivariate statistical algorithms, such as clustering or Principal Component Analysis (PCA).

We will illustrate how PCA can rapidly group m/z values which exhibit similar distributions throughout the tissue, without prior knowledge. This approach easily facilitates, for example, the differentiation of exogenous molecules from endogenous molecules, as well as the identification of structural features within the image.

Innovative aspects
- Use of PCA to interpret MALDI Imaging data sets
- Ion mobility provides an extra dimension of separation andnreduces interferences

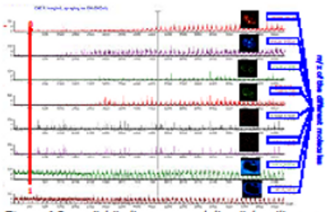

Figure 1 Scan distribution representation (intensities vs. pixel number) of eight molecules where molecules 1&2, 3&4, 5&6, 7&8 have similar scan distributions as a pair, but the pairs differ one from another.

M205

MALDI-Tissue Imaging at High Resolution and Speed: Towards its Applications in Histology

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Introduction
MALDI imaging permits extracting molecular information from tissue sections and enables the elucidation and detection of biomarkers directly from tissue. The resolving power of imaging – just like the resolving power of a chromatograph – determines the level of morphological correlation between the molecular image and the histological image. Therefore, increasing the MALDI image resolution at no loss in signal intensity, at increased speed of data acquisition, is paramount for a method characteristic that is meant to provide histological meaningful results. Increased acquisition speed is required to keep acquisition times of high resolution images reasonable.
We introduce a new laser design in an new axial MALDI-TOF instrument, which provides 20 µm image resolution for proteins at 1 kHz spectra acquisition speed.

Methods
Fresh-frozen rat testis and mouse brain was analyzed. Tissue sections were prepared on ITO coated glass slides. All tissue samples were covered by matrix using vibrational vaporization, creating crystal sizes around 20 µm in a homogeneous preparation.
An ultrafleXtreme MALDI-TOF/TOF with 1 kHz smartbeam II laser with 10-100 µm laser focus size was used for all measurements (Bruker).
After MALDI acquisition, the matrix was rinsed off the tissue, which was subsequently H&E stained and scanned for bimodal H&E+MALDI image analysis using a Zeiss Mirax scanner. Image co registration and analysis was done using flexImaging 2.2.

Results
Smartbeam solid state lasers have a structured, non Gaussian beam profile. This property is responsible for the efficient readout in particular of peptide signals up to higher molecular weight (~30 kDa). New methods were implemented for the modulation of the beam profile for very small focus diameters (~10 µm).
With classical Nd:YAG-solid state lasers with Gaussian profile we observed a dramatic loss in signal intensity from MALDI images – even at focus diameters exceeding 100 µm. The modulation of the beam profile as described in [1] counteracted this effect quite efficiently. However, imaging at spot diameters and raster distances below 80 µm suffered considerable loss in signal intensity, particularly of peptides exceeding 1 kDa.
The new generation smartbeam II laser provided now for the first time image analyses of the fine structured rat testis and the well known mouse brain model systems with a protein resolution of 20 µm without detectable loss of spectral quality and signal intensity loss. At the same time, these images were obtained with a laser repetition rate of 1000 Hz providing for a gross acquisition rate of 2 pixel/sec.
The 20 µm MALDI images were matched to the stained optical image scans and permitted the bimodal analysis in a quality not available before. This quality level is extremely helpful in the context of in-depth biomolecular or clinical research, as it approaches the single cell level – though it doesn't reach it for normal mammal cells. In many biological processes such as tumor development and progression, single cells represent distinguishable biochemical states that can only be tackled with if the MALDI image resolution approaches the 10 µm level.

Innovative aspects
- 1 kHz MALDI tissue imaging at 20 µm resolution for protein biomarkers
- Virtual microscopy (a la GoogleEarth) is merged with molecular MALDI imaging
- Near single cell imaging permits individual cell layer analyses for clinical research

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M206

EXPRESSION OF Human VITREOUS HUMOR PROTEINS IN RETINAL DETACHMENT

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Introduction
The retina is the inner layer of photoreceptor nerve cells lining the back, inside wall of the eye. Retinal detachment(RD) is a disorder of the eye in which the retina peels away from its underlying layer of support tissue. The proteomic changes that occur during RD cannot be measured in the human retina due to ethical reasons, but could be reflected by proteomic changes in vitreous humor. Thus, we considered that comparisons between the proteome profiles of the vitreous humors of RD with that of Proliferative Diabetic Retinopathy (PDR) controls could lead to the discovery of novel pathogenic proteins and clinical biomarkers.

Methods
Vitreous Sample of RD patients were collected during vitrectomy surgery. Total Protein estimation was done by BCA assay. Samples were Lyophilized. Vitreous Proteins were separated by SDS- PAGE and 2D-PAGE.Proteins spots were visualized by Coomassie brilliant blue and silver staining and analyzed by mass spectrometry.

Results
A significant negative correlation of total protein with age ($p<0.05$) was observed in patients with RD. Mass spectrometry analysis revealed Type II cytokeratin 1 and Serum albumin variant. Main objective of the study was to observe and compare the protein expression pattern in RD and PDR vitreous.We have developed a baseline comparison for Human vitreous proteins appeared in RD and PDR samples which could provide clues to new therapeutic approaches in eyes with RD.

Innovative aspects
- Total protein concentration was negatively correlated with age in RD vitreous while there was no correlation of age with total protein contents in control(PDR) vitreous.
- RD vitreous protein profile became more complex with age.
- A 25 KDa Protein was observed to be down regulated in RD vitreous with age.

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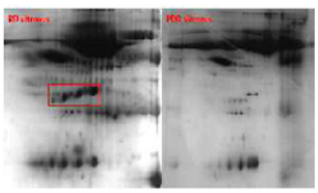

Figure 1: Difference in RD vitreous protein profile and control (PDR) vitreous profile. Highlighted spots were observed only in RD vitreous while they were absent in PDR vitreous. These were further analyzed by mass spectrometry.

Nanotechnologies and Cell Imaging

(N101 – N104)

N101

Optical and optico-acoustical biosensor application for protein-protein interaction in proteomics.

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Introduction
Implementation of nanotechnologies into proteomics will enable to make the proteomics methods more fast-acting and less costly. From this viewpoint, the following most efficient nanotechnological approaches were developed: 1) optical biosensors devices based on fast-acting and capable of revealing protein markers in real time and (2) systems based on compact disks.

Methods
Optical biosensor nanosystems allow ones to attain high throughput efficiency with very small amounts of analyzed sample without labels. The principle of optical biosensor action is based on the monitoring of the refraction coefficient of the medium upon complex formation. In this system, the nanolayer of one of the partner proteins is formed on the support of the cuvette's bottom to form a biochip. Formation of complexes between partner proteins brings about changes in the refractor index on the sensitive layer surface and these changes are registered in real time.
New optico-acoustical biosensor transforms directly intermolecular interaction into the informational 2-bit code. The sensitive element of this biosensor is compact discs with sensitive zones of immobilized probe proteins or other biomolecules. Analyzer is a standard reader of CD – ROM to personal computer.

Results
The application of "resonant mirror" optical biosensor for registration of protein-protein interaction within P450-containing monooxigenase systems were demonstrated. Formation of binary and ternary protein complexes in multiprotein soluble P450cam and membrane P450scc and P4502B4 systems were registered and association/dissociation rate constants and Kd were measured. The diagnostic optical biosensor biochips for revelation of the hepatitis B and C markers were developed also. These biochips allowed to register of HbsAg, anti-HCVcore, anti-HCvcore markers protein in sera in real time without labels.
We created new optico-acoustical biosensor for registration of protein–protein interaction. Protein complex formation on CD surface were registered based on monitoring of appearance of reading errors. which were presented in form of acoustic line or 'molecular" music. The musics for different protein, for example, cytochromes P450, cytochrome P450 reductase, ferrodoxin and protein complexes are demonstrated.

Innovative aspects
- Creation of optical biosensor biochips for registration protein-protein interaction in real time, measuring of association/dissociation rate constants and Kd simultaneously
- Creation of optical biochips for revelation of Hepatitis B and C markers.

Creation of new optico-acoustic biosensor for registration of protein protein interactions.

N102

AFM/MS irreversible fishing technology for detection of low- and extra-low copied proteins

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Introduction In the absence of an analog of PCR for proteins, the concentration detection limit (DL) becomes a real challenge. There is a problem of revelation and identification of low- (10^{-13}- 10^{-15} M) and extra-low (<10^{-15}M) copied proteins. We suggest to solve this problem by using the method combining two technologies: AFM-irreversible fishing and mass-spectrometry.

Methods In an early stage AFM-irreversible fishing/MS allows to concentrate the molecules of the reference analyte on the surface of AFM-chip and to identify proteins by mass-spectrometry in a second stage. AFM-irreversible fishing technology is based on the fishing of low- or extra-low copied proteins from the biological material on the activated atomic-smooth chip surface to AFM microscope and covalent immobilization of these proteins in that chip.

Results The advantage of irreversible AFM-fishing compared to the reversible one is in the fact that it takes away the concentration barrier of registration of complex-formation. Using proteins of HCVcore Ag, avidin and anti-α-phetoprotein as the examples, it was experimentally shown that irreversible fishing allows to perform AFM-registration not only of low-copied but also of extra-low copied proteins up to 10^{-18} M concentration with subsequent identification of fished proteins on the surface of AFM-chip.

Innovative aspects
- The new AFM-irreversible fishing/MS methods were created for revelation and identification of low- and extra-low copied proteins.

N103

Optico- acoustic biosensor technology for express analysis of proteins.

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Introduction
The most effective way to put nanobiosensor into proteomics might be to create the technology so that it can realize on "everyday" devices, rather than on specialized apparatus. New optico-acoustic biosensor technology can be used for registration of proteins. .

Methods
New optico-acoustical biosensor transforms directly intermolecular interaction into the informational 2-bit code. Analyzer is a standard reader of CD – ROM to personal computer. The principle of optico-acoustic biosensor action is based on the monitoring of the interference picture upon complex formation by CD-ROM. The protein probe is covalently immobilized on CD-biochip, creating this way a CD-biochip, and to carry a procedure of fishing of an protein-partner from solution, then the formation of protein-partners complexes on the surface of CD is registered. The number of complexes formed can be calculated from comparison of the distribution of 2-nd order errors that were caused by complex formation on the compact disk surface with the initial distribution of 1-st order errors on the compact disk with immobilized probe protein.

Results
Optico- acoustic study of soluble and membrane proteins P450 2B4, P450cam, P450scc and their redox partners in the cytochrome P450 containing systems was demonstrated using CD-ROM. The optico-acoustic signal dependence on protein concentration was obtained. Errors from these proteins and their playback as a frequency of acoustic signals on PC CD-ROM enables to obtain an acoustic melodies – molecular musics. This technology is developing for diagnostics of disease. For example, we have created the CD-biochip to optico-acoustic biosensor for registration of mioglobin - protein markers of cardio-vascular disease.

Innovative aspects
- Creation of new optico-acoustic biosensor for express registration of proteins and protein complexes.
- New optico-acoustic biosensor is based on standard CD-player.

N104

Nanozeolite-driven approach for enrichment of secretory proteins in human hepatocellular carcinoma cells

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Introduction

Due to the vital roles of secreted proteins in lots of important life processes, the research of secretome has been arousing considerable attentions. However, the analysis of secreted proteins represents a challenge for current proteomic techniques. One of the difficulties in secretomic study is to concentrate proteins from large volume of growth media, particularly, the low abundant and low molecular weight proteins (LMWPs, MW < 30KDa). Herein, we describe a novel strategy for harvesting secretory protein in the first time with nanozeolites LTL.

Methods

Four types of nanozeolite particles (LTL, BEA, ZSM-5 and silicalite-1) with different topological structures were used to optimize the pre-enrichment condition and material. Secretory proteins, as exemplified by human hepatocellular carcinoma cell line, were enriched by zeolite LTL nanocrystals, followed by 1D SDS-PAGE for protein fractionation and then by LC-ESI-LTQ-Orbitrap for protein identification (Fig 1). To enrich LMWPs specially, conditioned media (CM) were firstly filtered by ultra-filtration with 30 KDa molecular-weight cut-off membranes, and then the filtered solution was collected and treated with nanozeolite particles, followed by tricine-SDS-PAGE separation (Fig 2). For obtaining high-confidence identification, stringent criteria (such as precision=0.99, ΔCn > 0.1, Rsp=1) were used.

Results

After optimization of pre-enrichment condition and material, nanozeolite LTL was selected to enrich the secreted proteins from CM solution at pH 6.0. In total, 1474 unique proteins were confidently identified and 97 proteins of 1474 proteins were those notably with MW less than 15 KDa, which were seldom captured previously by traditional methods. Notably, the smallest MW touches 4 KDa. Many proteins such as alpha-fetoprotein (AFP), osteopontin (OPN) and matrix metalloproteinase (MMPs) were found to be involved in the processes associated with the cancer development and metastasis. These observations demonstrate the contention that secretomic approach is a great way to the discovery of potential biomarkers.

In summary, the proposed nanozeolite LTL represents a promising adsorbent material for secretomic analysis, and it permits detection and identification of a wide range of proteins including LMWPs and low abundant secreted proteins. Notably, for special enrichment of LMWPs, compared to conventional methods, this strategy was special and efficient. Furthermore, this enrichment strategy is expected to provide a new avenue for secretome analysis, and be available for most disease cell lines.

Innovative aspects

- Secretory proteins in large volume of growth media were enriched by zeolite LTL nanocrystals.
- LMWPs were captured specially and efficiently.

References

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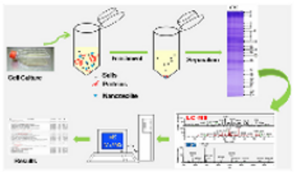

Figure 1. The flowchart of nanozeolite-driven approach for enrichment of secretory proteins.

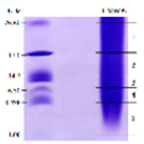

Figure 2. Tricine-SDS-PAGE analysis of enriched LMWPs. Ultra low range molecular weight markers are shown on the left, 5 bands of LMWPs were marked on the right.

Neurodegenerative Biology and Medicine

(N201 – N224)

N201

Proteome Analysis of Carbonyl Proteins in Cerebrospinal Fluid by Cy5-Hydrazyde labeling

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Introduction
The development of diagnostic markers for earlier stages of Alzheimer's disease (AD) is expected to improve the effectivenesss of therapeutic medication of AD. Oxidative stress has been implicated in neurodegenerative disorders including mild cognitive impairment (MCI) and senile dementia of Alzheimer's type (SDAT). Carbonylation is the most significant modification observed on various proteins under oxidative stress. Proteomic profiling of carbonyl proteins in cerebrospinal fluid (CSF) reported here would be a powerful tool for monitoring oxidative stress-induced neurodegeneration in the brain.

Materials and Methods
CSF samples collected by lumbar puncture for clinical diagnosis with informed consent were used in this study under the approval of the Ethical Review Board in Tokyo Metropolitan Institute of Gerontology and Kanto Medical Center. Carbonyl proteins were labeled with Cy5-hydarzide according to the method reported by Sawada et al (1) with slight modifications. Internal standard for quantitation of carbonyl protein was prepared by labeling of acetyl lysozyme with IC5-OSu. Cy5-labeleled CSF proteins were supplemented with the internal standard then separated by 2-D gel electrophoresis. Whole protein spots were stained with SYPRO Ruby and the carbonyl level of each protein spot was quantitated by laser-scanning fluoroimaging.

Results
The proteome analysis of carbonyl proteins in cerebrospinal fluid by Cy5-hydrazide labeling revealed that albumin, immunoglobulin light chain, transferrin and transthyretin were predominantly carbonylated, and the carbonyl levels of those protein spots were quantitated by image analysis with referring to the fluoresoence intensity of the internal standard spot. The carbonylation of albumin was observed in serum also, and the carbonyl level of albumin did not show significant variation with patients. Among those proteins, carbonyl level of the most acidic isoform of transthyretin showed significant variation with CSF samples. Interestingly, the carbonyl level of transthyretin was higher in CSF than in serum suggesting the carbonylation of transthyretin might occur in the brain. Native transthyretin is thought to have a beneficial side, in binding to beta-amyloid, thereby preventing beta-amyloid's tendency to accumulate into the plaques associated with the early stages of AD. The proteome analysis of carbonyl level of each protein spot by Cy5-hydrazide labeling suggested that it would be a powerful tool for evaluating the pathophysiological significance of carbonyl transthyretin in the process of neurodegeneration.

Innovative aspects
- Development of the method of quantitative proteome analysis of carbonyl protein in CSF
- Characterization of carbonyl proteins in CSF of MCI by our simple procedure of 2-D fluoroimaging in gel
- Focused proteomic approach to biomarker discovery for early diagnosis of AD

References
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N202

Biomarkers in animal model of Parkinson's disease- Relationship of cerebrospinal fluid and plasma

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Introduction The clinical manifestations of Parkinson's disease (PD) are apparent when about 80% of nigral dopaminergic neurons have died and clinical diagnose of PD relies on the identification of these cardinal motor signs. A preclinical diagnosis before the extensive death these neurons it is necessary. The neurological loss process may produce some change in the composition of cerebrospinal fluid (CSF), identification and characterization of CSF protein could be relevant to the identification of new markers that can be used for clinical purposes as diagnostic help and evaluation the response to a given therapy.

Methods Six groups of animals (Female Wistar rat) were established: control non-lesioned, sham, and unilateral and bilateral LPS-injected (2 µg into the SN) animals at 1 and 10 post-lesion days. Samples of CSF were collected in the cisterna magna as described previously[1] after uni- and bilateral intranigral injection of LPS and CSF was centrifuged at 900 × *g* for 4 min at 4ºC to remove blood contamination from the puncture point. CSF proteins were first analyzed either by 2D electrophoresis and MALDI-TOF at days 1 and 10 after the lesion to discern between potential prognosis and diagnosis protein markers. To undoubtedly confirm the LPS-induced changes on some protein, we performed a western blot analysis in CSF and plasma samples using specific antibodies.

Results Most significant changes from this analysis were early increases of haptoglobin (HP), transtyretin (TTR) and different spots further identified as prostaglandin D synthase in response to LPS. A delayed increase of α-2-macroglobulin was also detected. Most relevant identified biomarkers were HP and TTR since sustained elevations were detected in both plasma and CSF in response to intranigral LPS injections before the onset of degeneration of dopaminergic neurons (Fig 1). HP,an acute phase protein with antioxidant and antibacterial activity. TTR binds to myeloid β protein, preventing its aggregation and reducing the concentration of free myeloid β that is available to form fibrils in brain parenchyma. In a previous report, significant elevations of HP and TTR were found in plasma and CSF in a different animal model of PD induced by unilateral transections of the medial forebrain bundle[2]. Taking together, the fact of finding significant plasma and CSF elevations of HP and TTR in two different models of dopaminergic degeneration is clearly suggestive of i) potential involvement of both proteins in dopaminergic degeneration and ii) promising candidates for detecting presymptomatic PD.

Innovative aspects
- Use of haptoglobin and transthyretin as potential biomarkers for the preclinical diagnosis of parkinson's disease.

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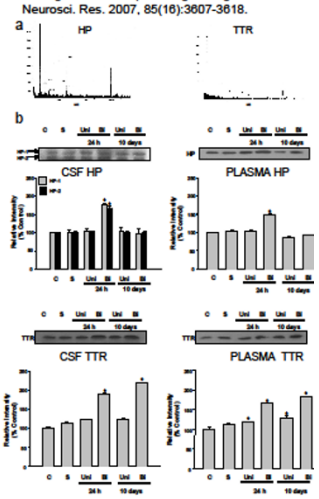

Figure 1. a. Typical MALDI-TOF spectrum of HP and TTR, obtained from a in-gel digestion of the gels of CSF of rat corresponding to 24 h after bilateral intranigral injection of LPS, **b**. Effect of LPS on CSF and plasma levels of HP and TTR. *Statistically different from control value (ANOVA followed of Tukey's Test, p<0,001).

N203

Profound Protein Expression Changes Precede Phenotype Onset in a Mouse Model of Huntington Diseases

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Introduction
Huntington disease (HD) is an autosomal dominantly inherited disorder triggered by mutation consists of an unstable, elongated cytosine-adenine-guanine trinucleotide repeats at the first exon of *Hungtintin* gene. Although late stage HD with visible disease phenotype has been studied extensively, studies on the early stages of disease have not been reported. The identification of early disease-related protein expression alterations will play a key role in our understanding of HD. In this study, we carried out a global analysis of brain proteins in a mouse model of HD, focussing on the kinetics at very early stages and during the course of disease.

Methods
We employed the large-gel two-dimensional protein electrophoresis based proteomics approach to analyse a well established murine HD model R6/2. Total brain tissues of 2, 4, 6, 8, and 12 weeks of age were investigated (n=5-8). This corresponds to the entire disease progression from the absence of disease to early, intermediate, and late stages HD. Altered proteins were identified by mass spectrometry and sequence database based approach. Pathway enrichment analysis was carried out in the protein data sets.

Results
Unexpectedly, the most HD stage-specific protein changes (71-100%) as well as a drastic alteration in protein expression (ca. 6% of the proteome) was observed as early as 2 weeks of age (205 protein isoforms). This number was almost as high as those detected at the end of disease (240 protein isoforms). The overlap of these two time stages exists at the levels of pathways as well as individual proteins. Respecting the relative amount of protein changes, 3.1% of the total spot volume (corresponding to protein concentration) was altered at 2 weeks, comparable to 3.2% at 12 weeks of age (Fig.1). Our finding suggests a period of highly variable protein expression that precedes the onset of HD phenotypes. Early changes included mainly the up-regulation of proteins involved in glucose metabolism and the down-regulation of actin cytoskeletal subunits. Components of the proteasome pathway were additionally up-regulated in symptomatic disease stages, reflecting the cell's effort to remove protein aggregates. Our observation hints that HD is characterized by a highly dynamic disease pathology not represented by linear protein concentration changes over the course of disease. These findings should aid in determining the optimal time points for HD therapies.

Innovative aspects
- Studied pre-symptomatic disease stages of HD
- Revealed that profound protein changes take hold early on in HD
- Offer insights to the optimal time window of HD therapy

References
Zabel C. et al, A large number of protein expression changes occur early in life and precede phenotype onset in a mouse model for Huntington disease. *Mol Cell Proteomics*. 2009 Apr; 8(4) 720-734

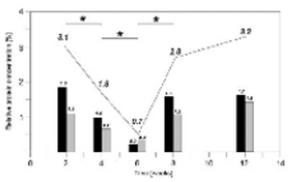

Figure 1. Relative protein concentration changes during HD progression. Brain proteins of R6/2 mice were studied at 2, 4, 6, 8 and 12 weeks of age. Protein concentration alterations were calculated for all significantly altered protein isoforms (p<0.5, <0.9- or >1.1-fold change), presented as percentage of changes. Y-axis indicates the amount of protein changed relative to a total protein concentration of 100%. Asterisks indicate statistical significance between stages (p<0.05). Black and gray bars indicate up- and down-regulated proteins, respectively. The line indicates the sum of all protein concentration changes.

N204

Proteome Wide Peptide Libraries For MS/MS Identification of Human Protease Cleavage Sites

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Introduction
The design of specific inhibitors for BACE1, a promising drug target in Alzheimer's disease, is complicated by the related anti-targets BACE2, Cathepsin D and Cathepsin E. Definition of protease cleavage site preferences is usually done by amino acid exchanges within a few known substrates or positional scanning libraries, which are slow and limited to either the nonprime or prime side sequences. We developed the first MS based proteomic method for the characterization of protease cleavage sites using proteome derived peptide libraries that are amenable for database searches.

Methods
Our laboratory has recently developed a novel mass spectrometric method that identifies dozens to >1000 cleavage sites for a protease of interest in a single experiment (1, 2). Termed Proteomic Identification of protease Cleavage Sites (PICS), this method is based in the use of whole proteome peptide libraries, generated from a human cell line by digestion with a highly specific protease such as Trypsin or GluC, as biologically relevant peptide libraries for digestion and characterization with the protease of interest. Carboxy-terminal cleavage products of substrate peptides are selectively enriched and identified by LC-MS/MS to specify the prime side residues. Residues amino-terminal to the scissile bond (non-prime side) are derived for each peptide from the human database.

Results
We used the PICS platform to characterize the cleavage site preferences of human BACE1, BACE2, Cathepsin D and Cathepsin E. For each enzyme we identified dozens to hundreds of cleaved sequences, which we summarised and visualized as sequence logos and heatmaps. In agreement with earlier studies we found that all four enzymes share relaxed sequence specificity and a preference for hydrophobic residues close to the scissile bond. However, the large number of cleavage sites identified by the PICS approach enables us identify quantitative differences among subsite preferences of these four proteases. For instance, all four enzymes prefer Ala at P1' position, but the second-best preference varies among the proteases: BACE1 and BACE2 additionally prefer Ser, while Cathepsin D and E prefer Val. This suggests that substrates with Ser at P1' will be cleaved more efficiently by BACE proteases, whereas Cathepins would be more active towards substrates containing Val at P1'. Combinations of similar observations at other positions thus reveals new opportunities for protease inhibitor and/or test substrate discrimination between these four important human aspartic proteases.

Innovative aspects
- First mass spectrometry based approach for determining protease cleavage site specificity
- Using database searchable proteome derived peptide libraries is the only approach for the simultaneous identification of prime-side and non-prime side protease cleavage site specificity in a single experiment
- Detailed characterization of cleavage site specificities within a protease family

References
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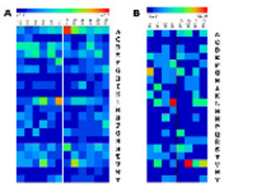

Figure 1. BACE1 cleavage site specificity. Heatmaps were generated from (A) 47 peptides identified in a single MS/MS analysis of a PICS experiment using a tryptic peptide library or (B) 15 known substrate sequences as summarized in the MEROPS peptidase database.

N205

Discovery And Validation of Human African Trypanosomiasis Staging Markers

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Introduction Human African trypanosomiasis (HAT), also known as sleeping sickness, is a parasitic tropical disease. It progresses from a haemolymphatic first stage to a neurological second stage due to invasion of parasites into the central nervous system (CNS). As treatment depends on the stage of the disease, there is a critical need for tools that efficiently discriminate the two stages of HAT. We hypothesized that markers of brain damage discovered by proteomic strategies and inflammation-related proteins could individually or in combination indicate the CNS invasion by the parasite.

Methods Cerebrospinal fluid (CSF) originated from untreated, parasitologically confirmed *Trypanosoma brucei gambiense* patients. They were staged on the basis of CSF white blood cell (WBC) count and presence of parasites in CSF. Discovery experiments were performed using either 2-DE (n=10), six-plex TMT™ isobaric labelling (n=6) or multi-plex bead suspension arrays (mBSA) (n=20). Sixteen markers with staging potential were verified using classical immunoassays (ELISA and mBSA) on a cohort of 21 stage 1 and 79 stage 2 HAT patients. Panel selection was performed by optimized threshold based calculations using all available markers. Each cut-off value was changed iteratively by quintile of 2% increment and sensitivity was determined after each iteration until a maximum of sensitivity was achieved for 100% specificity.

Results The combined discovery strategies have revealed more than 50 proteins that were significantly differentially expressed in stage 2 vs. stage 1 patients. Pre-validation experiments performed on 16 out of the 50 potential staging markers showed that CXCL10 most accurately ($p<0.0001$, Mann-Whitney U test) distinguished stage 1 and stage 2 patients, with a sensitivity of 84% and specificity of 100%. CXCL10 was also clearly associated to the severity of neurological signs ($p<0.0001$, Kruskal-Wallis). Rule induction like (RIL) analysis defined a panel comprising of CXCL10, CXCL8 and H-FABP that improved the detection of stage 2 patients to 97% sensitivity and 100% specificity.

Innovative aspects
- This study highlighted the value of CXCL10 as a single biomarker for staging potential of *T. b. gambiense*-infected HAT patients.
- Its further combination with H-FABP and CXCL8 resulted in a panel, which efficiently rules-in stage 2 HAT patients.
- The health care impact of this potential discovery on HAT, a neglected African disease that only affects the rural poor, is critical to allow more appropriate therapeutic interventions.

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N206

COMPARATIVE PROTEOMIC AND DIGITAL TRANSCRIPTOMIC INVESTIGATION OF PARKINSONS DISEASE RELATED CELL LINES

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Introduction
Despite numerous studies examining the etiopathological nature of Parkinsons disease (PD), especially the last ten years, no one has yet found a unifying pathway that ties the various hereditary form of PD to the dominating population of sporadic Parkinson's disease cases.

In this study our aim is to examine whether there are significant cellular confounding mechanisms between different pathologic variants of PD seen from a proteomic perspective.

Methods
To achieve this thorough comparative study we have examinined several human brain-type cell line models of PD using the mass spectrometry-based method, stabile isotope labelling with amino acids in cell culture (SILAC) in a quantitative mass spectrometry workflow, including differential centrifugation of lysates into four compartmental fractions and further separation by gel-electrophoresis followed by in-gel digest and LC-tandemMS.

Simultaneous with the proteomic investigation, the cells lines transcription patterns are characterised by a comprehensive tag-based transcriptomic analysis using our in-house Solexa gene sequencer using a highly optimized DeepSAGE protocol (1).

Results
At present we have profiled two PD model cell lines, neuroblastona SHSY-5Y and oligodendrocyte OLN-93, stable/transient transfested with one or several PD related proteins including a-synuclein, Parkin, P25a as well as induced cellular stress resulting in formation of aggresome-like inclusions.

We will here present both discovery and hypothesis driven analysis of the data sets indicating affected (regulated) proteins and genes and their assembly in celluar networks.

Innovative aspects
- Integration of quantitative and digital transcriptomics information to study PD

References:
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N207

Proteomic Analysis of Parkin Ubiquitin Ligase

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Introduction
Ubiquitylation is a major post-translational modification based on a network of about 500 E3 ubiquitin ligases in human. It is involved in several processes such as proteolysis, vesicle trafficking and DNA damage. Mutations in *PARK2*, which encode parkin ubiquitin ligase, account for half of early onset Parkinson's disease (PD)(1). Multiple *PARK2* mutations underlie the RING domain, which promotes ligase activity suggesting an inability for substrate degradation may trigger neurodegeneration (2). Our goal is to use a quantitative proteomics approach to identify parkin substrates and interactors.

Methods
Neuroblastoma over-expressing flag tagged-parkin versus endogenous parkin were subjected to flag immunoprecipitation and mass spectrometry to identify dynamic interactors. We have also undertaken a second approach relying on purification of the ubiquitin proteome using His-biotin tagged ubiquitin. The rationale is that over-expression of parkin should lead to an enrichment of putative substrates due to increased parkin mediated ubiquitination. The effect of mitochondrial uncoupling agents were also employed to investigate parkin ligase targets during stress as parkin is known to localize to mitochondria upon a reduction in mitochondrial membrane potential (3).

Results
Stable isotope labelling of amino acids in cell culture (SILAC) ratios after analysis of the immunoprecipitant show an enrichment of neuroendocrine chromogranin-secretogranin family proteins in parkin over-expressing cells. These results suggest a potential granin vesicle trafficking role for parkin since parkin is known to participate in vesicle shuttling. We found enrichment for DJ-1, a known parkin interactor which is implicated in PD. Several cytoskeletal proteins were enriched from flag-parkin cells treated with a mitochondrial membrane depolarizer suggesting a potential role for these proteins during parkin-mediated mitophagy. Non-conventional myosins were enriched and these are able to transport proteins and organelles while walking along actin filaments. I am currently validating if the cytoskeleton is required for the localization of parkin to the mitochondria. I have been able to identify proteins that were specifically enriched after proteasome inhibition with MG132 and am investigating whether parkin over-expression may alter the ubiquitin proteome profile.

Innovative aspects
- Quantitative proteomic analysis of parkin substrates and interactors
- Identification of ubiquitin conjugates by global pull-down
- Assessment of parkin's role in mitochondrial regulation upon membrane depolarization

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Development of a Protocol for a Rat Spinal Cord Proteome

N208

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Introduction: Several proteomic techniques have been developed for neurological disorder research[1]. Although brain and cerebrospinal fluid samples from patients with different central nervous system (CNS) disorders have been extensively studied, less research has been focused on spinal cord protein content and the changes induced after injury. In the present study we aimed to describe total protein content in the spinal cord of healthy rats, employing different proteomics tools (2DE, LC-MS). We have developed an easy and reproducible sequential protocol for the extraction of proteins from rat spinal cords.

Methods: Spinal cords were extracted from 10 week old male rats injected with an overdose of Pentobarbital (and subsequently sacrificed). The tissue was homogenized in two consecutive protein extraction buffers. The first buffer that allowed to extract most of the soluble proteins while the second buffer aided in the extraction of the more insoluble proteins. Protein concentration was determined by Bradford protein assay.
We employed conventional 2DE at different pH ranges (pH 4-7 and 3-11NL) to generated different spot maps that could be successful in the future of biomarker. Besides, together with LC-MS/MS for reducing the technical limitations of 2DE gels we expect obtain a more complete picture of rat's spinal cord proteome.

Results: This work presents a very simple, reproducible and efficient method for spinal cord protein extraction. In the 2DEs experiments with IPG Strip pH 4-7 and 17cm, approximately 400 spots were detected using PD Quest analyses software (Bio Rad). For improving the distribution, number of spot and resolution of them, 2DE gels were performed using IPG strip pH 3-11NL and 24cm. In this occasion more than 1000 spot were detected using PD Quest software analyses. In this case, spots presented a good distribution and resolution. 200 protein spots have been identified until now, corresponding to 128 different and unique proteins.
The 2DE limitations have partially been resolved by the application of other proteomics tools such as LC-MS/MS (Liquid chromatography coupled to mass spectrometer). These analyses shown a total of 18734 peptides identified that correspond with 41481 spectra that resulted in 387 unique proteins.

The application of new proteomics protocols to the spinal cord and the obtention of spot maps may be a useful tool for the study and identification of differential molecular changes associated with CNS pathologies such as spinal injury and/or neuropathic pain [1-3].

Innovative aspects:
-Sequential protein extraction protocol in Spinal Cord.
-Combination of proteomics tools (2DE-MALDI/TOF and LC-MS) in Spinal Cord analysis.

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Quantitative Analysis of Brain Proteins in an animal model of Multiple Sclerosis

N209

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Introduction Multiple sclerosis is a chronic inflammatory autoimmune disease of the central nervous system. The exact cause and working mechanism of the disease is not known yet, which makes specific treatment difficult. The aim of this study is to identify disease-related proteins that help us unravel the fundamental disease processes. To identify differentially expressed proteins in the brain stem of EAE-animals and control rats in different stages of the disease, a quantitative study by two-dimensional difference in-gel electrophoresis (2D-DIGE) was performed.
Methods Three rats were CFA injected controls, the other 9 rats were immunized with MBP to induce acute EAE. The EAE animals were divided into three groups, representing onset, top and recovery of the disease (3 animals in each group). 'Blood-free' brain stems of all 12 Lewis-rats were collected after perfusion with PBS. Detergent-soluble brain protein extracts were used for the 2D-DIGE analysis. Differential proteins were selected and thereafter identified using mass spectrometry.
Results We found 36 differentially expressed protein spots over the four conditions (control, onset, top and recovery) which are at least 1.5 fold regulated (1-ANOVA $\leq$ 0.01). With these proteins, we were able to discriminate between early and late groups (control and onset samples versus top and recovery samples). Moreover, a set of the 16 most discriminating proteins was selected from the discriminant analysis. This classifier was able to discriminate all four groups. Partition cluster analysis makes it possible to study proteins that are regulated in the same way. Apart from commonly identified differentially expressed proteins, also known brain markers and new candidates or interesting pathways were identified to be differentially expressed. Currently, a number of proteins are further studied to unravel their role in the disease process. Quantitative analysis of differentially expressed brain proteins in an animal model of Multiple sclerosis was the first successful step to identify interesting disease-related proteins that may be targets for further fundamental research.
Innovative aspects
New candidate markers or interesting pathways
Set of the most discriminating proteins

FTMS Reveals Tissue Transglutaminase Induced Crosslinking and Deamidation of Amyloid-beta

N210

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Introduction

Tissue transglutaminase (tTGase) catalyzes a calcium dependent transamidating reaction involving Q and K residues, that results in formation of covalent inter- & intramolecular crosslinks. tTGase activity may be involved in the formation of insoluble lesions in Alzheimer's disease (AD). In AD, the *in vitro* aggregation of amyloid-beta (Aβ) peptide is a spontaneous process that may be facilitated in the presence of tTGase. Here we report a tTGase mediated glutamine deamidation and intramolecular crosslinking of substance P and Aβ peptides and its implication in forming toxic aggregates.

Methods

$A\beta_{1-40}$, truncated $A\beta_{1-25}$ and substance P [25μM] peptides were incubated at 37°C for 1-24h in presence and absence (ctrl.) of tTGase. Samples were analyzed as a function of incubation time by direct infusion and LC-interfaced ESI LTQ Orbitrap FTMS/MS and ESI LTQ FT-ICR MS/MS with 12 T superconducting magnet. Transmission electron microscopy (TEM) was used for monitoring changes in peptide morphology. tTGase mediated Aβ peptide oligomerisation was monitored by MALDI-TOF and SDS-PAGE.

Results

Following tTGase crosslinking, we observed a shift in the isotopic distribution of monomeric Aβ m/z = 1082.5394 $[M+4H]^{4+}$ (Fig. 1b) and doubly charged substance P. This shift in mass of 0.984 Da may account for a tTGase induced deamidation occurring at residues Q_{15} for Aβ and $Q_{5,6}$ for substance P resulting in the conversion of glutamine to glutamic acid (E). The appearance of a new mass of m/z = 1078.5406 $[M+4H]^{4+}$ for Aβ with a mass shift of -17 Da [1], and increase in hydrophobicity by LC/MS indicates the presence of intramolecular cross-linked Aβ. This shift in mass was not observed in control incubations (Fig. 1a). Collision induced dissociation revealed residue Q_{15} as a preference substrate for deamidation. Crosslinking of truncated $A\beta_{1-25}$ confirmed the preference of Q_{15} deamidation, without resulting in intramolecular crosslinking, since K_{28} is no longer available as an amide acceptor substrate. Substantial $A\beta_{1-40}$ peptide oligomerisation was observed in the presence of tTGase, a feature reported previously [2].

Innovative aspects

- intramolecular crosslinked substance P and Amyloid-β peptides studied by high resolution MS
- CID & ECD fragmentation employed for analysis of residue deamidation occurred upon tTGase action

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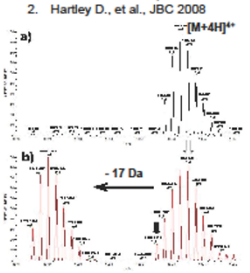

Figure 1. FTMS analysis of crosslinked Aβ peptide. a) High resolution mass spectrometry of the isotopic cluster of control $A\beta_{1-40}$ peptide $[M+4H]^{4+}$. b) The presence of tTGase, resulted in a shift of the monomeric cluster (left) m/z = 1078.5406 $[M+4H]^{4+}$, with a mass shift of -17 Da, indicating the presence of an intramolecular crosslink. The shift in mass of 0.984 Da of the most intense peak within the isotopic cluster (right; white arrow) m/z = 1083.5421 $[M+4H]^{4+}$, accounts for tTGase mediated deamidation at Q_{15}.

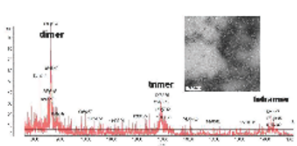

Figure 2. MALDI-TOF analysis of tTGase crosslinked $A\beta_{1-40}$ oligomers. Inset; TEM imaging of Aβ oligomer formation.

Proteomic analysis reveals the effect of *Dot1* silencing on Neuro-2a cells

N211

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Abstract

Dot1 (disruptor of telomeric silencing) can specifically catalyze the methylation of H3K79. It could involve in telomeric silencing, meiotic checkpoint control and DNA-damage response in budding yeast. However, its biological functions remain largely unknown, especially in mammals. In this study, *Dot1* silencing Neuro-2a (N2a) cells line was established via transfection with *Dot1*-siRNA and the role of Dot1 in N2a cells was investigated through proteomic studies. The results indicated that *Dot1* silencing did not affect cell growth, cell cycle or induce cell death. However, N2a cells lost its normal neuronal shape, and displaced shorter filopodia and less connection. Eight differentially expressed proteins were indentified after *Dot1* silencing. Among of them, stress-induced-phosphoprotein1, RAD23b homolog and peroxiredoxin 1 were up-regulated; while far upstream element-binding protein 1, nucleosome assembly protein 1-like 1, eukaryotic translation initiation factor3, nascent polypeptide-associated complex alpha subunit and prohibitin were down-regulated. The decreased expression of p53 was also observed after *Dot1* silencing. Our study suggested an explanation for the multifunction of Dot1 on axon outgrowth/synaptic plasticity, gene regulation, protein translation and folding, apoptosis and DNA double-strand break repair. The results demonstrated the possibility of Dot1 on the contribution to the brain dysfunction in the neurodegenerative disease or aging.

Keywords: Dot1 (disruptor of telomeric silencing); H3K79 methylation; Cell culture; Neuro-2a; Gene expression; Proteomics.

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N212

Quantitative Study of Phosphorylated Brain Proteins in a Model of Multiple Sclerosis

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Introduction Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system. The underlying molecular processes remain poorly understood, but are crucial in the search for new therapeutic options. Protein phosphorylation may be involved in the pathology of MS and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. To analyze differentially expressed phosphorylated proteins, brain protein extracts from EAE animals were fluorescently stained and detected on two-dimensional gels. Association with a previous 2D-DIGE experiment allows quantitative analysis.
Methods Protein extracts from 'blood-free' brain stems of control and EAE Lewis rats were separated by 2D-gel electrophoresis. In order to detect phosphorylated proteins, these gels were stained with a fluorescent dye, Pro-Q diamond phosphoprotein gel stain (Invitrogen). The Ettan DIGE Imager (GE Healthcare) was used for scanning. Mass spectrometry was used for identification of phosphorylated proteins. Quantitative data were obtained by comparison to a previous two-dimensional difference in-gel electrophoresis (2D-DIGE) study at different disease stages (control, onset, top and recovery, three biological replicates each).
Results The Pro-Q diamond phosphoprotein gel staining was optimized on 2D-gels. Post-gel total protein staining is used as quality control and thus normalization of the phosphoprotein signals. Moreover total protein staining is useful for matching different gels. Phosphorylated proteins are identified by mass spectrometry. Furthermore, we are able to study the expression profile of the identified phosphoproteins throughout the disease process via matching of the PTM-specific 2D-spotmaps with the spotmaps of a previous 2D-DIGE experiment, a quantitative study of the brain proteome dynamics during the disease course of EAE in which 36 differentially expressed proteins were identified (significance: ANOVA ≤0.01 and at least 1.5 fold regulated).
The identification of a panel of differentially expressed phosphorylated proteins in the disease course, could provide information about the global disease processes in EAE and MS. Protein phosphorylation is of great importance to the protein function. This global overview of phosphorylation during disease invites new studies to unravel the complicated molecular biological processes in the pathology of MS.
Innovative aspects Quantitative study of phosphorylated proteins due to comparison to a previous 2D-DIGE study

N213

Identification of novel protein-protein interactions linked to Alzheimer's and Parkinson's disease

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Title: Identification of novel protein-protein interactions linked to Alzheimer's and Parkinson's disease.

Introduction Alzheimer's and Parkinson's disease are the most common neurodegenerative disorders. These diseases are thought to result from a complex interaction among multiple predisposing genes and other factors. (1) The purpose of this study is to identify new players implicated in these diseases.

Methods Bait proteins were selected based on their link or potential link to Alzheimer's and Parkinson's disease using bioinformatic tools. Baits of interest were cloned into a Flag tagged vector using Gateway technology. HEK293T cells were transiently transfected with the bait of interest. Immunopurifications of the bait with its associated proteins were performed using anti-Flag M2 agarose beads and eluted with Flag peptide. The purified proteins were separated on 4-12% gels and silver stained. Bands were isolated and in gel digestion was performed with trypsin. Samples were analyzed by LC-MS/MS.

Results We curated the data in this study following similar principles established in our group previous work on large protein-protein interaction dataset (2). This has enabled us to remove background contaminants as well as commonly observed hits creating a high confidence dataset. From this initial set of tagged baits related to Alzheimer's and Parkinson's disease known interaction partners were indentified as well as new interaction partners. These new interactions are currently being validated by reciprocal immunoprification. Understanding these new interactions will be critical in deciphering their role in the disease.

Innovative aspects

- **Large scale functional proteomic study of protein-protein interactions in mammalian cells.**
- **Characterization of proteins implicated in Alzheimer's and Parkinson's disease.**

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N214

Alzheimer's Disease Proteomics: Expression of Membrane Proteins in Different Brain Regions

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Introduction
Proteomic analysis of brain is useful to comprehend the complexity of central nervous system and is also supportive to investigate its disorders. Study of regional membrane protein expression provides evidence not only to the developing and/or diseased stage, but also the changes occurring at molecular level under various disease conditions. The present work is aimed to investigate the brain expression proteomics of Alzheimer patients and an initiative to construct a regional brain protein profile that will support the identification of proteins involved in the diseased states.

Methods
The protein extracts of Hippocampus and Cortex were prepared. The tissues were homogenized in homogenization media (50mM Tris, 100mM KCL, 20% glycerol, and 0.1 M phosphate buffer, protease inhibitors, CHAPS, PMSF pH 7.1) and subjected to differential centrifugation. The membrane protein extracts were collected. Membrane proteins of Hippocampus and Cortex were separated by 2-DE, performed with IEF as the first dimension using Multiphor II (Amersham Biosciences) and SDS-PAGE as the second dimension. Gels were visualized with a Colloidal Coomassie brilliant blue staining method.

Results
Cell fractionation of the AD brain regions (cortex and hippocampus) was performed to yield membrane protein fractions and subjected to two-dimensional gel electrophoresis at both 3-10 pI and narrow pI ranges. Significant number of acidic and basic proteins was expressed, visualized by Colloidal CBB staining. The membrane protein expression of the two brain regions was compared and analyzed. 2DE expression profiles of brain regional proteins revealed exciting findings and interesting observations were made. Protein specificity for a particular region was also observed. Further characterization with MS provides the information of expressed proteins, like the specific function and role in a particular brain region. A total of 19 significant protein components were identified in the membrane fractions of these brain regions.

- The identification of region specific proteins.
- Identification of differentially expressed proteins, proteomic profile of normal and diseased brain region was compared.
- Characterization of proteins differentially expressed in two different regions of Alzheimer's disease brain.

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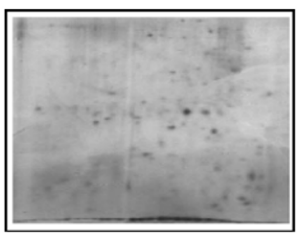

Figure 1. Zoom in fraction (4-7 pI range) of AD hippocampus membrane proteins separated on 12% SDS-PAGE followed with staining by colloidal CBB method.

Acknowledgement
We thank Professor Dr. Abid Azhar, Co-Director Karachi Institute of Biotechnology and Genetic Engineering, University of Karachi, Pakistan for providing the working facilities. We are indebted to Dr Robert Walker, MRC Sudden Death Brain & Tissue Bank Manager, The University of Edinburgh, Edinburgh, UK for kindly providing postmortem human brain tissues. This work was supported by a grant from the Higher Education Commission, Pakistan, No.20-560/R&D/07.

Innovative aspects

N215

Evolutionary descent of prion family genes from a ZIP metal ion transport ancestor

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Introduction
Prion diseases are fatal neurodegenerative diseases of humans and animals which, in addition to sporadic and familial modes of manifestation, can be acquired via an infectious route of propagation. Prion diseases include sporadic and variant forms of Creutzfeldt-Jakob disease in humans. In sheep, cattle and cervids, prion diseases manifest as scrapie, bovine spongiform encephalopathy, and chronic wasting disease, respectively. In the more than 20 years since its discovery, both the phylogenetic origin and cellular function of the prion protein (PrP) have remained enigmatic. Insights into the function of PrP may be obtained through a characterization of its molecular neighborhood.

Methods
Following a 'guilt-by-association' logic, we set out to identify the function of the cellular prion protein through a comprehensive interactome investigation. Investigations of this kind often result in long lists of candidate interactors. The challenge then remains to discriminate specific from unspecific binding partners. Here we incorporated quantitative mass spectrometry based on isotopic tagging of peptides into the workflow to overcome this limitation. Furthermore, investigations were extended to all three members of the mammalian prion protein family to further facilitate discrimination of potential interactors by differential interactome comparison. A murine neuroblastoma cell line (N2a), an established cell model for prion replication, served as the biological source material.

Results
Quantitative interactome data revealed the spatial proximity of a subset of metal ion transporters of the ZIP family to mammalian prion proteins. A subsequent bioinformatic analysis revealed the presence of a prion-like protein sequence within the N-terminal, extracellular domain of a phylogenetic branch of ZIPs. Additional structural threading and ortholog sequence alignment analyses consolidated the conclusion that the prion protein gene family is phylogenetically derived from a ZIP-like ancestor molecule. Our data explain structural and functional features found within mammalian prion proteins as elements of an ancient involvement in the transmembrane transport of divalent cations. The connection to ZIP proteins is expected to open new avenues to elucidate the biology of the prion protein in health and disease.

Innovative aspects
- Rare example of proteomics-based discovery of evolutionary linkage between protein families
- First and conclusive evidence that the mammalian prion protein evolved from a specific branch of highly conserved metal ion transporters
- Data explain structural and biological features within prion proteins as remnants of an ancient involvement in the sensing and/or import of metal ions present in the extracellular milieu

References

None

Quantitative analysis of Membrane Proteins and Glycoproteins in Activated Brain Cells using Label-Free LC-MS-Based Proteomics

N216

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Introduction. Biomarkers on the luminal surface of human vasculature are attractive targets (due to their accessibility) for non-invasive molecular imaging-based diagnosis. The vascular luminal surface of brain endothelium is decorated with proteins, glycoproteins and proteoglycans that form a thick coating called glycocalyx. The glycocalyx is significantly altered in response to ischemia-reperfusion and various inflammatory stimuli. For example, most of the endothelial surface and adhesion molecules that are up-regulated as hallmarks of cerebrovascular inflammation are glycosylated proteins. However, the complex nature of the brain endothelial glycocalyx, has so far hindered deeper understanding of its molecular alterations in pathological conditions. We have identified proteins and glycoproteins present in the membranes of human brain endothelial cells (HBEC) and their quantitative changes in response to inflammatory stimulus using label free quantitative proteomics.

Methods. HBEC (HCMEC/D3) were treated with ischemia/reoxgyenation (3 h/16 h) or IL-1b (100 U/mL). Total membrane proteins were isolated by ultracentrifugation and sucrose gradient. The membrane proteins were then treated with PNGase F, reduced, alkylated, trypsinized and fractionated on cation exchange columns. Each fraction was analyzed by nanoLC-MS using nanoAcquity UPLC coupled with Q-TOF Ultima (Waters). This was followed by quantification and normalization of peptide levels between control and activated HBEC using MatchRx software (1). Peptides were identified using a combination of data-dependent and target list-dependent nanoLC-MS/MS analyses on LTQ XL (Thermo) followed by semi-automated alignment of data with the corresponding original LC-MS data using MatchRx. Mascot was used as the database search engine.

Results. More than 9,000 high-quality unique peptide sequences have been identified in the total HBEC membranes, corresponding to about 2,500 proteins. More than 400 proteins were found to be glycoproteins as determined by PNGase F treatment (e.g., TMEM30A, various solute carrier family and ABC proteins). While most (68%) of the PNGase F-responsive proteins are known glycosylated proteins, many novel N-glycosylated proteins were identified. In addition, >750 proteins showed differential expression in response to IL-1b treatment (>2.5-fold up- or down-regulated, $p < 0.05$, 3 biological and 2 technical replicates). These include ICAM-1 (Fig 1), ALCAM, PECAM-1, various integrins, and ECM remodeling proteins.

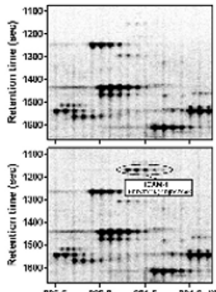

Fig 1. NanoLC-MS runs of total membranes from control (top) and IL-1b (bottom) samples. Label indicates a glycopeptide showing >14-fold overexpression (as determined by MatchRx) in treated sample. The peptide belongs to ICAM-1 protein.

Literature-mining and protein classification associated many of the differentially expressed proteins with signal transduction, cell-cell adhesion, transport, cytoskeletal processes and several other processes consistent with pathophysiology of neuroinflammation. Majority of the proteins have not been previously associated with neuroinflammation and >20% of the proteins are of unknown functions. In conclusion, these results demonstrate that the combination of membrane isolation, protein deglycosylation, label-free LC-MS analysis and MatchRx-based quantification represent a powerful approach to identify relative changes in proteins and glycoproteins on the surface of endothelial cells. The results demonstrate the complexity of brain endothelial glycocalyx and point to its important role in cerebrovascular physiology and pathology.

Innovative aspects

- Quantification of proteins and glycoproteins in membranes of brain endothelial cells using a combination of protein deglycosylation, label-free LC-MS and MatchRx analyses.
- Identification of potential diagnostic targets for molecular imaging agents for brain ischemia (stroke) and neuroinflammation.

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Proteomic Analysis of Stress Responses Associated to the Ubiquitin Proteasome System

N217

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Introduction

The ubiquitin proteasome system (UPS) plays a major role in the clearance of protein quality control targets in the cell. In this system, proteins to be eliminated are first ubiquitylated and then recognized and degraded by the proteasome. Impairment of the UPS has been associated to a myriad of pathologies including neurodegenerative diseases. We aim at understanding which proteins are targeted to the UPS, as well as which proteins are more susceptible to aggregation after disrupting the UPS.

Method

We employed a combination of enrichment methods to purify ubiquitylated proteins in yeast and human tissue culture cells, as well as induced proteins aggregates. More specifically, we used cells expressing an octameric histidine tag fused to ubiquitin to purify ubiquitin conjugates by nickel chromatography. Protein aggregates induced by chemical inhibition of the proteasome and containing ubiquitylated proteins were enriched by sucrose gradient. Quantitative mass spectrometry was performed using $^{14}N/^{15}N$ labeling in yeast cells and SILAC in human tissue culture cells. In solution trypsin digest was performed on whole protein mixture and peptides were analyzed by a LTQ-Orbitrap followed by MASCOT identification.

Result

We noticed that heat shock induced a general increase of poly-ubiquitylation in the cell. We determined that heat shock mainly affected cytoplasmic proteins in yeast cells after analyzing purified ubiquitylated proteins by quantitative mass spectrometry (MS). We then identified several candidate E3-ligases responsible for ubiquitylation of misfolded proteins after heat shock. We performed quantitative MS, to determine the substrate specificity of some of these E3s. Overall, our results indicted that there is likely a network of several E3 ligases responsible for the ubiquitylation of misfolded proteins in the yeast cytoplasm.

We noticed that ubiquitylated proteins were poorly soluble after stresses like heat shock. Similarly, chemical inhibition of the proteasome induced formation of ubiquitin positive inclusions in the cell. We isolated poorly soluble aggregates from neuronal tissue culture cells by using sucrose gradient and analyzed them by MS. We identified a large number of proteins that specifically co-fractionate with ubiquitin, suggesting that a large fraction of proteasome substrates are poorly soluble and that proteasome inhibition has a broad impact in the cell.

Innovative Aspects

- Purification methods to analyse ubiquitylated proteins by MS
- Identification of proteins targeted to the UPS by a protein quality control mechanism
- Enrichment method to analyse induced protein aggregates by MS

Comprehensive analysis of lipid extracts from hippocampus of human brain

N218

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Introduction

There is growing interest in understanding the roles of lipids in neurodegenerative diseases. This requires technologies for the identification and quantitation of different lipid species. We have developed a nanoflow chromatographic approach coupled to electrospray ionization mass spectrometry (ESI-MS) for the quantitation of individual lipid species in a lipidome. In this work, we report the comprehensive analysis of the lipidome of hippocampus derived from healthy and Alzheimer disease (AD), human brains.

Methods

Five samples of human hippocampus with AD and 5 healthy samples as controls were used. Lipids were extracted from brain tissues using a modified Bligh and Dyer procedure. Prior to lipid extraction, known amounts of lysophosphatidylcholine C13 (LPC13) and 4 deuterium labelled platelet activating factor (PAF) species (PAF C16, PAF C18, Lyso C16, lyso C18) were spiked into samples. Lipid identification and quantification were carried out on a QTRAP 2000 spectrometer via an ESI interface using direct infusion or frontend HPLC. Reverse phase C18 columns were employed. Precursor ion scan and neutral loss scan modes were employed to target specific lipid species. Peak area integration of individual lipid species was performed from its extracted ion chromatograph using Analyst 1.4.2.

Results

Lipid extracts were initially analyzed with LC/MS under positive ion mode between m/z 450 and 600, primarily owing to the great interest in the four PAF species (1). Precursor ion scan at m/z 184, was employed to selectively target phosphatidylcholines (PCs). The integrated peak area of each lipid species was then normalized to that of LPC13, to circumvent sample loss as well as the fluctuation of signal intensity of the mass spectrometer from run to run. Preliminary results indicated a higher abundance of LPCs and PCs, including four PAF species present in AD samples compared to controls (Fig 1). LC/MS was also carried with neutral loss scan of 141 to profile phosphatidylethanolamines (PEs). Lipid extracts from AD and control samples were also analyzed in an extended mass range (up to m/z 1000) via direct infusion to identify lipid species with long fatty acyl chain, including the high abundant phospholipid species (e.g., PCs, SMs, and PEs). Preliminary results proved that a combination of direct infusion and LC/MS is a powerful tool for comprehensive lipid identification and quantification.

Innovative aspects

- Comprehensive analysis of lipid species in hippocampus of human brain
- Combination of diverse MS techniques for exhaustive and complex lipid profiling

References

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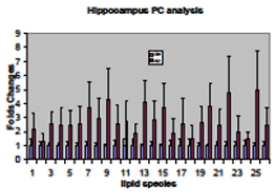

Figure 1. PC species in lipid extracts from the hippocampus of AD samples exhibit a higher abundance than those from healthy controls in the mass range of m/z 450 to 600.

A Proteomics Based Investigation of the Insoluble Proteins Implicated in Degenerative Neuropathy

N219

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Introduction:

The accumulation of insoluble proteins is a common feature of the neurodegenerative diseases. A growing body of literature supports concludes that these insoluble proteins are inherently toxic, and indeed, some intracellular protein aggregates grow to the point that they stop axonal transport and result in cell death.

Developing techniques to efficiently extract and analyze the insoluble protein fraction of affected neural pathways could play a key role in the identification of proteins associated with a wide range of neurodegenerative diseases.

Methods:

The insoluble native protein populations of healthy and diseased mice and were made by extracting representative tissue samples. Lumbar spinal cords of mice transgenic for the ALS-associated mutation, G93ASOD1 and control transgenic for WTSOD1 were dissected and the insoluble proteins fractionated and processed with standardized preparation protocols for proteolysis and isobaric labelling.

Nanoscale LC-MS/MS was performed with a novel FTICR equipped with programmable RF transport optics to facilitate efficient broadband transfer of the reporter and peptidic fragments. This arrangement has been shown capable of effectively passing mass ranges of greater than 30x (Th).

Results:

Comparisons of the extracted peptide populations of the insoluble proteins is based on optimization of a model experiment that requires hi-fidelity in the peak magnitudes measured for the representative reporter ions by the detector.

Instrumental optimization has produced a workflow that enables a novel FTICR to provide quantitative results comparable, and in some cases superior to conventional platforms used for relative quantitation of the commonly used low-mass isobaric tags.

Specifically, the system uses a multiplicity of secular frequencies for the transport of collisionally generated fragments to the detector. The initial results using available standards has been very promising, and will be applied to the system of interest after the protein extraction process has been optimized and executed.

Innovative aspects

- Comprehensive proteomics based study of relative quantitation of the insoluble protein components of motor neuron pathways
- Implementation of a novel FTICR-MS platform capable of performing low-mass reporter ion ratio measurements

Investigation of the role of Tau gene transcriptional regulation in neurodegeneration

N220

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Introduction
The H1c sub-haplotype drives the association of the human Tau gene (*MAPT*) with increased risk of progressive supranuclear palsy, corticobasal degeneration and Alzheimer's disease (1, 2). Two SNPs have been found as risk alleles which affect *MAPT* transcription and splicing (3). The objective of this project is to investigate *MAPT* transcription regulation and the precise role of the *MAPT* core promoter and predicted regulatory domains by identifying transcription factors (TFs) and TF complexes that bind to these domains.

Methods
We are using site-directed mutagenesis, promoter-luciferase- and electrophoretic mobility shift assays (EMSA) to assess the individual single nucleotide polymorphisms (SNPs) in the *MAPT* promoter, including a putative downstream enhancer containing one of the H1c-specific SNPs (SNP035). With immobilized double-stranded oligonucleotide probes (promoter trap) representing these regulatory domains we are affinity purifying TFs and TF complexes that bind to these domains to characterize them using gel electrophoresis and mass spectrometry (MS).

Results
Using site-directed mutagenesis in luciferase reporter vectors containing this conserved domain cloned downstream to the *MAPT* core promoter, we show that SNP035 is mainly responsible for the allele-specific elevation of *MAPT* promoter activity *in vitro*. With electrophoretic mobility shift assay (EMSA) we also observe quantitative allele-specific differences in intensity of bands representing TF complexes bound to the probes containing SNP035 (Fig 1). On protein gels we observe quantitative allele-specific differences of discrete protein bands of promoter trapped TFs and have identified these proteins using ESI-MS/MS (Fig 2).

Innovative aspects
- SNP035 shown to attenuate promoter strength
- SNP035 shown to alter DNA-binding proteins' binding affinities and specificities
- These DNA-binding proteins have been identified using a mixture of proteomic approaches

References:
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(2) A Myers et al, The H1c haplotype at the MAPT locus is associated with Alzheimer's disease; Hum. Mol. Genet. 2005 **14** 2399-404.
(3) A Myers et al, The MAPT H1c risk haplotype is associated with increased expression of tau and especially of 4 repeat containing transcripts; Neurobiol. Dis. 2006 **25** 561-70.

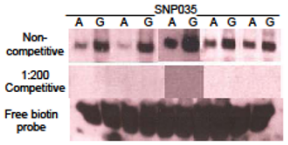

Figure 1. EMSA of SNP035 A/G variant. Biotin-labelled 16-mer oligonucleotides containing the A & G allelotypes of SNP035 were incubated with undifferentiated SH-SY5Y nuclear extracts. Incubation with cold, unlabelled probe showed specific interaction between probe and nuclear proteins. On average, the G allelotype pulled down 3x more nuclear proteins than the A allelotype. Replicated in 5 independent experiments.

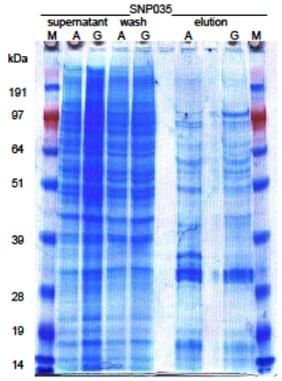

Figure 2. SDS-PAGE gel of DNA-binding proteins purified using oligonucleotides containing the A & G allelotypes of SNP035 incubated with undifferentiated SH-SY5Y nuclear extracts.

Characterization of TTR-Cys10 isoforms in CSF from AD patients by immunoaffinity–MS and ETD MS/MS

N221

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Introduction
Post-translational modifications (PTMs) contribute significantly to the complexity of proteins. PTM patterns on single proteins may be used as biomarkers alone or in multiplex combinations (1). The single free cysteine-residue (Cys10) in transthyretin (TTR) from cerebrospinal fluid (CSF) is prone to oxidation and might display mixed disulfides patterns possibly related to Alzheimer's disease (AD) (2).
In a search for such diagnostic TTR-modification profiles, we here investigate TTR isoforms directly immunoprecipitated (IP) from CSF by Orbitrap MS and ETD MS/MS analysis. The CSF specimens were sampled from patients with AD and appropriate controls.

Methods
CSF samples were obtained at the Memory Clinic at Copenhagen University Hospital.
TTR was IP directly by polyclonal rabbit anti-human-TTR (DakoCytomation A/S, Denmark) from CSF single AD-patient samples. An optional DTT reduction step was included to verify the oxidative modifications. After washing x3 (with 100 mM NH_4HCO_3) and buffer changed (to 50% methanol, 1% formic acid) the IP material was introduced by direct infusion into the Orbitrap MS instrument (Thermo Scientific Inc.).
In addition to high resolution MS analysis, ETD MS/MS fragmentation was performed for sequencing and characterization of different Cys10 modifications. To assign the putative modifications at Cys10, deconvoluted spectra were manually evaluated.

Results
Using Fourier transform MS and MS/MS combined with advanced fragmentation techniques, a number of oxidative modifications could be assigned to the cysteine residue with monoisotopic resolution. Cys-10 was observed to be modified by oxidation in numerous ways to sulfenic (RSOH), sulfinic (RSO_2H) or sulfonic acid (RSO_3H), alternatively by being S-cysteinylated, S-cysteineglycinylated, S-sulfated and S-glutathionylated. Reversibility of oxidation was readily demonstrated by reducing the samples with DTT. Additionally, the cysteine residue was also found to be modified into dehydroalanine and glycine. The relative distribution patterns of these TTR-Cys10 modifications from single samples were semi-quantitatively calculated and compared. Specific modification patterns could be tentatively assigned to TTR in CSF from AD patients.

Conclusion
Direct IP of TTR from CSF combined with state-of-the-art MS instrumentation and techniques produce high resolution results facilitating valid modification-specific analysis of TTR from CSF.
A very complex deconvoluted isoform pattern for TTR was seen in all samples, which was highly reproducible between runs. The TTR isoforms were characterized using ETD-MS/MS and the isoforms could be assigned to oxidative modifications in agreement with the disappearance of the peaks after reduction.
Unrivalled monoisotopic resolution of TTR molecular isoform species was obtained. The approach makes dissection of one specific molecular family possible directly from crude CSF and may be used for other molecules where monospecific antibodies are available.
The results are promising for CSF-based AD diagnostics. Further studies, also including CSF sample handling conditions (sampling, storage, and processing) for potential contribution to the Cys10-oxidation profiles are required to explore and validate these results.

Innovative aspects
- Easy purification of TTR isoforms directly from minute amounts of CSF by IP
- Detailed identification and characterization of TTR isoforms by sensitive high resolution Orbitrap MS and ETD-MS/MS
- Determination of CSF-TTR isoform-patterns in individual AD patients for qualitative comparison and development of TTR-modification specific AD diagnostics

References
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Protein expression variations in Cornelia de Lange Syndrome

N222

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Introduction
Cornelia de Lange syndrome (CdLS) is a rare genetically heterogeneous disorder characterized by distinctive dysmorphic facial features, upper limb abnormalities, growth retardation and neurodevelopment delay. CdLS has an highly variable phenotype, from moderate to severe, which appears correlated to mutations in cohesin complex and cohesin associated proteins. About half of the patients with CdLS carry mutations in the NIPBL protein, a regulator of cohesin loading and unloading onto DNA; while mutations in two cohesin structural components SMC1A or SMC3 (Structural Maintenance of Chromosomes 1A and 3) contribute to the 5% of cases and result in a mild phenotype, with absence of the major abnormalities.

Methods
In this study we have applied a proteomic approach to characterize the etiopathology of CdLS at protein level, and trying to correlate protein variations with previous data obtained mainly at a genomic level.
We have compared lymphoblastoid cell lines from CdLS patients affected by different mutations in SMC1A and SMC3 genes with healthy control individual cells, using the quantitative 2D-DIGE analysis. In DIGE gels we combined seven CdLS patients and seven control individuals according to age and type of mutation. Moreover, to avoid staining bias, CdLS patient and control individual samples were alternatively labeled with Cy3 and Cy5 fluors. The results were obtained using the Decyder 2-D Software. All statistically significant variations were then identified using mass spectrometry.

Results
After computer analysis, several differences were highlighted (Fig. 1) and successfully identified using MALDI-TOF MS and ESI-IonTrap MS. Many interesting proteins with defective expression in CdLS patients were related to different functional classes (Fig. 2), such as metabolism (glycolysis pathway, nucleotide catabolism and salvage), cytoskeleton rearrangement and chromatin organization. Further analyses, such as immunoblotting and functional assays, have been applied to validate our data. The identified proteins may provide new light on the functional role of cohesin subunits and their implication in Cornelia de Lange Syndrome onset.

Acknowledgment:
this work was supported by the FIRB project "Italian Human ProteomeNet" (RBRN07BMCT_013 - MIUR).

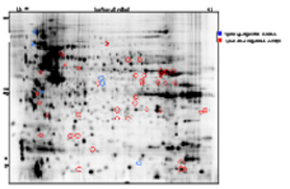

Figure 1: 2D-DIGE gel of CdLS with protein variations visualized by red (spots down regulated in CdLS) or blue (spots up regulated in CdLS) circles.

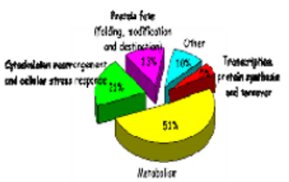

Figure 2: Schematic representation of the functional classes of protein variations present in CdLS.

Proteomic analysis of CSF from methamphetamine abusers and meth treated neural stem cells shows profound effects on oxidative stress pathways and growth factors.

N223

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Background: Methamphetamine (meth) abuse is steadily increasing. It appears to have long lasting wide spread effects on the brain but the effects at a molecular level are poorly understood. The goal of this study was to use a proteomics approach to identify key targets of methamphetamine that may alter adult neurogenesis.

Methods: Using iTRAC and LC-MS/MS (orbitrap) we analyzed pooled cerebrospinal fluid from patients with a history of meth-dependence and compared it to that of non-drug user age-matched controls (n= 5 each). Prior to labeling, the CSF was depleted of abundant proteins. We also used an in-vitro model in which meth treated neural precursor cells were analyzed. In this model, nitro-tyrosine modified proteins were isolated by an affinity column and proteins identified by LC-MS/MS. The nitrome was compared to untreated control cells.

Results: A total of 279 proteins were identified in the CSF. Six proteins were found exclusively in the meth abusers. Another 27 proteins were elevated 1.2 to 2 fold compared to the normal controls and 22 proteins were decreased which included growth factors and those with antioxidant properties. The in-vitro studies also showed meth was toxic to neural precursor cells independent of its effects on the dopamine system. We discovered nitrosylation of several mitochondrial proteins and heat shock proteins.

Conclusions: Methamphetamine has profound effects on neurogenesis, which are mediated in part by down regulation of growth factors, induction of oxidative stress and nitrosylation of mitochondrial proteins.

Proteomic Analysis of Human Brain Microdialysates of Stroke Patients

N224

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Introduction
Microdialysis samples of brain tissue are a unique source for the discovery of biochemical markers of brain damage (1). In particular, such pertinent samples are needed for the identification of early and specific diagnosis and prognosis markers of stroke.
To find innovative protein markers of stroke, comparisons of microdialysis samples collected simultaneously in two brain regions (from infarct core (IC), penumbra (P) or controlateral (CT)) of stroke patients were carried out using a shotgun proteomic approach including isobaric tagging and tandem mass spectrometric (MS/MS) analysis.

Methods
Microdialysis sampling of stroke patients was approved by the local institutional ethical committee. A microdialysis probe with a molecular weight cut-off of 100 kDa was used. Ten-hour samples were pooled for further analysis.
The 2-plex isobaric Tandem Mass Tag (TMT) technology (2) was used to label trypsin-digested extracts from two brain regions of six patients suffering stroke (Figure 1). Following the labeling, the pooled samples were first fractionated by off-gel electrophoresis (OGE). The fractions were then analyzed by reversed-phase liquid chromatography (RP-LC) and matrix-assisted laser desorption tandem time-of-flight (MALDI TOF/TOF) MS. The identification and quantitation of proteins were assessed with stringent criteria using Phenyx search in the Swiss-Prot human database.

Results
Microdialysis is a bioanalytical sampling tool to continuously monitor events occurring in living tissues. It is based on probing extracellular fluid and allows collecting endogenous substances from the extracellular space, which can diffuse through the semi-permeable membrane at the tip of the microdialysis probe. Such a technique is quite appropriate to search and follow biochemical markers in real-time in many organs.
The proteomic comparisons of human brain microdialysates showed significantly over-represented proteins (with a ratio superior to 2) in the IC compared to the CT and P counterparts. Proteins such as glial fibrillary acidic protein (GFAP), protein S100-B (S100B) have already been reported to be associated to brain damage and appeared to be increased in one or several IC microdialysis samples. The glutathione S-transferase P (GSTP1) increase was validated by home-made sandwich ELISA in the serum of 10 stroke patients and controls.
This study not only highlights changes between protein levels in these different microdialysis samples but also depicts an extensive proteome map of human brain microdialysate.

Innovative aspects
- Shotgun analysis of the human brain microdialysis proteome of stroke patients
- Quantitative analysis using TMT, OGE and RP-LC MS/MS
- Discovery of innovative potential biomarkers for stroke diagnosis

References:
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(2) L Dayon et al, Relative Quantification of Proteins in Human Cerebrospinal Fluids by MS/MS Using 6-Plex Isobaric Tags; Anal. Chem. 2008; 2921-2931.

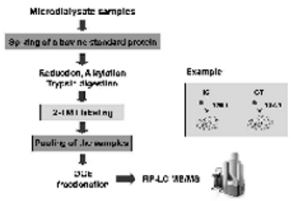

Figure 1: Proteomic quantitative workflow used for the analysis of human brain microdialysates of stroke patients.

Organelle Proteomics

(O101 – O124)

Proteomic study of Endoplasmic Reticulum from Jurkat Cells During Heat Stress

O101

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(Make certain you underline the presenting author's name)

Introduction
Endoplasmic reticulum (ER) is a subcellular compartment playing a pivotal role in cellular functions. Beside its role in calcium and signaling, the ER lumen is the compartment where the folding processing of all membrane and secretary proteins takes place. Proteomic analysis of ER may be important to understand the protein folding processes and their alterations during heat stress which leads Jurkat cells to apoptosis associated with hyper-phosphorylation of stathmin of an intracellular phosphoprotein by cdk-1 (1) and down regulation of splicing factors (2). The proteomic profiling of ER during heat stress in Jurkat cells was performed to find biomarkers of ER membrane proteins for unfolded protein response (UPR) and ER-associated degradation (ERAD). We will discuss the mechanisms of hest stress-induced cell death in ER protein level.

Methods
Jurkat cells of a human T lymphoblastic leukemia cell line were incubated at 43 degrees of Celsius for 30 min. ER membrane proteins were prepared by different centrifugation followed by sodium carbonate washing of ER (3) and a treatment with cold acetone-methanol. ER membrane proteins were separated by SDS-PAGE into 58 bands visualized with CBB R250. The protein bands which were increased or decreased by the heat stress were cut out to submit to in gel digestion and protein identification by nano-LC-MS/MS. The protein candidates were confirmed by immuno-blotting with antibodies specific for the candidate proteins and ECL plus Western Blotting detection system (4). The post-translational modifications of the proteins were also identified by the immuno-blotting and tandem mass spectrometry.

Results
The ER membrane proteins were separated into 58 bands having 128 proteins. Twelve of the bands were decreased by the heat stress and proteins in the bands were 60S, 40S ribosomal proteins, THO complex subunit 4, Histone H1.5, NADH-cytochrome B5 reductase, heterogeneous nuclear ribonucleoprotein A1, emerin, heme oxygenase 2, eIF4A-1, nuclear sensitive element-binding protein 1. While two of the bands were increased by the heat stress and proteins of eIF3 subunit 10, clathrin heavy chain 1, protein diaphanous homolog 1, filamin-A, spectrin beta chain, talin-1 were identified with ubiquitin concomitantly. The immuno-blotting showed that phosphorylated form of eIF2 alpha as UPR marker was increased at the bands showing molecular weights (MW) higher than its theoretical one. Further examinations of post-translational modifications such as phosphorylation and ubiquitination of the candidate proteins are being made by immuno-blotting and tandem mass spectrometry. In addition ubiquitinated histones were identified in two protein bands showing molecular weight between 10 to 15 kDa which were not changed by the heat stress.

Innovative aspects
- Membrane proteins in ER fraction from Jurkat cells were successfully identified by SDS-PAGE combined with nano-LC-MS/MS and Immuno-blotting.
- Novel proteins and their ubiquitinated /phosphorylated isoforms were identified for biomarkers of UPR and ERAD.

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Phosphoproteome Analysis Reveals Regulatory Sites In Major Pathways of Cardiac Mitochondria

O102

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Introduction
Mitochondria are power houses of cardiac cells playing vital roles in metabolism, cell survival, and Ca^{2+} load. The acute regulation of mitochondrial function is thought to be phosphorylation based, in response to various intracellular demands. Accordingly, the significance to delineate the cardiac mitochondrial phosphoproteome is high. Thus far, previous reports using various approaches have identified approximately 70 phosphorylated proteins in the mammalian mitochondria; in this study, we implemented an integrated LC-MS/MS platform combining the strength of both Collision Induced Dissociation (CID) and Electron Transfer Dissociation (ETD) to characterize residue-specific phosphorylation events in several key mitochondrial pathways in a non-bias fashion.

Methods
Phosphorylation based regulation of cardiac mitochondrial function was examined according to previous established methods [1]. To determine residue-specific phosphorylation events, proteins extracted from purified functional viable cardiac mitochondria [1] were processed in parallel for either CID or ETD based phosphorylation identification [2]. A complete trypsin digestion protocol was applied to support CID and a partial trypsin digestion protocol was optimized for ETD. The phosphopeptides resulting from either digestion procedure were enriched with TiO_2 resin and then sequenced by mass spectrometers. CID analyses were based on phosphorylation specific neutral loss and MS3 sequencing. ETD analyses took advantage of the preservation of phosphate moiety after fragmentation, thus sites of modification were pinpointed at MS2 level. Statistical analyses were conducted to ensure confidence of identification; each phosphorylation site was annotated.

Results
9 biological replicates ensured phosphorylation identifications with both CID and ETD reached plateau. 232 unique phosphorylation sites were identified in cardiac mitochondria; 220 of them were not previously reported. These sites were mapped to 199 phosphopeptides derived from 179 phosphoproteins. The complementary of CID and ETD technologies were illustrated by the observation that 42 phosphorylation sites were identified only by CID; whereas 184 phosphorylation sites were identified only by ETD. Trypsin digestion has been optimized individually for either CID or ETD; interestingly, only 6 sites of modification were detected by both ETD and CID approaches. Furthermore, the biological significance of these mitochondrial phospho-proteins was evaluated by functional assays. Phosphoproteins were located to all mitochondrial electron transport chain complexes, as well as other key enzymes in pathways regulating energy metabolism (e.g., pyruvate dehydrogenase, glycogen synthase kinase 3, citrate acid cycle, and fatty acid transport/oxidation; see figure below).

Innovative aspects
- Comprehensive characterization of cardiac mitochondrial phosphorproteome with 220 novel identifications;
- Identification of novel regulatory sites in essential mitochondrial pathways;
- Demonstrating combined CID and ETD as an effective approach for PTM analyses.

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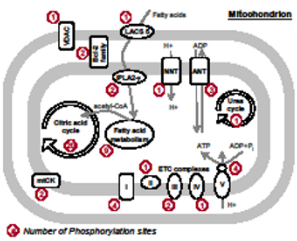

Figure 1. An illustration of phosphorylation sites in many key pathways of cardiac mitochondria. Identified phosphoproteins are involved in core mitochondrial functions. By combining both ETD and CID technologies, we defined a total of 20 phosphorylation sites for TCA and 12 sites for ETC. Furthermore, 12, 2, and 1 phosphorylation sites were also identified for fatty acid metabolism, mitochondrial creatine kinase and the urea cycle, respectively. Phosphorylation sites were also identified in adenosine nucleotide translocase (ANT), nicotinamide nucleotide transhydrogenase (NNT) and voltage-dependent anion channel (VDAC), which are major regulatory proteins involved in transport.

Proteomics of Mouse Liver Microsomes: Performance of Different Protein Separation Workflows for LC-MS/MS

O103

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Introduction
Quantitative comparison between series of biological samples requires a choice of appropriate separation strategy. In order to make a background for comparative experiments on mouse liver microsomes, we compared a performance of three separation procedures prior to ESI-MS-based measurements in terms of identified proteins number, run-to-run reproducibility and ability to detect membrane proteins. These latter, such as cytochromes P450 were often in a focus of clinical and functional mammalian liver studies (1).

Methods
Tree different separation techniques were used prior to MS-analysis: SDS-PAGE followed by slice trypsinolysis with reverse-phase HPLC-MS of resultant digests; 2D-HPLC-MS of microsome proteome digest; and 3D-LC-MS of the same where at first stage of procedure whole proteins were separated by heat-stable mRP HPLC. All MS measurements were conducted by ESI ion trap mass-spectrometer. Protein identification was performed using Spectrum Mill MS Proteomics Workbench by processing experimental data against Swiss Prot mouse subset database.

Results
Total number of proteins identified with selected search conditions in all experiments was as high as 4142. Comparison of datasets generated by 3 separation procedures illustrated that a majority of proteins (3703) from whole list was identified from mRP-based 3D-LC-MS. 2D-LC-MS and SDS-PAGE-LC-MS yielded 1410 and 519 proteins, respectively. Since only those proteins that appear in most and, preferably, in all replicates may be used for comparative study, SDS-PAGE-based method performed somewhat better (41% proteins identified in all 3 runs), than 2D-LC and 3D-LC supporting the evidence that the addition of chromatography steps decreases overall reproducibility (Table 1). Gel-based method yielded a number of predicted membrane proteins similar to LC-based workflows. At the same time, an absolute number of proteins identified by the gel-based technique remained notably smaller. A subset of proteins always identified by all methods in all runs contained 126 gene products. Of them, the most widely presented group was a cytochrome P450 superfamily, a fact, which could be explained by usage of drug-induced liver for this study. This also supports the opinion that LC-MS-based proteomics is a good tool for simultaneous measurements of cytochromes P450 in different biosamples (2).

Innovative aspects
- Derived dataset may help to select the best approach for both label-free and label-based quantitative proteomics.
- Especial attention was paid to ability of each separation scheme to find proteins with predicted transmembrane domains.

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Separation method	Number of proteins identified	Number of proteins identified in 3 runs of each method	Reproducibility	Number of proteins with predicted membrane helices (Ratio)	Number of proteins with predicted membrane helices in 3 runs (Ratio)
SDS-PAGE	519	213	41%	138 (27%)	93 (44%)
2D-LC	1410	451	32%	295 (21%)	122 (27%)
3D-LC	3703	764	21%	659 (18%)	118 (15%)

Table 1. Performance of SDS-PAGE, 2D-LC and 3D(mRP)-LC separation workflows coupled with ESI Ion Trap MS: run-to-run reproducibility of protein sets and a proportion of proteins with predicted membrane domain.

Analysis of exosome-like vesicles from rat urine as model for biomarker discovery

O104

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Introduction
Urine is an ideal non-invasive source of biomarkers because of the convenience of its collection in large amounts repeatedly over lengthy time periods. Urinary vesicles, including exosomes, are nanometer sized particles that have been described as good source for biomarker discovery unveiling promising results since they allow the detection of relatively low-abundant proteins and decrease the complexity of the urinary proteome (1). In this work we describe the urinary vesicles present in rat urine samples as an approach to identify potential biomarkers for diseases before attempting human trials.

Methods
Urine was collected from Wistar male rats using a metabolic cage during 12 to 14 hours. Urinary vesicles from rat urine were isolated by sequential centrifugation steps (2). Isolated vesicles were further fractionated by sucrose gradient ultra-centrifugation. For cryo-electron microscopy analysis, exosome preparations were analyzed using a JEM-1230 transmission electron microscope (JEOL, Japan). Protein identification was performed in a nano Acquity on-line coupled to a quadrupole-time of flight Premier (Waters, USA). The resulting data was searched with Mascot (Matrixscience, UK) against v52 of SwissProt database (Rattus: 5830 sequences). The search against the decoy database gave a false-discovery rate (FDR) on peptide level of 1%.

Results
The analysis of nanometer-sized vesicles present in rat urine samples by electron microscopy shows that the isolated material contains rounded-vesicles with a mean diameter of aprox. 90 nm. The presence of exosome-like vesicles was confirmed by checking the presence of Cd63, Cd81, Flotilin or Tsg10 by western blot.
The proteomic analysis of the exosome highly-enriched vesicles revealed the presence of 134 unique proteins (Fig 1). Among them, 28 proteins have been reported for the first time present in urinary vesicles. Many proteins identified have been associated with different diseases and may be considered as candidate urinary exosomal biomarkers.
In order to obtain indicators of hepatic damage in urinary vesicles, D-galactosamine (GalN) has been administrated to animals. Administration of GalN causes diffuse liver damage in rats closely resembling those seen in human viral hepatitis (3). Urinary vesicles from GalN-treated rats were purified and compared by western blotting to those obtained from a control group. While the presence of the protein Cd63 in urinary vesicles was not affected, we found that other exosomal proteins were dramatically reduced by the treatment.

Innovative aspects
- We show the proteomic analysis of highly purified exosome-like vesicles from rat urine samples.
- We show first cryo-electron microscopy of these urinary vesicles.
- These urinary vesicles may provide with potential biomarkers regarding to diagnosis and prognosis.

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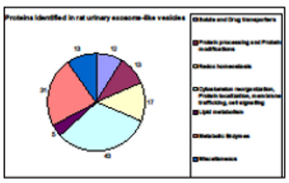

Figure 1. A total of 134 proteins were identified in rat urinary highly-enriched vesicles. Proteins are sorted by families.

Strategies for Membrane Proteomes. The AOHUPO Membrane Proteomics Initiative.

O105

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Introduction
AOHUPO represents 15 countries in the Asia-Oceania region (www.aohupo.org).The AOHUPO Membrane Proteomics Initiative (MPI) was established to carry out a systematic comparison of strategies for mining membrane proteomes (1,2). A membrane proteome standard (MPI standard) has been analysed independently by 17 laboratories using separation of intact proteins (differential detergent separation, 1D SDS PAGE, BN-PAGE, chromatography including PF2D, off-gel electrophoresis, and combinations thereof) or by separation of digest peptides (IEF, 1D and 2D HPLC) prior to on-line or off-line tandem MS of peptides.

Methods and Results
The MPI standard was distributed to participating laboratories as aliquots of a single preparation of carbonate-washed liver microsomes (3) from 10-11 week male C57BL/6J mice that had been maintained in germ-free conditions.
Initial comparison of the carbonate-washed MPI standard with the precursor microsomes shows that the MPI standard is enriched for integral membrane proteins (Figure 1).
The multi-laboratory dataset derived from the various experimental platforms consists of 192 Gb tandem MS files from TOF-TOF, 2D and 3D ion-trap, QuadTof, Orbitrap and FT instruments. Preliminary analysis indicates that several of the platforms including methods based on protein chromatography and methods using shotgun IEF or tandem MS give comparable high quality lists of integral membrane proteins.

Innovative Aspects
- The carbonate procedure substantially enhances detection of integral membrane proteins including proteins that are not well-represented in other reports of membrane proteomes.
- Further fractionation of the MPI standard enhances the detection of proteins containing transmembrane domains

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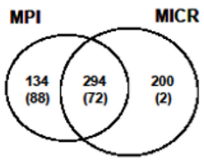

Figure 1 Analysis of the precursor microsomes (MICR) and carbonate-washed MPI standard using RP-LC MS MS of solution tryptic digests. Numbers in brackets are for proteins with predicted transmembrane domains.

The Human Proteome Project #X
Differential proteomic analysis of DRM (detergent resistant microdomains) proteins in Cystic Fibrosis.

O106

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Introduction
Proteomic approaches have been successfully used by us to study the pathogenesis of Cystic Fibrosis (CF), a genetic disease due to the mutation in CF transmembrane conductance regulator (CFTR) and characterized by defective fluid transport in secretory epithelia and chronic inflammation and infections. Detergent resistant microdomains (DRM or rafts) are specialized platforms involved in many cell processes, including inflammation signaling. DRM play a role in the response to inflammation in CF.
Our goal is to compare the DRM proteome of epithelial cells expressing wild type and mutant CFTR.

Methods
HeLa cells expressing either wild type or mutated CFTR (WT or deltaF508) and the corresponding control (empty vector) were labelled using $^{13}C_6$ L-Arginine and $^{13}C_6$ $^{15}N_4$ L-Arginine.
Thereafter DRM were prepared using a standard sucrose density gradient protocol. DRM proteins were concentrated using Centricon cut off at 10kDa, followed by in gel protein trapping (at the interface of the stacking/ resolving gel of a 1D SDS PAGE).
The proteins were then digested *in situ* and the peptides obtained were analysed by nano-HPLC MALDI-TOF-TOF (ABI 4800).

Results
We highlighted that a cutoff step augmented the number of identified proteins from 103 to 186. DRM markers Flotillin-1 and 2, were identified amongst them.
Differential quantitative proteomics of DRM was also performed using metabolically labelled tranfected Hela cells. We found differentially located proteins between WT and F508del-CFTR and we established their relation to CFTR as we simultaneously analysed the cells transfected with the empty vector.
Those proteins found as specifically recruited in the DRM were analysed using Ingenuity software.

Innovative aspects
- Creation of SILAC cell model to study CF
- Combination of enrichment of DRM proteins and SILAC in CF

Membrane Proteome of Cyanobacterium *Synechocystis* sp. PCC 6803

O107

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Introduction
Cyanobacteria are unique prokaryotes due to the presence of a differentiated membrane system. They contain the photosynthetic membranes (thylakoid, TM) in addition to an envelop consisting of an outer membrane (OM), a peptidoglycan layer, and a plasma membrane (PM). In this study, the three membrane fractions of cyanobacterium *Synechocystis* sp. PCC 6803 were completely purified by density centrifugation and polymer two-phase partitioning. The integral proteins in each membrane fractions were identified.

Methods
Each membrane fractions were purified by sucrose-density gradient centrifugation technique combined with an aqueous polymer two-phase partitioning method. Each membrane protein fraction was separated in 1D SDS-PAGE. After the excision of ~20 slice bands of 1-D gels followed by in-gel digestion with trypsin, membrane proteins in each fraction were identified by RP-LC-MS/MS analyses using LTQ-FT mass spectrometer, based on the highest score results by searching through the *Synechocystis* sp. database with Mascot 2.0. Putative signal peptides and their cleavage sites were predicted using SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP/).
Transmembrane helices were predicted using TMHMM program (www.cbs.dtu.dk/services/TMHMM-2.0/).

Results
Very few studies on integral membrane proteins from the membrane of cyanobacteria have been carried out. We focused on the identification of integral membrane proteins in the plasma, thylakoid and outer membrane of *Synechocystis* sp. PCC 6803. The immunosignal for Toc75, a marker protein for the outer membrane of cyanobacteria, could only be detected in outer membrane. Their fractions were separated on 1D SDS/PAGE, digested with trypsin and identified using RP-LC-MS/MS analyses using LTQ-FT mass spectrometer and with a database searching. Totals of 1549, 1334 and 678 proteins were identified in the plasma, thylakoid and outer membranes of *Synechocystis*, respectively. Enrichment of the integral membrane proteins from purified plasma, thylakoid and outer membrane allowed the identification of 496, 395 and 172 proteins containing 1–17 predicted transmembrane helices, respectively.

Innovative aspects
- Complete purification of TM, OM, and PM fractions of *Synechocystis* by density centrifugation and polymer two-phase partitioning
- Identification of a total of 593 integral proteins corresponding to 87 % of 707 integral membrane proteins predicted by whole genome-wide analysis
- A total of 282 peripheral proteins predicted by the SignalP program

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Table 1 Proteins identified in the plasma, thylakoid and outer membrane of *Synechocystis*.

	ID proteins	Integral proteins(%)	Signal peptides§
PM§	1549	496 (32)	234
TM	1334	395 (30)	182
OM	678	172 (25)	103

*Number and position of transmembrane helices predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/).
§Position of cleavage site predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

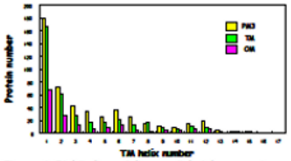

Figure 1 Distribution of the predicted transmembrane helices for integral proteins identified in the plasma, thylakoid and outer membrane of *Synechocystis*, respectively

O108

New Insight into Peroxisome Membrane Interaction Networks by Quantitative Proteomics

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Introduction
Biogenesis of functional peroxisomes is mediated by a highly coordinated (inter)action of membrane protein complexes and related defects induce severe, often lethal human disorders. For an in-depth study of the structural composition of membrane protein complexes and the intricate interplay between them, we designed a global, functional proteomics strategy based on epitope tagging and affinity purification combined with SILAC labeling of yeast and quantitative MS. Here we report new insights into peroxisomal membrane protein interaction networks providing new clues on peroxisomal biogenesis.

Methods
Distinct populations of yeast cells expressing the protein of interest fused to a TEV-Protein A tag or the native protein were differentially labeled with SILAC. SILAC-labeled populations were mixed after cell harvest ("PAM-SILAC"). After preparation of membrane fractions, protein complexes were affinity-purified via IgG, the resulting eluates were digested with trypsin and analyzed by nano HPLC/ESI-MS/MS on an LTQ-Orbitrap followed by bioinformatics data analysis. Alternatively, protein complexes were affinity-purified separately before joint MS-based analysis ("MAP-SILAC"). Relative quantitative analyses using MSQuant enabled both the accurate identification of "core" complexes and transient/weak interacting partners. For statistically sound data, biological triplicates were performed.

Results
We first applied quantitative proteomics strategy to the study of the interaction network of Pex14p, an integral membrane protein and part of the matrix protein import machinery in peroxisomes. Quantitative MS-based analyses (n=3) enabled the identification of specific interaction partners against a high background of contaminants. Using the PAM-SILAC strategy, we defined a Pex14p "core" complex consisting of the entire importomer, Pex11p and Dyn2p. We extended our analysis to the identification of transient interaction partners using MAP-SILAC enabling the identification of 10 further proteins (e.g., Pex5p) transiently interacting with Pex14p or other components of the membrane core complex. Most of these are hitherto unknown interaction partners of Pex14p. We next studied further membrane proteins with essential functions in the biogenesis of peroxisomes, such as Pex3p and Pex25p to establish successively the peroxisomal membrane protein interactome. Our results provide ample new information leading to better understanding the molecular mechanisms underlying peroxisomal biogenesis.

Innovative aspects
- Characterization of peroxisomal membrane protein complexes by quantitative MS.
- Identification of both cores and specific transient interactors of membrane protein complexes.
- Global strategy for the establishment of accurate and most comprehensive protein interaction networks of organellar membrane proteins.

O109

Subcellular proteome analysis discovering annexin A2 related to liver fibrosis caused by HBV

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Introduction
Liver fibrosis is a common sequel to diverse liver injuries, and can be reversed. However, little is known about its mechanism and non-invasive biomarkers. In this work, we used subcellular proteomic technology to dynamically study the proteins related to liver fibrosis. We found that Annexin A2 is a protein related to liver fibrosis and can be relocated by HBV. Our research suggests a possible mechanism by which HBV or immune alters protein expression and relocation to intense the liver fibrosis progress, and offered a new potential biomarker -Annexin A2 for non-invasive diagnosis of liver fibrosis.

Methods
A rat liver fibrosis model induced by pig serum was built. The liver plasma membrane was purified and proteins were separated by two-dimensional electrophoresis (2DE). The differentially expressed proteins were identified by mass spectrometry. The expression of Annexin A2, keratin 8 and keratin 18 was verified by western blot analysis. The up-regulation of Annexin A2 was verified by immunohistochemistry in liver biopsies of rat model and liver fibrosis patients infected by HBV. The relationship of Annexin A2 with HBV was analyzed through a vitro immunocytochemical experiment in cell line stimulated by HBV. ELISA was performed in plasma of patients and health controls to analyze the possible diagnosis value of Annexin A2.

Results
The liver fibrosis model was successfully built. At 6 weeks, hepatic fibrosis with formation of many small-sized pseudolobules was developed and enhanced further to 8 weeks. High purity PM was enriched. Annexin A2, keratin 8 and keratin 18 were found to be dynamically changed during the development of liver fibrosis (figure 1A) and verified by western blot analysis (figure 1B). Through immunohistochemistry, we found that the protein expression of Annexin A2 gradually increased in liver hepatocytes of rat liver fibrosis model from 2 to 8 weeks and also up-regulated in liver fibrosis patients with S3-4 compared with S0-1(figure 1C). Annexin A2 was detected to move from nuclear membrane and cytoplasm to plasma membrane through immunocytochemical analysis. Furthermore, annexin A2 was also found to be up-regulated for 2-fold ($p<0.05$) in the plasma of liver fibrosis patients with S1-2 compared with S0 and healthy through ELISA experiment (Figure 2).

Innovative aspects
- The first plasma membrane proteomic study of immunity liver fibrosis.
- Suggests a possible mechanism by how HBV or immune intense the liver fibrosis progress though altering the expression and relocation of proteins.
- Offered a new potential biomarker (Annexin A2) for non-invasive diagnosis of liver fibrosis.

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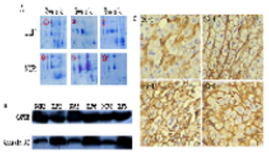

Figure 1 A. The different expression of Annexin A2 in 2-DE; B. WB verification of Annexin A2 expressed in rat liver fibrosis model; C. Immunohistochemistry analysis of annexin A2 in human liver biopsy. ILF, immunity liver fibrosis. NOR, controls.

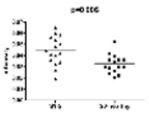

Figure 2 ELISA analysis of annexin A2 in patients with S0-2 and healthy donors.

O110

Organelle based proteomics of the caudate nucleus in simian immunodeficiency virus infected and methamphetamine treated rhesus macques

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Introduction
Drugs of abuse are a significant co-morbid factor in human immunodeficiency virus (HIV) infection, notably in within the central nervous system (CNS). Using simian immunodeficiency virus (SIV)-infected rhesus monkeys, we propose to examine by proteomics techniques the interactions between SIV and methamphetamine (METH) in the caudate nucleus. We posit that METH can exacerbate SIV CNS effects and in turn, neuronal viability. Since the striatum, which includes the caudate nucleus, is a METH target, we have initiated our studies by analyzing the **synaptic proteome of the caudate nucleus**. Isolating synaptosomes, which represent isolated synapses containing the complete pre and postsynaptic components including mitochondria and synaptic vesicles by employing classical subcellular fractionation techniques and quantitative mass spectrometry based proteomics; we opine such analysis will improve our understanding of synaptic molecular composition and function thus enabling us to explore distinct synergistic CNS disease mechanisms.

Methods
Purified synaptosomes from the caudate will be prepared from 6 monkeys (3 SIV+PBS, 3 SIV+METH) using a protocol as described previously [1]. Briefly, tissue will be homogenized in 10 volumes of lysis buffer (0.32M Sucrose, 20mM HEPES pH 7.4 containing protease inhibitors). The homogenate is centrifuged at 800xg for 10min at 4°C to separate out the cellular debris. The post nuclear supernatant is then centrifuged at 17,000xg for 20min at 4°C to obtain the crude synaptosomal pellet that contains microsomes and mitochondria as contaminants. To obtain a pure synaptosomal fraction, the crude fraction is loaded onto a sucrose gradient comprising of 0.8M, 0.8M and 1.2M sucrose and centrifuged at 53,000xg for 90min at 4°C. The pure synapatosomal fraction at the interface of 0.8 and 1.2M will be isolated, washed in lysis buffer and 100µg protein from each sample will be reduced, alkylated and trypsin digested. An equivalent amount of pooled sample comprising the six samples will serve as an internal standard. The respective tryptic digests will be iTRAQ labeled, pooled and fractionated by OFFGEL electrophoresis, a novel approach that fractionates peptides based on pI and has been shown to improve proteome coverage when analyzed by LC-MS/MS [2, 3]. For quantitation, all ratios will be normalized to the internal standard, followed by statistical analysis. Proteins significantly different ($p<0.05$) and 1.5 fold up/down regulated will be further validated.

Results
Pilot experiments performed with mouse synaptosomes identified a total of 1227 proteins (Figure 1). This preliminary data thus lends credibility to our approach. Data from the experiments proposed will be presented at the meeting.

Innovative aspects
- Mining the caudate proteome is the focal point of this study
- Application of OFFGEL electrophoresis for separation of peptides based on pI
- iTRAQ analysis using hybrid PQD/CID strategy on an LTQ

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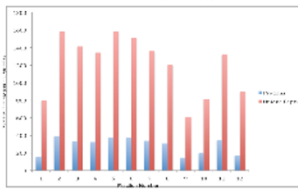

Figure 1. 100µg of mouse synaptosomes was reduced, alkylated and trypsin digested. The tryptic digest was subjected to OFFGEL electrophoresis prior to analysis by LC-MS/MS.

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O111

Phosphoproteome analysis of calcium-dependent cell adhesion dynamics in mouse EpH4 cells

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Introduction
Cell attachment is an essential component of cell morphology and has a critical role such as cell differentiation and tumor invasion. The formation of cell attachment is dependent on the organization of calcium-dependent cell-cell adhesions which are regulated by various signal transduction cascades. Despite the importance, the dynamics of the signal transduction network during the formation of the calcium-dependent cell-cell adhesion is not clearly known yet. In this study, we applied a quantitative phosphoproteomics approach to reveal the cellular signaling events on the cell-cell adhesion formation after calcium-switch in EpH4 cells.

Methods
Mouse mammary epithelial EpH4 cells were grown to confluence in DMEM containing 10% FBS (normal medium). For calcium switch experiments, EDTA was added for 30 min and this calcium-free medium was replaced with the normal medium. Then, cells were harvested for 0 min, 15 min, 60 min and 24 hours after the calcium-switch. Proteins were extracted with phase-transfer surfactant [1] and were digested with trypsin. Phosphopeptides were selectively enriched by aliphatic hydroxy acid-modified metal oxide chromatography [2] and were analyzed by nanoLC-MS/MS.

Results
The formation process of the cell-cell adhesion in EpH4 cells was observed after the calcium-switch. We identified 1590 unique phosphorylation sites (pS/pT/pY:1318/212/60) on 724 proteins, of which 85 proteins (151 phosphorylation sites) were adherence junction-, tight junction- or cytoskeleton-related proteins, defined by UniProt keyword description. The time course profiles of these phosphopeptides were clustered into seven groups. We found that the phosphorylation on S285 of junctional adhesion molecule A (JAM-1), which is a member of immnoglobulin superfamily expressed in tight junctions of epithelial cells, were highly up-regulated during the formation of the cell-cell adhesion. It was known that the human JAM-1 phosphorylated at S284 (corresponding to S285 in mouse) localizes at cell-cell contacts [3]. On the other hand, the phosphorylation on S1943 of myosin-9 was down-regulated during the formation of the cell-cell adhesion, supporting the reduction of the cell motility in S1943A mutant cells. In addition, we identified novel phosphorylation sites of adherens junction-related proteins.

Innovative aspects
- The first large-scale profiling of protein phosphorylation during the cell-cell adhesion formation.

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O112

Subcellular Fractionation in a Fluidic Microsystem by Dielectrophoresis (DEP)

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Introduction
The application of pre-fractionation techniques is a widely used strategy in proteomics to reduce sample complexity and to enrich for low abundance proteins. In this context, subcellular organelle fractionation is of particular importance, since functional units provide additional information with respect to the cellular localization of a particular protein (1, 2). Methodologies currently employed for subcellular fractionation lack specificity to provide sufficiently pure organelle preparations for specific down-stream applications such as comparative proteomics. To overcome these drawbacks of current organelle isolation methodologies we are developing a microfluidic system with integrated electrode arrays and a dedicated high through-put channel layout to isolate mitochondria from crude organelle samples based on dielectrophoretic sorting.

Methods
Mitochondria of a human lymphoblastoid cell line were stained with the fluorescent dye JC-1. Cells were homogenized with a douncer and the resulting cell homogenate was centrifuged at 1000xg for 10 min to remove cell debris and unbroken cells. The supernatant was centrifuged at 10.000xg for 10 min in order to obtain a crude mitochondrial preparation. The resulting pellet was suspended in a standard mitochondrial storage buffer at a concentration of 0.2 µg/µL and pumped through the microsystem at rates of 1 µl/min (Figure 1). Mitochondria were deflected by dielectrophoretic forces for 20 hours. Mitochondrial samples (before and after dielectrophoresis) were characterized either by 1-D electrophoresis followed by immunoblotting with organelle specific antibodies or by 2-D electrophoresis followed by mass spectrometric analysis.

Results
Dielectrophoresis (DEP) occurs when a dielectric particle – cell and organelles are among them – is subjected to a non-uniform electrical field. The strength of that force depends on the medium and the size and electric properties of the particle. In a microfluidic flow system with channels of 10 – 20 µm in height, DEP can be used to divert specific particles, in our case mitochondria, with great selectivity. We have explored the optimal experimental parameters for mitochondria DEP by varying buffer flow velocity, voltage amplitude and frequency. Deflection and subsequent collection of mitochondria was successfully demonstrated at frequencies of 400 kHz and a field of 4 kV/cm.

About 100 µg mitochondrial proteins were isolated during a total collection time of 20 hours. This protein sample was further analyzed by 1-D immunoblotting with organelle specific antibodies. Data evaluation of the immunoblots showed that the ratio between antibody signal intensity and protein amount loaded per lane differs significantly between the crude starting material and DEP sample. For the mitochondrial marker protein VDAC an enrichment factor of about 2.5 can be calculated. BiP, a marker protein for the ER, shows a six fold decrease in signal intensity. For LAMP1, a lysosomal marker protein, almost no signal was observed in the DEP sample. 2D-PAGE analysis followed by mass spectrometry confirmed the superior purity of the DEP sample in comparison to standard gradient centrifugation techniques for the isolation of mitochondria.

Innovative aspects
- High-through-put microfluidic chip based on dielectrophoresis technology for organelle separation successfully designed and fabricated
- Dielectrophoretic properties of mitochondria were determined
- Deflection of mitochondria derived from a crude organelle preparation was successfully demonstrated and the superior purity of the DEP sample was shown by 1-D immunoblotting and 2D-PAGE/MS analysis.

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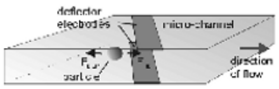

Figure 1: Dielectrophoretic deflector array. Separation is achieved by a competition between hydrodynamic friction forces and dielectrophoretic forces

O113

Acetylation of lysine, serine and threonine residues in mouse liver detected by Liquid Chromatography-Mass Spectrometry

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Introduction
Acetylation of amino acids is a reversible post-translational modification with potential significance both in prokaryotic and in eukaryotic cells. Lysine acetylation of histones was first reported in 1968 (1). Serine and threonine acetylation of YopJ (2) was demonstrated by Mukherjee S. recently. Proteomics surveys based on HPLC/MS/MS are efficient tools to identify proteins with acetylation modifications. We describe a proteomics survey of the serine, threonine and lysine acetylation from mouse livers by combing shotgun technology and nano-HPLC/MS/MS analysis.

Methods
Three milligrams of mouse liver protein was reduced with 10mM DTT, alkylated with iodoacetamide, precipitated with cold acetone, resuspended in 50mM NH4HCO3 (pH8.5), and then digested with trypsin. Peptides were resolubilized in 5% acetonitrile/0.1% trifluoroacetic acid/94.9% (v/v/v) buffer. Chromatographic separation was performed in a LC-20AD system equipped with a SCX column. The eluted peptides were collected every three minutes and dried in a SpeedVac. Resulting peptides were resolubilized in 5% acetonitrile/0.1% Formic acid/94.9% (v/v/v) buffer. Nano-HPLC/MS/MS tandam mass spectrometry analysis was performed in a LTQ Orbitrap mass spectrometer. The MS/MS spectrum was acquired in a data-dependent mode that determined the masses of the parent ions and fragment of the eight strongest ions. Tandem mass spectra were searched with SEQUEST and PeptideProphet by using the Tran-Proteomic Pipline against IPI mouse 3.45 database with the concatenated decoy database. Hierarchical clustering analysis of the residue preference for acetylation sites was performed.

Results
In the subsequent MS/MS spectrum of peptides with acetylated amino acid residues, typical fragment ions of y and b was produced. Figure 1 shows an example for acetylation of serine residues of peptides from adenosylhomocysteinase. The △m/z of b5-b4 and y6-y5 was all 129, which supported the acetylation on same residue.
We identified 71 acetylation sites in 60 distinct proteins, 35 serine-acetylated sites in 30 proteins, 25 threonine-acetylated sites in 23 proteins and 11 lysine-acetylated sites in 9 proteins. Twenty-eight serine-acetylated proteins, 22 threonine-acetylated proteins and 5 proteins have not been previously reported. Three of the proteins identified have functions related to acyl-coenzyme A. Therefore, it is interesting that whether these three proteins could serve as the enzyme or coenzyme responsible for acetylation need to be further studied.
The hierarchical clustering analysis of the amino acid residues surrounding acetylation sites indicated the significant patterns of residue preference. Methionine is significantly favored at the -1 and/or -2 positions flanking the acetylation site with serine, threonine and lysine acetylation.

Innovative aspects
- Acetylation of lysine, serine and threonine residues
- Characterization of acetylation protein
- 2D LC/MS/MS

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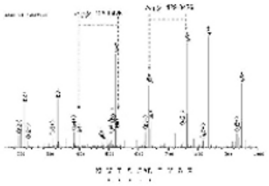

Figure 1. MS/MS spectra of serine acetylated peptide using ions m/z 581.3352 (z=2). The signals were identified from Cytochrome P450 2D26 (aa 187-196, NVIAS*LIYAR). The acetylation site located at serine residue S191.

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O114

Organelle proteomics of endosomal compartments in *Arabidopsis thaliana*

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Introduction: The knowledge of the protein composition of endosomal compartments will be most valuable for a good understanding of receptor mediated protein signalling in plants. This goal is complicated by the fact that endocytosis and downstream endosomal trafficking are highly dynamic processes, making it a real challenge to distinguish true residents from false positives. In this study we aim to give insight in the localization and dynamics of proteins residing in the endosomal system of whole roots Arabidopsis *thaliana* by way of LOPIT (Localization of Organelle Proteins by Isotope Tagging).

Methods: Post nuclear supernatant of *Arabidopsis thaliana* roots is applied to an iodixanol or sucrose based gradient. After ultracentrifugation, the distribution of organelle proteins along the gradient is visualised by checking for known marker proteins by western blotting. The assumption is that proteins that belong to the same compartment will comigrate which results in a specific organelle distribution pattern. 4-8 Gradient fractions are reduced, alkylated, trypsinised and labelled with iTRAQ reagents. The fractions are combined, run over a SCX column, and measured with a LC-ESI Q-TOF (premier, Waters). Peptides are identified, quantified and organelle residency is determined by a combination of Principal Component Analysis (PCA) and partial least square discriminant analysis (PLS-DA).

Results: Endosomal fractions were succesfully isolated from *Arabidopsis thaliana* roots. By using antibodies for endosomal marker proteins it was shown that different endosomal compartments were partly separated upon ultracentrifugation as shown by western blot. iTRAQ labelled fractions were analysed and projected on a plot by use of PCA. Proteins were assigned to organelles by use of PLS-DA. It is the first time that the LOPIT approach is performed on whole roots of *Arabidopsis thaliana*. The data generated provides novel information about the proteome composition of endosomes, and gives insight into the dynamics of protein trafficking in endosomes and the mechanisms of endosomal-based signalling.

Innovative aspects:
* LOPIT approach on whole *Arabidopsis thaliana* roots.
* LOPIT approach on endosomal compartments in *Arabidopsis thaliana*.
* Dynamics of proteins by use of LOPIT.

O115

The Protein Composition of Mitotic Chromosomes by Multidimensional Quantitative Proteomics

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Introduction
Mitotic chromosomes accomplish the critical segregation of the genome when cells divide, yet despite many decades of study remain poorly characterized with respect to their structure, mechanism of condensation and composition. The most comprehensive previous proteomic analysis of chromosomes identified 316 proteins, including 14 centromere proteins (1,2).

Methods
We isolated mitotic chromosomes from chicken DT40 cells by a variant of the polyamine method developed by Lewis and Laemmli (3). 250 µg of total chromosomal protein were digested, pre-fractionated using SCX, and analyzed on LC-LTQ Orbitrap. MS data were processed using MaxQuant and searched using Mascot against a chicken IPI database in target/decoy configuration. Enrichment, retention and SMC2-dependency of chromosomal proteins were determined using SILAC experiments.

Results
In the present study, mass spectrometry has identified 4024 polypeptides in prometaphase chromosomes isolated from chicken DT40 cells. Of these, 122 were previously annotated as centromere proteins and 14 as telomere proteins (Figure 1). Analysis of the spectra enabled us to determine accurate copy numbers for all chromosomal proteins, and revealed that chicken regional centromeres appear to resemble the point centromere of the budding yeast much more closely than previously appreciated. In order to identify novel proteins specifically associated with chromosomes, we used SILAC analysis to define three classifiers for every protein based on its level of enrichment in chromosomes, exchange rate on and off of chromosomes in cytosol, and dependency on condensin for chromosomal association. These classifiers distribute the ~4000 chromosomal proteins in a multi-dimensional space. We then identified regions of this "SILAC space" enriched in proteins important for chromosome structure (Figure 2). The approach presented here offers a powerful way to define the functional proteome of complex organelles and structures whose composition is not simple or fixed.

Innovative aspects
- Proteomic analysis of a complex organelle whose composition is not simple or fixed
- Multi-dimensional SILAC analysis
- Copy numbers of proteins in complex mixtures

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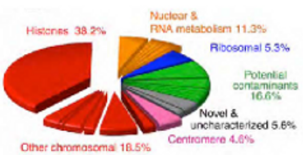

Figure 1. Functional classes of the 4024 proteins identified in mitotic chromosomes according to protein abundance.

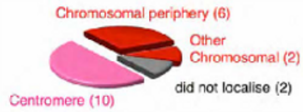

Figure 2. As a validation of our approach, GFP tagging of 20 previously unknown proteins selected from the "SILAC space" revealed 10 to be novel centromere proteins, and another 8 to occupy other chromosomal domains during mitosis.

Altered Proteins in Subcellular Compartments of Human Endothelial Cells during Dengue Virus Infection

O116

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Introduction
The hallmarks of severe form of dengue hemorrhagic fever and dengue shock syndrome are presented with the vascular permeability, hemorrhagic diatheses and vascular leakage. The endothelial cells which line blood vessels play an important role in haemostasis and in immune response to dengue infection. We applied proteomics to analyze a global change of proteins in subcellular localization of human endothelial cells during Dengue virus infection.

Methods
Human endothelial cells, EA.hy926 were infected with Dengue virus type 2, strain 16681 (DEN-2) for 24 h. The infected cells were extracted with Subcellular Proteome Extraction Kit (S-PEK; Calbiochem, EMD Biosciences, Darmstadt, Germany) into 4 fractions: cytosolic, membrane/organelle, nucleus, and cytoskeleton. The protein lysates derived from these 4 fractions were separated by 2D-PAGE and visualized by SYPRO Ruby fluorescence stain. Quantitative intensity analysis compared between DEN-2-infected cells and mock-control cells were performed using Image Master 2D platinum software and the altered proteins were then identified by MALDI-MS and MS/MS analysis.

Results
During 24 h post-infection and MOI of 10, the mass spectrometric identification revealed the 8, 4, 2, and 5 proteins that were up-regulated, whereas 5, 2, 5, and 1 proteins were down-regulated in cytosolic, membrane/organelle, nucleus and cytoskeleton, respectively. These proteins were functionally categorized and found that most of proteins involved in endocytosis system (programmed cell death 6 interacting protein, valosin-containing protein, and moesin) and enzyme metabolism (transglutaminase 2 isoform a, spermidine synthase, and purine nucleoside phosphorylase) were up-regulated, whereas the proteins involved in molecular chaperone (heat shock 70 kDa protein 1B, heat shock 90 kDa protein 1, alpha, heat shock 70 kDa protein 4, and heat shock protein 90 kDa beta, member 1) were down-regulated. Of these up-regulated proteins were included two viral proteins (NS1 and NS5).The function study of the proteins involved in endocytosis system would provide better understanding of viral replication in the early phase on Dengue infection.

Innovative aspects
- The altered proteins were identified and localized in sub-cellular compartments of human endothelial cells during Dengue virus infection

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Analysis of IMPs Based on Heat Gel-embedment and Improved In-gel Digestion Strategies

O117

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Introduction
Integral membrane proteins (IMPs) are important biological and pharmacological targets. (1). Contemporary genomic analyses indicate that 20-30% of all open reading frames encode for IMPs (2). Comprehensive and high throughput analysis of IMPs would facilitate our understanding of their roles. However, despite the rapid development of proteomic techniques in recent years, the analysis of IMPs still presents a special challenge due to their hydrophobic nature and low abundance(3). Here we report a new method for the analysis of this type of proteins.

Methods
The rat liver cellular membranes were enriched by sucrose density gradient ultracentrifugation and the membrane proteins were extracted with buffer containing 4% SDS. The detergent-containing membrane protein solution was mixed with acrylamide solution and the proteins were embedded when the gel polymerized. For comparison, the gel-embedment of proteins was made at different temperatures (25 °C, 35 °C or 45 °C), and, after removal of SDS from the gel by wash, in-gel digestions were performed in the presence of 0.1% RapiGest reagent (ALS), 0.1% sodium deoxycholate (SDC) and 10% ACN, respectively. The resultant peptides were extracted and then analyzed by CapLC-MS/MS.

Results
The results showed that, in contrast to that at 25 °C, gel-embedment at 45 °C could avoid residual protein solution and thus improve the protein embedment, with the identified IMPs increased by 27.3%; 0.1% SDC was more efficient than 0.1% ALS and 10% ACN in terms of improving the in-gel digestion and tryptic digest recovery of gel-embedded proteins particularly the hydrophobic IMPs. Out of the 326 IMPs identified by heat gel-embedment combined with improved in-gel digestion strategies, 149 (45.7%) proteins had at least two mapped transmembrane domains, most of which were ion channels, receptors or transporters, etc. These results indicate that our newly developed protocol was effective for high throughput analysis of integral membrane proteome.

Innovative aspects
- Gel-embedment of membrane proteins extracted by detergent-containing buffer so as to remove the detergent lest it should interfere with the subsequent digestion.
- Gel-embedment at higher temperature for improvement of protein embedment.
- Improved in-gel digestion for identification of gel-embedded proteins particularly the hydrophobic IMPs.

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Differentiation and activation of myeloid cells, as viewed by proteomics

O118

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Introduction :

Dendritic cells are the major antigen-presenting cells in upper vertebrates, and play an important role in the coordination of the immune system. Dendritic cells are activated by various microbial or endogenous ligands. Upon activation, they prduce cytokines and present antigens to T lymphocytes.
We investigated how the proteome of dendritic cells evolves, either when compared to their progenitors, or when immature and mature dendrttic cells are compared. We present here the results of such comparative proteomics at the level of the total cell proteome and at the secretome level.

Methods :

Proteomics
Either total cell extracts made under denaturing conditions or secretome extracts concentrated by the detergent/TCA precipitation method described previously (1) were analyzed by 2D electrophoresis, silver stained, and the proteins of interest were analysed by peptide mass fingerprinting or MS/MS.

Cell biology
Murine dendritic cells were obtained from bone marrow progenitors by culture in the presence of GMCSF, IL6 and FLT3L.
DC activation was monitored by the surface expression of MHC class II molecules and by cytokines production (ELISA)

Results
While the proteomic analysis of the secretome of LPS-activated dendritic cells showed the expected cytokines (1), the same analysis carried out on immature dendritic cells revealed proteins such as lipocalin 2 and the YM1 lectin. This led us to investigate if the ligands of these proteins, repectively bacterial siderophores and chitin derivatives, could modulate the activation of dendritic cells.
While bacterial siderophores did not show any specific effect, chitosan induced a partial activation of dendritic cells.
Indeed, no cytokines were produced, while the classical membrane markers (MHC Class II, CD80, CD86) were up-regulated as described for a LPS activation. The use of TLR-KO mice allowed us to show that this membrane aoctivation was TLR4-dependent.
However, the absence of cytokine production upon activation by chitosan, confirmed both by ELISA and by proteomics, prevented the chitosan-activated dendritic cells to activate T cells.
As chitosan is produced by the remodelling of the chitin exoskeleton of eukaryotic parasites (e.g. fungi) this inefficient dendritic cell activation could explain, at least in part, the survival of these parasites in infected hosts (2).

Innovative aspects

High sensitivity analysis of secretomes

New mode of action of fungal products on dendritic cells

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iTRAQ reagent-based quantitative proteomic analysis of age-related changes in mitochondrial proteome from mouse liver and kidney

O119

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Introduction
Over the past three decades, a large amount of evidence has accumulated indicating that mitochondria play an essential role in aging. Analysis of the protein profile of mitochondria and its age-dependent variation is a promising approach to unravel mechanisms involved in aging and age-related diseases. Moreover, in a recent paper (1) we have shown that age-related changes in subcellular proteome seem to be gender-specific. Therefore the aim of this study was to analyze age- and gender-related changes in mitochondrial proteome from mouse liver and kidney using iTRAQ and LC-MS/MS.

Methods
Mitochondria were isolated from liver and kidney (CD1-Swiss, 3 ages: 6, 12 and 18 months old; male and female specimens; 5 biological replicates per each group) by a standard density-gradient centrifugation protocol (2). Purity of mitochondria isolation procedure was controlled by Western blots and electron microscopy. Samples were cleaned up by acetone precipitation, and protein concentrations were measured using BCA protein assay. Equal protein amounts were reduced, denatured, alkylated, and and digested with trypsin. Protein digests corresponding to each biological replicate were labeled with different 8-plex isobaric iTRAQ (Applied Biosystems) mass tag labels. All supernatants were combined, fractionated by strong cationic exchange (SCX) HPLC and further analyzed by MALDI-TOF/TOF and 3200 Q TRAP.

Results
Mass spectra obtained by MALDI-TOF/TOF and 3200 Q TRAP are currently under analysis.

Innovative aspects
To our knowledge this is the first comprehensive age- and gender- related proteomic analysis of mitochondrial proteome.

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O120

Low Levels of Collapsin Response Mediator Protein1 (CRMP1), as Detected by iTRAQ Analysis, Promotes Invasion of Human Gliomas Expressing Mutant EGFRvIII

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Introduction: Glioblastoma multiforme (GBM) is the most common and lethal primary human brain tumor. GBMs are characterized by a variety of genetic alterations, amongst which oncogenic mutations of epidermal growth factor receptor (EGFRvIII) is most common. GBMs harboring EGFRvIII have increased proliferation and invasive characteristics versus those expressing wild-type (wt) EGFR. To identify the molecular basis of this increased tumorgenic phenotype, we utilized iTRAQ-labelling differential proteomic analysis. Amongst several differentially expressed proteins we selected CRMP1, a protein implicated in cellular invasion, which was markedly decreased in GBMs expressing EGFRvIII for further study.

Methods: The differential expression of CRMP1 was confirmed in a panel of human GBM cell lines and operative specimens which express wtEGFR or mutant EGFRvIII, by quantitative Real-Time PCR, western blot and immuno-histochemical analysis. The functional consequence of altered CRMP1 expression in GBMs was evaluated by knock down and rescue of CRMP1 in human GBM cells which express wtEGFR and EGFR-vIII respectively.

Results: In human GBM samples, decreased expression of CRMP1 correlated with EGFRvIII positivity. Knockdown of CRMP1 resulted in increased invasion of wtEGFR expressing human GBM cells (U87 and U373) to those found in isogenic GBM cells. Exogenous expression of EGFRvIII in these wtEGFR expressing GBM cells promoted their ability to invade and was accompanied by decreased expression of CRMP1. Rescuing CRMP1 expression decreased invasion of the EGFRvIII expressing GBM cells by tilting the balance between Rac and Rho.
Collectively, these results demonstrate that the loss of CRMP1 contribute to the increased invasive phenotype of human GBMs expressing mutant EGFRvIII. Further investigation is needed to elucidate the complete mechanism by which CRMP1 modulates glioma invasion and migration.

Innovative aspects:
Loss of CRMP1 in GBMs, which express EGFRvIII, is involved in their more aggressive tumor phenotype. Mechanistically this involves increased relative activation of Rac-GTPase and inhibition of Rho-GTPase.

Our study is also an early demonstration of how differential MS analysis of well-defined tumor sets can shed light into relevant alterations in their proteome.

Verification of the MS findings in human specimens followed by functional and mechanistic studies can contribute much to the understanding of the biological differences between the relevant tumor sets and hopefully elucidate novel biological targets.

O121

Organelle mapping of the human proteome – towards a subcellular atlas

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Introduction
One important aspect when characterizing the human proteome is to gain information on protein localization on an organelle level. This information can be used to better understand the cellular functions of proteins and to tailor functional assays accordingly. Here, we report on the development and progress of a confocal subcellular protein atlas, as part of the Human Protein Atlas portal (www.proteinatlas.org) (1). Currently, the subcellular data comprises approximately 24000 high-resolution confocal images corresponding to the analysis of 3500 proteins in three human cell lines.

Methods
Three human cell lines with different origin were carefully selected for the localization study: U-2OS osteosarcoma, A-431 epidermoid carcinoma and U-251MG glioblastoma. The sample preparation procedure was automated using a pipetting robot. Cells are seeded in glass-bottomed 96-well microscopy plates, fixed and permeabilized using an optimized formaldehyde/detergent treatment. The antibodies generated in the HPA program (2) were employed for immunostaining together with organelle markers for cytoskeleton (microtubules), endoplasmic reticulum and nucleus. Two high-resolution confocal images of each antibody/cell line were acquired using a confocal microscope and the microscope operator ensured that they were representative for the sample. All images were manually annotated in terms of protein localization, staining intensity and characteristics.

Results
We describe the first systematic, antibody-based, study to localize proteins into subcellular compartments using confocal microscopy (3). Currently, over 24000 high-resolution, four-color, confocal images are available in the atlas, corresponding to more than 3500 proteins. Along with the images comes a detailed annotation of the subcellular localization, staining intensity and characteristics. The obtained subcellular localizations were further assessed in comparison to literature and a reliability score (supportive, uncertain or non-supportive) was provided for all proteins. In each cell line around 70 % of the analyzed proteins could be detected and altogether could more than 85% of the analyzed proteins be classified into one or multiple compartments suggesting that this approach is a valuable tool for protein localization. The presented atlas aims to provide publicly available information on subcellular information for all human proteins with the ultimate aim to facilitate functional studies of proteins.

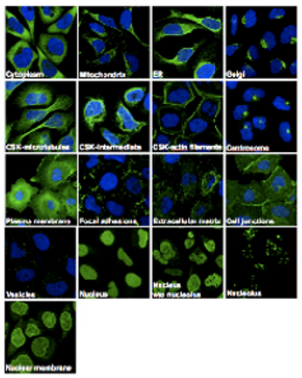

Figure 1: Representative confocal images of immunofluorescently stained U-2OS, A-431 or U-251 MG cells demonstrating the 17 different localizations that are annotated. The different localizations are shown in green and nuclei are showed in blue.

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O122

Proteome Analysis of RER from Acute Pancreatitis Models

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Introduction
Acute pancreatitis (AP) is a common clinical condition with a high morbidity and significant mortality. It is generally believed that the earliest events in acute pancreatitis occur within acinar cells, however, the initiating cellular mechanisms are not fully understood. At present it is generally accepted that initial phases of AP involve intrapancreatic digestive enzyme activation, disturbances of intracellular calcium, and inhibition of apical secretion followed by activation of transcription factors such as NF-kB, leukocyte infiltration and an intrapancreatic inflammatory reaction. In order to elucidate the initiating cellular events causing AP, several laboratory animal models mimicking human AP have been developed. The current study explores two of those models to elucidate their common features and differences using quantitative proteomics, immunohistochemistry and other techniques.

Methods
This study used an organellar proteomics approach to catalog the protein components and quantify changes in the pancreatic rough endoplasmic reticulum (RER) proteome during the onset of experimental pancreatitis using isobaric tags (iTRAQ). Acute pancreatitis was successfully induced using two rat models (caerulein and arginine). The high purity of the RER preparation has been confirmed by electron microscopy and Western blotting. Observations were validated by Western blotting and key details were further explored by immunohistochemistry.

Results
MS/MS analyses from four data sets revealed a total of 427 unique proteins over 95% confidence. These proteins belong to a large number of functional categories including ribosomal proteins, translocon subunits, chaperone proteins, secretory proteins, glyco- and lipid-processing enzymes. Less than 8% could be ascribed to potential carryover from other organelles. Our proteomic analyses identified a significant number of responsive proteins including translational regulators as well as fibrinogen and digestive enzymes whereas no major change was found in BiP or other luminal ER chaperones. These results indicate the inhibition of protein synthesis during the onset of acute pancreatitis as well as the very early deposition of fibrin, a key indicator of inflammation, with fibrin levels increased up to 10-fold.

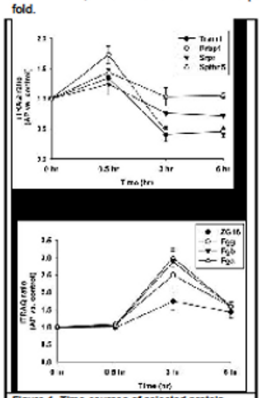

Figure 1. Time courses of selected protein responses in acute caerulein-induced pancreatitis. Fgx is fibrinogen, Rrbp1 is ribosome binding protein, ZG16 is zymogen granule membrane protein 16, Tram1 is translocon associated membrane protein, Srpr is the signal recognition particle receptor, and sptbn5 is non-erythrocytic spectrin 5.

Innovative Aspects
- Proteome analysis of two experimental models of pancreatitis, including a time course.
- Identified key steps in pancreatitis and differences in the mechanisms of the two experimental models.
- Identified very early deposition of fibrin and shutdown of protein synthesis as stages in pancreatitis.

O123

Proteomic characterization of rat gastrocnemius subsarcolemmal and intermyofibrillar mitochondria

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Introduction: Skeletal muscle contains two distinct mitochondria subpopulations, the subsarcolemmal (SS) and the intermyofibrilar (IMF) mitochondria with different capacities for mitochondrial respiration and with specific responses to several stimuli (1). Taking into account the absence of studies characterizing the proteome of mitochondrial populations, the aim of our study was to perform a mass-spectrometry-based proteomic analysis of fractions of SS and IMF mitochondria isolated with high purity from rat red *gastrocnemius* skeletal muscle and relate with their cell location.

Methods: Subcellular fractionation of *gastrocnemius* muscle was performed combining the polytron and protease treatment followed by ultracentrifugation on a density gradient with Percoll (2). Pure fractions of SS and IMF mitochondria were separated and several approaches were used: (i) mitochondria were further subfractionated in membrane, intermembrane space and matrix subfractions and 2DE-MS/MS was used to identify the proteins in each population and its subfractions; (ii) comparative TEM analysis of isolated with in-tissue mitochondria; (iii) BN-PAGE and 2D-BN-PAGE followed by MS/MS analysis was performed to identify the mitochondrial complexes composition; (iv) Histochemical staining of in-gel activity of complexes IV and V was used to assess mitochondrial functionality.

Results: Two populations of mitochondria were obtained from red *gastrocnemius* muscle by differential and Percoll gradient centrifugation. Their purity and integrity was confirmed by TEM, ensuring the quality and accuracy for the subsequent proteomic analysis. Moreover, morphometric evaluation showed significantly higher dimensions of SS mitochondria, similarly to the observed in tissue.
A total of 292 distinct proteins were identified by 2DE-MS/MS, most of which belong to the functional clusters "oxidative phosporylation", "metabolism" and "signal transduction". Although more spots were observed in SS mitochondria, 38 of the identified intact proteins were differently expressed among the SS and IMF subpopulations. With the exception of proteins from "protein binding/folding" and "lipid metabolism" clusters, an up-regulation of differently expressed proteins was observed in IMF mitochondria. 2D-BN-PAGE-MS/MS analysis increased the number of identified proteins and confirmed the up-regulation of OXPHOS subunits in IMF mitochondria, correlating with the higher respiratory chain activity observed in this mitochondrial population.

Innovative aspects:
- With a new methodological strategy, two pure mitochondrial populations with distinct dimensions were obtained and a positive correlation was established with in-tissue skeletal muscle mitochondria by morphometric analysis.
- Combining several proteomic approaches, more than 300 distinct proteins were identified in isolated skeletal muscle mitochondria populations.
- An association between the proteome profiles of mitochondrial populations and their functional specificity was recognized.

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Proteomic Investigation of *Leishmania* containing phagosomes

O124

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Introduction
Phagocytosis is a process by which macrophages engulf particles or microorganisms into specialized vacuoles, phagosomes.
Proteomic studies are performed to understand the process of phagosome maturation. A common methodology is the isolation by centrifugation on a sucrose density gradient of latex beads-phagosomes from macrophage cells. Although this provides a blueprint, it is neglecting the influence that pathogens likely have in this process.
Here we devised a proteomics methodology that use genetically modified parasites and murine primary macrophage cells to investigate the proteome of the parasite containing-phagosome.

Methods
A model system of phagocytosis was established by using genetically modified parasites (*Leishmania mexicana*), which express a red fluorescent protein, and murine primary macrophage cells derived from bone marrow (BMDM). After the cells internalized the parasites, they were gently lysed, nuclei were pelleted and the supernatant was taken for ultracentrifugation. The parasite containing fraction was collected and re-suspended in PBS supplemented with protease inhibitors and DAPI before sorting by FACS for $DsRed^{+}$ and $DAPI^{-}$ events[1]. Supernatant from the FACS sorting were precipitated before further treatment for proteomic analysis. Phagosomal proteins were tryptic digested and subject to 2D LC-MS/MS analysis for protein identification.

Results
Following isolation of parasite-containing phagosomes from BMDMs a total of 701 proteins were identified (above 95% confidence). Proteins identified included membrane proteins, subunits of the vacuolar ATPase, cytoskeletal proteins, early and late endosomal proteins, lysosomal membrane proteins, ER and Golgi proteins.
Comparison of our results with previous phagosomal studies (latex beads-phagosomes[2,3]) revealed known vesicular membrane markers, proton transporters and other proteins common to both studies. Importantly, new proteins were found here (in contrast to the latex-beads studies), including signaling factors involved in membrane trafficking and fusion, components in carbohydrate interaction and modification (integrin, lectin, galactosidase, etc.) and ion transporting system (Na+/K+-ATPases).
Bioinformatics analysis shows relevant information regarding functional categories, and could shed new light on our understanding of the complex interaction between the parasite and the host cell.

Innovative aspect
This is the first time that this methodology is applied to the study of phagosomal protein express under infection with transgenic parasites expressing red fluorescent protein.

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Proteomic Imaging of Endothelium and Caveolae for Targeted Penetration into Single Organs and Solid Tumors

O125

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Hundreds of disease biomarkers have been discovered. Accomplishing noninvasive targeted imaging and pharmacodelivery using biomarkers is challenged by *in vivo* barriers limiting access inside most tissues. For example, vascular endothelium prevents the penetration of intravenously injected biological agents into tissue where they can be effective. By integrating tissue subfractionation, subtractive proteomics, bioinformatic interrogation, antibody generation, expression profiling, and various imaging modalities, we can identify and actually validate the subset of biomarkers that are targetable *in vivo*. Tissue microenvironment extensively influences endothelial expression. Antibodies to select endothelial and caveolar proteins can not only very rapidly target a specific organ or tumor *in vivo* but also penetrate deep into the tissue. This greatly enhances the targeting of drugs, nanoparticles, gene vectors, and radioisotopes to facilitate disease imaging and treatment. This "organellar proteomic imaging" strategy creates a unique delivery system with many clinical opportunities to diagnose and treat a wide variety of diseases.

Other

(O201 – O207)

O201

Application of an Intact Protein Separation Space for PTM Characterization

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Introduction:

Post-translational modifications (PTMs) of proteins occur in-vivo both as a function of cellular processes and environmental/oxidative stresses and remain a substantial challenge for characterization. Multiple forms, differential % site occupancy, temporal flux and varied isoforms require non-standard MS and proteomics approaches for complete analyses. Here we describe our ongoing efforts in the development of an intact-protein fractionation workflow in order to maintain protein and PTM integrity throughout the analysis scheme. Our workflow allows for MS and MS/MS characterization at the peptide and protein level and takes into account the effects of PTMs on search space and data interrogation.

Methods:

1D and 2D-protein HPLC was performed with a Beckman Coulter PF2D. Fractions were analyzed directly by MS or subjected to trypsin digestion prior to MS and MS/MS. MALDI-TOF MS used a Bruker Reflex IV mass spectrometer. LC-MS/MS was performed with a Waters QoTOF MS or Thermo LTQ-Orbitrap, both coupled to Waters nanoAcquity UPLCs. Database searches used Mascot (Matrix Science) and Boston University Protein IDentifier (BUPID). Protein collation and validation was with the Trans Proteomics Pipeline (ISB). Additional analyses were performed using open source tools and tools written in-house including Software Tool for Rapid Annotation of Proteins (STRAP).

Results:

Our approach offers ease of automation and acceptable reproducibility for intact protein fractionation after the optimization of experimental parameters and the development of protocols. We applied our proteomics scheme to an on-going project involving the elucidation of differential PTMs on plasma proteins as a function of oxidative stress. Pooled plasma samples from controls and patients were depleted of albumin and fractionated via 2D-HPLC with chromatofocusing and C4. MALDI-TOF MS was used to rapidly screen fractions for changes in peak profiles at the intact protein level. Select fractions where then subjected to PMF and LC-MS/MS. By maintaining an intact protein separation space, PTMs of proteins did not lose their connectivity to the parent protein. We were able to apply sensitive and robust PTM search strategies. This allowed for better mapping of known and novel PTMs in PMF and LC-MS/MS results post database search. We observed distinct PTMs occurring on multiple amino acids in abundant and medium abundance plasma proteins and could correlate these findings directly to samples from patients who had been subjected to different types of oxidative stress. Multiple forms of oxidation were predominantly observed. Additionally, we found various PTMs as adducts known to be generated via free radical mechanisms. PTMs were readily mapped to specific locations along the topology of the parent proteins thus confirming in-vivo modification. In some cases PTMs were found close to catalytic centers where it is suspected that stress-induced PTMs would affect biological functions. Maintaining intact protein separation and data interrogation spaces and integration of the results from multiple forms of data analyses, we were able to improve our ability to identify and compare oxidative stress-induced PTMs present in clinically relevant plasma samples.

Innovative aspects:

1D- and 2- protein HPLC coupled with down-stream MS analysis for enhanced characterization and mapping of post-translational modifications

Acknowledgements:

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O202

Effect of high dose thiamine on the levels of protein biomarkers in diabetes mellitus type 2.

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ABSTRACT

Introduction:
This report describes the levels of protein biomarkers specific to diabetes mellitus type 2 and effect of high dose thiamine on these levels. Type 2 diabetic patients, age and sex-matched normal healthy controls were recruited from Sheikh Zayed Hospital, Lahore, Pakistan.

Methodology:
Biochemical parameters were assessed by standard protocols. Protein analysis was done by 2-D liquid chromatographic system in which samples were initially fractionated by chromatofocusing and the selected fractions were further fractionated through a reverse-phase column. The proteins which showed variation between test and control samples were identified by MALDI TOF TOF analysis.

Results:
Urinary albumin excretion arte was 35% decarased by the thiamine thaerpy. Levels of apolipoprotein A-I is decreased by 43 % in the diabetic group while apolipoproetin-E, leptin and C reactive protein (CRP) were found to be increased by 86, 69 and 89 %, respectively in the diabetic patients as compared to the controls. Analysis of all the samples belonging to the control, placebo and thiamine treated groups were then analyzed for these four proteins by ELISA. The level of CRP significantly decreased by 63% after thiamine therapy as compared to controls and placebo while other protein markers did not show a significant change after the therapy.

Conclusion:
Since CRP level variation has been reported in arrange of other pathological sates, role of thiamine may have a significant becoming on the prognosis of such diseases. This study will help in preventing diabetic nephropathy and inflammation and will suggest better treatment for diabetes.

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Key Words: High dose thiamine, protein biomarkers, diabetes mellitus type 2.

O203

A label-free method identified differentially abundant proteins in related *M.tuberculosis* Beijing strains

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Introduction:
Tuberculosis, the disease caused by *Mycobacterium tuberculosis* has been a major health concern over the last decade due to the appearance of drug resistance. In this study, we used protein extracts of two closely related, Hypo-and hyper-virulent, Beijing stains of *Mycobacterium tuberculosis*. We used a proteomic approach and a label-free quantification method to estimate differences in protein abundances between the two strains in the Beijing lineage.

Methods
Two closely related Beijing strains were selected for culturing and protein extractions. In-gel trypsin digestions were performed on complex protein mixtures and peptides were subjected to nano-LC-MS/MS system connected to a LTQ-Orbitrap. Protein abundance in each sample was calculated through emPAI analysis (1).

Results
Two *M.tb* Beijing subtypes, hypo-virulent^{BAWC507} and hyper-virulent^{BAWC5627}, were selected based on a reference database of an epidemiological field site near Cape Town, South Africa. We identified a total of 1443 proteins for the hypo-virulent^{BAWC507} strain and 1524 proteins for the hyper-virulent^{BAWC5627} strain. A total of 1670 proteins were found when both identifications were merged. From these, we found that 48 were over-represented in the hypo-virulent strain and 53 were over-represented in the hyper-virulent strain. Using a label free proteomic approach has shown a clear advantage because slow growth rates of *M. tuberculosis* do not facilitate metabolic labelling protocols in culture. Functional classification of these results shows that molecules of cell wall organisation may be of critical influence in virulence. We also identified 9 transcriptional regulatory proteins which only Rv3574 was previously investigated in depth. It was shown that Rv3574 controls the expression of 74 genes in H37Rv (2). Many of these genes regulated by Rv3574 are involved in lipid metabolism which may be a key feature when comparing Beijing strains. Our results illustates the full potential of using a proteomic approach to identify novel candidate molecules as potential drug targets.

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O204

Global Analysis of Small Molecule-Protein Interactions

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Extensive effort has been made to characterize the interactions of proteins with other proteins, and for the case of transcription factors, their interaction with DNA. Natural small compounds comprise the majority of the total cellular molecules, and have been shown to interact with proteins as substrates, products, cofactors and regulatory ligands. Although small molecules participate in such diverse processes, a large scale investigation of protein-small molecules has never been performed. We have developed a mass spectrometry assay for the large-scale analysis protein-natural small molecule interactions in yeast, and applied this method to the analysis of lipid pathway biosynthetic proteins and protein kinases. We find that a large number of proteins bind small molecules; some proteins bind their substrates and products and many key regulatory proteins and enzymes bind small molecules. Moreover, some small molecules such as ergosterol bind many targets. We further demonstrate that the activity of a Ypk1 a highly conserved homolog of the AKT protein kinase is stimulated by the presence of ergosterol. Overall, our study suggest the presence of a large number of natural small molecule-protein interactions in eucaryotes and thereby helps define biochemical functions of proteins, potential key regulatory steps in biosynthetic pathways, and identifies potential regulators and global coordinators of protein activity.

O205

A thiol reactive alkylhydroquinone exhibits cytoprotective effect via Keap1-Nrf2-ARE signaling

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Introduction
Nuclear factor-E2-related factor 2 (Nrf2), a transcription factor transactivating antioxidant responsive element (ARE), is sequestered by Kelch-like ECH-associated protein-1 (Keap1) in cytosol under unstimulated condition. Upon exposure of cells to electrophiles, which react with some cysteine residues of Keap1, Nrf2 is dissociated from Keap1 and is translocated to the nucleus. The Keap1-Nrf2-ARE signaling plays important role in electrophilic stress-induced expression of many cytoprotective enzymes. A botanical alkylhydroquinone HQ17(3) was previously found to be an anticancer agent, however it exihibited cytostatic effect on normal cells (1, 2). In this study, HQ17(3) was found to react with cysteine-thiols, activate Keap1-Nrf2-ARE signaling and, at low concentrations, protect cells from oxidative stress.

Methods
Reaction of HQ17(3) with cysteine-thiols of glutathione (GSH), γ-ECG, and peptide LNVRCESEVF were studied. The decreases in UV absorption peaks of HQ17(3) and formation of new absorption peaks were used to monitor the reaction with glutathione. The adduct formed by HQ17(3) with the peptide was identified by LC-MS/MS. Further, treatment of WS1 human skin fibroblasts with HQ17(3) led to activation of Nrf2 and induction of heme oxygenase-1 (HO-1), a cytoprotective protein regulated by Nrf2, were analyzed by IHC, Western and luciferase reporter assay. Cytoprotective effects of HQ17(3) from H_2O_2-induced cellular oxidative damages were analyzed by flow cytometry.

Results
According to UV absorption spectra, a peak at 303 *nm* was formed after 6-h reaction of HQ17(3) with GSH. This newly formed peak was referred to HQ17(3)-GSH adduct as compared with the reaction product of benzoquinone with GSH. Using LC-MS/MS, reaction of HQ17(3) with cysteine thiol was confirmed as indicated by the 341 Da mass shift of the peptide fragments. As a result of modification of Keap1, HQ17(3) treatment induced nuclear translocation of Nrf2 and expression of HO-1 in fibroblast WS1 cells. Furthermore, the pretreatment of WS1 cells with HQ17(3) reduced H_2O_2-induced cell death and oxidative damages, such as lipid peroxidation, membrane damage and DNA breaks. In conclusion, HQ17(3) is a thiol-reactive compound, it may alkylate cysteine thiol of Keap1 and contribute to the cytoprotective activity (Scheme 2).

Innovative aspects
- Reaction with cysteine-containing peptide and monitored by LC-MS/MS is a useful *in vitro* system for screening cytoprotective compounds.
- HQ17(3) is an electrophile capable of activating Keap1-Nrf2-ARE pathway.

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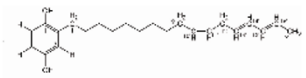
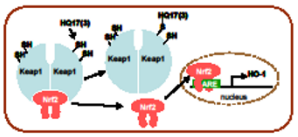


Scheme 1. Structure of HQ17(3) [10' (Z),13' (E),15' (E)-heptadecatrienylhydroquinone]



Scheme 2. HQ17(3) alkylates Keap1, activates Nrf2 and induces HO-1 expression.

O206

oPhylogenetic analysis of Hepatitis B virus

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Hepatitis B is a worldwide disease with an estimated 500 million carriers. The frequent occurrence of occult infection in close contacts of carriers, parenteral vertical transmission and high incidence of insidious chronic liver disease or cancer of the liver among the carriers signify a major public health problem. Study was carried out to analyze variability in the immunogenic regions of surface envelope genes of Pakistanian Hepatitis B virus isolates. The PreS region, a hypervariable and highly immunogenic region, containing at least five epitopes, was PCR amplified, cloned and sequenced. Sequence alignments and comparisons with other known HBV sequences from different parts of the world were performed in order to build a phylogenetic tree and to check if certain amino acids mutations tend to recur in certain places within the hypervariable region. On the basis of phylogenetic analysis of PreS1 region from Pakistanian HBV isolates, it appears that the most prevalent genotype in Pakistan is genotype C as 75% samples belong to genotype C and 25% belong to genotype D. It was also concluded that four mutations shared by NA004, NA018 and NA020 may be prevalent mutations in the Pakistanian ayw subtype. Six novel sequences and 13 mutations explored during this study may be specific to Pakistanian HBV isolates hence this information may be potentially valuable for diagnostic purposes and for HBV vaccine development in Pakistan

O207

Proteome Responses of Human Oral Epithelial Cell to HIV/AIDS and Drug Abuse

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Introduction
Oral manifestations of HIV have been associated with increased risk of progression of HIV disease in HIV-infected individuals. The severity of several HIV-related oral conditions has been lessened with the advent of highly active antiretroviral therapy (HAART) but not all oral conditions are restored. In fact some oral conditions appear to be worsen with HAART including apthous ulcers, salivary gland disease, oral warts (1, 2) and some co-infections such as hepatitis C virus (HCV), commonly found in drug abusive HIV+ individual. Such impaired responses of the host OECs could underline both the genomic and proteomic changes in HOEC in the setting of HIV infection that are not reverted by HAART treatment and/or induced by HAART treatment. This study was undertaken as a first step to elucidate the proteome changes that are mediated by HIV and HAART in human oral epithelial cells (HOECs) that are collected from HIV+ and treated with HAART and HIV- individuals using 2D-DIGE/MS platform.

Methods
Human gingival tissue samples were obtained from healthy (n=4) or HIV+ subjects treated with HAART (n = 4) undergoing third-molar extraction at Case School of Dental Medicine. Primary HOEC were isolated and grown in keratinocyte basal medium with supplements as previously described (3). Epithelial monolayer was grown to 80–90% confluence in serum-free EpiLife media (Cascade Biologics Inc, Portland, OR). Protein samples from four replicates per group were prepared independently and analyzed together across multiple DIGE gels using pooled internal standard sample to quantify expression changes with statistical confidence. The 2-D gel images were then analyzed using DeCyder software (GE/ Amersham Biosciences). Once the spots from the common standards were matched across the four gels, the ratios of the protein abundances (differences in expression) and their statistical level of significance (T-test) were computed. A total of 153 spots were determined to be 50% or more increased or 50% or more decreased with P-values of 0.05 in the HAART treated HIV+ compared to the healthy; these were assigned as proteins of interest. These spots were subjected to tryptic digestion with subsequent protein identification via mass spectrometry.

Preliminary results
Our preliminary proteomics assessment of HOECs from HIV+ compared to HIV- individuals identifies a total of 153 spots that were determined to be significantly changing between the two groups. Out of the 153 spots of interest identified, 137 (~90%) were down regulated. HIV has been shown to have a negative effect on net protein synthesis (4). Our results indicate changes in specific proteins consistent with this hypothesis. A subsequent mass spectrometry analysis of the 153 spots identified a total of 63 unique proteins. A network analysis of these proteins using ingenuity pathway analysis (IPA) suggested altered expression of transcriptional factors that are too low abundance to be detected by 2D- gel method and identified an enriched cluster of down regulated proteins that are involved in immunological, hematological, and respiratory diseases including, Ppp2r1a, Fkbp4, P4HB, Calr, and GSTP1. Our proteomic analysis captured changes in proteome expression in the host OECs, depicting changes in cellular processes that may contribute to HIV and/or HAART mediated oral manifestations. In addition to validating some of the targets from the 2D-DIGE and pathway analyses with targeted MRM and/or immunoblotting analysis to confirm the magnitude and the direction of the fold changes, continuing analyses are expected to focus on comparing proteome profiles of (HIV-) HOECs versus (HIV-) HOECs challenged *in vitro* with HIV, and opioids.

Innovative aspects
Proteomics identifies molecular signatures in HOECs in response to HIV infection and/or HAART.

Acknowledgments
This research was supported in part by The National Institute for Drug Abuse grant P20-DA26133 and NIDCR grant P01-DE019759

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Plant proteomics

(P101 – P105)

P101

Proteomics analysis of rice shoot treated with gibberellic acid and salt

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Introduction
Salinity stress is a major abiotic stress to most plant including rice and it has been reported that salt could decrease active gibberellins (GA_4) content in rice and GAs could exert a natural beneficial effect on salt stressed rice. However, the details of the mechanism of the interactive effects of exogenous gibberellic acid (GA_3) and salt stress in rice remains unclear. Here we describe the effect of salt stress on seed germination of rice (*Oryza sativa* L. cv. Nipponbare) and the effect of GA_3 on salt-stressed rice by observing the growth parameters at first, then we investigate the interactive effects of GA_3 and salt stress on germinated seed shoot of rice by employing a proteomic approach.

Methods
Seeds of rice (Oryza sativa L. cv. Nipponbare) were cultured in treatment solutions with different concentrations of GA_3 (0, 5, 25, 100 μM) with 0.5% NaCl, and the growth parameters were observed, then, the differentially expressed proteins from germinated seed shoots of rice which were treated or not treated with NaCl and GA_3 (5-day-old seedlings grown on H_2O, 0.5% NaCl, 0.5% NaCl + 100 μM GA_3, and 100 μM GA_3 for 48 h), were analyzed by 2D PAGE and MALDI-TOF MS (1,2).

Results
The results showed that the exogenous GA_3 counteracted the inhibition effect of salt on germination energy, shoot length and root length of rice significantly, and thirteen differentially expressed spots were repeatedly detected by 2D PAGE and eleven spots were identified by MALDI-TOF MS. Among these spots, three were up-regulated by salt but partially down-regulated when added with GA_3 (salt stress-induced protein, os09g0249700, and hypothetical protein osj_014066), five were down-regulated by salt but partially or completely up-regulated when added with GA_3 (putative chaperonin 21 precursor, hypothetical protein OsJ_025258, isoflavone reductase-like protein, phosphoglucomutase, and an unknown protein), one was down-regulated by salt and partially down-regulated by GA_3, but the down-regulation by salt or GA_3 could not be affected each other (glutamyl-tRNA reductase), two were up-regulated by salt but could not be affected by GA_3 (enolase; os04g0659300), one was down-regulated by GA_3 but the down-regulation could be blocked by salt (RuBisCO activase small isoform precursor), and one disappeared under salt stress but could be detected when added with GA_3 (unknown protein)(Fig. 1).

Innovative aspects
- New insight to reveal the interactive effects of GA_3 and salt stress on rice
- Find some differential expression proteins in germinated seed shoot of rice which cultured in treatment solutions
- These differential expression proteins may play important role in the mechanism of the relieving effect of gibberellin on the inhibition of rice germination by salt stress

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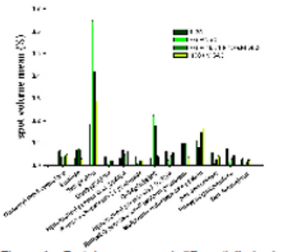

Figure 1. Proteins expressed differentially in rice shoot treated with H_2O, 0.5% NaCl, 0.5% NaCl + 100 μM GA_3, and 100 μM GA_3 for 48 h. This shows that these proteins may play important roles in the effects of gibberellic acid and salt stress on rice shoot.

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P102

In-Depth Analysis of the *Arabidopsis thaliana* Proteome Using a Novel Hybrid Linear Trap-Orbitrap Mass Spectrometer

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Introduction
Enormous improvements in mass spectrometry technology over the last few years have allowed for increasing the success in analyzing complex proteomes. However, many low abundance proteins remain undetected in most large-scale proteomic analyses due to the limits in acquisition rate and quality of MS/MS spectra. To improve the speed and depth of analyzing complex protein mixtures, we developed a new hybrid linear ion trap-orbitrap mass spectrometer that provides faster MS/MS scanning at low and high resolution and mass accuracy. Based on the improved data acquisition speed we were able to identify more proteins with increased sequence coverage and confidence in a highly complex protein digest.

Methods
Leaves from 5 week old *Arabidopsis thaliana* plants were harvested, frozen in liquid nitrogen and ground until homogenization. The material was centrifuged and the soluble proteins from the supernatant were concentrated and digested with a Serine protease cleaving C-terminal to Lys and Arg. The technological improvements implemented in the new hybrid ion trap-Orbitrap mass spectrometer are improved ion optics, a dual-cell differentially pumped ion trap, predicted injection times for increased scan speed; a HCD-collision cell with axial field; and an improved vacuum in the Orbitrap. Nano-LC-MS analyses of the complex protein digest from *Arabidopsis thaliana* whole cell lysate were performed using LC gradients of 70 and 150 min length. Database searches were performed with Mascot using the Proteome Discoverer software.

Results
NanoLC MS/MS analysis of 100-500ng of the highly complex protein digest from *Arabidopsis thaliana* was carried out using two different LC gradients. Data files were acquired in triplicate analyses and repeated using exclusion lists to identify less abundant proteins and to increase the dynamic range of the whole analysis. Overall, a two-fold increase in the number of MS/MS spectra acquired per unit time was observed resulting in the identification of a significantly higher number of proteins and peptides in the 75' runs than previously identified in 150' runs. Analyses were repeated using an exclusion list based on proteins identified in the first runs even which further increased the number of total proteins identified from one sample by ~20%. With the novel hybrid linear ion trap-orbitrap mass spectrometer, we were able to identify 30-40% more proteins overall and 50-60% more peptides (0.25% FDR) than in the previous experiments. These results demonstrate the benefit of high scan speed in combination with exclusion lists for a two stage analysis of complex proteome digest.

Innovative aspects
- Pushing the limits of protein identification from high-complexity protein digests based on high speed data acquisition combined with exclusion lists.

P103

Comparative tuber proteome in wild-type and transgenic potato expressing a seed albumin

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Introduction
The improvement in nutritional quality of crop plants, particularly in terms of protein and amino acid, is an urgent worldwide health issue. Proteins represent one of the principal constituents of a balance diet, which impart nutritional value to the food and fodder. In a previous study, we introduced a seed albumin into potato, which led to enhanced accumulation of protein, essential amino acids besides increased tuber yield (1). The transgenic plants also showed enhanced photosynthetic activity and aerial growth as determined by increase in carbon-dioxide assimilation rate and total biomass. To understand the molecular mechanism effecting the increased protein synthesis and reserve accumulation in genetically modified plants, a comparative proteomic approach has been applied at different tuber developmental stages.

Methods
Two transgenic events generated against the background of a diploied potato variety, A16 expressing *AmA1* gene under CaMV 35S (A16/1) and GBSS promoter (A16/6) by *Agrobacterium*-mediated transformation (1). The wild-type and transgenic tubers were grown in randomized plots according to the standard agricultural practice for 100 days, until tuber maturation. The tissues were collected from different developmental stages and stored at -80ºC after quick-freezing in liquid nitrogen. The tuber-specific soluble proteins were isolated from the wild type and the transgenic tubers at different developmental stages as described earlier (2). The proteins were resolved by 2-DE and analyzed by PDQuest software, version 7.2.0 (BioRad, CA). Differentially expressed protein spots that changed their intensities significantly for more than 2.5 folds, at least, at one developmental stage were excised. The protein spots were in-gel digested with trypsin and identified using LC/MS/MS.

Results
The differentially displayed proteomes revealed more than 150 protein spots that changed their intensities for more than 2.5 fold, most of which were developmental stage-specific. The LC-ESI-MS/MS analyses led to the identification of 115 proteins, some presumably associated with tuber cell differentiation, which regulate diverse functions like protein biogenesis and storage, bioenergy and metabolism and cell signaling. The homology based amino acid sequence search of the identified proteins did not show any homology with known candidates of antinutritional elements or allergenic proteins. A model network was constructed using the protein data-sets, which shows how target proteins might work in coordinated fashion, and attribute to increased protein synthesis, storage reserve accumulation and increased productivity in transgenic tubers.

Innovative Aspects
- Detection and identification of differentially expressed proteins at different tuber developmental stages and comparison of tuber-specific protein profile between wild-type and transgenic tubers.
- Investigating the possible mechanism of a seed albumin gene in increased protein accumulation and increased productivity in transgenic tubers.

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P104

Proteome analysis of differentially displayed proteins in extracellular matrix of rice

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Introduction
Water-deficit or dehydration impairs almost all physiological processes and greatly influences geographical distribution of many crop plants. Higher plants rely mostly on the induction mechanisms to protect cellular integrity during dehydration. Cell wall or extracellular matrix (ECM) serves not only as the repository for most of the cell signaling components but also as front-line defense thus, playing a predominant role in cell fate decision. To understand the molecular mechanism of dehydration response in plants better, we have developed a comparative ECM proteome in rice.

Methods
Rice seedlings (cultivar Rasi) were grown for 4 weeks at 28 ± 2 ºC, 70 ± 5% relative humidity under 16 h photoperiod. A gradual dehydration condition was then applied on the 4-week-old seedlings by withdrawing water and aerial tissues were harvested during 48-192 h, at every 24 h interval. The ECM protein fraction was essentially isolated as described earlier (1). The samples from the unstressed and stressed tissues were analyzed using the 2-D approach. The gels were stained and images analyzed by PDQuest software (2). Differentially expressed proteins spots that changed their intensities significantly for more than 2.5 folds, at least, at one time point during dehydration were excised, in-gel digested with trypsin and identified using LC/MS/TOF.

Results
Dehydration-responsive temporal changes of ECM revealed 203 proteins that change their intensities for more than 2.5-fold during dehydration. Mass spectrometry analysis led to the identification of 111 differentially regulated proteins, presumably involved in a variety of functions including carbohydrate metabolism, cell defense and rescue, cell wall modification, cell signaling and molecular chaperones, among others. A large number of proteins identified in this study are known to be localized in cell wall. However, a great many unexpected proteins were also found. The differential expression of the candidate proteins, in conjunction with previously reported results, may provide new insight into the underlying mechanisms of dehydration response in plants. This may also facilitate the targeted alteration of metabolic routes in cell wall for improved agricultural exploitation.

Innovative aspects
- Resolve extracellular matrix associated proteins from rice ECM.
- Detection and Identification of dehydration-responsive proteins.

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P105

Proteome analysis of apoplastic proteins in rice shoot respond to salt

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Introduction
In this work, a sub-cellular, comparative proteomic approach has been applied to study the changes of apoplast proteins in rice shoot under different time course of salt stress. A CyDye™ DIGE fluorescent saturation labeling method was used for the analysis of apoplast protein, and coupled with mass spectrometry were applied to identify 117 proteins successfully. Of these, 64 protein spots representing 37 proteins were typical apoplastic proteins involved in many different metabolic processes.

Methods
Plants were grown at a relative humidity of 50% under long-day conditions (16 h light/8 h dark cycle) at 28℃/25℃. After 10 days, seedlings were treated with 200 mM NaCl for 1 h, 6 h and 12 h, respectively. A vacuum infiltration procedure was deployed for apoplast extraction. To evaluate intracellular contamination of apoplastic proteins, malate dehydrogenase activity was examined. Twelve gels were run for each mixture of Cy3-labeled internal standard and Cy5-labeled control or treated samples. Decyder 2D (GE Healthcare) were used to eliminated Gel to gel variations and screen differential expression proteins. Protein spots of interest were analyzed by LC-MS/MS, proteins identified by Prospector (V 5.52, UCSF).

Results
Up to 2000 spots were detected in our gels. After DeCyder 2D Software analysis, 122 spots with 1-ANOVA p-value < 0.05 were considered to be statistically significant and excised for identification by LC-MS/MS. Finally, 117 spots were identified with positive results, among of which 64 spots were apoplastic proteins including 56 spots represented by proteins with a signal peptide, secreted through endoplasmic reticulum-Golgi pathway, and 8 spots represented by proteins lacking signal peptides and predicted to be secreted through non-classical secretory pathways. In addition, some apoplastic proteins were identified from multi spots on the gel, implying that posttranslational modifications (PTMs) may happen to these proteins or they may belong to multigene families. According to the biological process proteins involved in and their molecular functions provided by gene ontology tools and UniProtKB. All the identified apoplastic proteins fell into 8 main functional categories, including carbohydrate metabolism, oxido-reductases, protein processing and degradation, cell wall biogenesis and degradation, lipid metabolism, protein folding and assembly, energy metabolism and other mechanism.

Innovative aspects
(1) The DIGE gels revealed a total of 122 differentially expressed salt-responsive spots.
(2) Apoplast may be an important site for plant cells to respond to salt stress.
(3) According to the possible functions of the identified apoplast protein,we can reveal possible mechanisms involved in the initial perception of salt stress in rice apoplast.

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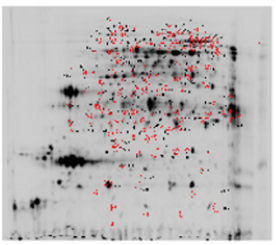

Figure. 1 preparative gel of apoplast proteins obtained from leaf sheath of rice seedlings. Spots number corresponds to position numbers listed in Table

Proteomics of Macromolecular Complexes

(P201 – P224)

P201

Membrane Complexome of *F. tularensis* and its Response to Iron Restriction Studied by BN-PAGE/DIGE

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Introduction
Francisella tularensis is causative agent of rare but severe contagious disease. However, the information about the pathogenesis of this gram-negative bacterium is still not sufficient. Investigation of protein complexes (complexome) residing in bacterial membrane enables getting functional data about the cooperating partners. Furthermore, the study of differences in protein-protein interaction under the iron depletion conditions (resembling host stress environment), could provide deeper insight into the functional changes occurring during process of infection.

Methods
Blue native polyacrylamide combined with sodium dodecyl sulfate gel electrophoresis (BN/SDS-PAGE) allows the analysis of hydrophobic membrane protein complexes and also enables studying their subunit composition and stoichiometry. Differential gel electrophoresis (DIGE) is based on fluorescence pre-labeling of diverse protein samples by cyanine dyes (Cy2, Cy3 and Cy5) and their subsequent co-electrophoresis within a single gel allowing the precise comparison of different samples. *Francisella tularensis* was cultivated both in complete and in iron-depleted Chamberlain medium and membrane protein complexes were isolated (1). These complexes were solubilized by mild non-ionic detergent, labeled by cyanine fluorescent dyes and separated by 2D (BN/SDS-PAGE (2,3).

Results
The effectivity of BN/SDS-PAGE in investigation of protein complexes was confirmed on structural analysis of F_1F_0-ATP synthase. This example demonstrated the ability of detection of 6 of 7 subunits of molecular complex of estimated Mw around 600 kDa. This assemble represents whole intact complex. However, protein subunits of F_1F_0-ATP synthase were found in other Mw regarding the partially disassembled complex. Moreover, other membrane complexes involved in bacterial physiology were analyzed e.g. cytochrome O ubiquinol oxidase and succinate dehydrogenase. Interestingly, many hypothetical proteins were detected and some interacting and/or functional schemes were proposed. In addition, using DIGE the expression of several membrane proteins was found to be significantly different under the iron depletion. These results highlight the importance of these proteins and their corresponding complexes in *Francisella* pathogenesis.

Innovative aspects
- Optimization of isolation of bacterial membrane protein complexes under non-denaturing conditions
- Analysis of membrane protein complexes of *Francisella tularensis*
- Characterization of changes in membrane protein complexes expression under iron-limiting conditions

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P202

Proteome-wide Identification of Poly(ADP-ribose)-binding Proteins and Poly(ADP-ribose)-associated Protein Complexes.

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Introduction Poly(ADP-ribosyl)ation is a DNA strand break-driven posttranslational modification of proteins catalyzed for the most part by poly(ADP-ribose) polymerase-1 and 2 (PARP-1 and PARP-2). Several recent studies revealed growing importance of the noncovalent protein binding to poly(ADP-ribose) (pADPr) in addition to covalent modification. Poly(ADP-ribosyl)ation is an important biological phenomenon with wide-ranging implications in several cellular pathways including DNA damage signalling and repair, chromatin remodelling, transcription, progression through cell cycle and apoptosis [1].

Methods *In silico* prediction of pADPr-binding proteins and large-scale mass spectrometry-based proteome analysis of pADPr binding proteins were used to establish a comprehensive repertoire of pADPr-associated proteins.

Results Based on a motif search of noncovalent pADPr-binding proteins [2], we derived a refined consensus sequence that more closely reflectsthe diversity of experimentally validated pADPr-binding proteins identified by large-scale immuno-precipitation of pADPr-containing multiprotein complexes coupled to high-throughput liquid-chromatography tandem mass spectrometry (LC-MS/MS). Visualization and modelling of these pADPr-associated proteins in networks (Fig 1) not only reflect the widespread involvement of poly(ADP-ribosyl)ation in several pathways but also identify protein targets that could shed new light on the regulatory functions of pADPr in normal physiological conditions as well as after exposure to genotoxic stimuli [3].

Innovative aspects
- Generation of a refined motif representing a new set of pADPr-binding proteins
- Selective purification of pADPr-associated proteins
- Proteome-wide identification of pADPr acceptor proteins from the converging analysis of experimental and prediction-based methods

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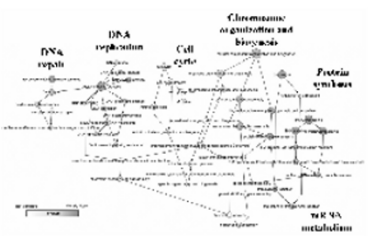

Figure 1. Network representation of pADPr-associated proteins identified by LC-MS/MS in anti-pADPr immunoprecipitates. Functional categorization of pADPr-associated proteins was performed using GO annotations. Over-represented categories were statistically identified using BINGO and visualized with Cytoscape. The size of the terms (circles) is representative of the proportional protein abundance in the dataset and the color shading indicates the degree of statistical significance (darker shades indicate stronger significance).

P203

Interaction network analysis: cross-comparison of methodologies, species and cell types for the PP2A interactome

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Introduction: The analysis of protein-protein interactions is essential to understand complex cellular processes. High-density protein interaction networks not only reveal connectivity between proteins that have different biochemical functions but also assign function to unknown proteins by association with protein modules and subcomplexes. There is a limited understanding of the plasticity of the interaction networks and the effect of the methodology selection on the resulting networks. Here we perform side-by side comparisons of 1) a stringent methodology for identifying protein interactors (Tandem Affinity Purification) and a more sensitive approach (single step FLAG) on the same set of human proteins; 2) comparison of the interaction networks (TAP) for yeast and human PP2A modules; 3) comparison of the list of interaction partners for PP2A (FLAG) in two human cell lines.

Methods: Tandem Affinity Purification from yeast and human cells was performed as described (1). FLAG-affinity purification from stably transfected HEK293 or K652 cells was performed as described (2). In all cases, samples were eluted in volatile buffers, lyophilized in a speed-vac, trypsinized in ammonium bicarbonate prior to resuspension in HPLC buffer A2 (2% acetonitrile, 0.1% formic acid). Samples were then directly loaded onto capillary columns packed in-house with Magic 5 mm, 100A, C18AQ. MS/MS data was acquired in data-dependent mode (over a 2 hr acetonitrile 2 - 40% gradient) on a ThermoFinnigan LCQ, LTQ or LTQ-Orbitrap, each equipped with a Proxeon NanoSource and an Agilent 1100 capillary pump. Data was analyzed by SEQUEST, Mascot or X!Tandem, and background was subtracted as described (1,2).

Results: Starting from the serine/threonine phosphatase PP2A we used iterative TAP AP/MS to generate a high-density interaction network that contained 34 proteins. The same baits were next analyzed by a less stringent FLAG AP/MS approach; strikingly, after removal of the background contaminants, there were now 92 high-confidence interactors centered around this phosphatase, several of which were further validated by reciprocal AP/MS and orthogonal approaches (e.g. **(2)** Goudreault et al., 2009). All of the interactors detected by TAP AP/MS were amongst the 92 interactors detected by FLAG AP/MS. Therefore, the choice of the methodology greatly influences the portion of the interactome that can be detected. To analyze the evolution of the interaction network, we performed TAP AP/MS on the same 12 baits in yeast and human, and detected the same pattern of interactions amongst the true orthologs; notably, the only interactions that were not detected was occurring with proteins restricted to one species (there were eight such protein families in humans). This indicates that the PP2A network detected by TAP has been largely conserved throughout eukaryotic evolution. Lastly, we used our more sensitive FLAG AP/MS detection technique to compare the interaction profiles for the catalytic subunit of PP2A from the human HEK293 cell line (our standard lab model for AP/MS study) and the myeloid leukemia cell line K562. The degree of overlap between the two cell lines for the high-confidence PP2A interactors was 72%.

Innovative aspects:
- The degree to which an interactome can be assessed is greatly influenced by the choice of affinity purification methodology.
- Comparative analysis across species can reveal conserved protein interactions.
- For the PP2A module the choice of cell line does not greatly influence the interaction network profile.

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P204

Molecular dissection by AP/MS of a signaling complex linked to cerebral cavernous malformations

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Introduction
Highly specific interactions of two or more proteins are critical for many different cellular processes. Protein interaction networks provide an invaluable resource towards understanding these cellular processes, but often do not provide information on the assembly or connectivity of specific proteins within a multi-protein complex. A comprehensive analysis of these protein interactions - which is often the key to understanding the mechanisms of disease - requires information on interaction modules or domains. Here we present affinity-purification coupled to mass spectrometry (AP/MS) as a strategy for molecular dissection of these interaction modules within a protein complex and show that the approach is complementary to more classical *in vitro* mapping.

Methods
Our test case for the approach is a newly identified large protein complex called STRIPAK (striatin-interacting phosphatase and kinase) in which resides most of CCM3 (a protein mutated in cerebral cavernous malformations, a condition associated with seizures and strokes). Our group has identified up to 20 different STRIPAK components, several of which assemble in the complex in a mutually exclusive manner (1). We have established stable cell pools in HEK293 that each express low levels of FLAG-tagged truncated forms of defined STRIPAK components, and have performed gel-free AP/MS analysis on each of these cell pools. To complement the method, we have analyzed binary interactions using purified proteins for a subset of the interactors and performed gel filtration chromatography.

Results
The large size of the complex and the absence of specific immuno-affinity reagents for many of the proteins prompted us to design approaches to systematically investigate protein complex composition resulting from mutation of key components. We have now identified direct physical interactions and defined minimal interacting regions within the STRIPAK complex, using full-length and truncated versions of various STRIPAK proteins. These mapping studies have elucidated a role for the striatin family of proteins as a scaffolding core within STRIPAK and the disease-linked CCM3 as an adaptor protein that recruits the Ste20 family kinases to STRIPAK. These studies shed light on the modularity of the STRIPAK complex and give possible new insight into CCM disease. They also provide an example of how dissection of a macromolecular assembly can uncover direct protein-protein interactions as well as interactions between specific protein modules and domains.

Innovative aspects
- AP/MS can be used as a strategy to identify minimal interaction modules within a protein complex.
- Dissection of interaction modules within a protein complex can aid in elucidating a role for proteins involved in disease.

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P205

Identification of novel interactors of Protein Phosphatase 4 using quantitative proteomics

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Introduction
Protein phosphatase 4 (PP4) is an evolutionarily conserved member of the PP2A subfamily of protein Ser/Thr phosphatases. Functional PP4 holoenzymes are composed of the catalytic subunit in association with various regulatory subunits (e.g. PP4R1, PP4R2, PP4R3 and IGBP1/alpha4). The central mode of action and substrate specificity of PP4 is likely dictated by the interplay between these different regulatory subunits. PP4 has been linked to apoptosis, centrosome maturation and formation of mitotic spindle; however targets and regulators of PP4 within these pathways largely remain to be identified.

Methods
Here, we have used a gel-free FLAG affinity purification coupled with mass spectrometry to identify interactors of PP4 with high sensitivity (Chen and Gingras, Methods, 2007). We coupled our strategy with quantitative proteomics using Stable Isotopic Labeling with Amino acids in Cell culture (SILAC) to efficiently filter the background from the immunoprecipitations.

Results
Our study recapitulated numerous previously known PP4 interactors, and in addition revealed several novel binding partners that effectively link PP4 to various cellular functions, such as apoptosis and DNA damage. Most of the new interactors are phosphoproteins, and may be putative substrates for PP4. This poses exciting revenue in identifying PP4 substrate, as to-date, limited number of direct targets has been found for PP4 (Nakada et. al., EMBO, 2008). Using this approach, we identified and further characterized a novel cytosolic PP4 regulatory subunit, KIAA1622, which we have renamed PP4R4 (Chen et. al., JBC, 2008). PP4R4 displays weak sequence homology with the A (scaffolding) subunit of the PP2A holoenzyme, but specifically associates with PP4. Unlike the PP2A A subunit, which plays a scaffolding role, PP4R4 does not bridge PP4 with previously characterized PP4 regulatory subunits. Our results demonstrate that gel-free immunoprecipitation coupled to quantitative proteomics can be used to detect specific interactors of serine/threonine phosphatases. This approach can also potentially be used to identify substrates for the enzymes.

Innovative aspects
- Using quantitative mass spectrometry to efficiently filter out backgrounds and identification novel interactors of protein phosphatases.
- Using quantitative mass spectrometry to identify potential substrates of protein phosphatases.

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P206

Benchmarking Affinity-Purification/Mass Spectrometry to define probability values for protein-protein interactions

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Introduction
Affinity-Purification coupled to mass spectrometry (AP/MS) is the method of choice to identify protein interaction partners and protein complexes in a near-physiological context. A community-driven project aims at using AP/MS to map the human interactome. As the experimental process will be performed across different laboratories, it is imperative to define the robustness and reproducibility of the approach, and to derive a methodology to integrate data from different platforms. Here we present an experimental and statistical benchmarking set.

Methods and Results
Robust AP/MS platforms based on a gel-free, single step FLAG affinity purification and a gel-LC-based Tandem Affinity Purification system have been developed and validated across our laboratories (e.g.[1,2]). Twenty baits (ten selected at random based on a previous benchmarking set for binary interactions[3], and ten selected from well-defined protein complexes) were tagged with either a FLAG and/or a Tandem Affinity Purification (TAP) epitope, and expressed stably as inducible clones in HEK293 cells. Expression levels were monitored for each bait. Initially, a set of baits was used for a reproducibility analysis within a laboratory or across different laboratories.

Biological and technical replicates (intra-lab and inter-lab) were analyzed by AP/MS to monitor the robustness and sensitivity of the methods. All data was entered into the ProHits interaction database, which allows us to record the data in a bait → prey format, and to provide experimental annotation. The non-filtered search results were used to train a statistical algorithm that models spectral count distribution for each of the *x* hits (or prey) across the *n* baits. Normalization factors include the length of the proteins; the relative bait/prey abundance in the lysate; the spectral count distribution for the hit across the negative control samples, and the reproducibility factors. The naïve scoring algorithm was then used to provide an initial p-value for the interaction, based solely on AP/MS data. In the next step, we are referencing this set to the literature and testing interactions detected at different p-values using orthologous approaches (LUMIER, Yeast-two-hybrid) to experimentally adjust the scoring algorithm.

Innovative aspects
- High quality cross platform comparison of AP/MS analysis pipelines sets the stage for large collaborative interactome projects
- The development of statistical analysis for significance of the interactomes establishes a framework for integrating AP/MS data from different laboratories and platforms
- The use of orthologous approaches to validate interactions helps to experimentally define the confidence values.

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P207

Nested Partition Clustering Algorithm for AP/MS-derived Protein Interaction Data

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Introduction
Reconstruction of protein-protein interaction networks using the Affinity Purification (AP) - Mass Spectrometry (MS) approach is a valuable tool for understanding complex mechanism of many cellular processes (1). While high-throughput AP/MS datasets are being generated with increasing popularity, data analysis and visualization often rely on existing methods such as hierarchical clustering that do not explicitly reflect the data structure intrinsic to the experimental platform of AP/MS. Here we present a novel clustering algorithm for reconstructing protein complexes from AP/MS data.

Methods
We present a model-based nested partition clustering algorithm (NPCluster) with non-parametric Bayesian inference estimation procedure for modeling AP/MS data. It takes as input the list of prey protein identifications and their spectral counts for each profiled bait and clusters bait and prey proteins into complexes. It not only clusters bait proteins into protein complexes based on the similarity of expression patterns of their interaction partners, but it also draws nested clusters of interaction partners within the complexes. The average expression level of the nested clusters is categorized into discrete bins so that the nested clusters of preys can be compared across the protein complexes.

Results
The method is illustrated using a published human protein interaction network dataset centered around two ATPases, Tip49a and Tip49b, and involving four major protein complexes, namely SRCAP, hINO80, TRRAP/Tip60, and Uri/Prefoldin complexes (2). NPCluster regenerated the network configuration of the four protein complexes connected by the Tip49a and Tip49b proteins, as was previously delineated in (1). Although the physical interaction map was nearly identical, the bait clustering part of NPCluster has provided additional insights such as dispersion of several key proteins across multiple bait clusters due to the heterogeneous expression profiles of their prey proteins.

We also demonstrate that prey proteins could be sorted within each bait cluster based on their abundance (spectral counts). The clustering also allows the comparison of interaction strength of a prey protein with bait proteins from multiple protein complexes. This, in turn, provides clues to the proximity of prey proteins to core components of different protein complexes and facilitates the arrangement of the nodes in the network for visualization purposes (Figure 1).

Innovative aspects
- Novel statistical algorithm for bi-directional clustering of AP/MS protein interaction data
- Direct translation of the clustering outcome into node arrangement for network visualization

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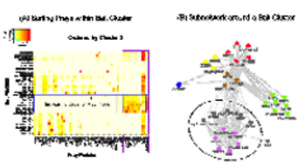

Figure 1. Nested clustering algorithm and heuristic approach to translate the results into node arrangement in the network visualization.

P208

Systematic Interrogation of the Budding Yeast Kinome

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Introduction
Kinases and phosphatases regulate many if not all cellular processes through reversible phosphorylation of myriad substrates. The systematic identification of the proteins that associate with kinases is crucial for understanding the global regulatory network that dictates cellular behaviour. Due to the difficulty in detecting the often weak or transient interactions between kinases and their substrates or regulators, the kinase interactome (kinome) is only poorly charted, even in yeast (1). We have developed a sensitive gel-free mass spectrometric method that has enabled detection of 1844 protein interactions with the complement of protein kinases and phosphatases in budding yeast.

Methods
The complete set of kinases was systematically interrogated using a variety of epitope tags (HA, FLAG and TAP) in small scale cultures. Kinase complexes were efficiently recovered on pre-coupled Protein A magnetic beads and rapidly washed before on-bead trypsin digestion. Samples were analyzed by nano-scale liquid chromatography coupled to an LTQ mass spectrometer. Data was analyzed using the Mascot search engine to generate a raw list of interactions. To identify which interactions were significant, we next developed an algorithm called SAINT (Significance Analysis of Interactome) that uses multiple variables to partition potential interactions between true and false probability distributions. Most interactions above the SAINT threshold could re-validated by immunoprecipitation followed by immunoblotting. Many predictions were functionally validated in by genetic and biochemical experiments.

Results
129/130 yeast protein kinases (as well as most protein phosphatases and kinase/phosphatase regulatory subunits) were successfully expressed and purified. Using SAINT, 1844 statistically significant (p>0.85) interactions were determined. This interaction dataset expands substantially on the current published set of kinase interactions. Visualization and reduction of the overall kinase network was performed by grouping baits in close binary proximity (based on the number and spectral count distribution of shared interactors) to reveal several super-hubs, notably the TOR kinases and the Cdc14 phosphatase. Novel functional connections between these super-hubs and a host of other kinases/phosphatases were validated genetically and biochemically. A statistically enriched kinase-backbone appears to link many parts of the proteome. In summary, sensitive gel-free mass spectrometry platforms for detection of protein interactions should enable an understanding of the transient interactions that comprise the dynamic proteome.

Innovative aspects
- Comprehensive coverage of the budding yeast kinome using gel-free nano-scale liquid chromatography and tandem mass spectrometric analysis to identify low abundance kinase interaction partners
- Novel algorithm called SAINT (Significance Analysis of Interactome) for statistically robust filtering of large datasets
- Network analysis reveals a kinase-kinase super-network and identifies Cdc14 and TOR as super-hubs

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P209

Development of a Selected Reaction Monitoring (MRM) Assay for the Adaptor GRB2 Proteome

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Introduction
Adaptor proteins are composed exclusively of interaction domains and binding motifs (1). The growth factor receptor-bound protein 2 (GRB2) is one such adaptor that modulates cellular responses by directing protein complex formation in signalling networks. Its two Src-Homology 3 (SH3) domains bind proline-rich motifs while its single SH2 domain connects to tyrosine phosphorylated (pYXN) sequences. We have identified over 100 proteins associated with GRB2 in human cells and have designed a scheduled MRM (sMRM) assay to quantify their abundance and changes in their phosphorylation state.

Methods
We have expressed a wild-type 3xFlag-tagged form of human GRB2 or its mutant forms in human embryonic kidney (293T) cells. The affinity-precipitated complexes were reduced, alkylated and trypsin-digested using a highly-controlled SCX solid-phase (proteome reactor) sample preparation protocol designed to ensure reproducibility (2). MRMs (n=1500) were selected from full scan MS/MS discovery data (QSTAR Elite) into the MRM Pilot software and programmed into 5 non-scheduled MRM/MIDAS runs to refine scheduling. The sMRM assay was designed using a cHiPLC-nanoflex system coupled to a 4000QTRAP or a 5500QTRAP hybrid triple quad. The resulting data was refined into a 90 minutes sMRM assay that quantifies around 100 proteins and 50 phosphorylation sites therein to constitute the GRB2ome assay.

Results
We have found over 100 proteins interacting with GRB2. Treatment of cells with a tyrosine phosphatase inhibitor revealed that half of these interactions are induced by tyrosine phosphorylation, and therefore likely involve binding of the GRB2 SH2 domain with pTyr-containing proteins. The others are constitutive and involve SH3-mediated interactions to Pro-rich motifs. In a proof-of-principle experiment, we have used a series of GRB2 point mutants where binding domains function was abrogated individually or in combination. Our results show that the interaction of several proteins known to bind the SH2 or SH3 domains may be completely abolished. For example, DNM1, 2 and 3 did not the proteins in our assay. These data show the high sensitivity and specificity of our MRM assay. We will further use the assay to better define the GRB2 network upon specific cell responses, e.g. growth factor stimulation or drug treatment of cells.

Innovative aspects
- Quantification of targeted proteins in cellular complexes following affinity precipitation
- Standardized nanoLC-chip combined with sMRM
- Statistical assessment of sMRM data

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P210

Proteomic Screen for Multi-protein Complexes in Synaptic Plasma Membrane from Rat Hippocampus

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Introduction
Neuronal synapses are specialized sites for information exchange between neurons. Many diseases, such as addiction and mood disorders, likely result from altered expression of synaptic proteins, or altered formation of synaptic complexes involved in neurotransmission or neuroplasticity. A detailed description of native multi-protein complexes in synaptic plasma membranes is therefore essential for understanding biological mechanisms and disease processes.

Methods
For the first time in this study, two-dimensional Blue Native/SDS-PAGE electrophoresis, combined with tandem mass spectrometry, is used to screen multi-protein complexes in synaptic plasma membranes from rat hippocampus. The aqueous two-phase partition method and a prefractionation strategy of adjusting the pH of the extraction solution were employed and optimized during the sample preparation.

Results
As a result, 514 unique proteins were identified, of which 36% were integral membrane proteins. In addition, 19 potentially novel and known and heterooligomeric multi-protein complexes were found, such as the SNARE and ATPase complexes. A potentially novel protein complex, involving syntaxin, synapsin I and Na^+/K^+ ATPase alpha-1, was confirmed by co-immunoprecipitation and immunofluorescence staining. As demonstrated here, Blue Native-PAGE is a powerful tool for the separation of hydrophobic membrane proteins. The combination of Blue Native-PAGE and mass spectrometry can systematically identify multi-protein complexes.

Innovative aspects
- Identification protein complexes in synaptic plasma membrane by 2D Blue Native/SDS-PAGE electrophoresis and tandem mass spectrometry
- Characterization of 19 heterooligomeric multi-protein complexes

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P211

Systems analysis of networks and complexes containing poly(ADP-ribose)-metabolizing enzymes

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Introduction
Poly(ADP-ribose) polymerases (PARPs) and poly(ADP-ribose) glycohydrolase (PARG) are responsible for the metabolism of the long and branched polymer poly(ADP-ribose). There is an accumulating evidence that poly(ADP-ribose) metabolizing enzymes are implicated in various signalling pathways (1). The present study is aimed at assessing the interplay between PARPs, PARG and their proteins interactors to better comprehends the biological dynamics and influence of poly(ADP-ribose) on cellular pathways.

Methods
Immunoprecipitations of PARP-1, PARP-2, PARP-3 and PARG were performed. Pulled down proteins were separated by SDS-PAGE prior to in-gel trypsin digestion. A linear ion trap and a quadrupole time of flight LC-MS were used for peptide analysis. Several proteins identified by MS were confirmed by western blot. PARPs and PARG associated proteins were submitted to Gene ontology classification. Overrepresented categories were organized in hierarchical tree to facilitate visualization.

Results
The combination of affinity enrichment and tandem mass spectrometry is a powerful method for revealing many potential interacting proteins associated directly or indirectly with proteins of interest. It has permitted permit to identify more than 200 proteins associated with poly(ADP-ribose) metabolizing enzymes. Given the biological importance of protein-protein interactions, we performed global analysis of the generated network providing a general trend in the possible roles of PARPs and PARG. Hence, comparison between poly(ADP-ribose)-metabolizing enzymes possible function through their identified partners. Gene ontology annotations of overrepresented categories highlighted 5 different biological processes. Indeed, RNA metabolism was found in all poly(ADP-ribose)-metabolizing enzymes analysis, DNA repair was present for all three PARPs associated proteins, PARP-1 and PARP-2 shared glycolysis and apoptosis annotations while PARP-1 interactors revealed a possible implication in cell cycle. This proteomic study reveals novel and significant information on PARPs and PARG interaction network which is a first step towards systems biology of poly(ADP-ribose) metabolism.

Innovative aspects
- First large scale proteomics study including major PARPs and PARG
- Novel hypothetic functions proposed for PARPs

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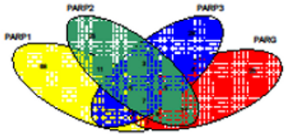

Figure 1. Venn diagram illustrating the number of associated proteins overlapping between all three PARPs and PARG described in this proteomic study.

P212

HMGA Oncoproteins in Double Strand Break Repair Network

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Introduction
HMGA proteins are chromatin architectural factors playing essential roles in organizing macromolecular complexes involved in many aspects of cell biology, from embryonic development to cancer growth. They are widely and dinamically post-translationally modified by several kinases, acetyl- and methyl-transferases. These modifications modulate their DNA- and protein-interacting properties. Our proteomic investigations revealed a link of HMGA with double strand break repair factors, namely DNA-PK, a member of the PIKK kinase family, which is one of the main players in Non Homologous End Joining.

Methods
The interactions of HMGA with NHEJ factors were detected by Affinity Chromatography- and Blot Overlay-based approaches (1, 2). In vivo protein-protein interactions were confirmed by Co-Affinity purifications. Phosphorylation of HMGA by DNA-PK was analysed by LC/MS analyses. Phosphorylation site mapping was performed combining the use of HMGA deletion mutants and LC/MS^2 analyses of wild-type and DNA PK phosphorylated HMGA proteins. Protein-protein and protein-DNA interactions were assayed by GST-pull down, Co-IP and EMSA assays. Influence of HMGA versus Ligase IV activity was assessed by in vitro ligation assays. Chromatin dynamics of HMGA and NHEJ factors during double strand break repair were studied by immunofluorescence analyses.

Results
Proteomic approaches aimed at identifying HMGA partners allowed us to find out a specific interaction of HMGA with the Ku70/Ku80 heterodimer. GST-pull down experiments showed that the interaction of HMGA with this heterodimer is mediated by the Ku70 subunit. In vivo Co-AP experiments allowed us to demonstrate that HMGA is part of a macromolecular complex including some of the main actors of NHEJ, i.e. Ku70/Ku80, Ligase IV and DNA-PKcs (Figure 1). In vitro phosphorylation assays showed that HMGA is a DNA-PK substrate. Despite the presence of three S/TQ DNA-PK consensus sites in HMGA sequence, our phosphate group mapping analyses demonstrated the presence of phosphates at the level of non canonical consensus sites. In addition, phosphorylation by DNA-PK turned out not to affect HMGA DNA binding properties, as resulted from EMSA experiments whereas it seems to be involved in protein-protein interaction modulation. In particular we were able to show a decrease in HMGA binding affinity towards well known partners and, of particular interest in respect to the DNA repair context, the Ku70 subunit. In vitro DNA ligation assays showed a positive effect of HMGA towards Ligase IV activity.

Innovative aspects
- Involvement of a chromatin architectural factor in DNA repair;
- Non canonical phosphorylation consensus sites for DNA-PK;
- Protein-protein interaction modulation by DNA-PK phosphorylations.

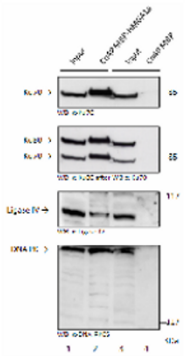

Figure 1. Association of HMGA with members of NHEJ pathway. Co-affinity purifications using an MBP tagged HMGA show that HMGA belong to a macromolecular complex including Ku70, Ku80, DNA-PKcs and Ligase IV.

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P213

LC-MS Analysis to Identify Ryanodine Receptor 1 (RyR1) Protein-Protein Interactions

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Introduction
Excitation-contraction coupling is defined as the process linking action potential to contraction in striated muscle. This process depends on a large macromolecular protein complex, the central element of which is the Ca^{2+} release channel, ryanodine receptor 1 (RyR1) (1). It has been demonstrated that protein binding partners regulate RyR1 via its cytosolic domain and mutations in RyR1 and its interacting proteins result in disease. Here, we describe the purification and LC-MS analysis of affinity-tagged RyR1 protein complexes with the overall aim of understanding calcium ion movement at a molecular level.

Methods
Full length RyR1 rabbit cDNA and a series of five truncated RyR1 constructs were N-terminally affinity-tagged with 6x-histidine (figure 1).These constructs were introduced into HEK293 cells and C2C12 mouse skeletal myocytes enabling column purification of RyR1 protein complexes under non-denaturing conditions. The purified proteins were subjected to immunoblotting experiments to verify stable expression and purification of clones. The purified complexes were analyzed using 1-dimensional gels and by gel-free LC-MS. A filtering algorithm was applied to all putative results to obtain a measure of the statistical reliability (confidence score) for each candidate identified and only proteins with confidence value ≥ 99% and identified with ≥ 2 peptides were accepted.

Results
Here, we present data using 6x-His affinity tagged proteins transfected into HEK293 and C2C12 muscle cells, followed by gel-free LC-MS to identify protein-protein interactions in skeletal muscle. Column purification of tagged proteins from transfected HEK and C2C12 shows stable expression and purification of clones by immunoblotting experiments. Figure 2 shows ryanodine receptor 1 (RyR1) from C2C12 myocytes, purified on a Ni-NTA column under non-denaturing conditions. LC-MS analysis of the purified protein complex showed that we have identified 5 novel RyR1 interacting proteins and we are currently validating these interactions using conventional co-expression and immunoprecipitation assays. These putative protein interacting partners were also subjected to fluorescence confocal imaging to determine co-localization with RyR1 within rat muscle tissue.This protein-protein interaction analysis provides a framework for a more detailed analysis of RyR1 function.

Innovative aspects
- To date, there has been no large scale protein-protein interaction analysis of RyR1.
- We have identified novel RyR1 interacting proteins.

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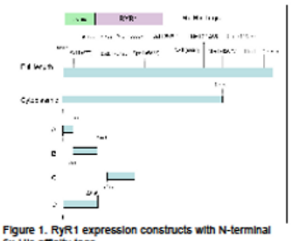

Figure 1. RyR1 expression constructs with N-terminal 6x-His affinity tags.

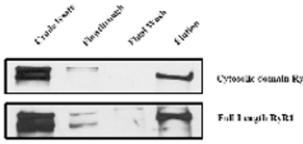

Figure 2. Histidine-tagged RyR1 purified from transfected C2C12 muscle cells using NI-NTA chromatography. Eluted protein complexes were immunoblotted and probed with an anti-RyR1 antibody.

P214

Four and a half LIM domains protein 1 (FHL1) interactions in mammalian tissue

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Introduction
Heart failure (HF) is a severe and progressive cardiac disorder. Recently, we performed a large scale comparative proteomic study using wildtype and a transgenic mouse model of HF (PLNR9C) (1). In the present study, we extend these results and hypothesize affected cellular processes are better understood by studying interactions between proteins. FHL1 was one of the most upregulated proteins in PLNR9C mice, has been implicated in muscle growth and differentiation, and is the causal gene for several myopathies (2,3). An interaction network for FHL1 could provide insight into its functions.

Methods
Dual affinity (streptavidin and calmodulin binding peptides) tagged FHL1 was generated and transfected into mammalian HEK293 kidney cells. The tags allow for tandem affinity purification of FHL1 and interacting proteins under native conditions. Purified samples were analyzed using 1-dimensional gels, and by gel-free liquid chromatography mass spectrometry (LC-MS). Validation of interactions were performed by co-immunoprecipitation (co-IP) and immunofluorescent colocalization studies from 1) cells co-transfected with FHL1 and dual tagged (V5 and 6x histidine) potential interactors (PI) or 2) PLNR9C ventricular tissue. Co-IP results from the latter were also analyzed by LC-MS. Expression correlations between PI and PLNR9C mice were assessed by proteomic data mining and immunoblotting.

Results
Tandem affinity purification successfully allowed for the enrichment of tagged FHL1 and interacting proteins, as detected by immunoblot analysis and MS of trypsin digested samples. MS datasets from multiple FHL1 purifications were first filtered to remove common contaminants found in negative and tag controls and then compared. Potential interacting proteins to further pursue were prioritized based on number of repeat detections, biological relevance, and previous predictions derived from online OPHID (ophid.utoronto.ca) and HPRD (www.hprd.org) database searches. Many proteins of high confidence detected can be classified as cytoskeletal scaffolding or LIM domain binding proteins. Multiple components of known complexes have also been detected in purifications. On-going validation efforts have proven successful for numerous interactions. In conclusion, our strategies allow for the purification and identification of *in vivo* protein interactions from mammalian cell systems. Since proteins assemble into dynamic complexes within the cell, delineation of interactions can provide insight into their functions by association with their interacting partners.

Innovative aspects
- Utilization and extension of proteomic derived data via molecular biology and mass spectrometry
- Enrichment and purification of protein complexes using tandem affinity purification in mammalian cell systems
- Elucidating protein-protein interaction networks could contribute to the identification of novel drug targets relevant to managing cardiac disease

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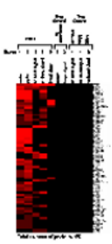

Figure: Heatmap representations of 62 proteins uniquely and repeatedly identified in multiple FHL1 purifications by MS. Color intensities depict total spectral counts. Proteins arranged based on frequency of detection and intensity.

P215

MRJP1-Binding Proteins from Honey Bee Brain

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Introduction
Major royal jelly protein 1 (MRJP1) is the main protein component of honey bee (*Apis mellifera*) royal jelly, but is also present in its brain. MRJP1 expression changes during ontogenetic and behavioral development of honey bee worker subcastes[1]. MRJP1 is 9-fold more abundant in nurse than in forager brain. The protein is located in the cytoplasm of brain cells, probably along filaments of the cytoskeleton, in the antennal lobe, optical lobe and mushroom body tissues. MRJP1 is also deposited on the rhabdom, a structure composed of numerous tubules, and in intercellular spaces between cells in mushrooms bodies, indicating that it may be both a binding protein of intracellular filamentous structures and a secreted protein[1]. It is also found as a calcium/calmodulin-binding protein[2]. However, its roles in the nervous system are still unknown. In this work, we identified possible MRJP1-binding proteins (M1BPs) in the nurse honey bee brain proteome.

Methods
Activated thiol-sepharose, with the ability to reversibly couple proteins, was used for preparing an affinity chromatography column. Purified MRJP1 was first immobilized to the resin as a bait. The nurse brain extract was incubated to this affinity column in order to bind the M1BPs. After washing, the whole MRJP1-M1BPs complex was eluted, TCA precipitated, and analyzed by 2D electrophoresis (2DE), followed by identification through MALDI-TOF and MALDI-TOF/TOF mass spectrometry.

Results
Several polypeptides were retained by the MRJP1 affinity column. These proteins were considered M1BPs since none of them was found to bind directly to the resin on control experiments. The eluted complex was separated by 2DE, and presented over 200 M1BPs spots when silver stained. A preparative 2DE was performed to produce a coomassie blue stained gel (Figure 1), which was destined for protein identification by peptide mass fingerprinting and MS/MS. The major spot #16 was MRJP1. Other main spots corresponded to 60 kDa heat shock protein (spot # 11), actin-87E isoform 2 (spots # 21-24) and arginine kinase (spots # 26-30). Identified MBP1s were also ATP synthase, glutamine synthetase 2, annexin IX, 14-3-3 protein, cofilin/actin-depolymerizing factor homolog and profilin, among others. As cytoskeleton proteins bound to MRJP1, comprobatory experiment will be the co-localization of MRJP1 and actin by immunocytochemistry and confocal microscopy.

Innovative aspects
- MRJP1 probably binds over 200 honey bee brain proteins;
- Actin and other cytoskeleton proteins are among these MRJP1 binding proteins;
- Some regulatory proteins may also be M1BPs.

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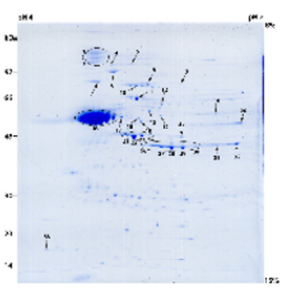

Figure 1. 2DE gel of MRJP1 binding proteins. Nurse honey bee brain extract was incubated to a MRJP1 affinity resin. MRJP1-M1BPs complex was then eluted from the affinity column and analyzed by 2DE (IEF under pH 4-7, SDS-PAGE under 8-15% T gradient) and CBB stained.

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P216

Quantitative analysis of the effect of methylation inhibitors on messenger Ribonucleoprotein complexes

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Introduction

mRNA synthesis, processing, nuclear export and translation are complex cellular processes requiring the coordinated and temporal action of many RNA binding proteins. Many of these proteins contain arginine and glycine rich motifs that are often Arginine di- or mono methylated by Protein Arginine Methyltransferases. Therefore, arginine methylation is likely to affect protein-RNA interactions. Here we analyse the in vivo effects of methylation inhibitors on mRNA-Protein complexes using a combination of Silac, UV crosslinking, mRNP capture and mass spectrometry.

Methods

Hek 293T cells were grown in DMEM medium or DMEM medium suitable for SILAC. Cells were UV irradiated on ice, lysed and denatured in oligo(dT) binding buffer (20 mM Tris-HCl, pH 7.5/1.0 M NaCl/1% SDS/0.2 mM EDTA). Next oligodT dynabeads were added an polyA capture was performed for 1 hour. The resin was washed 3X in binding buffer and crosslinked proteins were eluted by RNAse A and T1 treatment. Captured proteins were concentrated using a vacuum dryer and subjected to in solution trypsin digestion. LC-MS was then performed using a Bruker HCT Ultra PTM Discovery system.

Results

Using the mRNP capture assay we were able to identify approximately 150 mRNA binding proteins using a combined CID and ETD analysis. Abundant classes of proteins include heterogeneous ribonucleoproteins and splicing factors. Moreover, significant changes in protein abundance between the AdOx and non- AdOx treated cells were observed. Stable isotope labeling with amino acids in cell culture (SILAC) and western immunoblotting was used to confirm quantitative changes in arginine methylation and protein abundance. ETD significantly enhanced the identification of highly charged peptides containing GAR rich regions.

Innovative aspects

- Large scale analysis of mRNP complexes
- Quantitative analsyis of mRNP complexes using stable isotope labelling with amino acids in cell culture
- ETD analysis of mRNP complexes

P217

Studying Integrin β1 Complexes by Formaldehyde Cross-Linking and Mass Spectrometry

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Introduction

Integrins are membrane spanning heterodimeric complexes composed of α and β subunits. They play a major role in cell signalling as they relay signals across the membrane by interacting with intracellular adaptor proteins. Several interaction partners of integrins have been described, their binding being dependent on the activation status of the cell. However, the detailed mechanism of regulation of these signalling processes is not fully understood. Here, we apply formaldehyde cross-linking to stabilize integrin complexes and study them by mass spectrometry.

Methods

To cross-link protein complexes Jurkat cells were treated with 0.35% formaldehyde for 10 min at RT, before the reaction was quenched with 1.25 M glycine. Cells were lysed afterwards in RIPA buffer and immunoprecipitation was performed using monoclonal anti integrin β1 antibodies. The precipitation of the integrin complexes was confirmed by immunoblotting. To study the composition of these complexes, the immunoprecipitated samples were separated by SDS Page. The complex of interest was isolated, in gel digested, analysed by LC-MS/MS and the obtained data was sequenced using Mascot or the Global Proteome Machine respectively. To investigate activated integrin complexes, cells were treated with 25 ng ml PMA before cross-linking was performed.

Results

Formaldehyde cross-linking of cells paired with affinity enrichment and mass spectrometry is a powerful method to study protein complexes and was established succesfully in our lab to analyze myc-tagged proteins (1). However, in order to investigate native integrin complexes we wanted to use antibodies against the endogenous proteins. This requires that the corresponding epitopes are not destroyed by formaldehyde modification. We tested eight monoclonal antibodies for immunoprecipitation of integrin β1 from formaldehyde treated Jurkat cells. Two of them did not precipitate cross-linked complexes, whereas the remaining six were successfully applied. Repeated investigation of one cross-linked complex was performed by mass spectrometry and revealed that it was composed exclusively of integrin β1 and integrin α4, which is a known heterodimer present in Jurkat cells. This proves that formaldehyde cross-linking combined with immunoprecipitation by antibodies against endogenous proteins can be used for investigation of complexes. Applying this method to monitor changes in integrin complex composition after stimulation allows us now to study signalling processes involving integrins.

Innovative aspects

- Applying antibodies against endogenous proteins for immunoprecipitation of formaldehyde cross-linked complexes

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P218

Purification and Characterization of a novel PFOR:2-ADH complex from *Thermoanaerobacter tengcongensis*

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Introduction

Based upon the genomic annotation, pyruvate-ferredoxin oxidoreductase (PFOR) is localized at the fork point for generation of alcohol and acetic acid in *Thermoanaerobacter tengcongensis* (*T. tengcongensis*). It catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2, in turn acetyl-CoA is reduced to ethanol by alcohol dehydrogenase II (2-ADH) or oxidized to acetic acid by phosphate acetyltransferase (PAT)/acetate kinase (AK). The mechanisms of how acetyl-CoA goes the specific pathway at downstream of PFOR are still uncovered yet. PFOR with a homodimer structure was an up-regulated protein in response to the elevated temperature and actively interacted with other proteins (1,2). The fact prompts us to raise a question whether there is any interaction between PFOR and the enzymes participating in alcohol or acetic acid synthesis.

Methods

To examine if there was an interaction between PFOR and 2-ADH or PAT/AK *in vivo*, the bacterial lysates were loaded onto DEAE-52 column and the eluted protein fractions were collected during a NaCl linear elution. The enzyme activities for these enzymes were checked, and the abundances of PFOR and 2-ADH were detected using Western blot in all the fractions as well. Blue native gel electrophoresis (BNGE) was applied to separate the protein complexes in the lysates followed by Western blot inspecting the presence of PFOR, 2-ADH, or PAT/AK with the correspondent antibodies. Immunoprecipitation with antibody against 2-ADH and pull-down with recombinant 2-ADH were conducted to find the interactions between PFOR and 2-ADH in the bacterial lysates. To scrutinize whether there was an interaction between PFOR and 2-ADH or PAT/AK recombinant protein *in vitro*, the recombinants were incubated together at room temperature for a period, and the incubated mixtures were taken for BNGE to check the formation of protein complexes and for enzyme activity determination to monitor the effect to their catalysis caused by protein interactions.

Results

As shown in Figure 1, purification of 2-ADH appeared in two peaks, in the early fractions which mainly contained 2-ADH and in the later fractions which had two components, 2-ADH and PFOR. The 2-ADH activity was examined in all the eluted fractions, and found in two peaks as well, which were chromatographically coincided with the 2-ADH abundant fractions. Importantly, the early fractions exhibited higher 2-ADH activity whereas the later fractions showed much lower 2-ADH activity. The DEAE-52 chromatography for PAK/AK was not found in such similar pattern. Furthermore, the eluted fractions were loaded onto BNGE, while the separated electrophoretic bands were excised for the secondary SDS-PAGE followed by Western blot. In all the bands containing 2-ADH, PFOR was always present over there. Thus, the evidence revealed that an interaction indeed linked 2-ADH and PFOR in *T. tengcongensis in vivo*. When recombinant 2-ADH was incubated with recombinant PFOR, its V_{max} was attenuated more than 5 times, and the kinetic inhibition parameter (K_i) for PFOR was about 60nM in a non-competitive pattern. However, there was no interaction found between recombinant PAT/AK and PFOR.

Innovative aspects

- To identify for the first time the interactions between PFOR and 2-ADH in thermophiles.
- The interactions of 2-ADH and PFOR cause inhibition of the 2-ADH catalysis, which is likely to be a regulatory mechanism of alcohol formation in *T. tengcongensis*.

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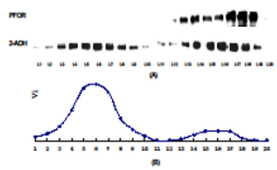

Figure 1. (A) The fractions from L1 to L20 were examined by Western blot for the abundances of the native 2-ADH and PFOR. (B) The 2-ADH enzyme activity was measured in the fractions from L1 to L20.

P219

The interplay between protein modification and protein complex assembly of a RNA helicase DDX3

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Introduction

DDX3 is a RNA helicase that unwinds double-stranded RNA in an energy-dependent manner. Previous reports showed that DDX3 interacts with and is regulated by hepatitis C virus (HCV) core protein, while it is dysregulated in hepatitis virus-associated hepatocellular carcinoma (HCC) and is involved in cell growth control (1). Functional alterations in tumours often involve protein structural changes at primary structure level, such as post-translational modification, as well as protein complex formation. Systematic characterization of DDX3 structures may help us understand the role of DDX3 in pathogenesis of human HCC.

Methods

Flag-tagged DDX3 from transiently transfected 293T cell was immunopurified with M2 anti-Flag beads and resolved in a polyacrylamide gel. The specific DDX3 interacting proteins were identified using an ^{18}O labeling method that facilitates the selection of peptides from specific proteins for MS/MS analyses, which identified proteins with distinct amounts between treated and control samples (2). The band corresponding to DDX3 was excised from the gel and subjected to in-gel digestion, which was analyzed by an Orbitrap tandem mass spectrometer. The acquired MS data was examined using our proprietary computer programs for identification of peptides bearing protein modifications of interest.

Results

Flag-tagged DDX3 protein was prepared from 293T cells under different culture conditions, such as regular, serum-starved and insulin-stimulated ones. We found that DDX3 was a highly methylated protein, as a total of six methylation sites, including one Lys and five Arg residues, have been identified. Tandem mass spectrometry verified that all of these residues have two methyl groups incorporated at one position. Intriguingly, quantitative MS analysis showed that most of dimethylation was enhanced particularly in serum-starved cells (Fig 1). Moreover, we appeared to discover two new types of amino acids in DDX3, namely acetylated arginine and nitro-lysine. With our new analytical method, we have also identified a group of DDX3-interacting proteins, most of which are known to participate in RNA metabolism. Most importantly, there seems to be a correlation between protein modification profile and the set of interacting proteins. This provides an excellent case exemplifying that protein modification may regulate protein complex assembly and consequently modulate cellular activity.

Innovative aspects

- DDX3 is a highly modified with dimethylation at Arg and Lys residues.
- Dimethylation status of DDX3 is altered under different culture conditions.
- A group of DDX3-interacting proteins are documented, which is in accordance with the role of DDX3 in RNA metabolism.
- Several novel amino acids appear to be present in DDX3 protein.

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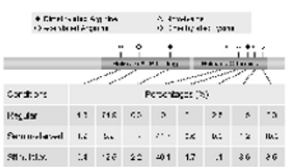

Figure 1. The locations of modified amino acid residues in DDX3 polypeptide and the percentages of these modifications under different culture conditions.

The Role of Protein Methylation in the Interactome

P220

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Introduction:
Interactome research has shown that proteins participate in a wide number and variety of interactions. Together, these form networks of great complexity. However, the dynamics of these networks remain virtually unknown. Post-translational modifications, such as protein methylation and phosphorylation, are one means of regulating protein-protein interactions. Whilst the predominance of phosphorylation in the proteome, especially for the model organism *S. cerevisiae*, is well understood, the post-translational modification of protein methylation is poorly understood both in its presence in the proteome and its possible role in controlling protein-protein interactions.

Methods:
We have developed two approaches to help detect protein methylation in the proteome. One approach is immonium ion scanning using a wide range of collision energies (1). This has been applied to the study of the *S. cerevisiae* proteome. We have also analysed ~26,000 peptide mass spectra of purified *S. cerevisiae* proteins for the presence of methylation, using the FindMod tool (http://au.expasy.org/tools/findmod/).

To understand the role that protein methylation plays in the control of protein-protein interactions, we have studied the effect of lysine- and arginine-methyltransferase knockout mutants on the formation of protein complexes. Blue native PAGE, and a prefractionation technique of continuous elution blue-native PAGE (2) have been used for this purpose.

The above results have been interpreted in the context of the yeast interactome. To do this, we have developed the GEOMI software platform to co-analyse protein-protein interactions in the context of the interactome (3).

Results:
We have undertaken some of the first proteome-wide analyses for protein methylation. We have found that the methylation, notably on lysine and arginine residues, is much more widespread than previously thought. It is enriched in proteins of specific function, such as those associated with protein translation, and is predominantly found in proteins of the cytoplasm, nucleus and nucleolus. It is found associated with particular sequence motifs.

The analysis of methyltransferase knockouts has revealed that these enzymes display redundancy. Double and triple knockouts, at least for yeast, are viable. However, BN-PAGE analysis of mutants has revealed a number of protein complexes which fail to form in the absence of methylation. Whilst further confirmatory analyses are required, this suggests that methylation can play a role in the control of protein-protein interactions.

Innovative aspects:
- Global methylproteome analysis
- Functional association of protein methylation with the control of protein-protein interactions
- investigating the dynamics of protein interaction networks by post-translational modification analysis.

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2D BLUE NATIVE SDS-PAGE ANALYSIS OF MULTIPROTEIN COMPLEXES OF HUMAN ERYTHROCYTE MEMBRANE

P221

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Introduction
Patients with chronic kidney disease in haemodialysis present low quality erythrocytes, showing higher rigidity. We hypothesize that membrane structure may play a role in the flexibility of erytrocytes. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) is an electrophoretic technique for high resolution separation of membrane protein complexes in the mass range of 10KDa to 10MDa.Here we describe a methodology to perform BN-PAGE of erythrocyte membrane complexes followed by a second dimension of tricine-SDS page for isolating and identifying individual proteins forming these complexes.

Methods
Erythrocytes were isolated from plasma after maturation, washed in 5mM Na_2HPO_4 pH 8, 1mM EDTA and 0.9% NaCl and lysed in 5mM Na_2HPO_4 pH 8, 1mM EDTA and 1mM PMSF. Membrane pellets were frozen at -80º C in 50 mM imidazole / HCl and 10% glycerol (pH 7) or immediately analyzed. For BN-PAGE, Samples were centrifuged and pellets were solubilized in 50 mM sodium chloride, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1mM EDTA (pH 7) and the appropriate detergent. After centrifugation, 5 µl of 5% glycerol and coomassie G-250 in a detergent/dye ratio of 8:1 were added to the supernatant. Membrane complexes were run in 4-13% acrylamide gradient gels. The second dimension was run in 10% tricine-SDS/PAGE and a silver staining protocol with modifications was used for tricine gels [1, 2].

Results
Membrane proteins complexes solubilization was carried out by testing two different detergents (digitonin and Triton X-100) at various concentrations (0.1-10% and 0.5-2 %, respectively). Solubilization was always performed for 1 h. 4% Digitonin resulted to be the best option in terms of first dimension resolution, as it was the lowest detergent concentration at which the highest number of distinct bands can be observed without disturbance from the detergent in excess. Prior to the second dimension, protein complexes disruption into individual proteins was optimized by treating the strips with SDS/ mercaptoethanol solution tested at various compositions (1-20% SDS; 1-5% mercaptoethanol). 12% SDS treatment for 1h prior to load the strip on the second dimension provided a higher number of protein spots detected; however, further increase of SDS amount negatively affected resolution. 1% β-mercaptoethanol was used, as its variation did not show any changes. Identification of erythrocyte membrane protein complexes isolated and analyzed by 2D-BN-PAGE is being carried out by mass spectrometry.

Innovative aspects
- For human erythrocyte, optimum experimental conditions are provided not only to visualize membrane protein complexes, but also proteins composition of each of them.
- An overview of membrane protein complexes present in the human erythrocyte is so provided.

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Comprehensive characterization of gene regulatory complexes in *Saccharomyces cerevisiae by quantitative SRM*

P222

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Introduction: Proper regulation of gene expression is essential for normal cell function and consequently misregulated gene expression plays a contributing role in many diseases. To understand how gene expression is regulated, the dynamic composition of the complexes that assemble at gene regulatory elements must be comprehensively determined. Here we describe a mass spectrometry based approach to alleviate the limitations of the current proteomics approaches and demonstrate its application to identify potential gene specific transcriptional regulatory proteins.
Methods: After selecting a set of proteins with known and predicted roles in promoter proximal transcriptional events involving RNA polymerase II (Pol II), we compiled a list of signature peptides for each protein. These peptides, either made by chemical synthesis or protein digestion, were used to generate and validate the most sensitive transitions for each peptide. To test the assays, proteins in yeast nuclear extracts were monitored for the entire set of targeted transcription factors. Yeast cells were grown in heavy and light SILAC media and were mixed in 2:1 (L:H) ratio and digested for analysis. Next, SRM assays were used to detect the potential regulators of the Flo11 gene. To identify potential Flo11 regulators, 200 base pairs (bp) segments of the FLO11 promoter were immobilized on magnetic beads and used to isolate proteins from yeast cells grown in heavy SILAC media. 5 random 200 bp DNA sequences were immobilized for control captures. Digested mix was then monitored by SRM for potential gene regulators.
Results: Our final assay included 9896 validated transitions for 1647 peptides representing 472 transcription factors and related regulators(1). Collectively, these measurements constitute a set of validated assays to detect and quantify all known and predicted factors with roles in Pol II promoter proximal transcriptional events. Two hundred and fifty eight of 463 targeted proteins were confidently detected from yeast nuclear extract by SRM, of which 72 were also detected by shotgun mass spectrometry using an LTQ-Orbitrap mass spectrometer. The detected proteins spread across 5 orders of magnitude in concentration and proteins with concentrations as low as 50 copy/cell were detected. The frequency of detection for low copy proteins was comparable to that for more abundant proteins. The SRM measurements showed better accuracy compared to MS (Fig 1). The coefficient of variation for L:H ratios for peptides of a protein was used as the measure of precision, by which the SRM measurements appears more precise as well.
MS: 1.66±0.95 SRM: 2.05±0.34

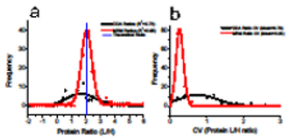

Figure 1. a. These plots show the level of quantification accuracy and precision by the SRM method and shotgun mass spectrometry

Next, SRM assays were used to detect the potential regulators of the Flo11 gene which encodes a cell-surface mucin protein that mediates filamentous-form growth of yeast. Thus far we analyzed proteins that are enriched on two of the 200 bp segments. Out of the 232 regulators that we assayed for, 23 and 22 were specifically enriched on segment 4 (-600-800) and 7 (-1200-1400) respectively. To prioritize candidates protein for follow-up experiments, we compared the SRM results with predicted consensus binding sites and ChIP-Chip data (Fig 2). In accord with the SRM results, both ChIP-Chip data and sequence prediction algorithms indicate that Mot3 can bind to sites in the Flo4 and 7 regions. To test whether Mot3 can affect *FLO11* expression, we deleted the *MOT3* gene and monitored *FLO11* transcription. *FLO11* transcription increased in the *MOT3* deletion strain compared to the wild type strain, and consistent with the prediction that Mot3 is a repressor of *FLO11* , *MOT3*-deletion strains are hyperfilamentous. Thus we have identified a *bona fide* regulator of Flo11. Other potential regulators detected by SRM are under investigation.

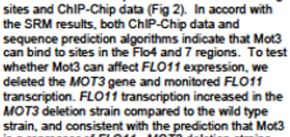

Figure 2. This figure shows the comparison between SRM data and predicted binding and ChIP-Chip data used to find potential regulators of *FLO11*.

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(1) P Picotti et al, Nat. Meth. 2008

Structural System Intraactomics by Peptide Arrays: Interrogating the Vacuolar ATPase

P223

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Introduction
The Vacuolar ATPase (V-ATPase) (Fig 1), a multi-subunit rotary acid pump powered by ATP hydrolysis, is found ubiquitously throughout eukaryotic life. While the structure of the related F-ATP synthase (F-ATPase) is well characterized, there are several features of the V-ATPase that are not well understood. Rather than traditional methods such as yeast one- or two-hybrid assays, use of peptide arrays should allow higher resolution and faster mapping of subunit-subunit interactions of the V-ATPase.

Methods
Peptide arrays were designed to cover 13 subunits of the V-ATPase at about 2x resolution per amino acid; each spot representing an amino acid 20mer. Subunits E and G of the V-ATPase peripheral stators were grown in *E. coli* with a FLAG tag on subunit G for detection on the array. Arrays were treated similar to a far-western blot, with the E+G complex used as a probe and an anti-FLAG antibody conjugated to HRP used for detection.
Spots detected by chemiluminescence were then grouped by intensity, filtered against an antibody-only control, and mapped against subunit structural data.

Results
We were able to find distinct spots for interactions pertinent to the EG heterodimer of the peripheral stator, as well as the G subunit alone.
Particularly useful in this data set are the interaction sites that are observed between the probe subunits and the subunit C of the V1 periphery. While interactions between these subunits have been reported in the literature, the specificity of the interaction has not previously been described at high resolution, nor has the stoichiometry been clear. We see in the data sets that the EG heterodimer has two distinct regions of interaction with the C subunit, which are separated in 3-dimensional space on the crystal structure of subunit C (Fig 2).
Furthermore, the data derived from probing the peptide arrays with only subunit G provide insight into the basis of these interactions to subunit C – in particular we see that complementary subunit G on its own has an interaction with subunit C, which supports a particular orientation of the EG heterodimer in the V-ATPase complex.
The same technique could also be used to explore interactions between the V-ATPase and other molecules of the cell.

Innovative aspects
- High throughput, high resolution mapping of inter-molecular interactions a macromolecular complex

References
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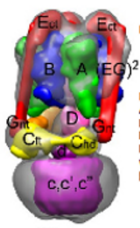

Figure 1. Model of the V-ATPase showing the proposed locations of subunits A,B,C,E, and G

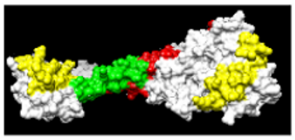

Figure 2. Crystal structure of V-ATPase subunit C, with interaction regions for the EG heterodimer colored. Structure is rotated 180 degrees on the vertical axis relative to its position in Figure 1

P224

Dynamic Processes in Proteins and Protein Interactions Studied by Mass Spectrometry Combined with Protein Chemistry

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The combination of tertiary structure selective chemical modification in proteins (like acetylation of lysine residues), mass spectrometric approaches, and molecular modeling methods has been used to determine structure-function relationships in proteins. One main focus of our research is to combine photochemical reactions, H/D exchange and chemical crosslinking of proteins with mass spectrometric methods. This concept has proven useful for identification of target proteins and components of protein complexes, structural characterization of protein-ligand and protein-biomolecule interactions, and elucidation of dynamic processes in proteins. The photochemical reactions of proteins used for this purpose can be classified as photoaffinity labeling, photo-crosslinking, or time-resolved photochemical reactions. The purpose of the research is to develop and apply these approaches to the study of proteins involved in a number of disease states and biochemical and pharmaceutical processes. Photoaffinity labeling, combined with mass spectrometric approaches and molecular modeling, is being used to determine the drug binding structures of HIV integrase, an important drug target in AIDS research. Determination of drug binding structures is essential for understanding the mechanism of binding to the inhibitor, and to provide information useful for generating more efficient anti-HIV drugs.

For the concept of crosslinking combined with MS to structurally elucidate protein-protein interaction, novel crosslinker and crosslinking strategies are being developed. In particular, crosslinkers and strategies are developed towards the enrichment, detection and identification of inter-peptide crosslinks. Their feasibilities have been shown with numerous model complexes and first applications to address biological and biomedical questions including the elucidation of interaction sites of membrane/cellular adaptor protein complexes. Within this research area, our special interest is to study the topology of the anaphase-promoting complex (APC), a multi-protein ubiquitin ligase involved in cell cycle regulation that is essential for the metaphase to anaphase transition and exit from mitosis using chemical crosslinking and mass spectrometry.

Proteomics Toolbox

(P301 – P339)

P301

Molecular tool-box for in vivo functional biology and proteomics: Application to DNA topoisomerase II

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Dedicated to the memory of C Mioskowski

Introduction
In 1999, the ABPP (Activity-Based Protein Profiling) concept was introduced to detect activity changes of proteins in cells.[1] Here we describe a molecular tool-box for protein profiling in living cells applied to Gyrase, a DNA topoisomerase II. The first tool is a modified novobiocine **1** keeping a high affinity for the protein: **1a** (photosensitive) for a covalent ABPP and **1b** to be used for non-covalent ABPP. The second tool **2a-b** is a fluorescent probe reacting with azide for labeling the tagged proteins. The third one **3** allows to catch by Staudinger reaction and release the tagged proteins.

Methods
Purified Gyrase was incubated with **1a** for 15 min then irradiation under UV at 365 nm for 2h at 4°C to form covalent bonding or incubated with **1b** for 15 min without UV. The protein was treated with **2a**, $CuSO_4$, sodium ascorbate and bathophenantroline for 1h at RT or with **2b** at RT for 4h. The crude reaction mixture was separated by SDS-PAGE or by native gel and analysed by fluorescence imaging then Coomassie blue. Alternatively a cell culture was submitted to **1a** for 15 min and irradiated. Cells were lysated. One part was treated with **2b** which react specifically with azide. The rest of the cells were treated with **3** and pulling down with streptavidin magnetic beads allow us to trap the azide-tagged proteins. Reductive cleavage of the "azo" function and analysis by mass revealed the tagged protein.

Results
The protein incubated with **1a** and treated with **2b** was separated by SDS-PAGE. The labeled protein was analyzed by fluorescence (fig 2). Only line **d** shows the fluorescence-tagged Gyrase. Control experiments do not show fluorescence and Coomassie blue revealed the presence of proteins. We showed that: UV irradiation didn't affect the protein (line **a**), there isn't non-specific labelling (line **b**), and we need a covalent bonding by photoactivation to tag the protein in denaturing conditions (line **c**). The tagged protein was also labeled with triphenylphosphine **3**. After purification and cleavage of the "azo" linker, the protein was digested, and identified by mass spectroscopy. The use of Staudinger ligation was preferred because it can be used *in vivo*. We then applied this method to develop a non-covalent approach by incubating the Gyrase with the probe **1b**. After treatment with **2b**, a non-denaturing gel separation, afforded the tagged protein visualized by fluoresoence. Based on this proof of concept for a non-covalent ABPP, we plan to use the probe **1a** or **1b** and modified QDots with phosphine in living cell for cell imaging.

Innovative aspects
- Non covalent ABPP
- DNA topoisomerase II profiling
- In cells Staudinger ligation reaction

References
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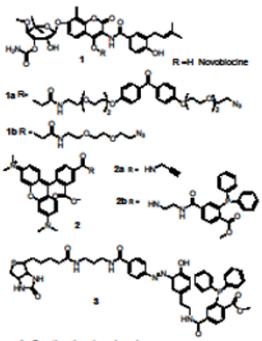

Figure 1: Synthesized molecules

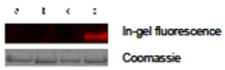

Figure 2: Purified Gyrase (1.25µg/mL) was treated: a) under UV irradiation 2h; b) under UV irradiation 2h then **2b** (50 µM); c) with **1a** (1µM preincubated 15 min) without irradiation then **2b**; d) with **1a** (1µM preincubated 15 min) then UV irradiation 2h then **2b**.

P302

An efficient lentiviral tagging system for the affinity purification of human protein complexes

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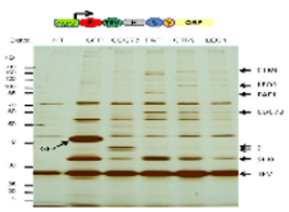
Introduction: Protein complexes (PCs) and protein-protein interactions (PPIs) are essential for many cellular functions. Several protein purification approaches have been developed and mainly applied to the studies of PCs and PPIs in lower eukaryotic systems. However, the procedure that enables efficient and systematic purification of PCs from mammalian cells is lacking. Here, we present an efficient protein tagging and purification strategy in human cells.

Methods: We established a Lentiviral delivered, Gateway-compatible and triple-Tag affinity purification System (LGTS).The LGTS integrates the lentiviral elements for efficient delivery of tagged genes into most human cell lines, the site-specific recombination elements allowed for easy cloning and the 3xFlag(F)-6xHis(H)-Strep(S) tags separated by a tabacco etch virus (TEV) protease cleavage site between F and H for complete dual affinity purification (Figure, upper panel). After the initial purification by F tag, either H or S tag could be used for the secondary purification. The expression of the tagged genes is controlled by the CMV promoter. After the open reading frames (ORFs) were cloned into the LGTS, lentiviruses containing the tagged genes were individually prepared, infected into HEK293 cells and a stable pool of infected cells was established by puromycin selection. Tagged proteins were then purified and identified by mass spectrometry.

Results: We utilized the PAF1 complex, a PC involved in transcription regulation, as a benchmark to test the newly developed LGTS. Four (i.e. CDC73, CTR9, LEO1, PAF1) of the 5 known human PAF1 complex subunits were N-terminally tagged and were expressed at the levels comparable, even though not identical, to their endogenous ones (ranged from 10 to 200% as compared with these of endogenous genes) in HEK293 cells. Approximately 40% of the tagged proteins were purified through the two step (F-H) purification. All the known PAF1 complex subunits (i.e. CDC73, CTR9, LEO1, PAF1 and WDR61) were identified (Figure), demonstrating the feasibility of this technique. Remarkably, several novel proteins that interact with PAF1 complex were identified. These results indicate that (1) LGTS is a capable new tool in mammalian proteomic study; (2) several novel proteins that interact with PAF1 complex may regulate transcription in human cells; and (3) the PAF1 complex or its subunits may have other novel functions. Next, we will characterize the functional roles of the newly identified factors.

Figure: Protein purification with LGTS procedure. Human ORFs of the genes in PAF1 complex were cloned into the LGTS (upper) and the purified proteins were visualized by silver staining (lower) and subsequently identified by LC-MS/MS. NT: untagged PAF1. GFP: irrelevant control. ?: novel factors. Y: Yeast proteotrypic peptide.



P303

Human Cell-Derived *in vitro* Coupled Transcription/Translation Systems

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Introduction
Cell-free translation systems derived from *E. coli* and wheat germ extracts have been used in the high-throughput production of thousands of gene products from cDNA libraries. Because mammalian cDNAs encode many glycoproteins and very large proteins, cell-free systems that can produce such "difficult to express" proteins are needed. Here we established human cell extracts-based *in vitro* coupled transcription/translation systems that allow expression of glycoproteins and large proteins from DNA in one tube.

Methods
HeLa cell and hybridoma (human) cell extracts were supplemented with translation enhancers. These extracts were incubated with T7 RNA polymerase and a plasmid that harbored a cDNA between a T7 promoter/terminator unit. The template plasmid also harbored the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) or the hepatitis C virus (HCV) IRES between the T7 promoter and the coding region of the cDNA to enhance the production.

Results
We found that these IRES-dependent transcription/translation systems were able to produce large proteins including Dicer (200 kD) and mTOR (260 kD) to levels detectable on SDS-PAGE by Comassie Brilliant Blue-staining (Fig. 1). We purified the synthesized proteins to near homogeneity, and validated their functionalities in the appropriate biochemical assays (1). Furthermore, glycosylated proteins including HIV-1 gp120 (Fig. 2) and human choriogonadotropin (hCG) subunits were produced to significant levels. In conclusion, the human cell-based *in vitro* coupled transcription/translation system using the EMCV or HCV IRES will be a convenient tool for the high-throughput production of human proteins including large proteins and glycoproteins.

Innovative aspects
- Efficient production of large proteins in vitro
- Efficient production of glycoproteins in vitro
- Convenient transcription/translation system to produce proteins in one tube

References
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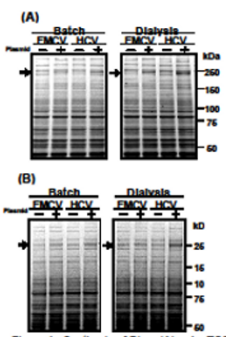

Figure 1. Synthesis of Dicer (A)and mTOR(B) proteins in the EMCV IRES or HCV IRES dependent *in vitro* coupled transcription/translation system. Arrows in each panel indicate synthesized proteins detected by CBB staining.

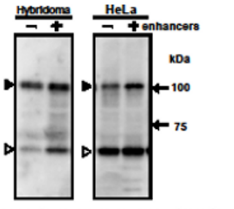

Figure 2. Synthesis of HIVgp120-HA protein in the Hybridoma or HeLa cell-based *in vitro* coupled transcription/translation system. Closed and open arrowheads indicate fully glycosylated and non-glycosylated forms detected by anit-HA antibody, respectively.

P304

DeepQuanTR: a software package for the MALDI-based label-free quantification of proteins

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Introduction

The label - free quantification of changes in protein abundance for complex specimens is of fundamental importance for the discovery of biomarkers and of targets for pharmaceutical intervention. Here we report on the use of LC - MALDI - MS and of the newly developed DeepQuanTR software for the identification of differentially expressed proteins in complex samples. The identification and validation of differentially regulated proteins in cancerous lesions compared to normal kidney was used to demonstrate the performance of DeepQuanTR for the comparative analysis of multiple closely related samples.

Methods

The two-dimensional peptide mapping technique is based on the separation of complex tryptic peptide mixtures by reverse-phase HPLC, the spotting of eluting fractions mixed with internal standard peptides on MALDI target plates and the analysis of these fractions by MALDI-TOF/TOF mass spectrometry. MS data are loaded into DeepQuanTR (1), normalized to the internal standard peptides and a feature extraction step is performed grouping consecutively eluting peaks with the same m/z ratio. Since the alignment of multiple samples is the crucial step for large scale proteomics comparison activities, we developed a novel alignment algorithm based on the identification of feature patterns. Finally MS2 data are added and the automated relative quantification of several hundred proteins is performed.

Results

We have used sixteen samples, corresponding to surgically - resected human kidney with cancer following ex vivo perfusion with a reactive ester of biotin (2), to evaluate the performance of DeepQuanTR and to discover tumor - associated vascular antigens. Tenascin - C, fibronectin and periostin were identified by the software as prominent antigens over-expressed in kidney cancer. These findings were experimentally confirmed by immunofluorescence staining of normal and cancerous kidney sections.
Two - dimensional peptide maps from complex biological specimens, such as the ones presented in this study, frequently comprise 5000–10000 features based on MS signals with S/N >3. When comparing 2D maps from sample replicates, the identification of cognate features not always relies on MS2 data supporting the assignment of each individual feature to a given peptide in all maps. DeepQuanTR establishes a correspondence between cognate features in homologous regions based on a pattern recognition algorithm. We experimentally checked the reliability of this procedure in our samples on the basis of features unambiguously identified by MS2 data, discovering a rate of correct identification >97%.

Innovative aspects

- Automated label-free relative quantification using MALDI-MS data
- Algorithm for the identification of cognate features in homologous regions of different samples based on pattern recognition
- Substantial reduction of analysis time

References

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P305

High resolution map of Prostaglandin E_2-dependent signaling networks

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Introduction

Prostaglandin E_2 (PGE_2) plays a crucial role in the normal physiological control of immune homeostasis as well as in inflammation and cancer immune evasion. PGE_2 is known to signal through four G-protein coupled transmembrane receptors, EP1-4, activating a multitude of potential signaling pathways. To analyze PGE_2 signaling in lymphoid cells, we introduce a multi-pronged strategy, combining temporal quantitative phospho-proteomics, stringent kinase prediction, and phospho flow cytometry to analyze signaling networks in high resolution.

Methods

Primary $CD3^+$ T cells were stimulated for 0, 10, and 60min with PGE_2 (10µM), trypsin digested, and stable isotope labeled by peptide dimethylation[1]. Labeled peptides were mixed at a ratio of 1:1:1, SCX fractionated, and subjected to online RP-TiO_2-RP-LC-MS/MS analysis. The eluted peptides were analyzed by MS^2 using a LTQ-Orbitrap mass spectrometer. Regulated phosphosites were subjected to three kinase prediction algorithms – NetworKIN, Scansite, and PHOSIDA. To multiplex the analysis we utilized phospho flow cytometry combined with fluorescent cell barcoding (FCB)[2]. Phospho-epitope specific antibodies were used to investigate signaling profiles in different cell populations from a complicated cell mixture, which were identified by using CD3, CD4, and CD8 as cell surface markers.

Results

A total of 796 unique (isotope labeled triplets) phosphopeptides could be confidently identified using online RP-TiO_2-RP-LC-MS/MS analysis. We describe the PGE_2-induced phosphoproteome by monitoring around 250 regulated phospho-epitopes. According to kinase prediction algorithms the regulated phosphosites originate from just a selected number of kinase networks. Phospho flow cytometry was used to further assess these signaling pathways, providing higher temporal resolution, at a range of PGE_2 concentrations, and in multiple subsets of lymphoid cells. Perturbation with protein kinase inhibitors identified PKA as the dominant kinase node, with CAMKII and PKB/Akt present as weaker nodes in the PGE_2 activated signaling network. The combined phospho-proteomic mass spectrometry, phospho flow, and kinase prediction analysis yielded high resolution information about the PGE_2 signaling network. Furthermore, this combinational approach allowed the mapping of temporal signaling events in several cell types simultaneously, identifying differences in signaling kinetics between $CD3^+$ and $CD3^-$ lymphocytes.

Innovative aspects

- Novel triple combination of three complimentary methodologies to study high resolution signaling networks.
- High performance phosphoproteomics is linked to high resolution phospho-signaling in single cells.
- Efficient sample use to allow the study of limited sample material such as patient material with minimal pre-analysis handling.

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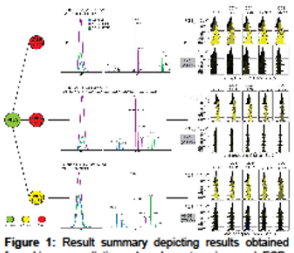

Figure 1: Result summary depicting results obtained from kinase prediction, phosphoproteomics, and FCB-phosphoflow. All three depicted substrates (VASP, GSK3α, and Hsp27) have been detected by MS and the relative changes in phospho-status at the specified sites have been determined and subsequently studied using FCB-phosphoflow to reveal high resolution kinetic information over extensive time courses in five lymphocyte cell populations in the absence and presence of a PKA inhibitor (H89). Warmer colors (yellow) indicate an increase in phospho-epitope specific antibody signal. Differences in antibody signal were calculated with the first lane in each column as reference.

P306

Automated 2D Peptide Separation on a 1D Nano-LC-MS System

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Introduction
Given the complexity of the mammalian proteome, high-resolution separation technologies are required to achieve comprehensive proteome coverage and to enhance the detection of low-abundance proteins. Among several technologies, Multidimensional Protein Identification Technology (MudPIT) enables on-line separation of highly complex peptide mixtures directly coupled with mass spectrometry-based identification.
Here, we present a variation of the traditional MudPIT protocol. We combine highly sensitive chromatography
on a nanoflow liquid chromatography system (nano-LC) with a two-dimensional pre-column in a vented column set-up. When compared to the traditional MudPIT approach, this nanoflow variation demonstrated superior first phase separation leading to the characterization of more proteins. In addition, the simpler instrumentation set-up and protocol were faster and easier to perform.

Methods
(To evaluate system performance, we analyzed a human multiple myeloma cell line proteome. Proteins were extracted, reduced and alkylated followed by trypsin digestion. The resulting peptide sample was purified and analyzed by 1D and 2D methods on a split-free nano LC-MS system (EASY-nLC, Proxeon, Odense, Denmark) coupled to LTQ-Orbitrap (Thermo-Fisher, Bremen, Germany). For the vented 2D LC approach, samples and the high salt (ammonium acetate) steps required to elute peptides from the strong cation exchange (SCX) material onto the reversed phase (RP) material were applied using the autosampler of the EASY-nLC. For each salt plug injection, eluted peptides were washed on the RP section of the two-dimensional pre-column, separated by gradient elution and subjected to MS/MS analysis.

Results
This study describes a simple automated approach to multidimensional peptide separation using SCX in the first dimension and RPC in the second dimension. We have compared this set-up to a traditional on-line MudPIT experiment and found that it characterizes more peptides, uses simpler instrumentation and hence is technically much easier to perform and automate.
In a direct comparison, it was possible to identify around 30% more proteins using the described nanoflow vented column set-up instead of the traditional MudPIT approach .
Importantly, the automated sample loading via the microplate autosampler enables the system to be operated "24/7", thus increasing sample throughput. In contrast, a manual sample loading process used for traditional MudPIT procedure is, in practice, limited to one sample per day (at least for a 9-step set-up). Many samples can be analyzed using the same pre-column/ column set-up whereas a new column is used more frequently in the traditional approach. Samples that have been run on the same column have chromatograms that are more easily aligned and make post-acquisition analysis easier.
Finally we present an abbreviated variant of this experiment which enables 3 times as many samples to be analyzed without significantly compromising data quality.

Innovative aspects
- Simple, fast nanoflow 2D-LC method for comprehensive peptide separation.

P307

Identification of Ub/Ubl chains in an integrated mass spectrometry/SpectraST/SUMmOn-based work-flow

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Introduction:

Ubiquitin (Ub) and the Ub-like proteins (Ubls) are polypeptides that are reversibly conjugated to target molecules to modulate their function. While MS is a powerful tool for the study of many types of PTMs, the identification of some types of Ubl conjugates has proven to be difficult using standard software. We developed the SUMmOn software tool and demonstrated its efficacy in the identification of Ub, SUMO and NEDD8 conjugation sites. Here we present a CPU-time optimized workflow for the identification of Ub/Ubl chains and conjugation sites using SpectraST and SUMmOn.

Method:

Ub and many Ubls (e.g. the SUMOs and NEDD8) may be conjugated to targets in a monomeric form or as polymeric "chains"; i.e. the Ub/Ubls can self-conjugate to form extended multimeric structures on protein targets. Using standard database search algorithms, along with SUMmOn analaysis, we have catalogued hundreds of collision-induced dissociation (CID) spectra corresponding to: Ub chains linked via K11, K48 and K63; SUMO-1 chains linked via K7, K16, and K17; SUMO-2 chains linked via K11 and K42; SUMO-3 chains linked via K11 and K41; yeast SUMO (Smt3p) linked via K54, and; NEDD8 chains linked via K22, K48 and K54. A library containing CID spectra of these Ub/Ubl chains was constructed and utilized to identify Ub/Ubl chains in biological samples.

Preliminary data:

A consensus spectral library was created for Ub/Ubl chains at multiple charge states, and cleaved with two different widely used proteases (trypsin and LysC). Using this spectral library, we found that SpectraST is routinely able to identify 5-20% more spectra matching Ub/Ubl chains than SUMmOn, while using much less computing power and time (~1min vs ~25min on a laptop equipped with a 2GHz Intel Core2 processor with 3GB of memory). Thus, spectral matching with our new Ub/Ubl library represents a highly efficient method for the identification of Ub/Ubl chains in a biological sample. Following SpectraST analysis, the SUMmOn algorithm may be utilized on the same dataset to identify new conjugation sites on target molecules. We thus propose that a routine MS analytical pipeline consist of; (a) a standard database search, (b) a spectral matching search and (c) SUMmOn analysis. This type of workflow can be easily incorporated into current pipelines with very little increase in analysis time, and dramatically enhances the user's ability to identify Ub/Ubl chains, while maintaining the capacity to identify novel Ub/Ubl conjugation sites on target proteins.

Innovative Aspects:

Creation and validation of a Ub/Ubl chain spectral library.

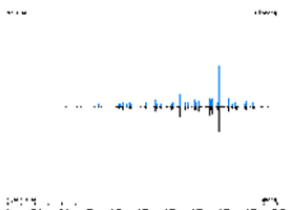

Figure 1: A sample SpectraST view of a SUMO-2 K11 linkage. The upper half illustrates the consensus spectra in the library, while the lower half shows the spectra being identified.

P308

Genetical and Chemical Proteomics

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Introduction
The sequencing of the human genome has turned our attention to the functions of the protein end-products. We want to know how proteins work together to make a cell, how they endow cells with the ability to respond to signals and organize into multi-cellular tissues of remarkable complexity, and how aberrations in protein expression or function lead to diseases such as cancer. Geneticists have traditionally approached these problems by trying to identify sets of genes that influence a trait in question.

Methods
Ten years ago, microarray technology opened a second front by making it possible to compare expression levels for genes active under a variety of physiologic, environmental or pathologic conditions. A typical study reveals up- or down-regulation of pathways associated with a phenotype (case/control) or condition (treated/untreated). Likewise, chemical genomics offers new ways to dissect protein function by producing probes that can selectively modulate protein function. In the past few years, several groups have started to combine proteomic studies with genetic and chemical-genomic procedures, with the aim of creating a new synergy between these approaches.

Results
Here, I explore methods that seek to interface protein mass spectrometry with the analysis of genetic variants, perturbagens and quantitative phenotypes to systematically explore biological systems with enhanced resolution.

Innovative aspects
- I present innovative methods to examine the effects of genetic mutation
- I present innovative methods to examine the effects of drug perturbation
- I present innovative methods to examine quantitative proteomic phenotypes

P309

Optimized Covalent Conjugation of Antibodies and Co-ImmunoPrecipitation using Magnetic Beads

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Introduction
Co-immunoprecipitation (co-IP) is a classical method used to isolate protein complexes from biological samples. Methods use antibody-antigen affinities to "pull down" a target protein that reacts specifically with an antibody from a mixture of proteins, such as cell lysates, so that the target protein's quantity, physical characteristics or interaction partners can be determined.
Traditionally, sepharose and agarose slurries have been used, but recently the use of magnetic beads has increased dramatically due to surface properties that give very low non-specific binding, shorter and simpler protocols, faster kinetics that enable the isolation of labile composites that might otherwise dissociate during long incubation times (or be damaged by proteases), and the absence of an upper size limit for the complex to be isolated. Here we show data that demonstrates optimized reagents and workflows that further improve the usefulness of magnetic beads for the efficient conjugation of antibodies and for the isolation of protein complexes.

Methods
Methods have been developed for the covalent conjugation of antibodies to surface activated magnetic Dynabeads. Conjugation efficiency and antibody functionality are followed and measured using radio-labeled assays and standard 1-D gel electrophoresis. Co-Immunoprecipitation of protein complexes is achieved using antibody-conjugated Dynabeads followed by 1-D gel electrophoresis, Western blotting and mass spectrometry.

Results
We have optimized protocols for covalent conjugation of antibodies to magnetic Dynabeads. These protocols enable an increased antibody conjugation efficiency, thus decreasing the quantity of antibody needed for co-IP workflows. In addition, the new coupling procedures maximize antibody functionality and minimize non-specific binding and antibody leakage to a level not detectable by silver staining. We also present data showing that the optimized protocols are compatible with common antibody additives and are applicable to a range of antibody species and concentrations.
Magnetic Dynabeads are ideal for co-IP. We show here the development of reagents and protocols for co-IP using Dynabeads. These scaleable, rapid procedures permit the isolation of labile protein complexes and ultra-low non-specific binding ensures the absence of contaminants. This is demonstrated by isolation of the E-cadherin complex. With Dynabeads there is no loss of beads, as there is with resins, a factor that is demonstrated to improve the reproducibility of co-IP experiments.

Innovative aspects
- Significant reduction in antibody consumption for co-immunoprecipitation workflows
- Rapid, optimized co-immunoprecipitation reagents and protocols result in ultra-low non-specific binding
- Isolation of transient labile complexes with no upper size limit

P310

Design of Recombinant Antibody Microarrays Targeting Complex Proteomes

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Introduction
High-throughput disease proteomics will play a key role to perform early and improved disease diagnostics, prognostics, and biomarker discovery. The issue of targeting complex proteomes, searching for low-abundant candidate biomarkers has, however, turned out to be a major challenge. By adopting a cross-disciplinary approach, we have successfully designed a state-of-the-art antibody microarray technology platform capable of targeting non-fractionated directly biotinylated proteomes.

Methods
The platform is based on human recombinant scFv antibodies, microarray adapted by molecular design, individually dispensed in the pL scale onto black polymer Maxisorb slides. The platform provides a miniaturized set-up (<1 cm^2) for profiling of low abundant (fM range) biomarkers in crude proteomes in a multiplexed and rapid (<3h) manner while consuming only minimal amount (uL scale) of the clinical samples.

Results
Recently, the repertoire of the platform was significantly expanded, and we are now capable of targeting most clinically relevant sample formats, including serum, plasma, urine, intact cells, cell lysates and tissue extracts. Notably, water-soluble proteins as well as membrane proteins can readily be targeted, either separately or simultaneously on the same microarray, in the end allowing true global proteome analysis to be pursued. The optimized platform, focusing on the sample formats, will be presented.

Innovative aspects
- The antibody microarray platform is capable of targeting most clinically relevant sample formats, including serum, plasma, urine, intact cells, cell lysates and tissue extracts.
- Using our optimized platform, we can simultaneously analyze water-soluble as well as membrane bound proteins on the same microarray.

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P311

A Multi-Laboratory Study Assessing Reproducibility of a 2D-DIGE Proteomic Experiment

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Introduction
Although 2DE-electrophoresis has been long used to study differential proteomics, its reproducibility has been always a major concern. In recent years, different methodological improvements have contributed to more robust 2DE workflows: use of immobilized IEF strips, fluorescence based difference gel electrophoresis (DIGE), new software tools, etc. In order to assess the reproducibility of 2DE experiments across laboratories, we set up a multi-laboratory study, performed at 11 laboratories of the ProteoRed network (Spanish network of proteomics facilities).

Methods
All participating labs received two protein extracts, prepared from cultured human adenocarcinoma MDA-MB-468 cells, treated or not with 50 ng/ml EGF (Epidermal Growth Factor) for 24h. Differential analysis was performed by a 4-gel 2D-DIGE experiment, using 4 technical replicates of each sample, with Cy dye swapping. Strictly defined 2DE conditions were followed by all labs. Each lab selected the 30 spots presenting the highest fold variations (with $p<0.05$), and attempted MS protein identification..

Results
The results demonstrate a very good within lab and across lab reproducibility. Within labs, 70-93% detected spots present %CV <20%, and 7-37 %CV <5%. Across all labs, around 72% and 32% of spots show %CV <20% and <10%, respectively. Selection of differentially expressed spots shows good reproducibility across labs, although there is a certain degree of subjectivity in the selection, as each lab applied its own filtering criteria. Overall, 19 spots were ranked among the top-30 by at least 3 labs, and 17 by at least 4. MS protein identification was, on average, 60% successful, with 13 spots identified by at least 3 different labs. In those cases, identical gel locations corresponded to the same protein Id.

Innovative aspects
- Wide multi-laboratory experiment, showing robustness of 2D-DIGE technology on a comprehensive experiment, from 2DE, to image analysis and MS protein identification.
- Demonstrating feasibility of across lab validation schemes and QC strategies in proteomic research.

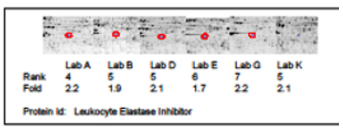

Figure 1. Identical gel location of a differentially expressed spot selected for identification by 6 different labs. An identical protein Id. (Leukocyte elastase Inhibitor) was obtained by all labs. Both the rank position and the measured fold change (Treated vs. Control) were very similar.

P312

Digital Microfluidics: An Automated, Integrated Platform for Protein Extraction and Processing

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Introduction

Clinical proteomics has emerged as an important new discipline, promising the discovery of biomarkers that will be useful for early diagnosis and prognosis of disease. While clinical proteomic methods vary widely, a common characteristic is the need for (i) extraction of proteins from extremely heterogeneous fluids (i.e. serum, whole blood, etc.) and (ii) extensive biochemical processing prior to analysis. Here, we report a new digital microfluidics (DMF) based method integrating several processing steps used in clinical proteomics. This includes *protein extraction, resolubilization, reduction, alkylation* and *enzymatic digestion*. DMF, a technique characterized by the manipulation of discrete microdroplets (100nL – 10μL) on an array of electrodes [1], is a good match for carrying out protein processing at room temperature, allowing parallel, automated analysis of samples. This represents a significant advance over conventional methods (relying on manual pipetting or robots), and has the potential to be a useful new tool in clinical proteomics.

Methods

DMF devices were fabricated using established methods [2,3]. Briefly, chromium electrodes were patterned on glass by photolithography; the electrodes were then coated with 2 μm of Parylene-C and 50 nm of Teflon-AF. Unpatterned indium-tin oxide (ITO) coated glass substrates were also coated with 50 nm Teflon-AF. Devices were assembled with an ITO-glass top plate and a patterned bottom plate separated by a spacer. Droplets were sandwiched between the two plates and actuated by applying driving potentials (80-150 V_{RMS}, 18 kHz) between electrodes on the top and bottom plates.

Fluorogenic assays were used to quantitatively evaluate recovery of extracted proteins and reproducibility of each reaction step. Quantitation was achieved by positioning DMF devices on multiwell plates and inserting them into a fluorescence microplate reader (Pherastar, BMG Labtech, Durham, NC). Electrospray ionization – mass-spectrometry (ESI-MS) and matrix-assisted laser desorption ionization – mass spectrometry (MALDI-MS) were used to assess purity of extracted proteins and reaction progress.

Results

To evaluate compatibility of protein extraction with DMF, both effectiveness and percent recovery were assessed; a full description of this work can be found in Jebrail and Wheeler [2]. Briefly, ESI-MS was used to assess purity of protein extracted from a mixture containing a "contaminant" phospholipid present at 80× excess. In addition, a fluorogenic assay was used to quantitatively evaluate recovery of extracted proteins. In all cases >80% recovery was obtained.

To illustrate the ability of carrying out multistep proteomic sample processes on DMF MALDI-MS was used for qualitative confirmation of reaction steps, including reduction, alkylation and digestion. Fluorogenic assays were used to quantitatively evaluate reproducibility and progress of each reaction step. A description of this work can be found in Luk and Wheeler. [3]

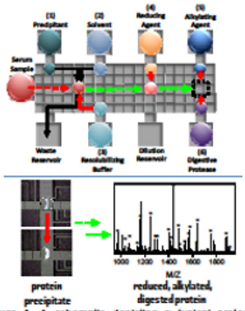

Figure 1. A schematic depicting a typical proteomic sample processing workup. (top) Images of protein precipitation on DMF and MS spectra of DMF-driven digest. (bottom)

Innovative Aspects:

- First DMF-driven method for processing protein(s) in heterogeneous mixtures
- Flexibility of integrating several processing step on one platform
- Great potential for automated clinical proteomic biomarker discovery

References

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P313

Workflow for maximizing protein identifications using multiple re-injections and multiple search engines

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Purpose: To develop strategies for both data acquisition and data mining for maximum proteome coverage

Introduction

Identifying a maximum number of proteins with the largest sequence coverage in a complex sample with a minimum of effort has always been a challenge. Hereby we describe a method that combines an automated intelligent acquisition method on an LTQ Orbitrap XL using different dissociation techniques with a data processing workflow that uses multiple search engines to increase the number of identified proteins to a maximum.

Methods

Use CID and ETD as acquisition strategies in combination with dynamic exclusion list generation for multiple sample injection, and multiple search engines for data analysis. The sample, a LYS-C digest of Yeast is injected multiple times. After each injection all precursors that have been selected for MS2 are added to the exclusion list of the next run automatically. The consecutive runs are searched with 4 different algorithms, Sequest, ZCore, Mascot and Peaks DeNovo and summarized.

Results

Use of complementary dissociation techniques, exclusions lists and use of multiple search engines increase proteome coverage.

References

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(2) Swaney et al., Decision tree-driven tandem mass spectrometry for shotgun proteomics, Nat. Methods, 5, 959 (2008)

P314

Optimization of Peak Capacity in One- and Two-dimensional nanoLC

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Introduction

The higher peak capacity of two-dimensional (2D) LC separations is often sacrificed for the ease of use of a one-dimensional (1D) approach. This has resulted in extremely long gradients (up to 4 hours) under conditions that waste analysis time, without significantly improving the separation. In order to obtain the best compromise between peak capacity and analysis time for highly complex proteomic samples it is necessary to optimize column technology and LC conditions in one- and two-dimensional LC. We use the "peak-capacity-to-analysis-time ratio" to characterize the efficiency of a chosen separation method.

Methods

The effects of gradient time, column temperature, flow rate, and column length were studied to maximize the peak-capacity-to-analysis-time ratio for peptide samples in one-dimensional RP gradient nanoLC. These findings were compared to an offline 2D-LC approach using SCX and RP in which effects of ^{1}D gradient time, sampling time, and ^{2}D gradient time on performance were studied. The 2D-LC approach was developed with a microfractionation option of the autosampler allowing automated fractionation and reinjection of the separated peptides providing maximum ease of use.

Results

Optimal 2D-LC conditions at undersampling conditions were obtained applying a short (10 min) ^{1}D gradient and 20 min ^{2}D RP gradients. For separations requiring a maximum peak capacity of 360 1D-LC was superior to the off-line 2D-LC approach in terms of analysis time. For separations requiring a peak capacity higher than 360 it is more profitable to perform a 2D experiment. With the right conditions this will provide a higher peak-capacity-to-analysis-time ratio. Finally, the potential of the optimized offline 2D-LC coupled to tandem mass-spectrometric detection is demonstrated by the analysis of a tryptic digest of a 20 protein mixture and an E. coli tryptic digest.

Innovative aspects

- Optimized 1D and 2D LC methods in proteomics.
- Evaluation of efficiency for various popular separation strategies in proteomics
- Automation of off-line 2D-LC

P315

Improving the Specificity of Peptide identifications from MS/MS Spectra Using a Novel Transition-based Correlation Algorithm (Tcorr)

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Introduction

Effective methods are needed to identify and quantify proteins present in complex biological samples with high confidence and sensitivity, for example putative biomarkers for clinical screens. Coupled with liquid chromatograph, tandem mass spectrometry (LC-MS/MS) is currently the main approach for unveiling the proteome of the complex biological samples. Newer, targeted driven procedures, such as TPM (Targeted Peptide Monitoring) and MRM (Multiple Reaction Monitoring), are increasingly attractive technologies for tracking low abundance targets (1). A major challenge is how to design multiplex assays that are capable of monitoring multiple targets in an optimal manner across different samples and how to interpret the resulting MS/MS data with high accuracy and high confidence. We report a breakthrough addressing each of these important issues.

Methods

In our previous study (2), we introduced a novel statistical scoring measure, termed Tcorr (Transition-based Correlation), to identify target peptides by matching MS/MS spectra to a reference spectral library that is based only on a pre-selected short list of particularly informative fragment ions. However, while it is exceptionally sensitive to ion intensity patterns, Tcorr can also generate incorrect matches, esp. when only a few ions are monitored (as is typical during MRM experiments). In the light of this, we use the normalized intensity sum from the monitored products ions (typically the b- and y-ions) as a secondary measure to cluster spectra of a particular LC-MS/MS run to markedly improve target specificity. In particular, the formula below is used to detect the apex of peptide elution:

$$T = \arg\max_t (Tcorr(t) * (normInstSum)$$

Where t is the retention time and NormInstSum is the intensity sum of transition ions, normalized by the total ion current of the whole spectrum at t for TPM spectra, or by the maximum transition sum throughout the LC/MS runs for MRM data.

Results

A reference MS/MS library was created using synthetic peptide arrays to monitor transcription factor regulatory networks in mouse embryonic stem cells (3). To compare targets across different samples, four 'sentinel' peptides were used as internal standards to align entire LC chromatographs using linear regression. To validate our methods, the sentinel peptides were analyzed in quadruplicate on 3 different microcolumns using 45 min gradient for a total of targeted LC-MS/MS experiments. Using the b-/y-ions were used as transitions, we applied our Tcorr algorithm to identify specific targets with the normalized intensity sum described above. **Figure 1** illustrates the results for one representative LC-MS/MS run. Stringent thresholds were imposed to Tcorr and the intensity sum ratio, respectively. Upon such constraints, the bottom subplot reveals the most reliable target identifications, falling with a small elution time window. Similar processing was likewise repeated for each of sentinel peptides in the 12 datasets. An apex was also determined using the above formula. **Figure 2** demonstrates the stability of peptide elution time, implying a linear relationship between different columns.

Innovative aspects

- Boosting the specificity of Tcorr identifications.
- Improving the prediction of target elution time for scheduled multiplex TPM/MRM aasays.
- Enabling 'on-the-fly' data dependent data acquisition for TPM/MRM assays.

References

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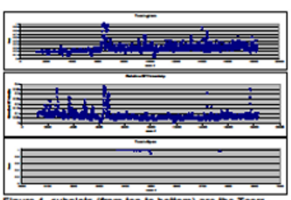

Figure 1. subplots (from top to bottom) are the Tcorr-gram, relative transition intensity sum graph and the resultant high confidence peptide identification in a elution time window (in terms of scan #).

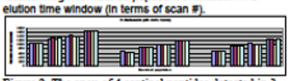

Figure 2. The apex of 4 sentinel peptides detected in 3 different columns (45 gradients, each with 4 repeats) validates the strong reproducibility of retention time patterns within different runs, and linear relationship between different columns.

P316

A red line not to cross: evaluating the limitation and properness of gel image tuning procedures

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Introduction
Presently results of gel electrophoresis are commonly documented in digital formats by image acquisition instruments. In this study, gel images tuned by a common image processing software package, Photoshop, were assessed to understand the transforming algorithms and their impacts on quantitative analysis. TotalLab 100, an electrophoresis gel image analysis software package, was applied for image quantitation and evaluation. Three most frequently used image tuning functions, including adjustment of the brightness, the contrast and the grayscale span (level) of images, were investigated using both data generated from a standard grayscale tablet and an actual electrophoresis gel image. The influences of these procedures were analyzed for the grayscale transformation between the input and output images.

Methods
(1) Software: Photoshop CS was used to adjust the brightness, the contrast and stretch the grayscale span of input images. TotalLab 100 (Nonlinear Dynamics, Durham, NC, USA) was used to analyze the quantitative data of all images.
(2) Preparation of the grayscale tablet image: To assess the algorithms of the above three tuning functions, a standard image of the grayscale tablet (No.2, Kodak) was used for evaluation.
(3) Preparation of the gel image: Two-fold serial dilution of a low molecular weight standard protein marker (GE Healthcare) was loaded from 8 µg to 15.6 ng per well and separated by 15% SDS-PAGE and then developed by imidazole-zinc reverse stain using VisPro 5 minutes protein stain kit (Visual Protein).

Results
(1) Although all three procedures differentially improved the visualization of the input image, adjusting the contrast of images disrupted the quantitative information because of its non-linear transforming algorithm (Fig. 1D and 1E).
(2) Under certain conditions, adjusting the brightness or the level of images could preserve the quantitative information because of the linear transforming algorithms (Fig. 1A 1B and 1F). It was found that when the minimum and maximum grayscale of a gel image were recognized, using a commercial software package to maximally stretch the level may significantly improved the quality of gel image without jeopardizing quantitative analysis (Fig. 1F).

Innovative aspects
A methodology for qualitatively tuning gel images with quantitative information preserved.

References
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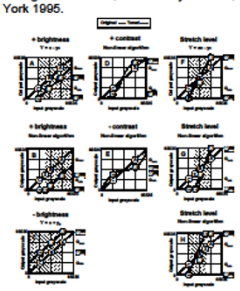

Fig 1. Diagram representations of the transforming algorithms for the three image tuning procedures. Possible transforming outcomes of an image that has been (A-B) increased for brightness by a linear (A) or a non-linear algorithm (B), (C) decreased for brightness by a linear algorithm, (D) increased for contrast, (E) decreased for contrast, or (F-H) stretched for the grayscale span by a linearly (F) or a non-linear algorithm (G-H). Symbol usages: White circles A, B, and C represent pixels in the original image with the highest (G_{max}), the medium, and the lowest (G_{min}) grayscale value respectively. Closed circles A', B', and C' represent the corresponding pixels in the tuned image with the highest (G'_{max}), the medium and the lowest (G'_{min}) grayscale value respectively. Solid lines are the non-transformed curves (Original, where Y=X); while dashed lines are transformed curves (Tuned). Hatched areas stand for the range of grayscale span in which a linear transformation can be obtained.

P317

Streamlined in-gel protein digestion with a mass spectrometry-compatible surfactant

From gel slice to mass spec analysis in 3 hours or less

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Introduction
In-gel protein digestion is a commonly used approach in preparation of samples for mass spectrometry-based identification of proteins. As a sample preparation method gel electrophoresis provides unparalleled fractionation of a mixture while simultaneously facilitating the removal of impurities such as protease inhibitors, salts, detergents or adjuncts added for sample isolation that are not compatible with MS analysis. While the typical in-gel digestion process is well-established, the procedure is lengthy and requires many manipulations that can lead to sample loss and provide the potential for contamination. We report here a streamlined process that eliminates post-digestion extraction without sacrificing sequence coverage or probability scoring reducing total process time from gel slice to mass spec analysis to 3 hours or less.

Methods
To substantially reduce process time and improve peptide recovery without additional extraction we have optimized several parameters for the in-gel digestion process through the use of MALDI and LC-MS compatible surfactant Sodium 3-((1-(furan-2-yl)undecyloxy)carbonylamino)propane-1-sulfonate (ProteaseMAX™). To demonstrate these improvements we have contrasted the new surfactant-assisted protocol steps against the conventional steps of in-gel digestion. Several models that range from a single globular protein (i.e. BSA) to mouse and human protein extracts have been used to demonstrate the ability of the streamlined in-gel digestion process to give consistent and reproducible results when compared to the conventional process. Mass spec methods included MALDI-TOF/TOF and LC-MS/MS (ion trap and LTQ). Relative performance of the contrasted methods is judged on sequence coverage and probability scoring.

Results
In-gel digestion normally involves overnight incubation followed by laborious peptide extraction. In the presence of the surfactant, digestion required no longer than one hour. In addition, during the digestion process the surfactant facilitates peptide extraction eliminating the need for post-digest extraction and drying. As a result, protein digestion and peptide recovery is complete in a single one hour step. In-gel digestion data quality is significantly improved by the recovery of longer peptides that typically remained trapped in gel under conventional conditions. With surfactant aided digestion and extraction peptides in the range of 2500 – 4000Da are routinely recovered in quantities sufficient for MS/MS analysis. An investigation of an assortment of proteins of mouse or human origin showed that this simplified protocol provided reliable and robust protein identification with increased sequence coverage in many cases.

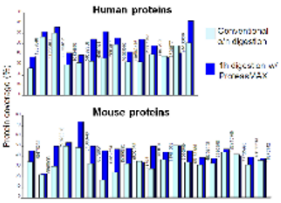

Figure: **In-gel protein digestion with ProteaseMAX versus conventional digestion.** Human or mouse proteins from cytoplasmic and membrane extracts were resolved with SDS-PAGE, stained with Coomassie Blue, in-gel digested and analyzed with an AB 4800 MALDI-TOF/TOF. Higher protein coverage was achieved with ProteaseMAX™ surfactant in most cases. This was due in part to improved recovery of longer peptides

Innovative aspects
- From protein gel band to mass spec analysis in under 3h due to rapid protein digestion and elimination of peptide extraction step
- Extraction of long peptides from polyacrylamide gels in the range of 2,500-4,000 Da in quantities sufficient for MS/MS analysis

P318

Efficient methods for avoiding human keratin contaminations during gel-based proteomics analysis

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Title:
Efficient methods for avoiding human keratin contaminations during gel-based proteomics analysis

Introduction
To avoid human keratins contamination happened, all of the steps of gel-based proteomics analysis were examined, to make sure of what mechanism was essential for the keratin contamination. By using the static eliminator combined with our modified procedures, the keratin contamination was drastically eliminated. In this presentation we report a practical, easy applicable and efficient procedure to avoid the keratin contamination during the in-gel digestion for proteomics analysis.

Methods
Protein of human glomerulus was extracted; 20ug proteins each was separated in several lanes on a 12.5% polyacrylamide gel. Each lane was divided into 15 aliquots followed with in-gel digestion, digested peptide samples were analyzed by mass spectrometry using Agilent 1100 series LC-MSD under a 120 min. -gradient of H2O-MeOH, and the MS data were processed by Spectrum Mill software, identified keratin in each analysis was considered as contamination.

Results
We modified some operations of in-gel digestion, and an efficient protocol for avoiding human keratin contamination by using static electricity eliminator was reported in this presentation.

Innovative aspects
- A practical, easy applicable and efficient procedure was reported
- Shorten in-gel digestion procedure
- Avoid the human keratin contamination satisfactorily

References
1. Two-dimensional electrophoretic profiling of normal human kidney: differential protein expression in glomerulus, cortex and medulla, Bo Xu, Ying Zhang, etc., pp 5-13, Vol. 49 (2005) , No. 1, *Journal of Electrophoresis*
In-Depth Proteomic Profiling of the Normal Human Kidney Glomerulus Using Two-Dimensional Protein Prefractionation in Combination with Liquid Chromatography-Tandem Mass Spectrometry. Masahito Miyamoto, etc., *J. Proteome Res.*, 2007, *6* (9), pp 3680–3690
Authors: Bo Xu, Shuichiro Shimada, Ying Zhang, Sameh Magdaldin, Hidehiko Fujinaka, Yutaka Yoshida, Eisin Yaoita, Tadashi Yamamoto
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Abstract Body:
Gel-based separation of large-scale proteins is the most popular procedure for proteomics analysis. During this critical procedure, contamination of human keratins to specimens becomes a frightful problem, especially for accurate mass analysis of small amount proteins. To solve this problem, we have tried many procedures generally described in the textbooks of proteomics and protocols reported form other laboratories. However, the keratin contamination was not able to eliminate satisfactorily. Therefore, we examined all of the steps of gel-based proteomics analysis from gel preparation, electrophoresis, staining, gel cutting, in-gel digestion to mass spectrometry and found critical steps to have the contamination. Of course, all reagents should be prepared without contamination, and researchers need to wear gloves, hair cap, lab coat, and mask. To avoid contamination, we minimized time of the procedure by using dispensers and vacuum suction for liquid handling, and washed fingers at several steps, and avoided adherence of dusts to sample tubes. We found that the keratin contamination mostly happened at the steps of in-gel digestion and that the course was considered to be adhesion of air dusts due to static electricity. To reduce the static electricity from the bench, equipments and sample tubes, a static eliminator was employed. We compared 2 sets of proteomics analysis data of human glomerulus proteins; one was obtained by using the static eliminator and the other was not. Human glomerular protein of 20 mg each was separated in several lanes on a 12.5% polyacrylamide gel, each lane was divided into 15 slices and each slice was subjected for in-gel digestion. Peptides were extracted, and analyzed by mass spectrometry using Agilent 1100 series LC-MSD and the data were processed by Spectrum Mill software. By using the static eliminator combined with our modified procedures, the keratin contamination was drastically eliminated. None or few human keratins were identified with very low level of peptide spectral intensity. A practical, easy applicable and efficient procedure to avoid the keratin contamination during the in-gel digestion for proteomics analysis is introduced in the presentation.

P319

High Throughput Antibody Development Platform

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Introduction
With the expansion of proteomics, exploring human proteomes is becoming a priority for the research community (1). To accompany this growing field, probes of high quality and specificity such as Monoclonal Antibodies are needed as well as processes enabling high throughput selection of these probes in a short time. In this way, our laboratory set up a unique robotic platform for the high throughput selection and characterization of high quality monoclonal antibodies (HT-MAb) useful to investigate function of protein and identify potential new biomarkers coming from proteomics.

Highlights of HT-MAb
- A unique process from immunogen design to MAb purification
- 8 automatic fusions in parallel (1920 seeded wells/fusion)
- Monoclonality ensured at seeding
- High throughput primary screening of hybridoma by protein microarray (4 antigens in parallel)
- Early antibody characterization: epitope mapping, antibody pairs identification by SPR directly on supernatant, isotyping
- Post fusion study
  - Study of sensitivity & specificity
  - Sub-cloning to get stable clones
  - Production and purification
  - Kinetics study by SPR (Ka, Kd, KD)

Total process to get MAbs: 4-6months

Deliveries:
Per target: up to 10 specific and sensitive monoclonal hybridoma stable in secretion (putified Mabs + frozen cells)

Projects and results presented on poster
- Microbiology: selection & characterization of high affinity MAb to C. Difficile: from immunization to diagnosis kit
- Diabetes: characterization of high affinity MAb to specific variants of Hemoglobin

Innovative aspects (maximum of 3 bullet points)
- Short cut in development of high quality and sensitive reagents to identify highly specific epitopes

References (maximum 3 references)
1- S. Hobet et al. Human protein atlas and the use of microarray technologies. Curr Opin Biotechnol. 2008, 19, 30-5
2- O. Nahshol et al. Parallel kinetic analysis and affinity determination of hundreds of monoclonalantibodies using the ProteOn XPR36. Anal Biochem. 2008, 383, 52-60

P320

New Developments in the ProteoWizard Open Source Software Library

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Introduction

The ProteoWizard open source software project has two components: 1) a set of C++ libraries to facilitate cross-platform proteomics tools development and 2) a set of cross-platform data analysis tools created using those libraries.

Since its initial release 18 months ago, the project has undergone significant development, with major contributions from the Center for Applied Molecular Medicine at University of Southern California, the Vanderbilt University Mass Spectrometry Research Center, the MacCoss Lab at University of Washington, Insilicos Software, and the Institute for Systems Biology. ProteoWizard is available for both commercial and non-commercial use, under the permissive Apache v2 open source license.

Methods

ProteoWizard was written from the ground up using modern software design principles and advanced C++ techniques. The codebase was designed to be testable and extensible, in order to maintain stability while undergoing continual development. ProteoWizard is cross-platform, using native compilers, including Microsoft Visual C++ on Windows, Xcode on OSX, and gcc on Linux. ProteoWizard uses the new HUPO-PSI standard mzML as its native data format. In addition, the ProteoWizard team has active, ongoing relationships with major instrument vendors to support their raw data formats. ProteoWizard's data abstraction layer handles the details of the underlying data file format and operating system, allowing the proteomics software developer to focus on data analysis algorithms.

Results

Many software tools are using ProteoWizard for mzML support, including the Trans-Proteomic Pipeline (TPP) and the Computational Proteomics Analysis System (CPAS).

ProteoWizard now has support for the data formats from the following instrument vendors: Thermo, Waters, Bruker, Agilent, and Applied Biosystems.

New analysis modules have been added to ProteoWizard, including data smoothing, peak picking and charge state calculation, intensity thresholding, precursor recalculation, chromatogram calculation, and ms1 feature finding. All new modules include general interfaces to facilitate use and comparison of multiple algorithms.

The ProteoWizard tools incorporate all the new features of the library. For example, MSConvert is a general data format conversion tool that also allows filtering and processing for subsets of spectra. MSPicture supports static visualization of ms1 data with demarkation of pepXML ms2 identification information. MSAccess allows scriptable command-line access to spectra and metadata. MSMatchMaker matches ms1 features to ms2 identifications across multiple runs. SeeMS is a Windows program that provides straight-forward browsing and visualization of any data format supported by ProteoWizard. Skyline is a Windows application for building SRM methods.

Innovative aspects

- Major instrument vendor formats are supported, including Thermo, Waters, Bruker, Agilent, and Applied Biosystems.
- Many new analysis modules have been developed.
- ProteoWizard provides a mature and open source framework for proteomics tools development and is being actively used and extended by a community of developers internationally.

References

(1) Darren Kessner, et al, ProteoWizard: Open Source Software for Rapid Proteomics Tools Development, Bioinformatics 2008
(2) http://proteowizard.sourceforge.net/

P321

Characterization of host cell protein patterns to increase process understanding

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Introduction

Many pharmaceuticals, such as monoclonal antibodies are produced from genetically engineered mammalian cell lines. Purity with regard to undesired host cell proteins of final product is essential for product safety. Process development by varying upstream conditions (cell clone and media composition) and analyze the effects on downstream processing (purification) is needed for optimal yield and purity. We have characterized host cell protein patterns in purification of monoclonal antibodies from CHO cells by using 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE).

Methods

The 2-D DIGE technology with two different samples and a pooled internal standard per gel pre-labeled with CyDye™ DIGE Fluor minimal Dyes, detects differences in protein abundance. Experimental variation is virtually eliminated and the quantitative data is very reliable. Samples from culture supernatant or MabSelect SuRe™ eluent fractions, were buffer exchanged into DIGE labeling buffer using a Vivaspin column MwCO 3 kDa. Protein samples, as well as a pooled internal standard were labeled with CyDye minimal DIGE fluors and subjected to 2-D electrophoresis using IPG strips pH 3-11NL and the new pre-cast DIGE Gels followed by scanning using Typhoon™ Imager. Differences in protein levels between the samples were analyzed using the DeCyder™ 2D differential analysis software v7.

Results

Differences in protein expression between culture supernatants grown with a set of altered media compositions were analyzed. Also, differences in the HCP patterns of MabSelectSuRe™ eluent fractions were analyzed. The results were related to yield of target protein and HCP levels obtained with ELISA assay.

Innovative aspects

- Use of a 2-D DIGE proteomic approach for improved process understanding during production of biopharmaceuticals
- Analyse host cell protein patterns in chromatography eluate fractions
- Linking complex samples spot maps with fractionated samples spot maps by using the pooled DIGE internal standard.

References

1) Ettan DIGE System User Manual, 18-1173-17, GE Healthcare
2) 2D Electrophoresis Principle and Methods, Hand book, 80-6429-60, GE Healthcare

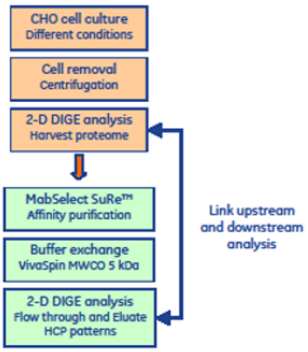

Figure 1. Workflow of experiments.

P322

Functional protein analysis using DNA based cell-free protein synthesis systems

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Introduction

In the post-genomics era, efforts are now being directed to investigate the proteins encoded by genomes, including efforts to elucidate the structure, function, and interacting networks of proteins. These analyses require expression systems to produce sufficient amount of protein and methods for efficient target recovery and detection. Cell-free protein synthesis, with its adaptability to high throughput operations, has emerged as a viable alternative for both functional (1) and structural proteomics, especially when combined with HaloTag® technology (2), a fusion tag that enables protein expression in multiple systems and efficient protein detection and recovery.

Methods

Using four DNA based cell-free protein expression systems prepared from different organisms (S30 extract from *E. coli*, insect cell extract, wheat germ extract and rabbit *reticulocyte* lysate), protein expression and function can be quickly analyzed. In these study, proteins were expressed as HaloTag® fusions for efficient purification, protein immobilization, and for studying protein function, such as enzymatic activity and protein-protein interaction.

Results

High level protein expression was achieved in the S30 T7 High Yield System, and protein can be purified when expressed with an N-terminal HaloTag® or metal affinity purification tag. TNT SP6 Wheat Germ High Yield System provided a platform for producing full-length, soluble mammalian protein. Stable isotope and seleno-methionine labeling also were achieved with a modified wheat germ extract (3). TNT T7 Insect Extract System enabled functional mammalian protein (e.g. kinase) expression and activity analysis. TNT T7 Quick rabbit reticulocyte system supported quick screening of protein targets when cloned into common T7 driven vectors. HaloTag® technology was used for target expression in cell-free systems to detect protein expression, immobilize protein targets and study protein function, such as protein-protein interactions.

Innovative Aspects

1. A comprehensive platform of DNA based coupled transcription/translational systems prepared from different organisms can greatly simply the process of protein expression and functional screening, with the following attributes:
   - Time savings: obtain protein in hours rather than days
   - Open system: possibility to add auxiliary components
   - Scalable reactions: adaptability to high throughput analysis
2. The combination of HaloTag® technology and cell-free protein synthesis enables quick protein expression, efficient protein immobilization and recover, which can significantly accelerate downstream analysis.

References

(1) P Braun et al, High throughput protein production for functional proteomics. Trends Biotechnol. 2003 21:383–388
(2) GV Los et al, HaloTag: a novel protein labeling technology for cell imaging and protein analysis. Chem Biol. 2008 3:373-82.
(3) KQ Zhao et al, Functional protein expression from a DNA based wheat germ cell-free system. J. Struct Funct Genomics 2007 8:199-208

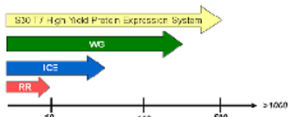

Figure 1. Range of Protein expression levels from different cell-free systems. WG: TNT SP6 Wheat germ High Yield System; ICE: TNT T7 Insect Extract System; RR: TNT T7 Quick rabbit reticulocyte

P323

Increased reproducibility and resolution with the new pre-cast DIGE Gels

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Introduction

The Ettan™ DIGE system is widely used for differential analysis proteins in complex samples because it delivers very statistically accurate and reliable data than traditional single post-stained gels. New pre-cast DIGE Gels from GE Healthcare is now available that improves reproducibility and ease of use for the user. The gels have a shelf life of 12 months. In this study we show the performance and features of the new DIGE Gels.

Methods

Samples and a pooled internal standard were pre-labeled with CyDye™ DIGE Fluor minimal dyes and subjected to 2-D electrophoresis using the new pre-cast DIGE gels followed by scanning using Typhoon™ Imager. Differences in protein levels were analyzed using the DeCyder™ 2-D differential analysis software v7.

Results

DIGE Gel provides high sensitivity and high resolution protein spot maps.
We show that DIGE Gel run with DIGE Buffer Kit resulted in spot maps with higher reproducibility compared to homecast polyacrylamide gels, thus reducing technical variation between gels. Using the DIGE Buffer Kit, together with DIGE Gels, resulted in superior protein spot maps with focused and clearly resolved protein spots, even in the low molecular weight region. The separation pattern of DIGE Gel and DALT Gel 12.5 resulted in very similar protein spot maps, and the DIGE Buffer Kit is compatible with both types of pre-cast gels. DALT Gel 12.5 is recommended for the preparative workflow.

Innovative aspects

- New DIGE Gel and DIGE Buffer Kit offer increased performance compared to home cast Laemmli gels.
- New DIGE gels and DIGE Buffer Kit have a shelf life of at least 12 months.

References

1) DIGE Gel and DIGE Buffer Kit, Data file, 28-9480-26, GE Healthcare
2) 2D Electrophoresis Principle and Methods, Hand book, 80-6429-60, GE Healthcare
3) Ettan DIGE System User Manual, 18-1173-17 03/2006 GE Healthcare

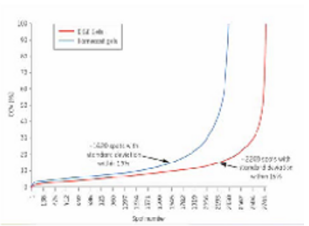

Figure 1. Higher reproducibility with DIGE Gel: DIGE Gels run with DIGE Buffer Kit or homecast polyacrylamide gels run with Laemmli buffer. Eight technical replicates of DIGE Gels, and six technical replicates of homecast polyacrylamide gels. Log abundance for all matched spots exported from DeCyder Biological Variation Analysis (BVA) module, recalculated to covariance (%) and plotted.

P324

NCI's Antibody Characterization Pipeline

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One of the most significant roadblocks of diagnostic techniques is a lack of high quality and well characterized protein affinity capture reagents (antibodies). This barrier was recognized by the NCI, which led to the development of an antibody characterization pipeline, as part of the Reagents & Resources component of its Clinical Proteomic Technologies for Cancer initiative (CPTC; http://proteomics.cancer.gov). The pipeline developed by CPTC involves production of cancer related proteins, generation of antibodies, and antibody characterization/validation. Presently, Argonne National Laboratory expresses and purifies cancer related proteins from a target set of 1261 candidates (Polanski & Anderson, *Biomarker Insights* 2006;2:1-48). Each of these purified proteins then goes to private laboratories for the generation of three monoclonal antibodies to each protein target. NCI-Frederick, Harvard Institute of Proteomics, Human Protein Atlas and the Tissue Array Research Program at CCR each perform characterization of the antibodies (including isotyping, ELISA, Western Blot, Immuno-Mass Spectrometry, Surface Plasmon Resonance, Tissue Microarrays and Immunohistochemistry).

Antibodies go to NCI-Frederick for standard operating procedure driven characterization assays. Promising antibodies are then evaluated by Human Protein Atlas and Harvard Institute of Proteomics for further analysis. Antibodies and hybridoma cells are made available to the research community through the Developmental Studies Hybridoma Bank at the University of Iowa. DNA clones will be available through Harvard Institute of Proteomics. To date, over 80 cancer-related antigens have been selected with partnerships involved in the antigen production, antibody production/characterization and distribution. We currently have over 65 mAbs to ~30 targets available on our reagents portal (http://antibodies.cancer.gov). This program acts as a catalyst to spur the development of pivotal resources (such as antibodies) that serves the research community, public and private entities, and international collaborations, needed to accelerate biomarker discovery and validation, translational research, molecular diagnostics, and therapeutic monitoring.

P325

Comprehensive Analysis of Extracellular Growth Substrate Matrigel Reveals Diverse Protein Content

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Introduction
Matrigel is the trade name for a gelatinous protein mixture secreted by mouse tumor cells. This mixture resembles the complex extracellular environment found in many tissues and is used by cell biologists as a substrate for cell culture including stem cells[1]. Matrigel consists mainly of laminin, collagen IV, and enactin. Several growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin growth factor 1 (IGF-1), and transforming growth factor beta (TGF-β) have been detected in Matrigel [2]. The discovery of these growth factors was antibody based, limiting the depth and comprehensiveness of the dataset obtained. In this study we employed a sensitive detection technique such as liquid chromatography coupled to mass spectrometry to provide a comprehensive proteomic profile of Matrigel samples.

Methods
Matrigel samples representing 3 separate batches were obtained from BD Biosciences (Mississauga, ON, CA). Two of the three batches were growth factor reduced (GFR) Matrigel, whereas the other was non-growth factor reduced (nGFR). Matrigel samples were divided into 1mg/ml aliquots based on protein concentration determined through Bradford assay. Aliquots were solubilized in solutions of 8M Urea and 200mM Ammonium Bicarbonate prior to separation using size-exclusion chromatography (SEC) on a BioSep-SEC-S2000 column (Phenomenex) equipped with a SecurityGuard precolumn (Phenomenex). SEC fractions were digested using in-solution digest with trypsin and desalted. Trypsin-digested peptides were injected into a UPLC-QTOF MS/MS system (Micromass) for characterization utilizing an iterative exclusion (IE) method for 6 rounds to obtain MS data on low abundance proteins. Alternatively, Matrigel aliquots were precipitated using a 15% $(NH_4)_2SO_4$ solution, the supernatant was collected, dried, and protein reconstituted in a 100mM ammonium bicarbonate solution. These samples were digested as above, or run on an SDS gel and extracted using an in-gel digestion method.

Results
Mass spectrometry analysis of the Matrigel samples resulted in the identification of several proteins not previously detected in Matrigel. These identifications were compiled through a combination of SpectrumMill, XTandem, and PEAKS 5.0 software. The most abundant peptides in all samples are those of laminin, regardless of fraction or extraction method used. As a result of the high number of laminin peptides present, numerous exclusion rounds had to be employed in order to detect lower abundance proteins after an in-solution digest protocol. Samples that were treated by the ammonium sulfate precipitation provided a larger number of protein identifications. From this dataset we were able to identify previously known growth factors, as well as growth factors such as Insulin Growth Factor-2 (IGF-2) which were not previously known. The dataset also contains a large number of other proteins that likely play a role in a diverse number of cellular processes.

Innovative aspects
- First comprehensive protein analysis of Matrigel using IE-MS.
- Dataset that will be valuable to a wide range of researchers.

Figure

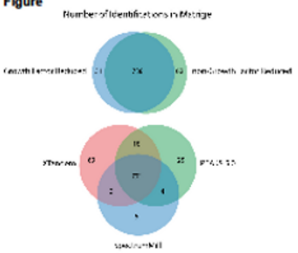

Figure 1 – Comparison of the number of protein identifications found in different types of Matrigel. Second graph depicts overlap between identifications made in the search engines used in the study.

References
1. Kleinman, H.K. *et al.* (1982) *Biochemistry*, 21(24): 6188-93
2. Vukicevic, S. *et al.* (1992) *Experimental Cell Research*, 202(1): 1-8

P326

Exploration of the Phospho-Tyrosine Proteome Using SH2 Domains

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Introduction
Src Homology 2 (SH2) domain-containing proteins are critical for mediating cellular signalling from receptor tyrosine kinases. Deregulation of SH2 domain-containing proteins have been implicated in human disease including multiple cancers, diabetes, human immunodeficiency and metabolic disorders. These modular domains of approximately 100 amino acids selectively bind proteins phosphorylated on specific tyrosine residues with binding affinities similar to antibody:antigen interactions (typically 0.01-10 μM). Here we employ a phospho-proteomic enrichment strategy using various human SH2 domains to capture and identify a subset of the phosphotyrosine proteome.

Methods
Cells were rendered quiescent through serum starvation followed by no stimulation or stimulation with growth factors (PDGF or EGF) or insulin. Whole cell lysates were prepared and incubated with various GST-SH2 domains overnight at 4°C. Protein complexes were then captured on immobilized glutathione resin beads, washed, eluted, and analyzed by SDS-PAGE and Western blotting or by mass spectrometry. The selectivity of SH2-phosphotyrosine binding was validated by identifying known interactions using phospho-specific antibodies.

Results
GST-fusion proteins were generated for 17 different human SH2 domains. Global phosphotyrosine binding was monitored by analyzing lysates from serum-starved or growth-factor treated cells by Western blotting using the general phosphotyrosine antibody pY4G10. We observed differences in the global pY banding patterns for each SH2-mediated pulldown reaction, indicating binding of these domains to different targets. The specificity of these interactions was further validated by analyzing known site-specific phosphotyrosine events on the EGF and PDGF receptors using phospho-specific antibodies. Time-dependent differential binding of various SH2 domains to PDGFR show virtually instantaneous binding upon PDGF stimulation, with maximal binding of Plcγ, Src, and Shc SH2 domains occurring at 1-5 minutes post treatment. In addition to Western blot analysis, the SH2 domain pulldown experiments can be easily integrated into mass spectrometry applications. Different SH2 domain-mediated pulldowns generated unique protein subsets, showing that this methodology can be used to identify and characterize SH2 interactomes.

Innovative aspects
- Utilization of GST-SH2 domains to pulldown phosphotyrosine-containing proteins
- Monitor tyrosine phosphorylation events using naturally occurring protein interaction domains
- Identification of SH2 interactomes

References (maximum 3 references)
(1) T Pawson et al, SH2 domains, interaction modules and cellular wiring; Trends in Cell Biology, 2001, 11(12):504-511.

P327

Global quantitative proteomic profiling through ^{18}O-labeling and MS/MS spectra analysis

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Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada

Introduction
Although well established, ^{18}O-based stable isotope labeling[1] has not yet seen widespread use in quantitative proteomic studies, possibly due to insufficient characterization and support. Here, we integrate the simplicity of ^{18}O-labeling and the low background and high signal-to-noise of MS/MS spectra with dedicated supporting software and combine them with global shotgun protein identification to create a robust quantitative pipeline.

Methods
Tryptic digests of BSA and soluble *C. elegans* protein extract were prepared in normal (^{16}O) water and 95%-pure ^{18}O-water. Pre-defined ratios of proteins were created by mixing proportional volumes of the unlabeled and ^{18}O-labeled digests. RP and tri-phase microcolumns were loaded with the mixtures and placed in line with an HPLC pump interfaced to a LTQ linear ion-trap MS. Over the course of either 1D or 2D chromatography, spectra were gathered in data-dependent mode using an isolation width sufficient to together trap both the labeled and unlabeled species of each peptide. The Sequest search algorithm[2] was invoked to match fragmentation spectra to peptide sequences. Our ySelect and yRatios programs were then applied to select high-confidence matches and compute relative ratios.

Results
Accurate relative quantitation was observed for different ratios (1:5 up to 5:1) of unlabeled and labeled proteins, whether for BSA in isolation or spiked into a complex *C. elegans* extract digest, or for proteins of the complex digests themselves (Fig 1). Ratios based on two or more peptides were found to be quantifiably more accurate than single-peptide results. Individual peptide bias was indicated and suggests a need for reverse labeling. A consistent bias in favour of the unlabeled term of ratios was observed for singly-charged species. Despite long tails, the distributions at different expected ratios were shown to be similar; accordingly, confidence levels for two-fold and five-fold change were empirically derived. Examination of the variability of unlabeled and labeled *y*-ion pairs relative to the ratio calculated for a spectrum allowed spurious results to be flagged, including nearly all false positive matches. Our results demonstrate that ^{18}O-labeling coupled with systematic interpretation of MS/MS spectra using dedicated software tools can form the basis of a routine global proteome quantitation method and provide accuracy within an experimentally useful dynamic range.

Innovative aspects
- Use of ^{18}O-labeling in combination with shotgun MS/MS spectral analysis to simultaneously identify and determine the relative abundance of multiple proteins present in two samples, with a correction for partial labeling.
- Confidence levels for significance of fold-change established through the systematic characterization of data distributions.
- Reduction in the False Discovery Rate (93% elimination of false positives) achieved through *y*-ion duplet consistency checking.

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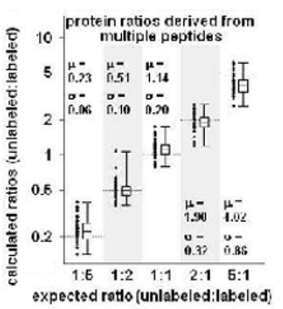

Figure 1. *C. elegans* protein digests mixed in unlabeled: ^{18}O-labeled portions 1: 5, 1: 2, 1: 1, 2: 1 and 5: 1 were analyzed by MS/MS. Calculated ratio distributions are shown with means (μ) and standard deviations (σ).

P328

SILVER: a Tool for the Stable Isotopic Labeling Quantitative Data Analysis in LC-MS based Proteomics

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Introduction
Large scale protein quantification becomes routine in proteomics. Different stable isotopic labeling techniques were developed for the sample comparison on the mass spectrometry (MS) platforms, such as LTQ/FT and LTQ/Orbitrap. Several software tools have been developed for single or multiple labeling techniques, and typical workflows of the quantitative MS data analysis are entering into agreement. However, a common software tool, which supports most of widely used labeling techniques, and has easily accessed graph user interface (GUI) and fairish calculation speed, is absent.

Methods
Here, we present an effective tool for the protein quantification based on the stable isotope labeling MS data, which introduces a compact Index MS raw data file (IRawF) to improve data analysis speed, a least square fitting algorithm with nonlinear optimization for the universal quantitative calculation of different isotopic labeling techniques. In this tool, batch processing for the large scale data analysis and GUI for the manual inspection were integrated. The user can easily browse the protein quantification and trace back the extracted ion chromatogram (XIC) for each peptide and the paired isotopic peaks in the MS spectra.

Results
We verified the merits of this tool (named as SILVER) by applied it on 3 datasets with different isotopic labeling methods and discussed some important issues related on the quantitative algorithm development. The software SILVER, the user guide and the sample datasets are available freely by connecting us for academic and nonprofit use.

Innovative aspects
(1) SILVER can support most labeling types currently used and also the user defined labeling methods by introducing the least square fitting algorithm with nonlinear optimization.
(2) SILVER provides both the GUI operations for the manual inspection and the batch processing for the large scale data processing, the work state can be stored and loaded to facilitate the long time data analysis.
(3) SILVER support the database search results loading from Mascot *.htm files and *.dat files, and can retrieval the raw data form the Thermo *.raw, *.mzXML, *.mzData and *, mzML files.

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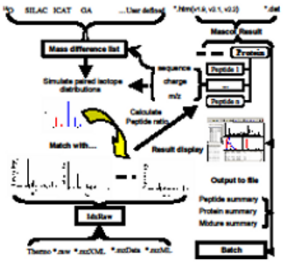

Figure 1. the framework of SILVER. The protein and peptide identifications can be loaded form the *.html files and *.dat files of Mascot (v1.9, v2.1 and V2.2). Different labeling methods were converted to a mass difference list, and then the isotopic pattern was predicted to match wit the peaks in MS spectra, which from the IdxRaw file. The IdxRaw from servers as the fast data accession interface of the Thermo *.raw, *.mzXML, *.mzData and *.mzML file. The protein identifications, peptide list, the XIC and the paired peaks are organized in the GUI of SILVER, the user can easily browse the quantification results and exclude the outliers by deselect them in the table. The quantification results can be exported to a plain text file in the peptide summary, protein summary and mixture summary format, which can be opened in the Microsoft Excel.

P329

HUPO-PSI Transitions Markup Language (TraML) for Exchange of SRM Transition Lists

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Introduction The use of selected reaction monitoring (SRM; also referred to as multiple reaction monitoring, MRM) has long been used in the analysis of simple compounds, but now is becoming increasingly common in quantitative proteomics experiments [1]. A necessary part of such SRM experiments is the selection of transitions, the signatures that are meant to uniquely identify and quantify peptides. Currently lists of these transitions are communicated in simple tabular formats of many different styles, and this makes the exchanging of transition information and writing software to facilitate this difficult.

Methods In order to address this problem, the Institute for Systems Biology (ISB) and the Proteomics Standards Initiative (PSI) have collaboratively designed and implemented a format, TraML, for encoding SRM transitions, both predicted and validated along with annotations about their source (Figure 1). The format is implemented in XML using schema definitions in conjunction with a controlled vocabulary implemented within the Open Biological Ontologies framework. Semantic validation, in addition to standard XML validation, is achieved via a same PSI semantic validator tool used for other formats such as mzML.

Results A draft version of TraML has been developed by the PSI Mass Spectrometry Working Group for submission to the PSI document process, so that wider community review and participation can be solicited. The format can support encoding information about proteins and peptides as well as small molecules or other compounds, in addition to the transitions that are intended to identify them. After community review and subsequent revision, TraML 1.0 will be released for recommended adoption

Innovative aspects
- TraML is a new XML format that is proposed to unify the many ad-hoc tabular lists for encoding SRM transitions.

References
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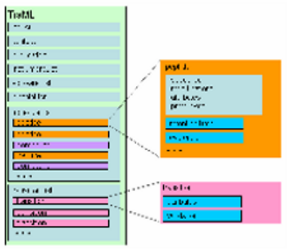

Figure 1. Outline of the TraML schema. First some overall metadata about the list can be encoded, followed by option protein, peptide, and compound lists. Finally the list of transitions along with rich annotations and validation information can be encoded.

P330

Enhance proteomic detection limitation by random nucleotide library

In most biological sample, the few high abundant proteins are usually composed to 95% of overall protein content. Although there are many methodologies for depletion of high abundant protein, unfortunately many defects still occur. For example, the immunodepletion method by antibody also removes other low abundant proteins that bind the depleted species simultaneously. Here, we provide a new method to reduce the dynamic range of complex biological samples by DNA library. The DNA library is composed of 25 random sequences for sufficient diversity to match a ligand to every protein in complex biological sample. Therefore, according to the saturation-overloading principle, an abundant protein will saturate all its available ligands and leave the majority of the same protein unbound. After binding the library, the concentration range of complex sample will decrease and the low abundant proteins will be enrich by the library on the beads. In 1D and 2D PAGE experiments, we show that both peptide and aptamer libraries can remove high abundant protein, and consequently enrich low abundant proteins. Following the analysis of the LTQ-Orbitrap mass spectrometry, we can identify more low abundant proteins such as insulin-like growth factor-binding protein. Therefore, from this technology, we can identify the low abundant protein for deep proteome and may also applied to biomarker discovert.

P331

Protein profiling of malignant B cells using reversed phase protein microarrays

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Introduction
Reversed phase protein microarray is an emerging high-throughput approach within proteomics allowing protein profiling of complex samples in a multiplexed and miniaturized manner. However, high technical demands will be placed upon the platform to be able to perform global proteome analysis in a specific and sensitive manner. Here, we have designed and optimized a set-up based on genome selected PrEST antibodies and reversed cell lysate arrays on ZeptoMARK slides, interfaced with the planar wave guide technology for visualization.

Methods
32 cell lysates derived from cell lines and primary cell samples were generated and arrayed onto ZeptoMARK slides. Next, the cell lysate microarrays were probed with 43 different PrEST antibodies, selected by gene expression analysis to be mantle cell lymphoma associated. Detection of bound PrEST antibodies was performed using biotin labeled secondary antibody combined with Alexa-647 labeled streptavidin. The microarrays were imaged using the ZeptoREADER.

Results
The results showed that the platform could be used to detect differentially expressed soluble proteins as well as membrane proteins in malignant B cells vs. normal B cells and non B cell control samples in a highly specific, sensitive and reproducible manner. Targeting either cell lines or primary cells, the data showed that the malignant B cells could be classified with a specificity and sensitivity of >90% based on 43 PrEST antibodies. Further, significantly up- and down-regulated proteins, originating from different compartments of the cells, could be identified and verified using immunohistochemistry. Thus, we have a reverse phase protein cell lysate microarray set-up at hand highly suitable for high-throughput oncoproteomics.

Innovative aspects
- Protein profiling of malignant B cells were performed using reversed phase protein microarrays.
- Differentially expressed proteins originatin from different cell compartments could be identified and malignant B cells could be classified with a high specificity and sensitivity.

References
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P332

Solubilization and digestion of integral membrane proteins in ionic liquid solvent system

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Introduction
Integral membrane proteins (IMPs) play unique roles in mediating cellular processes. Up till now, four different kinds of buffers, including chaotropes, detergents, organic solvents and organic acids (1), have been applied to improve the solubility of membrane proteins. In this work, ionic liquid (IL), 1-butyl-3-methyl imidazolium tetrafluoroborate, was used to dissolve membrane proteins extracted from Rat cerebellum, and more proteins were identified by μRPLC-ESI-MS/MS comparing to those dissolved by SDS and methanol.

Methods
Rat cerebellum was firstly homogenized in 2 M NaCl, and then after centrifugation, with supernatant discarded, the residual was suspended in 0.1 M Na_2CO_3 (pH 11.3). Subsequently, after removing the supernatant, the sediment was sonicated for in-depth disruption of membrane. Finally, 200 μg membrane proteins sample was precipitated with methanol/chloroform, and re-suspended in different buffers for trypsin digestion.
For SDS and methanol methods, the dissolution and digestion of membrane proteins were performed according to published protocols (2). For IL method, membrane proteins suspended in IL were firstly heated for thermal denaturing, and then reduced and alkylated, followed by tryptic digestion. The digests by three methods were analyzed by μRPLC-ESI-MS/MS (LCQ IT mass spectrometer, ThermoFinnigan).

Results
After analysis by μRPLC-ESI-MS/MS, as shown in Fig. 1, 61 IMPs out of 245 proteins (25%), 86 out of 311 proteins (28%), 20 out of 64 proteins (31%), and 15 out of 50 proteins (30%) were identified from proteins dissolved by 10% (v/v) IL, 1% (v/v) IL, SDS and methanol, respectively, which demonstrated that 1% IL was more effective than 10% IL, SDS and methanol for IMPs study. The distribution of identified membrane proteins by 1% IL, SDS and methanol methods is shown in Fig. 2. A total of 87 IMPs were identified. Those identified by methanol method are totally covered by that identified by IL method, and 95% IMPs identified by SDS method are overlapped with those identified by IL method. Such results could be due to two main factors. One is the property and structure of 1-butyl-3-methyl imidazolium tetrafluoroborate. The polarity of such IL is close to that of methanol, and the structure is similar to detergents, containing a hydrophobic side and a hydrophilic side. Another reason might be the better biocompatibility of such IL with trypsin than methanol and SDS. All these results demonstrate that IL might be promising for the identification of membrane proteins by increasing the solubility and decreasing the negative effect on tryptic digestion.

Innovative aspects
- Ionic liquid applied for membrane proteins analysis with improved identification capacity, contributed by improved solubility and biocompatability for trypsin

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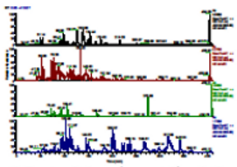

Figure 1. Base peak chromatograms of trypsin digests of membrane proteins dissolved in 10% IL, 1% IL, SDS and methanol (from upper to bottom)

Figure 2. Comparison of integral membrane proteins identified by 1% IL, SDS and methanol methods.

P333

Middle down peptide analysis using an LTQ Orbitrap and ProSightPC 2.0

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Introduction. Middle down peptide analysis identifies polypeptide chains that weigh more than 3000 Da and are the products of enzymatic or chemical digestion of proteins. Such longer, heavier peptides are commonly produced by proteolytic reagents that exhibit single residue specificity, in contrast to the double residue specificity of trypsin. Analogous to "top-down" workflows, middle down search programs consider molecular masses in addition to fragment ions, and thus are sensitive to protein modifications. Identification of the polypeptides also provides identification of proteins and the species of origin.

Methods. Ribosomal proteins were isolated by published methods from MCF cancer cells cultured in our laboratory. Microwave-accelerated acid digestion was carried out in a solution of 12.5 % acetic acid and 5 mM DTT on a Discover Benchmate microwave system (CEM Corp., Mathews, NC) with a temperature of 140 ± 5° for 20 min, as previously reported (Swatkoski et al, J. Prot. Res. 2007). The resulting peptide mixtures were fractionated on an Accela HPLC (ThermoFisher, Pittsburgh, PA) using a 1mm i.d. x 150mm Biobasic-C-18 column and a 90 min 0.1% formic acid/ 97.5% acetonitrile gradient. Five repetitive analyses were made. Automatic high resolution analysis (30,000) of both precursor and product ion was provided on the chromatographic timescale by the LTQ Orbitrap system (ThermoFisher, San Jose, CA) equipped with an electrospray ion source. Helium was used as the target gas. The three most abundant ions in every precursor scan were automatically selected for CID and MSMS analysis. Ions were scanned through m/z values between 300 and 2000. Selected ions were subsequently excluded for 10 s. Spectra were automatically processed using the high-throughput mode of ProSight PC 2.0 (Boyne et al, J. Prot. Res. 2009), configured to decharge each MSMS spectrum using THRASH, and search a database constructed from putative acid-digest peptides using ProSight's absolute mass search. These searches were conducted using a search filter, which only accepted identifications when a minimum of 5 fragment ions were found. The precursor search window was set at 10Da and the fragment tolerance at 15ppm.

Results. Two hundred and seventeen distinct peptides were identified, representing 63 of 84 proteins in the database. Seventy-one had masses greater than 3000 Da. Charge states ranged up to +11. Protein coverage ranged from 5 % to 100%. When the same data sets were analyzed by MASCOT, a program optimized for analysis of smaller tryptic peptides, 105 distinct peptides were identified from 45 proteins. five peptides had masses above 3000 Da. Analysis of longer peptide products was also shown to provide higher protein coverage and more reliable protein identifications. Successful analysis of proteolytic peptides in the mass range above 3000 Da demonstrates that acquisition of accurate mass measurements automatically on an HPLC- LTQ Orbitrap, combined with processing and searching by ProSight PC 2.0, provides a viable workflow for high throughput middle down analysis on the chromatographic timescale. Low energy collisionally induced dissociation was found to produce searchable sequence tags and fragmentation patterns from many polypeptides in this mass range. Databases constructed from predicted peptide cleavage products allowed reliable identifications to be made.

Innovation. The workflow demonstrated here allows automated identification of polypeptides in the mass range 3000 to 10000 Da, without implementing the technically more complex electron transfer dissociation or charge reduction.

P334

Generating high affinity phage-antibodies to multiple SH2 domain proteins by chain shuffling

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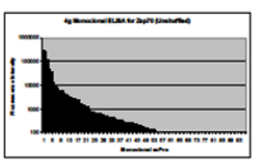
Introduction
Generating high affinity antibodies on a proteome-wide scale will provide a wealth of reagents generating consistent, direct information on protein interactions, protein expression levels, modifications and sites of action. We have previously demonstrated the feasibility of selecting on multiple antigens in parallel[1]. Here we demonstrate generation of specific antibodies to 20 different human SH2 domain proteins. Furthermore we show that affinity maturation can be carried out in parallel on multiple antigens, resulting in a scalable method to generate antibodies with high affinity and utility.

Methods
Using an antibody phage display library of over 10 billion clones, we have carried out 2 rounds of selections on 20 bacterially produced human SH2 domain proteins. Positive clones which were specific for only one SH2 domain were identified by ELISA.
For 8 of the antigens, chain shuffled libraries of 10^9-10^{10} clones were constructed by cloning VH domains from the selected antibodies into a repertoire of VL domains. These chain shuffled libraries were subjected to stringent selections using biotinylated antigens. The output populations were assessed by polyclonal and monoclonal ELISA. Affinity was measured by BiaCore and specificity demonstrated on protein arrays.

Results
Antibody selections were carried out on 20 different human SH2 proteins. In total 1200/3800 of the resultant clones were shown to bind the target antigen. A sub-set of these were tested in specificity ELISA against all 20 SH2 domains and 385 antibodies were identified which recognised only the target antigen.
Using phage display it is possible to affinity mature selected antibodies by creating a diversified library of variants from a lead antibody clone or population. Here we have diversified by recombining selected heavy chains with a new population of light chains. This simple "chain shuffling" approach is followed by stringent selection using limiting amounts of antigen. Chain shuffled libraries were prepared for 8 antigens and selected with 1-100nM antigen. Polyclonal phage ELSA demonstrated that the average affinity of the populations had increased by up to 10 fold. In monoclonal ELISAs a higher average signal was achieved compared with the original selections. The success rate overall increased from 31% in the original screen to 74% after chain shuffling (787 positives from 1056 screened). Specificity was demonstrated on protein microarrays containing 350 different proteins.

Innovative aspects (maximum of 3 bullet points)
- Generation of antibodies to 20 human SH2 targets
- Demonstration of simple, multiplexed antibody affinity maturation by chain shuffling
- Demonstration of high affinity in resultant antibodies by Bia-Core and high specificity by protein arrays

References: List references cited in text as such (max 3 references allowed):
(1) DS Schofield DS et al (2007) Application of phage display to high throughput antibody generation and characterisation. Genome Biology. 8 (11) R254



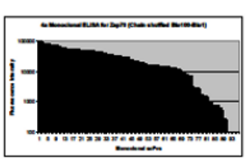


Figure 1. Improved performance of antibodies after affinity maturation. Profile is shown of results from monoclonal ELISA screen before (top) and after (bottom) chain shuffling

P335

Improving the Human Protein Atlas Coverage by Re-Designing Protein Epitope Signature Tags

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Introduction
The Human Protein Atlas Program (HPA) was initiated more than five years with an aim to characterize the human proteome by immuno-histochemistry (1). A mono-specific antibody is generated using a unique protein fragment called PrEST (Protein Epitope Signature Tag) as antigen in immunization. Today, PrESTs are designed with the in-house software PRESTIGE (2), subsequent cloning and protein expression. A set 96 PrESTs previously failed in the HPA cloning process were re-designed with the latest version of PRESTIGE to investigate re-designing strategies.

Methods
PrESTs were amplified by reverse transcriptase PCR (RT-PCR) using the specific oligonucleotide primers designed with PRESTIGE and total RNA either from different human tissues or from 10 different cell lines as a template. Amplicon size were verified by agarose gel electrophoresis and digested using an automated solid phase restriction set up with magnetic beads. Restricted fragments were ligated with the pAff8c-vector and transformed into *E.coli* Rosetta. The clones were sequence verified by Sanger sequencing before approval for downstream expression and protein purification.

Results
The 96 PrESTs in the test set were distributed on 17 different human chromosomes and new PrESTs and corresponding PrEST primers were re-designed with the PRESTIGE software. Of these 96 PrESTs, 67 passed the cloning successfully and 30 failed the cloning process (Fig 1). With a success rate of 70 % in the test set, a re-design of all PrESTs earlier failed in the cloning has been initiated. More than 2 300 PrESTs, randomly distributed over the human genome has been re-designed. So far 1303 PrESTs has been started in the HPA cloning pipeline and 249 has generated sequence verified amplicons. Of these 1303 re-designed PrESTs only 73 has failed to generate an amplicon.
When previously designed, the great majority of the test set PrESTs failed in the RT-PCR. The increased RT-PCR success rate for the test set PrESTs re-designed in PRESTIGE can be an effect of altered primer design criteria. The results of the 96 PrEST test set showed that iterations of PrESTs can be worthwhile as new in silico methods evolve. It also showed the importance of continuously evaluating and re-considering present methods and techniques to improve the success rate and enable full coverage of the Human Protein Atlas.

References:
(1) M. Uhlén et al. Mol Cell Proteomics, 4 (12), 1920-32, (2005)
(2) L. Berglund et al. Proteomics, 8 (14), 2832-9, (2008)

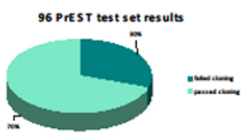

Figure 1. Cloning results for the 96 PrESTs re-designed in PRESTIGE.

P336

Peptide Modification Strategy for Optimizing Electron Transfer Dissociation Fragmentation

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Introduction
Peptide coupling chemistry has often been used to amidate carboxylate residues prior to MS analysis. The derivatizations have been used for many purposes; including quantitaion, cross linking, and ionization enhancement. Additionally, with the right reagent choice this simple, powerful chemistry can markedly enhance ETD fragmentation via the introduction of added positive charge. Here we apply this chemistry to enhance ETD spectra using conditions mild enough to permit direct introduction in to the mass spectrometer, while still providing many of the other benefits of derivatization.

Methods
Whole proteins were reduced, alkylated, digested, desalted and dried prior to amidation. Peptide samples were dried prior to reaction initiation. Net peptide content of derivatized samples ranged between 1 and 100 pmols, individual peptides contributed 1 fmol to 1 pmol. Peptides were amidated with one of two protocols. The first used EDC (a water soluble carbodiimide) and polyamine in a pyridine-HCl buffer. The second protocol utilized EDC and HOAt in DMF with TFA, followed by drying. All samples were analyzed utilizing reverse-phase nanoflow LC to gradient elute peptides into an ETD equipped mass spectrometer. Individual spectra of peptides known to fragment poorly via ETD and CAD were inspected both with and without modification to qualitatively evaluate fragmentation improvement.

Results
A wide variety of aliphatic and aromatic amines were screened, yielding a reaction optimized to produce short reaction times, desirable HPLC characteristics, and superior ETD fragmentation of otherwise poorly fragmented peptides. The mild conditions were achieved by using EDC, a water soluble reagent that produces water soluble by-products that are easily rinsed from LC columns. Assessment criteria evaluated include: ETD fragmentation improvement, reaction speed, reaction simplicity, compatibility with LC and MS, and column retention time effects. Using this methodology, the relative merits of different polyamines were established.

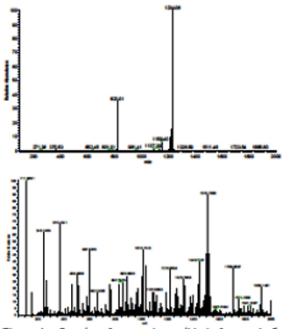

Figure 1 – Spectra of a sample peptide before and after amidation.

Next, the chemistry was tested in complex biological applications. Specifically, this chemistry allowed the identification of two sub-stoichiometric O-GlcNAc sites on an AspN generated peptide from RelA. The peptide generated was 38 residues long and included nine potential modification sites. Spectra required to positively assign the site were unavailable using CAD, ETD with supplemental activation or even high-resolution ETD. However after derivatization, ETD spectra included a robust z ion series that permitted unambiguous assignment of the site.

Innovative aspects
- The derivatization provides enhanced ETD fragmentation
- The reaction is applicable to small volumes of complex mixtures (fmol - pmol)
- Application of the chemistry preserves posttranslational modifications, and is amenable to direct introduction to LC-MS

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P337

Improving the Reproducibility of Proteomics Using MassQC

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Introduction:

The further growth and acceptance of Proteomics requires improvements in experimental reproducibility. Most tandem mass spectrometry labs run standards to ensure a base level of function for their instrumentation. Hand analysis of these standards leaves much room for improvement. The National Institute for Standards and Technology (NIST) has developed a series of metrics for LC-MS/MS standards. We have incorporated these metrics into MassQC, a software package that tracks instrument performance to ensure reproducibility and diagnoses hardware anomalies that lead to degraded performance.

Methods:

RAW data from a simple or complex standard run is loaded into MassQC. The data is scored using multiple metrics developed by NIST. The user can examine a history of specific metrics and determine whether the current LC-MS/MS state is acceptable. MassQC alerts the user when a run deviates from the historical range of the instrument, diagnosis can be performed using the metric data. Twenty Eight metrics are divided into the following categories; Chromatography, Ion Source, MS1, Dynamic Sampling, MS2, and Identifications.

Results:

MassQC allows users to monitor multiple aspects of their LC-MS/MS pipeline for reproducibility. Runs which deviate from the norm for an instrument are highlighted and diagnosis can be performed to narrow down possible causes of error. Once corrective measures have been taken the pipeline can be reverified using MassQC. We will show successful diagnosis of instability in the ion source, column degradation, polymer contamination, and chromatographic variation. Users can identify problem areas in their pipeline before wasting expensive or difficult to acquire samples.

Innovative aspects:

- Detailed analysis of standard LC-MS/MS runs
- Identify when an instruments is performing is sub-optimally
- Diagnose problems in an instrument before they effect samples

P338

Simple Enrichment of Phosphopeptides Using TiO_2 Magnetic Beads

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Introduction
Phosphorylation is a common reversible post-translational modification involved in the regulation of many essential biological processes for example cell signaling, which is of prime importance for the study of various disease states such as cancer. Knowledge about phosphorylation may help to understand disease progression. Phosphoproteins and phosphopeptides are usually present at very low concentrations and ionize poorly, making their detection by mass spectrometry (MS) difficult. In this study we have used porous agarose TiO_2 beads with paramagnetic properties as a tool to enrich phosphopeptides.

Methods
At first pure phosphopeptides of different sizes and a phosphatase assay was utilized to investigate the binding capacity of the TiO_2 magnetic beads (TiO_2 Mag Sepharose™, GEHC, Sweden). Then a sample mixture of α-casein, β-casein, and bovine serum albumin (BSA) was trypsin digested and applied to the TiO_2 magnetic beads according to the manufacturer's instruction. The eluates with enriched phosphopeptides were lyophilized, dissolved in 20% acetonitrile with 0.1% trifluoroacetic acid and analyzed with MALDI-ToF MS. Finally, a trypsin digested lysate from a leukemia cell line, corresponding to 500 µg protein, was applied to TiO_2 magnetic beads to enrich phosphopeptides prior to LC-MS/MS analysis (LTQ FT, Thermo Electron, Germany)*.

Results
By using a small amount of TiO_2 magnetic beads enrichment of phosphopeptides was achieved and about five phosphopeptides including the tetraphosphopeptide from β-casein were found from the sample mixture of α-casein, β-casein and BSA, see Figure 1. When the eluate was diluted 100 times two phosphopeptides could still be detected. Moreover, of all detected peptides from the leukemia cell line sample 16% were phosphorylated. In total, 15 phosphopeptides and 14 phosphorylation sites were found. Without enrichment no phosphopeptides were detected (analyzed on non-enriched peptide material corresponding to 100 µg protein). This initial experiment shows the usefulness of TiO_2 magnetic beads as a tool to study phosphorylation patterns.

Innovative aspects
- Paramagnetic, spherical, highly cross-linked agarose particles with TiO_2.
- Convenient tool for enrichment of phosphopeptides from cancer cells.

Acknowledgement
*This data was kindly provided by PhD Sara Lind, Lu Lu, Lioudmila Elfineh and Prof. Ulf Pettersson, Department of Genetics and Pathology, Uppsala University; and PhD Konstantin Artemenko and Prof. Roman Zubarev, Department of Cell and Molecular Biology, Molecular Biometry Group, Uppsala University.

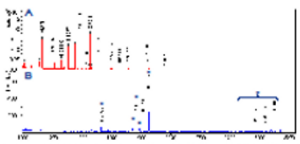

Figure 1: MALDI-ToF spectra of start material with 50 pmol α-casein, β-casein and BSA, respectively (A) and the corresponding eluate (B). The enriched phosphopeptides are marked with an asterisk and also a metastable phosphopeptide is seen, marked with a dot.

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Membrane Nanodiscs for Identification of Membrane-Protein Interactions

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Introduction: The function of membrane associated molecules is often difficult to *study in vitro* because of low solubility in the absence of detergent. Many proteins, for example, loose function in the presence of deterent due to loss of the phospholipid bilayer. Some membrane proteins can be reconstituted as proteoliposomes, but often with problems of heterogeneity and aggregation. As a homogeneous alternative for soluble reconstitution of membranes we explore the use of discoidal phospholipid bilayers termed nanodiscs to identify interaction partners.

Methods: Nanodisc synthesis: we reconstitute membranes that contain glycosphingolipids in phosphatidylcholine phosphatidyl lipids by mixing the lipids with cholate and an amphipatic helical membrane scaffold protein (MSP) that stabilizes the membrane as a discoidal structure upon removal of cholate by biobeads SM2. The homogeneity is checked by gel-filtration chromatography.
Screening for interaction partners: Biacore employing immobilized nanodiscs (see reference 1) is used for identification of fractions of E. coli cultures that contain binding activity specific for glycosphingolipids.
Identification of interaction partners: The nanodiscs are used for co-immunoprecipitation to identify protein interaction partners of glycospingolipids by mass spectrometric techniques.
Results: We demonstrate that tags engineered into the MSP can be used for immobilization of the nanodiscs. By testing different specific elution conditions we demonstrate that the nanodiscs can be solubilized by certain mild detergents or eluted as intact nanodiscs by competitive elution from immobilized antibodies. The knowledge from the development of methods to bind and elute was used to fish out interaction partners of glycosphingolipid containing nanodiscs. Heat labile toxin was identified as an interaction partner of the glycolipid ganglioside GM1.
Innovative aspects:
- Affinity purification with reconstituted membranes as baits

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Proteomics and Epigenetics

(P401 – P411)

P401

Proteomic Analysis of a Novel HKMT and its Role in Regulating Gene Expression

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Introduction
Histones are subject to a number of post-translational modifications, mainly on their N-termini which allows control of the accessibility to the underlying DNA. Modifications to the histone proteins change their affinity toward DNA and thus control gene expression. Histone methylation is the result of histone-lysine N-methyltransferase and histone-arginine N-methyltransferase enzyme activity. Most histone lysine methyltransferase contain the SET domain. SMYD2 is a member of the SMYD protein family that contains SET and MYND domains.

Methods
In order to understand the role of SMYD2 in the cell, we mapped its interactome using two approaches. The first approach, termed IP-HTMS, looked at the global interactors of SMYD2 [2]. In this approach, FLAG tagged SMYD2 was transiently transfected in 293T cells 48 hours after the transfection, the FLAG tagged SMYD2 was Immunoprecipitated, and its protein interactors identified by mass spectrometry .One limitation of the IP-HTMS method is a bias against nuclear proteins. Therefore, a second approach, termed ChIP-HTMS, was used. The approach consists of a modified chromatin IP protocol coupled to HTMS to enrich SMYD2 interaction partners from the nucleus and from SMYD2 bound to DNA. The ChIP-HTMS method enlarged the list of SMYD2 nuclear proteins interactors.

Results
We report the cytosolic and nuclear interactome of SMYD2 using a combination of IP-HTMS, ChIP-HTMS, and co-immunoprecipitation methods. In particular, we report that SMYD2 interacts with HSP90α independently of the SET and MYND domain; with EBP41L3 through the MYND domain; and with p53 through the SET domain. We demonstrated that the interaction of SMYD2 with HSP90α enhances SMYD2 histone methyltransferase activity and specificity for histone H3 at lysine 4 (H3K4) *in vitro*. Interestingly, histone H3K36 methyltransferase activity is independent of its interaction with HSP90α similar to LSD1 dependency on the androgen receptor. We also show that the SET domain is required for the methylation at H3K4. We demonstrated using a modified chromatin IP protocol that the SMYD2 gain of function leads to an increase in H3K4 methylation *in vivo*, while no observable levels of H3K36 were detected. We also report that the SMYD2 gain of function is correlated with the up-regulation of 37 and down-regulation of 4 genes, the majority of which are involved in the cell cycle, chromatin remodeling, and transcriptional regulation. TACC2 is one of the genes up regulated as a result of SMYD2 gain of function. Up-regulation of TACC2 by SMYD2 occurs as a result of SMYD2 binding to the TACC2 promoter where it methylates H3K4. Furthermore, the combination of the SMYD2 interactome with the gene expressions data suggests that some of the genes regulated by SMYD2 are closely associated with SMYD2 interacting proteins.

Innovative aspects
- SMYD2 is a histone methyltransferase that methylates H3K4 by its SET domain in presence of HSP90α
- SMYD2 interactions with other proteins that have the PXLXP occure through the MYND domain
- SMYD2 is localized in both cytoplasm and nucleus where it bind to DNA sequences of TACC2 promoter specific

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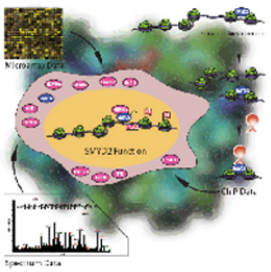

Figure 1. An illustration of the combined methods used to understand SMYD2 function. At the center of these methods is the use of IP-HTMS and ChIP-HTMS used to identify SMYD2 protein interactions. Affect of SMYD2 gain of function on gene expression were investigated by Microarray. The ability of SMYD2 to bind to promoter regions of regulated genes was tested by ChIP.

P402

Demethylation Increased the Expression and Transcription of *SOCS*-1 in Leukaemic Cell Lines

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Introduction
Genetic alterations such as point mutations and chromosomal translocations have been identified to transform normal haemopoietic progenitor cells into leukemia. Acute myeloid leukemia (AML) is a multistep process in which more than one genetic lesion is required to result in full leukemic transformation. These diverse genetic events may serve as targets for drug treatments. In addition, epigenetic may play a major role in the pathogenesis of AML where aberrant methylation is a major mechanism for silencing of tumour suppressor genes. Hypermethylation of the suppressor of cytokine signalling-1 (*SOCS*-1) gene has been reported to cause inactivation in leukaemogenesis. However, there is an issue in the use of different locations of *SOCS*-1 gene being amplified (exon 2 and 5'UTR) that gave discrepant methylation results in AML. *SOCS*-1 hypermethylation of 72% AML cases (1) was reported in contrast to another report that showed that methylation of the *SOCS*-1 promoter region was absent in AML (2). The former used sets of primers inside exon 2, instead, the latter claimed that they used primer sets in the untranslated promoter region (5'UTR). Previously, we have investigated both regions of *SOCS*-1 gene in AML, myelodysplasia (MDS) and juvenile myelomonocytic leukaemia (JMML) patients (3). Aberrant methylation in *SOCS*-1 was found in 40% AML patients at exon 2 region while methylation was not detected at the 5'UTR. Thus, in this current study, the aim was to determine the role of *SOCS-1* exon 2 methylation in haematopoietic cell lines and the relative expression of the gene after the addition of either azacytidine or deoxyazacytidine as the conversion agent of methylcytosine.

Methods
The relative expression level of *SOCS-1* gene in four leukaemic cell lines was compared to determine the gene expression in *SOCS-1* exon 2 methylated and unmethylated cell lines. Demethylation technique was established by treating the cell lines using either azacytidine or deoxyazacytidine. Methylation-specific PCR (MS-PCR) was employed to determine if demethylation using the conversion agent would convert methylcytosine into cytosine in the *SOCS*-1 exon 2 methylated cell lines. Quantitative reverse transcription PCR (RQ-PCR) were used to analyse the relative expression of *SOCS*-1 in haematopoietic cell lines and calculated using the $2^{-\Delta\Delta CT}$ equation.

Results
There was a decrease in the relative expression level of *SOCS*-1 gene in *SOCS*-1 exon 2 methylated cell lines (ME-1 and Jurkat) compared to unmethylated cell lines. The demethylation technique was established using 1μM of azacytidine and harvested after three days. The use of azacytidine is favoured instead of deoxyazacytidine because of the greater toxicity of the latter to the cells. The demethylation experiments showed that there was a marked increase in relative expression of *SOCS*-1 in ME-1 and Jurkat cell lines after the addition of azacytidine with MS-PCR results showed evidence of cytosine conversion in both. The mean expression level of *SOCS*-1 gene in ME-1 and Jurkat cell lines were 17 folds and 7 folds higher respectively compared to untreated controls. RQ-PCR results showed that *SOCS*-1 exon 2 methylation is associated with the transcriptional silencing of *SOCS-1* gene in haematopoietic cell lines.

Innovative aspects
- A prove that methylation of *SOCS*-1 exon 2 but not 5'UTR may lead to epigenetic silencing of *SOCS*-1 in haematopoietic cell lines.
- Treatment using demethylating agent azacytidine resulted in the re-expression of *SOCS*-1 may contribute to the possibility of targeted therapy for patients with AML.

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P403

Mass spectrometry analysis of the variants of histone H3 and H4 of soybean and their post-translational modifications

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Introduction
Histone is prone to be modified and histone modifications and histone variants are of importance in many biological processes, ranging from regulating gene expression, modulating cell cycle to maintaining genome stability. Histone modifications and variants in mammals, *Drosophila*, *Tetrahymena* and yeast are well studied; however, they remain mysterious in plant except in *Arabidopsis thaliana* (1-3). Here, we elucidated the variants and post-translational modifications of histones in soybean, a legume plant with a much bigger genome than that of *A. thaliana*.

Methods
Soybean histone mixture were isolated with acid extract method and then separated by reversed phase high-performance liquid chromatography (RP-HPLC). Purified histones were then digested by trypsin or Lysine-C. Digested peptides were further separated by Nano-LC and histone modifications were identified by MALDI-TOF/MS-MS.

Results
In soybean trifoliate, mono-, di- and tri-methylation at Lysine 27 and Lysine 36, mono-methylation at Lysine 4, and acetylations at Lysine 14, 18 and 23 were detected in histone H3. Lysine 27 was prone to be mono-methylated, while tri-methylation was predominant at Lysine 36. We also observed that Lysine 27 methylation and Lysine 36 methylation usually excluded each other in H3. Interestingly, although methylation at H3 Lysine 79 was not reported in *A. thaliana*, mono- and di-methylated H3 Lysine 79 were detected in soybean. Acetylations at Lysine 8 and 12 of H4 were identified. With the combination of mass spectrometric and nano-liquid chromatography, two variants of H3 were detected and their modifications were determined. They were different at the position of $A^{31}F^{41}S^{87}S^{90}$ (histone variant H3.1) and $T^{31}Y^{41}H^{87}L^{90}$ (histone variant H3.2), respectively. The methylation patterns in these two H3 variants also exhibited differences. H3.1 lacked the Lysine 36 methylation and the mono-methylated Lysine 4 presented in H3.2, suggesting that histone variant H3.2 was associated with actively transcribing genes. Surprisingly, two novel variants of H4 were also detected, which were missing in other organisms. In the variant H4.1 and H4.2, the amino acid 60 is isoleucine and valine, respectively.

Innovative aspects
- Isolation of the two variants of histone H3 of soybean
- Characterization of the histone modification patterns in soybean H3 and H4 and their variants

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Generation of highly specific synthetic antibodies targeting chromatin remodeling protein domains

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Introduction
Chromatin organization is shaped in large part by the activities of multi-protein histone remodeling complexes composed of functional domains with histone binding and/or modifying activities. Despite high structural similarity and sequence identity, individual members of chromatin remodeling protein classes often exhibit distinct cellular roles and interaction partners. Here, we use a synthetic antibody approach to rapidly isolate binding reagents sensitive and specific enough to discriminate between subtle differences in highly similar members of chromatin remodeling domain classes.

Methods
To efficiently develop highly selective binding reagents against targeted chromatin remodeling protein domains, we turned to an in vitro synthetic antibody selection process based on a phage library displaying restricted side-chain diversity antibody fragments, similar to those previously reported to yield high affinity antigen binding[1]. We used phage display to screen for binding reagents against recombinant SET domains of histone methyltransferases G9a and GLP, chromodomains of CBX3, CBX4, CBX5 and CBX6, and MBT domains of L3MBTL1, L3MBTL2, and L3MBTL4 and SCMH1 and SCML2. After multiple (3-5) rounds of phage panning, we conducted a rigorous cross-reactivity screen to compare binding to target domains, closely related domains and non-related domains.

Results
Using our in vitro selection approach, we have identified several unique Fab reagents displaying target domain binding preferences with limited or even no residual binding even for closely related members of a class. For the G9a and GLP SET domains, Fab binders were obtained that selectively recognize the particular SET domain used for panning and not related family members. Likewise, Fab binders selectively targeting each instance of the chromo and MBT domains were identified with a high degree of specificity (low cross-reactivity against highly similar and non-related domains). Importantly, the DNA sequences encoding these highly specific binders are easily accessible, which permits engineering of tailor-made recombinant forms adaptable to a wide array of biochemical applications[2]. The stringent binding properties of these synthetic antibodies will permit high resolution targeted proteomic characterization of individual chromatin remodeling proteins and their interacting protein partners in different cell lines, diverse patho/physiological conditions and in healthy and diseased cellular contexts.

Innovative aspects
- Our in vitro screening approach can quickly generate renewable and modifiable binding reagents with high binding selectivity in high-throughput format.

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P405

Large-Scale Proteomic Characterization of Chromatin in Budding Yeast by mChIP

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Introduction: Chromatin, the complex packaging of DNA with proteins, is in many ways the master control centre for the cell. Despite the critical role of chromatin on most cellular functions, chromatin remains poorly studied by proteomic means due to significant technical hurdles. We have developed a novel approach, termed modified chromatin immunopurification (or mChIP), for the purification of protein-DNA macromolecules and their subsequent analysis by mass spectrometry in *Saccharomyces cerevisiae* (1).

Methods: *S. cerevisiae* strains expressing a bait of interest with a C-terminus TAP tag under the control of their native promoter were used for all mChIP purifications. The mChIP procedure consists of a single affinity purification step, whereby chromatin bound protein networks are isolated from mildly sonicated and gently clarified cellular extracts using magnetic beads coated with antibodies. Previously reported proof-of-principle studies based on five chromatin associated baits demonstrated that the mChIP procedure can successfully isolate protein/DNA macromolecules, referred to as chromatin associated protein networks, and further our understanding of the diverse functions of chromatin associated baits (1).

Results: mChIP purifications of ~100 chromatin associated baits were performed and their associated protein networks were analyzed by LC-MS/MS. This dataset includes mChIP data for most known histone chaperones, lysine acetyl transferases, lysine methyl transferases, histone deacetylases found in the budding yeast as well as many other proteins associated with chromatin. Following bioinformatics curation of the mChIP data to remove contaminant proteins, high abundance ribosomal proteins as well as chromatin background (defined in reference 1), numerous analyses of the mChIP data were performed. Direct comparison of the mChIP association data with the previously reported TAP tag datasets (2-3), demonstrated the superior sensitivity of the mChIP technique when studying chromatin associated proteins. Furthermore, 2-D hierarchical clustering of the complete dataset revealed a large diversity in the bait/prey associations showing the specificity of the new method. The improved specificity and sensitivity of the mChIP method derives from its ability to purify both soluble protein complexes as well as chromatin associated protein networks, something that classical AP-MS schemes cannot achieve.

Innovative aspects:
- First large-scale functional proteomic study of chromatin associated proteins in any organism.
- Demonstration of the significant gain in the amount of associated proteins observed by mChIP over classical AP-MS methods due to the sampling of both soluble and chromatin associated protein networks.
- Identification of the protein networks associated with most histone chaperones, lysine acetyl transferases, lysine methyl transferases, histone deacetylases and numerous chromatin remodelers.

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P406

Differentiating phenotypic response to environmental stimuli from individual Daphnia microcrustacea by two-dimensional gel electrophoresis

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Daphnia pulex and *D. magna* are parthenogenic microcrustaceans capable of rapid adaptive response to adverse environmental stimuli such as hepatotoxins produced during cyanobacterial blooms. Hence, these organisms are useful monitors of water toxicity. Under normal conditions, *Daphnia* populations are exclusively female and reproduction is clonal. However, in response to environmental changes, the appearance of males and the shift to sexual reproduction facilitates genetic recombination and rapid phenotypic response. Pressure cycling technology (PCT) used in combination with two-dimensional gel electrophoresis (2DE) elucidated 904 ± 7 proteins over the pI 4-7 range from individual *D. pulex* (0.05 ± 0.01 mg anhydrous biomass). From pools of five organisms, 1,267 ± 3 proteins were detected over the same pI range. For reasons unknown, only 690 ± 4 proteins were isolated over the pI 4-7 range from the larger *D. magna* species (0.23 ± 0.06 mg anhydrous biomass). From pools of multiple *D. magna* organisms, 382 ± 27 proteins were isolated over the pI 7-10 range representing over 50% of the 746 proteins predicted by the genome sequence in that pI range. 2DE enabled the putative identification of multiple phenotypes from single *D. magna* organisms, as demonstrated (*i*) in populations transitioning from clonal to sexual reproduction, (*ii*) under normal and hypoxic conditions, and (*iii*) in two genotypically distinct clones. In comparing *D. magna* clones Iinb1 and Xinb3, 57 and 79 proteins were upregulated, respectively.

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Proteomics Unraveling Honeybee Drone (*Apis mellifera* L.)Embryonic Development

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Introduction
The honeybee is becoming an increasingly important model insect and its egg is very amenable to laboratory manipulations. Drones are haploid, inheriting just one set of chromosomes from their mother. Every gene is effectively homozygous without dominant and recessive effects. Thus, all drone genes are open to selection pressures. The aim of this study was to identify the protein profile, build up some biochemical networks and find some key node proteins that help us better understanding this model organism.

Methods
The characterization of the proteome profile of embryonic development of the honeybee drone, *Apis mellifera*, was investigated by employing two-dimensional gel electrophoresis, MALDI-TOF and Nano LC-MS/MS, protein engine identification tools that were applied to MASCOT search engines and biological network software (pathway studio). Protein expression were validated by using real-time PCR at mRNA level.

Results
200, 242, and 233 proteins were detected at 24, 48, 72 hours with 94, 98, 97 proteins were unambiguously identified. respectively,These proteins were involved in the metabolism of carbohydrates and energy production, lipid, and proteins; cytoskeleton proteins, nutrition metabolism, development regulation, antioxidant system, molecule transporters, regulation of transcription and translation and protein folding. Twenty proteins were validated at mRNA level by real-time PCR, with 11 of them following the same expression thread between mRNA and proteins. This suggests that the embryonic development of the honeybee drone involves in multiple functions of proteins that take part in a variety of biological processes for which the energy production is the most. After placing all the identified proteins and differentially regulated proteins into the biology network, 42 key node proteins were detected, while 31 had altered their expression significantly. These key node proteins probably play an important role in embryogenesis and provide us some target proteins when genetically manipulating the embryo of the honeybee drones. Furthermore, 14 novel proteins have been identified in the honeybee genome in this study. This is the first large scale and comprehensive research in this regard so far.

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P408

Network regulating of antibiotic-resistant outer membrane proteome in *Escherichia coli*

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Introduction
The antibiotic-resistant bacteria that were once susceptible to antibiotics are becoming more and more difficult to be treated by antibiotics, and thus pose a serious threat to human health today. Previous reports have indicated that several outer membrane (OM) proteins, an OM proteome, rather than single OM protein are responsible to the resistance in the intrinsic resistance of Gram-negative bacteria. However, little is known about whether the proteome are functionally interacted in response to antibiotics.

Methods
Proteomic methodologies were utilized to characterize altered OM proteins in antibiotic-resistant *E. coli* K12 BW25113 strain (streptomycin-R: SM-R; ciprofloxacin-R: INN-R) which was selected from its original strain (SM-R-O or INN-R-O) by the use of ten sequential propagations in LB medium with 1/2 minimum inhibitory concentration (MIC) of antibiotics. The changes at the level of protein expression of the altered OM proteins were validated by Western blotting, and their possibly functional characterization and interaction were respectively investigated using gene-deleted *E. coli* K12 BW25113 strains with antibiotic resistance (△SM-R; △INN-R) and their original strains.

Results
Proteomic methodologies were utilized to characterize altered OM proteins in antibiotic-resistant *E. coli* K12 BW25113 strains (for example: SM-R) which was selected from its original strain (SM-R-O) by the use of ten sequential propagations in LB medium with 1/2 MIC of antibiotics. The changes at the level of protein expression of the altered OM proteins were validated by Western blotting, and their possibly functional characterization and interaction were respectively investigated using gene-deleted *E. coli* K12 BW25113 strains with SM resistance (△SM-R) and their original strains (△SM-R-O). TolC, OmpT and LamB were found to be up-regulated, and FadL, OmpW and an unknown location protein Dps were down-regulated in the SM-resistant *E. coli* strain. The interaction network among the altered OM proteins was characterized based on effect in absence of each of the six altered proteins on the other five in SM-resistant genetic modified strains with gene deletion. Decrease of TolC was detected in Δ*ompW*-R, elevation and decrease of FadL respectively in Δ*ompT*-R, Δ*dps*-R, and Δ*ompW*-R, Δ*lamB*-R, elevation and decrease of OmpT respectively in Δ*ompW*-R, Δ*dps*-R, and Δ*tolC*-R, Δ*fadL*-R, Δ*lamB*-R, elevation and decrease of OmpW respectively in Δ*tolC*-R, Δ*lamB*-R, Δ*dps*-R, and Δ*ompT*-R, elevation and decrease of LamB respectively in Δ*fadL*-R, Δ*dps*-R, and Δ*tolC*-R, Δ*ompT*-R, Δ*ompW*-R, elevation and decrease of Dps respectively in Δ*tolC*-R, Δ*fadL*-R, Δ*ompT*-R, and Δ*lamB*-R. Of the six altered proteins, TolC mainly regulates other proteins, and OmpT, LamB and Dps may be middle factors at a center of the network.

Innovative aspects
- Interaction network exists in OM proteins in response to antibiotic-resistant bacteria.
- TolC, OmpT, and Dps may play more key roles in positive and negative regulation of SM resistance than the others respectively.
- Our results provide novel insights into mechanisms of SM resistance

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P409

Proteomic and Bioinformatic Identification of Hyperthermostable proteins in Hyperthermophilic Archaeon *Thermococcus onnurineus* NA1

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Introduction
Thermococcus onnurineus NA1 was the hyperthermophilic Archaeon, which was isolated from a deep-sea hydrothermal vent area. Recently, genome sequencing of *T. onnurineus* NA1 was completed. Recently, we performed 2-DE/MS-MS analysis in order to characterize abundant *T. onnurineus* NA1 proteins expressed in enriched medium. Here we identified heat stable proteins using bioinformatic and proteomic approaches.

Methods
In order to screen the thermo stable proteins, proteins extract *T. onnurineus* NA1 was treated with heating at high temperature. Protein extract in 20mM Tris-HCl (pH 8.0), 1mM PMSF was incubated for 2 hr at 80 °C and 100 °C. The denatured and coagulated proteins were precipitated and removed by centrifugation at 18,000 x g for 20min. Proteins in the supernatants were analyzed using both 2-DE/MS-MS and 1D /LC-MS-MS. Also thermostable proteins were predicted from bioinformatic analysis with 10 different genomes using TargetStar analysis.

Results
In vitro heat treatment result showed that 13 proteins including intracellular protease I and thioredoxin reductase are remained as soluble in 2-DE gel and thus considered to be heat-stable. Then Approximately 63.7% of protein extract were remained in soluble fraction after heat treatment at 80°C. Also we identified more than 270 proteins using LCQ MS after heat treatment corresponding to 80, 100°C. The highly abundant proteins such as phosphoenolpyruvate synthase, elongation factor Tu, and glutamate dehydrogenase were mostly removed after heat treatment at 100°C. Interestingly, most of putative peroxiredoxin and thioredoxin remained at 100°C. In addition transcriptional regulator, proteasome, intracellular protease and translation initiation factor were included in the heat stable proteome identified on the 2-DE/MS-MS analysis. Also we performed TargetStar analysis, which can predict the relative thermal stabilities for given proteins based only on their amino acid sequences, with total 10 genomes containing 5 different thermophilic genomes including NA1 and 5 mesophilic genomes. 42 proteins considered to be thermostable in LCQ MS data was predicted as thermostable by TargetStar analysis. Further studies are under way to confirm thermostabilities of these proteins by measuring enzyme activities.

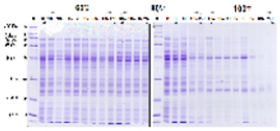

Fig. 1. 1-DE of heat treated protein extract

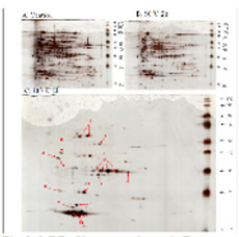

Fig. 2. 2-DE of heat treated protein Extract
T. onnurineus NA1 control, 3-10NL 182μg, B. NA1 80℃, 2hrs, 3-10NL 116μg, C. NA1 100℃, 2hrs 3-10NL 35μg,

Innovative aspects
(1) Construction of heat stable proteome of thermostable Archaea using 1D/LC-MS-MS and 2D/MS-MS
(2) Prediction of heat stable proteins from 5 different hyperthermophilic and 5 mesophilic bacteria, respectively

References
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P410

Histone Post Translational Modifications Associated with Transcription

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Introduction
Post translational modifications (PTMS) of histone tails play significant roles in the organization and functional state of chromatin.[1] Trimethylation of H3K4 has been shown to be characteristic of active transcription.[2] The TAF3 PHD containing subunit of TFIID can selectively bind and isolate nucleosomes containing the H3K4-me3 mark in native chromatin.[3] High resolution mass spectrometry (MS) and tandem MS (MS/MS), using CAD and electron transfer dissociation (ETD) was used to characterize modification patterns on H3 and the other core histone proteins in the enriched nucleosomes.

Methods
Nuclear extracts were prepared from murine embryonic stem cell homogenates and mononucleosomes were then generated using micrococcal-nuclease digestion. Mononucleosomes were affinity purified using TAF3. The eluates were then solubilised in HCl and the histones were TCA precipitated. Histone protein mixtures were digested with endoproteinase GluC. In addition aliquots of the peptide mixture were chemically modified to propionylate unmodified and monomethylated lysines and further digested with trypsin. The peptide mixtures were analyzed by nanoflow-HPLC micro ESI on a Thermo Scientific LTQ-FT modified for front-end electron transfer dissociation (FETD).

Results
The use of the TAF3 domain of TFIID was successfully used to enrich for nucleosomes containing the H3K4-me3 mark. Analysis of the short peptides generated from trypsin digestion provided the relative abundances of different modifications present on specific residues of histone H3. ETD analysis of the longer, highly charged, histone peptides resulting from endoproteinase GluC digestion provided information about combinations of modifications present on the same histone tails. In addition it has been possible to identify a number of isoforms and variants of each of the four core histones present. For example, H2A.Z was found to be present at equal relative abundance to canonical forms of H2A. This is significant because H2A.Z has been shown to localize to areas upstream of actively transcribing genes. Determination of the combinations of PTMs present on a single histone tail and the patterns of modifications found within the same nucleosome could lead to better understanding of the roles of these modifications. Selectively isolating nucleosomes that carry the gene activation marker K4-me3 on H3 allows for characterization of all core octamer histone isoforms present and the modifications present, which are presumed to be associated with actively transcribing genes.

Innovative Aspects
- First analysis of nucleosomes enriched for transcription active mark H3K4-me3.

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P411

DISRUPTION OF THE DIRECT INTERACTION BETWEEN PLURIPOTENT PROTEIN CTCF WITH Y BOX BINDING PROTEIN 1 (YB-1) BY HPV TYPE 16 AND 18 E7 ONCOPROTEIN, UP-REGULATES C-MYC GENE.

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Human Papillomaviruses (HPVs) type HPV-16 and HPV-18 are known as high risk HPV, which causes 95% of cervix cancer. E7 protein of HPV interacts with cellular protein pRB and inactivates pRB thus allowing progression of the cell cycle, further triggers cancer development. CTCF is a multivalent transcription factor which has been found to interact directly with YB-1. Both binds synergistically on *c-myc* gene promoter and down regulates the gene. Cell lysate were prepared from HeLa and Caski cell lines using RIPA buffer, which were then used for co-immunoprecipitation assay using CTCF and YB-1 antibody, respectively. Full domain of HPV 18 and 16 E7 proteins, and truncated CTCF and YB-1 proteins were produced in bacterial expression system and purification. These proteins were coupled to sepharose beads and preceded with pull-down assay. EMSA assay was performed to confirm the pull-down assay. CTCF was found to bind with HPV 16 E7 but did not have interaction with HPV 18 E7. Meanwhile, YB-1 interacts with E7 protein vice-versa to CTCF. HPV 16 E7 bind with Zn-domain of CTCF specifically and HPV-18 E7 bind with CSD-domain of YB-1 specifically. EMSA reveals negative shift in the promoter region of *c-myc* gene, which indicates that HPV 16 & 18 E7 disrupt the interaction of YB-1 and CTCF. HPV 16 E7 bind to CTCF at Zn-domain and HPV 18 E7 bind to YB-1 at CSD domain. However CTCF and YB-1 have no binding with HPV 18 E7 and HPV 16 E7, respectively. It is known that CSD-domain of YB-1 binds to Zn-domain of CTCF and produce synergistic effect towards *c-myc* promoter by down-regulation the *c-myc* gene expression. EMSA has shown that interaction of HPV E7 protein towards CTCF/YB-1 complex have release CTCF/YB-1 complex from *c-myc* gene promoter site. Therefore it is postulate that the interaction of E7 protein with CTCF and YB-1 significantly reduces the interaction of YB-1 and CTFC. Thus, enhances the up-regulation of *c-myc* promoter and promotes tumourogenesis of cervix.

Quantification

(Q101 – Q155)

Q101

Quantitative Proteomic Analysis of *Sulfolobus solfataricus* P2 under Different Temperatures

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Introduction
In this investigation, *Sulfolobus solfataricus* P2 (a member of archaeal domain) was grown under elevated temperatures ranging from 65°C, 70°C and 80°C. Cells were then extracted for quantitative proteomic analysis using iTRAQ (isobaric tag). We here successfully applied the iTRAQ technique for quantitation of both membrane and soluble proteins using modified protocols for protein extraction, protein digestion as well as iTRAQ labelling. Moreover, the dynamic changes of cell metabolisms were performed at proteomic level. These data provided rich information for understanding of *S. solfataricus* under high temperatures.

Methods
Briefly, cell was disrupted by sonicating, and then centrifuged at ultra-speed to separate soluble and insoluble proteins. While soluble fraction was used for iTRAQ experiment based on protocol offered by Applied Biosytem, membrane proteins were delipidated (by methanol and chloroform) before resuspended in iTRAQ buffer (0.5M TEAB pH8.5 +0.095%SDS). After alkylating and cysteine blocking, membrane proteins were digested with 2 steps; firstly samples were added with SDC for enhancing trypsin digestion. Peptides (digested by trypsine) were then collected, whilst remained membrane proteins were applied for a second digestion using combination of both trysin and chymotrypsin. After that, peptides collected from the second digestion were combined with peptides from the first digestion steps before labelled with iTRAQ reagents. Sample was submitted to tandem mass spectrometry, data were then analysed using Phenyx program for interpreting proteomic data.

Results
By applying modified protocol as mentioned above, analysing separately soluble and insoluble proteins, we detected 1037 proteins where 307 hydrophobic proteins were identified (for iTRAQ 4-plex). Furthermore, by applying iTRAQ 8-plex for samples collected at 65°C, 70°C and 80°c, we can detected 999 proteins where 117 and 82 proteins were observed as up-regulation at 65°C and 70°C compared to 80°C respectively; and 94 and 56 proteins were down-regulated at 65°C and 70°C compared to 80°C respectively. Moreover, most important proteins relating to central carbon metabolism were found, offering rich information for understanding of *S. solfataricus* in responding to different temperatures. From these data, we observed that most up-regulated proteins under low temperature belonged to t-RNA synthases, ribosomal proteins, cofactor biosynthesis proteins, DNA-directed RNA polymerases, Redox balance, transporter proteins, conserved hyphothetical proteins and hypothetical proteins. Most of down-regulated proteins were permease and metabolite permease, binding proteins, amino acid metabolism, conserved hyphothetical proteins and hypothetical proteins.

Innovative aspects
- Protocol for quantitating membrane proteins using iTRAQ technique.
- Provide rich information for understanding the response of *S. solfataricus* P2 under different temperatures.

Acknowledgments
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Q102

Progress in Label-Free Protein Quantification Tool Development *T3PQ* in comparison with Superhirn, APEX and emPAI

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Introduction
Tandem mass spectrometry allows for fast protein identification in complex samples. As machines get faster, more sensitive and more accurate, methods were devised by a variety of academic research groups and commercial suppliers that allow protein research also in quantitative respect. Since accurate label-free methods are an attractive option for proteomics researchers, we evaluated several open-source methods for their performance on a reference data set, which was generated explicitly for this purpose. For the evaluation we took protein centric (APEX, Mascot-emPAI) as well as peptide centric (SuperHirn) methods into account.
Furthermore, we extended the collection of available label-free quantification software tools by introducing another protein centric tool: T3PQ (Top 3 Protein Quantification). T3PQ is based on the idea of Silva, using the intensities of the precursor ions of the three most efficiently ionized and identified peptides of a protein to provide a measure for its abundance.

Methods
A reference dataset was generated consisting of a complex yeast background (cytosolic fraction digested with trypsin) and peptides of a digested spike-in protein in different concentrations (0 fmol – 300 fmol loaded on column). Mass spectrometry was performed in triplicates with an LTQ-FTICR instrument.
The data was searched with Mascot and processed with several published label-free methods. We implemented the idea of Silva in a workflow which makes use of the Mascot search results and of the information content of mzXML data format to find the three most efficiently ionized peptides per protein. The three most intensive precursor ions are selected and averaged to calculate the protein abundance.

Results
Our analysis show that the selected publicly available label-free protein quantification methods are limited in dynamic range, variance, and accuracy of protein abundance values. Although all tested methods are able to capture the increasing concentration of the used spiked in protein, there are differences with respect to linear responsiveness and variance of the protein abundance values. We show that spectral counting based methods suffer substantially from undersampling issues at higher protein concentrations (>100fmol) and changed sample complexity, whereas the *T3PQ* method is robust if at least three peptides per protein are detected.
The software T3PQ is available under GNU public license at http://www.sourceforge.org.

Innovative aspects
- extend existing software toolbox with T3PQ
- show effectiveness of selected label-free quantification tools

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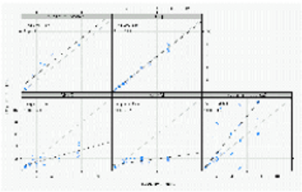

Figure 1. This figure displays the correlation of theoretically calculated ratios versus the experimentally measured abundances of spiked in Fetuin versus Fetuin abundance of 40 fmol. The read-out of the *T3PQ* method is linear over the full dynamic range. For the emPAI method and the APEX method the read-outs show saturation above a fold change of 2.5 (corresponding to 100fmol). The grey line indicates perfect linearity whereas the black line represents the linear fit of the calculated ratios. The peptide centric SuperHirn method indicates the in-crease of the spiked Fetuin but when the individual peptide ratios are plotted it shows a larger variation. In the SuperHirn (protein) panel the peptide ratios per condition are averaged to conclude for the protein.

Q103

Quantification of total peptide amount by an optimized micro-LC/UV method

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Introduction
Proteome sample preparation plays a key role in ensuring maximum proteome coverage in shotgun proteomics [1]. This is particularly true in dealing with small samples amounts, such as rare tumor cells captured from human blood, stem cells isolated from a large population of cells, and primary cells procured from laser capture microdissection of a tissue. In working with these types of samples, a rapid and non-destructive technique for quantifying small amounts of peptides during sample workup leading to MS analysis becomes critical. In this work, we report a micro-LC/UV method to quantify small peptide samples (0.1 µg-10 µg) for applications in proteome analysis.

Methods
A four protein mixture solution containing equal moles of myoglobin, cytochrome C, lysozyme, and ß-casein was prepared. After reduction with dithiothreitol and alkylation with iodoacetamide, the mixture was digested with trypsin. The four protein digest was used as the standard solution for generating a quantitative calibration curve. Breast cancer MCF-7 cells were cultured and subjected to flow cytometry for cell sorting and counting. Cells were lysed and digested and the peptide mixture was injected onto a RPLC microcolumn on an Agilent 1100 LC/UV system. After the optimal step gradient was developed for peptide elution, the measured peak area was compared against the calibration curve for quantification. Columns of different inner diameters were examined for quantifying different ranges of peptide amounts.

Results
A step gradient was used to rapidly elute peptides from a LC column to generate one integral peak from which the peak area could be related to the amount of peptides injected. However, with a step gradient, a system peak from the blank injection was observed at 214 nm and must be subtracted. It was found that system peaks were reproducible when 4.6- and 2-mm columns were used, while using a 1-mm column required careful optimization of the flow rates and solvent conditions to achieve reproducible results. The linear range for all columns was about two orders of magnitude. For example, the 2-mm column had a linear range from 0.1 to 10 µg. With a 1-mm column, the limit of quantification was <0.05 µg. The relative standard deviation in signal response at different sample loading amounts for each column were <10%. We are currently using this method to optimize the sample preparation process related to proteome analysis of <1000 cells. For the MCF-7 cells, the peptide amount from the whole cell digest of 1000 cells was about 0.3 µg. And by using this optimized method, we could measure the total peptide amount from the digest of as low as 250 cells. We experimented with different cell lysis methods, protein precipitation/solubilization reagents, and trypsin digestion conditions, in conjunction with the UV-LC quantification method, to develop an optimal protocol with minimal sample loss for MS profiling.

Innovative Aspects:
A micro-LC/UV method using step gradient elution was developed for rapid quantification of total peptide amount in proteomic digest samples

References:
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Q104

Identification of *miR-17-92* Targets in lung epithelial cells using a quantitative proteomic approach

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Introduction
Lung cancer is the leading cause of cancer death in most economically developed countries. Recently amplification and over-expression of the *miR-17-92* microRNA (miRNA) was found in lung cancer. This miRNA has been suggested to play a role in B-cell lymphoma development. Therefore the *miR-17-92* over-expression in human cancers may have important roles in cancer development, but only a few targets for the *miR-17-92* cluster have been identified thus. In this research study, we investigated target proteins for *miR-17-92* using iTRAQ® reagents followed by QSTAR® LC/MS/MS system.

Methods
Lung epithelial cells BEAS2B were lysed with 100µl of 0.5M triethylammonium bicarbonate in 0.1% (w/v) SDS. Cell lysates (100 µg) expressing either the control or *miR-17-92* cluster were reduced, alkylated, and digested with trypsin. Each digested sample was labeled with iTRAQ reagents according to standard procedures. The combined peptide mixtures were analyzed by 2D-LC-MS/MS system. Data was processed using ProteinPilot™ 2.0 Software to identify proteins and determine relative expression levels. Expression of unique proteins was confirmed by Western blot analysis.

Results
479 proteins were identified. The number of proteins which was more than a 2-fold down-regulation in the *miR-17-92* transfected clone was eight. Four of the eight proteins with significant down-regulation, PGK1, ENO2, ENO1, TP11, are in the glycolytic pathway and six of the eight proteins lacked potential target sites for the miRNAs comprising the *miR-17-92* cluster. Down-regulation of multiple proteins lacking potential target sites for *miR-17-92* suggested the possibility that *miR-17-92* could target a key transcription factor that commonly regulates their expression. We consequently discovered that HIF-1a, a well-known molecule regulating the glycolytic pathway, was computationally predicted to be a target for multiple miRNAs comprising the *miR-17-92* cluster. Western bolt analysis revealed a significant down-regulation of HIF-1a protein in BEAS2B cells stably overexpressing *miR-17-92*.

Innovative aspects
- Combined iTRAQ reagents and QSTAR LC/MS/MS system is an effective method to investigate the target proteins for microRNA.

References
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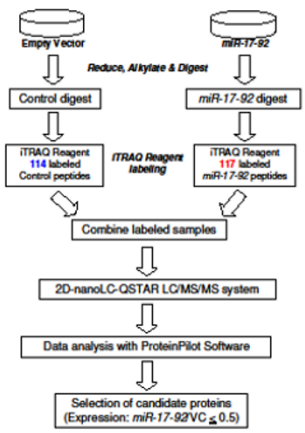

Figure 1. Workflow for identification of differentially expressed proteins between *miR-17-92*-introduced and control cells of BEAS2B. ProteinPilot™ 2.0 Software is a program designed for maximizing information from iTRAQ reagent labeling and MS/MS quantification.

Q105

Proteomics to Study the Roles of ROS and Src Kinase Inhibitor, PP1, in Ischemia Reperfusion Injury

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*These authors contributed equally to this work

Introduction
Ischemic heart disease remains a great killer in Taiwan. While the restoration of blood flow to the myocardium during heart attack is a major therapeutic aim, the process of ischemia followed by reperfusion is often followed by an injurious cascade in terms of Ischemia reperfusion injury. Myocardial damage induced by ischemia-reperfusion is largely due to the generation of reactive oxygen species (ROS). Herein, we confirm that oxidative stress in response to hydrogen peroxide (H_2O_2) treatment of rat myocytes (H9C2) induces the robust tyrosine phosphorylation of multiple cellular proteins. An anti-phosphotyrosine purification and LC-MS/MS approach was then utilized to characterize these H_2O_2-induced tyrosine phosphorylation.

Methods
Sepharose-conjugated anti-phosphotyrosine mAb was used to enrich H_2O_2-induced tyrosine phosphorylated proteins (Figure 1). Subsequent elution with phenyl phosphate and precipitation with TCA followed by LC-MS/MS sequencing was used to identify the purified phosphorylated proteins. Interaction networks of identified proteins were also generated using the EMBL Search Tool for the Retrieval of Interacting Proteins (STRING) database. Validation and functional characterisation of identified targets was performed by western blotting, immunostaining, cell adhesion and cell viability assays. The role of specific kinases was investigated using small molecule inhibitors.

Results
A proteomics approach using anti-phosphotyrosine affinity purification and LC-MS/MS was used to identify 24 tyrosine phosphorylated or associated proteins. Importantly, we show that Src kinase is the primary upstream regulators of many of these identified proteins and that their activation after H_2O_2 treatment is responsible for the vast majority of the observed tyrosine phosphorylation. Around 75% of the identified proteins have functions in maintaining cell adhesion, cell-cell junctions and the actin cytoskeleton, prompting us to examine oxidative stress-induced changes in adhesion. Immunofluorescence and flow cytometry analysis showed that H_2O_2 alters cell adhesion structures and the actin cytoskeleton and results in the loss of adhesion and the enhancement of apoptosis. Notably, these H_2O_2-induced cellular changes and apoptosis could be alleviated by Src kinase inhibition, PP1. The peroxide and PP1-dependent proteome changes in H9C2 cells were elucidated by 2-dimensional differential electrophoresis (2D-DIGE) and MALDI-TOF MS analysis. While the pathogenesis of ischemia-reperfusion has not been completely understood, we expect this study provides a possible strategy to develop a therapeutic model for heart ischemia-reperfusion injury.

Innovative aspects
Src and EGFR play crucial roles in oxidative stress-induced tyrosine phosphorylation and cell damage and provide a general model for redox stress signaling in ischemic heart disease.

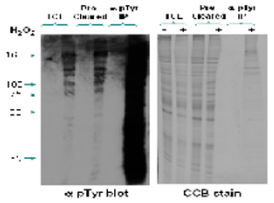

Figure 1. Purification of H_2O_2-induced pTyr proteins by anti-pY antibody enrichment.

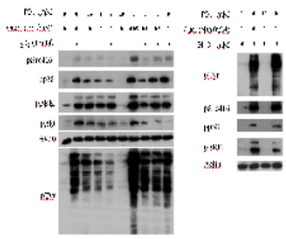

Figure 2. Effect of Src kinase inhibitor and reductant (Quercetin) on H_2O_2-induced signaling events.

Q106

Toxicoproteomics: Clostridial Toxins Induce Changes Of Proteinprofiles In Colonocytes

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Introduction
Toxin A and B are the causative agents of *Clostridium difficile*-associated diseases (CDAD) like diarrhoea and pseudomembranous colitis. These toxins glucosylate and inactivate Rho-GTPases leading to reorganization of the actin cytoskeleton and cell rounding. Toxin-induced morphological changes are accompanied by only few alterations in mRNA level (1). Hence, we started to analyse the proteomes of toxin-treated and untreated colonic cells. Proteins were labelled using the ICPL technique that is based on heavy and light stable isotopes and enables the relative quantification of proteins (2).

Methods
Toxin A-treated and untreated Caco-2 cells were lysed and extracted proteins were labelled with the heavy or the light ICPL reagent. Labelled proteins were combined, separated by SDS-PAGE and the whole gel lane was cut into 35 pieces which were processed by tryptic digestion. Formed tryptic peptides were separated by nanoscale RP-LC and directly fractionated onto a MALDI target. Peptides were analyzed off-line in an Ultraflex MALDI-TOF/TOF MS (Bruker Daltonics) and identified and quantified using WARP-LC software (Bruker Daltonics) and the MASCOT search engine.

Results
Proteins were extracted from untreated and toxin A-treated colonic cells (Fig. 1) separated at protein and peptide level and subsequently identified and quantified by LC-MALDI-MS. Overall 495 different proteins were identified and for 286 proteins (58%) regulation factors were calculated (Table 1). In control experiments similar protein extracts were labelled 1:1 with ICPL and regulation factors were clearly below 1.2 showing the capability of the ICPL approach. Thus, proteins exhibiting a regulation factor exceeding 1.4 were termed regulated. Nine proteins were up-regulated and only one protein was down-regulated after toxin A treatment. Among these proteins fructose-biphosphate aldolase A, Tubulin beta-2A and Actin-related protein 2 play a role in actin filament organisation and regulation of cell shape. Fructose-biphosphate aldolase A is also involved in glycolysis. The up-regulation of these proteins may be linked with the effects of toxin A-induced cytoskeletal reorganisation. Applying the ICPL technique we were able to identify several proteins that have not yet been recognized to be involved in toxin A-triggered cellular changes.

Innovative aspects
- Identification and relative quantification using ICPL-technique
- System biological analysis of toxin impact on colonic cells

References:

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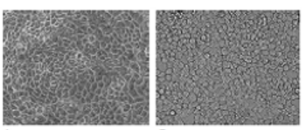

A B

Figure 1: Toxin A treated colonic cells.
(A) Caco-2-cells
(B) Caco-2-cells treated with toxin A

	Protein	Accession	Q (H/L)
1	Fructose-bisphosphate aldolase A	P04075	2,12
2	Vesicle-associated membrane protein-associated protein A	Q9P0L0	2,00
3	Putative mitochondrial inner membrane protein import receptor	Q9UPE4	1,85
4	Actin-related protein 2	Q8IY98	1,68
5	Apolipoprotein E	P02649	1,66
6	Tubulin beta-2A chain	Q13885	1,47
7	60S acidic ribosomal protein P0	Q3B7A4	1,46
8	Heterogeneous nuclear Ribonucleoprotein C-like 1	Q6PKD2	1,45
9	Polymerase delta-interacting protein 2 (p38)	Q9Y2S7	1,45
10	Ribophorin 1 protein	Q6IBR0	0,70

Table 1: Toxin A responsive proteins in colonic cells

Q107

Quantitative phosphoproteomics of the insulin and IGF-1 signaling pathways in adipocytes

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Introduction
Analysis of the insulin signaling pathway in adipocytes can provide new insights into the mechanism of insulin resistance.
A cellular signaling cascade is dependent on the series of induced phosphorylations on tyrosines and serines/threonines as it is integrated with internal state information and other signals that the cells receive (1).
Direct comparison of the phosphoproteomes of the insulin and IGF-1 signaling cascades is of great importance for identification of specific regulatory sites and critical nodes that control the differential signaling by these closely related growth factors.

Methods
To quantitatively compare the global phophoproteomes of the insulin and IGF-1 signalling pathways we employ a triple encoded SILAC strategy (Stable Isotope Labeling by Amino acids in Cell culture) to the murine fibroblast cell line 3T3-L1 (2). The cells were differentiated to mature day 6 adipocytes using dexamethasone, methyl-isobutylxanthine and insulin and before stimulation with either insulin or IGF-1 the cells were serum-starved to reduce the background phosphorylation level.
Following stimulation the cells were lysed, combined in 1:1:1 ratio, reduced, alkylated and digested. The resulting peptides were fractionated using strong cation exchange chromatography followed by enrichment of the phosphopeptides by titanium dioxide. The samples were analyzed by tandem mass spectrometry.

Results
We have successfully applied the SILAC approach to 3T3-L1 preadipocytes. The cells were cultured for 5 passages in different versions of labeled media which contained either the light, medium or heavy weight stable isotopes of the amino acids arginine and lysine.
To create a temporal activation profile of the proteins involved in the two closely related signaling pathways the cells were stimulated for either 0, 5, 10, 20 or 30 min. with either insulin or IGF-1.
The samples were analyzed on an LTQ-FT-Orbitrap, a high mass accuracy mass spectrometer and the generated data were processed, analyzed and identified by using the Mascot search engine and the quantitative software tool MaxQuant.
Our strategy resulted in identification and quantitative assessment of the global phosphorylation dependent cascades initiated by insulin and IGF-1 that can help in understanding the differential cellular response to these two growth factors. Furthermore, the temporal activation profile provides information about the early and later interaction players in the two signaling cascades as well as duration of the signal transient.

Innovative aspects
- Quantitative analysis of proteins involved in the insulin and IGF-1 signaling pathways in 3T3-L1 adipocytes.
- Temporal activation profiles of proteins regulated by insulin and IGF-1.
- Identify regulatory sites that could be used as drug targets in treatment of insulin resistance.

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Q108

A Simplified Method for microRNA Target Identification by Label-Free Mass Spec Analysis

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Introduction
Over the course of the last decade, microRNAs (miRNAs) have emerged as important regulators of protein expression in a wide variety of organisms, including humans. Through mRNA-targeted inhibition of translation, miRNAs have been shown to play a major role in normal cellular function and development, as well as disease. Many gaps still remain in our understanding of how miRNAs function, from rules governing mRNA targeting to their effects in various pathways of the cell. In order to fully elucidate the mechanisms by which miRNAs operate, a greater knowledge of real functional targets is needed.

Methods
We have developed a simple high-throughput method for the identification of miRNA targets by mass spectrometry (MS). Utilizing the APEX method of label-free protein quantitation previously developed in our lab (1), we can determine relative changes in protein abundance across samples by analyzing the frequency of observations for a given protein in an MS experiment. Comparing the proteomic profiles of cultured cells transfected with a miRNA of interest to a control sample subject to mock-transfection, we identify proteins exhibiting a relative decrease in abundance upon miRNA treatment. These down-regulated proteins are then cross-referenced with computationally derived target predictions to generate a list of putative targets.

Results
We have applied our method to several miRNAs of interest, including hsa-miR-1 and hsa-miR-124, and compared the results to previously published research from other groups in order to evaluate the effectiveness of our method in identifying valid functional targets. When compared to previous label-based MS approaches (2,3) our method yields similar depth of coverage (~3000 proteins identified per experiment), as well as a large overlap of putative targets identified between the different analyses when evaluating the same miRNA. Confirmation of several putative target mRNAs by luciferase assay further validated the accuracy of our method. Thus, the resulting lists of experimentally-derived targets generated by our method can be considered high-confidence target sets. We evaluated the accuracy of several computational target prediction algorithms against our target sets and those generated by similar experimental methods. Our analysis identified distinct differences in the accuracy of the tested algorithms. This work demonstrates the effectiveness of our method to identify valid targets of miRNAs, and highlights the importance of generating such high-confidence target sets to improve our understanding of how miRNAs function.

Innovative aspects
- High-throughput miRNA target identification
- Quantitative label-free mass spectrometry analysis
- Evaluation of computational target prediction algorithms

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Q109

A Label-free Quantitative Analytical Approach for Phosphopeptide Profiling

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Introduction
Reversible phosphorylation represents a major mechanism of signal transduction in a variety of cellular functions. Quantitative phosphoproteomics is necessary to identify phosphorylation events that may be regulating biological response to cellular perturbation or disease states. Currently most studies employ stable-isotope labeling methods to quantify phosphoproteins. However, there is still lack of a general label-free quantitative strategy for enriched phosphopeptides in a large-scale study.

Methods
Here we have developed a novel phosphorylation quantification approach by spectral counting and proteotypic peptide analysis. Enriched phosphopeptides with titanium dioxide and zirconium dioxide were analyzed by nano-LC-MS/MS with automatic cross-validation of MS/MS and MS/MS/MS (MS2 + MS3) data-dependent neutral loss method. For quantitative analysis, spectral counting was employed on a MS2-only scan implemented successively after each MS2 + MS3 scan. We applied a normalized spectral abundance factor approach to quantify phosphoprotein expression. Only those proteins, both identified by MS2 + MS3 scan and statistically significant by spectral counting analysis, were considered. Then we employed proteotypic peptide analysis to improve the confidence of quantification.

Results *(Arial 10, 1200 char. max. incl. spaces)*
We identified 700 phosphorylated sites on 182 unique phosphoproteins with false discovery rate less than 3% in human breast cancer malignant cell line M-4A4 and non-malignant cell line NM-2C5, in which 339 sites (48.4%) were not reported in the Phosphosite Plus database as of November, 2008. With the spectral counting method on MS2-only scans, 108 proteins were identified with p value less than 0.05. Forty-two proteins were confirmed by MS2 + MS3 and MS2-only data. By adopting peptides with positive proteotypic scores, we obtained 24 proteins as significantly changed phosphoproteins. Our integrated phosphorylation quantitative approach addresses the issue where spectral counting cannot apply to the MS2 + MS3 scan which is necessary for high-reliability phosphopeptide identification with the low mass accuracy MS mode. It also tackles the problem where most phosphoproteins have only one peptide detected in MS after enrichment which is not appropriate for spectral counting. This label-free quantification approach is completely generic for identification of key phosphorylation events in signaling pathways and is applicable to any cell culture system, tissue or body fluid.

Innovative aspects
- Development of a novel label-free phosphorylation quantification approach on enriched phosphopeptides in a large-scale study.

References
(1) K Rikova et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell. 2007, 131, 1190-1203.
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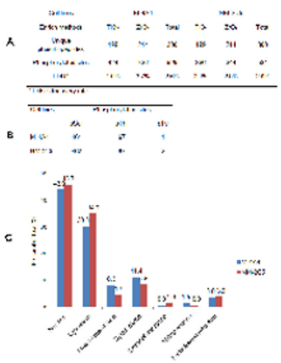

Figure 1. A. Summary of identified unique phosphopeptides and phosphorylated sites in human M-4A4 and NM-2C5 cell lines; B. Distribution of phosphorylation sites by amino; C. Gene ontology component.

Q110

Relative quantitation of plasma proteins using next generation MALDI TOF/TOF instrumentation

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Introduction
The LC MALDI workflow on TOF/TOF MS systems in combination with relative quantitation techniques like iTRAQ reagent labelling has become a powerful platform for biomarker discovery. LC MALDI benefits from decoupling of MS acquisition and chromatographic separation which provides higher quality MS/MS spectra. As iTRAQ quantitation is based on MS/MS generated reporter ions, experiments require 1000s of MS/MS spectra to determine changes in protein expression which previously required days of instrument time. The faster laser with software and hardware improvements on the 5800 MALDI TOF/TOF increases throughput and sensitivity of LC-MALDI analysis while maintaining quantitation reproducibility.

Methods
Four depleted plasma samples were digested with trypsin and labelled with the iTRAQ reagent 4Plex. Labelled samples were pooled into one iTRAQ multiplexed sample and fractionated by SCX chromatography. Collected fractions further separated by nano flowrate RP chromatography in quadruplicate and automatically spotted on MALDI targets every 60s. Sample spots overlaid with CHCA matrix. Duplicate sample sets analyzed by either a 4800 system (UVic Genome BC Proteomics Centre) or 5800 TOF/TOF System (AB SCIEX) using the best acquisition modes for each instrument. Data from both instruments analyzed by ProteinPilot software 3.0 with integrated false discovery rate determination by searching against a SwissProt database with a Human species filter.

Results
Previous work has demonstrated that the faster laser (1kHz), continuous stage motion, and smart acquisition software (DynamicExit™ Algorithm) provided a significant increase in the numbers of proteins and peptides identified in a much shorter time frame (>10x faster) for protein ID workflows. For quantitative proteomics, however, the number of identifications which yield high quality quantitative results is the most important factor and was investigated here. The iTRAQ optimization feature built into DynamicExit™ Algorithm ensures that that the ion statistics of the iTRAQ reporter ions are obtained at a high quality. In this 2D LC MALDI experiment on human plasma, we found that the 5800 system identified 66% more proteins and 39% more peptides over the previous generation instrument in half the time. The effects on quantitation quality were investigated by comparing the iTRAQ ratios measured for the intersecting set of proteins identified by both instruments (65%). Figure 1 shows considerable agreement between $\log_2$ iTRAQ ratios. The average relative percent difference between protein iTRAQ ratios were 11.31%, 11.35%, and 11.34% for 115:14, 116:114, and 117:114 respectively.

Innovative aspects
- 5800 instrument identified 66% more proteins and 39% more peptides than the 4800 MALDI TOF/TOF, in half the acquisition time
- Smart acquisition software ensures high quality MS/MS without consuming each MALDI spot for high quality quant and more IDs
- iTRAQ ratios of proteins identified by both instruments differed by 11.3% on average

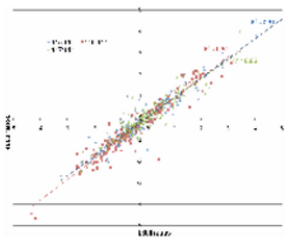

Figure 1 Agreement plot of protein iTRAQ ratios ($\log_2$) from proteins identified by both instruments.

Q111

An Efficient Label-free Strategy for Phosphoproteomics in Depicting Mechanisms of Cancer Invasion

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Introduction
Aberrant protein phosphorylation plays important roles in cancer-related cell signaling. However, label free quantitative analysis of protein phosphorylation presents an analytical challenge. With the goal of achieving multiplexed and fully automated quantitation of site-specific phosphorylation, we present a simple label-free strategy combining pH/acid-controlled IMAC and SEMI (Sequence, Elution time, M/Z and Internal standard)-assisted algorithm. We will demonstrate the high quantitation accuracy and precision and application of this approach on lung cancer metastasis model to explore the metastasis-associated phosphoproteomic alteration in lung cancer.

Methods
The simple multiplexed label-free approach involving only three steps: (1) gel-assisted digestion[2]; (2) pH/acid controlled IMAC purification and (3) LC-MS/MS analysis. A phosphoprotein, β-casein, was spiked into each sample as internal standard prior to gel-assisted digestion. The sample along with the spiked β-casein was incorporated into polyacrylamide gel matrix in eppendorf without electrophoresis and subjected to in-gel digestion. Any interfering components, including lysis buffer and reduction/alkylation reagents, can be removed by subsequent in-gel washing steps, which bypassing the addition desalting step. Finally, the phosphopeptides were purified by automatic IMAC purification and followed by triplicate LC-MS/MS without addition peptide fractionation.

Results
We first evaluated the quantitation performance on standard phosphoprotein, ovalbumin. Reliable linear correlation can be obtained with 4000-fold dynamic concentrations (R^2=0.99). On the proteome-scale, high quantitation accuracy (1±0.32) and precision (8.9%) can be routinely obtained by SEMI algorithm. Furthermore, the SEMI strategy effectively increased the number of quantifiable phosphopeptides among all identified phosphopeptides; the number of quantifiable phosphopeptides can be increased from 262 to 1171 in replicate cell lysate. This application of the approach was demonstrated on lung cancer metastasis model. Without sample fractionation, the SEMI algorithm enabled quantification of 1770 unique phosphopeptides corresponding to 889 phosphoproteins from a series of NSCLC cell lines. Validation for subset of phosphoproteins by western blotting and microarray not only demonstrated consistent data but also revealed the dramatic changes in phosphoproteome are mostly from translational or post-translational regulation. Mapping of these differentially expressed phosphoproteins in multiple cellular pathways related to cancer metastasis suggests that the site and degree of phosphorylation might have distinct pattern or function in the complex process of cancer progression.

Innovative aspects (maximum of 3 bullet points)
1. We present a simple label-free strategy combining automatic pH/acid controlled IMAC procedure and SEMI assisted algorithm for quantitative phosphoproteomics.
2. This approach was demonstrated on the first delineation of differential phosphoproteomics profiles of lung cancer metastasis model.

References (maximum 3 references)
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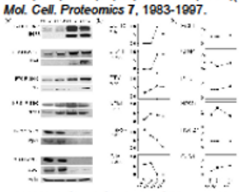

Figure 1. Verification of the differential expression levels of subset of phosphoproteins, EGFR (T693), CD44 (S706), NPM1 (T199), HSP27 (S82), GJA1 (S368) and PTK (S910), by western blotting and mRNA levels. The quantitative MS data were confirmed by western blot analysis and mRNA array. (a) The differential phosphorylation levels and protein expression levels were determined using both protein antibody and phosphorylation site-specific antibodies across all four CL1 sublines. (b) Normalized peak area obtained by mass spectrometry and SEMI strategy. (c) The mRNA levels were also measured by microarray hybridization.

Q112

Quantitative proteome profiling of obesity-induced type-2 diabetes mice using LTQ-OrbitrapXL

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Introduction
Obesity is a complex trait, reflecting interactions of a network of genes with environmental factors, such as diet, age and gender [1]. We therefore applied high accuracy mass spectrometry for the identification and quantification of proteins from liver and white adipose tissue of mice developing high-fat diet-induced obesity and diabetes.
The obtained proteomics data is currently being used for advanced mathematical modeling of the involved cellular processes and exploring the potential of bioinformatics methods promoting the general understanding of the disease.

Methods
Proteins were carefully extracted from mouse adipose and liver tissue, tryptically digested and labeled with iTRAQ™ reagents. Fractionated peptides were analyzed on the LTQ OrbitrapXL using HCD fragmentation resulting in robust precursor ion fragmentation down to m/z 98. Since ion trap CID fragmentation usually produces best quality spectra for peptide identification, we have developed a method where spectral merging of CID and HCD of two consecutive scans from the same precursor ion was applied. The mascot generic files were generated using Mascot Distiller 2.2 and protein identification and quantitation was performed using Mascot version 2.2.0 (Fig 1). We have analyzed three biological replicates of each strain and treatment with switched labels in each experiment.

Results
From initial comparisons between platforms we can conclude that the OrbitrapXL in our lab is, in terms of quantifiable proteins, at least 3x more sensitive than LC-MALDI-TOFTOF.
We therefore established LC-MS/MS compatible protocols for the extraction and the labeling of proteins from mouse adipose tissue with very high lipid content, as well as liver tissue. Data was searched against a SwissProt mouse database with 10ppm peptide tolerance for +2 and +3 charged peptides, 0.8 Da MS/MS tolerance, a variable oxidation on Methionine and two missed cleavage sites, mascot mowse score > 59 and p<0.05. The high sensitivity and signal-to-noise ratio for the reporter ions yielded in the identification of roughly 900-1200 proteins in each iTRAQ experiment of which 65-75% were quantifiable with two or more peptides (Fig 1).

Our main interest was not only to identify as many proteins as possible, but also the accurate and reproducible determination of their relative concentrations. We employed a set of statistical tool to test the quality, consistency and reproducibility of the data in order to generate regulation plots shown in Figure 2.

Innovative aspects
- Large scale quantitative analysis of white adipose and liver tissue
- Novel parallel acquisition method for iTRAQ samples on OrbitrapXL
- Setup of workflow for statistically consistent and reproducible datasets

References
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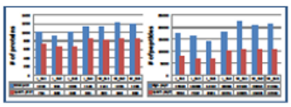

Figure 1. Absolute number of identified (blue) and quantified (red) proteins and peptides of all iTRAQ experiment of liver and white adipose tissue.

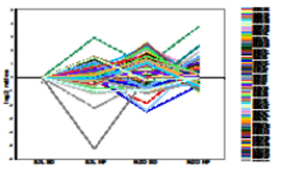

Figure 2. Regulation plot of iTRAQ experiment number 3 of white adipose tissue. Log2 ratios of quantified proteins of two different mice SJL (Swiss Jim Lambert) and NZO(New Zealand Obese) with two different diets; HF (high fat) and SD (standard diet).

Q113

Measurement of Phosphorylation Stoichiometry in Tumors by Mass Spectrometry

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Introduction
Measuring the extent, or stoichiometry, of phosphorylation at a protein site in malignant tumors is desirable because it may reveal the activated kinases in cancer. Determination of relative MS response rates of cognate peptide and phosphopeptide pairs enables stoichiometry measurements, and can be done by label-free MS methods[1]. To achieve increased sensitivity for phosphorylation stoichiometry determination in unlabeled (e.g. tumor) samples, a selective reaction monitoring (SRM) based method was developed. As proof of principle we measured Lyn phosphorylation stoichiometry in myeloma tumor.

Methods
A biological protein mixture, enriched for Lyn by immunoprecipitation (IP) when applicable, was protease digested and analyzed by SRM. An integrated peak area under the curve was derived for summed transition intensity chromatographs for each peptide, which was a linear function of the peptide's SRM response rate and quantity (i.e. peak area ∝ SRM response rate x peptide quantity). The SRM response rate of Lyn phospho-site-containing phospho-peptide, relative to the response rate of its cognate unphosphorylated peptide, was measured and termed the response rate ratio (RRR). The RRR was applied to the observed peak areas as a correction factor to derive the relative abundance ratio of the cognate Lyn phospho-peptide pair, from which, the phosphorylation stoichiometry can be calculated.

Results
By applying this method, the phosphorylation stoichiometries of two Lyn phosphotyrosine sites (Y194; Y397) were determined in a multiple myeloma tumor-derived cell line, treated with or without a phosphatase inhibitor, and in corresponding xenograft tumors. The SRM-derived data were consistent with Western blots that showed a dramatic increase in the level of pY397 in inhibitor treated cells, and a moderate increase in the tumor, compared to the control cells (Fig. 1). The total Lyn level showed no significant change. Additional experiments were performed on Lyn-derived peptides to measure the phosphorylation stoichiometries of Lyn during a phosphatase time course assay. The observed decreasing stoichiometry is consistent with phosphatase treatment (Fig. 2). This result demonstrated the sensitivity of the measurement assay in that small changes in phosphorylation at a low stoichiometry (< 1%) site (Lyn Y194) were clearly distinguished. This methodology has potential applications in analyzing clinical samples and may facilitate new therapeutic strategies.

Innovative Aspects
Applied selective reaction monitoring to measure phosphorylation stoichiometry, which reduced the effect of background interference and led to enhanced sensitivity.

References
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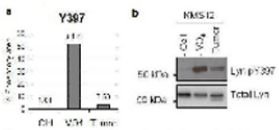

Figure 1. Lyn Phosphorylation Stoichiometry Determined in Multiple Myeloma (KMS12) Cells and Tumor. a. Bar graph of pY397 stoichiometries in KMS12 control cells (1.01 %), cells treated with phosphatase inhibitor (51.4 %), and mice xenograft tumor (3.68 %). b. Western blot showing levels of pY397 and total Lyn in KMS12 control cells (left), inhibitor-treated cells (middle), and mice xenograft tumor (right).

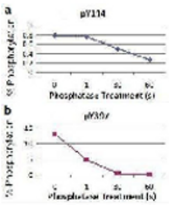

Figure 2. Lyn Phosphorylation Stoichiometry Determined During a Phosphatase Time Course. Line graph of phosphorylation stoichiometries of Lyn Y194 (a) and Y397 (b) sites measured, by SRM, for GST Lyn fusion protein-derived peptides incubated with phosphatase for indicated times.

Q114

Protein Quantification in Label-Free LC-MS Experiments

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[2]Technical University Munich, Germany
[3]Indiana University, School of Medicine, Indianapolis, IN, USA
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Introduction
The goal of quantitative LC-MS proteomic investigations is to quantify and compare the abundances of proteins in complex biological mixtures. However, the output of an LC-MS experiment is not a list of proteins, but one of quantified spectral features (1). There is no generally accepted method for making protein-level conclusions from the features. Typically, researchers continue the per-feature analysis, or average feature abundances to obtain a single protein-level quantity for each sample. We argue that, from a statistical perspective, both approaches are inadequate for protein quantification.

Methods
We distinguish two types of statistical analysis for quantifying and comparing protein abundance. The first is *group comparison*, i.e. determination of proteins that change in abundance across samples of multiple types. The second is *subject quantification*, i.e. quantification of proteins separately in each sample.
We introduce two statistical models, namely fixed and mixed effects analysis of variance (ANOVA), which can be used for both group comparison and subject quantification. The models incorporate the fact that individual features are replicate estimates of a protein's abundance, and explicitly account for this redundancy. Abundances of individual features are decomposed into contributions of (disease) group, feature-specific abundance, subject-specific abundance, and random error.

Results
Performance of the models is evaluated using two datasets. The first is a controlled mixture, where six proteins were spiked into human serum in known concentrations (2). The dataset is used to evaluate the ability of the models to detect known differences in abundance in group comparisons of mixtures. The second dataset is from a clinical experiment, and contains LC-MS features on 246 patients in five stages of cardiovascular disease (3). The dataset is also used to compare the models with respect to the sensitivity of detected differences between disease groups. Furthermore, it is used to evaluate subject quantifications of the models, by comparing the abundances of 11 proteins with independent assay quantifications on the same subjects. The best model will provide subject quantifications that best correlate with the assay data.
We demonstrate that, in comparison with the per-feature quantification and averaging of abundances, the proposed ANOVA models

1) Improve both sensitivity and specificity of detecting true changes in abundance
2) Improve the accuracy of subject quantification
3) Provide a more consistent and robust quantification in the presence of missing data.

Innovative aspects
We introduce statistically rigorous methods for protein-level quantification on the basis of quantified LC-MS features.

References
(1) L. Mueller et al, *J. Proteome Res.*, 7, p. 51-61, 2008.
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Q115

Quantitative Profiling of the Secretome during Skeletal muscle cell Differentiation

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Introduction
Systematic characterization of secreted proteins that regulate muscle cell differentiation (i.e. conversion of proliferative myoblast into differentiated myotubes) may help identifying new therapeutic targets for various muscle pathologies. In our previous study, 26 secreted proteins were identified in the C2C12 skeletal muscle cell culture system (1). To further characterize the proteins secreted by muscle cells, quantitative profiling via Stable Isotope Labeling by Amino acids in Cell culture (SILAC) was validated and implemented.

Methods
Two C2C12 cell pools were inoculated in either $^{13}C_6$-K (H) or $^{12}C_6$-K (L) supplemented serum-containing culture media for 5 cell divisions. Lysates collected from each cell pool were then mixed in 1:1, 1:4 and 4:1 (L:H) for quantification validation. The H and L labeled cells attained above were thereafter switched to serum-free culture media for differentiation. Conditioned media (CM) were collected from each cell pool for every 24 hr up to 120 hr. The progress of differentiation was revealed by immunocytochemistry. Tryptic peptides generated were analyzed by RPLC-LTQ linear ion trap MS. Quantitative profiling of secreted proteins during muscle differentiation was determined by comparing the H-labeled peptide intensity at 120 hr (myotube) versus its L counterpart at 24 hr (myoblast).

Results
In our study, a preponderance of secreted proteins was identified. Many of these proteins, such as fibronectin, nidogen, biglycan, procollagen, moesin and heparan sulfate proteoglycan (HSPG) are involved in both morphogenesis and tissue regeneration (1). To further our insight into the secreted proteins during muscle differentiation in a global view, quantitative profiling via SILAC was validated and implemented. By MALDI-QqTOF, it was observed that 5 cell division period was sufficient for the incorporation of $^{13}C_6$-K into the cells with efficiency greater than 95 %. Fig 1 shows the comparison of experimental and theoretical protein ratios which were determined by the average ratio of H- versus L-labeled peptide peak intensity. The R^2 of >0.98 ascertain the linearity of the SILAC experiments. In addition, well differentiated myotubes were observed in H labelled cell pool at 120 hr by immunocytochemistry of the myosin heavy chain (Fig 2). Differentially expressed proteins, their functional roles, and the implications of these findings will be reported and discussed.

Innovative aspects
- The first study in quantitative profiling the secretome during muscle cell differentiation

References
(1) C.Y. Chan, et al, Identification of Secreted Proteins during Skeletal Muscle Development; *J. Proteome Res.* (2007) 6, 698-710

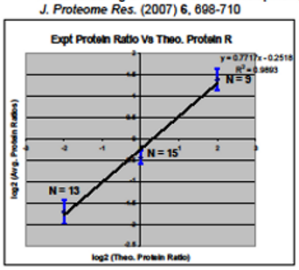

Figure1. Comparison of experimental and theoretical protein ratios in 1:1, 1:4 and 4:1 (L:H) mix. N represents the number of quantified proteins.

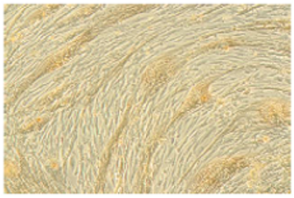

Figure2. Visualization of muscle differentiation in serum-free culture by immunocytochemistry at 120 hr. Brown stain indicates the expression of myosin heavy chain, a muscle differentiation marker.

Q116

Targeted MRM expression profiling of 45 proteins in a cohort of 60 CVD plasma samples

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Introduction
Cardiovascular disease (CVD) is the leading cause of death in North America. To date, >150 proteins have been described in the literature as biomarkers of CVD. Using MRM to simultaneously quantify hundreds of proteins in a single analysis will facilitate rapid protein expression profiling of large sample sets. By looking at multiple proteins in a large number of samples, multiplexed MRM-based quantitation will allow elucidation of protein expression patterns that correlate with disease states and be used to complement established blood and metabolic panels.

Methods
We have created a mixture of 45 stable isotope labeled standard (SIS) peptides that have been used to develop and characterize multiplexed MRM assays for simultaneous determination of the endogenous levels of all 45 plasma proteins (31 of which are putative CVD biomarkers) in a single LC-MRM/MS analysis (**1**). A cohort of 60 CVD plasma samples containing 20 samples from patients that are coronary artery disease (CAD) positive (one or more lesions with >50% stenosis), 20 from CAD-negative patients (lesions of ≤10% stenosis), and 20 from individuals who did not present symptoms consistent with CAD upon angiography. Expression profiling of trypsin digests of these samples was performed by LC-MRM/MS analysis with SIS peptides.

Results
60 clinical plasma samples were trypsin digested and prepared for LC-MRM/MS quantitation of 45 plasma proteins using an Tecan Freedom Evo150 liquid handling workstation. LC-MRM/MS analyses performed on 3 different days, on 3 different analytical runs of plasma trypsin digests of a standard sample yielded inter-assay CV's of <20% for 43 of the 45 MRM protein assays. Additionally, concentrations for 39 of the 45 proteins are within 2-fold of reported literature values.

Trypsin digests were analyzed in triplicate using 30 minute LC-MRM/MS analyses with retention time scheduled MRM's. Average analyte concentrations from all 60 samples were subjected to various multivariate statistical analyses (ex. principle component analysis, support vector machine) to explore the utility of MRM based expression profiling using this *initial panel* of putative CVD makers to stratify/segregate these patient samples into discrete groups. The specificity and sensitivity of these statistical results will be directly compared to existing clinical data and diagnosis.

Innovative aspects
- Multiplexed quantitation of 45 human plasma proteins in a single LC/MS MRM analysis
- Sub-femtomole, absolute quantitation of 45 proteins using isotopically labelled internal standards
- Quantitation of these 45 proteins in 60 CVD clinical plasma samples to determine disease state

References
(1) M. A. Kuzyk, D. Smith, J. Yang, T. J. Cross, A. M. Jackson, D. B. Hardie, N. L. Anderson & C. H. Borchers. MRM-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. & Cellular Proteomics*. Published online May 1, 2009.

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Q117

Quantitative proteomic changes by activation of PPAR-γ with the agonist troglitazone.

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Introduction
The nuclear receptor and transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) affects transcription of proteins involved in a wide range of cellular processes. Troglitazone (TGZ) is a well-studied agonist of PPAR-γ. Studies of TGZ-response has shown induction of apoptosis in several cell lines in both a PPAR-γ-dependent, and -independent manner(1, 2). We want to supplement these targeted studies with a proteome-wide view of the regulated proteins. This will provide new insight into the complex mechanisms underlying ligand-activation of PPAR-γ.

Methods
The human colon carcinoma cell line Caco-2 was used as a model. The cells were grown in SILAC-labelled RPMI-1640 (deprived of phenol red). The cells were split in two populations and grown in either light or heavy labelled medium. Five population doublings were allowed to ensure complete incorporation of the heavy isotopes. After having reached a confluency of about 80%, the cells were stimulated with a total concentration of 1 μM TGZ for 24 hours.

Total cell protein was fractionated according to cellular compartment. The fractions were separated by SDS PAGE, in-gel digested, and analyzed using a nano-LCLTQ Orbitrap setup. The raw files were analyzed and quantified using the MaxQuant computational platform.

Results
A total of 778 proteins were identified and quantified, of which 129 were regulated. As reported in other studies, a significant overlap of proteins from the different fractions was observed. This is probably due to the sensitivity of the instrument, which detects sub-femtomol levels of peptides. A large number of the detected proteins were abundant proteins involved in cellular processes. The dynamic range is limited, and further separation would no doubt increase the depth of protein identifications, yielding more information regarding the less abundant proteins, which are often the most interesting ones.

Interesting protein identifications
- Translational activator GCN1 (downregulated)
- Serine/threonine-protein kinase PAK 2 (upregulated)
- Gastric-associated differentially-expressed protein YA61P (Drug-sensitive protein 1) (downregulated)

This work identified regulated proteins as a result of TGZ-stimulation, including proteins directly involved in apoptosis or in other ways influencing cell proliferation. This information can aid the understanding of the mechanisms induced by troglitazone, and may aid in the development of novel drugs targeting specific cellular processes.

References
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Q118

Quantification of Light-Regulated Proteins by ICPL Quadruplex Labeling

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Introduction
Quantitative proteome analysis is very challenging especially when membrane associated proteins in different biological states have to be quantified. As biological system retinal proteins from rod outer segments (ROS) were analyzed in a dynamically regulated light-induced and dark-adapted state. The proteome analysis of rhodopsin-associated proteins was performed with mass spectrometric measurements and quantification was achieved, using the quadruplex ICPL-technology.

Methods
The ICPL technology (1) allows the relative quantification of up to four biological samples in parallel. A selective labeling of primary amino groups of intact proteins is achieved by this method. The labeled and combined protein samples were separated by 1-D SDS-PAGE and were in-gel digested with trypsin and endoproteinase GluC. The resulting peptides were separated by reversed phase HPLC and directly spotted onto MALDI targets. The identification and quantification was performed with MALDI TOF/TOF measurements. An in house developed software tool ICPL*Quant* allows the accurate quantification of the four samples.

Results
The identification and quantification of proteins located in the rod outer segments was performed with quadruplex ICPL labeling. The relative quantification of proteins identified in the light-induced and dark-adapted states could be easily determined in one experiment. The identified proteins were correlated to an extended rhodopsin signaling network, which was derived by a combined experimental and literature/structure based approach. The ICPL results indicate that S-arrestin is mostly present in the light-induced ROS, while transducin is present in the dark-adapted ones. Further proteins known from the rhodopsin-network could be identified and relatively quantified – for example phosphodiesterase 6G, neurocalcin delta and peripherin-2 are found to be more than 1.5-fold down-regulated in the light-induced state, whereas enolase-3 is found to be more than 1.5-fold up-regulated in the same state.

The results of the ICPL proteome analysis were compared with iTRAQ and DIGE experiments and discussed regarding the reproducibility, accuracy and proteome coverage.

Innovative aspects
- First study which describes the use of the ICPL quadruplex labeling for a more complex proteome analysis.
- Besides the determination of relative protein quantification also the behaviour of light-regulated protein-interactions was analyzed.
- Comparison ICPL, iTRAQ and DIGE technology.

References:
(1) A. Schmidt et al, A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics. 2005, 5(1):4-15.

Q119

Isotope coded protein labels for quantitative analysis of encephalitogenic CD4+ T lymphocyte cells

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Introduction

The molecular basis for the autoimmune disease multiple sclerosis is not well characterized. It is believed that central nervous system infiltration of CD4+ T lymphocytes is an important stage in both human and animal disease. At this stage, no quantitative proteomics technologies have been applied to the spinal CD4+ cell population. This study applied isotope coded protein labels (ICPL) and liquid chromatography coupled to matrix assisted laser desorption/ionization time-of-flight mass spectrometry (LC-MALDI MS) to characterize spinal CD4+ cells during murine experimental autoimmune encephalomyelitis (EAE), a relapsing-remitting mouse model of human multiple sclerosis.

Methods
CD4+ T lymphocyte cell lysates from both EAE immunized and naïve mice were independently pooled, acetone precipitated, labeled with ICPL light and heavy reagent ($^{12}C/^{13}C$-Nicotinoyloxy-succinimide), mixed together and separated by LDS-PAGE. Separated proteins of all molecular weights were excised and each gel band (~30) was in-gel digested and peptides were extracted for LC-MALDI MS analysis using an Ultra Flex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics). Peptides were screened for regulation where the light: heavy modifications were ≤0.66 or ≥1.5 using WARP-LC software (V3.2, Bruker Daltonics) and differentially regulated peptides were submitted to fragmentation for protein identification. Experiment was repeated and datasets compared.

Results
Protein bands, as shown in Figure 1, were analyzed by LC-MALDI MS for both ICPL labelling experiments. Comparison indicates that regulatory differences are apparent for peptides, which can be positively identified from a low amount (ICPL1:~19 μg and ICPL2:~35 μg) of complex sample. MASCOT database searches submitted to NCBI showed positive identification of peptides chosen by WARP-LC software for fragmentation. We identified several important, similarly regulated proteins in both experiments, including Annexin A6, Cyclophilin A and Histone H3.3. Of these proteins, their significant regulations (outside the calculated regulation window) for ICPL labellings one and two respectively were 2.5 and 5.6 (Annexin), 0.54 and 0.73 (Cyclophilin) and 0.4 and 0.16 (Histone). These results indicate an inactive CD4+ cell population in the spine of animals at peak disease. Suggesting that deleterious cell function has already occurred and animals are entering remission.

Innovative aspects
- Identification plus quantification of proteins from relatively small numbers of specifically purified cells
- Reproducible identification of regulated proteins by LC-MALDI MS.

References
(1) A. Schmidt et al, A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics, 2005. 5(1): p.4-15.
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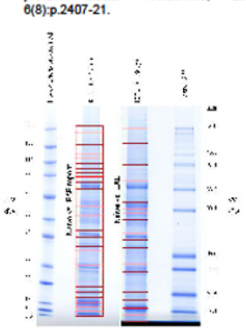

Figure 1. ICPL labelled encephalitogenic and naïve CD4+ T lymphocyte lysates separated by LDS-PAGE. Excised gel bands indicated by red boxes. Similarity in protein band patterns is indicative of method reproducibility.

Q120

The Development of Targeted MRM Assays from Multiplexed Discovery Phase Protein Results

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Introduction

Profiling biological samples using LC-MS^E on a Q-ToF mass spectrometer, produces lists of proteins differentially expressed between conditions. Specific peptides from these proteins may be targeted as markers for each protein and screened using a triple quadrupole mass spectrometer operating in multiple reaction monitoring (MRM) mode. The challenge of determining which MRM transition to monitor is overcome by interrogating and filtering the MS^E data inventory of precursor and product ions ensuring unique MRM transitions are scheduled for analysis on a triple quadrupole mass spectrometer.

Methods

Standard digests were spiked into *Echerchia coli* digest and separated using nanoUPLC coupled to a QTof mass spectrometer. The replicate analysis provides an input file for the Verify^E informatics, which filters the data to generate unique protein specific MRM signatures. These data populate a compound database from which MRM methods are generated and implemented on the tandem quadrupole mass spectrometer. A unique scan function is incorporated into the workflow, whereby the mass spectrometer performs an enhanced product ion scan for selected transitions during the MRM experiment. This high sensitivity full scan MSMS acquisition provides sequence confirmation of the peptide, thereby confirming that the correct chromatographic peak is selected for quantitation.

Results

Identity^E processing produced an inventory of 47912 potential MRM transitions. These were filtered to remove any which share a common sequence prior to being assessed for m/z and retention time overlap. The two most intense precursor ions per protein and their two most intense fragment ions were retained, providing a confirmatory ion for quantitation. An MRM experimental file which scheduled 484 functions to monitor 963 transitions was produced.

PIC scanning was performed on the Xevo TQ MS, to acquire a scanwave enhanced full scan MSMS acquisition at 5000amu/sec for 0.4secs.

Duplicate calibration curves for spiked yeast alcohol dehydrogenase 1 (ADH1) peptide IGDYAGIK (418.7>723.3) showed good linearity when four peptides from E coli proteins were used as internal standards. The limit of quantitation for this ADH peptide was 18 attomoles and is linear over the analysis range 18 attomoles to 20 femtomoles on column.

The chromatogram for the IAAANVPAFVSGK peptide contains two peaks in the two minute retention time window. PIC scan allows matching of observed masses to the theoretical fragment data for the peptide confirming that the first eluting peak is the correct one for quantitation

Innovative aspects

- Rapid development of targeted MRM assays
- Sequence confirmation with PIC scanning

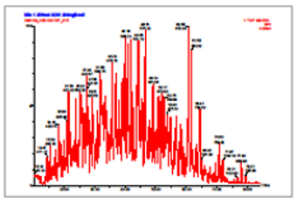

Figure 1. Low energy LCMS^E chromatogram obtained for the analysis of 25fmoles of Mix 1 spiked into 200ng of E. coli digest. Identity^E processing and database searching of the triplicate datafiles, produced a comprehensive inventory of 47912 potential MRM transitions which were imported into Verify^E to determine the 'proteotypic' peptides for these proteins.

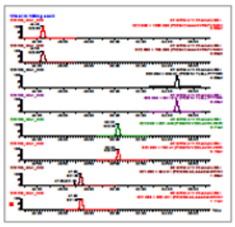

Figure 2. Selected MRM chromatograms from four E coli peptides. Each peptide is represented by two transitions.

Q121

Optimization of Sample Processing and LC/MS/MS Proteomic Analysis of Complex Field Samples

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Introduction

LC/MS analysis of tissue samples collected in the field presents unique challenges. Our Genome BC-funded project *Apis mellifera* Proteomics of Innate reSistance (APIS) aims to identify disease resistance biomarkers in honey bees and involves collection of samples from a large number of colonies in British Columbia (BC) and Alberta (AB). Initial sample processing occurs in the field, followed by further sample processing, LC-MS/MS analysis and data interpretation at UBC. Here we describe the evaluation and optimization of protocols for sample collection, processing and LC-MS/MS analysis.

Methods

Honey bee samples were collected from different sites at Grand Forks, BC and Beaverlodge, AB, including larvae, larval hemolymph (the equivalent to human plasma), midgut and antennae. The method described by Chan et al (1) was adapted for collection and initial processing of larval hemolymph from over 750 larvae. Several methods for midgut and antennae sample processing were evaluated. Complex peptide samples were fractionated by strong cation exchange chromatography after digestion. A triplexed reductive dimethylation labeling protocol was implemented based on (2). Labeled peptides were separated by online capillary reversed phase HPLC prior to tandem mass spectrometric analysis with either an LTQ-Orbitrap and LTQ-FT. Relative quantitation was performed using MSQuant and in house scripts.

Results

The collection and analysis of large number of tissue samples was achieved utilizing modified methods for sample processing and LC-MS/MS analysis. For midgut samples, protein isolation with trichloroacetic acid was found superior to the ethanol/acetate method to address the proteolytic degradation challenge of these samples. The number of identified and quantified peptides and proteins were affected by the different peptide separation protocols and the instrument used (Figure 1). Combining the results of a single LC run on an LTQ-Orbitrap and an LTQ-FT before relative quantitation yielded similar numbers of peptides compared with the combined results from five strong cation exchange fractions analyzed with the LTQ-FT.

Sample independent and tissue-specific conclusions were drawn to balance the use of resources and the quantity and quality of the data obtained.

Innovative aspects

- Comparison of field sample collection and processing for LC-MS/MS
- Combination of data from MS runs using LTQ-Orbitrap and LTQ-FT after Triplex formaldehyde labelling for protein quantitation

References

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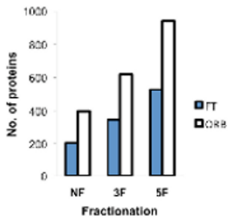

Figure 1. Number of quantified proteins from honey bee antennae using different fractionation protocols and analysed on either an LTQ-FT (FT) or LTQ-Orbitrap (ORB). (NF – no fractionation, 3F – 3 fractions, 5F – 5 fractions).

Q122

Unbiased Quantitation of Membrane Proteome Using Phase-Transfer Surfactants

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Introduction

Despite the importance of cell surface biology, the conventional shotgun proteomics strategy has often resulted in underrepresentation of membrane proteome because of the difficulty in solubilization and protease digestion. The goal of this study is to establish the membrane proteomics method unbiased to protein solubility, hydrophobicity and abundance. So far, there have been few reports on the quantitative assessment for the recovery of membrane proteome. In this study, we employed the phase-transfer surfactant(PTS) protocol (1), and evaluated the bias to quantifying membrane proteome.

Methods

Proteins were extracted from whole cell lysates and membrane-enriched fractions of *E. coli* K-12 strain and human HeLa cells with 12 mM sodium deoxycholate and 12 mM sodium lauroylsarcosine in 50 mM ammonium bicarbonate. The protein mixtures were 5-fold diluted with 50 mM ammonium bicarbonate and digested by loading onto pipette tips packed with the immobilized trypsin beads at 100 g for 15 minutes. An equal volume of ethyl acetate was added to the eluent solution and the mixture was acidification with the final concentration of 0.5% TFA according to the PTS protocol(1). The mixture was shaken for 1 min and centrifuged at 15,700 g for 2 min, and then the aqueous phase was collected for subsequent nanoLC-MSMS analysis with LTQ-orbitrap XL (ThermoFisherScientific).

Results

We previously developed a sample preparation protocol for rapid and unbiased analysis of membrane proteome using an alimentary canal-mimic system where proteases are activated in the presence of bile salts(1). In this study, we modified the rapid and unbiased protocol, where immobilized trypsin was used in the presence of deoxycholate and lauroylsarcosine to facilitate the digestion efficiency as well as to increase the solubility of the membrane proteins. Using 22.5 microgram of *E. coli* whole cell lysate, we quantitatively demonstrated for the first time that membrane proteins were extracted and digested at the equal level of soluble proteins without any solubility-related bias and the recovery of membrane proteins was independent of the number of transmembrane domains per protein. In the analysis of the membrane-enriched fraction from 22.5 microgram of the *E. coli* cell lysate, we found that the abundance distribution of the membrane proteins was in agreement with that of the membrane protein-coding genes. In addition, we applied the PTS protocol to estimate the copy numbers per cell for soluble and membrane proteins simultaneously, based on emPAI(2).

Innovative aspects

- Unbiased quantitation of membrane proteome independent of protein solubility and hydrophobicity.
- Rapid protocol for sample preparation for shot-gun proteomics.

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Q123

A common processing and statistical framework for label-free quantitative proteomics analyses

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Introduction

Label-free quantitation is increasingly used in proteomics, but it requires software to be able to extract meaningful features from MS data, a difficult task. Different groups have developed open-source softwares in recent years, e.g. SpecArray, SuperHim and MsInspect (1). They are based on different algorithms, each having its own strengths and weaknesses. Our goal is to develop an analysis pipeline to compare data obtained by different softwares and to build a common downstream processing and statistical framework.

Methods

Samples were prepared with known protein digests to provide baseline results in order to validate the analytical pipeline.
Cell lysates preparation and protein extraction were carried out according to standard procedures. Proteins were digested overnight with trypsin, and peptide mixtures analyzed by LC-MS/MS on a LTQ-Orbitrap. Protein identification was performed by Mascot. Raw LC-MS/MS files, converted to mzXML format, were processed by SpecArray and SuperHim for quantitation. Statistical analyses were performed with R. Four test samples were prepared with protein extracts from *E. coli*, spiked with different concentrations of four standard proteins.

Results

An analytical pipeline including SpecArray (2) and SuperHim (3) has been developed, performing automatization of the LC-MS/MS data submission and the extraction of features (*m/z*, retention time, intensity) within a common output for downstream statistical analyses. It allows the optimization of parameter settings and the side-by-side comparison of softwares. Validation of the pipeline was based on test samples, injected in triplicates, containing known ratios (0.7 to 14 fold changes) of spiked proteins in *E. coli* extracts. Quantitation softwares were evaluated based on their sensitivity (true positive rate) and specificity (true negative rate). For example, when comparing two samples, with minimum intensity threshold set to zero during feature extraction, SpecArray shows better specificity but lower sensitivity than SuperHim (Figure 1). This validation dataset was also used for optimizing statistical analyses.
For further testing, we will use this pipeline to analyze MS data from BJAB human cells stimulated by heat-shock. We plan ultimately to provide a free access to the developed pipeline, and to extend it to include other label-free quantitation programs.

Innovative aspects

- Development of an analytical pipeline with common processing and statistical framework for label-free quantitative analyses.
- Test and comparison of SpecArray and SuperHim on both test and biological samples.

References

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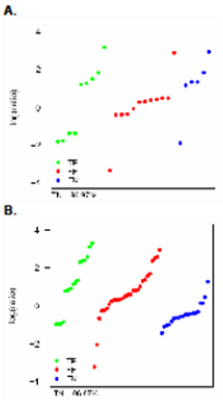

Figure 1. Ratios of peptides with different abundance between two test samples, according to SpecArray (A) and SuperHim (B). True positives (•) and false positives (•) are significantly differentiated peptides (log(ratio) ≠ 0, t-test with p<0.01) from spiked and *E. coli* proteins, respectively. False negatives (•) are identified peptides from spiked proteins, which were not statistically differentiated. True negatives (not shown) are peptides from *E. coli* proteins not statistically differentiated.

Q124

SILAC Based Quantitative Phospho-Proteomic Study of TGF-β signaling in Colon Cancer Cells

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Introduction
Transforming growth factor-beta (TGF-β) is a negative regulator of cell growth. Canonical signaling involves Smad proteins, with Smad4 being the key signal transducer regulating gene expression. In cancer, TGF-β often exhibits a dual function showing an inhibitory and supportive effect (1). Recent studies suggest that this dual function of TGF-β may be attributed to the phosphorylation of proteins and activation of signaling pathways that operate independently of Smad proteins (Fig. 1). The aim of our research is to characterise TGF-β signaling that occurs independently of Smad4 interactions.

Methods
In this study, two sets of SW480 colon carcinoma cells lines were used, one carrying a wild type Smad4 deletion and another where Smad4 has been rescued using retroviral transduction. Western blotting and proliferation analyses were performed to demonstrate a functional TGF-β pathway in these cells. For quantitative analysis, the cells were metabolically labeled *in vivo* with 13C and 15N versions of L-lysine and L-arginine using the Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) method. Following SCX, phosphoproteins were selectively enriched using TiO_2 and p-Tyr affinity columns and analysed by nano-LC-MS/MS. Mascot Distiller was used to identify and characterise differentially expressed phosphoproteins in response to TGF-β.

Results
Western blots with anti-Smad4 confirmed that the rescued cell line was successfully transformed. Western blots with anti-phospho-Smad2 indicated that the cells were responsive to the TGF-β cytokine. Similarly, proliferation analysis showed that a lower number of cells were present when treated with TGF-β indicating also that the cells were responding to the TGF-β cytokine. In the Smad4 deleted cell line, following TGF-β treatment for 30min and enrichment of phosphoproteins, 298 proteins containing phospho-serine/threonine residues and 26 proteins containing phospho-tyrosine residues were identified. These phosphoproteins included cell division control proteins, cell division kinases and MAPK proteins which have been previously described in the context of cancer and Smad independent TGF-β signaling (2). In addition, phosphoproteins have been described in this context for the first time in this study. The use of SILAC demonstrated an efficient and accurate method for phosphoprotein quantitation. A comparison between the two SW480 colon carcinoma cell lines enables the identification of alternate signaling networks that may operate alongside the canonical Smad dependent TGF-β pathway.

Innovative aspects
- Our study is the first to apply SILAC based quantitation to study TGF-β not only as a linear pathway but as an entire network involving proteins that operate independently of Smad4.
- This study describes for the first time phosphoproteins that may operate in Smad independent TGF-β signaling.
- This study uses stably transfected cell lines that are expressing the Smad4 retroviral transduction product continuously, comparing its phospho-proteome to the Smad4 deleted wild type cell line.

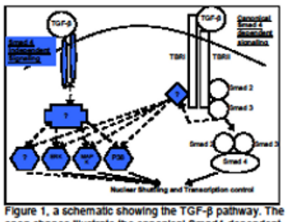

Figure 1, a schematic showing the TGF-β pathway. The open shapes illustrate the canonical Smad4 dependent TGF-β pathway. The shaded shapes show the poorly defined Smad4 independent signaling pathways.

References:
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Q125

Performance Factors of an Ultra-high Resolution API-Qq-TOF for Label-free Proteomics Studies

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Introduction
Label-free protein quantitation approaches became a significant complement to established quantitation methods like 2D-GEL, label or MRM based methods for biomarker studies. However, identifying and quantifying trace compounds based on accurate mass LC/MS in complex matrices such as serum, plasma or complete cell lysates requires very high resolution. The scope of this work was to evaluate the performance factors of a targeted label-free quantitation approach on a further improved ultra-high resolution API-Qq-TOF and to apply the instrumentation in targeted label-free proteomics studies.

Methods
The experiments have been performed with a nano-HPLC connected to an ultra-high resolution API-Qq-TOF that has been further improved regarding mass resolution, mass accuracy and dynamic range. While quantification is purely obtained from MS data, identification is done as subsequent step involving a targeted approach to obtain sequence information of relevant peptides and proteins from MS/MS data. Using a newly developed bioinformatics platform we analyzed the performance factors of our label-free quantitation approach. As suitable model system we have chosen digested E. coli cell lysate spiked with protein standards in a dilution series. For every concentration level we have performed 6 technical replicates. We further applied the approach to elucidate protein changes in biological systems.

Results
We have evaluated the following performance factors: precision of quantitation, limit of quantitation, limit of identification, linearity, and dynamic range as well as the mass accuracy in MS and MS/MS. As expected most performance factors could be improved significantly compared to a previous API-Qq-TOF platform with lower resolution. The study covers the current status, the limitations and chances of MS based label-free quantification and show that the used instrument and bioinformatics platform will become an excellent tool for this application. The goals of the ongoing work are identification and validation of potential biomarkers in clinical samples. This is shown using samples from cell lysates.

Innovative aspects
- Improved performance factors of a new targeted label-free quantitation approach on a further improved ultra-high resolution API-Qq-TOF
- Application of new instrumentation in label-free proteomics studies

Q126

MatchRx – A software tool for label-free mass spectrometry protein quantitation

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Introduction
The quantitative proteomic profiling of complex biological samples for the purposes of target and biomarker discovery is a key challenge in proteomics. The comparison of protein expression levels between two or more samples via liquid-chromatography mass spectrometry (LC-MS) can contain thousands of proteins which makes manual analysis virtually impossible. To assist in this analysis, we have developed a software tool called MatchRx (Haqqani et al, 2008).

Methods
MatchRx is a series of perl scripts wrapped via a Java GUI (Figure 1) that automates the steps involved in the analysis of nanoLC-MS runs and the matching of multiple runs. The steps include peak detection, peak quantification, neighbouring peak finding, run matching and normalization. Statistical analysis for differential expression is performed using existing statistical tools and validation is performed using virtual images of each run (Figure 2) either within MatchRx or through the use of MSight (Palagi et al, 2005). For peptide identification, a peak list file from a nanoLC-MS/MS run is searched using the Mascot search engine or any other probability-based engine.

Results

For each nanoLC-MS run, MatchRx takes centroided data and identifies the isotopic pattern of peptide peaks and calculates the charge states. Each peptide is quantified by finding its elution profile and integrating the intensities with background subtraction. For each peptide peak, additional peaks within its surroundings are identified for use in the alignment step.
Once these steps are completed for all runs, run matching is performed. For the initial matching, peptides from two runs are considered matched if they have the same charge state, their m/z values are within 0.2Da, the neighbouring peaks align and their retention time values are within 200s. From this matching, the relative shift in retention time between 2 runs can be determined. This allows the retention times to be adjusted for one of the samples and the matching is repeated under more stringent tolerance for retention time. (±10s)
After all the runs are matched using the above method, MatchRx combines all the matches into one matrix for further analysis.

Innovative aspects
- User-friendly, automated analysis of label-free proteomic data
- Ability to use external statistical analysis tools of one's choosing.
- Enhanced validation through integration with multiple visualization tools

References
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P Palagi et al, MSight: An image analysis software for liquid chromatography-mass spectrometry; Proteomics 2005, Vol5, p2381-2384

Figure 1 – Java GUI for alignment step within MatchRx

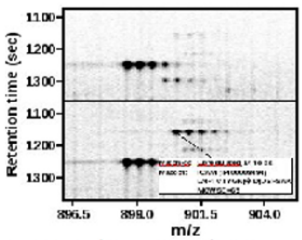

Figure 2 – Differential expression validation by Image visualization

Q127

Comparison of Sample Preparation Methods in Amine-based Isotope Labeling for Quantitative Proteomics

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Introduction
As large scale MS-based experiments move from qualitative analysis to quantitative profiling of proteome changes, careful sample handling becomes important. Considering the small sample amounts involved, variations in sample preparation can influence the accuracy and reliability of quantification results. Since amine-based labeling techniques such as acetylation, reductive dimethylation, and certain commercial products (iTRAQ™/TMT™) have become widely available, preparation methods that adversely affect labeling should be avoided. In this work, we compare several commonly used protein sample handling methods for their compatibility with the quantitative work.

Methods
Equal aliquots of *E. coli* cell lysate were precipitated using acetone. Protein pellets were solubilized with various solutions (ammonium bicarbonate, urea, SDS, methanol, Rapigest™, ProteaseMax™, and AALS). After determining the protein amount using the BCA assay, proteins were reduced using dithiothreitol and alkylated using iodoacetamide. A small aliquot of each solution was labeled using reductive dimethylation, followed by desalting and quantification prior to MS analysis to ensure consistent sample comparison. Samples were analyzed by LC-MS in triplicate to detect potential interferences from the sample workup procedure.

Results
While hydrolysis of cleavable detergents such as ProteaseMAX™ is limited under non-acidic pH, the relatively high concentration detergent to peptide can influence labeling reaction efficiency, even if only a small percentage of detergent is hydrolyzed. To prevent reagent quenching, certain commercial kits use triethylammonium bicarbonate as the reaction buffer instead of ammonium bicarbonate. In our study, it was found that ammonium bicarbonate was a reasonable choice for solubilization and labeling, but was limited in the number of proteins that could be solubilized. The extended reaction time of the *E. coli* digest in 2 M urea resulted in a detectable amount of carbamylation on peptide N-termini, potentially leading to inaccurate quantification. SDS was the best reagent for solubilizing proteins. However, SDS removal by strong cation exchange chromatography resulted in sample loss during the SDS removal process. In contrast, most of the cleavable detergents examined were found to provide reasonable performance without additional workup steps.

Innovative aspects
Examination of sample handling methods that may adversely affect the efficiency of amine-based labeling chemistry and quantification in proteomics experiments

Q128

Selected Reaction Monitoring Cubed (SRM^3) : a new strategy for validation of putative biomarkers

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Abstract content:

Stable isotope dilution-selected reaction-monitoring mass spectrometry (SID-SRM-MS) carried out in triple quadrupole instruments has emerged as a promising alternative to ELISA for validation of putative protein biomarkers discovered during proteomics projects. However, mass spectrometry-based quantitation of plasma proteins in the low nanogram/mL range currently requires extensive sample fractionation steps which hampers its implementation within clinical laboratories.

In the present work, we describe a new method called Selected Reaction Monitoring Cubed (SRM^3) that significantly improves the specificity and sensitivity of detection of proteotypic peptides in comparison with SRM.

The method consists of : i) Q1 selection at unit resolution of a proteotypic peptide ion ii) Q2 CID activation of the selected proteotypic peptide ion iii) Q3 trapping at unit resolution of the most intense fragment ion that defines the SRM transition iv) activation of this species to generate fragment ions of the second generation, v) mass selective scan of the second generation fragment ions, vi) extraction of specifically defined second generation fragment ions to reconstruct a chromatogram.

As an illustration, five proteins (TP171, TP435, TP574, Core NS4 and PSA) were spiked into human plasma samples at concentrations ranging from 5 nanograms to 1000 nanograms/mL. SRM^3 reconstructed ion chromatograms were used to generate a standard concentration curve for each protein and from this, a limit of detection (LOD) in the low nanogram/mL range (between 5 and 50 ng/ml, depending on the protein) was observed. On average, we estimated a 5 fold improvement of this limit as compared to SRM mode because of the significant reduction in the background interfering species. Furthermore, SRM^3 dramatically improved both the linearity of the response and the precision for the weakest concentration points because of the improved fidelity of the peak.

In conclusion, SRM^3 quantitation strategy provides much higher confidence towards the specificity of detection of peptides in complex mixtures and allows protein quantification at low nanogram/mL range in crude serum or plasma.

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Q129

Identification of Molecular Pathways Underlying the Development of Psoriasis Using iTRAQ-2DLC-MS/MS

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Introduction

The use of isobaric tags for relative and absolute quantization (iTRAQ) followed by multidimensional liquid chromatography (LC) and tandem mass spectrometry (MS/MS) analysis is emerging as a powerful methodology for biomarker and drug target discovery. We have used this method to identify molecular events in keratinocytes underlying psoriasis development and progression. A simultaneous deletion of *c-jun* and *junB* (DKO*) in keratinocytes leads to a severe psoriasis-like phenotype in mice[1]. In human psoriasis and in our mouse model, S100A8/S100A9, two chemotactic molecules that effectively activate immune responses, are up-regulated in (and released by) keratinocytes. To investigate if this up-regulation is a key event in the development of psoriasis we generated triple-knockout mice (TKO*) also lacking S100A9. These mice show a clear amelioration of the phenotype. To discover molecular pathways associated with S100A8/A9 protein expression and to identify proteins that are differentially expressed in TKO*, DKO* and control mice, we carried out a comparative analysis of mouse tail epidermis labelled via iTRAQ.

Methods

Samples for each genotype group were prepared by pooling equal amounts of proteins from three different mice. Protein extracts were digested with trypsin and peptides were labelled with iTRAQ reagents. Samples were subsequently fractionated by SCX chromatography, RP LC and analysed by MALDI TOF/TOF MS. Three software packages were utilized for data analysis: ProteinPilot for identification and quantification of differentially expressed proteins, Protein Center for gene ontology (GO) analysis and Ingenuity Pathways Software to provide insight into biological networks.

Results

Comparative analysis among different genotypes have identified a high number of non-redundant proteins with high confidence (>99%). A panel of proteins showing consistent differential expression among different genotypes has been discovered. The involvement of the identified proteins in biological networks has served to characterize molecular pathways associated with *c-jun, junB* and S100A8/9 expression in keratinocytes triggering psoriasis and to select critical candidates for validation analyses by Western Blot using independent replicates. As part of complementary clinical validation strategies, inmunohistochemical analyses of potential biomarkers in psoriasis development have been performed.

Innovative aspects

- Our study not only has served to reveal molecular mechanisms associated with the role of *c-jun, junB* and S100A8/9 in psoriasis, but also to identify novel potential biomarkers for patients affected with psoriasis.
- We believe this is the first report to profile the protein changes in psoriasis development utilizing the iTRAQ proteomic technique

References

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Q130

The quantitative tyrosine phosphoproteome analysis of the IGF-1 signaling pathway in glioma C6 cells

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Introduction

The insulin-like growth factor 1 (IGF-1) plays critical roles in cell survival, proliferation, and differentiation in the nervous system. The early tyrosine phosphorylation signaling initiated by IGF-1 is closely associated with many important downstream biological events. In the present study, we investigated the temporal dynamics of the IGF-1 mediated tyrosine phosphorylation in rat glioma C6 cells combining phosphotyrosine-containing peptide enrichment, stable isotope labeling by amino acids in cell culture (SILAC), and high resolution LTQ Orbitrap mass spectrometry. Our quantitative phosphoproteomics strategy may provide some valuable information to dissect the role of the IGF-1 signaling in the nervous system.

Methods

Rat C6 glioma cell line were labeled using a SILAC Protein Quantitation kit (Pierce) and stimulated with IGF-1 (100 ng/ml) at different time point (0, 1 min, 5 min, 25 min). 2×10^8 cells were lysed and trypin-digested. The digested peptides containing phosphotyrosine were enriched using a monoclonal antibody P-Tyr-100 (Cell Signaling Technology)[(2)]. Samples were analyzed using a nanoLC LTQ Orbitrap mass spectrometry. Mascot and MSquant were used for data processing [(1)].

Results

To characterize the IGF-1 signaling targets in glioma C6 cell line, we stimulated the SILAC-labeled C6 cells with IGF-1 (100 ng/mL) at different time point (0, 1 min, 5 min, 25 min). The treated cells were lysed and digested with trypsin. The phosphotyrosine-containing peptides were enriched by anti-pY immunoprecipitation. Using high resolution LTQ Orbitrap mass spectrometry, we identified 80 phosphorylation sites in 73 tyrosine phosphopeptides with a false positive rate of less than 1%. The results may help to understand the role of the IGF-1 signaling pathway in the nervous system.

Innovative aspects

First quantitative tyrosine phosphoproteome analysis to study the IGF-1 signaling in rat glioma C6 cells by combining phosphopeptide enrichment, SILAC, and high resolution mass spectrometry.

References

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(2) John Rush et al. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol. 2005 Jan; 23(1): 94-101.

Q131

Characterization of Cytokine-dependent Protein Stability by Comparative Pulse-Chase SILAC Analysis

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Introduction

Many cells of the immune system require cytokines to sustain growth and survival, as their withdrawal initiates apoptosis. An initial comparison of cells grown in light and heavy SILAC medium, with cytokine GM-CSF present in only one, helped us identify proteins that change in concentration in response to GM-CSF withdrawal. As this did not provide information on the origin of these changes, we developed and applied an alternative approach to determine whether changes in steady-state concentrations are due to altered protein half-lives caused by changed synthesis and/or degradation rates.

Methods

Human erythroleukemic TF-1 cells were grown in heavy SILAC medium (13C6-Lys, 13C6-Arg) for six doublings. Cells were washed, and light SILAC medium (12C6-Lys, 12C6-Arg) replaced heavy medium (1), with cytokine GM-CSF present in only one set of cells. 0.5 to 15 hours after GM-CSF withdrawal, aliquots were taken from both sets of cells and lysed. Equal amounts of protein lysates were separated by SDS-PAGE. Each lane was cut into 27 bands, subjected to tryptic in-gel digestion, and extracted peptide mixtures were analyzed by LC-MS/MS on a LTQ-FT instrument (ThermoFisher) coupled to an Agilent 1100 series LC system. Extracted data were analyzed by MSQuant, MaxQuant, and Mascot Distiller to identify proteins and to determine SILAC incorporation rates.

Results

Initial analysis identified ~1,500 proteins from each set of cells. For a subset of 359 and 343 proteins contained in eight bands across the entire MW range, their SILAC ratio was determined and converted into a calculated protein half-life. Proteins with half-lives shorter than 15 hours, or longer than 24 hours, underwent additional data analysis. This identified 43 short-lived and 89 long-lived proteins in the control sample, compared to 24 short-lived and 101 long-lived proteins in the starved sample, indicating an increase in rapid protein turnover upon starvation. 20 short-lived and 48 long-lived proteins were common to both, 13 and 8 of which showed half-life differences of greater than 30%. Comparison with protein concentration changes revealed that half-life changes did not always result in altered protein concentration. Classification based on level 4 gene ontology suggested synergistic effects for mitochondrial proteins and proteins involved in translation initiation and elongation. Data processing for the remaining gel bands and time points is currently underway, and will provide additional insights into the regulatory processes underlying early apoptosis.

Innovative aspects

- Comparative pulse chase labelling of proteins are possible with SILAC
- Changes in protein stability are identified by calculating protein half-lifes
- Cytokine withdrawal can alter protein stability independent of changes of the steady-state concentration

References

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Low Cost, Automated Chemical Derivatization for Relative Proteome Quantification

Q132

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Introduction
Recent commercialization of isotopically labeled reagents for MS-based proteome quantification has simplified sample preparation. However, the cost of specialized reagents can be very high for routine or large scale analyses.

We report the automation of a chemical labeling strategy for quantification experiments using inexpensive isotope reagents and a liquid handler for reagent dispensing. This method was used to compare proteome profile changes in *E. coli* grown under aerobic and anaerobic conditions. In preliminary experiments, 748 proteins were identified, with 598 quantified, using 2D-LC MS.

Methods
E. coli cells were lysed using a French press. Proteins were precipitated using acetone, reduced, alkylated, and digested with trypsin. Using a commercially available liquid handler, peptides were labeled using O-methylisourea and either "light" formaldehyde (CH_2O) or "heavy" formaldehyde ($^{13}CD_2O$). Samples were kept at 37 °C using a heating block and the entire labeling time was three hours. Samples were desalted and quantified by RPLC-UV and mixed 1:1 by weight. The mixed digests were separated into 8 fractions using strong cation exchange chromatography, desalted, and analyzed by LC-MS/MS using a Waters Premier ESI-QTOF. MS/MS spectra were processed using Mascot Distiller and searched using the Mascot search engine. Quantification results were reported using Mascot Distiller.

Results
To evaluate labeling efficiency, proteins from *E. coli* grown under aerobic and anaerobic conditions were digested and derivatized. MS/MS spectra were searched by allowing known side-products as modifications. Correctly labeled peptides accounted for >95% of all identifications. Common side products included guanidination of glycine/alanine N-terminal peptides and dimethylation for peptides with missed lysine cleavages. Peptide digest solutions between 0.1 µg/µL and 2 µg/µL were labeled with >95% efficiency.

Quantitative performance was evaluated by comparing "light" labeled aerobic digest with "heavy" anaerobic digest and the reversed labeled sample. Preliminary results from 2D-LC MS/MS experiments identified 748 proteins from 2128 peptides. 1614 peptides (602 proteins) were identified in the "light" aerobic/"heavy" anaerobic mixture and 1647 unique peptides (628 proteins) in the complementary mixture. While 72% of all identified peptides were quantifiable, only 50% of all peptides were quantifiable and common to both mixtures.

To identify a greater number of proteins with common peptides, we have increased the starting materials used and will separate the peptide mixtures into more fractions prior to MS analysis.

Innovative Aspects
A low cost, automatable MS-based quantification method was developed

High quantification coverage of *E. coli* cells grown under different conditions

General applicability of described method to other types of proteome quantification experiments

Endometrial Cancer Biomarker Discovery by a Drill-down Strategy using iTRAQ and LC-MS/MS

Q133

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Introduction

In a previous study comparing tissues from patients suffering from endometrial carcinoma to healthy tissues, we identified a dozen differentially expressed proteins (1).
However, sample complexity, coupled with the tendency for proteins of interest to be low-abundance regulatory proteins, may result in potentially important proteins being missed during routine proteomics analysis.
The current study, comparing 10 tumour and 10 non-malignant endometrial tissues, is aimed at confirming our previous results, while seeking additional potential biomarkers by iterative sample analyses.

Methods

Clarified tissue homogenates were digested by trypsin, individually labelled with isotope-coded mass tags (iTRAQ), and fractionated by strong-cation exchange HPLC (SCX). Each SCX fraction was separated and analyzed by reverse-phase nano-HPLC tandem mass spectrometry that simultaneously provided both sequence and relative abundance of the eluting peptides.
Data files were analyzed by Proteinpilot (2), using a reversed concatenated human Swissprot / Panther database. Resulting Peptide reports from the first and second iterations were used to add identified peptide ions to an exclusion list that was used during the subsequent acquisitions of the same fractions. Finally, data from all sets was processed to obtain and compare protein iTRAQ ratio across samples.

Results

The second run added 34% more proteins to those identified during the first run, whereas the third iteration contributed an additional 14%. However, 40% of the proteins of a set were identified in each of the three datafiles. Most of the peptide redundancy (72.6%) was due to an elution time shift, emphasizing the importance of LC reproducibility. The reversed database was used to measure the false discovery rate (FDR) of the samples, a quality check in addition to the original confidence scoring system used by ProteinPilot (3). This crosswise examination reduced the list of confidently identified proteins; however, the approach provided a more robust degree of confidence.
Twenty-nine proteins were found significantly differently expressed by our statistical analysis (t-test, 5% threshold). Encouragingly, our results confirmed that chaperonin 10 (mean ratio of 1.5) and pyruvate kinase (1.61) appeared over-expressed in tumour samples, as previously reported (1). Similarly, alpha-1-antitrypsin (0.51) and creatine kinase B (0.59) were again found to be under-expressed. New potential biomarkers were also revealed, including PCM 88 (2.09), PCM 13 (1.87), PCM 87 (1.58), and PCM 92 (1.64).

Innovative aspects

Drill-down analysis of tissue samples
False-discovery rate data analysis

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Abstract categories:
Quantification
Cancer Proteome Biology and Medicine
Systems Approach to Biomarker Discovery

Absolute Quantitation of Phosphorylation Dynamics in Breast Cancer Using MRM-MS Detection

Q134

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Introduction
Breast cancer is often related to genetic defects resulting in aberrations in the function of the epidermal growth factor receptor (EGFR) and estrogen receptor (ER) signaling pathways. Phosphoproteins are centrally involved in cancer development and progression, and individualized treatment selection and drug development, will require a thorough and quantitative understanding of protein expression and phosphorylation dynamics. We describe a novel mass spectrometry (MS)-based proteomic assay to directly investigate phosphorylation levels of select signal transducing cancer-related phosphoproteins with absolute quantitation for clinical relevance.

Methods
Our method, termed phosphatase-directed phosphopeptide quantitation (PPQ) with multiple reaction monitoring (MRM) MS detection, can obtain absolute quantitation of select proteins and their site-specific phosphorylation levels. In PPQ, sample proteins are digested with trypsin prior to addition of isotopically labeled standard peptides representing the non-phosphorylated forms of target phosphopeptides. Samples are then divided and treated with either phosphatase or a buffer-only control. Two separate MRM MS analysis are then conducted to quantify peptide phosphorylation. Absolute level of phosphorylation is determined by measuring the change in the area of each peptide's natural peak versus the area of each peptide's internal standard of known amount, before and after phosphatase treatment.

Results
Phosphopeptide detection has been a challenge in MS-based proteomics and our novel approach of phosphatase treatment and quantitation of the resulting de-phosphorylated peptide (versus quantitation of the less detectable phosphopeptide) promises to increase the sensitivity of detection and provide higher quality quantitation. This was tested by comparing the PPQ-MRM method to an MRM method that detected the phosphopeptides of interest directly. We tested a number of phosphopeptides of interest from ER and EGFR pathway proteins. These were synthesized as natural and heavy-isotope-labeled standards in both, phosphorylated and non-phosphorylated form, and then combined at different ratios and amounts in different sample matrix backgrounds. The PPQ-MRM method was able to absolutely quantify levels of phosphorylation with CV's similar to the direct MRM approach, with sensitivity in the fmol range. This method could potentially allow the profiling of EGFR and ER signaling networks at a high throughput, quantitation accuracy, and sensitivity, allowing the creation of detailed profiles or 'maps' of signaling networks in breast cancer tumour samples.

Innovative aspects

- PPQ-MRM provides absolute quantitation of phosphorylation dynamics.
- The PPQ-MRM method has greater sensitivity due to detection of non-phosphorylated peptide.
- PPQ-MRM can be used for profiling large signaling networks with high-throughput in an absolutely quantitative manner.

Achieving pg/mL Peptide Sensitivity in Plasma using Electrophoretic Enrichment and nanoLC-MS/MS

Q135

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Introduction
The high sensitivity (pg/ml) and specificity of nanoLC-MS/MS makes it an attractive choice for the quantitation of endogenous peptides. However, traditional sample preparation methods such as protein precipitation, though inexpensive, often provide poor selectivity, requiring long chromatographic runs. The high relative expense of mass spectrometers, long chromatographic cycles, and serial analysis make it difficult for nanoLC-MS/MS to compete with multiplexed immunoaffinity assays in a clinical setting. Electrophoretic enrichment combined with nanoLC-MS/MS was thus evaluated for sample quality, chromatographic throughput, and matrix interference removal.

Methods
Enrichment of peptide containing plasma samples was accomplished using off-line gel elution liquid fraction entrapment electrophoresis in a 96 well plate format. Anionic acid labile surfactant was used to perform the PAGE separation. Following a 30 minute parallel separation/elution under 50V/2mA, all 96 samples enriched for peptides <5kDa were injected directly and analyzed using an ABI/Sciex 4000 triple quadrapole mass spectrometer in MRM mode. Model peptides were used to investigate assay performance, and endogenous peptide levels were measured from human plasma. Optimization of the sample preparation conditions was performed to evaluate sensitivity, chromatographic cycle times, and matrix effects.

Results
nanoLC/MS/MS conditions for quantitation of endogenous peptides, leu enkephalin, angiotensin II, and neurotensin, as well as known agents of signal suppression in plasma, were optimized. Lysophosphatidyl cholines (SRM = 496-184/524-184) and phosphatidyl cholines (SRM =704-184/758-184/786-184/806–184) were monitored following protein precipitation and parallel electrophoretic preparation. Precipitated samples are shown to contain extensive levels of phospholipids, dramatically reducing sensitivity and precision. Analysis of electrophoretically-prepared samples indicate phospholipid exclusion >99.5%. Chromatographic runs ranging from 60 minutes to 10 minutes were compared using precipitation and electrophoresis (10uL injections). Due to the presence of phospholipids, salts, proteins, and other signal suppression agents in the precipitated samples, LC gradients <20 minutes are shown to result in significant retention time shifts, reduced reproducibility, and higher LLOQ. Electrophoretic processing results in retention time stabilization (RSD 12%). Quantitative analysis of leu enkephalin from human plasma after peptide enrichment using parallel electrophoresis is <10 pg/mL.

Innovative Aspects

- Novel parallel electrophoretic enrichment of peptides
- Removal of matrix interferences and direct injection of samples for nanoLC/MS/MS
- Demonstrated sensitivity <10pg/mL

Quantitative mass spectrometric analysis of PKA phosphorylation of purified CFTR

Q136

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Introduction
The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel belonging to the ABC superfamily of proteins. Absence of functional CFTR at epithelial membranes results in cystic fibrosis (CF). CFTR is regulated by protein kinase A (PKA) phosphorylation, and a cycle of ATP binding and hydrolysis. An unstructured intracellular segment of CFTR known as the R region contains 11 putative PKA phosphorylation sites. As it remains unclear which of the sites are modified by PKA phosphorylation in the full length protein, we investigate sites of PKA phosphorylation in purified CFTR, and quantify the relative change in phosphorylation at each site before and after treatment with PKA.

Methods
CFTR purified from an Sf9 cell expression system was reconstituted into proteoliposomes (1). CFTR is phosphorylated with purified, catalytic subunit of PKA (200 nM). Samples were digested by trypsin and subjected to LC-MS/MS using an LTQ orbitrap mass spectrometer. Peptides containing phosphorylated PKA consensus sites were selected for quantitative analysis using a multiple reaction monitoring (MRM) approach on a triple quadropole mass spectrometer. Resulting signal intensities of PKA treated proteins were compared to PKA untreated CFTR. Signals were normalized to 4 peptides without phosphorylation sites. Increases in phosphorylation were measured as a fold change after PKA treatment.

Results
Phosphorylation was detected at ten sites, regardless of whether the sample was PKA treated or not. Of these ten sites, nine were in the R region, and one in the neighbouring NBD1 domain. The sites detected with phosphorylation included: S422, S660, S670, S700, S712, S737, S753, S768, S795 and S813. Residues S422 and S670 had never before been detected as phosphorylated in the context of the full length protein. In order to establish if PKA treatment induces a change in phosphorylation levels at these sites, we designed a MRM method to quantify the abundance of the phosphopeptides containing each of these sites. At each site, there was a significant increase in phosphorylation. Of particular interest is the increase in phosphorylation at the poorly characterized monobasic PKA consensus sites S670 and S753. The increase in phosphorylation at these sites was measured as 26 and 500 fold increases in phosphorylation, respectively. Functional consequences of mutagenesis at these sites will be evaluated to determine their role in channel gating.

Innovative Aspects
-quantitative phosphorylation analysis on a membrane protein by mass spectrometry
-novel phosphorylation detected

References:
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Application of an Immuno MALDI (iMALDI) Mass Spectrometry Assay for Diagnosis of Hypertension Related Diseases

Q137

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Introduction
The renin-angiotensin system plays an important role in regulating arterial pressure. Hypertension can be diagnosed by determining plasma renin activity, which quantifies the amount of angiotensin I generated through enzymatic cleavage of angiotensinogen by renin. Determination of plasma renin activity by radioimmunoassay is a commonly employed method of diagnosing hypertension.
Here we demonstrate application of immuno-MALDI (iMALDI) as a highly sensitive and specific method for absolute quantitation of angiotensin I through incorporation of stable isotope standards, and compare the technique to radioimmunoassay.

Methods
Custom synthesized isotopically labeled angiotensin I peptide was spiked into human plasma as an internal standard. Anti-angiotensin I polyclonal antibody was immobilized on magnetic Protein G Dynabeads and used to immunoprecipitate endogenous angiotensin I and the isotopically labeled internal standard from human plasma. Antibody conjugated beads with bound peptides were placed directly on a MALDI plate. An Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer was used to detect and quantify angiotensin I in MS and MS/MS modes.

Results
Employing the iMALDI approach, we show an improved limit of detection of angiotensin I by 80-fold compared to angiotensin I spotted directly on a MALDI target. By direct MALDI MS analysis, endogenous angiotensin I in normal human plasma is not detectable. However, using the iMALDI approach we detected endogenous angiotensin I in normal human plasma corresponding to a concentration of approximately 25 pg/mL. The sequence of angiotensin I was confirmed by tandem mass spectrometry. A concentration curve, created by varying the abundance of natural to isotopically coded forms of angiotensin I, indicates the assay is linear over a 20-fold concentration range with a correlation coefficient of 0.995.
Radioimmunoassay for plasma renin activity requires lengthy incubation periods and the use of radioactive molecules. Our iMALDI approach represents an improved method for determining plasma renin activity through increased specificity, ease and robustness. Furthermore, the assay's multiplexing capabilities permit the detection and quantitation of angiotensin I in conjunction with other clinically relevant disease markers related to hypertension and cardiovascular disease.

Innovative aspects
- Immuno-affinity approach combined with MALDI-MS analysis
- Detection and absolute quantitation of angiotensin I for determination of plasma renin activity

References (maximum 3 references)

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Improving Fragmentation Efficiency of TMT Labeled Peptides Using Stepped Higher Energy Collisional Dissociation

Q138

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Introduction
The importance of quantitative proteomics has driven the development of various mass spectrometry based quantation methods in the past few years. Among them, isobaric stable isotope tagging reagents such as TMT and iTRAQ enable multiplexed quantitation of peptides via reporter ions in the low mass range of MS/MS spectra. Efficient fragmentation with detection over a wide m/z range makes Higher Energy Collisional Dissociation (HCD) of LTQ Orbitrap XL a powerful tool for identification and quantitation of isobaric labeled peptides. Usually at optimized collision energy (CE) most peptides will be fragmented effectively but not all of them will produce enough reporter ions for quantitation. In this study, we evaluated the improvement of fragmentation efficiency using stepped CE.

Methods
Digest of reduced and alkylated 10 standard protein mixture from Sigma was divided into 6 aliquots and each of these was labeled according to manufacturer provided protocol with 126, 127, 128, 129,130 and 131 TMT tags. Samples were subsequently mixed in ratios one to one, and diluted five times with 5 % formic acid before LC-MS/MS analysis. All the experiments were run on an Orbitrap XL with a full scan in orbitrap at 60,000 resolution followed by three data dependent HCD at 7,500 resolution and three data dependent CID in LTQ. HCD CE was optimized and a fixed CE or stepped CE was used for different experiments. The results were compared after data analysis using Proteome Discoverer 1.0.

Results
For quantitation using reporter ions from iTRAQ or TMT, the ion statistics of reporter ions are important factor with respect to the precision of quantitation. Several instrument parameters can influent the ion statistics including CE of HCD, the AGC target of MS/MS scan and the number of microscans. The CE not only needs to be optimized to generate enough fragments for identification but also need to be adjusted to get reasonable reporter ion abundance for quantitation. Using a fixed CE at 40%, we observed good MS/MS spectra for most peptides but some of them did not have detectable or enough reporter ions. Increasing CE to 50% improved the reporter ion abundance but it also caused over fragmentation of some peptides at the same time. Here, we used 2 stepped collision energy (40% and 50%) during HCD to improve the reporter ion abundance while not to lose identification information. The first step with CE at 40% will produce same fragmentation information and the second step with CE at 50% will add more fragment ions in the lower m/z region and also increase the abundance of reporter ions. As a result, richer fragmentation information will be obtained along with more reporter ions.
Using stepped CE also allows us to increase the AGC targets to achieve better sensitivity and reporter ion statistics. During stepped CE, the number of ions in each stepped HCD is actually equal to AGC divided by the number of steps. So when using 2 stepped CE, we can double the AGC target without worry about the space charging effects. The advantages of stepped CE for HCD listed above make it an attractive method to improve quantitation results of iTRAQ and TMT experiments.

Innovative aspects
- Using stepped collision energy to improve quantitation results of TMT labeled peptides.

New Algorithm for Label-Free Protein Quantification

Q139

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Introduction
Quantitative proteomics analyses using a label-free is a flexible approach enabling the profiling of protein expression across different data sets. The success of this approach relies not only on the efficient detection of peptides over a wide range of ion abundance but also on the capability of correlating their precise coordinates in different LC-MS runs. Several approaches have been previously applied to achieve these goals including the use of normalized LC retention time for data acquired on high resolution mass spectrometry instruments. To overcome some of the challenges in features detection and data clustering we report a new approach termed "tuple" that analyzes multiple samples simultaneously to increase the accuracy of feature detection and the protein coverage.

Methods
Our detection algorithm includes the following key steps: 1) The alignment of data sets using a novel algorithm enabling the normalization of retention times across data sets. 2) Feature detection using theoretical isotope distribution and correlation across adjacent scans. Features from different samples defined by the same mass and similar normalized retention time form a "tuple". 3) A tuple containing at least one identified peptide determines the ratio of the peptide feature which are then averaged together after removing outliers to compute protein ratios. Peptide digests from a mixture of 6 standard proteins (200 fmols each) were used as training set. All nanoLC-MS/MS data were acquired using a hybrid LTQ-Orbitrap mass spectrometer.

Results
Six protein standards, DHE3_BOVIN, PERL_BOVIN, ALDOA_RABIT, GLPK_ECOLI, CAS1_BOVIN, and ALBU_BOVIN, are mixed with nine different abundance configurations and spiked into nine identical J774 protein extracts, respectively. Our program is used to compute the quantity ratio of each spiked protein in each sample, relative to the first sample.

There are about 1000 features detected in each sample and about 85% of them can be observed in all of the other samples. By grouping these features into tuples, about half of these tuples contain at least one peptides identified by PEAKS protein database search. A small fraction of the tuples containing different peptide identification results is removed. The other tuples are used to compute the peptide ratios.

The LC-MS/MS experiment is repeated three times on each sample. The following is the result on one replicate, where each number in parentheses is the known ratio in each of the 8 samples, and the number outside of parentheses is the ratio computed by PEAKS Q.

DHE3_BOVIN: 0(0), 23.2(20.5), 14(9.8), 6.8(4), 2.9(2.1), 0.4(0.5), 0.2(0.2), 0(0)

PERL_BOVIN: 0(0), 17(20.5), 14.6(9.8), 4.7(4.0), 1.8(2.1), 0.4(0.5), 0.3(0.2), 0(0)

ALDOA_RABIT: 0(0), 0.8(1), 1.1(1), 1.1(1), 0.9(1), 0.9(1), 1.2(1), 1.2(1)

GLPK_ECOLI: 0(0), 0.7(1), 1(1), 1.1(1), 1.1(1), 1(1), 1.1(1), 0.9(1)

CAS1_BOVIN: 0(0), 0(0), 0(0.01), 0(0.25), 0.5(0.5), 2.6(2), 11(5.1), 17.2(10.1)

ALBU_BOVIN: 0(0), 0(0), 0.18(0.01), 0.25(0.25), 0.5(0.5), 2.3(2), 8(5.1), 16.7(10.1)

These values show that the quantification accuracy is well within experimental error, and significance of abundance changes between samples is easy to see.

For each spiked protein and each sample, we calculated the relative standard deviation (RSD) of computed ratio across the three replicates of the LC-MS/MS experiment. The average of the RSD in all spiked proteins and samples is approximately 8%, showing excellent reproducibility in both the LC-MS/MS experiment and our quantification algorithm.

Novel Aspects
New algorithm for label-free quantification

Q140

Global proteome analysis of *Leptospira interrogans*

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Introduction
Non-gel-based and gel-based proteomic analyses were conducted on an organismal level for *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130. Comparative analyses were conducted on total protein extracted from bacteria that had been grown under conventional *in vitro* conditions and those mimicking *in vivo* conditions (iron limitation and serum presence, defined as *in vivo*-like conditions). These studies provide the first comprehensive, comparative and quantitative analysis of *Leptospira* total proteome alterations in response to environmental conditions that mimic those found within the host.

Methods
Proteomic analyses were conducted using iTRAQ and LC-ESI-tandem mass spectrometry complemented with two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry. Quantitation of select proteins was confirmed via western blot experiments and their expression during infection confirmed via ELISA and immunofluorescence experiments.

Results
Combined, the iTRAQ and 2DGE experiments identified a total of 563 expressed leptospiral proteins. Altered expression was observed for 65 of these proteins. These include upregulation of the previously identified virulence factor, outer membrane lipoprotein Loa22, and 5 novel potential virulence factors that exhibit sequence similarity to proteins shown to play key functional roles in other pathogens, including the outer surface lipoprotein ErpY, coagulase, catalase, TolC and glycosyl hydrolase. Analyses also revealed conditional methylation of glutamic acid recidues of a putative outer membrane protein (Figure 1). Lastly, ELISA analyses using serum from patients with leptospirosis and immunofluorescence studies performed on liver sections derived from *Leptospira*-infected hamsters combined to verified expression of 4 of the upregulated potential virulence factors during infection.

Innovative aspects
- Glutamic acid methylation of an outer membrane protein in *Leptospira*
- Identification of 5 proteins with potential for novel roles in *Leptospira* virulence

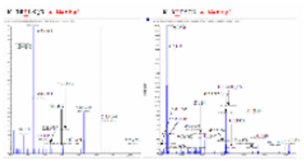

Figure 1. Representative MS/MS spectra utilized for manual sequencing of methylated protein . MS/MS spectra were obtained from manual injection of in-gel digests into an ESI-QSTAR MS. All matching peptide spectra were manually sequenced and the two examples shown above represent the signal to noise ratios in the spectra. Only peaks with a signal to noise ratio > 2 were used to sequence peptides and to detect methylation sites.

Q141

Quantitative Nano-Proteomics for Resolving Protein Complexes (QNanoPX) Related to Estrogen Transcriptional Action

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Purpose
Gold nanoparticles (AuNPs) can be easily modified with many choices of functionality by self assembly monolayer (SAM) technology for increasing solubility that could greatly improve interfacial interactions and for a variety of application. Moreover, the surface-only binding of AuNPs leads to no upper size limit for the protein complex and no limit on reaction kinetics for pore penetration, both of which are useful for IP experiment. Thus, we would like to take the advantage of AuNPs in developing an efficient IP method. We will investigate the protein-DNA interactome related to estrogen response element (ERE) located in the promoter region of the target gene. ERE is known to be regulated by its binding transcription factors: Erα or β. We propose to functionalize AuNPs with the consensus sequence of ERE using SAM technique, and combine the IP method with stable isotope dimethyl labeling, statistics and informatics, for unraveling the pull-down proteins associated with the ERE complex. We term such approach as Quantitative Nano-Proteomics for resolving protein complexes (QNanoPX). QNanoPX is expected to improve the ways protein complexes are analyzed by MS and helps to resolve complexes related to the transcriptional action of estrogen.

Disclosure

In this approach, AuNPs were functionalized with the consensus sequence of ERE (AuNP-ERE) as the positive probe and without the ERE as the control probe using thiolated self assembly monolayer (SAM) technique to quest for estrogen transcriptional actions. AuNP-ERE probe was shown to pull down about nine times more ER from the total lysate of MCF-7 cells than that by the control probe. Moreover, the AuNP-ERE probe was easy to track by color change and was shown to have lower nonspecific binding, higher solubility, and comparable loading capacity compared to gel beads. We combined the IP method with MS-based quantitative proteomics using stable isotope dimethyl labeling, statistics and bioinformatics analyses for revealing the components of the ERE complex pulled down from 17 estradiol (E2)-treated MCF-7 cells. The surface-only binding allows the AuNP-ERE probe to capture a large protein complex. A total of 304 proteins were identified; 72% of identified proteins exhibited significant binding based on statistics criteria defined for quantification ratios and some of them were further confirmed by Western blotting. A majority of identified proteins with significant binding are involved in genomics pathway including known and unknown coactivators/corepressors, and proteins involved in gene transcription and translation. Moreover, non-receptor ERE binding proteins and proteins involved in nongenomics pathway and cell cycle were also identified to be in the complex. Based on bioinformatics analyses, the identification of many novel proteins in the complex may indicate cross interactions among pathways and worth further investigations.

Comments

It is particularly worth noting that the identification of many novel proteins such as TRI25, HSP27, and NDPK-B, to be in the ERE complex of MCF-7 cells suggests cross interactions between transcriptional activation and multiple pathways, as well as between two receptor isoforms. We have also demonstrated a successful QNanoPX method that combines chemically modified AuNPs and quantitative proteomics for revealing a large protein complex using IP method. The method is superior to the traditional gel beads and is expected to hold great promises for analyzing other protein complexes *in-vivo* for a diverse of applications in resolving protein interactome.

Q142

Protein Concentration Estimates from Mass Spectrometer Peptide Intensities

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Introduction: (maximum 600 characters)
The typical time-of-flight mass spectrometer used in proteomics provides a linear response of peptide intensity to changes in peptide concentration. The linear response has been exploited considerably in quantitative proteomics. However, peptide intensity information has been used almost exclusively for comparative measurement as in the isotopic/isobaric tagging methodologies. We investigate the more direct approach of using peptide intensities to estimate protein concentration and we show that this approach is successful for protein concentration prediction in our dataset.

Methods: (maximum 800 characters)
A set of highly purified proteins were combined to form 16 sets of protein mixtures with protein concentrations varying over two orders of magnitude. Each set was analyzed in MS and MS/MS mode, and each analysis repeated 5 times.
The peptide intensities were extracted from the MS dataset and used to model the individual peptide "intensity coefficients" (response to changes in concentration of parent peptide. All peptide ions were used; ie, different change states and different ptms were considered as unique peptides.
The peptide intensity coefficients are then used to predict peptide concentration from peptide intensities; the set of peptide concentrations stemming from a single protein are combined as an estimate of protein concentration.

Results: (maximum 1200 characters)
Single peptide linear modelling success was tested by calculating the %VAF (variance accounted for) of each peptide. Across all 200+ peptides the VAF averaged approximately 82%. The distribution of all VAFs show that the large majority of intensity coefficients are fit quite well, and that only a small handful account for the poor tail (figure 1).
Leave-one-out cross validation testing showed that the intensity coefficients are quite stable, as most changes were less than 10% across any of the five replicates.
Peptide abundance predictions from the intensity coefficients for a single protein showed significant dispersion; however the scatter appeared to be normally distributed so a single protein concentration was easily estimated.
Figure 2 shows the predicted protein abundance versus the designed abundance for a single protein in all 16 mixes. All points (save one at low intensity) predicted the true protein abundance quite accurately.

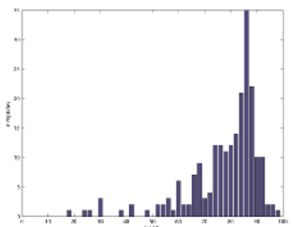

Figure 1: VAF Distribution of peptide intensity coefficients

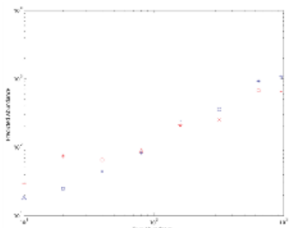

Figure 2: Predicted protein concentration versus true protein concentration

Innovative aspects: (maximum of 3 bullet points)
- Concentration from intensity
- Direct measurement of concentration

Q143

The Effects of Ionizing Radiation and Radiprotectors on Lymphoblastoid Cells

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Introduction
Ataxia Telangiectasia, Mutated (ATM) is a nuclear protein kinase at the core of the cellular response to double-stranded DNA breaks induced by ionizing radiation (IR) (1). Loss of functional ATM results in neurodegeneration, cancer predisposition and sensitivity to IR. Comparative proteomics using Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC) is employed to study DNA damage repair following IR insult, and to explore the cellular effects of potential radioprotector compounds in lymphoblastoid cell lines (LCLs) with functional or aberrant ATM.

Methods
SILAC has been employed to encode the cellular proteomes of normal (NAT8) and ATM-deficient (LEPU) LCLs with light isotopes or heavy isotopes of arginine and lysine. LCLs were exposed to 5 Gy of radiation and the cells harvested at specific points over the course of seventy-two hours following radiation exposure. Proteins from harvested LCL cultures were isolated, digested, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Quantification of peptide and protein abundance from MS-based measurements were achieved by a combination of in-house and readily available automated software packages to yield a detailed quantitative picture of the changes in protein abundance in LCLs in response to IR and radioprotector compounds.

Results
Using LC-MS/MS we have analyzed LCL sets prior to irradiation, in order to establish a baseline of differential protein abundance, as well as LCL sets harvested one hour after exposure to 5 Gy radiation. Cellular proteins were subjected to in-solution digestion or in-gel digestion, following separation of proteins in a 1D-SDS PAGE gel. Manual quantification and automated quantification using the Trans Proteomic Pipeline (Seattle Proteome Center) produced quantification data for in-solution digests of LEPU and NAT8 LCLs prior to irradiation and 1, 4 and 8 hours following 5 Gy of radiation (see Figure 1). In LEPU LCLs, prior to irradiation, a number of proteins are upregulated (glutathione S-transferase (GST) and a variety of histone proteins) and downregulated (mitochondrial ATPase, plastin-2, actin and L-lactate dehydrogenase B) relative to NAT8 LCLs. Following irradiation, there is a general trend towards degradation of these proteins in both NAT8 and LEPU LCLs, with the exception increasing levels of GST and some histone proteins in LEPU LCLs.

Innovative aspects
- SILAC-based quantitative proteomics provides a first look at the affects on protein expression by ionizing radiation and the cellular effects of radioprotector compounds

References
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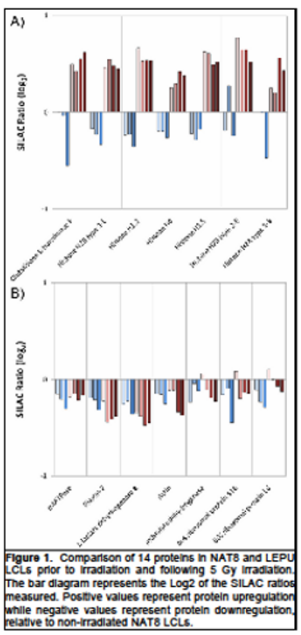

Figure 1. Comparison of 14 proteins in NAT8 and LEPU LCLs prior to irradiation and following 5 Gy irradiation. The bar diagram represents the Log2 of the SILAC ratios measured. Positive values represent protein upregulation while negative values represent protein downregulation, relative to non-irradiated NAT8 LCLs.

Q144

hypopharyngeal gland of newly emerged honeybee workers could secret royal jelly: a proteomic analysis

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Introduction

The hypopharyngeal gland (HG) of honeybee workers plays an important role in honeybee nutrition and caste differentiation. The study of HG is mainly focusing on its age-dependent morphological, physiological and biochemical characters. However, these findings are mainly derived from transcriptional data reflected by expressed genes, which can not describe the real developmental picture well. So proteomics was used to mine the protein information in the development of HG, expecting to find individual genes or proteins and understand how gene networks enable complex functions to be carried out.

Methods

HG samples of 1-, 3-, 6-, 12-, 15-, and 20-day-old honeybee workers (*Apis mellifera*. L) were prepared. Proteins were separated by two-dimensional gel electrophoresis. After in-gel digestion, proteins were identified by using MALDI-TOF, MALDI-TOF /TOF and MASCOT search engines. Identified proteins were subjected to biological network analysis by pathway studio software. Then protein expression was validated by employing western blot.

Results

A total of 175, 189, 209, 190, 168 and 151 protein spots were detected with 92, 91, 95, 102, 79 and 71 proteins were successfully identified on each development stage, respectively. These proteins were involved in the metabolism of carbohydrates and energy production, lipid and proteins; cytoskeleton proteins, development regulation, antioxidant system, molecule transporters, regulation of transcription/translation, protein folding and major royal jelly proteins (MRJPs), secretions from HG. This suggests that the development of HG is involved in multiple functions of proteins that take part in a variety of biological processes for which the energy demand is the most. The most interesting is that MRJP that have been detected in HG of the newly emerged worker bees, suggesting that the worker bee could secrete royal jelly (RJ) since they born. This expands our knowledge that worker bees start secret RJ 6 days after they born. MRJP1, 2 and 3 were validated by western blot, which coincide with the expression trend from the 2D-gel, respectively, implying HG secrete RJ at peak within 3-12 days after eclosion. All the identified proteins (excluded MRJPs) and differentially regulated proteins were applied to biological network analysis, 35 proteins were assigned to key node proteins, while 13 proteins had altered their expression significantly. These key node proteins probably play an important role in the development of HG and provide us some target proteins even genes for further analysis.

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Q145

Modified Spectral Count Index (mSCI) for Estimation of Protein Abundance by Protein Relative Identification Possibility (RIPpro): A New Proteomic Technological Parameter

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Introduction

Peptides Count (SC) was widely used for protein abundance estimation in proteomics, Based on that, Mann (1) corrected the SC by dividing spectrum counts through the number of observable peptides per protein. Here we present modified spectral count indexe (mSCI) for protein abundance estimation, which defined as the number of observed peptides divided by protein relative identification possibility (RIPpro), a new proteomic technological parameter. Also, multiple linear regression were performed to quantitative assessment of several factors (including RIPpro) have on the mRNA/protein abundance correlation.

Methods

RIPpro was derived form 6788 human liver protein expression data, and related to proteins' three physical and chemical properties (Mw/pI/Hp). First, the entire range of each property (Mw, pI or Hp) in the IPI Human_3.07 database was divided into 100 bins and the numbers of proteins in each bin were counted and denoted as $C^p_{All}(i)$ where i is the index of the bin. The number of human liver proteins in each bin was also calculated and denoted as $C^p_{Exp}(i)$. $R^p(i) = C^p_{Exp}(i) / C^p_{All}(i)$ was calculated for each bin. The maximal value of the ratios is set equal to 1 and all values were normalized to this ratio, i.e. $RIP^p(i) = R^p(i) / R^p_{max}$. Where R^p_{max} is the maximal value of $R^p(i)$. Thus the minimal RIP value between the 3 generated (Mw, pI and Hp) for each protein is used to measure the identified possibility of the protein; this is known as RIPpro.

Results

For 46 proteins in mouse neuro2a cells and 30 proteins in human liver, mSCI show a linear relationship with the actual protein concentration, similar or better than determination of abundance by PAI (1) and other similar methods.
Multiple linear regression were performed to quantitative assessment of the role several factors have on the mRNA/protein abundance correlation. Our results shown that the primary factor affecting protein levels was mRNA abundance (36%), followed by RIPpro, variability in protein measurement, and protein turn-over (11%, 2% and 8%, respectively). Interestingly, we determined that the concordance between levels of mRNA transcripts and protein expression was not consistent across all classes of proteins; this correlation was lower for signaling proteins as compared to metabolism genes. It was determined that RIPpro was the primary factor affecting signaling protein abundance (23% on average), followed by mRNA abundance (17%). In contrast, only 5% (on average) of the variability of metabolic protein abundance was explained by RIPpro and mRNA abundance had a much greater effect (40%) on the expression of these proteins.

Innovative aspects

- Introduce mSCI for estimation of protein abundance
- RIPpro score provides useful information for characterizing the identification possibility of each protein in MS/MS
- Provide the impetus for further investigation of the biological significance of mechanisms regulating the mRNA /protein abundance correlation

References (maximum 3 references)

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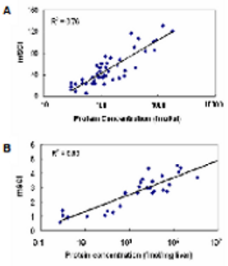

Figure 1. Relationship between mSCI and protein concentration. A, relationship between protein concentration and mSCI for 46 proteins in neuro2a cells (1); B, relationship between protein concentration and mSCI for 30 proteins in human liver.

Q146

Development and validation of a multiplexed panel of MRM assays for clinical use

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Introduction

A list of 30 candidate protein markers of Alzheimer's disease (AD) was developed from previous observations in CSF and through literature mining. To assess whether these proteins are qualified as markers of AD, an effort was initiated to develop multiplexed MRM assay panel for these 30 proteins. Following the initial development, we have sought to carry out the technical validation of the multiplexed panel so that it may be used on clinical samples.

Methods

Normal human CSF was separated on an SDS-PAGE gel then analyzed nanoflow LC coupled to a hybrid a LTQ-Orbitrap. MRM candidate peptides for the 30 proteins were selected based on earlier observations for further testing. The performance of each of the peptides was assessed in an iterative manner using a Quantum Ultra TQMS to obtain a set of peptides to bring forward to validation. The %CV, LOD, LOQ, linear dynamic range and interference for each peptide in the multiplex was determined using the SOPs established during the iterative assessment. Standard curves for absolute quantification were constructed in a randomized manner to above batch affects.

Results

In the initial phase, 29 of the 30 targeted proteins were identified in normal human CSF. From the 29 proteins, a set of 57 peptides was brought forward. This represented two peptides for all but one of the proteins. This preliminary set of peptides was evaluated for interference in either the light or heavy channel in neat solvent or in CSF. Calibration curves covering the dynamic range were constructed each of the peptides. The linear regression values were greater than 0.9 for 51 of the 57 peptides analyzed. Problems were observed with the detection and linear response of the remaining six peptides. These six peptides were discarded. The 51 peptides surviving the secondary screen were moved to technical validation. To determine technical and biological performance of the peptides, a small cohort of human CSF samples from both normal and AD patients was analyzed. This sample set also enabled an initial assessment of the possible range of protein expression. Eight point calibration curves were constructed for each peptide over three orders of magnitude in proxy matrix. Assay validation is being carried out by adopting guidelines developed for the analysis of xenobiotics by LC/MS. To establish the robustness of the assays, the analyte peptides were evaluated for freeze/thaw stability.

Innovative aspects

- Multiplex MRM assay to qualify candidate proteins as biomarkers of AD in CSF
- PK/PD level validation of multiplex MRM assay

Q147

Quantitative analysis of lectin-enriched aberrant glycoprotein by mass analysis

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Introduction

Protein glycosylation, one of important post-translational modifications of protein, is playing a variety of biological functions. Variations in glycosylation levels or in glycoprofile of a certain glycoprotein in tumor-related proteome have been widely reported and can be used as a means for studying of biological functions of protein glycosylation in cancer disease. However, quantitative mass analyses of glycoproteins, especially tumor-related aberrant glyco-isoforms, are difficult because of their low mass sensitivity and high structural complexity. Therefore, more powerful technologies are required for separation and enrichment of these low-abundant aberrant glyco-isoforms, and their high-sensitive mass quantification.

Methods

Among a variety of lectins employed for enrichment of unique glycan form, L-PHA was used for enrichment of aberrant glyco-isoforms up-regulated as a result of aberrant addition of β-1,6-N-acetylglucosamine (β-1,6-GlcNAc) by N-acetylglucosaminyltransferase-V (GnT-V), which is reportedly up-regulated in invasive/metastatic cancer cells. Using trypsin-digested samples L-PHA-enriched from control cell and GnT-V treated cell, quantitative analyses for some interesting glycoproteins like tissue inhibitor of metalloproteinase 1 (TIMP-1), were conducted by LTQ-FT MS using a MRM mode.

Results

The analytical results reveal that the abundance of aberrant glyco-isoform of TIMP1 is greatly increased in GnT-V treated cell rather than in control cell, although the abundance of total TIMP1 in GnT-V treated cell is slightly increased than that in control cell. The dramatic variation in abundance of the aberrant glyco-isoform can be interpreted as glycosylation of TIMP1 is playing an important role in biological function. And to detect and quantify the aberrant glyco-isoform of TIMP1 from complex serum samples of cancer patients, Anti-peptide TIMP1 antibody was prepared and immobilized on a magnetic bead. Antigen obtained from L-PHA-enriched serum by the immobilized anti-peptide antibody was quantified with MRM mass analysis.

Innovative aspects

- Quantification of lectin-enriched aberrant glyco-isoform by anti-peptide antibody and mass analysis

References

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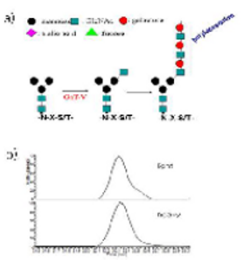

Figure 1. a) Schematic representation of aberrant addition of β-1,6-GlcNAc by GnT-V, up-regulated in invasive/metastatic cancer cells. b) MRM mass analysis for quantification of lectin-enriched aberrant glyco-isoform.

Q148

Optimizing the Peptide Quantitation Workflow from Biomarker Discovery to Validation

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Introduction

Quantitative proteomics often requires monitoring of hundreds of target peptides in thousands of biological samples. Acquiring MRM transition ion data only during the retention window for each peptide reduces the overall number of ions monitored at any one time and therefore improves sensitivity. Optimizing the collision energy for the selected transitions also improves sensitivity. In this study, standard peptides were optimized, then these peptides were spiked into human plasma for quantitation. The impact of retention time triggering of MRMs (dynamic MRM) on quantitation was assessed.

Method

Tryptic digests were prepared for a mixture of standard proteins, depleted human serum and yeast lysate. LC/MS analyses were performed on a triple quadrupole (QQQ) or Q-TOF mass spectrometer interfaced to a microfluidic-based nanoflow LC system. Software was used to help design and optimize MRM transitions from the standard peptides. Additional MRM transitions were selected from discovery data (Q-TOF) collected from the complex samples. These transitions were used to examine the impact of the number of dynamic MRMs in a method on quantitation of the target standard peptides.

Results

For the standard peptides, an automated MRM optimization routine was applied that primarily determined the optimal collision energy for selected transitions (Figure 1) and results were validated against manual optimization results. The spiked plasma digest was first analyzed using a Q-TOF system for protein identification and retention time determination. The average standard deviation for retention time was less than 0.1 minutes from triplicate runs. Two proteotypic peptides from each selected protein based on the peptides identified from the Q-TOF experiment. The software selected the peptides based on the amino acid requirements (length, absence of Cys and Met etc.), missed cleavages and the uniqueness in the database. For the selected peptides, two MRM transition pairs were selected for each peptide based on intensity. A total of more than 400 MRM transition pairs were generated to be analyzed in a single QQQ run. The same gradient as in the Q-TOF analysis was used with QQQ so the retention time value could be used to trigger MRM acquisition. The quantitation results were measured in 5 replicates with and without retention-time triggering of the MRM transitions. The performance of these two experimental conditions will be reported and the effect of the two methods on the quantitation results of the spiked proteins will be compared.

Innovative aspects

- High-throughput protein quantitation using MRM triggered by retention time
- Selection of peptides and transition from protein identification results
- Automated routine for MRM optimization

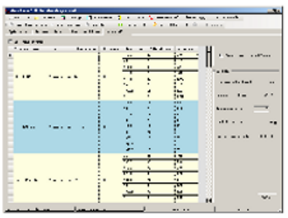

Figure 1: Results of automated MRM optimization routine showing transitions and optimal collision energy.

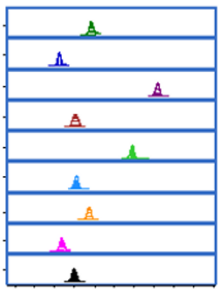

Figure 2: Analysis using retention scheduling of MRMs. Each transition was collected only over a narrow time window.

Q149

Multiplexing proteomic quantification: Application to Autism biomarkers evaluation

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Introduction

Hypothesis-driven approach combining an effective method to reduce sample complexity and selected reaction monitoring (SRM) was demonstrated to be highly sensitive and specific, allowing quantification of low abundant proteins. However, throughput remains a challenge, and in this context we combined SRM and multiplexing samples via labeling (iTRAQ).This strategy was specifically applied to samples originated from autism patients to detect, quantify, and confirm the identity of putative plasma biomarkers.

Methods

Plasma samples were collected from autism and control patients. To circumvent the high complexity and large dynamic range of blood plasma proteins, we focused on two subproteomes: N-glycoproteins and proteins containing cystein peptides using solid phase extraction methods. Peptides were then labeled with mTRAQ reagents or iTRAQ 8plex reagents and analyzed on a triple quadrupole instrument. For iTRAQ analyses, due to the isobaric tag, the quantification of the mixed samples is obtained through transitions conferred by the eight reporter ions.

Results

Preliminary transcriptomic and peptide microarrays experiments on Autism samples allowed us to identify many putative candidates. SRM assays were built for selected proteotypic peptides (of N-glyco or cys peptides subproteomes). The analyses allowed the measurement peptides in a tryptic digest of plasma proteins labeled with multiplex reagents by monitoring transitions for quantitative measurements. For iTRAQ labelled samples, ten transitions were selected for each peptide, including the two most abundant fragments for identification (b or y ions) and the eight reporter ions for relative quantification. Several of the biomarker candidates were detected, identified and precisely quantified simultaneously in different plasma samples. It established the basis for high throughput systematic quantitative and qualitative analysis in large cohorts of plasma samples to gain specificity and selectivity for the detection of biomarker panels.

Innovative aspects

- High throughput qualitative and quantitative analysis of biomarkers in plasma using SRM.
- Multiplexing proteomic approaches in conjunction with SRM.

References

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Q150

An Hsp90 Dependent Folding Defect in CFTR NBD1 contributes to Cystic Fibrosis

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Introduction

Defective folding of the ΔF508 variant of the multi-membrane-spanning protein, cystic fibrosis transmembrane conductance regulator (CFTR), results in its degradation and loss of its chloride channel function at the cell surface leading to onset of the childhood disease cystic fibrosis (CF). Phe 508 is located in the structure of an aromatic rich ligand binding cleft (F508-LCB) that has been shown to interact with the cytoplasmic loop domain 4 (CLD4), a critical folding step in CFTR biogenesis[1]. Recent studies have implicated multiple chaperones in the folding of CFTR. We have applied absolute quantification and crosslinking strategies coupled with mass spectrometry to determine the critical folding steps associated with the disease.

Methods

CFTR and its interacting partners were immunoprecipitated from cell lines expressing ΔF508 and wt CFTR at 30ºC (permissive) and 37ºC (physiological temperature). Fully labelled chaperone proteins were used as internal standards for absolute quantification. Mass spectrometry data was obtained using LTQ Orbitrap and a TSQ Quantum Ultra Triple Quad. Zero Length crosslinkers in combination with mass Spectrometry were used to examine the CFTR /chaperone binding interactions.

Results

Absolute Quantification measurements of CFTR at 30ºC and 37ºC and its interacting partners revealed the accumulation of ΔF508 in a folding intermediate in the ER containing near stoichiometric levels of heat shock proteins (Hsp) 40, Hsp70 and Hsp90, and the Hsp90 ATPase activator Aha1 (Figure 1). We proposed that these heat shock proteins may facilitate the folding of F508-LCB in a fashion similar to recognition of other client LCBs by chaperones. In *vitro* binding assays of purified NDB1 with CLD4 showed binding was inhibited by the addition of heat shock chaperone proteins supporting our model. Previous work we performed on Hsp90 activator AHA1 implicated Hsp 90 to play a critical role in folding of CFTR[2]. Crosslinking analysis of NDB1 and Hsp90 revealed differential binding patterns between ΔF508 and wt CFTR supporting its role in the folding process and additional degradation experiments provided evidence for the role of Hsp 90 in CFTR stability.

Innovative aspects

- Using absolute quantification strategies to measure stoichiometric interactions of CFTR and its binding partners
- Using crosslinking strategies in combination with mass spectrometry to elucidate key folding interactions in CF
- Combining key proteomic tools with cell biological assays to elucidate key regulators of CFTR folding

References

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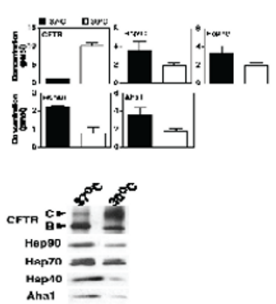

Figure 1. Shows absolute measurements of CFTR and interacting chaperones at permissive and physiological temperature by absolute quantification (above) and western blotting analysis confirming observation.

Q151

Method development for quantification of bacterial integral membrane proteins

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Introduction

We established a single consecutive strategy which assigned the most comprehensive number of integral membrane proteins from Gram-positive bacteria to date. Integral membrane proteins are of great interest as potential drug targets, and their analysis appears to be quite challenging. For this purpose we adapted a biphasic partitioning system for the biotechnologically intensively used *Corynebacterium glutamicum* and proved for the first time that such a system is well suited for quantitative comparisons.

Methods

A two-phase partitioning system, containing PEG and dextran, was optimized to purify integral membrane proteins of *C. glutamicum* (1). Optimization was conducted stepwise and verified by using protein determination assays and western blotting. Analyses were performed by means of the MudPIT (2) technique on an LTQ-XL-Orbitrap. For quantification a published stable isotope labelling strategy was used (3).

Results

297 integral membrane proteins were identified by our integrated approach, which depletes stringently cytosolic proteins. In combination with our previously developed SIMPLE strategy, our data comprise 65% (426 integral membrane proteins) of the entire membrane proteome, which aims towards an almost close-to-complete coverage. Wild type and a production strain of *C. glutamicum* were compared by ^{15}N metabolic labelling and quantitation was obtained by spectral counting and peak areas. Compared to spectral counting, the ProRata algorithm seems to attenuate regulation, whereas stronger regulation and therefore a higher dynamic range is more favourably monitored by spectral counting. We highlight some of the candidates, which prove that our approach helps to give a deeper quantitative insight towards the understanding of transport and other membrane associated processes, important for strain development of *C. glutamicum*.

Innovative aspects

- The two-phase partitioning system allows to detect a high number of integral membrane proteins (297) in a single purification workflow.
- A large number of membrane embedded peptides could be detected (301 related to 141 IMP).
- Also reliable quantitative data can be obtained using this method.

References

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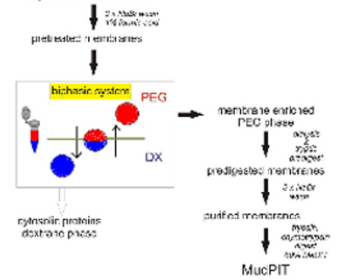

Figure 1 Workflow of the single consecutive integral membrane purification strategy

optimized biphasic system
6% PEG-6000
6% Dextran T-500
500 mM Li_2SO_4
15 mM Tris-HCl pH 7.4

Figure 2 Composition of the optimized two-phase partitioning system

Q152

Proteolytic ^{18}O-Labeling For Quantitative FSHD Biomarker Discovery Using PALeO Strategy

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Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common forms of skeletal myopathy in humans. The molecular mechanism leading to the disease was poorly understood, with no treatment for FSHD ever developed. Searching for the FSHD protein biomarkers is in much need to achieve a better understanding of FSHD development and progression, and identification of pharmacological targets. We developed proteolytic ^{18}O labeling using PALeO strategy for comparative FSHD Biomarkers discovery, which can be applied to clinical sample.

Methods

Proteins were digested by trypsin (substrate: enzyme – 1:20) for 24 hrs at 37°C in the presence of normal water or 50% ^{18}O water. Tryptic peptides were isolated from intact trypsin by 5K ultrfiltration spin column. Peptides were analyzed by LC-MALDI-MS/MS using Tempo LC MALDI Spotting system and 4800 MALDI-TOF/TODF (Applied Biosystems). MS data were analyzed using in-house information system (PALeO) that utilizing MASCOT Distiller software (Matrix Science) for spectral processing, peak detection and automatic determination of ^{16}O incorporation ratio. Peptides were identified by using the MASCOT algorithm to search against mammalian proteins in the Swiss-Prot database *via* trypsin digestion.

Results

PALeO has been used before to characterize proteolytic activities in complex biological samples (1). Now, we are extending this method in ^{18}O-labeling based relative quantitative proteomics. Here we use BSA (Bovine Serum Albumin) as the standard protein to test PALeO strategy in M-S based protein quantitation analysis. BSA tryptic peptides digested in ^{16}O and ^{18}O water were pooled as following ratios: 10:1; 5:1; 2:1; 1:1; 1:2; 1:5; and 1:10 in triplets, respectively. Compared to the literature, calculated ^{18}O labeled BSA ratio based on PALeO showed highly reproducibility with tighter and more accurate calculated ratio (Figure 1). Additionally, we further proved the reproducibility and precision of PALeO strategy in more complex biological samples using 3T3 mouse fibroblasts cell lysate. In conclusion, our method allows us to perform MS-based comparative quantitation analysis in two different samples using 50% ^{18}O enriched H_2O (half of the literature suggested amount). This will provide a cheaper and faster alternative method to perform MS-based quantitative analysis by *in vitro* labeling.

Innovative aspects

- No additional chemical reaction step involved with universally labeling of all peptides from proteolytic digestion
- label efficiency can be monitored for more accurate quantification
- faster and lower cost

References

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Theoretical ratio	U quan	U Stdev	S quan	S Stdev	D quan	D Stdev	S/D quan	S/D Stdev
0.1	0.202	0.024	0.202	0.049	0.201	0.001	0.202	0.025
0.2	0.242	0.035	0.285	0.059	0.195	0.033	0.240	0.036
0.33	0.353	0.015	0.421	0.021	0.286	0.018	0.354	0.015
0.5	0.480	0.024	0.602	0.022	0.382	0.048	0.492	0.023
0.67	0.625	0.012	0.745	0.027	0.504	0.005	0.625	0.012
0.8	0.746	0.015	0.926	0.009	0.565	0.029	0.746	0.015
0.9	0.815	0.002	0.919	0.002	0.714	0.006	0.814	0.002

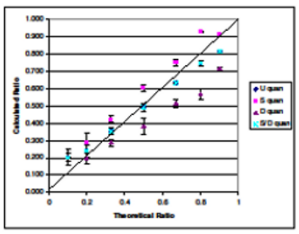

Figure 1: Comparison of calculated ^{18}O labeled BSA ratio based on PALeO to theoretical ratio with different calculation formula. U quan utilized the unlabeled and doubly labeled ratio; S quan only utilized the singly labeled ratio; D quan only utilized the doubly labeled ratio, and S/D quan utilized the unlabeled and doubly labeled ratio. Both U quan and S/D quan utilized 2 out of 3 PALeO ratios and represent a more accurate calculation.

Q153

Proteomic analysis of Microtubule Associated Proteins during Macrophage Activation

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Introduction

Macrophages are key regulators of the immune system connecting innate and specific immune responses. Activation of macrophages induces a range of signaling and vesicle trafficking events through rearrangement of microtubule (MT) cytoskeleton. The structure of MT cytoskeleton is dynamically regulated by interaction of MT with various microtubule associated proteins (MAPs). We took a shotgun proteomics approach to analyze and relatively quantitate (by spectral count) the composition of MAPs in macrophage stimulated with the inflammatory cytokine, IFN-γ, and the endotoxin, lipopolysaccharide (LPS).

Methods

The RAW264.7 mouse macrophage cell line was activated by a combination of 100 U/ml IFN-γ and 1 μg/ml LPS for 12 h. MT pellets were isolated and resuspended in buffer containing 1 mM GTP, 20 μM taxol at 37°C. 0.35M NaCl was added to release MAPs, which were collected as supernatant after 30,000 x g for 25 min (1). Intact MAPs were fractionated into 24 fraciotns by C4 reversed phase, digested, and analyzed by LC-MS/MS. Database search were performed by Agilent SpectrumMill and the spectral counts were used for relative quantitation (2). Two-tailed Student's *t*-test were used to test whether the mean base 2 logarithmic (log2) values of the ratios of spectral count of the same protein are different from zero (reported as significant if P value < 0.05).

Results

Three biological replicates were analyzed and resulted in identification of 409 MAPs by minimum 2 unique peptides and other criteria. The false positive rate was estimated to be < 3%. Out of these, 52 were up regulated two-fold or greater and 42 were down regulated two-fold or greater upon IFN-γ/LPS stimulation (Figure 1). Bioinformatics analysis based on binary protein interaction data from public domain produced a putative interaction network of these identified MAPs in activated macrophages. We confirmed the up regulation of several MAPs by immunoblotting and immunofluoresecence analysis. Further analysis of one up-regulated protein, HSP90β, revealed its role in stabilizing the MT cytoskeleton upon macrophage activation.

Innovative aspects

- Reveal the composition of microtubule associated proteins in mammalian macrophages
- Relatively quantitate the change of MAPs in response to LPS/ IFN-γ stimulation

References

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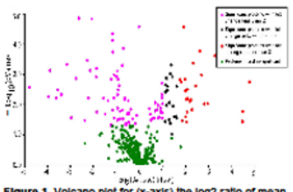

Figure 1. Volcano plot for (x-axis) the log2 ratio of mean spectral count of the MAPs in the treated cells vs. the control cells and (y-axis) – log10 (pValue). From a total of 409 identified proteins, 140 proteins were significant in differential expression (p<0.05). Among them, 52 proteins exhibited a greater than two-fold increase in MT binding, whereas 42 proteins exhibited a greater than two-fold decrease in MT co-sedimentation as a result of IFN-γ/LPS stimulation.

Q154

The Membrane Proteome of *Leishmania mexicana*

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Introduction

Proteomic analyses tend to be biased against the plasma membrane proteome, as these proteins are often of low abundance and hydrophobicity. The project's aim is to develop reproducible methods for comparative membrane proteomic analysis on the human pathogen *Leishmania mexicana*. Gel-based isoelectric focusing (IEF) is highly resolving and the first dimension separation for 2DE, and for quantitative approaches such as Difference Gel Electrophoresis (DiGE). As gel-based IEF is not suited to hydrophobic membrane proteins and such proteins are rarely seen on conventional 2D gels, as alternative, we are testing solution-based IEF for the separation of membrane proteins.

Methods

Leishmania mexicana promastigotes were subjected to subcellular fractionation, monitoring recovery of plasma membrane proteins by Western blot to detect a homologous myc-tagged surface glucose transporter. The plasma membrane enriched fraction was separated by solution-phase isoelectric focusing using an Agilent Off-Gel fractionator, followed by SDS-PAGE. We plan to combine this with a fluorescent tagging approach to look for changes in the transporter proteome in response to nutrient availability and drug selection.

Results

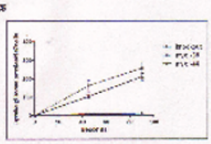

Figure 1. Glucose uptake of Myc-tagged glucose transporter (myc-39, myc 40) and ΔImGT (knockout).

A glucose transporter knockout *Leishmania* line transfected with a myc-tagged glucose transporter was used as a marker for the recovery of integral plasma membrane proteins upon various procedures for subcellular fractionation that is currently being tested. The myc-tagged glucose transporter is active, and complements the glucose transport lesion in the ΔLmGT, confirming that the tagged transporter is localised to the plasma membrane (Figure 1). Crude plasma membrane fractions by solution IEF and analysed fraction by SDS-PAGE, to screen for changes in the membrane proteome between wild-type and ΔLmGT *Leishmania* (Figure 3).

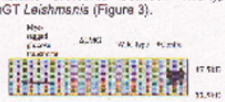

Figure 2: Western blot shows myc-tagged glucose transporter expression with Mouse Anti-myc mAb.

A selection of bands were excised for analysis by tandem MS. Many of the identified proteins were structural components, indicating that the plasma membrane enrichment methods need to be optimized. However, some membrane proteins were identified, including a sulphate transporter, indicating that solution IEF is able to separate integral membrane proteins.We plan to repeat this novel 2 dimensional separation after differential tagging of the two samples using CyDyes, in order to high-light proteins that are differentially represented.

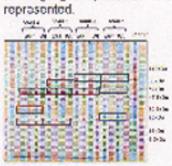

Figure 3: SDS-Gel cage picture stained with Coomassie. Protein samples were recovered from IEF fractionation from wild type and glucose transporter knock-out sample. Samples from the same well number were place side by side. Black-coloured lines/ boxes indicate the bands that of interest for in-gel trypsin and characterisation through mass spectrometry.

Innovative aspects

- Quantitative enrichment strategy for integral plasma membrane proteins.
- Novel solution IEF dimension for modified DiGE analysis.

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Q155

Driving Biological Discovery Using Quantitative Mass Spectrometry

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A component to understanding biological processes involves identifying the proteins expressed in cells as well as their modifications and the dynamics of processes. Several major technologies, but especially mass spectrometry, have benefited from large scale genome sequencing of organisms. The sequence data produced by these efforts can be used to interpret mass spectrometry data of proteins and thus enables rapid and large-scale analysis of protein data from experiments. Advances in multi-dimensional separations as well as mass spectrometry have improved the scale of experiments for protein identification. This has improved the analysis of protein complexes, and more complicated protein mixtures. Quantitative mass spectrometry can be used to study biological processes such as protein-protein interactions, development or the effects of gene mutations on pathways. Recent studies on the interactions of the Cystic Fibrosis Transport Regulator as it progresses through the folding pathway will be presented. Metabolic labeling of whole organisms can now be readily accomplished using 15N labeled proteins as a food source for studies of *in vivo* biology. The use of this method in combination with Shotgun proteomics was used to measure protein and phosphoprotein expression in organelles over several developmental time points in the rat brain (P1 to P45).

Stem Cell Proteome Biology

(S101 – S112)

S101

Comparative 2-D gel analysis of embryonic carcinoma 2102Ep cell line and three human stem cell lines

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The potential use of human embryonic stem cells (hESCs) for the treatment of a number of diseases in regenerative medicine has led to increased research in many disciplines. Proteomic technologies facilitate studies that reveal the global protein expression of pluripotent hESCs and that of their differentiated progeny or any endpoint of interest. Method standardisation will play an important role in validating this research and the establishment of robust reference materials for such purposes is essential. It has been reported that the embryonic carcinoma cell line 2102Ep, derived from a primary human testicular teratocarcinoma, could prove a good reference standard for data normalization, since it fulfils the requirements of a potential reference material e.g. there are no intellectual property constraints, it is easy to grow, stable, and resistant to spontaneous changes in phenotype. The aim of this study is to investigate whether this cell line is suitable as a reference material for use in gel-based proteomics analysis. The 2D-DIGE approach is used to compare the 2-D gel profiles of the embryonic carcinoma cell line to three hESC lines; SHEF1, hES-NLC5 and MEL-1, all deposited in the UK Stem Cell Bank.

S102

Applying FT-ICR MS in a Glycomics Approach Toward Understanding Cancer Stem Cell Differentiation

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Introduction
Cancer stem cells (CSCs) may account for relapse of Glioblastoma multiforme (GBM), a fatal primary brain tumor. As for other stem cells, CSCs possess the ability of unlimited self-renewal and multi-lineage differentiation. NSC11 glioma stem cells are isolated by CD133 affinity purification and subsequent characteristic growth as tumorospheres. STAT3 inhibition and serum-induced differentiation both lead to loss of CD133 but with different phenotypic differentiation. Differential analysis of the cellular glycomic response of NSC11 may elucidate the underlying molecular processes.

Methods
Glycolipid analysis (1): ~$2x10^6$ cells were extracted. 1/50 of the polar lipid fraction was separated by nano-LC in a self-packed phenyl hexyl column with a gradient system of H_2O, methanol and 10 mM NH_4OAc at 400 nL/min. Effluent was on-line analyzed by (-)-microelectrospray by LTQ 14.5 T FT-ICR MS (2). Structural assignment was based on accurate mass and tandem mass spectrometry. Signal magnitudes reflect polar lipid relative abundances.
Glycoprotein analysis: Cells were homogenized; extracted proteins were separated by 2D GE; and glycoprotein spots were stained with Pro-Q Emerald and quantified by PDQuest. The glycoprotein spots were in-gel digested and peptides separated and analyzed by nano-LC (+) LTQ 14.5 FT-ICR MS. Proteins were identified by MASCOT search.

Results
Highly sensitive differential analysis of NSC11 CSCs before and after induced differentiation revealed distinct modulation of glycolipid profiles. Elevation of GM3, GM2α, GM1b, GD2, and asialo-GM1 glycolipids in NSC11 cells under serum-induced differentiation correlates with increased glycotranscripts of UGCGL2 and ST6GALNAC6. In contrast, in STAT3 inhibitor treated cells, decrease of GM3, GM2α, GM1b, and GD2 glycolipids may due to the increased transcripts of NEU1.
Proteomic analysis showed a dramatic decrease of protein glycosylation level after STAT3 inhibition.
A class of endogenous glycosphingolipids (GSLs) was identified in the NSC11. This GSL shares the same/similar epitopes with several nervous system glycoproteins, MAG, L1 NCAM, etc., and may be a biomarker to account for the unusual phenotype of CSCs.
Nano-LC separation and high-resolution FT-ICR detection are crucial for analysis of endogenous GSLs.
Work supported by National FT-ICR MS Facility at the National High Magnetic Field Laboratory (NSF DMR 00-84173), the State of Florida, the Falk Foundation, and the Sorensen Family Fund from the Celebrate Life Charitable Organization.

Innovative aspects
- First report of correlating glycolipidomics, glycoproteomics and glycotranscriptomics in describing cancer stem cell differentiation.
- First report of endogenous sulfated GSL in CSCs

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S103

Quantitative analysis of surface proteome in mesenchymal stem cells cultured with or without bFGF

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Introduction
Bone marrow mesenchymal stem cells (MSCs) are multipotent stromal cells derived from bone marrow. bFGF is a potent mitogen that stimulates growth and differentiation of various cell types. The objective of this study was to compare the expression of surface proteome of MSCs cultured with or without bFGF. We identified 15 surface proteins that were differentially expressed in human MSCs cultured with or without bFGF condition. Among them, several actin-related proteins were identified, which may play an important role in bFGF-induced morphological changes and differentiation potential.

Methods
Human mesenchymal stem cells (hMSCs) were culture-expanded in DMEM containing 10% fetal bovine serum (FBS), 100 U penicillin, 100 mg/ml streptomycin and, in some experiments, 10 ng/ml bFGF. Biotinylation of hMSCs with EZ-link sulfo-NHS-SS-biotin was performed according to the manufacturer's instructions. The first-dimension isoelectric focusing (IEF) was performed using an Ettan IPGphor 3. Surface proteins (100 μg) were resuspended with rehydration buffer. IEF was conducted at 101,000 Vh/h. The second dimension was carried out using a standard 12.5 % SDS-PAGE protocol. Gels were analyzed using the Progenesis 2-DE analysis program. Proteins were identified by Q-TOF-MS and database searching. Validation was performed using western blot and immunohistochemistry.

Results
We have used two-dimensional electrophoresis (2-DE) coupled to mass spectrometry to identify differentially expressed proteins at the cell membrane level in MSCs growing in bFGF containing medium. The cell surface proteins isolated by the biotin-avidin affinity column were separated by 2-DE in triplicate experiments (Figure 1). A total of 15 differentially expressed proteins were identified by quadrupole-time of flight tandem mass spectrometry. Nine of the proteins were up-regulated and six proteins were down-regulated in the MSCs cultured with bFGF containing medium. The expression level of three actin-related proteins, F-actin-capping protein subunit alpha-1 (CAPZA1), actin-related protein 2/3 complex subunit 2 (ARPC2), and myosin regulatory light chain 2 (MRLC2), was confirmed by Western blot analysis. The results indicate that the expression levels of CAPZA1, ARPC2, and MRLC2 are important in bFGF-induced morphological change of MSCs.

Innovative aspects
- Biotinylated cell surface proteins were analyzed by 2-DE and Q-TOF MS/MS.
- Basic fibroblast growth factor (bFGF) modulates actin filament remodeling through the up- or down-regulation of actin related proteins such as CapZ, ARPC2, and MRLC2.

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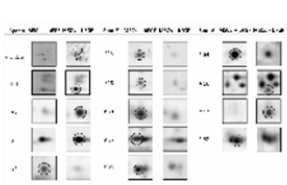

Figure 1. Proteins showing different expression in the human bone marrow derived MSCs cultured with or without bFGF containing medium.

S104

Glioma Stem Cell quantitative proteomics: A "Warburg proteomo-metabolic" deregulation

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Introduction
Glioma stem cells (GSC) realize a minor but motor cell compartment, now understood as a key physiopathological actor and therapeutical target. Deciphering GSC using global proteomic approaches has not been done previously. CD 133 has been described as a GSC biomarker, however this has been recently questioned, supporting the investigation of proteomic biomarkers. We investigated proteins expressed at different level of human GSCs with different clinical aggressiveness. The goal was to find new biomarkers, as well as new physiopathological and therapeutical pathways.

Methods
The use of relevant cell models is a crucial issue: the real oxygen concentration detected in the brain and glioblastoma *in vivo* is lower than 3% contrasting with the 20% oxygen concentration used for classical cell culture. After surgery removal, primary cultures of both aggressive Glioblastoma (survival 15 months) and oligo-astrocytoma (survival 5 years) were established under 3 % O2. GSC were preferentially selected in serum free-medium supplemented in EGF and FGF. CD133 detection was confirmed. Total cellular extracts of neural cancer stem cells were prepared by mechanical lysis.
iTRAQ differential labeling of the corresponding glioblastoma and oligodendroglioblastoma protein samples was performed and the resulting mixture was analyzed by nano-LC-MALDI-TOF/TOF after off-gel fractionation.

Results and discussion:
910 proteins were detected, 264 were over-expressed in lower aggressive GSC and 293 in the aggressive GSC. 50% are cytosolic proteins, 31% nucleus proteins, 9% Endoplamsmic-reticulum and golgi proteins and 10% from cell organites.
The strategy applied, combining offgel fractionation and nanoLC-MALDI-TOFTOF appeared to be efficient for the quantitative analysis of such samples as up to 910 proteins were identified.
Proteins identified provided interesting cues related to GSC biomarker, for example FABP7 [BLBP] = Fatty Acid Binding Protein 7 (Ratio Oligo / Glio = 0.25 - 9 peptides identified) - FABP7 is strongly predictive of poor prognosis in grade IV astrocytoma (=Glioblastoma). Its expression is correlated with increased migration and invasion of tumor cells into the surrounding brain parenchyma.
Proteins, providing potential therapeutical targets have been also individualized, such as PTPRZ1 265 kDa protein = Receptor-type tyrosine-protein phosphatase zeta (Ratio Oligo / Glio = 2.63 - 3 peptides identified). This has been previously described as the ligand for pleiotrophin, hyperexpressed in embryonic stem cells and glioblastoma with a potential role in migration and gliogenesis and pluripotency maintenance. An antitumoral activity of PTPRZ1 antibodies has been demonstrated.
More globally, we explored the pathways that may underlie the GSC phenotype. Among the differentially expressed proteins GO annotation and pathway analysis pointed out proteins involved in glycolysis, carbohydrate catabolic process, glucose, hexose, hexose and lipid metabolism. This can be correlated to the « Warburg phenomenon » (1920s) which consists of an increase in glycolysis that is maintained in conditions of high oxygen tension (« aerobic glycolysis ») and gives rise to enhanced lactate production (Warburg et al., 1924).
These peculiarities of cancer cell metabolism constitute an advantage for growth and/or tumor invasion and survival.

Innovative aspects
The quantitative proteomic approach of GSC provided:
- New biomarker for the stem cell phenotype
- New physiopathological insight in GSC biology
- New therapeutical targets

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S105

Quantitative Proteomic Analysis of Human Embryonic Stem Cells Using 8-plex iTRAQ Labeling

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Introduction

Gene expression analysis of human embryonic stem cells (hESCs) will help to uncover or further define signaling pathways and molecular mechanisms involved in the maintenance of self-renewal and pluripotency. We used isobaric tags for relative and absolute quantification (iTRAQ) to discover hESCs associated proteins.

Methods

The hESCs were prepared in undifferentiated state and different stages of spontaneous differentiation (the embroyid bodies of day 6, 12, and 20 after initiation of differentiation) in three biological replicates. After protein extraction and trypsin digestion, the peptides of each samples were desalted using HLB columns and labeled with eight different iTRAQ reagents (113-121) as follows: iTRAQ 113, 117 for ESC; iTRAQ114,118 for EB6; iTRAQ115,119 for EB12; and iTRAQ 116,121 for EB20. Then pooled and fractionized by SCX chromatography. Following injection to the LC-MS/MS, the relative concentrations of the peptides were deduced from the relative intensities of the reporter ions in the spectra. Each MS/MS spectrum was searched for species of *Homo sapience* against the UniProt database.

Results

With this approach, we successfully identified 168 proteins present in all six biological and experimental replicates, including different proteins involved in cell proliferation, cell apoptosis, transcription, translation, mRNA processing and protein folding. The differential expression pattern was significantly observed among several important proteins such as down-regulation of Lin-28 homolog A and Galectin which are taking part in regulating pluripotency and proliferation in ES cells, respectively, as well as up-regulation of various proteins like Ezrin, Plastin, Serpin B9, Nidogen and Cathepsin B. The further analysis is in progress.

Innovative aspects

- Using 8-plex labelling for hESCs to study different stages of spontaneous differentiation in one experiment to reduce any experimental errors.
- Identification of major transcription factors and membrane proteins as ESC associated proteins.

S106

SILAC Quantification to a Depth of 2500 Proteins from a Double Knockout GSK-3 of Mouse Embryonic Stem Cells

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Introduction
SILAC experiments provide a powerful avenue to extract more significant information from a proteomic dataset. In small scale experiments dealing with the analysis of relatively few proteins, quantification can be performed manually. However, the analysis of experiments resulting in the identification of thousands of proteins remains a significant challenge. Currently there is only one platform for large-scale quantitation independent of data file format, the Trans-Proteomic Pipeline. Here we will demonstrate quantification of a mouse embryonic stem cell dataset using PEAKS 5.0 software.

Methods
Mouse embryonic stem cells (mESCs) were generated containing knockouts in the genes controlling glycogen synthase kinase 3 alpha and beta (GSK-3αβ). mESCs were grown in a suitable medium for performing stable isotope labeling in cell culture (SILAC) experiments. Whole cell lysate extracted from labeled and unlabeled mESCs were separated by 1D gel electrophoresis as well as by MuDPiT. Trypsin- digested peptides were injected through a UPLC-QTOF MS/MS system for characterization utilizing an iterative exclusion method for 6 rounds to obtain MS data on low abundance proteins. Protein identification was performed with PEAKS 5.0 and relative protein quantification was performed by the PEAKS Q module.

Results
By combining the results from in-gel digestion and in-solution digestion followed by MuDPiT, and with six rounds of iterative exclusion on each set, we generated a total of 512, 455 MS/MS spectra. These spectra have resulted in the identification of 13,865 peptides and ~3500 validated proteins after the removal of single peptide hits. The utility of IE-MS is demonstrated once more in this dataset in significantly increasing the number of identifications of low abundance proteins.
PEAKS uses a *de novo* method for spectral identification and achieved an identification rate of 35% of MS/MS spectra in our dataset. Using the quantitation module built in to PEAKS, we quantified over 2500 proteins. PEAKS processed this large dataset of ~500 gigabytes with no difficulty. With the ability to perform quantification from raw data of very large datasets, PEAKS 5.0 increases the potential applications of SILAC, as well as other quantitative proteomics techniques. The ability to compare peptide and protein abundance levels between knockout and wild-type cell lines, even for low abundance species, will be essential for pathway reconstruction.

Novel Aspect
- A dataset of this size has not been reported to have been successfully processed in literature to date.
- Full quantitative information and comparison using available software on a

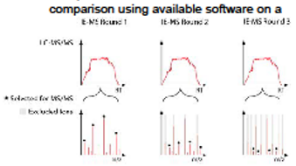

Figure 1 - Example spectra demonstrating iterative exclusion and the ability to identify low abundance peptides in complicated samples which would be missed using single injections

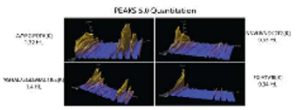

Figure 2 – Example quantitation spectra for four peptides found in SILAC labelled samples evaluated using PEAKS software

S107

Quantitative Proteomics of Leukemic Stem Cells Reveal Novel Regulators of Self-Renewal

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Introduction:
Most leukemia include a rare population of stem cells that are responsible for tumor development and recurrence after treatment. Leukemic stem cells (LSC) are maintained through self-renewal, a specialized cell division in which one or both of the daughter cells remain undifferentiated and retain the same replication potential of the parent. To date, pathways regulating the maintenance of these LSC remain poorly understood. In the present study, we used a combined proteomics and phosphoproteomics approach to characterize different LSC and identify important regulators of self-renewal.

Methods
We used retroviral transfection to create a series of *Hoxa9+ Meis1* LSC derived from purified fetal liver cells. LSC displaying significant changes in their self-renewal capabilities were selected for proteomics analyses. Cytosol and nuclear protein extracts were prepared for each LSC. To identify novel regulators of self-renewal, we performed phosphoproteome analyses following TiO2 enrichment and 2D-LC-MS analyses on an Orbitrap mass spectrometer. The same protein extracts were separated by SDS-PAGE and analyzed by LC-MS to determine protein profiles. Changes in protein abundance and phosphorylation stoichiometry (n=3) were determined using label-free quantitation and strict statistical thresholds were applied for subsequent bioinformatic network and functional analyses.

Results
We produced several *Hoxa9+Meis1 LSC* displaying similar phenotypical characteristics in terms of their homogeneity, cell death and cell cycle profile [1,2]. Two of these leukemias showed distinct differences in leukemic stem cell frequency, ranging from ~1 in 350 (FLB1) to 1 in 1.4 (FLA2). These LSC revealed no difference in morphology, cell phenotype and transcriptomic profiles. Comprehensive proteome analyses of nuclear and cytosol extracts of these LSC revealed more than 2,300 different proteins including 400 showing differential abundance. Phosphoproteome analyses of both cell populations enabled the identification of more than 11,000 unique phosphopeptides of which 1,000 were differentially regulated. Bioinformatics analyses identified significant differences in gene ontology (GO) terms for biological processes involved in chromatin silencing and chromatin modifications. For example, several proteins from the polycomb complex (PRC2) showed a different distribution between cytosol and nuclear extracts. This study also confirmed that specific phosphorylation of EZH2, Suz12 and EED from PRC2 impair the translocation of the PRC2 complex to the nucleus for LSC displaying lower self-renewal capabilities. Data will also be presented on the use of *in vitro* kinase assays combined with mass spectrometry analyses to identify kinases involved in the phosphorylation of specific substrates of the PRC2 complex.

Innovative aspects:
- First comprehensive sub-cellular phosphoproteomics study on leukemic stem cells
- Key regulators of stem cell self-renewal identified by proteomics analyses.
- Phosphorylation of the PRC2 complex impairs its nuclear translocation

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Quantitative proteomic analysis of hESCs by a novel "Rare cell proteomic reactor" S108

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Introduction

Human embryonic stem cells (hESCs) provide a novel model system to study early human development and also hold strong therapeutic promises. In this study, we developed a novel proteomic platform, termed "rare cell proteomic reactor" (RCPR), to achieve large-scale hESCs proteome profiling (more than 2,000 proteins) by using only 50,000 cells. Stable isotope labelling by amino acids in cell culture (SILAC) based quantitative proteomic approach was integrated into this platform for exploring potential proteins governing differentiation of hESCs.

Methods

Based on our previous reported proteomic reactor design (1), RCPR was developed to further simplify the protein processing and reduce the sample lose. As shown in Fig. 1, intact cells or cell lysate is loaded onto SCX monolith column by nitrogen pressure for in-column cell lysis, protein denaturing, reduction and digestion. After that, the monolith column with digested peptides is directly connected with reversed phase column for on-line two dimensional LC-MS/MS analysis.

To realize SILAC based quantitative proteomic study of hESCs, a novel chemically defined media is developed for hESCs culture. After getting enough incorporation of $^{13}C_6$-lysine, labelled hESCs are differentiated by drug treatment for quantitative proteomic analysis based on RCPR.

Results

A novel hydrophilic monolith capillary column carried with SCX group is developed for RCPR. As compared with SCX beads packed column, SCX monolith column demonstrates higher binding capacity and efficiency for cell analysis. By introducing only 510, 67 proteins can be identified. Further increasing the hESCs number to 50,200 results in more than 2,000 identified proteins using 13 salt fractions. Within the identified proteins from 50,200 hESCs, 8 hESCs markers can be found. The results obtained above demonstrate the possibility to use RCPR for large-scale hESCs proteome profiling with limited starting material.

To apply RCPR for quantitative proteomic analysis of differentiation of hESCs by drug treatment, a novel chemically defined SILAC media is developed for SILAC cell culture. As compared with recent reported dialyzed SILAC media (2), the chemically defined SILAC media is easy to prepare and has clean background. More than 95% incorporation of $^{13}C_6$-lysine is obtained within 10 culture days with clear self-renewal characteristics. Quantitative data are obtained with Q-TOF MS and discussed.

Innovative aspects

- The entire sample processing steps for proteomics, including cell lysis, protein denaturing, reduction, digestion and peptide fractionation are integrated into one single capillary column with novel monolith matrix.
- Large-scale hESCs proteome profiling is realized with only 50,000 cells.
- SILAC based quantitative proteomic analysis for hESCs is realized by developing a novel ready-to-use and chemically defined SILAC media.

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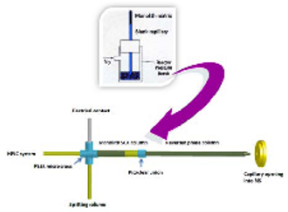

Figure 1. hESCs processing steps done using RCPR.

Proteomic analysis of human embryonic stem cells in hypoxic conditions S109

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Introduction

Human embryonic stem cells (hESCs) are pluripotent, which means they have the ability to self-renew indefinitely in culture or to differentiate into cells from any of the three embryonic germ layers. Many experiments and therapeutic applications of require the cells to be pluripotent. However, hESCs have a tendency to differentiate in culture [1]. Hypoxia promotes the sustained pluripotency of hESCs but the mechanism by which this occurs is unknown. We hypothesize that hESCs alter their protein expression in response to decreased oxygen levels, and that these changes result in pluripotency.

Methods

We used iterative exclusion (IE)[2] mass spectrometry to determine which protein factor(s) are expressed differently in hESCs after 3 time points of culture under hypoxic conditions (1% O_2). H9 hESCs were cultured under 1% O_2 or 20% O_2 in a feeder free system. After 12, 24 and 72 hours, whole cell lysate was extracted and separated by 1D gel electrophoresis. Each lane was divided into 10 fractions and the proteins were digested with trypsin. The peptides were extracted form the gel and injected through a UPLC-QTOF MS/MS system for characterization utilizing an iterative exclusion method for 6 rounds to obtain MS data on low abundance proteins. Protein identification was performed with PEAKS 5.0 and Spectrum Mill.

Results

After 48 hours, 90 proteins were expressed differently between cells cultured under hypoxia and cells cultured under normoxia, with 53 proteins upregulated in hypoxia. After 72 hours, 174 proteins were expressed differently between the oxygen conditions, with 118 proteins upregulated in hypoxia. There were differences in the expression of some proteins involved in transcription and epigenetic regulation, including TRRAP, PRMT1, PRMT5 and SPEN. There were also differences in expression of some proteins involved in translational regulation, such as ABCE1 and proteins involved in actin remodeling, such as NMT1, Arp2/3 and Coronin 1B. The identification of proteins that are expressed differently in hypoxia and therefore, proteins that may promote pluripotency could be important for the development of methods to maintain hESC pluripotency in culture without the use of animal factors.

Innovative aspects

- First comparative proteomics study of hECs under grown under hypoxic conditions
- Protein expression levels in this study do not correspond completely to mRNA levels

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Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins S110

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Although the classification of cell types often relies on the identification of cell surface proteins as differentiation markers, flow cytometry requires suitable antibodies and currently permits detection of only up to a dozen differentiation markers in a single measurement. We use multiplexed mass-spectrometric identification of several hundred N-linked glycosylation sites specifically from cell surface-exposed glycoproteins to phenotype cells without antibodies in an unbiased fashion and without a priori knowledge. Our **cell surface-capturing (CSC) technology**, which covalently labels extracellular glycan moieties on live cells, enables the detection and relative quantitative comparison of the cell surface N-glycoproteomes of T and B cells, as well as monitoring changes in the abundance of cell surface N-glycoprotein markers during T-cell activation and the controlled differentiation of embryonic stem cells into the neural lineage. A snapshot view of the cell surface N-glycoprotein will enable detection of panels of N-glycoproteins as potential differentiation markers that are currently not accessible by other means.

Innovative aspects

- Phenotyping cells without antibodies

References

Wollscheid B, Bausch-Fluck D, Henderson C, O'Brien R, Schiess R, Bibel M, Aebersold R, Watts J (2009) Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. Nature Biotechnology, 2009 April; 27(4):378-86

Delineating the Cardio-Myogenic Hierarchy during Development S111

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Introduction

Pluripotent stem cells are a promising source of cells as they can differentiate into cardiac progenitor (CP) cells [1, 2]. Although many CP stages can be generated, the hierarchal relationship is uncertain. The ability to isolate and enrich for specific CP types is also limited. To address this problem, new markers and assays to screen the cardiac potential of these cells have to be defined. The resultant CP characterisation can be integrated into a cellular interaction network, which then can further our knowledge of the system through perturbation experiments and model simulations.

Methods

Mouse embryonic stem cells are cultured in serum-free bioreactors and mesoderm differentiation is induced using Activin, BMP and Wnt. At d0, d2, d3, d3.75 of differentiation, surface proteins are isolated [3]. Briefly, surface proteins are labelled with a biotin linker. After cell lysis and tryptic digestion, the biotin linked peptides are enriched using streptavidin beads. Using enzymatic digestion, the peptides are released and analyzed using mass spectrometry. Resultant spectra are analyzed using SEQUEST and semi-quantitative data is extracted using SuperHirn. Bioinformatic analysis of expression profiles using a gene database, Panther, identified interesting candidate markers. Expression profiles of newly identified markers were validated using flow cytometry.

Results

The identified proteins covered a wide range of molecular functions, pathways and biological processes as annotated in the online Panther database. Using hierarchal clustering, protein expression profiles were clustered into five main groups: protein expression peaked at d0, d2, d3, d3.75 and an unchanged group. Each of the clustered peak proteins showed an increase in cell signaling and differentiation proteins. The unchanged cluster was significantly enriched for transport and metabolic proteins, which are essential to all cell types.

The data include large subsets of known and unknown markers. Among the known markers at d0, ciliary neurotrophic factor receptor was identified. It is known to be a co-receptor for leukemia inhibitory factor receptor and together they signal for stem cell maintenance. Platelet derived growth factor alpha and kinase insert domain receptor, markers for early cardiogenic mesoderm, have also been identified to peak at d3.75. Flow cytometry of select markers was used to validate the surface protein expression profiles as determined by mass spectrometry. Proteins were selected to include at least one known and one unknown protein for each time point.

Innovative aspects

- The highly integrated approaches herein proposed have the potential to provide fundamentally important pre-clinical insights for cardiac regenerative therapies.
- Detailed knowledge of the cardiac differentiation hierarchy will accelerate the development of protocols that efficiently generate the desired cell populations that then can be classified with a larger set of markers to ensure specificity. These sub-populations can then be systematically assayed to determine their in vivo regenerative potential.

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Phosphoproteomic Analysis of Human Embryonic Stem Cells

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Introduction

Human embryonic stem cell (hESC) fate determination, and protein phosphorylation were not well defined. We performed a multidimensional liquid chromatography-(MDLC) MS/MS based phosphoproteomic analysis of undifferentiated hESCs and their retinoic acid-differentiated derivatives for identification of protein phosphorylation sites in these cells. Pathway analyses of the phosphoproteins suggested responses of hESCs to perturbations of receptor tyrosine kinase-(RTK) driven signaling pathways, so we also treated hESC cultures with selected agonists or antagonists of these pathways.

Methods

Feeder free cultures were in conditioned medium containing 8 ng/ml bFGF. Differentiation was with 5 µM RA and no added bFGF. Cells were lysed, centrifuged, proteins precipitated with $(NH_4)_2SO_4$ and pelleted by centrifugation. Proteins were digested with trypsin and peptides desalted. Peptides were separated by SCX, phosphopeptides were enriched by IMAC and analyzed by nanoflow HPLC-ESI-MS/MS. Database searches used SEQUEST. Accepted phosphopeptides were manually verified. Ingenuity Pathway Analysis, Metacore, NCBI, Gene Ontology and peer-reviewed literature were used to identify signaling pathways. PDGF-AA and sub-threshold bFGF were used for culturing hESCs. JNK inhibitors were used and controls were included for each experiment.

Results

OCT4 staining suggested that our cells represented two populations, undifferentiated hESCs or differentiated hESC derivatives. Using XICs, phosphopeptides had higher normalized abundances in when they were identified than when they were not identified. Antibodies against phosphorylation sites identified in MDLC-MS/MS recognized expected bands on Western blots. Western signal intensities and frequency of phosphopeptide identification by MDLC-MS/MS correlated. Of 2546 phosphorylation sites, 472 were on proteins with more phosphorylation site IDs in undifferentiated hESCs; 726 were on proteins containing more site IDs in derivatives. Tyr phosphorylation was relatively prominent in undifferentiated hESCs (Fig 1). Transcription factors were the most abundant phosphoprotein category; second were kinases. Phosphoproteins grouped into 41 canonical and metabolic pathways. To test 1 pathway prediction, we decreased bFGF to 4 ng/ml and supplemented cultures with PDGF. PDGF-AA at 10 ng/ml and bFGF (4 ng/ml) maintained undifferentiated hESCs in chemically defined medium (CDM) for >15 passages (Fig 2). Our analyses also suggested JNK facilitates maintenance of undifferentiated hESCs.

Innovative aspects

- Comparative phosphoproteomics: hESCs and differentiated derivatives
- Pathway analyses using phosphoproteins as input
- Demonstration of the value of the phosphoproteomic predictions

References

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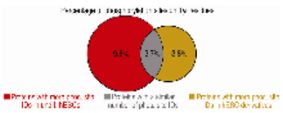

Figure 1. Percentage of non-redundant Tyr phosphorylation sites, among the sites for which the phosphorylated residue could be defined as Ser, Thr or Tyr (94% of all sites), that were on proteins containing more identified sites in undifferentiated hESCs, differentiated hESC derivatives or on proteins with a similar number of identified sites between undifferentiated and differentiated cells.

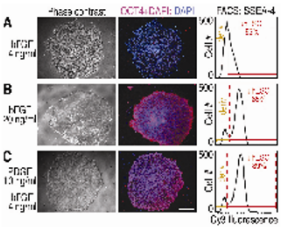

Figure 2. PDGF and sub-threshold bFGF sustained long-term culture of hESCs. Imaging conditions or FACS analyses of SSEA-4 expression is indicated above the columns, and additives that were varied are indicated beside the rows. Dotted lines delineate fluorescence intensity ~indicative of cells as undifferentiated hESCs ("uhESC"), and differentiated hESC-derivatives ("deriv"). Final culture was for 4 d in CDM lacking PDGF and containing bFGF at 4 ng/ml (A) or 20 ng/ml (B), or in the continued presence of bFGF at 4 ng/ml and PDGF-AA at 10 ng/ml (C). All photomicrographs were at the same magnification, and the scale bar (bottom center panel) represents 100 µM.

Structural Proteomics

(S201 – S214)

S201

Structural Mass Spectrometry of a Molecular Machine: ClpAP Protease Conformational Changes

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Introduction
ATP-dependent proteases are highly-regulated molecular machines that perform essential cellular functions in all organisms. These functions include involvement in processes required for cell growth, stress response mediation, and protein quality control. Understanding conformational changes that enable these proteases to manipulate substrates is important in providing an experimental basis for the design of agents to modulate their physiology. We have used synchrotron protein footprinting (SF), enzymatic assays and mass spectrometry to study conformational changes in the *E Coli* ClpAP protease.

Methods
SF probes the solvent accessibility of side chains, allowing protein interaction sites and conformational changes occurring upon complex formation to be mapped to specific areas of the protein. Hydroxyl radicals are generated by x-ray irradiation of a protein solution over milliseconds; these radicals oxidatively modify protein side chains in solvent-exposed protein regions, while areas of the protein not exposed to solvent are protected (1). Protease digestion of the sample followed by liquid-chromatography-coupled mass spectrometry and tandem mass spectrometry allows identification of affected regions and often the specific modified residue. Changes in modification rate with the addition of complex components identify interprotein interactions and conformational changes.

Results
We have investigated two aspects of ClpAP; the ATP-driven formation of the ClpA hexamer and the role of the N-terminus of the ClpP protease in substrate interaction and processing. SF experiments were performed in the absence of nucleotide, where ClpA is disassembled, and in the presence of ATP S, where it is hexameric; the results were compared to solvent-accessibility predictions based on two existing hexameric computational models and the ADP-bound monomeric crystal structure. The data suggest conformational changes upon hexamer formation requiring modification of the models and also provide structural support for a previously proposed mechanism for substrate translocation via the ClpA D2 loop (2). Surprisingly, footprinting experiments also revealed that the N-terminus of ClpP (peptide 1-12) becomes significantly more solvent-exposed upon interaction with ClpA and formation of the ClpAP complex. These footprinting studies and parallel biochemical experiments using both wild type and an N-terminal mutant of ClpP reveal the importance of the ClpP N-terminus in both substrate gating and enzymatic activity (figure 1) in the ClpAP molecular machine (3).

Innovative aspects
- Hydroxyl-radical footprinting of a 1.3MDa macromolecular complex
- Use of structural mass spectrometry to improve a computational model
- Combining footprinting, mass spectrometry and enzymatic assays to formulate a mechanistic model

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Figure 1. Model of ClpAP active site chemistry (3). The ClpP N-terminus acts as a "gate" controlling substrate access to the active sites; binding of ClpA opens this "gate," allowing substrate entry and formation of the acyl-enzyme intermediate, and closing of the N-terminal "gate" stimulates acyl-enzyme hydrolysis.

S202

Universal Integrative Approach to Study 3D-proteomics

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Introduction
Chemical crosslinking of proteins provides residue distance constraints, revealing structural and interaction information with residue resolution. Combining crosslinking data with spatial data generated by SAX or EM, it is possible to tether gross tertiary structural (volumetric) data to primary sequence. We have identified sites of close proximity in the *S. cerevisae* chromatin-remodeller Chd1 using chemical crosslinking and mass spectrometry with the aim of integrating MS data, structural prediction and SAX data to model its topology.

Methods
50 µg *S. cerevisae* Chd1 was crosslinked with BS3 (Pierce) at a ratio of 1:1000 (protein:crosslinker) and separated by PAGE. Crosslinked protein was in-gel digested with trypsin (Sigma). The extracted peptides were de-salted and SCX-separated on a combined C18-SCX StageTip column(1). The two high-salt fractions (100, 500 mM NH_4AcO) were injected on a nanoLC-coupled LTQ-Orbitrap (Thermo-Electron). Samples were separated on a 150 min gradient and FT-MS and FT-MS2 scans were acquired. Cross-linked peptides were identified and validated using in-house developed software.

Results
Crosslinking Chd1 and analysing tryptic peptides using our platform, we identified 34 crosslinks corresponding to 20 unique linked residue pairs in *S. cerevisae* Chd1. The crosslinks span very close, as well as very distant (>1000) residues in the primary sequence. We find that residues in the proximal DNA-binding domain, make contact with distal residues close to the predicted helicase domain(2). This is consistent with SAX data placing fragments containing the crossinked sites in close proximity. Consequently, our data suggests that histone-binding and helicase function is closely coordinated, over long stretches of primary sequence, and that Chd1 forms a circular structure. We are now in the process of more closely integrating the structural data obtained from SAX and MS. We show that the protein proximity data obtained by chemical crosslinking and MS can be integrated with other low-resolution technologies to create structural models with properties which could not have been inferred from either method alone. Both crosslinking/MS and SAX are high-throughput technologies, suggesting the possibility of gaining structural data on a proteomic scale.

Innovative aspects
- Identifications of protein-protein crosslinks in large proteins
- Integration of protein-proximity MS data with other structural data to model protein topology

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S203

Affinity purification of covalently-linked peptides following CNBr cleavage of proteins

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Introduction
The topology of a protein and the interactions it engages in are important determinants of its biology. Whereas the tools for the identification of proteins and the characterization of their posttranslational modifications are well-developed, gaining insights into the folds of proteins and contact sites between proteins remains a challenging undertaking.

Many studies advocate the application of chemical crosslinking combined with downstream mass spectrometry to fill this void. A critical limitation of this approach represents the observation that informative through-space covalent linkages are present in such protein samples at sub-stoichiometric levels, often masked by an overabundance of unmodified, derivatized or internally crosslinked peptides.

Methods
To overcome this limitation, we developed a generic method for the enrichment of covalently-linked peptides following chemical cleavage of a protein. The method capitalizes on the availability of homoserine lactone moieties at the C-termini of cyanogen bromide (CNBr) cleavage products which support selective conjugation of affinity tags. The availability of two C-termini within covalently-linked peptides allows for the conjugation of two distinct affinity tags and thereby enables subsequent removal of unmodified peptides by tandem affinity chromatography. Here we demonstrate successful implementation of this method using a polyhistidine and a biotin tag for the two-step enrichment of covalently-linked cyanogen bromide fragments of test peptides and proteins.

Results
Current data validate the concepts of this approach. Unlike alternative methodologies, which have been described in the literature and employ crosslinkers with built-in enrichment functionalities, this strategy is not conceptually limited in its application to a specific type of covalent linkage. It thereby avoids the unintended co-purification of derivatized or internally-linked peptides. Our data document the relative ease with which covalently linked fragments can be identified amongst a large excess of uncrosslinked or modified protein material.

Innovative aspects
- Compatible with in vivo applications
- No requirement for specialized data mining software
- Not conceptually limited to a specific type of covalent linkage

References
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S204

Probing Protein Dynamics and Higher Order Structure with a Novel ETD-FTICR

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Introduction

Given their non-ergodic nature, electron-induced dissociation methods, such as electron capture dissociation (ECD) and electron transfer dissociation (ETD) are ideally suited to supplement hydrogen/deuterium exchange (HDX) of peptides and small proteins in solution to obtain local information on conformation and dynamics. We have developed and tested a robust workflow for top-down/bottom-up HDX of a large protein on a newly developed hybrid ETD-FTICR. The desirable figures-of-merit provided by the ICR detection scheme facilitate the top-down approach by offering both selectivity (high mass resolving power) and specificity (high mass accuracy) of detected fragments.

Methods

The experimental design was based on an 18 kDa model protein, pseudo-wild type Cellular Retinoic Acid Binding Protein I (CRABP), that is structurally characterized by a robust β-barrel domain and 21 residue unstructured His-tag at the N-terminus (Figure 1). The HDX reactions were carried out by diluting the fully deuterated protein in 10 mM NH_4OAc (aq) at pH 3.0 and 25°C. The reaction was subsequently quenched at 0°C before being infused into the ESI interface. The ETD reactions were performed in a novel FT-ICR consisting of a Qh based front end with the source octopole segmented to allow injection of fluoranthene radical cations produced by an integrated nCI source. The ETD reaction occurs after Q mass isolation in the adjacent collision cell.

Results:

The ETD reaction of the intact protein yielded a multiplicity of c/z type ions consistent with those typically produced by electron-induced dissociation. Most of the observed fragmentation occurred in the His-tag region with over 1/3 of the fragments generated in the structurally ordered regions of the protein. Comparing the deuterium content of the fragment ions from both the structured and non-structured regions of the protein indicates a notable difference in the amount of labile deuterium atoms retained after the exchange reaction (Figure 2). Even after brief exchange, isotopic distributions of all fragment ions derived from the His-tag are nearly indistinguishable from those representing the HDX endpoint. Contrary to this, all fragment ions derived from the protein beyond the unstructured N-terminal segment exhibit significant retention of deuterium. These results provide conclusive evidence that proton mobility in protein ions undergoing ETD is very low and does not affect the outcome of local HDX MS measurements.

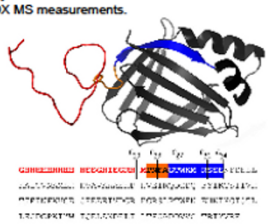

Figure 1: Tertiary structure and sequence of wt*-CRABP I showing the His-tag (red), the unstructured N-terminal segment of the wild-type protein (orange) and the first N-terminal segment forming a stable element of its secondary (strand β1, blue).

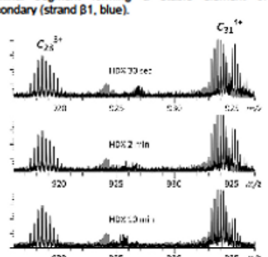

Figure 2: Evolution of isotopic distributions of c_{28}^{3+} and c_{31}^{4+} fragment ions in ETD mass spectra of *wt**-CRABP I undergoing HDX in solution (black traces). The gray traces represent the HDX end-point

Innovative aspects

- ETD successfully used as a fragmentation method for HDX protein in a model system with no detectable back-exchange
- First report of HDX/ETD MS of a protein > 10 kDa

S205

New developments in crosslinking technologies for structural proteomics.

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Introduction

Valuable structural information about proteins and protein complexes can be obtained through crosslinking followed by mass spectrometry analysis. Recently considerable efforts are being made in the bringing together crosslinking with mass spectrometry to the level of a routine method for structural proteomics. Here we present new developments in reagents design, experimental techniques and data analysis software for mass spectrometric crosslinking technologies aimed for structural analysis of proteins on an "omics" wide level.

Methods

Isotopically-coded protein modifying and crosslinking reagents were synthesized using deuterium-labeled starting materials from C/D/N Isotopes. The novel crosslinkers are distinguished by possessing special groups enabling cleavage of crosslinkers e.g. through CID and photolysis and affinity enrichment of crosslinked peptides. Proof-of-principle experiments were performed using model crosslinked peptides and proteins. Enzymatic digests of the crosslinked proteins were analyzed by MALDI LC-MS followed by tandem mass spectrometry of the detected crosslinks. Software suite for the analysis of the isotopically-coded cleavable crosslinking reagents were developed in house.

Results

New crosslinking reagents allowing affinity purification with protein-free affinity sorbents were developed and validated. A novel technique based on MALDI MS3 analysis of MS-cleavable inter-peptide crosslinks was developed allowing cleavage of crosslinked peptides in the mass spectrometer followed by sequence examination of individual peptides constituting inter-peptide crosslinks through tandem MS. Furthermore, a new technique for specific identification of the inter-peptide crosslinks was developed by a combination of crosslinking and N-terminal modification reagents employed after proteolysis of crosslinked proteins and protein complexes. . Using isotopically-coded crosslinker and modification reagents creates specific signature for the inter-peptide crosslinks, thus omitting the analysis of peptides like dead-end, intra-peptide crosslinks which are of limited value for providing structural information. The applicability towards structural proteomics of these novel crosslinkers and techniques has been shown on model systems and first biological applications.

Innovative aspects

- Novel crosslinking reagents possessing small molecules affinity purifyable groups.
- MALDI MS3 analysis of MS cleavable affinity purifyable crosslinks.
- Novel technique for selective detection of the inter-peptide crosslinks by combination of the N-terminal modification and crosslinking with isotopically-coded reagents.

S206

Mass Spectrometric Identification of Recombinant Phosphoproteins Expressed in *Escherichia Coli*

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Introduction

Protein phosphorylation regulates numerous cellular functions in organisms including eukaryotes, however, is less intensely studied in bacteria [1]. To characterize protein 3D structure, His-tag recombinant proteins expressed in *E Coli* are often used to purify sufficient amount of materials for crystallization. Mass spectrometry facilitates the structural evaluation of the target protein which allows detection of sequence errors, mistranslation, unwanted modifications, impurities and degradation products [2]. We present here the results based on a systematic examination on the phosphorylation states of over twenty recombinant proteins.

Methods

The proteins were expressed as a fusion with an N-terminal His_6-tag in *E. Coli* strains. Purification of the His_6-tagged proteins was carried out using a metal-chelate affinity chromatography (IMAC) on nickel affinity resin (Qiagen). Following reduction with dithiothreitol and alkylation with iodoacetamide, proteins were dialyzed with 10mM ammonium bicarbonate solution. Purified protein was subsequently digested with sequencing grade trypsin (1:100, w/w) or endoproteinase Lys-C at desired time intervals and incubated at 37 degrees. Protein molecular mass measurements and peptide sequence analyses were performed on ESI/MALDI QStar QqTOF (Applied Biosystems) or Ultra LTQ-FT MS (Thermo Electron) mass spectrometer. MS/MS data was routinely searched by Mascot, and all spectra were then manually validated.

Results

Recombinant protein kinase reveals strong self-phosphorylation activity for autophosphorylation. Although a complete phosphoprotein form was observed in the intact protein, however, no phosphopeptide fragments were retrieved in the MALDI MS spectrum after overnight digestion with trypsin. While using a short-time digestion (e.g. 2 hours) and endoproteinase Lys-C, we were able to identify two phosphorylated serine residues which located at lipid-binding sites of protein kinase C *eta* C2 domain, and seven tyrosine-specific phosphorylation sites at the C-terminal tail of the protein tyrosine kinase domain.
Overall analysis of twenty His_6-tagged proteins (protein serine/threonine/tyrosine kinases, protein transporters, altronate hydrolase, cystathionine beta-synthase domain proteins, tumor suppressor p53 and Mus81 protein) has shown a common character of high abundance protein adducts with mass differences of +42Da, +80Da, +178Da, +258Da, corresponding to protein modifications of acetylation, phosphorylation, and phosphogluconoylation analogs at the N-terminal His-tag [3]. Furthermore, we have also identified the *in vivo* modifications of +76Da (S-adenosylmethionine) in the recombinant protein Mus81 and +26Da increase (pyruvate) of a therapeutic protein synthesized from *E. Coli*. All exact sites were identified by tandem mass spectrometry with both CID and ECD. We can therefore conclude that the heterogeneous post-translational modifications at the N-terminal polyhistidine tag of recombinant proteins appear widespread in *E coli*, and our results also indicate that the protein phosphorylation level at the N-terminus is enhanced by nucleotide-binding domains (AMP, ADP, ATP, etc) in the protein sequences.

Innovative aspects

- Experimental strategies using simple enzymatic digestion and MS analysis for high sensitivity MS detection of phosphopeptides without the need of further separation.
- Accurate identification of new phosphorylation sites by both CID and ECD.

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S207

Creation and X-ray structural analysis of TNFR1-selective mutant TNF with antagonistic activity

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Introduction

Tumor necrosis factor (TNF) is a major inflammatory cytokine that plays a central role in host defense and inflammation via two receptor subtypes, TNFR1 and TNFR2. Currently, TNF-neutralization therapies have proven successful as a strategy for treatment of Rheumatoid arthritis (RA). However, these therapies can cause serious side effects, such as bacterial and virus infection and lymphoma development because they also inhibited the host defense function mediated by TNFR2. In this study, we generated the TNFR1-selective mutant TNF with antagonistic activity (R1antTNF).

Methods

TNFR1-selective antagonistic mutant TNFs were isolated from a phage library that displays structural human TNF variants in which six amino acid residues (amino acid positions 84-89) at the receptor binding site were replaced with other amino acids. Additionally, we used surface plasmon resonance and X-ray structural analyses to elucidate the underlying cause for the antagonist activity of this mutant. To extend the half-life of R1antTNF in plasma, we applied our site-specific PEGylation to R1antTNF. Next, the anti-inflammatory effects of R1antTNF on a murine collagen-induced arthritis (CIA) model have been investigated. We compared the effects of PEG-R1antTNF and Etanercept, which is clinically applied for TNF-blockade, on antiviral immunity using a recombinant adeno virus vector.

Results

Using phage display technique, we have succeeded in identifying TNFR1-selective mutant TNF with antagonistic activity (R1antTNF). R1antTNF neutralized the TNFR1-mediated bioactivity of wtTNF without influencing its TNFR2-mediated bioactivity. Despite the crystal structure of the R1antTNF closely resembling that of the wtTNF, the receptor-bound R1antTNF did not transmit any signal via the TNFR1. Docking simulations suggested that binding mode of R1antTNF was changed from the hydrophobic to the electrostatic interaction. This change in the interaction surface could result in a different binding mode between the wtTNF and R1antTNF. Indeed, the association and dissociation kinetic constants for R1antTNF binding to TNFR1 were considerably higher than those of the wtTNF, indicating a difference in the TNFR1-binding pattern between the wtTNF and R1antTNF (1).
Using site-specific PEGylation technology, the *in vivo* half-life of R1antTNF was improved without loss of antagonistic activity. In the CIA model, the clinical score of the mice given PEG-R1antTNF was improved equally or even more effectively than with Etanercept treatment. Next, we assessed whether PEG-R1antTNF and Etanercept had similar deleterious effects in adenoviral infection model. The data suggest that PEG-R1antTNF has less negative effects on viral clearance than Etanercept.

Innovative aspects

- Selective inhibition of TNF/TNFR1-mediated signaling could be effective in treating RA and avoid undesirable side-effect of inhibiting antiviral immunity.
- PEG-R1antTNF could serve as a novel anti-inflammatory drug.

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Analysis of PTM Changes in EGFR under Normal and Hypoxic Conditions using Extended Range Proteomic Analysis

S208

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Introduction
Successes in proteomic analysis are often dependent on the availability of samples either from cells or tissues that are prepared without inducing changes. Often, proteins from a cell line are easy to obtain and use as a model system prior to the analysis in tissue. However, cell culture at ambient 21% oxygen does not represent the predominantly hypoxic microenvironments of either normal, inflamed or cancerous tissues. In tissues, the environment often contains oxygen below 7%, and further reduced or completely abolished for tumor tissues due to the blockage of oxygen delivery in microvasculature. Thus, it is important to evaluate if a cell line could mimic cells grown in tissues.

Methods
Lysed samples from A431 cell line or mouse tissue (lung) are immunoprecipitated (IP) with anti-EGFR. The IP samples are further digested using Lys-C and subsequent for LC-MS using ERPA approach, which uses the FTMS for precursor ion scan and LTQ for CID-MS2 and MS3 fragmentation. In addition, a linear ion trap consisting of an electron transfer ionization (ETD) source will also be used to comprehensively characterize the phosphorylation and glycosylation, as described in our recent publications (1-3).

Results
To evaluate if a cell line could mimic cells grown in tissues, we used A431 cell line grown at normal (21% O_2) and different hypoxic conditions (O_2 at 7 and 1%), and compared with mouse and cancer mouse tissues. Initially, we examined the expression of EGFR and the post-translational modifications (PTMs) of EGFR. In our preliminary data, A431 cells grown at normal or hypoxic O_2 conditions, the expression of EGFR did not change, however, several phosphorylation sites of EGFR changed either with or without EGF stimulation. For example, phosphorylation at pT669 site started at 20 % occupancy at the normal O_2 level, compared to 10 % at hypoxic O_2 conditions. After EGF stimulation (treated with EGF at 50 ng / mL for 10 min), phosphorylation at pT669 increased to 45 % at normal and 20 % at hypoxic conditions. On the other hand, the phosphorylation at pY1148 started at undetectable level for normal O_2 but at 25 % for hypoxic conditions. However, after EGF stimulation, pY1148 increased to 17 % at normal but decreased to 10 % at hypoxic conditions. For site pY1173, similarly to pT669, the extent of phosphorylation was lower at the hypoxic than normal O_2 conditions. Interestingly, in a homologues position of EGFR (pT669) from mouse lung tissue, the extent of phosphorylation is similar to EGFR at hypoxic O_2 conditions. The glycosylation modifications are currently under investigation. Eventually, this important information obtained using middle down (ERPA) method, may lead to the development of a model system that more accurately mimic the tissue or even cancerous tissue environment.

Innovative aspects
A model system for cell line study could mimic tissue or cancerous tissue environment. EGFR phosphorylation in hypoxia is different from EGFR cultured at ambient 21% oxygen condition.

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Algorithms for Probing Biomolecular Surfaces using Structural Mass Spectrometry

S209

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Introduction
The potential of mass spectrometry based structural proteomics is limited because of the lack of analytical methods for the automated analysis of the generated data. Here we present FootWare, a suite of algorithms developed for the automated analysis of mass spectra obtained for studying protein structure using covalent labelling. The structures of monomeric and hexameric forms of insulin were investigated by oxidative labelling followed by mass spectrometry. The resultant data, analyzed using FootWare, was found to be in close agreement with predictions from the crystallographic structure.

Methods
Human insulin samples in monomeric and hexameric forms were exposed to synchrotron X-ray beam for intervals ranging from 0 to 20 milliseconds. Selective oxidative modifications dominate on these time scales(1). The exposed samples were subjected to tryptic digestion followed by liquid chromatographic separation coupled with electrospray ionization mass spectrometry using Thermo Scientific LTQ-FTMS instrument. FootWare was used for analyzing the resulting spectra from two independent datasets. It includes modules for data format conversion, mass spectrum interpretation, detection and verification of all peptide species using tandem mass spectra, and quantification of the extent of peptide modification. VADAR was used to calculate solvent accessibility of individual residues of the protein.

Results
Insulin, a metabolism-regulating hormone, exists as a monomer, dimer, or a hexamer depending upon the solution conditions. FootWare was able to detect oxidized and unoxidized forms of four tryptic peptides of insulin: 1-10, 11-22 and 23-29 segments of B-chain, and 1-21 segment of A-chain, providing 99% sequence coverage. Oxidative rate constant was calculated for each of the modified peptides, which is a measure of the Solvent Accessibility (SA) and reactivity of the peptide residues. The rate constants for hexameric form dropped by 1.9x, 3.7x, and 1.7x for 1-10, 11-22, and 23-29 segments of the B-chain from its monomeric counterpart (Fig 1). This is consistent with the crystallography data, which revealed a decrease in the SA of F1 in 1-10, Y16 and L17 in 11-22, and F24, Y26 and P28 in 23-29 segment of B-chain by 5-30 folds upon hexamer formation. Only L13 in the segment 1-21 of the insulin A-chain showed a drop in SA, minimal change (1.3x) was observed in the oxidation rate of this peptide. Also, SA of F24 (B-chain) drops to 0 Å, and its oxidized form was not observed in the hexamer. The drop in oxidation rates indicates substantial structural reorganization during the hexamer formation.

Innovative aspects
- Converted manually intensive data analysis to a high throughput, automated process
- Characterized human insulin structure using FootWare
- FootWare output found to be consistent with crystallography data

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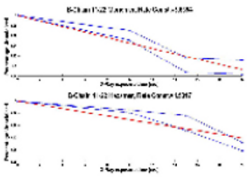

Figure 1: FootWare generated dose response plots for peptide 11-22 (B-chain) for monomeric and hexameric form from two independent experimental runs (blue) fitted to theoretical curve (red). Note that the rate constant drops from 5.6 to 1.5 indicating the reduced solvent accessibility of the peptide during the transition from monomeric to hexameric form.

Structural Determinants of SH2 Domain Specificity

S210

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Introduction
The Src homology 2 (SH2) domain specifically recognizes phosphotyrosine (pY)-containing sequences of ligand proteins, and is involved in intracellular signal transduction pathways. Our peptide-array approaches combined with literature data have determined SH2 recognition motifs for 85 SH2 domains out of 120 human SH2 domains. While most of them show specificity at the P+3 (third residue from pY) position, BRDG1 SH2 possesses unusual specificity at P+4 (Figure 1A). Here we present crystal structure of BRDG1 SH2-ligand complex, with implication to understanding specificity of other SH2 domains.

Methods
The BRDG1 SH2 domain was purified to homogeneity. We have identified that BRDG1 SH2 recognizes pY-136 of a membrane protein NTAL in vivo. The NTAL ligand peptide ANSpYENVLIAK was synthesized and purified. BRDG1 was co-crystallized with NTAL and X-ray diffraction data were collected. The structure was determined at 1.9 Å resolution. For peptide-binding studies, fluorescein-labelled peptides were prepared and dissociation constants were derived from fluorescence polarization assays.

Results
The crystal structure unambiguously determined NTAL phosphopeptide bound on the surface of the BRDG1 SH2 domain. The SH2 surface provides an unusual hydrophobic pocket at the ligand P+4 position. Instead, the conventional P+3 pocket region is blocked by its EF loop residue (L240) to disable regular P+3 ligand recognition (Figure 1B). Elimination of this residue (L240A mutation) dramatically changed its specificity from P+4 Leu/Ile to P+3 Trp/Phe. Structure comparison with other SH2 domains revealed that residue arrangement equivalent to the BRDG1 P+4 pocket is conserved among most SH2 domains; however this P+4 pocket architecture is permanently blocked by their own BG loop (Figure 1). In other words, the P+4 pocket of BRDG1 SH2 is unveiled to solvent due to its unusually short BG loop, which cannot cover the pocket. Our preliminary analysis on all available SH2 structures suggests both EF and BG loops are highly variable, yet could be clustered into groups. More detailed investigation on analyzing correlation between loop classification and specificity motifs is in progress.

Innovative aspects
- A novel SH2-ligand complex structure revealed unusual P+4 binding pocket.
- The P+4 pocket is conserved among SH2 domains but permanently covered by their BG loop.
- Loops significantly contribute to defining ligand specificity of SH2 domains.

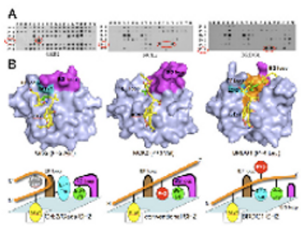

Figure 1. Structural basis of SH2 specificity differences. (A) Peptide array library screening for Grb2 (P+2 Asn), NCK2 (P+3 Val/Pro) and BRDG1 (P+4 Leu/Ile) SH2 domains. (B) SH2-ligand complex structures of these SH2 domains (upper panel) and their cartoon description (lower panel). Peptide binding modes are driven by EF and BG loop structures.

Isotopically coded N-terminal modification for identification of inter-peptide crosslinks

S211

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Introduction [565]
Crosslinking combined with mass spectrometry has great potential for determining three-dimensional structures of protein and protein assemblies. A main challenge is the detection of inter-peptide crosslinks from a crosslinked and digested protein complex. We use N-terminal modification (NTM) combined with isotopically coded crosslinker (ICCL) for specific detection of inter-peptide crosslinks. By combining ICCL with NTM, and using our in-house software, we have developed a method for the rapid generation of a small list of inter-peptide crosslink candidates.

Methods [773]
Human Immunodeficiency Virus Reverse Transcriptase (HIVRT) was crosslinked with ICCL Bis[sulfosuccinimidyl] suberate (BS^3-H12/D12) then digested with trypsin. Half of the reaction (ICCL) was kept as is, while the other half (ICCL + NTM) was mixed with an equimolar mixture of in-house synthesized istopically coded N-terminal modifying reagent Pyridine Carboxylic Acid Succinimide (PCAS-H4/D4). Treatment with 1.2M ammonium hydroxide (NH_4OH) was used to reverse the modification at serine, threonine, and tyrosine residues. Samples were separated by HPLC. MS spectra of fractions were analyzed by visual scanning of spectra and by use of in-house software for detection of "isotopic signatures". MSMS analysis was used to confirm assignment of interpeptide crosslinks.

Results [1079]
Inter-peptide crosslinks contain doublets of "tridents" of peaks with 1:2:1 intensity ratios. C-terminal lysines also react with PCAS-H4/D4, resulting in interpeptide crosslinks containing three or four PCAS-H4/D4 modifications. Inter-peptide crosslinks with three or four PCAS-H4/D4 modifications exhibit a 1:3:3:1 or 1:4:6:4:1 isotopic signature based on binomial distribution, correspondently. In-house software was developed which compares ICCL and ICCL + NTM HPLC fractions and creates a small list of inter-peptide candidates. Such candidates have two peaks separated by 12Da on the ICCL fraction and a corresponding peak set on the ICCL + NTM fraction. With HIVRT and BS^3-H12/D12, our final list of candidates created by the software contained seven peaks of interest. One was an intra-peptide crosslink, two were dead end crosslinks, and four were inter-peptide crosslinks. This method allows us to focus on analysis of inter-peptide crosslinks, excluding most of the cumbersome free peptides, dead-end crosslinks, and intra-peptide crosslinks from the analysis.

Innovative aspects
- N-terminal modification with isotopically coded reagents used for specific identification of inter-peptide crosslinks.

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S212

TGF-beta vs. BMP4: The Basis for Antagonistic Smad Signaling in Early Development

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Often, highly homologous transcription factors bind to short and indistinguishable DNA motifs yet regulate distinct downstream effects.

Currently, we try to resolve this conflicting notion for the Smad (homolog of Mothers Against Decapentaplegic (MAD) of *Drosophila*) family of transcription factors. Regulatory Smads 2, 3 (R-Smads) are regulated by the Transforming Growth Factor-β (TGF-β) pathway and Smads 1,5,8/9 are regulated by the Bone Morphogenetic Protein (BMP) pathway. These proteins are responsible for very different functions *in vivo* (eg.Smad2 TGF-β signaling endoderm differentiation and BMP4 Smad1 signaling mouse embryonic stem cell pluripotency) though the sequences of the DNA binding domain (MH1) of these proteins are very similar and DNA contacting amino acids are invariant. To understand how TGF-β and BMP4 Smads exert their antagonistic functions, the DNA binding domain of Smad1 was co-crystallized with a palindromic Smad Binding Element (SBE). The structure was solved using crystals diffracting to 2.8A° resolution. I will present a detailed structural, biophysical and biochemical comparison of TGF-beta, BMP4 and Co-Smads.

S213

Top-down Analysis of Native and Crosslinked Proteins to Locate Modifications and Investigate 3-Dimensional Structures

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In biological systems, most proteins undergo one or more forms or post-translational modification; these changes strongly affect protein localization, interactions and turnover. In addition, multiple sequence variants can occur, and proteins associate into functional complexes. Proteolytic digestion of protein mixtures risks the loss of valuable information, since critical details regarding the extent and interrelationships of these important features is lost. We are pursuing top-down and crosslinking as methods to extract the maximum information from intact proteins that are identified as significant in proteomic investigations and are applying new strategies and software tools for this purpose. Top-down sequencing of native and crosslinked proteins has been carried out online and offline, on LTQ-Orbitrap, FT, Q-o-TOF and ion trap MS systems and multistage MS^n, including in-source fragmentation, CID, IRMPD, ECD and ETD. The spectra have been interpreted using the Boston University Protein IDentifier – Top Down (BUPID-TD), and publicly available programs.

For the crosslinking experiments, we employed BS^3, a homobifunctional crosslinker that contains an amine-reactive *N*-hydroxysulfosuccinimide (NHS) ester at each end of an 11.4-A spacer arm. Conditions for the crosslinking reaction were carefully optimized to minimize the number of crosslinks, to focus on the most significant through-space interactions. Proteins of particular interest to our investigations of oxidative stress in cardiovascular disease, *e.g.*, hemoglobin, and/or systemic amyloidoses, *e.g.*, transthyretin, were chosen as the central objects of the research; other well-characterized proteins were used for method development and evaluation of results. Our results are consistent with published X-ray crystallographic data. When fully developed, the method should be applicable to novel proteins and sequence or post-translationally modified forms of known proteins, which occur in very small amounts and have no X-ray data.

Acknowledgements:

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S214

Mass Spectrometry and its Role in Structural Biology

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Standard proteomics techniques are unable to describe the stoichiometry, subunit interactions and organisation of assemblies since many are heterogeneous, present at low cellular abundance and frequently difficult to isolate. Using methods designed to maintain non-covalent complexes within the mass spectrometer we show how we can provide definitive evidence of interacting subunits based on the masses of complexes and subcomplexes generated by perturbation both in solution and gas phases. Structural models will be presented for a number of oligomeric protein complexes including the human U1snRNP and eIF3 complexes.

Developments in mass spectrometry have added a further dimension to the study of protein complexes. Using ion mobility mass spectrometry we have been able to add spatial restraints to our models with measurements of collision cross–sections.

Very recently we have had a considerable breakthrough which has enabled us to preserve intact membrane complexes in the gas phase . This enables us to establish lipid and nucleotide binding and to define the stoichiometry and post translational modifications within the intact transmembrane regions of a number of complexes.

Study Design and Standards for Clinical Proteomics

(S301 – S311)

S301

Deficient Triosephosphate Isomerase(TPI) Affect Micturition Dysfunction from Neurogenic Bladder

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Introduction
Neurogenic bladder refers to abnormal bladder function that is due to damage to the bladder nerve supply. It can arise in various conditions including spinal dysraphisms, spinal cord injury, transverse myelitis, cerebral palsy etc. TPI deficiency in humans causes neurological impairment and significant reductions in ATP levels(1). To reveal molecular factors associated with neurogenic bladder dysfunction, we compared the difference between neurogenic bladders(NB) and control bladders(CB) in VUR patients.

Methods
The data of 5 patients with neurogenic bladder (M:F=3:2) with mean age of 9 years 3 months (range: 4y 3m - 15y 3m) and 7 patients with VUR serving as controls (M:F=4:3) with mean age of 10 months (range: 9m - 2y 1m) were compared. Protein spots separated by 2D gel electrophoresis were matched to analyze differentially expressed spots using PDQuest software and Q-TOF MS/MS approach. Specific spots were investigated through Western blot and immunohistochemistry such as Masson's trichrome and TUNEL staining.

Results
Thirty six spots from 2D gel electrophoresis were differently expressed between NB and CB. Sixteen spots were overexpressed and 20 spots were underexpressed in NB. Differentially expressed protein spots were identified through Q-TOF MS/MS and database searching. In Q-TOF results, several protein spots which are associated with defective nerve system and bladder function were selected. Triose-phosphate isomerase(TPI) and smooth muscle(SM) protein spots in NB were relatively underexpressed . In western blotting, TPI and smooth muscle protein levels were significantly decreased in NB compared to CB (Figure 1). The density of stained smooth muscle in neurogenic bladder was significantly lower than control. The number of apoptotic cells was significantly greater in the smooth muscle layer of neurogenic bladder compared to control (Figure 2).

Innovative aspects
- This work was the first investigating of molecular factors associated with neurogenic bladder dysfunction by proteomics study.
- Deficient TPI causes significant reductions in ATP levels.
- Deficient TPI probably induces apoptosis of myocytes in the smooth muscle layer of the neurongenic bladder, potentially affecting micturition dysfuctions in neurongenic bladder.

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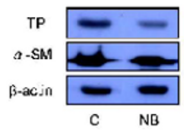

C (control bladder)
NB (neurogenic bladder)

Figure 1. In western blotting to confirm the protein level, TPI and α-SM were significantly decreased in NB compared to CB.

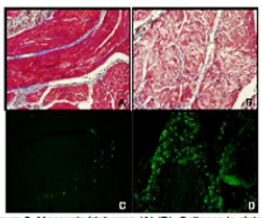

Figure 2. Masson's trichrome (A),(B) Collagen is stained in blue and muscle in red, Reduced from x400. TUNEL (C), (D) apoptosis cells are stained green, Reduced from X200.

S302

Proteomics of Cerebellar Floccules during Vestibular Compensation of A Rat Vertigo Model

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Introduction
Unilateral labyrinthectomized (UL) rats are used as a human vertigo model. After UL, imbalance of signals from inner ear brings a statical equilibrium disorder of vestibulooccular and vestibulospinal system, and then results in spontaneous nystagmus and disequilibrium. However, these symptoms are gradually improved within approximately 48 hr. The improvement of these symptoms is called vestibular compensation (VC). Several reports indicate that VC is accomplished with plasticity of central nervous system and that cerebellar floccules (CFs) are involved in VC. However, molecular mechanisms of VC are still not clear. Aim of this work is elucidation of the molecular mechanisms of VC and surveillance for proteins that would be a therapeutic target for vertigo.

Methods
Ipsilateral and contralateral CFs from UL- and sham-operated rats at the acute VC stage (48 hrs after UL) and the chronic stage (one week) were removed and proteins were extracted. The protein samples were separately labeled with Cy5 fluorescent dye. As a standard for a quantitative assay, equal amounts from each of the protein samples were mixed and labeled with Cy3. Then, each of the Cy5-labeled samples together with the Cy3-labeled standard for comparison was separated with 2D-DIGE. Intensities of the detected protein spots from UL- and sham-operated rats were normalized by the intensities of the standard. Further, the normalized intensitieis from the UL rats were compared to those from sham-opearted rats. As for protein identification, 2-DE gel fragments, which corresponded to protein spots of interest, were recovered. The protein in the gel pieces were digested with trypsin, and the digested peptides were extracted. Masses of the digested peptides were determined using a MALDI TOF/TOF MS.

Results
To clarify molecular alterations during VC, we compared proteins extracted from CFs at 48 hr and one week after UL with those after sham operation. Totally, 967 protein spots were detected on 2D-DIGE. Then, protein spots whose intensities were not less than 1.3 folds higher or not more than 1/1.3 fold lower in UL rats than in sham operated rats were selected. Thereby, 99 protein spots changed in the acute (48 hr) and chronic (one week) stages of VC. Detailed analysis of changes of the 99 protein spots revealed that temporal changes of proteins in contralateral side of CFs would be imporant in the acute stage, whereas proteins of both sides would be involved in VC of the chronic stage.
Next, among the 99 protein spots, we selected 32 spots containing enough amounts of protein for identification and successfully identified 11 proteins out of 13 protein spots. They are vesicle-fusing ATPase, mitochondrial aconitate hydratase, brain glycogen phosphorylase, heat shock congnate 71 kDa protein, lamin-B1, alpha-internexin, heterogeneous nuclear ribonucleoprotein K, protein disulfideisomerase A3, mitochondrial ATP synthase subunit beta, mitochondrial ATP synthase subunit b. These proteins should be investigated from the aspect of VC.

Innovation aspects
- Significant involvment of the contralateral side of cerebellar floccules in acute stage of vestibular compensation
- Significant involvment of the both sides of cerebellar floccules in chronic stage of vestibular compensation

S303

Thermal inactivation prior to 2DGE stabilizes brain proteome

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Abstract content
Protein degradation post-sampling in biological samples causes large problems in proteomic analysis. Rapid heat inactivation of intrinsic enzymatic activity has been evaluated using murine brain and 2DGE. It is found to be fully compatible as well as limiting the post-sampling degradation and improve results.

Introduction
The change in proteomes post-sampling caused by degradation may obscure important information of a sample's biological state and prevent discovery of biomarkers. In 2DGE, chemical inhibitors are routinely added to urea-based buffer used for protein extraction to ensure minimal protein degradation. An alternative is to use rapid heat inactivation to eliminate enzymatic activity. In this work heat inactivation has been evaluated and shown to avoid degradation and improve results by reducing proteolytic fragment spots.

Methods
Mice brains (n=3) were extracted after cervical dislocation, dissected into halves, and were either immediately frozen or stabilized in the heat inactivation system. Proteins were extracted in 7M urea, 2M thiourea, 4% CHAPS, and 5 mM TBP. Insoluble material was pelleted by centrifugation. Proteins were alkylated two hours with 10 mM acrylamide monomer and the alkylation reaction quenched by the addition of 50 mM DTT. IPG strips pH 3-10 were hydrated with 0.1 mg protein followed by IEF for 100 kVh. Second dimension gels were 8-16% polyacrylamide gradients. Digital gel images were captured and image analysis performed.

Results
The use of heat denaturation for avoiding post-sampling degradation in tissue samples has been evaluated. Protein extraction from heat stabilized tissue was done at comparative levels to non-stabilized tissue. Comparative image analysis of 2D gels were done and a PCA analysis was performed for the significantly altered proteins from the three treatments ($p<0.05$). Gels from stabilized and non-treated sample show two well separated clusters where the stabilized gels cluster closer together. Significantly altered spots separate into three main groups according to their protein expression profiles. A clear correlation between group and gel position can be seen. Proteins that increase in intensity after heat treatment are mainly found in the upper parts of the gel while proteins that increase in non-treated samples are mainly found in the lower part of the gel.
Identifications of representative spots from each groups reveals that proteins that increase in non-treated spots are mainly protein fragments that appear much lower in the gel then theoretically predicted. Increased spots in stabilized samples are mainly intact proteins found near or above their theoretical gel position.

Innovative aspects
- The problem of *post-mortem* degradation for proteomic analysis can be minimized through the usage of the rapid heat inactivation technology
- Rapid heat inactivation of samples following sampling eliminates post sampling protein degradation permanently from the moment of treatment and thus reduces the degradation associated problems drastically.

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S304

Identification and characterization of major ABO blood group-carrying proteins in human kidney

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Introduction
ABO(H) blood group antigens are linked to lipids and proteins. While glycolipids carrying ABO antigens have well characterized in the human kidney, glycoproteins carrying ABO antigens are largely unknown and their molecular properties remain to be elucidated.

Methods
All the ABO-carrying proteins were partially purified by HPA lectin affinity chromatography, and further purified by immunoprecipitation with anti-A antibody. The purified proteins were separated on a SDS-PAGE gel, and cut into slices, which were subjected to in-gel N-glycosidase F digestion followed by in-gel trypsin digestion. These tryptic digests were analyzed by LC-MS/MS to identify proteins and N-glycosylation sites. Protein candidates were further confirmed by immunoprecipitation and double-labeled immunofluorescence microscopy.

Results
All the glycoprotein linked to ABO antigens were Asn-linked glycoproteins, and presented as multiple bands with molecular masses in the range of 60-270 kDa. Of 147 proteins identified by LC-MS/MS, 17 proteins were selected as most probable candidates on the basis of 3 criteria: membrane proteins as annotated by Gene Ontology, existence of N-glycosylation sites as identified in this study and confirmed by annotations of Swiss-Prot with experimental evidence. Among them, most abundantly expressed proteins as estimated by spectral counts were further validated by immunoprecipitation and double-labeled immunofluorescence microscopy by using either anti-A or B antibody and antibodies specific to the candidate proteins. We successfully identified most of ABO-carrying proteins and their localization. These proteins include PECAM1, PLVAP, von Willebrand factor, NCAM1, CD34, and integrin alpha 3, which exhibited distinct feature in localization to most of vascular endothelial cells, distal convoluted tubules, and collecting ducts.

Innovative aspects
- All the ABO-antigen proteins in the human kidney are N-linked glycoproteins, and most of the proteins are identified with their N-glycosylation sites.
- The ABO-antigen proteins exhibit distinct features in localization to vascular endothelial cells, distal convoluted tubules and collecting ducts.
- These results provide basic knowledge for further understanding their physiological and pathophysiological roles, especially in ABO-incompatible kidney transplantation.

Reference
M Tasaki et al, Identification and characterization of major proteins carrying ABO blood group antigens in the human kidney. Transplantation, 2009; 87, 1125-1133.

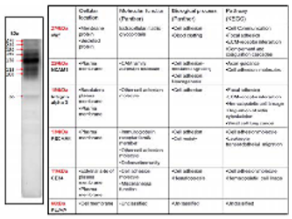

Fig. 1 Western blotting analysis of blood type A-carrying proteins of normal human kidney (Left) and proteins identified in this study.

S305

Understanding cross-lab reproducibility for SRM-based mass spectrometry clinical assays

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Introduction
Targeted SRM assays for protein biomarkers are increasingly being applied in the areas of disease prognostics and therapeutic drug monitoring because they are typically robust and selective, even in complex matrices (1). However, in order to develop effective assays for diseases or pathologies with multiple intersecting metabolic and/or signaling pathways, multiplexed assays (monitoring panels of peptides) will have to be developed. Unfortunately, designing effective SRM assays still remains a challenge, because the choice of peptides/transitions and the optimization of sensitivity, selectivity and throughput is typically slow, labor intensive and costly. More important, it is currently difficult to transfer assays between labs while retaining a high degree of inter-lab reproducibility. In this study, we used a panel of 64 synthetic peptides to standardize LC and SRM assays across four different laboratories demonstrating that inter laboratory peptide quantification can be highly reproducible.

Methods
SRM assays were developed on a TSQ Quantum Ultra triple quadrupole mass spectrometer, Surveyor MS pump, Micro Autosampler and an IonMax Source equipped with a low flow metal needle (ThermoFisher Scientific). Reverse phase separations were carried out on a 1mm X 50mm Hypersil Gold 1.9µm C18 particle. Solvent A was LC-MS grade water with 0.2% (v/v) formic acid, and solvent B was LC-MS grade 30% (v/v) acetonitrile with 0.2% (v/v) formic acid (Optima grade reagents, Fisher Scientific). Pinpoint software (ThermoFisher Scintific) was used for developing SRM assays. The software algorithm predicts candidate peptides and facilitates the choice of multiple fragment ions for SRM assay design, instrument method development, automatic peptide identity confirmation and quantitative data processing.

Results
A variety of SRM assays were monitored across four different laboratories with identical LC/MS-triple-quadrupole platforms. Initially, data were collected from single peptide infusions with analysis of MS and MS/MS under various conditions. Next, a mixture of 64 synthetic peptides was analyzed in order to understand and optimize numerous parameters that affect LC-MS SRM of a moderately complex but defined sample. The 64 peptide mixture was initially optimized in one laboratory and later the method was transferred to the other three locations. In a final series of experiments, the 64 peptide mixture was analyzed by all four laboratories in a background of digested plasma providing level of detection (LOD) and level of quantification (LOQ) information in a complex matrix for all 64 peptides.

Figure 1: Figure showing the total ion chromatogram with all 64 synthetic peptides being analyzed in a single HPLC run on the triple quadrupole.

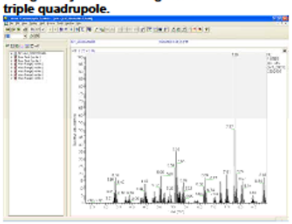
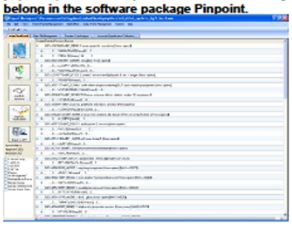


Figure 2: Figure showing the list of 64 peptides, and the proteins to which they belong in the software package Pinpoint.



Innovative aspects
- Achieving cross-lab reproducibility through properly controlled LCMS setup, sample preparation and sample analysis protocols.

S306

Fixing Proteomics Initiative: A Global Quest for Reproducibility

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Introduction
Researchers have long experienced a love-hate relationship with two-dimensional electrophoresis (2DE), arising from the ability to develop reproducible data. This in turn lead to the creation of a global initiative for the development of standardized protocols to validate the performance and reproducibility of 2-D processes, 2DE test standards, and kits, and establishment of global education (wet lab training) programs. Critically important is the reproducibility of the experimental data. Hence the quest for this reproducibility, run by the "Fixing Proteomics Campaign."

Methods
Samples of total protein extracts from HeLa cells were provided to all participants in the study. These extracts were then subjected to 2DE analysis in each of the 15-20 participating labs. This involves focusing the proteins on 24cm, pH 4-7 IPG strips, followed by 2nd dimension separation using 12% SDS-PAGE. The proteins were detected using visible or fluorescent stains followed by image acquisition. Image analysis of the protein spots was performed using Progenesis SameSpots v3.3 (Nonlinear Dynamics). The images were then compared to a "Gold Standard" image to determine the reproducibility of methodology between the 19 2DE labs around the globe.

Results and Deliverables;
Cross-lab reproducibility has been successfully demonstrated by the 2D gel Reproducibility Study in Phase I and data was presented at HUPO 2008. Continuing on this effort the Phase 2 initiative has enrolled 15-20 participating labs to analyze the HeLa Cell lysate as the reference sample using a standard protocol. Data generated by the participating labs will be compared to the "Gold Standard" image as exhibited in Figure 1. Results will be presented comparing (a) all images vs. gold standard as they were submitted (b) all images vs. gold standard after corrective changes following recommendations. Successful results will demonstrate that the 2-D gel image analysis from the participating labs can match the standard image after corrective actions using two measures (1) clustering within 95% confidence bounds using a PCA plot and (2) p-values <0.05 using a Chi^2 Goodness-of- Fit test. This initiative will highlight the value of standardization, quality control and generating data that is meaningful for publication. All the raw data plus methods from this study will be made publicly available for the community after publication of a paper.

Innovative aspects
- Global reproducibility study involving 15-20 labs (Phase II).
- Ability to further advances in proteomics by enhancing the reproducibility and credibility of scientific techniques.
- Study facilitates the development of global standards, kits and global education.

Figure 1: Fixing Proteomics Comparative Analysis

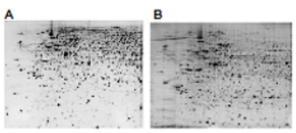

Fig.1. Gold standard gel image (A) compared to an image from participant laboratory 1 (B). This study participant followed the standard protocol and generated a 2-D gel image that is comparable to the gold standard.

S307

The proteomic profile of endometriosis – A comparative study between endometrium and serum

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Introduction
Endometriosis is a highly heterogeneous condition, affects 10-15% of women of reproductive age and can be associated with severe, debilitating pain and infertility (Ulukus et al., 2006). Despite intense research, pathogenesis remains elusive. Proteomics is a useful tool for analyzing complex tissue structures such as the endometrium. This study aims to investigate the proteome profile of controls vs. endometriosis. Observing consistent differences could assist in understanding endometriosis initiation and development into peritoneal, ovarian and deep-infiltrating endometriosis.

Methods
Proteins were extracted from endometrium and serum samples collected from women who underwent laparoscopy to diagnose or exclude endometriosis (peritoneal, ovarian and deep-infiltrating lesions). Protein extracts were then separated first by isoelectric focusing (IEF) on immobilized pH gradient (IPG) strips (pH 4-7, 11cm), then the proteins' molecular weight (M_r) on SDS-PAGE gels. Expressed proteins were visualized by staining overnight using colloidal Coomassie Blue.
Difference-in gel electrophoresis (DIGE) is also being performed on endometrial tissue and serum samples by fluorescently labeling the protein extract prior to 2D separation. 2D gel images will be analysed to detect differential protein expression using the Phoretix™ software and characterized via MALDI-TOF MS.

Results
Promising preliminary results from classic 2D gels indicate many proteins are aberrantly expressed, comparing the eutopic endometrium and serum from women with and without endometriosis (Fig 1 and 2). Prior pilot functional analysis from our group (ten Have et al., 2007) has illustrated molecular signaling, cell structure, adhesion, proliferation, immune, angiogenesis, neurogenesis and others to be disturbed in women with endometriosis. Possibly indicating a number of coordinated molecular pathways to be functionally disrupted leading to endometriosis initiation and development into its highly heterogeneous presentations on laparoscopy. Further testing and identification via DIGE and MALDI-TOF MS is currently underway.

With the advent of proteomics allowing analysis of complex tissue structures, like the endometrium, protein analysis of gene translation end-products will further our understanding of the complex molecular pathways interacting to form the foundation of dysfunctional mechanisms of cells and tissues in the pathogenesis of endometriosis. This provides further evidence that endometriosis may primarily be an endometrial disease (Al-Jefout et al., 2009).

Innovative aspects
- Compares the many different presentations of endometriosis (peritoneal, ovarian and deep-infiltrating lesions), strict criteria for sample inclusion utilizing DIGE study design.
- Develops further understanding of how endometriosis initiates and develops into multiple presentations.
- Furthers evidence that endometriosis may be an endometrial disease.

References
(1) M Ulukus et al. The role of the endometrium in endometriosis 13(7):467-76; J Soc Gynecol Investig. 2006.
(2) S Ten Have et al. Proteomic analysis of protein expression in the eutopic endometrium of women with endometriosis 1(10):1243-51; Proteomics Clin Appl. 2007.
(3) M Al-Jefout et al. The microanatomy and function of the eutopic endometrium in women with endometriosis 4(1):61-79; Expert Rev Obstet Gynecol. 2009.

S308

Proteomic analysis of Small Intestinal Bacterial Overgrowth

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Title: Proteomic analysis of Small Intestinal Bacterial Overgrowth

Introduction
The human small intestine (SI) is continuously exposed to microbial challenges. Paneth cells Defensins (HD5 and HD6) expressed in the SI crypts and secreted upon infectious stimulus. Both defensins are potent antibiotics and play critical role in host defense against microbial colonization/infection of the SI. Downregulation of defensins is hypothesized to predispose towards SI (bacterial) colonization manifested in Small Intestinal Bacterial Overgrowth (SIBO) and lower HD5 mRNA has been correlated with Inflammatory Bowel Disorder (IBD). However the possible differences in SI defensin(s) content at proteomic level have not been well explored.

Methods Small intestinal biopsy samples (tissues and luminal aspirates) from patients diagnosed for SIBO using the Hydrogen Breath Test and control (healthy individuals; breath test negative) analyzed for total antimicrobial potential and relative/quantitative expression of innate immune determinants including intestinal defensins.

Results Microscopic analysis of paneth cells in SIBO and control groups did not show any significant differences in terms of numbers or stored cationic granules. Mass analysis clearly identified trypsin digested mature HD5 signature in all sample sets. Quantitative proteomics on IBD and healthy SI samples showed no significant difference in the tissue (storage) levels of HD5. However, there was ~3 fold difference in luminal (secreted) levels of HD5 in SIBO vs controls. This indicates possible post-translational impairment of HD5 secretion (and not storage content) may be a determinant for predisposition to SIBO.

Innovative aspects First analysis of human intestinal innate immune determinants in SIBO.

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S309

Building a Robust and Reliable Proteomics Biomarker Pipeline: NCI's CPTAC Network

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Introduction Better biomarkers are urgently needed to improve diagnosis, guide molecularly targeted therapy, and monitor activity and therapeutic response across a wide spectrum of disease. Proteomics methods hold special promise for the discovery of novel biomarkers that might form the foundation for new clinical blood tests, but to date their contribution to the diagnostic armamentarium has been largely unsuccessful. While the scientific literature annually reports many biomarkers, few prove sufficiently resilient, reproducible, and real to translate into clinically useful assays. Advances in methods and technology now enable conceptualization and evaluation of a comprehensive biomarker pipeline from the following essential process components: candidate discovery in tissues or proximal fluids, prioritization of candidates using targeted screening approaches, verification of candidates by quantitative assays in patient (100s) samples, biomarker validation and clinical assay development in large population studies, and commercialization.
Method The primary goal of NCI's Clinical Proteomics Technology Assessment for Cancer (CPTAC) is to establish an integrated pipeline that will analyze cancer-relevant proteomic changes in human clinical samples. To do this, CPTAC has focused on bridging the gap between candidate discovery and clinical validation. The first step toward completing this bridge is to develop technology platforms that reliably detect changes in the human proteome. These technologies must be reproducible both within a lab and across labs. CPTAC has explored mass spectrometry and affinity-based platforms and developed reference materials and metrics to evaluate instrument performance. These materials and metrics are transferable across proteomic technologies, thus enhancing multiple components of the biomarker pipeline. The second step is to employ these technologies in the evaluation of each platform's ability to detect cancer-relevant changes. This larger step involves analyte (biomarker candidate) prioritization, clinical sample collection, assay development, and assay evaluation.
Results CPTAC has recently completed the first phase of technology evaluation and standardization. Multiple inter-laboratory studies have been able to demonstrate that specificquantitative proteomic assays are reproducible between labs. CPTAC is implementing this overall strategy to verify biomarker candidates for their ability to discriminate breast cancer prior to biopsy. Such a pipeline will either provide credentialed biomarker candidates for a large clinical validation study or provide an efficient means of attrition indicating that an analyte cannot be effectively verified using current technology. If successful, implementation of this pipeline will streamline the biomarker development process, shorten the timeline, and improve the success rate of clinical validation for biomarkers of any disease type.

S310

AMT tag Strategy for Bladder Cancer Biomarker Candidates Discovery in Urine

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Introduction
Advanced proteome profiling methodologies, provide quantitative information on hundreds of proteins in each analysis. This analytical power can be harnessed to discover biomarker candidates in clinical studies.
Urine is available using non-invasive collection procedures and contains a great variety of proteins. For bladder cancer, being directly in contact with the diseased organ, urine can act as a local sink for proteins or peptides secreted, shed or leaked from the tumor. For these reasons, urine is an ideal diagnostic analyte for bladder cancer recurrence detection.
Methods
Urine samples from patients with characterized bladder cancer recurrence or remission were collected and treated according to standard operating procedures (SOP). Briefly, urine was spiked with a proteases inhibitor cocktail and centrifuged at room temperature. The supernatant was freezed at -80°C.
After thawing, urinary proteins were desalted and concentrated using ultrafiltration. Extracts were deposited on SDS Page gels and separated (for MS/MS analyses) or stacked as a single band (for MS analyses). Bands were excised and proteins digested in-gel. Peptides mixtures were analysed by LC-MS/MS for AMT database generation or by LC-MS for quantitative analyses. All experiments were performed on a Thermo-Fisher Orbitrap XL coupled to a Dionex Ultimate 3000 nano-HPLC system.
MS/MS data were processed by Mascot and identifications compiled using proprietary in-house software: IRMa. MS data were processed using the Decon-2LS/VIPER software suite provided by PNNL [1].
Results Particular emphasis was put on evaluating the influence of sample collection and preparation protocols on the quantitative performances of the LC-MS analysis.
An AMT tag database was then generated from more than 250 LC-MS/MS analyses of gel fractions from pooled patient urine samples. A total of ~640,000 MS/MS spectra were submitted to the Mascot® search engine and ~150,000 peptide spectral matches were returned. Identifications were filtered (Rank=1, Score>identity threshold, p<0.05) and grouped into an AMT tag database using IRMa, resulting in a total of 6645 distinct peptides pointing to 4699 protein accessions. Grouping proteins on the basis of their peptides coverage helped to reduce redundancy to a conservative list of 918 protein groups.
Using this database, a quantitative study on a restricted cohort (20 patients) was undertaken. Statistical data analysis revealed substantial inter-individual variability that dramatically impaired the detection of differentially abundant species, stressing the need for better characterization of urine samples before MS analysis. As a result, rigorous SOPs covering all aspects from sample collection to MS analysis were established, including a method to accurately dose protein in urine for normalizing total injected quantities. These SOPs are now implemented in the clinical setting to ensure the best quantitative performances for a candidate biomarkers discovery study underway in the context of our European FP7 DECanBio research program [2].

Innovative aspects
- Human urine AMT database.
- Evaluation of post-collection treatment effects on the quantitative ability of LC-MS analysis.
- Improvement on quantitative performances using SOPs.

References (maximum 3 references)
[1] http://ncrr.pnl.gov/
[2] http://www.decanbio.eu/

S311

Shotgun Proteomics Identifies Protein Biomarkers Specific for Acute Renal Transplant Rejection

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Introduction: Availability of genome data has facilitated high throughput studies including proteomics. Proteomics can help in biomarker discovery for different diseases including kidney transplant dysfunction. Renal transplantation is the ultimate treatment for patients with end stage kidney disease, but there is no current non-invasive means to monitor for acute graft rejection. Since, acute rejection (AR) still remains the primary risk factor for the renal transplant outcome; development of non-invasive diagnostic tools through the discovery of biomarkers is important.

Methods: We analyzed a test-set of 40 samples, with 10 samples in each of biopsy proven AR and stable grafts (STA), nonspecific proteinuria (NS), and healthy controls (HC) and an independent verification-set of 72 urine samples with AR (n=20), STA (n=20), BK virus (n=10), chronic allograft nephropathy (CAN) (n=10), NS (n=6) and HC (n=6). The fractionated samples were analyzed by LC MS. Proteins were identified using SEQUESTTM. Protein-level spectral counts were obtained by addition of peptide-level spectral counts which were used to get quantitative information. To compare relative protein abundances, we used a weighted fold-change like statistic. ELISA assays on Tamm-Horsfall protein, pigment epithelium-derived factor (PEDF) and CD44 were performed to verify the trueness of discovery.

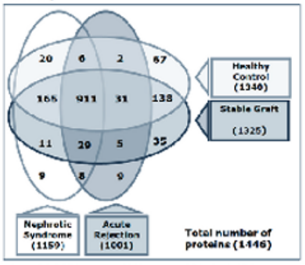

Figure1. Urinary proteins were identified from urine collected from healthy as well as renal patients with or without kidney transplant.

Results: A total of 1446 urinary proteins were identified along with a number of NS specific, renal transplantation specific and AR specific proteins (**Figure 1**). Relative abundance of identified urinary proteins was measured by protein-level spectral counts adopting a weighted fold-change statistic, assigning increased weight for more frequently observed proteins. We have identified alterations in a number of specific urinary proteins in AR, primarily relating to MHC antigens, the complement cascade and extra-cellular matrix proteins. A subset of proteins (UMOD, SERPINF1 and CD44), have been further cross-validated by ELISA in an independent set of urine samples, for significant differences in the abundance of these urinary proteins in AR.

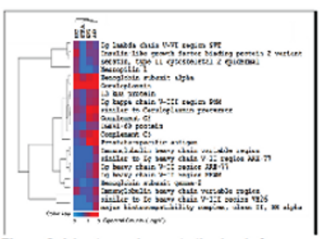

Figure 2. A heat map demonstrating level of elevated proteins in AR compared to STA healthy urine and NS.

Innovative aspects:
- Successfully attempted to identify biomarkers in AR of kidney transplant using high throughput LCMS method.
- Used label-free, semi-quantitative approach in urinary proteomics in normal and disease states.
- Demonstrated that this approach may provide a robust and sensitive method for detection of urinary proteins for serial, non-invasive clinical monitoring in kidney transplantation.

Systems Approach to Biomarker Discovery

(S401 – S443)

S401

Proteomics of Invasive and Metastatic Human Breast Carcinoma

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Introduction
Metastasis includes loss of cellular adhesion, invasion into extracellular matrix, entry into circulation, exit into new tissue and colonization of a distant site. The primary objective of this study is to establish an isogenic model of invasiveness and study mechanism of cancer invasion by proteomics and systems biology approaches.

Methods
By selecting the cells invaded into gelatin, highly invasive clones of MCF-7 cells were obtained for further study.
2D gels of parental MCF-7 cells, invasive MCF-7o46 cells and invasive, metastatic MDA-MB-231 cells were generated, as described earlier (1). Differently expressed proteins were selected and identified by mass spectrometry.
Systemic analyses were performed, included functional clustering, pathway and network analysis. Networks formed by identified invasiveness-related proteins were used for selection of proteins for further study.
Selected proteins were validated by immunoblotting with specific antibodies. To manipulate levels of selected proteins, overexpression and siRNA knockdown experiments were performed, and cell invasiveness, proliferation and apoptosis were measured.

Results
We isolated a highly invasive clone of MCF-7 cells, MCF-7o46. Invasion assay showed that their invasiveness across matrigel barrier is 10-fold higher than their parental cells (fig.1).
The subsets of differentially expressed proteins from 2D gel were defined to extract invasiveness-specific proteins, i.e. MCF-7 v.s. MCF7o46 (non-invasive v.s. invasive), MCF-7 v.s. MDA-MB-231 (non-invasive v.s. invasive, metastatic), and MCF-7o46 v.s. MDA-MB-231 (invasive v.s. invasive, metastatic).
By functionally analysis, all three subsets of proteome profile showed significant similarity in affected functional domains, but ECM organization and biogenesis were more represented in non-invasive v.s. invasive subset, i.e. MCF-7 v.s. MCF7/46, and MCF-7 v.s. MDA-MB-231.
Systemic analysis of invasive-specific network, which is a overlapped network of the subsets MCF-7 v.s. MCF7o46, and MCF-7 v.s. MDA-MB-231, revealed several major hubs, e.g. TGFbeta, EGF, VEGF, IGF, NFkabbaB and HNF. The network analysis showed also relations between these regulatory processes (fig. 2).
Subsequently, HNF4a, BRMS1 and DOCK10 were selected for validation and functional and study.

Innovative aspects
- New isogenic model of invasiveness of human breast epithelial cells has been generated.
- Major hubs in the invasiveness-specific network have been identified. They include TGFbeta, EGF, VEGF, IGF, NFkabbaB and HNF signaling mechanisms in defined relations, e.g. these mechanisms are involved in regulation of invasiveness in a defined order and hierarchy.
- HNF4a, BRMS1 and DOCK10 might be used as targets for prediction of invasiveness of breast tumors.

References
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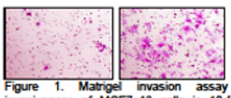

Figure 1. Matrigel invasion assay showed that invasiveness of MCF7o46 cells is 10-fold higher than parental MCF7 cells.

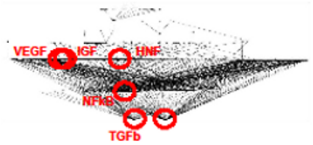

Figure 2. Network analysis showed that several major hubs, included TGFbeta, EGF, VEGF, IGF, NFkabbaB and HNF signaling mechanisms are involved in regulation of invasiveness in a defined order and hierarchy.

S402

Proteomic Profiling of Prostate Cancer and Benign Prostatic Hyperplasia from 1ml of Urine

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Prostate cancer is the most frequently occurring cancer and is the second highest cause of mortality by cancer for the male population. Infection or inflammation of the prostate (prostatitis) may increase the chance for prostate cancer. In particular, infection with the sexually transmitted infections Chlamydia, gonorrhea, or syphilis seems to increase risk. Serum prostatic specific antigen (PSA) has proven to be a generally reliable indicator for the diagnosis and management of prostate cancer. Patients with a serum PSA between 2.5ng/mL and 10ng/mL will often undergo prostate biopsies to confirm prostate cancer. However, only 20–30% of these men will have positive biopsies, meaning that the majority of men underwent these invasive investigations with little benefit. The development of new diagnostic tests is therefore highly needed to increase diagnostic accuracy and to reduce the number of negative biopsies. The objective from this study is to study the urinary proteome of patients with Prostate Cancer (CaP) as well as Benign Prostatic Hyperplasia (BPH) in comparison to the urinary proteome of normal healthy individuals. This may lead to the discovery of a novel and a reliable non-invasive urinary biomarker for the early detection of Prostate cancer, where therapeutic intervention will be more efficient. Therefore, urine samples were collected from eight Prostate cancer patients, 12 BPH patients and 10 normal health individuals. Urinary proteins were isolated from 1 mL of each urine sample. Total urinary proteins showed significant differences when resolved on 1D SDS-PAGE gel. Total urinary proteins were significantly higher in both prostate cancer patients and BPH patients, in comparison to that isolated from the health normal individuals. Furthermore, the bottom-up proteomic approach was used to investigate the differential protein expression among the three groups. The preliminary analysis from the LC/MS/MS data identified 85 different proteins that are differentially expressed among the three groups. Some proteins were found to be either over-expressed or suppressed in prostate cancer in comparison to both BPH patients and the normal healthy individuals. These proteins might be of a great significance and of a great value for the early and accurate detection of prostate cancer using only 1 ml of a urine sample.

S403

Gene-Centric View to the Proteins of 18th Chromosome Identified in Blood Plasma: Potential Biomarkers

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Introduction
Gene-centric direction of the Human Proteome Project leads to the urgent need in re-estimation of already received proteomic data and new way of obtaining future data in context of the specific chromosome. In the recent KHUPO meeting, the Russian proteome community has chosen the 18th chromosome as a part of the project. It is therefore necessary to know how many proteins of this chromosome were included to the state-of-the-art plasma proteomes and what biological role they may play.

Methods
Plasma protein datasets published by HUPO Plasma Proteome Project [Omenn et al, 2005] and by Anderson et al [Anderson et al, 2004] were taken to search for proteins from 18th chromosome. Moreover, own data were included in the study. A sample of healthy human plasma was treated by Agilent Multiple Affinity Removal System depleting 6 major human plasma proteins. The depleted fraction was digested and six repeats of 2D-LC-MS/MS were done using Agilent XCT Ultra ChipCube LC-MS system. All these data were used for the information analysis.

Results
Chromosome distribution of proteins from the plasma subsets of 3020 (HPPP), 1175 (Anderson) and 1585 (own data) non-redundant gene products has shown that plasma proteins are, in general, evenly distributed throughout the genome, with Pearson correlation coefficient between lengths of chromosomes and numbers of their plasma proteins was as high as about 0.9.
HPPP, Anderson's and own protein lists contained 46, 16 and 24 proteins of 18th chromosome, respectively. Only 10 of them are included at least in 2 datasets. Of 150 highly abundant plasma proteins only human transthyretin (TTR) gene is located in the chromosome of interest.
The list of 18th chromosome proteins contains a number of potential biomarkers for several diseases. Of them, the myelin basic protein, important adhesion and signaling molecules and several cancer-related antigens should be mentioned. A presence of these clinically relevant proteins in plasma-related mass-spectra provides a background for the multiplex quantitative analysis of them in plasma samples in scope of Human Proteome Project.

Innovative aspects
- Generation of the list of human proteins encoded by 18th chromosome and identified in plasma using published and own data
- Analysis of their possible biomarker properties for further quantitation

References
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S404

Discovery of KPNA2 as a NSCLC potential biomarker by integration of cancer cell secretome and tissue transcriptome

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Introduction

Lung cancer is one of the most prominent causes of cancer death over the world. Discovery of tumor markers those are biological parameters and can be measured in body fluid of suspected cancer patients would be a good opportunity to find a method for early cancer detection and metastatic prognosis in lung cancer. We herein integrated two lung adenocarcinoma cell lines (CL_{1-0} and CL_{1-5}) secretome datasets with one pulmonary adenocarcinoma microarray dataset to find targets that are highly up-regulated in lung cancer tissues and also secreted/released from lung cancer cells (Figure 1). Importin subunit alpha-2 (also known as karyopherin subunit alpha-2, KPNA2) represents one of the novel candidates and was chosen for further validation using clinical specimens from lung cancer patients.

Methods

We examined the tissue expression of KPNA2 protein in 66 non-small cell lung cancer (NSCLC) specimens with adjacent normal tissue by immunohistochemistry. Serum and pleural effusion levels of KPNA2 were determined by homemade ELISA. Gene knockdown was used to reduce the expression level of endogenous KPNA2, and the effects of depletion of KPNA2 on migration ability and survival were examined by transwell migration assays and MTT assay, respectively.

Results

We identified 35 potential biomarkers, including 16 well-known potential biomarkers and 19 novel candidates for lung cancer based on this integrated approach. Immunohistochemical staining revealed the overexpression and altered subcellular localization of KPNA2 in tumor cells as compared with adjacent normal cells. Higher serum levels of KPNA2 were detected in NSCLC patients versus healthy controls. The protein levels of KPNA2 in pleural effusion from NSCLC were significantly higher than those from breast cancer or colon cancer. Importantly, a combination of serum KPNA2 and serum CEA displayed a higher diagnostic capacity than either marker alone. Moreover, knock-down of KPNA2 inhibited the migration ability and reduced viability of lung cancer cells (Figure 2).

Innovative aspects

- Protein levels of KPNA2 were up-regulated significantly in cancer cells as compared to their adjacent normal counterparts.
- KPNA2 could be detected both in serum and pleural effusion, and the serum levels of KPNA2 were significantly higher in NSCLC patients than in healthy controls.
- KPNA2 was involved in the migration ability and cell viability in lung cancer cells.
- The integration of cancer cell secretome and transcriptome datasets provides an efficient way for discovery of novel body-fluid accessible biomarker.

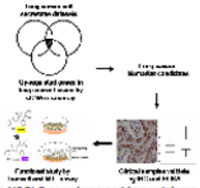

Fig.1 The NSCLC secretome and transcriptome datases were analyzed. KPNA2, a protein secreted by NSCLC cells and over-expressed in NSCLC patients, was selected and verified as a potential NSCLC serum biomarker using clinical specimens by immunohistochemistry and ELISA.

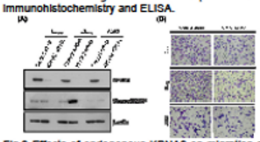

Fig.2 Effects of endogenous KPNA2 on migration ability and cell survival of lung cancer cells *in vitro*. (A) Lung cancer cell lines were transfected with control siRNA and KPNA2-specific siRNA, respectively. At 48 h after transfection, cell lysates were prepared and proteins detected by Western blotting. Simultaneously, cells were subjected to the migration assay and MTT assay as described in Materials and Methods. (B) Representative microphotographs of filters obtained from the migration assay. Original magnification: 100×

S405

New screening method for autoantigen protein based on biotinylated protein library

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Introduction

Autoimmune diseases are a heterogeneous group of diseases characterized by immune reactions against one or more autoantigens of the body itself. Thus identification of autoantigens reacting to patient serum would be a first step to understand the autoimmune disease. Here, we describe new screening method for autoantigen proteins using biotinylated protein library (BPL) produced by wheat cell-free protein production system (WCFS) (1,2), in which interaction between an individual biotinylated protein and the autoantibody in sera is detected by commercially available luminescence assay (Fig.1).

Methods

Biotinylated p53 protein was generated by WCFS (2), and was used for determination of optimal condition by luminescence assay (AlphaScreen®) with various concentration of anti-p53 monoclonal antibody from 5×10^{-3} to 5×10^{2} pg/µl. From well-known autoantigens and in the mouse autoimmune susceptibility loci, 229 mouse genes were selected, and used for generation of non-purified BPL by WCFS. The sera of MRL/lpr mouse, autoimmune model mouse, were used to identify the autoantibodies. For detection of antigen-antibody reaction, BPL and commercial available luminescence beads were simply mixed, and then luminescent signal of the mixture was measured. Validation of autoantibody was performed by using immunoblot and immunoprecipitation using the sera of MRL/lpr mouse.

Results

Biotinylated p53 protein was detected by the luminescent assay with low amount of the monoclonal antibody (<0.5 pg/µl). By screening of autoantigen proteins reacting autoantibodies in the sera of MRL/lpr mouse, 141 biotinylated proteins were showed high luminescent signals. From detailed analysis, 96 proteins specifically reacted with MRL/lpr mouse sera although 45 proteins did by normal mouse sera. Next, all eight of randomly selected from identified autoantigen proteins were confirmed by immunoblot and/or immunoprecipitation in the sera of MRL/lpr mouse. These results show that the new screening method

Innovative aspects

- Biotinylated protein library generated by wheat cell-free protein production system.
- Comprehensive screening system for autoantigen proteins in liquid phase.
- This screening method could detect antibodies reacting to structurally fold-state antigen proteins.

References:

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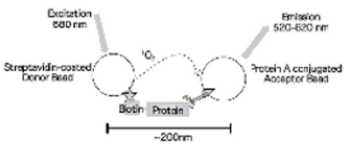

Figure 1. Detection principle of autoantigen proteins using biotinylated protein. When biotinylated protein is interacted with autoantibody in the sera, streptavidin-coated donor and proteinA conjugated acceptor beads produce a complex including antigen-autoantibody interaction. By proximity of acceptor and donor beads, the complex generates the luminescence signal upon irradiation at 680 nm.

S406

Pancreatic Cancer Cell Line and Pancreatic Juice Proteomics: A Search for Biomarkers

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Introduction

With an estimated 37,680 new diagnoses and 34,290 deaths in the United States last year alone, pancreatic cancer is a rare but deadly disease [1]. Current biomarkers lack the necessary sensitivity and specificity for adequate early detection and disease monitoring of the general population. In the present study, we analyzed the proteomes of conditioned media from seven pancreatic cell lines in conjunction with pancreatic juice from adenocarcinoma and chronic pancreatitis (control) patients to better capture the heterogeneity of the disease and generate candidate tumor markers.

Methods

Cell lines were grown in serum free media and growth conditions (incubation period and seeding density) were optimized to increase protein secretion and minimize cell death. A volume of conditioned media corresponding to one milligram of total protein was dialyzed and lyophilized. The lyophilized sample was then denatured, reduced and alkylated, followed by a desalting step utilizing a NAP-5 column. The samples were then trypsin digested and fractionated using strong cation exchange (SCX) chromatography. Each fraction was zip-tipped and run on an Orbitrap mass spectrometer. MASCOT, X!Tandem and Scaffold software was used for subsequent data analysis. Pancreatic juice was also analyzed in a similar manner. All samples contained three biological replicates.

Results

Between 1200 – 1700 proteins were identified in each of the cell lines, of which approximately 30% belonged to the plasma membrane and extracellular components. Pathway analysis identified cancer and gastrointestinal disease as the top two disorders associated with the cohort of proteins, and a large number of previously studied pancreatic cancer markers were amongst our candidates, serving to validate our approach. The large lists of proteins identified were subjected to a filtering algorithm to generate a shortened list of candidate biomarkers for validation in serum using multiple reaction monitoring (MRM), product-ion monitoring (PIM), or ELISA approaches. To date, effective markers or marker panels have proven elusive in the large majority of cancers due to the inherent heterogeneity of the disease and lack of reagents for validation studies. While the bottleneck in candidate validation still exists, we utilized a relatively large number of samples for discovery and combined proteomic data from multiple sources (cell lines and proximal biological fluids) to more adequately capture disease heterogeneity for delineation of stronger candidates for validation.

Innovative aspects

- Largest known proteome of pancreatic cancer cell line conditioned media.
- Novel filtering algorithm for generation of candidates.
- Integration of pancreatic juice and cell line conditioned media proteomes for biomarker elucidation.

References

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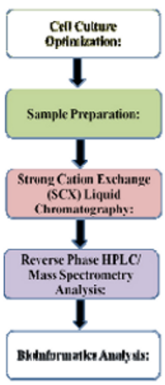

Figure 1. General workflow for cell line conditioned media analysis. Pancreatic juice analysis was similar. The 'sample preparation' step involves denaturing, reduction, alkylation and trypsin digestion. All samples were analyzed in triplicate.

S407

Proteomic and bioinformatic analysis of cancer-cell secretome for biomarker identification

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Introduction

Analysis of proteins secreted from cancer cells is important for understanding the cancer environment, and may be useful to identify potential tumor markers. Previously, we have identified collapsin response mediator protein-2 as a novel potential biomarker of colorectal carcinoma (CRC) on the basis of comparative analysis of CRC and other cancer cell line secretomes (1).

Methods

We therefore sought to analyze cancer cell secretome by one-dimensional SDS-PAGE in combination with the nano liquid-chromatography tandem mass spectrometry (GeLC-MS/MS) procedure. In this study, the secretomes of 23 cancer cell lines, derived from nasopharyngeal carcinoma (NPC-TW02, -TW04, and -BM1), colon cancer (Colo205, SW480, and SW620), hepatocellular carcinoma (SK-Hep-1, Hep G2, and Hep 3B), oral cancer (OEC-M1 and SCC-4), bladder cancer (U1 and U4), breast cancer (MCF7 and MDA-MB-435S), cervical cancer (C-33A and HeLa), lung cancer (CL_{1-0} and CL_{1-5}), pancreatic cancer (PANC-1 and MIA-PaCa-2), epidermoid carcinoma (A-431), and T cell lymphoma (Jurkat) have been analyzed.

Results

In this dataset, a total of 31,180 protein identities accounting for 4,584 non-redundant proteins were detected, with an average of ~1,300 proteins per cell line. Using protein secretion predictive algorithms, 55.8% of the proteins could be assigned to be released or shed off from cells. The identified proteins were further classified as potential tumor biomarkers according to the three strategies: (i) the proteins apparently secreted by one cancer type but not the others (cancer-type specific marker candidates), (ii) the proteins released by most cancer cell lines (pan-cancer marker candidates), or (iii) rational discovery through putative linkage with cancer relevant pathways. Next, we took the advantage of protein expression profiles existed in the Human Protein Atlas database to efficiently narrow down the list of identified proteins to find useful candidates. This analysis yielded ~30 potential markers specific for each tumor type and 71 potential pan-cancer markers that warrant further validation.

Innovative aspects

- A comprehensive secretome dataset from various cancer cell lines has been established.
- Comparative analysis of cancer cell secretome is a feasible strategy for identifying potential cancer biomarkers.

References

(1) Wu et al. Identification of collapsin response mediator protein-2 as a potential marker of colorectal carcinoma by comparative analysis of cancer cell secretomes. Proteomics 2008, 8:316-332

S408

Proteome profiling of invasive ductal and lobular carcinomas

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Introduction

Breast cancer is one of the most frequent malignancies, with more than 350.000 cases detected in Europe every year. To identify cancer-related changes in expression of proteins, we perform proteome profiling of samples of human breast tumors

Methods

Samples of tumors and histologically non-malignant peripheral tissues from the same patients were used to generate two-dimensional gels. Two to 3 replicas of 2D gels were generated for each tumor and "normal" peripheral tissues sample. Gel image analysis was performed to identify differentially expressed proteins.
In 2D gel electrophoresis, proteins were separated using Immobiline strips pH 3-10 and 10 % SDS-PAGE.

Results

We generated 64 2D gels from 10 cases of human breast cancer, and identified from 100 to more than 200 differentially expressed cancer-specific proteins/protein spots in 2D gels, depending of the studied case. The samples consisted of cases of invasive ductal and invasive lobular carcinomas.
We observed significant variability of 2D gels from various cases. To address the issue of variability, we performed histological examination of studied cases, by staining sections of the same tumors and "normal" peripheral samples with haematoxylin/eosin. We observed correlation of histological features of samples and type of protein presence and separation in 2D gels, e.g. presence of malignant cells, tumor stroma, adipocytes, etc.
We found that comparison of proteome profiles within the same case, i.e. tumor vs peripheral, provided more accurate evaluation of differentially expressed proteins, as compared to generation of a common for all tumor cases 2D protein map.
Currently, proteins in differentially expressed protein spots are being identified using MALDI TOF mass spectrometry (more than 100 proteins). Some of the proteins already known as breast cancer markers for diagnostic: (keratins as CK 2, CK12, CK8 etc). Systemic analysis of the network formed by the cancer-specific proteins indicated also HSP60 which was proposed as a plasma marker. Most of the identified proteins are involved in processes of apoptosis, cell cycle, cell differentiations, proliferation and metabolism.

Innovative aspects

- We showed that a case-by-case study is more informative, as compared to a combined all cancer vs all normal cases study. In combination with histological and immunohistochemical evaluation of tested samples, case-by-case approach allows to minimize a negative impact of inter-patients variability.

S409

Proteomics of Serum and CSF of Monozygotic Twins Discordant for Schizophrenia

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Introduction

Schizophrenia and bipolar disorder are lifelong diseases prevalent in 1% of the population. Biomarkers are desperately needed in this field to assist the practicians in the objective diagnosis and in the drug response monitoring of these psychiatric disorders. Considering the high inter-individual variability of these disorders, a carefully chosen sample set (e.g. discordant twins) might be particularly valuable to identify consistent proteomic differences for a biomarker discovery phase.

Methods

This biomarker discovery study relied on the screening of serum and CSF samples originating from ten pairs of monozygotic twins discordant for schizophrenia. The serum and CSF samples were processed either (i) by enrichment of their N-glycosylated protein content accordingly to a published protocol (1), or (ii) by depletion of their highest abundant proteins and subsequent enrichment of their cystein-containing proteins. The samples were then analyzed by an LC-MS map comparative approach and by an additional targeted analysis of ~50 potential candidates by selected ion monitoring and selected reaction monitoring.

Results

Establishment of a robust biological and analytical platform was a prerequisite to this biomarker discovery study. LC-MS map analysis of the glyco-captured serum sample of the twins showed an outstanding level of reproducibility (in terms of retention times and MS signal intensities, Figure 1) for each of the twin pairs and across all the pairs in general. Over 3000 MS signals/features were consistently monitored across the whole sample set and were used to perform a comprehensive comparative analysis of their relative intensities. The regulated features detected by automatic software analysis were all manually verified in the raw data with the help of a visualization software specifically developed for this purpose and further validated with targeted MS analyses.
Overall, the discordant twins sample set exhibited only very few differences consistent within more than five pairs. The level of reproducibility achieved in this study facilitated the detection of consistent changes in protein abundances and substantiated the relevance of proteomics to acquire high quality data sets for biomarker discovery.

Innovative aspects

- Exclusive set of samples from monozygotic twins discordant for schizophrenia.
- Outstanding reproducibility of the biological and analytical workflows (Figure 1).
- Extensive verification of the differentially expressed candidates by raw data visual inspection and targeted MS analyses.

Figures

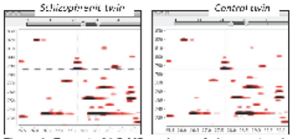

Figure 1. Excerpt of LC-MS analyses of glyco-captured serum samples of twins discordant for schizophrenia; note the outstanding reproducibility achieved with this sample set, which facilitates the detection of differentially expressed proteins

References

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S410

Building Tools for the Analysis and Visualization of Metabolomic Data

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Introduction

Metabolomics is a rapidly emerging field that is joining other high-throughput "omics", such as proteomics and transcriptional profiling. It promises to be a powerful systems approach for studying metabolic profiles pertinent to a variety of normal and disease states. Transcriptional profiling and proteomics have established data analysis tools; a metabolomics analytical toolkit is yet to be developed. We are creating a set of tools that will allow the user to examine experimental metabolomic data in the context of human metabolic networks and to combine them with other high-throughput data.

Methods

A number of public sources contain information about human metabolic networks (KEGG, BIGG, EHMN) (1,2,3). We used these data to trace the connections between metabolites and genes. Compounds, reactions, enzymes, genes and the relationships between them provide an initial framework for the analysis of metabolomic data. The Michigan Molecular Interactions database (MiMI) developed by the National Center for Integrative Biomedical Informatics (NCIBI) integrates protein interactions data from a number of public sources and thus can supply broader context for the analysis of the experimental data. The data are stored locally in a Microsoft SQL Server database.

Results

Users can browse the pathways, genes, enzymes and metabolites using web interface or Metscape, a new plug-in for Cytoscape that we developed. Both tools allow users to upload a list of metabolites, identify reactions, genes and pathways that are associated with them, and explore their relationships. The web interface is integrated with Michigan Molecular Interactions database (MiMI) that consolidates the wealth of information about protein-protein interactions.
Metscape allows users to import normalized experimental metabolite data and display them in the context of metabolic reactions (Fig. 1). Users can display the entire metabolic network, or pathways-specific networks, or apply a pathway filter to a network and create sub-networks from resulting subsets of compounds. Two different types of experimental values can be uploaded. The first is represented by changing node color; the second is represented by changing node size.
Web interface and Metscape access the same database and are interconnected. We are working on bringing normalized proteomics and expression profiling data into Metscape and displaying them side by side with metabolomic data.

Innovative aspects

- We developed a new plug-in for Cytoscape for visualization and analysis of metabolomic data.
- Our database consolidates the wealth of information about human metabolic networks from several public sources and enables comprehensive view of the data.

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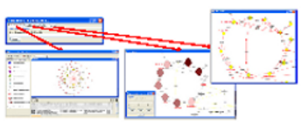

Figure 1. Metscape has three tabs that allow users to upload one or more compounds of interest, upload files containing experimental data, or display pathway-specific networks.

S411

High-speed Proteomic Signature by Informatics-Assisted Label-free Quantitation

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Introduction

Although mass spectrometry (MS) is one of the principal technologies for biomarker discovery, bottlenecks are present referring to relatively scarce amounts of biomaterials and low throughput. Due to the complexity of proteome and incompatible low acquisition speed of MS, additional time-consuming fractionation step is a critical prerequisite to enable profiling protein at low abundant levels. Here we report a high-speed label-free quantitation strategy combining LC alignment and informatics tool towards large-scale multiplexed proteomic profiling, allowing high throughput biomarker discovery.

Methods

Total cell lysate is proteolysed by gel-assisted digestion[1]. Phosphopeptides are further purified by pH/acid controlled IMAC[2] and analyzed by LC-MS/MS. The quantitation procedure is shown in Figure.1 (a). First, a reference peptide database obtained in long gradient LC-MS/MS is established. Comparative quantitation profile for peptides from different samples is constructed by shorter gradient LC-MS/MS, allowing rapid analysis of large number of samples. (b) The elution profiles of all peptides between long- and short-gradient experiments are aligned using sequence, elution time, and *m/z* information. The unidentified peptides in short-gradient experiments were detected and assigned by corrected elution time. (c) The areas of XIC were computed and normalized by internal standard.

Results

Using THP-1 cell as a model study, we demonstrated a high-speed label-free strategy on the quantitation at protein level as well as at extent of phosphorylation. For alignments across LC-MS/MS run with different LC gradients. The strategy utilizes long LC gradient for protein *identification* and short LC gradient for *quantitation*. Base on this algorithm, the XIC of identified peptides in 210-min LC-MS/MS can be successfully located in 40-min LC-MS/MS using the information of peptide sequence, *m/z* and aligned elution time. A total number of 5678 peptides was quantified in 40-min LC-MS/MS runs with a standard deviation less than 0.3 (SD=0.3 and CV <30% at 95% confidence level) in biological replicate experiments. Furthermore, we also applied this approach to phosphoproteomic study. High linearity on quantitation working curve can be achieved (r^2=0.991) over 200-fold dynamic range using a standard phosphoprotein β-casein. At complex proteome scale, high quantitative accuracy (log_2 ratio=0±0.27) and precision (CV=13.6%) were routinely obtained for 850 quantified phosphopeptides in 40-min LC-MS/MS. Its application on the biomarker discovery in cancerous tissues is on going.

Innovative aspects

- A rapid label-free quantitation strategy for high throughout biomarker discovery is developed.
- This approach can be applied to analyze individual proteomics profiles of large cohort of patents.

References

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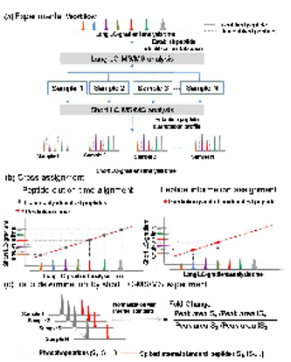

Figure 1. Workflow of swift label-free quantitation strategy.

S412

Metabonomic and proteomic research for discovering new biomarkers of liver failure caused by HBV

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Introduction
It is estimated that approximately 350 million people worldwide and 120 million in China alone are infected with hepatitis B virus (HBV). Liver failure is the most emergent clinic complication which leads to high mortality. To look for new biomarkers for predicting liver failure, LC/MS-based metabonomic and proteomic strategies were used to analyze metabolites and proteins from plasma of HBV infected patients with different status of disease progression including hepatitis and liver failure.

Methods
For metabolomic analysis, metabolites from patients with liver failure (26), hepatitis (10) and healthy controls (16) were analyzed by LC-MS, followed by principal component analysis (PCA) [1] to select differential compounds. Four differential compounds were analyzed through multiple reaction monitoring (MRM) in 167 clinical samples to select possible biomarkers and through an animal model experiment to exclude the influence of medicines taken by analyzed patients. Lastly, two verified compounds were purified and identified through ESI-Q-TOF (Bruker Company).
For proteomic analysis, plasma proteins from liver failure and healthy controls were analyzed by 2DE-MS according to our previous work [2]. One differential protein was verified by ELISA experiment.

Results
For metabonomic analysis, a LC/MS-based metabonomic platform was presented which combined the discovery of differential metabolites through PCA with the verification by MRM. A pattern recognition profile that had significantly difference between liver failure, hepatitis and healthy control ($P < 0.05$) was obtained. Four different peaks with m/z of 414.5, 432.0, 520.5 and 775.0 were verified by MRM experiment in 40 clinical samples (Figure 1) and detected not to be induced by the medicines taken by patients through rat models. Through 167 clinical subjects including healthy donors, hepatitis and liver failure verification, compound with m/z of 414.5 might be a new biomarker of liver failure with specificity and sensitivity of 95.7% and 86.5% in predicting liver failure. The qualitative analysis of 414.5 will be carried.
For proteomic analysis, 25 differential protein spots were identified including 11 non-redundant proteins such as Alpha-1-antitrypsin precursor, and fibrinogen alpha chain precursor. Alpha-1-antitrypsin precursor was verified to be up-regulated in failure liver through ELISA and consistent with the result from 2DE.

Innovative aspects
- A set of LC-MS methods for selecting metabonomic biomarkers was established and verified in nearly 200 samples with liver disease.
- Animal model experiment was used to exclude the influence of medicines.
- A possible new metabolite biomarker (m/z of 414.5) and protein biomarker (Alpha-1-antitrypsin precursor) for liver failure diagnosis were verified.

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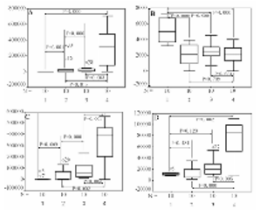

Figure 1 Mann-Whitney Statistic analysis of peak area of m/z 775.0 (A), 520.5 (B), 414.5 (C) and 432.0 (D) through MRM analysis in human plasma. 1, 2, 3 and 4 represents healthy controls, hepatitis, cirrhosis and liver failure.

S413

SNU4 KO mice is resistant to diet-induced obesity

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Introduction
SNU4 protein was secreted from adipocyte during adipocyte differentiation. Recently we developed SNU4 KO mice. The body weight of SNU4 KO mice were decreased with growth retardation due to decreased abdominal body fat but not of other tissues.

Methods
To validate the anti-obesity role of SNU4, HFD (High fat diet) and LFD (Low fat diet) were fed for 12 weeks from 6 weeks after newborn to 18 weeks to SNU4 KO mice with wild type control. Body weight and cumulative weight gain were monitored weekly for 12 weeks.

Results
Even though SNU4 KO mice showed reduced body weight at birth compared with the wild type mice, major organ weight ratio to body weight was almost same of wild type mice except abdominal body fat weight. The body weight of HFD-fed SNU4 KO mice was significantly lower than HFD-fed wild type control mice despite the daily food intake of HFD-fed SNU4 KO mice was same of HFD-fed wild type control mice. In HFD-fed SNU4 KO mice, epididymal fat mass are significantly decreased due to small size of adipocytes compared with HFD-fed wild type mice. However, the fatty liver change was observed both two groups. Also the expression level of transcriptional factors and enzymes leading to lipogenesis including Acrp30, aP2 and PPARr was decreased in HFD-fed SNU4 KO mice.

Innovative aspects (maximum of 3 bullet points)
- Characterization of new role of SNU4 in adipogenesis
- Investigation of new anti-obesity animal models (SNU4 KO) by diet-induced obesity

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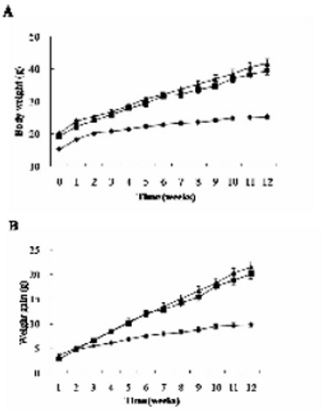

Fig 1. Effects of a high fat diet (HFD) on body weight and weight gain in SNU-SNU4 KO and wild type mice. Body weight changes were found to be significantly lower in HFD fed SNU4 KO (diamond◆, n=6) mice than in wild type (triangle▲, n=9) and hetero KO (square■, n=6) mice (A). Body weight gains were significantly lower in HFD fed SNU4 KO mice than in wild type and hetero KO mice (B).

S414

Analysis of Acyl-CoA dehydrogenase (ACAD) deficiency products in human urine

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Introduction
Acylcarnitines in urine may provide significant insight into the genetic variations of enzymes involved in the oxidation of fatty acids. For example, they provide a direct indication of ACAD activities and genetic states regardless of observed phenotype. Neonates homozygous for a gene coding for medium-chain acyl-CoA dehydrogenase accumulate higher amounts of octanoylcarnitine than those with missense variations although they might possess a similar phenotype (1). In this work we present our research efforts to analyze known as well as unknown acylcarnitines by tandem mass spectrometry.

Methods
Microsomal incubates of acylcarnitine standards were used as a signature for the identification of acylcarnitine phase I metabolites in urine. Sample clean-up and analyte extraction were performed on microsomal incubates and urine samples using cation-exchange solid phase extraction (SPE). Chromatographic separation was done on a Waters ACQUITY Ultra-Performance Liquid Chromatography (UPLC) system using a C_{18} column. Information-dependent acquisitions were performed using Multiple Reaction Monitoring (MRM) as a survey scan on an Applied Biosystems/Sciex 4000 QTRAP system. Eighty-three MRM transitions were obtained by combining data from two previous survey scans: precursor ion of *m/z* 85 and neutral loss of 59. The urine of six healthy individuals was analyzed using this method.

Results
A sensitive, selective and highly reproducible UPLC tandem mass spectrometry method was developed for the analysis of urinary acylcarnitines. By performing a high-resolution chromatographic separation, combined with selective SPE at the front-end, more than 300 species can be screened for in a single run. Acylcarnitines were identified based on their fragmentation pattern as well as retention time information. Five characteristic peaks were searched for, including the neutral losses of 59 and 161 Da, as well as peaks at *m/z* 60, 85 and 144. Compounds were identified as acylcarnitines when at least 3 of these characteristic peaks were present in their product ion spectra. A total of 329 acylcarnitines were detected, only 40 of which have been previously reported in the urine of healthy individuals. Urine specimens from six healthy individuals were collected and analyzed. It was found that their overall urinary acylcarnitine profiles were quite similar. However, some subtle differences in the relative intensities of certain peaks were observed. Future work will focus on the application of this method for potential use in newborn screening for inherited metabolic disorders.

Innovative aspect
- This method allowed for the identification of previously undocumented acylcarnitines which could potentially be used as biomarkers, providing information on the genetic severity of metabolic disorders.

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S415

A streamlined pipeline for plant metabolomics by FTMS

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Introduction

In untargeted metabolomics, dozens to hundreds of biological and technical sample replicates are usually analyzed in parallel to generate statistically significant results. Given the magnitude of the samples as well as the complexity of biological samples, high throughput analytical platforms and cutting-edge bioinformatics tools are necessary for successful metabolomics studies. Herein we present a streamlined pipeline for plant metabolomics by ultrahigh resolution FTMS via direct infusion.

Methods

Plant tissues or cells were pulverized in liquid N_2 and extracted with water/methanol/chloroform or water/ethyl acetate/acetonitrile. The total metabolite extracts were either directly infused into a 12-T FTICR mass spectrometer or further separated into aqueous/organic phases prior to infusion analysis. MS datasets were acquired in both (+) and (-) ESI modes with or without mass spectral stitching acquisition. A comprehensive software package was developed for automation of mono-isotopic peak picking, background mass exclusion, post-acquisition internal mass calibration, adduct ion/charge states discrimination, normalization, peak alignment as well as molecular formula generation/metabolome database searching. This pipeline was tentatively used to profile the metabolites in chitosan-elicitated spruce cell culture and transgenic *Brassica* seeds as the proof-of-principle studies.

Results

We routinely detected thousands of metabolite features in each plant sample and successfully assigned most features to metabolite candidates in plant metabolome databases. Our results have showed that liquid-liquid extraction and mass spectral stitching efficiently enhanced the metabolite detection and quantitation; the integrated software pipeline rendered reliable metabolite data processing and elucidation in a high-throughput manner. At least dozens of primary and secondary metabolites were detected correlation to the specific treatment and the genetic modification. This study has demonstrated the usefulness of the platform for high throughput-compatible plant metabolomics based on chromatography-free FTMS.

Innovative aspects

- The outlined pipeline provides an integrated strategy for high throughput plant metabolomics by infusion – FTMS

References

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S416

A proteomics approach to understanding Cryptococcus gatti infection

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Introduction
Fungal diseases are an emerging problem worldwide. Unfortunately, current therapies are limited in scope, toxic, expensive and resulting in resistance. Unlike bacterial pathogens, where a single gene can determine virulence, fungal pathogens require a minimum set of interacting virulence-associated properties for establishing and maintaining infection. The interplay between these in the infectious process is difficult to unravel by conventional genetic methods. Global approaches using genomics or proteomics allow differential gene expression to be studied at the whole organism level.

Methods
In vivo infection used a mouse model, which has similar susceptibility to human pulmonary cryptococcosis. Mice were infected while under anesthesia with 10^6 Cryptocal cells in 100ul PBS injected into the trachea. Mice were euthanased and infected lungs harvested at 14 days (Mid-stage infection). Control and infected lung tissue was homogenized in 7M urea, 2M thiourea, 1% C7bZ0, 40mM Tris in an Ultraturrax blender with sequential washes to separate the unbroken *C.gattii* from the lung tissue. *C.gattii* cells remained unbroken and were pelleted and stored at -70 C until required for analysis. The lung protein supernatant was labeled with Cy Dyes and analyzed by 2-D gel separation. Lung protein extracts were run on a pH 4-7 ReadyStrip IPG strip and midi sized (Criterion) gels from Bio-Rad. Gels were stained with Flamingo fluorescent stain for total protein detection.

Results and Discussion;
Elevated levels of chitinase 3-like protein 1 (Chi3L1) are associated with disorders exhibiting increased connective tissue turnover, such as rheumatoid and osteoarthritis, scleroderma, and cirrhosis of the liver. The molecular processes governing the induction of Chi3L1 are currently not known.
Recent studies indicate that intratracheal inoculation of Cryptococcus neoformans induces chitinase activity within the lung and bronchoalveolar lavage fluid of infected rats. Chitinase activity is also elicited by pulmonary infection with other fungi (e.g. C. albicans), but not by the inoculation of dead organisms. Enhanced chitinase activity reflects increased chitinase expression by airway epithelial cells and alveolar macrophages[1].

Innovative aspects
- Global profiling to identify changes in protein expression levels in Cryptococcus gatti infection.
- Exhibiting the increased levels of Chitinase 3-like protein 1 (Chi3L1) in infected lung tissues.
- Expanding the research work to compare C.gatti from infected lungs compared to in-vitro grown C. gatti as well as analysis of lung proteome with immune sera from infected rats/mice.

Reference
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Figure 1: 2DE analysis of lung proteins.

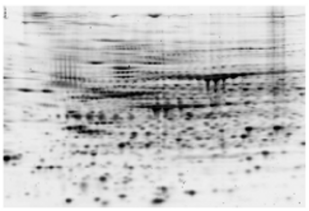

Fig.1. Representative gel of the control (non-infected) lung protein. Circles highlight the differentially expressed proteins between the infected lungs versus the non-infected lungs.

Supported by National Health and Medical Research Council (NHMRC)

S417

Mitochondrial Fatty Acid Oxidation (FAO) Enzyme Deficiencies: A Urinary Acyglycines Study

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Introduction
Elevated levels of acylglycines are directly related to the accumulation of acyl-CoA esters due to acyl CoA dehydrogenase deficiencies. Common genetic variations, along with diet, health, and environmental factors may influence enzymes, and thus disease expression. A combination of clinical, biochemical and metabolic studies can provide useful insight into genetic-phenotypic relationships (1). Discovery of new acylglycines can lead to biomarkers of enzymes for which deficiencies have not yet been discovered. Herein, we outline a sensitive tandem mass spectrometry method to detect acylglycines.

Method
Urine samples were collected from six healthy volunteers, with no known diagnoses of metabolic disorders. Seventeen acylglycine standards were incubated using human liver microsomes to generate hydroxylated and carbonylated acylglycines. Acylglycines were extracted from urine and microsome incubates using mixed-mode anion-exchange solid phase extraction. The collected fractions were analyzed by Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC MS/MS) using a Waters UPLC system coupled to a Applied Biosystems hybrid linear ion trap mass spectrometer. The mass spectrometer was operated in the positive and negative electrospray ionization mode. Mass spectral scans were obtained using information-dependent data acquisition (IDA) experiments.

Results
Most acylglycines produce an intense fragment ion of *m/z* 76 in the positive ion mode and a fragment ion of *m/z* 74 in the negative ion mode. Positive ions scans yielded more structural information in the MS/MS scans than the negative scans. Due to higher selectivity and sensitivity, MRM scans were used as survey scans, using the most intense transitions observed in the precursor or neutral loss scans. The higher sensitivity and resolution of the UPLC were used to separate isomeric and isobaric peaks and to enhance detection of the low-level acylglycines present in urine. The acylglycines detected were identified and confirmed using standards, while tentative identification was performed using retention time and fragmentation patterns. Common neutral losses of masses 18, 46, 60, 75, 93,103 and 117 were observed for most of the acylglycines. In order to identify an unknown acylglycine, at least five of seven neutral losses must be observed. A total of 65 acylglycines were detected, 38 of which were previously unreported in the urine of healthy individuals. The current work demonstrates a highly selective and sensitive method for the detection of acylglycines in human urine.

Innovative aspects
- A UPLC MS/MS has been developed for discovery of previously unreported acylglycines in human urine that may potentially be used as biomarkers for metabolic diseases.

Reference
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S418

Immuno-proteomic Approach for Antigenic Characterization of *Clonorchis sinensis*

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[3]Department of Environmental and Tropical Medicine, Konkuk University School of Medicine, Seoul 143-701, Korea

Introduction
Clonorchis sinensis, the Chinese liver fluke, causes clonorchiasis responsible to liver and biliary diseases. In this study, we have established a 2-D proteome map of total lysates of *C. sinensis* adult worms and identified diagnostic candidates for clonorchiasis through an immuno-proteomic approach. This work will help to develop a proteomic based methodology to find pathogenic proteins related to host-parasite interaction. And also, it will be possible to significantly increase the prospects of identifying the diagnostic antigens, effective vaccination candidates and drug targets.

Methods
C. sinensis adult worms were collected from bile ducts of the infected rabbits at 2 months post infection with metacercariae. To obtain total lystaes, adult worms were homogenized in buffer (7 M urea, 4% CHAPS, 2M thio-urea, 0.3% DTT). The total lysates were focused using an IPGphor system in the pI range of 3-11. After equilibration, the IPG strips were applied onto 12% SDS-polyacrylamide gels. Protein spots on 2-DE gels were transferred to NC membrane and blotted with clonorchiasis patients' sera. The interesting protein spots were manually excised from the stained 2-DE gels and subjected to mass spectrometry analysis (1). Sequences of uninterpreted mass spectra were identified by correlation with *in silico* peptide sequences translated from EST database of *C. sinensis* using Mascot software.

Results
Total lysates of *C. sinensis* adult worms were subjected to separation by 2-DE. Under optimized conditions of sample preparation, aiming at the prevention of proteolysis and limitation of artificial modifications, 2-DE gels of the total lysates yielded approximately 494 protein spots with silver staining in a molecular mass range of 10-150 kDa and pI range of 3-11 (Fig. 1). Through immune-proteomics analysis, 22 antigenic protein candidates were selected and identified by LC-coupled ESI-MS/MS and EST database of *C. sinensis*. These included the following: Cysteine proteinase, Papain, Heat shock protein 70, tegumental calcium-binding EF-hand protein, Actin, beta-tubulin, alpha-tubulin, gelsolin, myosin light chain, prohibitin, GST omega, Disulfide isomerase, carboxylase, fumarate hydrates, phosphoglycerate kinase, Adenylate kinase, Carboxyl transferase, Arginase, Coproporphyrinogen oxidase, pyruvate dehydrogenase, aldehyde dehydrogenase, 14-3-3 . This identification of antigen proteins may facilitate the development of serodiagnostic for human clonorchiasis.

Innovative aspects
- The profiles of antigenic candidate protein of *C.sinensis* and evaluated analysis of abundant antigenic protein.
- Immuno-proteomic approaches are novel and valuable tool for identifying serodiagnostic antigens in the parasitic diseases.

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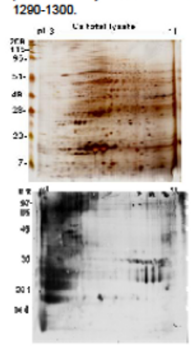

Figure 1. Proteome map of total lysates obtained from *C. sinensis* adult worms and immunoblot analysis against clonorchiasis patients' sera.

S419

Secreted protein expression from HepG2 cells exposed to Benzyl butyl phthalate

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Introduction
Benzyl butyl phthalate (BBP) is widely used plasticizer of polyvinylchloride plastics and BBP has adverse effects on liver, kidney, testicle and the reproductive, and development systems. BBP is also known as endocrine distrupting agents. In this study, we determined the effects of BBP on secreted proteins from HepG2 cells by using proteomic analysis and developed toxicological biomarkers, which would be used for human bio-monitoring.

Methods
HepG2 cells (ATCC) were treated with varying concentrations (0, 2.5, 5, 10, 25, 50, 100, 250 uM) of BBP for 24 h and 48 h and cell cytotoxicity was measured using an MTT assay. The effects of BBP on DNA damage was determined using a Comet assay which was performed as described previously (1). Proteomic analysis was carried out using two different pI ranges (i.e., 4-7, 6-9) and a large size 2-DE system. Image analysis was performed using Progenesis Discovery analytical system after silver staining (2). All MS/MS experiments for peptide identification were carried out using a nano LC/MS system consisting of an HPLC system (Surveyor, USA) and an ESI-quadruple IT MS (LCQ Deca XP-Plus, Thermo Finnigan, USA) equipped with a nano-ESI source.

Results
MTT and comet assays were carried out to determine cytotoxicity and genotoxicity, respectively. The MTT assay showed that 25 uM of BBP was the maximum concentration that did not cause the cell death. DNA damage in HepG2 cells exposed to BBP was found to be dose dependently increased. BBP treated HepG2 cells using the two different ranges of pI strips (at 4-7, 6-9), 1748, and 1523 protein spots were observed in BBP gels, respectively. A total of 30 proteins were found to be up- and down- regulated in cells expose to BBP, 20 spots were up- and down-regulated in a dose dependent manner. Ten spots were up- and down-regulated time dependently, respectively. The identification of these proteins was carried out by ESI-MS/MS which allowed the identification of 25 proteins, including proteins involved in apoptosis, Signaling, and cell growth and proliferation (Figure 1). Among these, the identities of DEK(protein names), Rho-GDI, RKIP and Cystatin C were confirmed by western blot assay (Figure 2).

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Figure 1. Proteins identified by MS/MS amino acid sequencing using an ESI-quadrupole IT MS

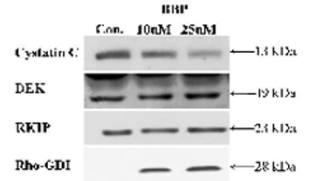

Figure 2. Western blots of Cystatin C, DEK, RKIP, and Rho-GDI. Secreted proteins from 0 uM (control), 10 and 25 uM BBP exposed cells were loaded in each lane.

S420

The identification of antigenic proteins in *Clonorchis sinensis* using by Multi Dimensional Fractionation System

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Introduction
C. sinensis cause the clonorchiasis to a person in China and Southeast Asia including Korea. Proteomic researches about clonorchiasis have been lacked, and other antigenic proteins have been reported little except cysteine protease. We have studied to find of novel antigen proteins by 2-DE and immunoblotting. But many candidate antigenic proteins didn't separate on the 2DE gel. So we had a try the other protein separation using the proteomeLabTM PF2D system. We conducted an investigation of antigenic candidates for serodiagnosis of clonorchiasis using immuno-proteomic approaches.

Methods
C. sinensis adult worms were collected from bile ducts of the infected rabbits at 2 months post infection with metacercariae. To obtain total lystaes, adult worms were homogenized in buffer (7 M urea, 4% CHAPS, 2M thio-urea, 0.3% DTT) and centrifuged. The supernatant was collected as crude extract. To apply to proteomeLabTM PF2D protein fractionation system from Beckman Coulter, buffer of crude extract was changed through PD-10 column. The separation of the enriched proteome was performed on the PF2D utilizing isoelectric focusing and RP-HPLC. The antigenicity of resulting fractions was analyzed by immuno-blot. 1D gel electrophoresis was performed to separate and identify antigenic proteins using LC-MS/MS approach (1).

Results
The total lysates of *C. sinensis*, prepurified by PD-10 column, were applied to 2-DLC to separated proteins. During 1-D HPCF (High pressure chromatofocusing), A pH gradient ranging from pH 8.5-4 was established and the protein fractions were eluted by intervals of 0.3 pH units (Fig.1). The pH gradient region (pH 8.5-4) is from fraction number 9 to 27. The basic region (pH 8.5 over) is from fraction 1 to 8 and the acidic region (pH 4 under) is from fraction 28 to 41. Elution proteins after HPCF were screened using dot blot technique to determine the antigenicity for clonorchiasis patients' sera. The results showed that the antigenicity of acidic fractions 32, 38 was stronger than that of gradient region between pH 8.5 and 4. This result is consistent with that of 2D-electrophoesis based immune-proteomics approach.
When sufficient protein of the fraction 32, 33 was enriched, 1D gel electrophoresis was performed and target proteins were identified using LC-MS/MS analysis. These proteins included Paramyosin, myosin heavy chain, Bowman-Birk Type protease inhibitor, hypothetical protein, actin, enolase, fibrillarin, Ewsr1a protein.

Innovative aspects
- Identification of antigenic proteins through 2D-LC coupled to immunological analysis and mass spectrometry
- Identification of proteins to develop serodiagnostic kit for clonorchiasis

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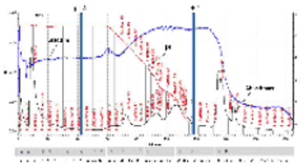

Figure 1. Dot-blot analysis of each fractions after HPCF of proteins from *c. sinensis*. To ascertain antigenicity of each fractions were spotted on PVDF membrane and were probed with mixed clonorchiasis patients' sera and anti-human IgG.

S421

Quantitative and qualitative screening of urinary biomarkers by selected reaction monitoring

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Introduction
The selected reaction monitoring (SRM) technique allows sensitive quantitative analysis of selected proteins, in tum peptides, in bodily fluids such as blood or urine. Recent developments of this technique enabled the analysis of a large several hundred analytes in one single LC-MS by time scheduling the measurement of each peptide. Further development of the SRM capability expands its application to beyond pure quantification into a hypothesis driven discovery tool. This technique was applied to detect, quantify, and confirm the identity of urinary biomarkers of bladder cancer.

Methods
To establish proof-of-principle, urine samples were collected from patients with bladder cancer and control patients without bladder cancer (N=5). Proteins (isolated from urine) were submitted to trypsin digestion, and peptides were then separated on C18 reversed phase column. LC-MS analyses were performed on a triple quadrupole instrument (TSQ Vantage) operated in i-SRM mode. The peptides of interest were targeted for quantitative and qualitative analysis by recording multiple transitions in parallel, in a data dependent manner. Typically 8-10 transitions were measured for each peptide (dwell time 10 ms/transition).

Results
A set of 250 proteins related to bladder cancer combining literature data mining, information extracted from the Human Protein Atlas, and previous transcriptomics results was selected for the screening. Corresponding proteotypic peptides (sequence unique to a protein) and their fragment ions were derived from MS/MS spectra to build the SRM assays, or predicted if no information was available. MS/MS spectra from LC-MS/MS discovery experiments or from data repository such as PeptideAtlas were used to identify the eight most intense transitions. If no experimental data were available, m/z values of mass y-ions, and retention times were predicted. The SRM data acquisition occurred by scheduling the elution and measuring transitions 'in parallel' for each peptide. The method allowed the qualitative and quantitative analysis of 500 peptides in one single LC-MS/MS run. Candidates were detected, identified and quantified in the urine samples from both case and control. Qualified candidates represent basis for a systematic quantification including isotopically labeled standards of large cohorts of urine samples to determine the robustness of the markers (sensitivity and selectivity).

Innovative aspects
- Novel SRM data acquisition method applied to biomarker discovery.
- Hypothesis-driven screen of biomarkers in urine for bladder cancer.
- High number of transitions measured ensures simultaneous qualitative and quantitative measurements.

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S422

Secreted protein expression from HepG2 cells exposed to Di-2-ethylhexyl phthalate

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Introduction
Di(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer of polyvinylchloride plastics and DEHP has adverse effects on liver, kidney, testicle and the reproductive, and development systems. DEHP is also known as endocrine distrupting agents. In this study, we determined the effects of DEHP on secreted proteins from HepG2 cells by using proteomic analysis and developed toxicological biomarkers, which would be used for human bio-monitoring.

Methods
HepG2 cells (ATCC) were treated with varying concentrations (0, 2.5, 5, 10, 25, 50, 100, 250 uM) of DEHP for 24 h and 48 h and cell cytotoxicity was measured using an MTT assay. The effects of DEHP on DNA damage was determined using a Comet assay which was performed as described previously (1). Proteomic analysis was carried out using two different pI ranges (i.e., 4-7, 6-9) and a large size 2-DE system. Image analysis was performed using Progenesis Discovery analytical system after silver staining (2). All MS/MS experiments for peptide identification were carried out using a nano LC/MS system consisting of an HPLC system (Surveyor, USA) and an ESI-quadruple IT MS (LCQ Deca XP-Plus, Thermo Finnigan, USA) equipped with a nano-ESI source.

Results
MTT and comet assays were carried out to determine cytotoxicity and genotoxicity, respectively. The MTT assay showed that 10 uM of DEHP was the maximum concentration that did not cause the cell death. DNA damage in HepG2 cells exposed to DEHP was found to be dose dependently increased. DEHP treated HepG2 cells using the two different ranges of pI strips (at 4-7, 6-9), 1748, and 1523 protein spots were observed in DEHP gels, respectively. A total of 37 proteins were found to be up- and down- regulated in cells expose to DEHP, 27 spots were up- and down-regulated in a dose dependent manner. Eleven spots were up- and down-regulated time dependently, respectively. The identification of these proteins was carried out by ESI-MS/MS which allowed the identification of 31 proteins, including proteins involved in apoptosis, transportation, and cell proliferation (Figure 1). Among these, the identities of DEK(protein names), Rho-GDI, RKIP, Gelsolin, Cystatin C and RBP were confirmed by western blot assay (Figure 2).

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Figure 1. Proteins identified by MS/MS amino acid sequencing using an ESI-quadrupole IT MS

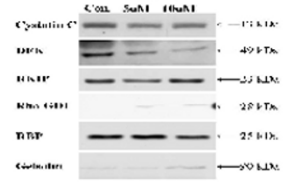

Figure 2. Western blots of Cystatin C, DEK, RKIP, Rho-GDI, RBP and Gelsolin. Secreted proteins from 0 uM (control), 5 and 10 uM DEHP exposed cells were loaded in each lane.

S423

Translating Protein Expression Results into Targeted MRM Assays to Study Kidney Osmotic Regulation.

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Although many biochemical functions in the mammalian kidney require specific inorganic ions, increasing the concentrations of these ions above those typically found in cells perturbs protein expression, protein modification, and protein translocation. Hypertonicity, as a result from high NaCl, causes osmotic flux of water out of the epithelium, elevating osmolyte stabilizing proteins and the transcription of genes encoding proteins involved in cell membrane regulation. To quantify the effects of tonicity on a renal medullary cell type, cultured cell lines were incubated with varying concentrations of NaCl and proteomic profiling was carried out using an LC-MALDI TOF/TOF approach employing iTRAQ® reagents 8Plex quantitative workflow. Improvements to TOF sensitivity combined with intelligent data acquisition functionality enabled the detection and quantification renal proteins for in-depth protein discovery in much shorter analysis times. Protein database searching using ProteinPilot™ Software 3.0 uncovered an up-regulated subset of transcription factors, i.e. TonEBP, and the discovery of several phospho-Ser/Thr sites that appear to be modulated by the exposure to high salt. To further validate these findings, a quantitative MRM assay using mTRAQ™ reagents was developed and used to obtain further relative quantitative data between control samples and low/high salt incubated samples. An easy translation of discovery data into a robust MRM assay is achieved by using the unique MRM triggered MS/MS workflow on a hybrid triple quadrupole linear ion trap platform, where full scan MS/MS for peptide sequencing and phospho-site assignment is obtained for the MRM transitions investigated. In most cases, the changes in expression observed in the iTRAQ reagent discovery experiment were confirmed by the mTRAQ reagent MRM quantitation with high accuracy and reproducibility. This study describes a complete discovery to verification workflow for identifying proteins and post-translational-modifications implicated in osmotic regulation.

S424

Improvement of 2D-PAGE resolution of Mammalian follicular fluid

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Introduction
Follicular fluid (FF) accumulates into the avascular antrum of mammalian growing follicles. It originates from the surrounding cell layers which produce factors related to their metabolic activity and permit the free diffusion of < 500 kDa plasma proteins into the antrum of follicle. FF contains several high-abundant plasma proteins (HAP) which represent approximately 85-90% of the total plasma protein mass. The aim of our study was to remove such HAP from FF because they may preclude the identification of other follicle specific proteins, present at lower concentration in FF that may be involved in the physiology of the follicle.

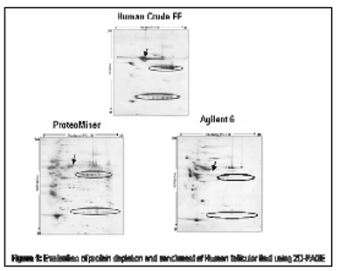
Methods
Follicular fluids were collected by ovarian puncture in human, equine, bovine, porcine and canine and individually stored at -80°. ProteoMiner and Multiple Affinity Removal Spin Cartridge - Human 6 (Agilent 6) columns were used to remove HAP from FF samples. All depletion and enrichment steps were performed at room temperature as recommended by the manufacturers. 100µg of proteins were absorbed actively into ReadyStrip™ IPG (11 cm, pH range 3–10, Biorad) and were then subjected to 10% SDS-PAGE. Gels were stained with silver nitrate. The 2D images were scanned and analyzed with Progenesis SameSpot software. Protein spots were excised from the gel, tryptically digested and analyzed by Mass spectrometer.

Results
To overcome the masking effect of HAP present in follicular fluid and to get a better resolution in 2D-PAGE analysis, we compared in the present study, the efficiency of depletion (Agilent 6) and enrichment (ProteoMiner) columns that are known to be effective for filtering out the HAP in Human plasma. To our knowledge, it is the first time that such comparative strategies are performed on follicular fluid of different mammalian species.

Our results clearly showed that the Agilent 6 depletion column which contains a mixture of antibodies against six HAPs can remove successfully these proteins from FF of different species. The depletion efficiency of this column in our study was 68.88 ± 4.90 % compared to 98.64 ± 0.14 for ProteoMiner. The technology of ProteoMiner, recently described by Righetti et al[1], is based upon a combinatorial hexapeptides hexapeptides capable of interacting with most proteins in any given proteome. Figure 1, clearly shows that 2-D PAGE profiles of Human follicular fluid samples enriched by ProteoMiner lead to the appearance of several proteins that were not detectable in the crude and depleted follicular fluid by Agilent6.

Innovative aspects
- It is the first time that such comparative analysis is performed in follicular fluid of different mammalian species.
- ProteoMiner is a more efficient method to increase the concentration of lower abundant proteins in biological fluids like follicular fluid.

I. Righetti, P. G.; Boschetti, E.; Lomas, L.; Citterio, A., Protein Equalizer Technology: the quest for a "democratic proteome". *Proteomics* **2006,** 6, (14), 3980-92.



S425

Comparative secretome-based identification of novel pancreatic cancer biomarker

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Introduction
Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal malignancies in the world, and less than 5% of the patients survive for 5 yr after diagnosis. The poor prognosis and late presentation of PDAC patients emphasize the need for developing efficient biomarker for PDAC detection. In 2005, we have developed a simple and efficient strategy to find potential serum cancer biomarker via analysis of cancer cell secreted proteome (secretome) (1). Here we describe a comparative cancer cell secretome approach to identify novel PDAC serum biomarkers, which can be preferentially detected in the secretome of PDAC cell lines and in elevated levels in serum samples from PDAC patients.

Methods
Twenty-one cancer cell lines derived from 12 cancer types (including pancreas, colon, bladder, lung, nasopharynx, liver, cervix, epidermis, ovary, uterus, breast and lymphoma) were cultured in serum-free media for 24 hr, and the resulting conditioned media were harvested separately. Proteins were resolved in SDS-gels and silver-stained, and 925 protein bands were excised for protein identification using MALDI-TOF MS, which resulted in 795 protein identities, accounting for 325 identified non-redundant proteins (2). We compared the protein list identified in each cell line, and choose protein(s) selectively detected in two PDAC cell lines (MIA-PaCa-2 and PANC1) for further validation in tissue and serum samples from PDAC patients using immunohistochemistry and sandwich ELISA.

Results
Among the 325 proteins, brain-type glycogen phosphorylase (BGP) was chosen as a candidate for PDAC biomarker evaluation, as it was not detected in the secretomes of other 19 cancer cell lines examined. To this end, we have generated specific anti-peptide antibodies against BGP. The presence of BGP in the conditioned media of PDAC cell lines was confirmed by Western blotting. The expression of BGP in PDAC tissues (N=87) was examined by immunohistochemistry; 68% (59/87) of the examined tissues showed moderately to strongly positive for BGP expression (Fig. 1). A fluorimetric sandwich ELISA was developed to measure the BGP level in serum samples from PDAC patients and healthy controls. BGP levels were significantly higher in PDAC (1.52 ± 0.61 µg/ml, n=154) than those in healthy controls (2.18 ± 0.81 µg/ml, n=159) ($p < 0.001$). The receiver operating characteristic (ROC) curve analysis in this case-control study further showed that the AUC (area under the ROC curve) for BGP was 0.745. Our results collectively suggest that BGP may represent a useful serological marker of PDAC.

Innovative aspects
- Identification of novel PDAC serum biomarker by a comparative cancer cell secretome analysis

References
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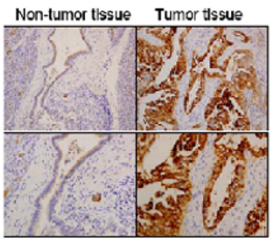

Figure 1. Overexpression of BGP in PDAC tissues examined by immunohistochemistry. Shown here are two representative cases containing paired non-tumor and tumor sections.

S426

Cell Secretome Analysis Using a Hollow Fiber Culture System Leads to the Discovery of CLIC1 Protein as a Novel Plasma Marker for Nasopharyngeal Carcinoma

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Introduction
Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in Southeast Asia. Unfortunately, most NPC victims have had metastasis when first diagnosed due to its deep location and vague symptoms. To date, discovery of sensitive and specific biomarkers for improving detection of NPC remains a challenge. Our previous study established a strategy for cell secretome analysis by using a hollow fiber culture (HFC) system combined with liquid chromatography mass spectrometry (LC-MS) (1). Herein, we used the above platform to collect NPC secretome for the discovery of relevant clinical biomarkers.

Methods
The NPC secretome was collected using the HFC system and analyzed by LC-MS. The HFC system provides high-density 3D cell cultures that mimic solid tumors *in vivo*, and in addition, collects cell secretome with improved efficiency, which is critical when the concentrations of the secreted proteins are low. Immunohistochemistry (IHC) and sandwich ELISA were then performed to confirm the protein expression levels in the tissue and plasma samples from human subjects, respectively.

Results
A total of 66 proteins were identified as NPC secretome. We have developed a strategy to sieve out chloride intracellular channel 1 (CLIC1) for intended use as a potential tumor marker candidate. More than 75% (30/40) of the NPC tissue specimens showed strong positive CLIC1 staining by IHC. The plasma levels of CLIC1 in NPC patients (N=33) were significantly higher than those in healthy controls (N=32) (mean ± SD, 6.43 ± 2.21 vs. 4.05 ± 1.36, µg/mL; $p < 0.00001$) (Fig. 1A). Using a cut-off point at 4.1µg/mL, CLIC1 levels comparatively discriminated NPC from benign healthy control groups, with a sensitivity of 84.9%, and a specificity of 71.9%. The area under the Receiver Operating Characteristic curve (AUC) was determined to be 0.85 (Fig. 1B). The expression levels of CLIC1 in plasma indicated that there were statistically significant differences between the different TNM stages of the NPC patients and the healthy controls (Fig. 1C).

Innovative aspects:
- Cell secretome analysis using a hollow fiber culture (HFC) system provides several advantages for the discovery of tumor-relevant clinical markers.
- CLIC1 is a novel plasma protein marker for nasopharyngeal carcinoma (NPC).

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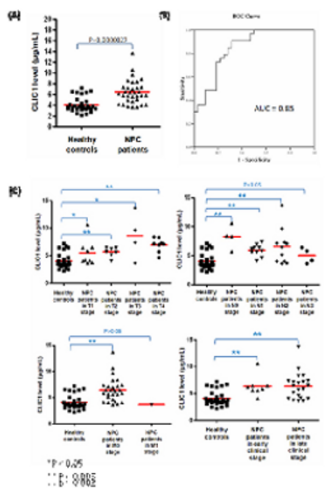

Figure 1. Elevated CLIC1 levels in NPC plasma samples. (A) The plasma levels of CLIC1 in the 32 healthy controls and the 33 NPC patients were measured by sandwich ELISA using 5 µL of plasma. (B) ROC curve analysis of the diagnostic efficacy of CLIC1. (C) The correlation of CLIC1plasma levels between the healthy controls and the different TNM stages of NPC patients.

S427

Quantitation of Carbonyls in Biofluids Using ^{14}N-/^{15}N-dansylhydrazine Labeling and LC/FTMS

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Introduction
Metabolomics and proteomics are complementary for disease biomarker discovery. We have developed a differential ^{14}N-/^{15}N-dansylhydrazine derivatization strategy for the quantitative analysis of carbonyl compounds in human biofluids. This is a universal technique specific for the identification and quantitation of ketones and aldehydes by the formation of easily ionized, hydrophobic dansylhydrazone derivatives. This strategy has been applied to the profiling of ketoacids in biofluids as indicators of ketoacidosis for conditions such as diabetes, as well as in the analysis of short chain aldehydes as biomarkers of lipid peroxidation.

Methods
Dansylhydrazine derivatization was applied to carbonyl standards, human urine and plasma. Prior to derivatization, plasma was deproteinized and both urine and plasma were reduced to dryness. Samples were prepared in methanol, and then combined with 3% trifluoroacetic acid and synthesized ^{14}N-/^{15}N-dansylhydrazine. The reaction mixtures were heated at 60 °C and shaken at 300 rpm for 1 hour. Further concentration of derivatized carbonyls and sample clean-up was achieved using SPE. Analyses of the derivatized samples were carried out using a capillary LC system at a flow rate of 1 µL/min interfaced to an FT-ICR-MS. Derivatized carbonyl metabolites were identified by comparison with derivatized standards and use of the Human Metabolome Database [1].

Results
The detection of carbonyls in biofluids is a challenging task as these analytes are poorly ionized by ESI. The conversion of these carbonyls to their dansylhydrazone derivatives has shown to improve the ESI surface activity and the chromatographic properties of the analyte making it amenable for analysis by LC/MS. The enhanced analyte signal allows for significant reduction of sample size. To this end, nano flow LC methods appropriate for the analysis of derivatized biological samples have been developed which are suitable for the interface with nanoESI-MS in a 9.4 Tesla FT-ICR-MS. This method of derivatization can be used to generate a ^{15}N-stable isotope labeled internal standard for each corresponding quantification of carbonyls in biofluids is being established. Results of differential analysis of biofluids from healthy individuals and individuals with ketone/aldehyde abnormality, such as in diabetes patients, will be presented.

Innovative aspects
- Synthesis of novel ^{14}N-/^{15}N-dansylhydrazine derivatization reagents
- Use of differential ^{14}N-/^{15}N-dansylhydrazine derivatization strategy for quantification of ketones and aldehydes in human urine and plasma using nanoLC/FT-ICR-MS
- Application of the quantitative strategy for discovering biomarkers of diabetic ketoacidosis

References
[1] Wishart DS et al., HMDB: the Human Metabolome Database. Nucleic Acids Res. 2007 Jan;35(Database issue):D521-6.

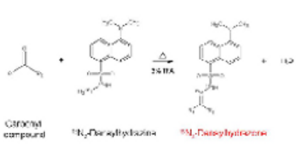

Figure 1. ^{15}N-stable isotope labeled dansylhydrazine derivatization scheme.

S428

Lipidomics in a Systems Biological Approach to Cancer Research: Response, Targets and Biomarkers (maximum 90 characters, now 86)

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Introduction (maximum **600** characters, including spaces, now **596**)
Glioblastoma multiforme (GBM), malignant primary brain tumors are characterized by low survival rate, high invasivity and resistance to apoptosis, necessitating a better understanding of the disease on a molecular level. Galectin-1 (Gal-1) levels correlate with decreased invasivity and greater tendency toward apoptosis (1) and have been defined as a therapeutic target for GBM. Gal-1 is known to bind terminal beta-galactose on both glycoproteins and glycolipids. A method for lipid profiling has been developed and implemented in the lipidomic studies of cell culture and biological fluids.

Methods (maximum **800** characters, including spaces, now **796**)
The polar lipid extraction, chromatography and high resolution mass analysis procedure for cell culture have previously been described (2) and applied to blood and CSF. In brief, ~$2x10^6$ cells, 250 µL blood or 500 µL CSF were chloroform/methanol extracted. Phase separation was established by titration with water. The polar lipid fraction was separated by nano-LC in a self-packed phenyl hexyl capillary column (75 micron i.d. X 8 cm long) with true unsplit gradient at 400 nL/min (85% methanol/ H_2O, 10 mM NH_4OAc to 98% methanol/ H_2O, 10 mM NH_4OAc in 10 min). Effluent was on-line analyzed by (-)-microelectrospray with an LTQ 14.5 T FT-ICR MS (3). Structural assignment was based on accurate mass and tandem mass spectrometry. Signal magnitudes reflect the polar lipid abundances.

Results (maximum **1200** characters, including spaces, now **1183**)
This novel lipidomics methodology was used to further access potential Gal-1 ligands in cultured GBM cell lines in response to gene and/or chemotherapy treatments. This method permits not only the identification of the targeted gangliosides, but also identifies phospholipids, sphingomyelin and sulfatides of various ceramide lengths and with varying degrees of modification (hydroxylation and double bond saturation). GBM cultures induced to apoptosis by gene and chemotherapy show high expression of short-chain sulfatides and increased hydroxylation of the phospholipid fatty acyl tails.

Lipidomic analysis has been extended to cancer stem cells (CSCs), patient blood samples and patient cerebrospinal fluid (CSF) samples. These lipidomic analyses are a major portion of our inter-institutional, multidisciplinary, systems biological approach to the study of glioblastoma, which correlates phenotypic, genomic, transcriptomic, proteomic and lipidomic data sets. The systems biological approach is essential to discover new biomarkers and identify novel therapeutic targets for treatment of glioblastoma.
Funded by NSF DMR 00-84173, the Falk Foundation and the Sorensen Family Fund.

Innovative aspects (maximum of 3 bullet points)
- Lipidomic analysis that enables identification of polar head groups, various ceramide lengths, and ceramide modifications.
- Correlation of phenotypic, genomic, transcriptomic, proteomic and lipidomic data sets to identify novel therapeutic targets and biomarkers.

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S429

Comparison of Protein Subcellular Location in Normal and Cancerous Tissues

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The early diagnosis of cancer can enhance the efficiency of a treatment and the chance of recovery. Lately, researchers have put a lot of effort into identifying cancer biomarkers for early cancer diagnosis and to distinguish between different cancer profiles. Common approaches seek genomic changes and expression variations associated with the disease process.
Here we seek to use location proteomics methods to identify changes in subcellular location of proteins associated with cancer. We have previously demonstrated that machine learning approaches can systematically distinguish 8 major subcellular locations in 45 different tissues with 83% accuracy (1).

The Human Protein Atlas is a large collection of protein images. These images were acquired using tissue microarray to immunohistochemically stained for proteins in human tissues (2). More than 6000 antibodies have been catalogued in normal and cancer tissue types. In this study, we focused on 14 pairs of tissue types.
We applied a blind unmixing method to separate the specific protein staining from hematoxylin staining in each image. We calculated a set of features combining multiresolution decomposition and texture descriptors to numerically capture the distribution of proteins in tissues without requiring the segmentation of images into single cell regions. Finally, a classifier previously trained to distinguish the major subcellular patterns (1) gave the probabilities that each image belongs to an organelle class. We used this output to assign proteins patterns to organelles.

Given the confidence level of the prediction by the classifier and the multiple images of a protein for a same tissue, several approaches were compared to assign a subcellular location to a protein in a given tissue. When only the most reliable predictions and the most selective way of voting are used, we were able to identify a set of proteins with different locations in normal or tumor samples (see Figure 1 for an example). A search for these proteins in the literature showed that some of them are already used as tissue specific cancer biomarkers (typically mutated gene or modification of the expression level), some others are known as cancer biomarkers in other tissue types, and some have not previously been suggested a tumor biomarkers. This study shows how location proteomics method can identify new potential biomarkers by comparing protein distribution in normal and cancerous tissues.

The innovative aspects of this study are
- the high-throughput analysis of protein location in tissue microarrays
- the development of a new approach to discover potential biomarkers for cancer

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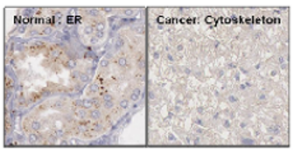

Figure 1: Cytokeratin-15 is classified as showing an endoplasmic reticulum (ER) pattern for normal kidney samples and a cytoskeletal pattern for kidney tumor samples. The literature reports on an over-expression of this protein in skin and cervical cancers, but nothing in regard to kidney tissue.

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2D-LC Fractionation and Dansylation Labeling Combined with FT-MS and NMR for Human Urine Metabolome Identification

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Introduction
There is much interest in proteomic analysis of urine since human urine is a good source for discovery of disease biomarkers. Metabolomic analysis of urine complements to the proteome analysis. Thus the combination of the two techniques holds a considerable promise for the discovery of new and more specific chemical markers of a disease. However, urine metabolome analysis is a challenge task, as many of the metabolites detected are unknown. To address this issue, we are developing efficient separation and compound identification techniques. In this report, we present a method of two dimensional LC fractionation followed by FT-MS and NMR analysis for unknown metabolite identification.

Methods
The urine samples were firstly fractioned by semi-Pep HPLC (Agilent 1100 HPLC system equipped with a 9.4mm Rx-C18 column) using HFBA as ion pair reagent. Individual fractions were subjected to dansylation which mainly labels the amino acids, amine and phenolic hydroxyl [2]. The dansyl labelled metabolites were further fractioned by HPLC using the same column. The fractions were analysed using LC-FT-MS (Acquity BEH C_{18} column 2.1 x 50 mm, 1.7 µm, and Bruker 9.4-Tesla Apex-Qe FTMS). In addition, MS/MS spectra were obtained using hybrid quadrupole/time-of-flight MS (Qstar). A standard library including 250 dansylated metabolites was first used to confirm the identification of the known metabolites. The unknowns were subjected to chemical formula calculation, MS/MS analysis and NMR (Varian 500 MHz.). The XCMS program and an in-house written Excel Macro were employed to process the LC-MS data.

Results
A novel two dimensional (2D) HPLC fractionation strategy was developed. 10 fractions were obtained from a high-resolution ion pair HPLC separation. The individual fractions from the first dimension separation were dansylated by ^{12}C-dansyl chloride. A total of 6-10 fractions were obtained from the second dimensional reversed-phase LC. Each resulting fraction was dried and re-dissolved in deuterated solvent for NMR structural analysis. A dansylation metabolite standard library containing 250 compounds was constructed and the library consists of NMR and MS spectra of each labelled metabolite. This library was used to confirm the identity of known metabolites in the fractionated urine samples. We are in the process of analyzing a large set of data to deduce chemical structures of unknown metabolites. These results will be described.

In a parallel experiment, a data processing strategy was developed for processing the dansylation labelled metabolites to facilitate peak identification in both MS and NMR. The integration of MS and NMR results may potentially be semi-automated for unknown metabolite identification.

Innovative aspects

- Develop a two dimensional HPLC fractionation approach for comprehensive urine metabolome characterization using a combination of MS, MS/MS and NMR.
- Employ a Dansylation labelling strategy which facilitates the identification of amines and phenolic hydroxyl metabolites by FT-MS and NMR.

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Understanding Pathogenesis of Down Syndrome by Quantitative Proteomics Analysis of Amniotic Fluid

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Background: Down syndome (DS), caused by an extra chromosome 21, affects 1 in 750 live births, and is characterized by cognitive impairment as well as congenital defects and increased risk for several diseases such as leukemia. Three major objectives of DS research include: 1) discovery of more sensitive and specific markers for prenatal screening, 2) understanding molecular mechanisms of DS phenotypes, and 3) finding therapeutic targets. Unfortunately, little is known about the molecular pathogenesis of DS, and no direct genotype-phenotype relationship has yet been confirmed.

Methods: Proteomic analysis based on mass spectrometry (MS) allows discovery of a large number of proteins in complex biological samples. We have recently generated the most comprehensive list of proteins present in human amniotic fluid (AF) by using high resolution MS, since AF contains the most information of the developing fetus (1). Based on this result, we hypothesize that quantitative analyses of AF proteins, as well as proteins from amniocytes, will reveal novel biomarkers and/or clues to altered molecular mechanisms of a developing fetus affected by DS and. We compared the proteome of pooled AF from normal and DS-affected pregnancies (n=10 ea.) and performed spectral counting for quantitative comparison. Additionally, we did quantitative analysis of secreted, intracellular, and membrane proteins from amniocytes obtained from normal and DS-affected pregnancies by SILAC.

Results: A total of 575 proteins from normal and DS AF samples were identified and quantified by LTQ-Orbitrap mass spectrometer based on spectral counting. Candidate biomarkers were selected based on the spectral counting difference between the conditions and consistency between the triplicates of each condition. Several candidates have been confirmed, and two candidates have been verified by ELISA, showing a two-fold increase in DS AF samples compared to the normal samples. We have also proteomically analyzed the conditioned media of metabolically labeled amniocytes from normal and DS-affected pregnancies, and at least 1200 proteins have been quantified from three pairs of normal-DS amniocytes, based on MS/MS spectra of peptides containing isotope-labeled amino acids. At least 5 of these quantified proteins consistently show differential expression between euploid and trisomy-affected amniocytes, indicating that they may play a role in DS phenotypes.

Innovative aspects
- First study to quantitatively analyze amniotic fluid from disomy and trisomy pregnancies
- Use of SILAC for proteomic analysis of amniocytes to discover altered expressions at the protein level due to Trisomy 21

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Examination of the Human Plasma Phospholipid Profile of Patients with Metabolic Syndrome

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Introduction
Irregularity of lipid abundances, dyslipidemia, is associated with prevalent diseases in North America such as diabetes, metabolic syndrome and cardiovascular disease. The identification and study of various lipids and their irregularities in key human tissues as well as blood plasma is important to a complete understanding of the role lipids play in these diseases. The application of a comprehensive profile of lipids, such as phospholipids, to human plasma samples from patients with metabolic syndrome will pave the way towards accurate evaluation of diseases associated with dyslipidemia.

Methods
Phospholipids were isolated from 3 different human plasma samples by liquid-liquid extraction. These phospholipids were analyzed by direct infusion- and LC-FTMS on a 12-Tesla FTICR mass spectrometer in (+) and (-) ESI mode. After internal mass calibration, custom software was used for monoisotopic peak pick up and peak alignment. Rational molecular formulae were generated using accurate masses and searched against lipid and metabolome databases. A model list of human plasma phospholipids was generated. Internal phospholipid standards were added to 20 whole human plasma samples from healthy patients and patients with metabolic syndrome. LCMS was conducted on a QTOF mass spectrometer and extracted ion chromatograms were generated for these samples to compare their phospholipid compositions.

Results
Results have shown that >200 and >500 phospholipids have been assigned using direct infusion- and LC-FTICRMS respectively. The phospholipid class distribution remained well correlated across LCMS and direct infusion data. By combining all unique masses obtained from all spectra,over 500 phospholipids were identified with mass errors of <2ppm. Approximately 80% of these had associated errors of <1ppm. The most abundant lipid classes in human plasma were found to be glycerophosphocholines, glycerophosphoethanolamines and glycerophosphoserines. In positive ion mode, these classes represented approximately 50%, 30% and 10% of total peak composition, respectively. In negative ion mode, these classes made up approximately 25%, 15% and 50% of total peaks respectively. In addition to those identified, there were also more than 900 peaks for which no hits were returned. A preliminary analysis of a number of these peaks revealed highly probable candidates for unidentified phospholipids. These phospholipid identities were used as a model list for targeted screening of potential phospholipid biomarkers for dyslipidemia against samples from patients with human metabolic syndrome.

Innovative aspects
-first comparative study that comprehensively profiles human plasma phospholipids of patients with metabolic syndrome against healthy patients with the aim of identifying lipid biomarkers.
- results have shown that this method has provided one of the most comprehensive profiles of human plasma phospholipids in a single experiment.

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Extending the Dynamic Range for Multiplexed Plasma Proteomics based Biomarker Discovery

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Introduction: The tandem IgY14-SuperMix depletion strategy has proven to substantially increase the dynamic range in proteomics studies [1]. In order to integrate this depletion strategy into a biomarker discovery workflow which precisely measures even small changes between patient cohorts, we examined: 1) the analytical reproducibility by introducing the second depletion column (SuperMix) by processing a number of technical replicates depleted in various experimental configurations, and 2) the consistency of the proteins collected after the second depletion stage in a pilot project with 50 primary samples.
Methods: The protein biomarker discovery platform at BG Medicine is based on the use of the 8Plex iTRAQ reagent, two-dimensional LC separation, and off line LC-MALDI MS/MS. For objective 1), twelve technical replicates from single and tandem depletion were compared in order to assess the variability of quantification and consistent retention of individual proteins on the SuperMix column. For Objective 2) above, 100 µl plasma aliquots from the Copenhagen Heart Study were depleted in two stages, followed by reduction, alkylation, digestion, and iTRAQ labeling. The iTRAQ mixes were separated into eight ion exchange fractions and analyzed by HPLC-MSMS.
Results: Comparison of the coefficient of variations of iTRAQ ratios in technical replicates indicated a slight loss of analytical precision when using tandem depletion instead of single-stage IgY depletion (mean CV%s from 7% to 7.5%). In the 50-sample study each iTRAQ mix accommodated two identical reference samples analyzed as technical replicates. Comparing nine pairs of replicates the mean CV% of iTRAQ ratios was found 8% that was consistent with findings from the reproducibility experiments (Objective 1)).
Our pilot project with 50 primary samples established that there is an excellent level of consistency in terms of proteins detected from sample to sample (**Figure 1**). In nine iTRAQ mixes of this project we detected and quantitated 831 proteins, with 80% (665) of these proteins being detected in 50% of all samples (**Figure 2**). We demonstrated that the platform can measure plasma proteins as low as 10 ng/ml, such as leptin, myoglobin, and IGF-IA. The dynamic range enhancement by tandem depletion over single stage depletion was estimated as 5-10fold resulting in a 2.5x increase in the number of proteins that could be reproducibly quantified across a larger number of individual samples.
Innovative Aspects :
- Systematic examination of the analytical variability in tandem depletion strategies
- Verify the viability and utility of tandem depletion for the quantitative analysis of large number of clinical samples

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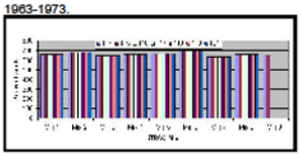

Figure 1 Comparison of each iTRAQ mix versus the number of proteins in each lane. The colors represent the iTRAQ lane (i.e., primary sample). Not all proteins are expected in all mixes.

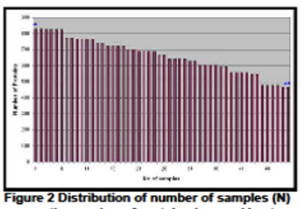

Figure 2 Distribution of number of samples (N) versus the number of protein observed in at least N samples. *Protein counts in at least 1 sample. **Protein counts in all the samples.

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Protein Signatures in Plasma for Stratification of Ovarian Cancer Subtypes

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Introduction
Recently, different pathogenetic pathways have been proposed for the development of ovarian cancer (OC) subtypes. Since these subtypes are characterized by distinct clinicopathologic patterns, it can be inferred that distinct protein networks are perturbed in different types of OC. Here we describe a targeted proteomic strategy for the detection of OC subtype specific protein signatures. The initial discovery is carried out in tissue of genetically engineered mice. Subsequently, these protein signatures are monitored in human plasma with a highly sensitive targeted MS based approach (1).

Methods
To increase the detection sensitivity of tissue-specific protein signatures, we have developed a method for the solid phase extraction of *N*-glycopeptides (SPEG) from tissue and plasma (2). Glycopeptides extracted from control and diseased tissue of a conditional Pten deleted and Kras activated mouse model for endometrioid OC were compared by means of label-free quantification using an LTQ-FT mass spectrometer (Thermo). The data obtained was analyzed using the software tool, SuperHirn (3). These findings were verified in human plasma by targeted mass spectrometry using scheduled selected reaction monitoring (SRM) on a QTrap 4000 (ABI).

Results
A mouse model reflecting the endometrioid OC subtype was employed for this study. Expecting that proteins of interest would be enriched at the tumor site, a quantitative comparison of the glycoproteomes was carried out in diseased versus control tissue. Out of 492 quantified glycoproteins, 152 showed significant up or down regulation and were therefore selected for further verification. Amongst these were Thrombospondin 1, Metalloproteinase inhibitor 1 and Leucin-rich alpha-2-glycoprotein, proteins that have previously been associated with OC, as well as new candidates with no prior relation to OC.
After mining other human ovarian cancer studies, 109 additional glycoprotein candidates, primarily for the serous OC subtype, were selected. In order to verify distinctive protein signatures for serous and endometrioid OC, SRM assays were established for 95 proteotypic peptides corresponding to 73 candidate proteins. These peptides were precisely quantified in plasma samples of serous and endometrioid OC patients using stable-isotope labelled internal standards. Combining the SPEG method with SRM, multiple peptides could be quantified in human plasma reaching a sensitivity of low ng/ml.

Innovative aspects
- Biomarker discovery using an in vivo model system and label-free quantification
- Verification of ovarian cancer subtype specific protein signatures in plasma by SRM

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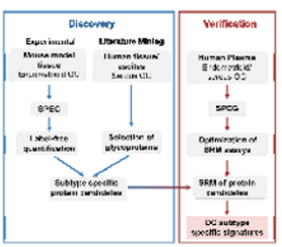

Figure 1: Workflow for discovery and verification of protein signatures in human plasma for stratification of ovarian cancer subtypes

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Discovery of RbAp46 as a novel prognostic marker for distant metastasis in NSCLC

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Introduction
Lung cancer is one of the most prominent causes of cancer death over the world. Discovery of tumor markers those are biological parameters and can be measured in body fluid of suspected cancer patients would be a good opportunity to find a method for early cancer detection and metastatic prognosis in lung cancer. Here, we simultaneously analyzed the lung caner cell secretome and proteomic profiles of pleural effusion from adenocarcinoma patients for lung cancer serum biomarkers discovery. Retinoblastoma associated binding protein 46 (RbAp46), one of the novel serum proteins was chosen for further evaluation as a biomarker for non-small cell lung cancer (NSCLC) and its involvement in modulating NSCLC cellular functions.

Methods
We examined the expression patterns of RbAp46 mRNA in 48 lung tissue cDNA samples by Q-PCR and RbAp46 protein in 154 NSCLC specimens as well as adjacent normal tissues by immunohistochemistry. Serum levels of RbAp46 in 108 lung cancer patients and 71 non-cancer controls were measured with ELISA. The role of RbAp46 in motility was examined using specific siRNA and transwell migration assays.

Results
RbAp46 mRNA and protein levels were significantly upregulated in cancer cells, compared to their normal counterparts. RbAp46 was located exclusively in the nuclear fractions of non-tumor epithelia, but detected in both the nuclear and cytoplasmic fractions of tumor cells (Table 1). Serum levels of RbAp46 were markedly higher in lung cancer patients than in healthy controls, and a combination of RbAp46 and CEA displayed a higher diagnostic capacity than either marker alone. Importantly, elevation of RbAp46 in serum was highly correlated with distant metastasis in NSCLC patients (Fig. 1). Moreover, knockdown of RbAp46 inhibited the migration ability of lung cancer cells.

Innovative aspects
- Combined analysis of lung cancer cell secretome and protein profiles of pleural effusion form lung adenocarcinoma patients will help us to identify the body fluid-accessible biomarkers efficiently.
- Our data collectively suggest that RbAp46 serves as a novel biomarker and prognosticator for NSCLC, and is involved in lung cancer cell migration.

Table 1. Immunohistochemical analysis of RbAp46 expression in 154 NSCLC tissue specimens containing both tumor and non-tumor cells.

RbAp46 localization	Patient No. (%)			
	RbAp46 -*	RbAp46 1+*	RbAp46 2+*	RbAp46 3+*
Non-tumor nuclei	11 (7.1)	138 (89.6)	5 (3.2)	0 (0.0)
Tumor nuclei	0 (0.0)	38 (24.7)	102 (66.2)	14 (9.1)
Non-tumor cytoplasm	140 (90.9)	14 (9.1)	0 (0.0)	0 (0.0)
Tumor cytoplasm	69 (44.8)	69 (44.8)	16 (10.4)	0 (0.0)

* -: negative staining. * 1+: weakly positive staining. * 2+: moderate expression. * 3+: strong expression.

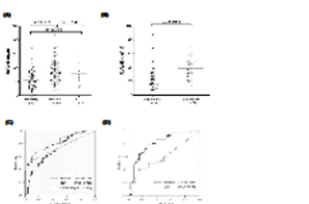

Fig. 1. The serum level of RbAp46 is significantly increased in NSCLC with distant metastasis. (A) Serum levels of RbAp46 in healthy controls and lung cancer patients were measured using ELISA. (B) Serum levels of RbAp46 in NSCLC patients with no metastasis (stages I-III) and distant metastasis (stage IV) were measured using ELISA. (C) ROC curve analysis of RbAp46 (black line), CEA (dashed line) and a combination of both proteins for discrimination between healthy controls and lung cancer patients. (D) ROC curve analysis for discrimination between lung cancer patients with no metastasis (stages I-III) and distant metastasis (stage IV) in NSCLC. AUC, area under the ROC curve.

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Quantitative proteomics for metastatic biomarker discovery in the microdissected oral cancer tissues

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Introduction
Oral squamous cell carcinoma (OSCC) remains one of the most common cancers worldwide, and the mortality rate of this disease has increased for decades. No molecular markers are available to assist the early detection, and therapeutic and outcome evaluations. Systematic identification and quantification of protein expressions in frozen tissue specimens of primary tumor and lymph node metastasis by quantitative proteomics provide an opportunity to discover the biomarker candidates and understand the molecular mechanisms of OSCC pathogenesis.

Methods
Three pairs of specimens of surgically resected primary and lymph node metastasis OSCC lesions were obtained from oral cancer patients who had signed informed consent forms prior to participation in the study, which was approved by the Institutional Review Board of Chang Gung Memorial Hospital at Lin-Ko, Taiwan. After fixing and hematoxylin staining, the tissue surrounding the selected area was cut using a UV laser, and the internal areas were irradiated via soft IR laser pulses in the Veritas Laser Capture Microdissection and Laser Cutting systems. Several selected areas were then adhered to a CapSure LCM Cap and immediately transferred to a 0.5 mL microcentrifuge tube for protein extraction.
The proteins extracted by RapiGest solution were trypitically digested and then labeled in ^{16}O or ^{18}O-buffer system. The equally mixed $^{16}O/^{18}O$-labeled peptides (150μg) were then fractionized in the first dimension by SCX chromatography into 30 fractions, and then subjected to second-dimensional online LC-ESI and off-line LC-MALDI analyses. The emerging spectra were identified by searching against SwissProt database using MASCOT searching engine, and quantified by in-house developed $^{16}O/^{18}O$ quantification software as previous report (1).

Results
The proteins extracted from microdissected primary tumor (T) or lymph node metastasis (M) tissue epithelia of three oral cancer patients were pooled and then performed enzymatically ^{16}O or ^{18}O labeling via trypsin digestion. Then, the equal amounts of ^{16}O- and ^{18}O-labeled primary tumor and metastatic digests (16T18M) were mixed for LC-ESI MS/MS and LC-MALDI-TOF/TOF analyses. The repeat experiments were implemented in the swap-labeled sample sets (16T18M and 16M18T). This strategy successfully identifies and quantifies 673 and 549 proteins, respectively, with at least two unique peptides identified and quantified per protein. Then, the protein with a normalized protein ratio (M/T) higher than 1.5 or lower than 0.67, and consistently determined in two experiments was selected as OSCC candidate. Due to the less reproducibility of protein identification in shotgun proteomics, the proteins solely identified in one experiment with a protein ratio higher than 1.5 and a relative standard deviation less than 30% were also filtered into the candidate list. Finally, 41 protein candidates were selected. Moreover, some proteins have been reported to involve in OSCC invasion and metastasis such as periostin, tenascin and collagenase 3 etc, which demonstrate the reliability and significance of this discovery. To deeply understanding the roles and the application, the level of these dysregulated proteins will be further examined via immunohistochemical validation experiments and enzyme-linked immumosorbent assay.

Innovative aspects
1. Using Laser capture microdissection to reduce the heterogeneity of cells isolated from tissue samples
2. Using stable-isotope labeling and multi-dimensional separation to improve the sensitivity and accuracy of protein identification and quantification.
3. Some protein candidates identified in this study have been proven to play a role in tumor invasion and metastasis, which demonstrates the potential of the metastatic protein signature discovery in this study

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Establishing sera metabolic fingerprinting and recognition models of NPC

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Introduction
Nasopharyngeal carcinoma (NPC) is a malignancy with an unusual geographical and ethnic distribution across the world. It is prevalent in southern China and Southeast Asia, and it has been a serious healthcare problem in these regions for decades. Early diagnosis and early treatment will greatly improve prognosis of NPC. And finding effective biomarkers or biomarker pattern for NPC will benefit its diagnosis, treatment effect, prognosis judgement and mechanism study.

Methods
Combination of hyphenated chromatographic instruments and effective chemometric approaches were adopted in this study. Gas Chromatography-Mass Spectrometry (GC-MS) technology was adopted to analyze the metabolites fingerprinting of sera from 102 cases of NPC at first diagnosis and 107 cases of normal healthy people; The database search strategy was adopted to identify endogenous compounds. Classified Characteristic Variable Method was adopted to find potential biomarkers; model recognition methods (PCA, PLS-DA and ULDA) were adopted to build recognition models for discriminating between NPC and healthy controls. 10-fold cross validation analysis evaluated the prediction ability of these models.

Results
GC-MS analysis acquired the spectrum data of each case, and established the metabolites fingerprinting of NPC and healthy control separately. The spectrum data (totoal ion current, TIC) were input and searched in the NIST107 database, 47 endogenous compounds were identified. Classified Characteristic Variable Method find 4 potential biomarkers, they are lactic acid, L-Lysine, glycine and glucuronolactone. Model recognition methods (PCA, PLS-DA and ULDA) were adopted to build recognition models for discriminating between NPC and healthy controls, recognition ability were 79.43%, 91.39% and 100%, separately.10-fold cross validation analysis evaluated the prediction ability of these models, and prediction ability were 77.51%, 88.04% and 97.61%, separately. Results turn out to be perfect. Additionally, double blind experiment validated the reliability and stability of recognition model ULDA. These results provide a new way for NPC diagnosis and a new idea for finding biomarkers，NPC-associated metabolomic pathways.

Innovative aspects
- Apply GC-MS and chemometric approaches to NPC study for the first time.
- Establish sera metabolic fingerprinting and recognition models of NPC for the first time.

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DBP as a potential biomarker for Multiple Sclerosis (MS) research

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Abstract content:
Introduction
Multiple sclerosis (MS) is one of the most common inflammatory central nervous system diseases. However, there is still a lack of specific biomarkers for diagnostic tests or medicine targets for therapy. In this condition, we analyzed individually the CSF and serum proteomes of patients with MS (the experimental group) and control subjects suffering from other neurological disorders (ONDs group) with two-dimensional difference gel electrophoresis (2D-DIGE) followed by MALDI-TOF and UPLC/Q-TOF technology. We found that DBP could be a potential biomarker for MS disease research and compared the capacities of the two different quantitative proteomic technologies.
Methods
This study was undertaken by two quantitative proteomic technologies (2D-DIGE followed by MALDI-TOF MS and UPLC/Q-TOF MS). ELISA and western-blot was also employed to validate the quantitative proteomic results. We drew a comparison between the capacities of two methods in protein separation to determine the relative overlap in protein separation. The identified proteins expressed differentially were further investigated to reveal their biological pathways regarding the pathogenesis of MS. MetaCore (GeneGo, St. Joseph, MI), which is an integrated software suite for functional analysis of experimental data, was used to build the differentially expressed proteins into biological networks.
Results
In the overlapped gel, 43 protein spots (39 proteins) were identified by MALDI-TOF, of which 17 spots showed more than 1.5-fold difference in abundance between MS and ONDs (Fig. 1). By UPLC/Q-TOF, there were 45 proteins with difference more than 1.5-fold in abundance between the two groups(Fig. 2). We compared the result of 2D-DIGE and UPLC/Q-TOF MS analysis, and found 23 proteins were common in two methods, having the same regulation tendency. The expression of DBP were reduced distinctly in CSF of MS group and was one of the most significant differential proteins among the total. ELISA and western-blot results also support it.
Innovative aspects
(1) It was confirmed that there's an association between up-regulated DBP levels and MS.
(2) Two quantitative proteomic technologies were applied and the capacities of them were compared.

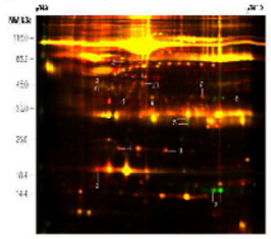

Fig. 1 Representative CSF overlapped gel by 2-D DIGE

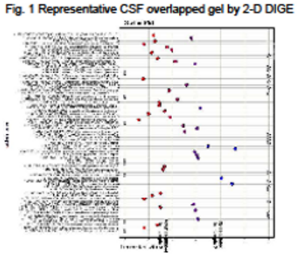

Fig. 2 Distribution map for the expressien levels of proteins identified by UPLC/Q-TOF MS.

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S439

Development of the platform for comparative analysis of the tear proteome based on the AMT approach

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Introduction
Tear fluid is a informative object actively used in ophthalmologic practice. Its protein composition could be used for eye diseases diagnostic like the tests for blood and urine in somatic disorders. Recently 491 proteins were identified in the human tear fluid proteome by mass spectrometry methods coupled with different sample preparation techniques (1). In this work the platform for high-throughput comparative analysis of different tear fluid proteomes was developed. We used the Accurate Mass Tag (AMT) approach in the frame of different mass spectrometry technologies and sample preparation techniques for comparative analysis of the human tear proteomes.

Methods
All tear fluid samples were collected at the Helmholtz Moscow Research Institute of Eye Diseases (Moscow, Russian Federation). On the first stage of the filling AMT tag database tear fluid samples were analyzed using LC/MS/MS method realized on a nano-HPLC system (Agilent 1100) coupled to a 7T Finnigan LTQ FT mass-spectrometer. Samples for LC/MS/MS analysis were prepared in two ways: without prior separation and with 2-D gel separation. Proteins were sedimented by lyophilization and/or by organic solvents, and then desolved in buffers containing chaotropic agents(urea, thiourea) in different concentrations. Proteins were identified against the IPI_human protein sequence database using Mascot. Protein identifications were accepted if they contained at least 2 identified peptides each with a Mascot Score = 24. All accepted identifications were added to the AMT tags database which was developed using MySQL version 5.0.45.

Preliminary results
The new platform for high-throughput comparative analysis of the human tear fluid proteome based on the AMT tag approach was developed. The platform enables to normalize all peptides retention times when new data is loaded into AMT tag data base: normalization is performed with the use of internal calibration peptides common for all the experiments which are stored and just loaded into data base.
The protein composition of the tear fluid from 10 healthy donors aged from 20 to 35 was investigated. From prognostic and pathogenic viewpoints, among the identified proteins the main attention are given to lysozyme, tear lipocalin, alpha-1-antitrypsin, alpha-1- and beta-2-microglobulins, chitinase, cystatins and lactoferrin.

Innovative aspects
- The new AMT tag data base was developed as a platform for comparative analysis of the human tear fluid proteomes.

References
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Acknowledgements
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S440

A Blood Protein Signature for Hepatotoxicity – Systems Strategy for Organ-specific Biomarker Discovery

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Introduction
A protein signature of 11 liver-specific blood biomarkers for hepatotoxicity was identified across mouse and human by antibody based and MS based methods. All the 11 proteins are liver specific at mRNA and protein level. In the mouse model of acetaminophen induced liver injury, there is good correlation between the biomarkers and the pathology along the progress of the injury and against different toxin dosage. Some of them have been validated in liver injury patient samples. The new signature has higher liver specificity, and better sensitivity than the current clinical panel. Working alone, or with the known markers, the novel signature has potential to give better prediction for drug toxicity in preclinical study and for clinical diagnostics.

Methods
Through the analysis of our human and mouse transcriptome database (multiple parallel signature sequences or MPSS), we identified 163 liver-specific transcripts. We purchased 119 commercially available antibodies for 65 liver specific proteins and also generated rabbit polyclonal antibodies for 75 liver specific proteins with multiple epitopes. C57BL/6J mice were administered with high and medium dosage of acetaminophen by i.p. injection. Mouse blood was drawn from acetaminophen treated or control mice at 1h, 3h, 12h, 24h, 48h, 72h and 196h post-injection. We established a high-throughput, label-free method for analyzing serum using Surface Plasmon Resonance Imaging (SPRI) of antibody microarrays (Plexera Nanocapture Gold™).

Results
In the search for biomarkers of hepatotoxicity, SPRI is profiling the serum proteome of mice exposed to toxic compounds. Relative expression profiles were generated, recorded and compared with the histopathology data. Hierarchical clustering of the serum protein levels revealed a distinct hepatotoxic profile corresponding to different levels of severity of liver damage. SPRI results were verified by western blots and MS.

Innovative aspects
Surface Plasmon Resonance Imaging (SPRI) is a high throughput, label-free method of protein interaction analysis. We are using Plexera Nanocapture system in protein biomarker discovery.

References
(1) C Lausted, etc. Quantitative serum proteomics from surface plasmon resonance imaging; Mol Cell Proteomics. (2008); 7(12): 2464--2474

S441

Six protein molecular signature to diagnose tuberculosis from human serum.

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Introduction:
Tuberculosis is one of the biggest global health problems. One third of the world's population is latently infected with tuberculosis. The tuberculin skin test which is commonly used to diagnose tuberculosis infection is inaccurate and has less specificity[1]. Most deaths by tuberculosis are preventable by early diagnosis and treatment. The present study addresses variations in the circulating proteins in serum of tuberculosis patients with the healthy control subjects to find out the biomarkers, which may help in disease diagnosis and prognosis.
Methods:
We hypothesized that serum proteins of human in response to the infection will display several similarities and differences. Further the change in serum protein in patients was monitored at different time frames of drug treatment. Sera were collected from diseased as well as control subjects from our collaborative hospitals. Sera were immunodepleted using Agilent Immuno affinity columns to remove the 7 high abundant proteins. The depleted serum samples were analyzed using high resolution proteomics approach. iTRAQ, a two dimensional liquid chromatography and tandem mass spectrometry approach, was used simultaneously to identify and quantify proteins from the infected and treated versus uninfected controls. The quantification was carried out using ProteinPilot software (2.0).
Results:
The serum proteome was identified using highest stringency (> 99%). The differentially expressed proteins in diseased samples were filtered using a cut off either >2 fold increase or < 0.5 fold decrease in comparison to the healthy samples. Leucine-rich alpha-1 glycoprotein, Serpin peptidase inhibitors, Fibrinogen alpha chain precursor, Alpha-1-acid glycoprotein precursor, Amyloid protein A and Alpha-1-antitrypsin precursor were found to be elevated in disease condition. With treatment the markers come to the normal level as seen in healthy from the elevated level at different period of treatment. In case of healthy contacts increased level of Fibrinogen alpha chain precursor is seen, which may be used as indicator of increased susceptible to the disease. The discovery of these biomarkers might play a significant impact on diagnosis of tuberculosis. These markers could also be used as a molecular signature to monitor the progression of disease and response to therapy. Appropriate utilization of biomarkers can facilitate early decisions about the ability of a new therapeutic to modulate its target and guide patient selection for future clinical trials[2]. These biomarkers might help in developing successful strategy in the development of novel anti-tuberculosis drugs.

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2. RS Wallis et al, Biomarkers for tuberculosis disease activity, cure and relapse, Lancet Infect Dis 2009; 9:162-72.

S442

Combining methods improves plasma proteomic analyses in patients with coronary artery disease

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Introduction. Coronary artery disease (CAD) is the leading cause of mortality in Canada. Biomarkers are precious tools to diagnosis and risk stratification, and the identification of new candidates may substantially impact on public health and health economics. Due to its complexity, the human blood proteome remains challenging to explore. Our study aims at combining various complementary methods to determine the plasma proteome signature of patients at various stages of CAD. Herewith, the key steps and preliminary results are presented.
Methods. The total cohort includes 1006 French-Canadian, 500 controls with a normal coronary angiogram, and 506 symptomatic patients with documented CAD. Two pools were reconstituted to represent the extremes of our population: 1)patients with a previous myocardial infarction (MI) and a recurrent MI over a 5 years follow-up and 2)controls without CAD and no events during follow-up matched for age and sex to the CAD pool (N=18). After a depletion of highly abundant proteins, pools were analyzed using 3 different proteomic methods:1.PF2D system followed by a liquid chromatography tandem mass spectrometry analysis (LC-MS/MS); 2.1D-SDS-PAGE followed by LC-MS/MS; 3.Further depletion of proteins followed by 1D-SDS-PAGE and LC-MS/MS analysis of both flow through (3a) and retained fractions (3b).
Results. Methods 2, 3a, and 3b allowed identification of 108, 125 and 91 proteins respectively. Overlapping proteins (23 between methods 2 and 3a, 44 between 2 and 3b and 3 between 3a and 3b showed significant absolute and relative expressions with various methods. A total of 12 proteins were significantly ($p<0.05$) different in amounts (number of peptides identified) between the 2 pools. Preliminary analyses with the PF2D elution spectra (method 1) displayed numerous different profiles between the two groups. Over a total of 1156 fractions (control: 1172 peaks ACS: 926 peaks) generated, 370 fractions (674 peaks) overlapped between the two pools. 15 fractions (23peaks) differed significantly ($p<0,05$) in amounts while some other peaks were uniquely present in one pool, representing biomarkers of potential interest. Plasma proteome signatures are significantly modeled by the methods serving their recognition. Taking profit of this finding, we are now testing in our population the hypothesis that a combination of methods will sharpen our ability to detect clinically relevant proteins tailored to various clinical situations and to disease staging.
Innovative aspects.
Possibility of selecting the most performing method from a cost/effectiveness perspective:
- Reduced cost with the PF2D method by performing a pre-selection before MS analysis.
- Improved detection of less abundant proteins with further depletion of samples.

S443

sPossibility of neuroprotection to toxic-induced retinal damage by synuclein-beta

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Introduction
Use of dye compound indocyanine green (ICG) to stain transparent retinal surface in vitreo-retinal surgery has facilitated success in the surgical procedure, however it is now known that the use damages retinal tissue (1). Though mechanism of the ICG-toxicity on retina is unclear. To see alternation of retinal proteins affected by ICG, a proteomics study using 2D-DIGE was conducted.

Methods
Sprague-Dawley rats were intravitreously injected with ICG (20 ul: concentration 0.01-25 mg/ml). The retinas were dissected 1, 5, 30, 90 days after the exposure, and the extracted proteins were assessed by 2D-DIGE and MALDI-TOF-MS. Western blot and immunohistochemistry followed.

Results
By the proteomics analysis, over 40 proteins significantly altered in the quantity over 2-fold in a day of the exposure. Among them, synuclein-beta (SNCB) was further focused. Protein level of SNCB gained in retina only when exposed low-dose ICG. Immunohistochemistry on paraffin-embedded retinal sections showed that SNCB is expressed at the cytoplasm of ganglion cell layer, inner plexiform layer, and inner nuclear layer in the retinas of ICG-treated, the controls, and other disease models. Intense expression of SNCB was simultaneously observed at spotted zone, continuously exists at the outer nuclear layer (ONL) only on the retinas that exposed low-dose ICG.

Innovative aspects
- SNCB is a member of synuclein family, co-express together with synuclein-alpha (SNCA) which is a major component of Lewy body, and is suggested to be involved in neuroprotection in Parkinson's disease and Alzheimer's disease (2). Synucleins in human diseases are now intensely studied. Exposure of low-dose ICG induced SNCB at ONL in retina. The phenomenon was not observed in other disease models or by exposure of high-dose, lower-dose, or longer exposure of ICG. This observation may suggest an acute neuroprotective role of SNCB to toxic-induced neural damage in retina involving the spotted zone.
- 2D-DIGE was an ideal approach to screen candidate proteins associated with retinal toxicity.

References
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1 Relative expression level of SNCB (1: control retina 2: retina exposed 0.1 mg/ml ICG for 1day 3: exposed 2.0 mg/ml ICG for 1 day).
2 Intense expression of SNCB at ONL in rat retina after exposure of 0.1 mg/ml ICG for 1 day.

Systems Biology

(S501 – S528)

S501

Phosphoproteome Dynamics Reveals Heat Shock Protein Complexes Specific To the *Leishmania* Infectious Stage

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Introduction. The protozoan parasite *Leishmania* is exposed to a sudden increase in environmental temperature during the infectious cycle that triggers stage differentiation and adapts the parasite phenotype to intracellular survival in the mammalian host. The absence of transcriptional regulation and constitutive expression of heat shock proteins (HSPs) in these human pathogens raise important unresolved questions as to regulation of the heat shock response and stage-specific functions of *Leishmania* HSPs. Here we identify stage-specific phosphorylation of *Leishmania* HSPs as being an important factor in post-translational control of the parasite heat shock response.

Methods. We investigated the signaling networks underlying the *Leishmania* differentiation process and quantified changes in protein phosphorylation across the parasite life cycle sytages utilizing two-dimensional differential gel electrophoresis (2D-DIGE). IMAC-enriched phosphoextracts from promastigotes and host-free amastigotes of three independent repeat experiments were differentially labeled with the spectrally resolvable CyDye™ fluors Cy3 and Cy5 and separated by two-dimensional electrophoresis (2DE) on 11 cm pH4-7 IPG strips and 12.5% polyacrylamide gels.

Results. We identified 189 phosphoproteins corresponding to 94 distinct database entries by MS-MS, which we associated by GeneOntology analysis to six major biological process including protein turn-over, translation initiation, and protein folding. Based on protein quantification and statistical analysis, 38% of the *Leishmania* phosphoproteome showed stage-specific differences with a strong bias towards increased protein phosphorylation in amastigotes. Amastigote-specific phosphoproteins were nearly exclusively implicated in chaperone function, including the co-chaperones HOP/Sti1, cyclophilin 40, and various isoforms of HSP83 and HSP70. Network analysis of the amastigote phosphoproteome perdicted a putative multiprotein complex formed between these chaperones termed foldosome. We confirmed this interaction in *L. donovani* and revealed the foldosome complex by 2D Blue Native electrophoresis. In conclusion, in contrast to the nearly constitutive expression of the *Leishmania* global proteome, our data show that the parasite phosphoproteome is highly stage-regulated emphasizing the importance of post-translational modifications in parasite differentiation. Increased phosphorylation of protein chaperones in amastigotes indicates the presence of stage-specific protein kinases, that use these proteins as substrates and may modulate their activities and interactions. The potential role of chaperone phosphorylation in the formation of the foldosome complex and the role of putative foldosome client proteins in *Leishmania* differentiation will be discussed.

Innovative aspects.

- First quantitative phosphoproteomic study in the human pathogen *Leishmania*
- Identification of parasite-specific phosphorylation sites in highly conserved HSPs
- Identification of a stage-specific heat shock complex implicated in ribosomal biogenesis

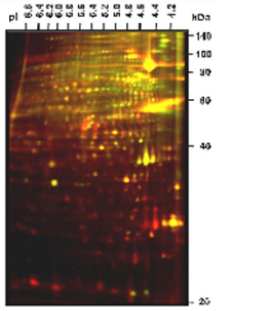

Figure 1: Quantitative analysis of the *L. donovani* stage-specific phosphoproteome by 2D-DIGE analysis using affinity-enriched phosphoextracts from promastigotes and host-free amastigotes, differentially labeled with the spectrally resolvable CyDye™ fluors Cy3 and Cy5.

S502

Global Functional Atlas of Escherichia coli Encompassing Previously Uncharacterized Proteins

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Introduction
One-third of the 4,225 protein-coding genes of *Escherichia coli* K-12 remain functionally unannotated (i.e. functional orphans). Many map to distant clades such as Archaea, suggesting involvement in basic prokaryotic traits, whereas others appear restricted to *E. coli*, including pathogenic strains.

Methods
To elucidate the orphans' biological roles, we followed the guilt-by-association concept, and performed an extensive proteomic tandem affinity purification survey of tagged *E. coli* strains to determine protein participation in physical interactions and multimeric complexes (**Figure 1**).

Results
A high-confidence network consisting of ~6,000 interactions among ~1,750 proteins, including ~450 orphans, or roughly two-thirds of the entire water-soluble proteome was determined. Flux simulated-based clustering of this network allowed us to delimit ~450 putative heteromultimeric complexes. Orphan membership in such complexes suggested participation in widely conserved core biological processes, like protein translation and DNA-replication, as well as in other more specific traits, such as biogenesis of the cell envelope and pathogenic processes, like the biosynthesis of lipopolysaccharide. Further integration of this proteomic-derived network with genomic context information using machine learning algorithms enriched the set of high confidence orphan functional predictions. Genetic and chemogenomic assays were used to validate many of these assignments.

Innovative aspects

- This work provides the **largest** high-confidence network of physical protein interactions in Bacteria.
- Roughly half of the orphans could be assigned to a **specific** functional category.
- All our results are **freely available** in a dedicated wed portal called 'eNET' (1), which contains not only protein interactions and groupings (as other databases do) but also specific function prediction 'labels' useful for mechanistic follow-up studies.

References
(1) Hu *et al.* (2009) PLoS Biology 7(4): e1000096.

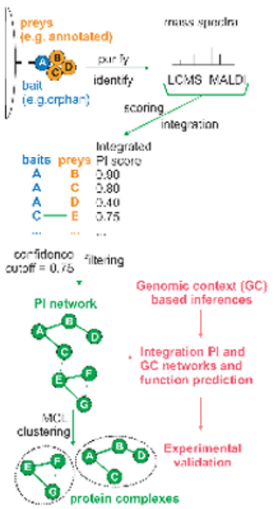

Figure 1. Generation and Integration of High Confidence Physical and Functional Networks and Orphan Function Prediction. Construction of a protein-protein interaction network (PI) based on protein copurification and detection by mass spectrometry (LCMS and MALDI). The joint datasets were scored using a logistic regression with references from low-throughput studies curated in DIP, BIND, and IntAct (gold positives) and proteins in different subcellular localizations (gold negatives),and integrated using a probabilistic model. The resulting interaction network, with edge weights corresponding to likelihood ratios, was clustered using MCL algorithm to delimit multimeric complexes. Genome context methods and machine learning were used to assign functional 'labels' to orphans for following up studies.

S503

A Targeted Protein-Protein "Interact-ome" of the Insulin Signaling Pathway in Drosophila and Compared to Human Cells

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Introduction. The insulin signaling pathway in eukaryotes is central to gene translation, cell growth and proliferation. Drosophila is a sophisticated model system for signal transduction and systems biology studies. We have dissected the insulin signaling pathway through immunprecipitations of key nodal proteins and shotgun mass spectrometry of the prey proteins. These bait-prey experiments produced a protein interact-ome showing many canonical interactions in addition to many novel interactions. Bait proteins such as phosphoinositide-3-kinase (PI3K) and insulin receptor substrate (IRS) were also carried out in human cancer cells and compared to the fly data. This revealed several novel pathway members.

Methods. For the drosophila insulin signaling pathway, tandem affinity purification (TAP) and shotgun LC/MS/MS using an EASY-nLC and Orbitrap XL from twenty bait proteins were performed under basal, insulin and EGF stimulation including TAP controls. The protein-protein interaction network was assembled by label-free quantification of the bait-prey interactions using MS/MS based average TIC and spectral counting. Proteins were identified via Sequest searches versus the reversed FlyBase database. For human cells, multiple myeloma and small cell lung cancer cell lines were prepared by immunoprecipitating (IP) several insulin signaling bait proteins including PI3K, IRS and TSC from basal and drug treated states. The bait-prey interaction results between human and fly were compared with a focus on quantitative differences of signal changes between stimulating conditions using both Scaffold and our in-house developed NakedQuant software. Bait-prey protein lists were compared by reverse BLAST and in-house developed tools in order to find similar novel interacting proteins between fly and human cells.

Results. A comprehensive interactome has been assembled (Fig. 1). Since PI3K is a central lipid kinase in the insulin signaling pathway and the activation of AKT, the protein complex surrounding the Insulin Receptor – IRS (chico in drosophila) – PI3K p85 regulatory subunit in humans was compared to the PI3K p60 regulatory subunit (drosophila). The membrane level of the pathway revealed several proteins that are novel and critical components of the activation pathway in addition to many canonical interactions (example in Fig. 2). One example is the Eps15 (Epidermal growth factor receptor substrate 15) as well as several other novel binders. The criteria for true novel protein binding are 1.) a significant change in signal upon stimulation or drug treatment and 2.) the prey protein must be present from IPs from both drosophila cells and human cells. This ensures that the interaction is conserved over evolutionary time. In addition, we have obtained information based on a genome wide RNAi screen about which bait-prey interactions negatively or positively regulate the pERK and pAKT pathways.

Innovative aspects

- Comprehensive and quantitative bait-prey insulin signaling network in drosophila
- Comparisons of signaling networks in drosophila and human cancer cells
- Novel signaling proteins revealed

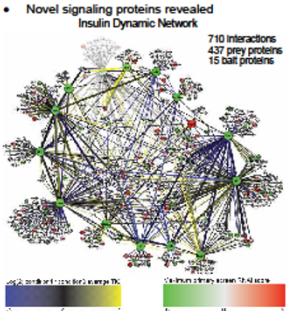

Figure 1. MAPK branch of the dynamic insulin signaling protein interaction network in drosophila.

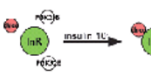

Figure 2. Mass spectrometry based dynamics of bait-prey interactions surrounding insulin receptor.

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S504

Cellular proteome of *Aspergillus fumigatus* reactive with sera of rabbits after experimentally induced aspergillosis

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Aspergillus fumigatus causes invasive aspergillosis, a life-threatening lung and systemic infection in the immunocompromised host. Immunobiology of *Aspergillus fumigatus* is not completely understood. The conidial proteins may be decisive for the Aspergillus-related immunological responses in the host. Proteome analysis to study the properties of functional proteins including those which may elicit protective immunity and the virulent factors involved in host pathogen interactions remains attractive. We previously reported new vaccine candidates and potential new allergens of this important fungal pathogen. In the present study, a protective immune response against the disease was achieved in rabbits and the cellular fungal antigenic proteome that mediated such a response was investigated against the background of vaccine development efforts. 51 different Aspergillus proteins reacted with the sera from the two infected rabbits. Most of these proteins are described in this context for the first time. The results indicate a continuous response of pathogens to escape from the host defense system. Among the proteins, which caused protective immunogenic reactions are proteins related to oxidative stress management, glycolysis and other metabolic pathways. Proteins with suspected cell surface association or crucial function for fungal cell development were also present. As these antigens are newly recognized during the process of the developing immunoprotection, they may not only represent valuable infection markers but also importantly broaden the list of possible vaccine candidates.

S505

Comparative semi-quantitative proteome analysis of five species

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Introduction

While for many years transcriptome data (i.e., the collection of transcribed mRNAs) has been used to approximate the proteome, a number of studies have demonstrated that the correlation between mRNA and protein expression is surprisingly low because of post-transcriptional regulation and variable protein half-lives.
We recently reported (1) the first comparison of two animal proteomes (C. elegans and D. melanogaster) where we showed that abundances of orthologous proteins in metazoans correlate remarkably well, better than protein abundance vs. transcript abundance within each organism. This hints at significant neutral drift of transcript abundances at long evolutionary time scales.
To investigate this further, we now included publicly available (Peptide-Atlas, PRIDE) data of three more species into our comparative proteome analysis: H. sapiens, A. thaliana and S. cerevisiae.

Results

First results confirm the strong correlation of protein abundances observed between worm and fly. Furthermore, there is a negative correlation between median abundance and variation of abundance.

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S506

Molecular Systems Biology of Aging and Anti-Aging

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Introduction

Aging of multicellular eukaryotic organisms affects numerous processes within cells. A challenge is to understand whether aging is the final step of a developmental program or merely a result of the lifelong accumulation of unrepaired cellular damage. The yeast *Saccharomyces cerevisiae* is a valuable model for unveiling mechanisms of cellular aging in multicellular eukaryotes. We use proteomics, metabolomics and chemical biology to study mechanisms of aging in yeast and to define how several recently identified anti-aging drugs extend yeast longevity by targeting these mechanisms.

Methods

Using a high-throughput screening of extensive compound libraries, we identified five groups of novel anti-aging small molecules that greatly extend yeast longevity. Yeast aging can be also slowed down by certain single-gene mutations and by calorie restriction (CR), a low-calorie dietary regimen that extends life span and delays age-related disorders in various organisms. We assessed the effect of several newly identified anti-aging drugs, numerous life-span extending mutations and CR on the age-dependent dynamics of cellular and organellar proteomes, metabolomes and lipidomes, interorganellar metabolic flow, concentration of reactive oxygen species, frequencies of nuclear and mitochondrial DNA mutations, mitochondrial morphology, stress response, apoptosis, and necrosis.

Results

We found that aging yeast merge a number of cellular processes, which we call modules, into a longevity network. Our findings provide evidence that 1) yeast establish a diet-, genotype- and drug-specific configuration of the network by setting up the rates of the processes taking place within each of its modules; 2) the establishment of a network's configuration occurs before yeast enter a non-proliferative state; and 3) different network's configurations established prior to entry into a non-proliferative state define different rates of survival following such entry. We therefore concluded that, by designing a specific configuration of the longevity network prior to reproductive maturation, yeast define their life span. Thus, aging in yeast is a developmental program that progresses through several checkpoints. We found that one of the anti-aging drugs that we identified extends yeast longevity by targeting a distinct set of proteins that function in 1) mitochondria-controlled, age-related apoptosis; 2) the evolutionarily conserved nutrient-signaling network governed by protein kinase A; and 3) lipid metabolism in the endoplasmic reticulum, peroxisomes and lipid bodies.

Innovative aspects

- Aging is a developmental program that progresses through several checkpoints
- Novel concept of a modular longevity network
- Identification of novel anti-aging small molecules and establishment of the mechanisms by which they extend longevity

S507

A Structured Syntax for Minimotifs

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Title: A Structured Syntax for Minimotifs

Introduction: Short linear peptide sequences (minimotifs) impart various molecular functions to cells such as mediating protein-protein interactions, molecular trafficking, and post-translational modifications (1). As the body of information related to minimotifs increases, also does the need to unify and normalize their annotation linguistics (2). We present a minimal functional syntax representing all minimotifs in the MnM 2 database which is understandable by both humans and machines.

Methods: To implement the minimotif syntax we developed a relational model of minimotif functionality which is based on a set of 20 minimotif attributes This relational database explicitly reflects minimotif functions.

Results: The normalized set of attributes was utilized to automatically generate text -based annotations for all Minimotifs in the MnM 2 database (3). We constructed a web site which enables mining of sequences based on these 20 attributes. This tool enables the grouping of sequences from multiple minimotif instances and automates the process of generating consensus sequences and position specific-scoring matrices.

Innovative aspects

-Presentation of an explicit relational model of minimotif function.

-Implementation of the data model for the Mnm 2 database.

-A new web tool for grouping minimotif instances, and building consensus sequences and position-specific scoring matrices.

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S508

Signatures of Human Protein and mRNA Expression Levels – Lessons on Translation and Protein Degradation

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Introduction: In addition to transcription and mRNA stability, translation and protein degradation play a major role in gene expression regulation. However, large-scale datasets on matching protein and mRNA concentrations in human are still rare, although they inform greatly about the combined outcome of translation and protein degradation and global signatures of their regulation (1). Most studies have focused on individual regulators and targets, and we lack, particularly for human, system-wide, quantitative analyses that evaluate the contribution of regulatory elements encoded in the sequence. We developed a method to measure absolute protein concentrations at large-scale using shotgun proteomics data (2) and integrate these with mRNA expression values to comprehensively identify correlates of protein production and turnover for >1,000 human genes.

Methods: Performing microarray and quantitative shotgun proteomics (2) experiments in lysate of the Daoy medulloblastoma cell line, we obtained high-confidence measurements of mRNA and protein expression levels for 1,025 human genes (3). We examine the number of protein molecules produced per mRNA as a measure of the combined outcomes of steady-state translation and protein degradation. We focus on a set of sequence features that play dominant roles in translation regulation: length, nucleotide composition, and structure of the coding sequence and untranslated regions (UTRs), composition of the translation initiation site, presence of upstream open reading frames (uORFs), putative target sites of miRNAs, codon usage, amino acid composition, and protein degradation signals.

Results: Protein and mRNA concentrations correlate surprisingly well (Fig. 1): ~30% of the variation of protein expression levels is explained by variation in mRNA expression, the other ~70% can be explained by translation and degradation regulation, and by noise. Strongest single correlates of the protein-per-mRNA variation are listed in Tab. 1. We discuss these features and their interpretation in detail. For example, much of translation and protein degradation is influenced by sequence lengths (Tab. 1) which in turn is a correlate of translation fidelity, protein folding, presence of secondary structures and regulatory binding motifs in the UTRs. We also identify genes of strong regulation, e.g. with uORFs repressing translation.

Innovative aspects:

- Large-scale quantitation of protein expression in human cells
- Comprehensive analysis of sequence-encoded correlates of protein-per-mRNA relationship - comparative assessment of aspects of global translation and protein degradation regulation
- Identification of strong candidates of regulatory mechanisms

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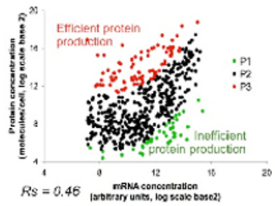

Figure 1. Protein and mRNA concentrations correlate significantly at a log-log scale. Genes with extremely high (red) or low (green) protein-per-mRNA ratios are likely regulated at the level of translation or protein stability.

Correlation with	Protein-per-mRNA (Spearman Rank)
Sequence lengths (coding strand, 3'UTR, 5'UTR)	-0.53***
Protein stability (PEST motif)	-0.49***
mRNA stability (mRNA degradation)	-0.37***
Amino acid composition (S, Q, L, G)	-0.24***
Translation initiation (upstream AUG)	-0.19***

Table 1. Features with the strongest partial correlation with protein expression values, fixing mRNA expression values. *** P-value<0.0001

S509

Integrating Genomics and Proteomics Data Using Cross-Linking Databases

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Introduction
Databases such as Entrez and iProClass (1) cross link various protein and gene identifiers. Researchers use these databases to match proteins and genes, and integrating their proteomics and genomics data. However with each new version of a database, records and their identifiers could be added, modified, or removed. Synchronizing these changes in the cross-linking databases inevitably leads to problems. Here we determine how the use of different identifiers and cross-linking databases affect the accuracy and completeness of the integration process.

Methods
We first linked the 4016 proteins from a previous proteomic experiment (2) to records in the cross-linking database using common external protein identifiers. To evaluate this procedure, we determined how many proteins were linked to the iProClass database (1) if we used different identifiers such as GeneInfo identifier and UniProt accession. Then we used the protein-gene identifier links in the cross-linking database to establish the link from the internal protein identifier to Entrez gene identifier. To evaluate this procedure, we determined how many proteins were linked to their genes through two cross-linking databases: Entrez and iProClass. Figure 1 illustrates these two evaluations. We performed sequence alignment to verify all protein-gene pairs.

Results
For the first evaluation, we linked 92% (1,325/1,443) of the proteins by their UniProt accession to the iProClass database. In contrast, we linked 71% (2,807/3,955) of the proteins by GeneInfo identifier to the iProClass database.
For the second evaluation, we linked 86% (3,388/3,955) of the proteins to their genes through the Entrez database. In contrast, we linked 69% (2,744/3,955) of the proteins to their genes through the iProClass database. In all but 8 cases, the protein was linked to the same gene regardless of the cross-linking database used.
In summary, 15-20% proteins were lost when linking was done across sequence repositories. Most data were retained when both the database and identifier used throughout the experiment were from the same sequence repository. The great majority of the protein-gene linkages were correct; only a few mismatched pairs were detected by sequence alignment. If used correctly, we found using identifiers and cross-linking databases is a very effective way to integrate proteomics and genomics data. Next we will evaluate if peptide sequences can be used to rescue protein-gene pairs lost due to incorrect identifiers.

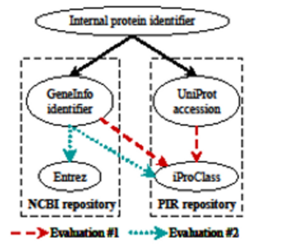

Figure1. Linking internal protein identifiers to the cross-linking databases. Evaluation #1 (dashed) was designed to evaluate effects of using different identifiers. Evaluation #2 (dotted) was designed to evaluate effects of using different cross-linking databases. National Center for Biotechnology Information, NCBI; and Protein Information Resource, PIR.

Innovative aspects

- Evaluate how the use of different identifiers or cross-linking databases affects the accuracy and completeness in genomics and proteomics data integration.
- Effects of linking identifiers across sequence repositories.

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S510

pSILAC in the zebrafish as a tool to study posttranslational regulation by microRNAs *in vivo*

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Introduction
Animal microRNAs (miRNAs) are small endogenous RNA molecules that regulate gene expression by destabilizing transcripts and/or by inhibiting protein translation. While computational approaches predict that a large fraction of genes are regulated by miRNAs, experimental analysis of miRNA targets remains a major challenge. We recently developed pulsed SILAC (pSILAC) as a novel method to study cellular protein production at a global scale. Using pSILAC we found that a single miRNA induces widespread changes in protein synthesis in cultured Hela cells (1). However, the impact of miRNAs on protein production *in vivo* is not known. The zebrafish *Danio rerio* is a powerful model system that is widely used to study early vertebrate development and miRNA function (2). Importantly, the zebrafish zygote develops rapidly with all organs forming within a few days post-fertilization. Here, we developed pSILAC in the zebrafish to analyze protein synthesis during embryogenesis. Combining pSILAC with miRNA depletion experiments allows investigating for the first time how miRNAs affect protein synthesis *in vivo*.

Methods
One-cell stage Zebrafish embryos were micro-injected with either medium (Lys4) or heavy (Lys8) labelled lysine. For microRNA depletion experiments one-cell stage embryos were co-injected with morpholino oligos targeting specific microRNAs (Fig. 1). Mixed Lys4/Lys8 protein extracts of at least 20 pooled embryos per time point/treatment were fractionated by SDS page, followed by LysC in-gel digest and LC-MS/MS analysis on an LTQ-Orbitrap instrument. Protein identification and quantification was performed using MaxQuant software and Mascot database search (3).

Results
Lysine labeling (Lys4, Lys8) was chosen, as no conversion into other amino acids occurs. Critical parameters such as time and site of injection, as well as amino acid concentration were optimized, precluding any obvious effect on animal growth and development, and allowing consistent and reproducible protein quantitation. Lysine incorporation rates ranged from 80% at 24 hours to 55% at 96 hours post-fertilization, demonstrating that pSILAC can be used to study *in vivo* protein synthesis during early embryonic development. Importantly, the zebrafish system also lends itself to studying miRNAs in vivo as it is possible to knock-down endogenous miRNAs with morpholinos. Hence, pSILAC in the zebrafish model offers the unique possibility to investigate the effect of miRNAs on protein production in vivo.
In conclusion, our *in vivo* application of pSILAC in zebrafish embryos represents a novel quantitative proteomic method to study early vertebrate embryogenesis. This method can reveal important aspects of animal development, such as posttranscriptional regulation of gene expression.

Innovative aspects

- first analysis of miRNA targets at the proteome level *in vivo*
- first application of SILAC to the zebrafish model

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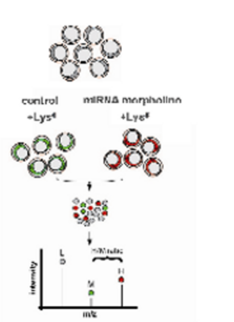

Figure 1. Zebrafish embryos differentially labeled with medium (M) or heavy (H) lysine allow for analysis of miRNA mediated regulation of gene expression by quantitative mass spectrometry.

S511

Cytoprotective effects of glutamine in mesothelial cells after exposure to peritoneal dialysis fluid

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Introduction
Peritoneal dialysis (PD) is a frequently used treatment modality especially for children with end-stage renal disease. We have recently shown that peritoneal dialysis fluids (PDF), which harm the mesothelial cell layer of the peritoneum by their cytotoxicity, lead to upregulation and conditioning of chaperones such as the heat shock proteins. PDF stress involves molecular processes, relevant for the stress response [1]. However, these cellular counter-measures may be insufficient. Aiming to enhance chaperone protection, we tested the addition of glutamine to PDF for cytoprotective effects.

Methods
Confluent immortalized mesothelial cells (MeT-5A) were subjected to either normal medium as control or 1:1 mixtures of medium and PDF (Dianeal PD4, Baxter) for 24 hours at varying levels of glutamine. Cells were harvested and equal amounts of 70µg of total protein (250µg for preparative gels) were loaded onto 24cm pH3-10NL IPG strips (Bio-Rad). Isoelectric focusing was carried out by increasing voltage gradually to 8000V (20°C, max. 50µA/gel) using a Protean IEF cell (Bio-Rad). SDS-PAGE was performed on a Dodeca Plus cell (Bio-Rad). Spots were visualized using RuBPS fluorescent dye [2] following the protocol of Lamanda *et al.* [3]. The gel-images were analyzed using Delta2D 3.6 (Decodon) and significantly altered spots were picked automatically for identification by MALDI-TOF MS and MS².

Results
Two independent biological experiments were carried out using three different glutamine levels (glutamine deficiency, normal glutamine, pharmacologic level), each of them under control and PDF stress conditions. Gels were run on a large format vertical 2D gel electrophoresis system. Image fusion in Delta2D led to a common pattern of 1028 spots. Spots with differentially altered abundance were identified using MS techniques. The resulting protein lists were completed in the biological context using next neighbor expansion [1] and analyzed with the aid of gene ontology databases. Bioinformatics exploration of MS-identified proteins indicates involvement of already described biological processes and pathways regarding control glutamine levels. Glutamine deficiency and addition of glutamine to a pharmacologic level lead to a shift of elicited pathways showing the involvement of starvation, cellular repair and stress response mechanisms as well as immunity and defense mechanisms in cytoprotection of mesothelial cells after PDF exposure. Because these findings we conclude that glutamine represents a potential cytoprotective additive to PDF to enhance cytoprotective mechanisms in PD.

Innovative aspects

- Use of proteomics techniques in clinically relevant setting of peritoneal dialysis.
- Addition of cytoprotective agent glutamine and analysis of elicited cellular processes.
- State-of-the-art bioinformatics techniques to interpret the mechanisms of cytoprotection in mesothelial cells.

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S512

Identification of proteins regulated by dDAVP and ANG II in kidney IM using systems biology

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Introduction
Vasopressin(dDAVP) and angiotensin II(ANG II) are the peptide hormones which have relation to renal water and sodium reabsorption, and these are generally involved in cAMP/PKA and phosphoinositide pathways, respectively. The relationship of these hormones is not clearly understood even if angiotensin II is known to increase vasopressin-stimulated urea permeability and aquaporine-2 trafficking. By combining proteomics and systems biology, we found the cross-talk between vasopressin and angiotensin II in kidney inner medulla (IM).

Methods
Four groups of experiments were performed. Two groups were treated by dDAVP (40ng/hr for 5 days, n=3) and ANG II (100ng/min/kg for 5 days, n=3) to study action of vasopressin and angiotensin II, respectively. For the third group, dDAVP (40ng/hr for 5 days) and ANG II (100ng/min/kg for 5 days n=3), was treated simultaneously, to examine the relationship between vasopressin and angiotensin II. The fourth group was vehicle infusion as a control (n=3). We isolated kidney IM from rat, and analyzed proteome of IM by label-free quantification with nanoUPLC Q-Tof mass spectrometry (Waters). The mass spectra were analyzed using ProteinLynx Global Server 2.3(IDENTITY^E ,Waters inc.). The proteomic results were analyzed systems biology tools.

Results
Kidney IM was isolated and analyzed between control and hormone treated rat. We identified approximately 200 proteins related to various signal pathways from rat kidney. The proteomic results were analyzed by systems biology approach and we could gain protein network model of vasopressin and angotensin II pathway. This result would help to know the relationship between vasopressin and angiotensin II.

Innovative aspects
- Understand the interaction between vasopressin and angiotensin II in kidney inner medullar in proteome levels
- Identification of novel target proteins related to these two hormone actions

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S513

Endoplasmic reticulum (ER) targeting of Phospholamban: The di-arginine motif in ER retention

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Introduction: Phospholamban (PLN) is an inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (1,2). The lack of a classic ER retention signal in PLN led us to investigate the di-arginine motif in the cytoplasmic region of PLN. PLN di-arginine mutants showed that PLN retention is dependent on an intact di-arginine motif (figure1). Bioinformatic analysis determined that the di-arginine motif is found in a significant number of all ER/SR gene ontology annotated proteins. By mutating candidates identified in the bioinformatic screen we indicate that the di-arginine motif maybe an ER retention sequence.

Methods: We performed site directed mutagenesis to generate arginine motif PLN mutants and analyzed the subcellular localisation of an N-Flag tagged wildtype and mutant PLN by confocal microscopy and by subcellular fractionation using a 20-60% linear sucrose gradient. The bioinformatic screen retrieved ER proteins with retention motifs KK and RR within the first 25 residues and ER retrieval motif XDEL in the C-terminal of proteins were from the Gene Ontology (GO) database from proteins with GO identifications as ER (GO:0005783) and SR (GO:0016529). Proteins were then analysed using Protein Centre (Proxeon, Denmark). Candidate proteins were selected from the screen and their subcellular localisation determined using the 20-60% linear sucrose gradients.

Results: In the present study we generated arginine motif mutants and analyzed the subcellular localisation of wildtype and mutant PLN. We showed that PLN retention in the ER is dependent on its R13/R14 di-arginine motif. Co-immunoprecipitation of endogenous PLN from cardiac tissue identified the minimal machinery necessary to form retrograde transport vesicles ARF1 and COP I coat to be co-immunoprecipitated with wild type PLN but not with PLN R Δ14. This data then implies that COP I requires the intact di-arginine motif in PLN since mutation of this motif results in COP I not binding to PLN RΔ14 and its mislocalization from the ER. The importance of the di-arginine motif led us to bioinformatically analyse its frequency in all ER and SR gene ontology (GO) annotated proteins and found that a significant number of proteins have a di-arginine motif within the first 25 amino acids, indicating that it may be used as an ER retention sequence. We demonstrated this by mutating the di-arginine motif in two candidates identified in the bioinformatic screen by subcellular fractionation that mutation of the di-arginine motif caused mislocalization of the resident ER proteins.

Innovative aspects:
- The di-arginine motif is an ER retention motif for PLN and may be a general ER retention signal.

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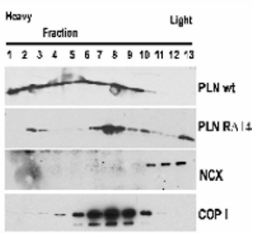

Figure 1: PLN arginine mutants are not retained in the ER: Subcellular fractionation of wild type NF-PLN and the RΔ14 PLN construct. HEK cells were transiently transfected with tagged WT-PLN or PLN mutants and cell lysates run on a continuous 20-60% sucrose gradient. Fractions 1-13 contain the highest to lowest sucrose concentrations respectively. The plasma membrane protein Na/Ca exchanger (NCX) and the ER marker COP I were detected as markers to monitor the fractionation procedure.

S514

System-wide in depth analysis of protein half-lives in mammalian cells

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Introduction
Protein and RNA half-lives are critical parameters that determine dynamic properties of gene expression. While large-scale data sets for mRNA stability are now available(1), protein turnover times have thus far only be determined for a few 100 proteins(2). Accordingly, a systematic comparison of protein and mRNA half-lives has not yet been performed. Here, we present a system-wide analysis of protein turnover in mammalian cells by employing metabolic pulse of newly synthesized proteins.

Methods
3T3 mouse fibroblasts were metabolically pulse labelled with heavy stable isotope versions of essential amino acids (SILAC(3)) for different length of time to assess *de novo* protein. Protein samples were analyzed by LC-MS/MS using an LTQ-Orbitrap instrument followed by quantitation of protein synthesis using the MaxQuant software package.

Results
In total, we quantified more than 4,800 proteins with high confidence (false discovery rate < 1%), thus allowing for half-live calculation of a large fraction of the proteome. Overall, protein half-lives range from 0.3h to 500h with a median half-life of 19.7h. Protein half-lives were analyzed with regard to physicochemical properties as well as in terms of functional categories over- or underrepresented. Preliminary data suggests a correlation between turnover times and both protein length and amino acid composition.
The PANTHER classification system was used to categorize proteins with highest and shortest half-lives according to their biological process. We observed that high and low turnover proteins are inversely enriched in functional categories (Fig.1).
By integrating protein data and mRNA half-lives obtained by pulse labeling with the nucleoside analogue 4-thiouridine (4sU) and next-generation sequencing we are currently trying to directly compare protein and transcript half-lives on a global scale in mammalian cells. In addition, we are expanding the analysis to PC12 cells expressing mutant huntingtin (htt) prone to accumulation as a model system for polyQ expansion-based neurological disorders. We seek to elucidate how mutant htt affects mRNA and protein dynamics thus contributing to disease progression.

Innovative aspects
- Large-scale analysis of protein half-lives in mammalian cells
- Integrative analysis of global protein and transcript half-lives under physiological and pathophysiological conditions

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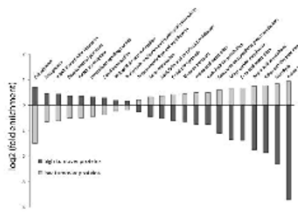

Fig.1. Correlation between high and low turnover proteins based on PANTHER Biological Process Ontologies

S515

A novel method to characterize the NuA4 lysine acetyltransferase and its substrates

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Introduction
The extent and biological consequences of lysine acetylation are poorly characterized outside the context of histone proteins. NuA4 is an essential acetyltransferase in budding yeast and genetic studies have linked it to a myriad of cellular processes beyond its traditional chromatin roles mediated by histone acetylation (1). In order to identify new acetylation targets of NuA4 we have developed a method that generates both a protein interaction map and identifies acetylated lysine residues. This method has dramatically expanded our view of the targets and pathways regulated by NuA4.

Methods
We have coupled NuA4 modified chromatin immunopurification, or mChIP (2), to an *in vitro* lysine acetyltransferase (KAT) reaction. The mChIP technique enhances the solubility of chromatin-associated proteins in clarified yeast cell lysates, thereby increasing the depth of coverage of NuA4 physical interactions. As acetylation levels may be low and/or rare, the subsequent KAT reaction enriches acetylation levels of the resulting NuA4-associated protein network *in vitro*. Samples were processed using the proteomic reactor (3), a microfluidic device enabling sample enrichment, clean-up, and enzymatic processing for an efficient sample preparation prior to analysis by LC-MS/MS. Interactions and acetylated lysine residues were identified using the Mascot search engine.

Results
Proteins that physically interact with NuA4 *in vivo* suggest pathways by which NuA4 may function and further represent putative acetylation targets. Our NuA4 interaction map includes ~200 novel interaction partners for NuA4, supporting its role in chromatin dynamics, and further implicating NuA4 in hitherto unknown processes such as cytoskeleton architecture. Coupling a KAT reaction to the NuA4 immunopurification adds a unique layer of information onto the interaction data, and allowed us to identify several novel NuA4 substrates, importantly discovering that NuA4 acetylates at least six of its own subunits. Analysis of this data revealed a previously unknown associated between NuA4 and the spindle pole body. The cellular implications of this association are currently being explored in our laboratory. Our novel methodology provides a unique paradigm for the identification and analysis of KAT substrates.

Innovative aspects
- The development of a novel methodology to characterize KATs and their substrates.
- The first NuA4 physical interaction map, providing key mechanistic insights into NuA4's impact on chromosome segregation via its association with the spindle pole body.
- The simultaneous identification of putative NuA4 substrates along with the specific lysine residue that is modified.

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Quantitative proteomic profiling of the interaction of *Francisella tularensis* LVS with macrophages

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Introduction
Francisella tularensis is a highly infectious intracellular pathogen that is preferentially internalized by professional phagocytes. The mechanisms that initiate bacterial uptake are still poorly understood. Recently, it has been published that *Francisella tularensis* enter macrophages via specialized membrane domains, so-called membrane rafts (1). Participation of Fc receptor, complement receptor 3, class A scavenger receptor and mannose receptor in bacterial uptake have been already reported. Here, we employed mass-spectrometry based approach in order to elucidate alteration of the raft protein composition upon *F. tularensis* internalization.

Methods
We have performed an iTRAQ workflow to identify and quantify proteins associated with detergent – resistant membrane domains (DRMs) of macrophages during the early stage of *Francisella tularensis* infection, using the macrophage-like cell line J774.2 and *Francisella tularensis* live vaccine strain. 4 samples of different time of infection were labeled by iTRAQ according to the manufacturer's iTRAQ reagents protocol from Applied Biosystems with slight modifications. Protein identification and quantification for iTRAQ experiments was carried out using the Protein Pilot software v2.0 (Applied Biosystems MDS Sciex). Only proteins identified with at least 95% confidence, or a ProtScore of 1.3 were reported.

Results
As a result, we were able to identify a total of 150 proteins associated with DRMS, out of these 28 showed significant alteration upon infection. The majority of the modulated proteins belonged to the raft structural, cytoskeletal and signaling proteins. The identification of the cytoskeletal proteins including actin and actin-related proteins give the evidence, that the actin skeleton play important role in the control of membrane domain organization. The second group is comprised of proteins known to be involved in a range of signal transduction pathways, such as heterotrimeric and small G proteins and Src family tyrosine kinases.

Conclusion
In summary, the data reflect the macrophage immune defence mechanism against invading pathogen as well as how bacteria modulate immune processes of macrophages in order to invade host cells.

Innovative aspects
- Using quantitative proteomic approach to characterize host-pathogen interaction

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Proteomic Profiling of Protein Kinases

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Introduction
Dysregulation of protein kinases is often intimately connected with severe diseases, especially cancer (1, 2). Gene expression data alone have not been sufficient to elucidate the complexities of such diseases. Proteomic profiling of kinases would thus represent a powerful technology for displaying the physiological state of a cell or tissue, and potentially be a revolutionary diagnostic. Here we employ a targeted highly specific and sensitive mass spectrometry-based approach towards the generation of multiplexed quantitative kinase profiles in cultured cell lines.

Methods
The identification of protein kinases that are expressed in different human cell lines has been performed with high resolution and mass accuracy using the LTQ Orbitrap. For this purpose total cell lysate was digested and subjected to OffGel Electrophoresis (OGE). LTQ Orbitrap profiling data as well as data collected in PeptideAtlas (www.peptideatlas.org) were used to establish a set of peptides that ideally unambiguously identifies each kinase of interest. Subsequently, we developed selected reaction monitoring (SRM) assays for these peptides to profile kinases in total cell lysates on a 4000 QTRAP mass spectrometer for the purpose of quantification with heavy labeled synthetic peptides. The SRM transitions were generated via libraries of synthetic peptides (3).

Results
More than 100 protein kinases per human cell line were detectable using extensive LTQ-Orbitrap profiling. This LC-MS/MS profiling was performed on tryptic digests of total cell lysate and after OGE separation into 24 fractions (pH 3-10). For a comprehensive dataset, fractionation of lysate is necessary. Most kinase peptides were detected in the acidic pI range, consequently basic fractions were pooled. As an example, an LTQ Orbitrap analysis of 16 pooled fractions of T47D cells resulted in the detection of 4,222 proteins, including 116 protein kinases. This compared with 1,032 proteins and only 11 protein kinases, with omission of the OGE step.
SRM assays were established with the synthetic versions of the target peptides and TIQAM software to generate SRM transitions and collision energies. Several of these assays were successfully applied to total cell lysate using a 4000 QTRAP, examples will be shown. More detailed analyses are currently in progress, with the aim of significantly expanding the list of kinases to profile, and the use of isotopically labeled synthetic peptide standards for absolute quantification.

Innovative aspects
SRM-based targeted proteomic approach for profiling of low abundance protein analytes, such as protein kinases, in complex biological samples.

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A systems biology approach to dissecting host-pathogen interactions

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Introduction We have carried out a series of studies to dissect *Salmonella*-host interactions using modern mass spectrometry-based methods. We compared expression of proteins and transcripts by sample matched proteomics and transcriptomics. Gene expression was analyzed both *in vitro* under 4 different growth conditions and *ex vivo* in primary murine macrophages.
Methods The proteomics identifications were initially made via MS-MS and subsequent identification via the AMT (accurate mass and time) approach to increase experimental depth. Approximate quantitation of proteins was determined by spectral counts using MS-MS and peak areas using the AMT tag approach. Separate analyses were used to identify small proteins and peptide degradation products as these are often missed.
Results Overall 3400 *Salmonella* proteins were shown to be expressed under at least one condition. The conclusions of this series of studies are that: 1) A very limited set of proteins were identified that were not previously annotated. 2) Conditions that mimic intracellular growth result in higher apparent protein turnover and/or selected degradation of a subset of proteins to peptides, which have higher apparent homology to mammalian proteins (Fig 1). 3) Proteins were identified that show similar expression profiles to known virulence factors and, in a few cases, were shown to be virulence factors. 4) Inside primary macrophages, *Salmonella* abundantly expresses proteins that were never identified in any of the four growth conditions employed including conditions that partially mimic the *Salmonella* containing vacuole [1]. 5) More than 20% of all *Salmonella* proteins are post-transcriptionally regulated under at least one growth condition [2]. Our most recent efforts have focused on how key transcriptional and translational regulators that are required for virulence alter expression under selected growth conditions and during intracellular growth.

Innovative aspects:
- A utilizing a large multi-pronged "systems" analysis of the pathogen *Salmonella*
- Sample matched proteomics and transcriptomics provides a richer view of complex host-pathogen interactions

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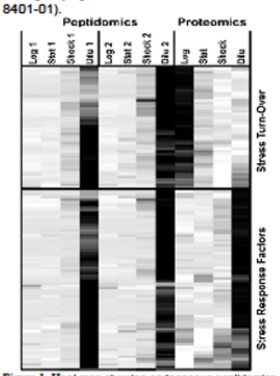

Figure 1. Heat map showing endogenous peptidomics results compared to global proteomics results, scaled using the Z-score across protein (with black representing a Z-score of 2.5 and white a Z-score of -1.0.

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Proteomics to Interactomics: a streamlined approach to identify high-yield hits

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Introduction:
Although recent preclinical and clinical studies strongly suggest that pirfenidone (PFD), a small molecule prevents fibrosis in various diseases, the mechanisms underlying its antifibrotic action are incompletely understood. To uncover novel biochemical pathways operative in mediating PFD drug action as an anti-fibrotic, we used LC/MS/MS proteomics to generate proteins unique to normal and diabetic (± PFD treatment) mouse kidneys. 47, 41 and 21 proteins respectively were found to be unique to normal, diabetic and PFD-treated kidneys.

Methods:
In order to take advantage of the human protein protein interaction (PPI) data, we generated the human orthologs of the above unique proteins by Blastall and Inparanoid algorithm. In the human PPI database, 32, 27 and 14 proteins were respectively found to be unique to normal, diabetic and PFD-treated diabetic groups. Subsequently, we used an in-house MySql repository comprising approximately 87000 interactions, to list all the putative and known interactions for the above proteins. The entire set of proteins in each analyzed PPI network was considered as background set to calculate enrichment score with a cognate p-value.

Results:
1347 interactions for normal, 397 for diabetic and 655 for PFD-treated diabetic group were pulled out, and subjected to pathway enrichment analysis. From this bioinformatics analyses, RNA processing, splicing and processed RNA localization were shown to be significantly altered in the PFD treatment group. By immonoblotting, we validated that PFD affected the phosphorylation levels of an RNA translation initiation factor (eIF4E), and an RNA translation elongation factor (eEF2), thus directly inhibiting RNA translation. In conclusion, systems biology approach can be used to elucidate the anti-fibrotic mechanism of PFD action. Directed bioinformatic analyses assists in framing and addressing questions relevant to progressive organ disease, and to identify mechanisms of novel therapeutics using proteomics.

Innovative aspects
- Proteomics high-throughput data integration
- Identification of pathways relevant to PFD targets
- Generation of hypothesis and experimental validation

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The chromatin proteome and a systems approach to understanding genetic stability

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Introduction
To understand how different processes involved in the organisation, replication, repair and segregation of DNA are coordinated, we have performed quantitative 'time-lapse' proteomics on chromatin progressing: (I) - from anaphase through S to G2; and (II) – from G2 to M to early G1. To date we have examined how interphase chromatin (I) changes in response to replication inhibition. This revealed a number of distinct functional modules that are synchronised with, and respond to, the progression through interphase of the cell cycle.

Methods
Nuclei were assembled in vitro in Xenopus cell free extracts and associated proteins were eluted from chromatin at different time points, digested with trypsin and subjected to LC MS/MS analysis. The proteins abundance was estimated from the extracted ion chromatograms (XIC) of their corresponding peptides. Using fuzzy c-mean clustering (FCM) proteins were grouped into clusters based on the dynamics of their association with chromatin. Each group was analysed for functional enrichment by collecting ontological terms associated with the participating proteins.

Results
~75% of proteins associated with interphase and metaphase chromatin in the *Xenopus* system varied in abundance by >15%, suggesting that the chromatin proteome is highly dynamic. To date the detailed analysis was performed on replicating chromatin [1]. We have assigned proteins to different clusters based on their pattern of chromatin association (Fig.1). GO annotation analysis demonstrated that each cluster contained functional groups of proteins involved in different nuclear processes related to progression through interphase (Fig.1a). We also blocked DNA replication by inhibiting either replication licensing or S phase CDK activity. This revealed an unexpectedly broad system-wide effect on the chromatin proteome, indicating that the response to replication inhibition extends to many other functional modules in addition to the replication machinery. Several proteins that respond to replication inhibition (including nuclear pore proteins) co-precipitated with the Mcm2-7 licensing complex on chromatin, suggesting that Mcm2-7 play a central role in coordinating nuclear structure with DNA replication.
On the basis of their dynamics on chromatin several proteins were selected for further study. Bod1 selected on strict association with mitotic chromatin appeared to be a novel kinetochore protein required for chromosome biorientation [2]. Association of ELYS/Mel-28 with chromatin matched the dynamics of various nuclear pore proteins and its behaviour changed when replication licensing was inhibited. We provided evidence that ELYS plays an essential role in coordinating nuclear assembly and DNA replication licensing. These results suggested a novel mechanism by which these early cell-cycle events are coordinated with one another [3]. Several other proteins from our analysis of the interphase chromatin proteome are currently under further investigation. We continue analysis of chromatin proteome dynamics during G2-M-G1 cell cycle transition and its responses to number of genotoxic stresses.

Innovative aspects
- Systems approach to study chromatin response to metabolic stress.
- System-wide effect of replication inhibition on the dynamics of the chromatin proteome
- Central role of Mcm2-7 licensing complex in coordinating nuclear structure with DNA replication

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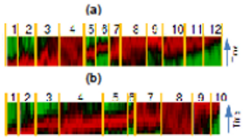

Figure 1. FCM clustering of temporal profiles of polypeptides associated with (a) - replicating chromatin, (b) – M-phase progressing chromatin

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Towards a Systems Approach to Understanding Breast Cancer

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Introduction

It is increasingly evident that breast cancer outcome is strongly influenced by signals emanating from tumor-associated stroma. However, little is known about how gene expression changes in this tissue affect tumor progression, nor how changes in the tumor proper modulate the associated stroma. A better understanding of these signaling interactions especially with respect to disease progression may suggest a broad spectrum of new and radically different therapeutic avenues that promise to be more robust than traditional epithelial-specific therapies. However, there are considerable technical and quantitative challenges that must be addressed, if we are to achieve this insight.

Methods
Over the past five years, we have become proficient with the use of laser capture microdissection (LCM) followed by Agilent-based gene expression profiling of epithelial, endothelial, and stromal cells both within the tumor proper and from morphologically normal adjacent tissue. Recently, we have begun to explore our human clinical samples and our mouse models of breast cancer using various forms of tiling arrays for epigenetic and microRNA based studies. Moreoever, we have developed collections of all available mouse and human breast cancer related high-throughput profiles. In fact, we now have close to 6,000 patient or mouse profiles within our platform that can be explored through a host of traditional and de novo bioinformatics methodologies.

Results
Over the past five years, we have developed a collection of breast cancer-associated molecular signatures analogous to MSigDB but tailored to breast cancer in addition to a statistical toolkit entitled BreSAT (Breast Signatures Analysis Tool). Whereas traditional methods such as GSEA require an a priori partition of the samples into two or more classes, our approach makes better use of the broad panels of samples that span this heterogeneous disease. Succinctly whereas GSEA is a supervised pathway analysis tool, BreSAT represents an unsupervised pathway analysis tool. Moreover our collections of profiles with these tools have allowed us to conduct several meta-analyses towards predictors of patient outcome & recurrence. A very recent result from our lab confirms that the majority of prognostic predictors are primarily accurate for the luminal subtype of breast cancer, & only slightly better than random within the other subtypes. Finally, we used these tools to compare the molecular profiles of approximately 50 mouse models against the human disease. We find that no mouse model reflects any single human subtype of the disease, & typically includes elements of 2 or more subtypes.

Innovative aspects
- First unsupervised pathway analysis tool.
- First stroma derived prognostic predictor and meta-predictors with two orders of magnitude better accuracy than previous efforts.
- Comprehensive human clinical samples versus mouse models of breast cancer comparisons.

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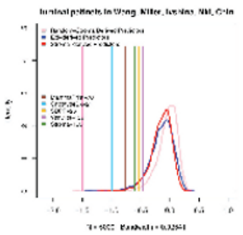

Figure 1. A comparison of different predictors across approximately 1000 human clinical samples. The epithelial-stroma combined predictors outperform all available predictors. Existing predictors are only slightly better than random sets of genes for predicting patient outcome (light pink curve).

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An Integrated Pipeline for Identification and Quantitation of Metabolites from Accurate Mass LC-MS Data

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Introduction
The complexity exhibited by modern proteomics and metabolomics samples necessitates the use of high performance separations as well as high resolution and sensitivity mass spectrometry techniques. The resulting datasets are equally complex with coeluting species and overlapping isotopic distributions present. Advanced tools are required for reliable data extraction, processing and mining. The integrated pipeline presented here intelligently processes the data and allows for both characteristic and quantitative information to be obtained from a single experiment.

Methods
Experiments are performed with a Dionex Ultimate 3000 capillary LC system interfaced to the front end of a Bruker Daltonics 12T apex-Qe FTMS instrument. The LC eluent is electrosprayed into the FTMS system after post-column addition of internal mass calibrants via a T-mixer union. The datasets are initially processed with custom Visual Basic scripts within the Bruker DataAnalysis (DA) package and then transferred to the custom processing pipeline software written in LabVIEW. Metabolomics mimic samples consisting of real matrices of urine spiked with known small molecules are used to simulate real samples pre and post treatment, for example. These mimic samples allow for the controlled analysis of the performance of the data processing pipeline.

Results
The processing pipeline can be broken up into four main steps. The first involves recognition of monoisotopic masses within each mass spectrum in an LC-MS dataset. Next, chemical species having a unique pair of retention time and mass values are identified within the composite LC-MS dataset. Lists of unique chemical species from a set of samples are then aligned allowing results from multiple samples to be filtered to identify metabolites of interest. Finally, using accurate mass and measured isotope distributions, rational molecular formulas are generated using the SmartFormula tool within DA. The formulas are directly searched against available databases to positively identify interesting metabolites.
The pipeline has been able to identify thousands of unique chemical species within a single LC-MS run. A set of six mimic species have all been located and accurately identified within a matrix of almost 3000 species using the pipeline (Fig 1). Mass accuracies are typically at or below 1 ppm and species can be detected down to column loadings of 2 fmol. Additional reprocessing algorithms are currently being developed to extend the detection limit of the technique.

Innovative aspects
- Use of a single set of data to locate, quantitate, and chemically identify biologically interesting species within real matrices
- Fully integrated pipeline requiring minimal user interaction

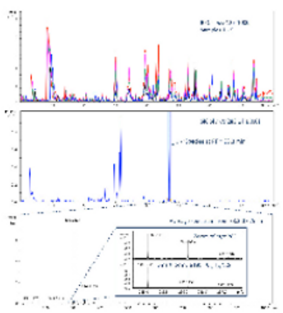

Figure 1. Location and identification of one mimic compound (Promethazine) present in a group of complex LC-MS datasets. The LC peak for this species is completely lost in the base peak chromatogram and it is a very low intensity species within the mass spectrum where it is observed. Yet, the accurate molecular formula was found by the SmartFormula algorithm and the experimental monoisotopic mass has an error of only 1.4 ppm.

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Network Dynamics during Embryo Development in Canola and Arabidopsis

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Introduction
Embryogenesis is an important developmental phase in the life cycle of seed plants. During this phase, the zygote undergoes a well-ordered series of cell divisions, followed by region-specific cell differentiation leading to an embryonic body plan to establish shoot and root apical meristems, central axis, cotyledons, and the hypocotyl. Despite advances in the identification embryo patterning mutants in recent years, the global genetic program involved in the regulation of early events after fertilization are largely unknown.

Methods
To address gene expression patterns during the key stages of embryogenesis, embryos isolated from single cell zygote to mature stages have been used in model system Arabidopsis and crop plant *Brassica napus*. The ESTs, microarray, and proteomic based approaches have been applied to generate genome-wide expression datasets for key stages of embryo development.

Results
Analysis of large datasets generated using systems approach identified developmental and stage specific programs that are connected to gene expression and metabolic regulation during embryogenesis. These studies also showed coordinated, regional and chromosome-specific expression patterns in the different stages of embryo development. By combining the omic data with KEGG metabolic database, we constructed stage-specific metabolic sub-networks mapped with differentially regulated genes in canola and *Arabidopsis*. Comparative analysis of these networks revealed that the network-associated structural, topological features, pathway interactions and gene regulations change significantly during canola and *Arabidopsis* embryogenesis. Our findings indicate that during plant embryogenesis, changes in metabolic activities are associated with changes in network structures and topologies, and the interactions of core-pathway, pathway-pathway and gene/expression-networks. Together these studies provide new regulatory insights into embryogenesis in model plant *Arabidopsis* and canola crop.

Innovative aspects
- Integrated systems approach revealed the dynamic gene expression and metabolic networks during embryo development in plants
- Identification and functional characterization of regulatory nodes

S524

High-Resolution Mapping of the Protein Interaction Network for the Human Transcription Machinery Identifies Novel Complexes That Regulate RNA Polymerase Biogenesis

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Introduction

Thirty years of research on gene transcription has uncovered a myriad of protein factors that regulate, directly or indirectly, the activity of RNA polymerase II (RNAPII) during the act of transcription (*e.g.* during mRNA synthesis on chromatin DNA). Yet many regulatory factors remain to be discovered and characterized. For example, little is known about the cellular machinery that regulates the biogenesis (*e.g.* subunit folding and assembly, nuclear import, Etc.) of this multi-subunit nuclear RNA polymerase.

Methods and Results

In previous work, we reported the development and use of a multistep procedure for the systematic analysis of the protein interaction network for the human RNAPII transcription machinery (1,2). This proteomic procedure couples (*i*) affinity purification of tagged proteins (TAP-tagging) from the soluble cell fraction in gentle conditions in order to preserve weak, transient interactions, (*ii*) the identification of co-purified proteins using sensitive, high-accuracy mass spectrometry, (*iii*) the validation of protein-protein interactions using a computational algorithm trained using machine learning to minimize the rate of false positive and false negative interactions and (*iv*) the schematic representation of protein-protein interaction networks to visualize protein connectivity. A key aspect of our procedure relies on the reciprocal tagging of preys identified in our experiments. This step is important to confirm some interactions and to expand the dataset. In this procedure, the accuracy of the network that we end up mapping increases proportionally with the number of baits used in our AP-MS experiments. As compared to the dataset we published in 2007 (1), which was built using 32 baits, the dataset used in the current study uses 77 baits. Coupled to other technical improvements of the procedure, especially regarding MS accuracy and sensitivity, the dataset used here permits a higher resolution in mapping the human RNAPII transcription network. Most interestingly, this high-resolution interaction network uncovers novel interactors, many of which being previously uncharacterized or poorly characterized proteins.

Using various biochemical and functional assays, we were able to characterize a number of novel proteins that interact, directly or indirectly, with RNAPII. These proteins were named the RNAPII-Associated Proteins (RPAPs).

We show that one of them, RPAP4, now referred to as XAB1/GPN1, is member of a unique class of GTPases containing a conserved GPN-loop that is involved in the biogenesis of RNA polymerase II (RNAPII) by regulating its nuclear import. XAB1/GPN1 is physically associated with RNAPII in the soluble cell fraction and is essential for cell growth. Accumulation of RNAPII in the cell's cytoplasm was induced upon (*i*) depletion of XAB1/GPN1 by siRNA silencing, (*ii*) overexpression of a XAB1-GFP dominant negative fusion protein, (*iii*) mutation of the GTP-binding or GPN motifs of the XAB1/GPN1 homologue in yeast, NPA3, and (*iv*) treatment of cells with leptomycin B, an inhibitor of XPO1/CRM1-dependent nuclear export. Yeast strains with mutations in the NPA3 gene showed hypersensitivity to benomyl, a specific inhibitor of microtubule assembly, suggesting a role for microtubules in RNAPII biogenesis. Together, these results indicate that XAB1/GPN1 shuttles between the cytoplasm and the nucleus to mediate nuclear import of RNAPII.

Another RPAP, RPAP3, is part of an 11-subunit protein complex that contains human homologues of the yeast R2TP complex, including RPAP3 (Tah1, Spag), PIH1D1 (Pih1, Nop17), RUVBL1 (Tip49) and RUVBL2 (Tip48). A protein called WDR92 (Monad) is also present but unlike R2TP, this complex comprises PFDN2 and PFDN6 (HKE2), two subunits shared with the canonical prefoldin complex, and three prefoldin-like proteins, UXT (ART-27), PDRG1 and C19orf2 (URI, RMP). The latter is a well-characterized protein known to bind POLR2E and has a conserved role in TOR signaling.

Innovative aspects

- High-resolution mapping of the protein interactome involving the human transcription machinery.
- Identification and characterization of novel protein factors, the RPAPs, that regulate the biogenesis of multisubunit RNA polymerases.

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Global Interaction Atlas of Escherichia coli Encompassing Previously Uncharacterized Proteins

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Introduction

One-third of the 4,225 protein-coding genes of *Escherichia coli* K-12 remain functionally unannotated (i.e. functional orphans). Many map to distant clades such as Archaea, suggesting involvement in basic prokaryotic traits, whereas others appear restricted to *E. coli*, including pathogenic strains.

Methods

To elucidate the orphans' biological roles, we followed the guilt-by-association concept, and performed an extensive proteomic tandem affinity purification survey of tagged *E. coli* strains to determine protein participation in physical interactions and multimeric complexes (**Figure 1**).

Results

A high-confidence network consisting of ~6,000 interactions among ~1,750 proteins, including ~450 orphans, or roughly two-thirds of the entire water-soluble proteome was determined. Flux simulated-based clustering of this network allowed us to delimit ~450 putative heteromultimeric complexes. Orphan membership in such complexes suggested participation in widely conserved core biological processes, like protein translation and DNA-replication, as well as in other more specific traits, such as biogenesis of the cell envelope and pathogenic processes, like the biosynthesis of lipopolysaccharide. Further integration of this proteomic-derived network with genomic context information using machine learning algorithms enriched the set of high confidence orphan functional predictions. Genetic and chemogenomic assays were used to validate many of these assignments.

Innovative aspects

- This work provides the **largest** high-confidence network of physical protein interactions in Bacteria.
- Roughly half of the orphans could be assigned to a **specific** functional category.
- All our results are **freely available** in a dedicated web portal called 'eNET' (1), which contains not only protein interactions and groupings (as other databases do) but also specific function prediction 'labels' useful for mechanistic follow-up studies.

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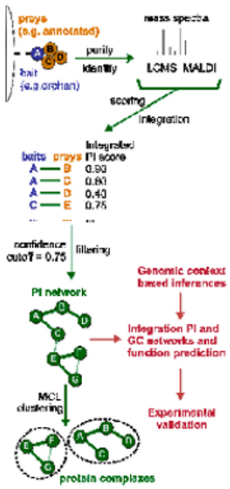

Figure 1. Generation and Integration of High Confidence Physical and Functional Networks and Orphan Function Prediction. Construction of a protein-protein interaction network (PI) based on protein copurification and detection by mass spectrometry (LCMS and MALDI). The joint datasets were scored using a logistic regression with references from low-throughput studies curated in DIP, BIND, and IntAct (gold positives) and proteins in different subcellular localizations (gold negatives),and integrated using a probabilistic model. The resulting interaction network, with edge weights corresponding to likelihood ratios, was clustered using MCL algorithm to delimit multimeric complexes. Genome context methods and machine learning was used to assign functional 'labels' to orphans for following up studies.

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Interactome mapping – where are we and how to move forward?

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Introduction

Protein-protein interactions are an essential constituent of all living systems and interactome mapping is an elementary aspect of all areas of systems biology. Interactome maps aid systems biology efforts on several levels: they provide network information for topological analysis as well as the development of large scale dynamic models (e.g. flux balance), and they provide numerous functional hypotheses for previously uncharacterized gene products that are key for subsequent quantitative studies and thus quantitative pathway models. Lastly, protein interaction information has very successfully been combined with other types of large scale data to further our understanding of pathways and biological networks.

Methods and Results

CCSB engages in interactome mapping for *H. sapiens*, *A. thaliana*, *C. elegans*, and *S. cerevisiae*. In this presentation we will introduce interactome mapping and its application to study human biology and disease. Using the example of *S. cerevisiae* – the best characterized map, for which large scale datasets have been assembled using literature curation, Y2H and AP/MS to date we will compare the network models produced by these different approaches. Furthermore, we will estimate the size of the interactome to estimate progress over the past decades and provide an estimate for the magnitude of systematic large-scale interactome mapping projects. Furthermore, we will introduce a strategy to derive an experimental-data based confidence score that can be used to assess the quality of individual interactions in future datasets. Lastly will demonstrate how network information can be used to study biology and human disease on both global and small scale.

Innovative aspects

- Empirical, internally-controlled quality assessment shows high precision of existing Y2H datasets.
- Y2H and AP-MS based network exhibit topologically and biologically different properties
- A novel conceptual framework enables size estimation of interactomes
- A method for empirical confidence scoring of individual interactions in large scale datasets.

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Computational methods for AP/MS protein-protein interaction data

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Introduction

Affinity purification followed by mass spectrometry (AP/MS) has become a commonly used method for the identification of protein-protein interactions and protein complexes [1]. Common computational challenges presented by AP/MS datasets include the need to access the statistical significance of protein interactions and filter out non-specific (background) proteins, clustering algorithms for reconstruction of protein complexes, and quantitative analysis of changes in the composition of protein complexes under different conditions. Here we review the existing approaches and present our recent developments in this area.

Methods

Computational methods for AP/MS data presented here take advantage of the semi-quantitative protein abundance information based on peptide or spectral counts [2]. This information can be easily extracted from AP/MS data, and does not require high mass accuracy instrumentation or significant modification in the experimental protocols. While spectral counting is semi-quantitative in nature and arguably not as accurate as ion intensity-based quantification, with proper normalization it provides extremely valuable information for improved analysis and modeling of AP/MS data.

Results

We will illustrate the utility of peptide or spectral counting for improved analysis of AP-MS data in several contexts. First, we are pursuing several strategies for addressing the problem of false positive interactions. We will describe a novel statistical approach, Significance Analysis of Interactome (SAINT), which utilizes spectral counting for assigning a confidence measure to individual interactions in large-scale studies. Essentially, the method compares normalized spectral count distributions for each of the hits (or prey) in the dataset and across the purification of each of the baits and models these distributions.

We are also developing a new resource - the Contaminant Repository for Affinity Purification (CRAP). Our main objective here is to create a central repository to store, annotate, statistically analyze, and disseminate lists of protein contaminants likely to be observed in AP/MS studies. The contaminant repository should significantly improve the ability of individual researchers, especially in small-scale studies, to filter out likely false interactions based on the frequency of protein identification across multiple control experiments annotated in the database.

Finally, we will discuss the use of spectral counts in clustering of AP/MS data for improved reconstruction of protein complexes, as well as for detection of quantitative changes in the composition of protein complexes as a function of the cell state.

Innovative aspects

- Methods for accessing statistical significance of AP/MS-derived protein-protein interactions
- Label-free protein quantification using spectral counts and its use in AP/MS studies

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Family reunion - encounter of molecular cousins uncovers founder and ancient function of prion gene family

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Introduction

Prion diseases are fatal neurodegenerative diseases of humans and animals which, in addition to sporadic and familial modes of manifestation, can be acquired via an infectious route of propagation. Prion diseases include sporadic and variant forms of Creutzfeldt-Jakob disease in humans. In sheep, cattle and cervids, prion diseases manifest as scrapie, bovine spongiform encephalopathy, and chronic wasting disease, respectively. In the more than 20 years since its discovery, both the phylogenetic origin and cellular function of the prion protein (PrP^C) have remained enigmatic. Insights into the function of PrP may be obtained through a characterization of its molecular neighborhood.

Methods

Following a 'guilt-by-association' logic, we set out to identify the function of the cellular prion protein through a comprehensive interactome investigation. Investigations of this kind often result in long lists of candidate interactors. The challenge then remains to discriminate specific from unspecific binding partners. Here we incorporated quantitative mass spectrometry based on isotopic tagging of peptides into the workflow to overcome this limitation. Furthermore, investigations were extended to all three members of the mammalian prion protein family to further facilitate discrimination of potential interactors by differential interactome comparison. A murine neuroblastoma cell line (N2a), an established cell model for prion replication, served as the biological source material.

Results

Interestingly, this work not only established a similar physiological environment for the three prion protein family members, but also suggested direct interactions amongst them. Furthermore, multiple interactions between PrP^C and the neural cell adhesion molecule (NCAM), the laminin receptor precursor (LRP), Na/K ATPases and protein disulfide isomerases (PDI) were confirmed, thereby reconciling previously separate findings.

Surprisingly, this work further revealed the spatial proximity of a subset of metal ion transporters of the ZIP family to mammalian prion proteins. A subsequent bioinformatic analysis revealed the presence of a prion-like protein sequence within the N-terminal, extracellular domain of a phylogenetic branch of ZIPs. Additional structural threading and ortholog sequence alignment analyses consolidated the conclusion that the prion protein gene family is phylogenetically derived from a ZIP-like ancestor molecule. The connection to ZIP proteins is expected to open new avenues to elucidate the biology of the prion protein in health and disease.

Innovative aspects

- First evidence of biochemical interactions amongst members of mammalian prion protein family
- A simple hypothesis is presented which accounts for the majority of interactions observed and suggests that PrP^C organizes its molecular environment on account of its ability to bind to adhesion molecules harboring immunoglobulin-like domains, which in turn recognize oligomannose-bearing membrane proteins.
- Rare example of proteomics-based discovery of evolutionary linkage between protein families
- First and conclusive evidence that the mammalian prion protein evolved from a specific branch of highly conserved metal ion transporters
- Data explain structural and biological features within prion proteins as remnants of an ancient involvement in the sensing and/or import of metal ions present in the extracellular milieu

References

None

Tissue Proteomics

(T101 – T144)

T101

Differential Proteomics Approach Yields Inventory of Domain Specific Epididymal Sperm Proteins

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In mammals, morphological structures of sperm such as head and flagella are formed in testis. Spermatozoa leaving testis are non motile, immature and incapable of fertilizing an egg. Spermatozoa attain functional maturation in terms of acquisition of forward motility by the flagellar domain and ability to bind, to penetrate and fuse with the egg investments by the head domain which is a prerequisite for successful fertilization. To understand the basis of sperm maturation and pursue epididymis as target for contraception, there is a need to identify and characterize epididymis specific sperm proteins. It is well established that proteins on different domains have specific roles to perform and hence the present study aims at identifying such domain specific proteins relevant to sperm maturation in the epididymis. In the present study, rat testicular sperm proteome (TSPome) was compared with cauda epididymal sperm proteome (CSPome) as well as with proteome from different domains of caudal epididymal sperm (HCSPome, FCSPome) using Image Master 2D-PAGE analysis software. As many as 701 proteins spots were resolved from TSPome and 742 from CSPome. Of these 335 protein spots were unique to CSPome, which are probably acquired during epididymal transit. Comparison of these 335 protein spots with HCSPome and FCSPome revealed 111 and 98 protein spots to be localized on sperm head and flagella domain respectively. Further analysis revealed that 48 protein spots are present on both head and flagellar domain whereas 63 and 50 protein spots were specifically localized on sperm head and flagella respectively. All the 335 epididymis specific proteins were cored, sequenced and classified with reference to sperm domain. GO annotation revealed that the identified protein majorly localized in intracellular (19%), mitochondria (17%), membrane (15%) and cytoskeleton region (11%). Molecular functions of the identified proteins have been classified into two major categories such as binding (50%) and activity (50%). The annotation for biological process revealed the involvement of these proteins in various processes (51%), cell (13%) and transport (12%). This comprehensive inventory of domain specific sperm proteins could be useful to select post testicular targets for contraception or as markers for infertility diagnosis.

T102

Large-Scale Proteomic Study of Chronic Kidney Allograft Rejection from Tissue Biopsies

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Introduction

The most common cause of kidney transplant failure is defined as Interstitial Fibrosis Tubular Atrophy (IFTA). There are no known unifying mechanisms, no effective therapy and no proven strategy to prevent it. Some evidence argues for chronic immune rejection, drug toxicity or chronic kidney injury. We conducted a large-scale proteogenomic study of kidney transplant biopsies with histological IFTA of varying severity. Tandem mass spec data was acquired via MudPIT, followed by quantification, analysis of differential protein expression and functional annotations to known molecular networks.

Methods

Biological samples consisted of kidney transplant biopsies from transplant recipients treated with either calcineurin inhibitor-based immunosuppression or a de novo rapamycin therapy. Mass spectrometry data were acquired using a linear ion trap mass spectrometer interfaced with 2D HPLC. Raw data were searched using SEQUEST and post-processed using DTASelect version 2.0. A measure of normalized amino acid coverage was used as label free quantification. One-way ANOVA and a two-tailed, independent Student's t-test were used for hypothesis testing. Ingenuity Pathway Analysis tool (IPA) (Ingenuity Systems®) was used to generate functional annotations of identified proteins.

Results

We identified over 1,400 proteins with unique expression profiles in IFTA from normal transplant biopsies to biopsies with mild and moderate to severe disease with the relative abundance levels that span three orders of magnitude. The identified sets of proteins were mapped to populate different functional pathways related to cellular distress: immune responses, inflammatory cell activation and apoptosis – all consistent with the chronic rejection mechanism hypothesis. The following pathways become upregulated during later stages of IFTA: acute phase response signaling, actin cytoskeleton and chemokine signaling, coagulation and complement systems, regulation of actin-based motility by Rho, integrin signaling, hepatic fibrosis and PI3K/AKT signaling among others. Two examples were the extensive population of the alternative rather than classical complement pathway, previously not appreciated for IFTA, and a comprehensive network for actin cytoskeleton control and acute phase response cell signaling that has also not been characterized at the global protein level in kidney transplantation. Our proteomic results are in agreement with the microarray gene expression data.

Innovative aspects

- First large-scale kidney tissue proteomic effort
- Discovery of several key biological processes during IFTA

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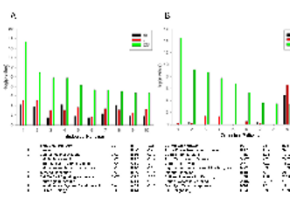

Top biological functions (A) and canonical pathways (B) of differentially expressed and unique proteins among b0, b1 and b23 stages of CAN. Each data series consists of proteins upregulated during b0 (black), b1 (red) and b23 (green) stages of CAN. Each enumerated annotation is assigned a probabilistic significance score represented as p-value. The table lists enumerated annotations as well as the fractions of identified proteins that belong to that particular biological function category (A) and the number of identified proteins that belong to a particular canonical pathway (B).

T103

A STUDY OF THE LIVER AND PANCREAS POST MORTEM DEGRADOME

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Abstract content

Pancreas and liver are two multifunctional, and two of the most well studied, organs in the body. The effect of *post mortem* time on the proteome composition was studied on tissue from snap frozen mouse liver and pancreas compared to rapid heat stabilized tissue using two-dimensional difference gel electrophoresis (2D-DIGE) and a label free peptidomic approach by LC-MS. We report a number of proteins and peptides that exhibit high degradation sensitivity, for instance superoxide dismutase in liver, and peptidyl-prolyl cis-trans isomerase and insulin C-peptides in pancreas. We also show that solely snap freezing related degradation can be attenuated by subsequent heat stabilization.

Introduction

The main factors influencing sample quality is storage temperature of the body until tissue removal (foremost a problem in clinical settings and extraction of less accessible tissue samples from model organisms) and *post mortem* time interval. *Post mortem* degradation is a well known problem when studying endogenous peptides and has also been proven to affect the results in proteins studies.

Methods

Liver and pancreatic tissue from mice was isolated and treated in one of three ways: directly snap frozen (F), heat inactivated from fresh state (D), or heat inactivated from frozen state (FD). Samples were divided for proteomic or peptidomic analysis. Proteins (liver and pancreas) were extracted in lysis buffer (8 M urea, 30 mM Tris, 5mM magnesium acetate and 4% (w/v) CHAPS) and analyzed on 2D-PAGE. Peptides (pancreas) were extracted using a 0.25% HAc aqueous buffer and isolated using a 10 kDa cut-off filter. The filtrate was separated on a nanoLC system coupled to a Q-tof mass spectrometer for profiling or LTQ FT mass spectrometer for identification.

Results

2D-DIGE revealed a number of proteins subjected to degradation in the proteome of pancreas. The analysis revealed 88 proteins that were significantly altered between the groups. In both tissues, the D and FD groups are more similar to each other than the F group. Some proteins such as Translation initiation factor 4B are especially sensitive to freezing and are more similar between FD and F than with D samples, indicating that they can not rescued by heat inactivation in already frozen samples. Using nanoLC ESI MS/MS and database matching approximately 100 peptides were identified. Besides C-peptides, we also detected endogenous peptides derived from chromogranin I, II and III, glucagon, islet amyloid polypeptide (IAPP), neuroendocrine convertase II, somatostatin and thymosin beta-4. The intensity of the intact C-peptide were found to in approximately 4 x higher levels in D and FD samples, again demonstrating that freezing without following heat inactivation (D, FD) cause more degradation The results show that it is possible to extract the peptidome after having stabilized tissue by rapid heat inactivation and that snap frozen samples can be stabilized before sample preparation.

Innovative aspects

- The problem of *post mortem* degradation for proteomic analysis can be minimized through the usage of the rapid heat inactivation technology

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Heat stabilization prior to shot-gun proteomics improve quantification & reduce non-tryptic peptides

T104

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Abstract content

Protein degradation post-sampling in biological samples causes large problems in shot-gun proteomics. Traditional methods to stop degradation include chemical protease inhibitors, which are severely restricted in the shot-gun methodology. Rapid heat inactivation of intrinsic enzymatic activity has been evaluated and found to be fully compatible with shot-gun proteomics as well as limiting the post-sampling degradation and improve results.

Introduction

Post-sampling degradation is a problem in proteomic studies. The change in proteome may obscure important information of a sample's biological state and prevent discovery of biomarkers. Steps must be taken to limit *ex vivo* proteolysis. For bottom-up, or shot-gun, proteomics, using chemical inhibitors is limited due to subsequent steps of proteolytic cleavage of extracted proteins with trypsin. An alternative is to use rapid heat inactivation of enzyme activity. This work show how heat inactivation can be used to avoid degradation in order to improve quantification and reduce non-tryptic peptides.

Methods

Mice brains were extracted after cervical dislocation and were either immediately frozen, stabilized in heat inactivation system or frozen and then stabilized. Proteins were extracted in 10 x volume in 8M Urea/4%CHAPS. Insoluble material was pelleted by centrifugation. Solubilised proteins were reduced and alcylated prior to dilution to a final 2M urea concentration and digested over night with trypsin. Samples were labelled with different iTRAQ masstags. After labelling, all samples were pooled and treated as a single sample. Part of the sample was used to enrich for phosphopeptides using a calcium phosphate precipitation followed by IMAC enrichment while the rest of the sample was analysed directly using nano-LC-MS on an OrbiTrap.

Results

Six different sample treatment protocols have been compared using mice brain and shot-gun proteomics. The resulting data have been analysed with special focus on detecting non-enzymatic degradation effects, effect on identification and especially relative quantification. 4 measures have been used: **1.** Overall detected and identified tryptic, as well as nontryptic, peptides which showed increased number of detected non-tryptic peptides in non-stabilized samples while the identification rate was similar or slightly lower in stabilized samples. **2.** Number of pairs of tryptic peptides and non-tryptic daughter peptides from corresponding tryptic peptide which show that the number of such peptide-pairs is much higher in non-stabilized samples. **3.** Intensity and detectability of phosphopeptides were increased in stabilized samples with several phosphopeptides only detectable in stabilized samples. **4.** Differences in intensity ratio between peptide pairs compared between stabilized and non-stabilized samples coming from the same protein indicating differential post sampling protein cleavage, thereby risking making relative quantification ambiguous with several potentially different peptide ratios to base quantification on.

Innovative aspects

- Post-sampling protein degradation is a problem in shot-gun proteomic analysis, especially for relative quantification.
- Rapid heat inactivation of samples following sampling eliminates post sampling protein degradation permanently from the moment of treatment and thus reduces the degradation associated problems drastically.

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Phosphoproteomic Pattern in Normozoospermic and Infertile Teratozoospermic Men

T105

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In mammalian system, spermatozoa are not able to fertilize the oocyte immediately upon ejaculation, thus they undergo a series of biochemical and molecular changes which is termed capacitation. Capacitation is generally accompanied by increases in phosphorylation of sperm proteins in response to activation of signal transduction pathways. In this study, we employed one dimensional and two dimensional SDS-PAGE techniques to characterize the phosphoproteomic pattern in sperms isolated from normozoospermic and infertile teratozoospermic men referred to Avicenna Infertility Clinic in Tehran, Iran. Semen samples were collected and spermatozoa were isolated using percoll gradient centrifugation. Spermatozoa were then incubated up to 6 h at 37° C in 3% Bovine Serum Albumin-supplemented Ham`s F10 for capacitation following a standard protocol. To characterize the phosphoproteome of sperm, we employed an antibody (PY99) specific against phosphorylated tyrosine residues. Results from 2D SDS-PAGE showed a phosphorylation pattern at tyrosine residues in the normozoospermic samples undergone capacitation. Results from western blots showed that: 1) the tyrosine phosphorylation patterns between the normozoospermic and teratozoospermic samples were different 2) the intensities of protein phosphorylation for certain proteins appear to have increased during capacitation in the normozoospermic samples but not in the teratozoospermic samples. These results suggest that the differences in the types of proteins and diminished tyrosine phosphorylation efficiency of sperm from teratozoospermic men may be responsible for compromised capacitation and low fertilization success in this category of infertile men.

Key words: Capacitation, Tyrosine phosphorylation, Normozoospermic Men

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HEAT STABILIZATION OF THE TISSUE PROTEOME FOR IMPROVED PROTEOMICS

T106

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Abstract content

A system for stabilization of the proteome in biological samples has been tested. The system which utilizes rapid heat inactivation was shown to eliminate enzymatic activity in tissue and thereby enable detection of a stabilized proteome including proteins, endogenous peptides, and preserved protein phosphorylations.

Introduction

After sampling, proteases and other protein modifying enzymes can rapidly change the proteome composition. Subsequent analytical results reflect a mix of *in vivo* proteome and degradation products. Post-sampling produced peptides affect the results either directly by modifying the protein from which they derive and/or indirectly by a serious contribution to sample complexity in bottom-up peptidomic analyses. In the latter case a dense proteolytic background in the mass spectra tend to mask the analytical signals from the *in vivo* proteome by ionic suppression or dynamic range restrictions.

Methods

Using tissue samples from brain, heart muscle, and liver, the stabilizing effect of the rapid heat inactivation system was assessed by Nano-LC-MS, MALDI and western blotting. This inactivation method should preserve the proteome and enable detection of endogenous peptides and monitoring of important post-translational modifications, such as phosphorylation of peptides and proteins. The technology is based on reproducible and rapid inactivation of enzymes through a combination of heat and pressure under vacuum. The system can process all solid tissues without the limitations associated with other enzyme inactivation technologies, e.g. microwave irradiation or chemical inhibitors.

Results

Inadequate sample handling normally causes an increase in protein degradation fragments which has been shown in these experiments. However, after immediate sample stabilization, no protein degradation fragments were detectable. Proteases, phosphatases and other enzymes were effectively inactivated. The mass spectrometrically identified peptide peaks in the stabilized samples consisted of several known neuropeptides, endogenous peptides, and novel potentially biologically active peptides. Most peaks detected in an untreated group originate from proteins such as hemoglobin, cyclophilin, NADH dehydrogenase, synuclein and other highly expressed proteins. The levels of the phosphorylated forms of CREB, GSK and MAPK were unchanged after 2 hours in room temperature after stabilization treatment as the levels of the same proteins in untreated tissue decreased in only 10 minutes.

Innovative aspects

- Enables analysis of proteins and peptides in their *in vivo* composition
- Degradation prevented during sample preparation

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Development of Off-line 2D-LC Tandem Mass Spectrometry for Comprehensive Tissue Proteome Analysis

T107

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Introduction

Comprehensive proteome profiling of tissue samples is challenging, as the samples contain many proteins with varying concentrations and modifications. Due to the limitations of current analytical techniques, only part of the entire proteome is profiled in a typical proteomics application. In this presentation, we describe the development of new techniques related to proteome sample preparation and mass spectrometric detection to increase proteome coverage. Applications of these methods for zebrafish liver and human breast tumor tissue proteome analysis are presented.

Methods

Figure 1 shows the workflow. The zebrafish liver tissue or human breast tumor tissue was lysed. The proteins were acetone precipitated and subjected to sequential buffer/MeOH/urea/SDS-assisted solubilization, followed by trypsin digestion and microwave-assisted acid hydrolysis [1]. The protein digests were fractionated by SCX LC. Desalting and quantification of each SCX fractions were carried out by using RPLC with step gradient elution and UV detection. An optimal amount of peptides from each SCX fraction was injected into nanoLC ESI QTOF MS/MS for sequencing [2]. After running the first SCX fraction, subsequent runs involve the exclusion of precursor ions found in the previous run(s) [3]. Database search was done using MASCOT with an estimated false positive rate of less than 1% for protein identification.

Results

Sequential solubilization of the proteins from the tissue lysates simplifies the protein contents for downstream analysis. After tryptic digestion of the proteins solubilized in each reagent, the peptide mixtures were subjected to SCX separation and each fraction was de-salted and accurately quantified using an automated RPLC-UV setup [2]. To improve the peptide identification efficiency by RPLC MS/MS, we investigated the effect of sample loading amount on the numbers of peptides identified and found that 1-µg sample injection allowed the identification of a maximum number of proteins with high scores and without causing column saturation problems. To further improve the peptide identification efficiency, MS/MS sequencing of redundant peptide ions was minimized by using precursor ion exclusion (PIE) during the LC-MS/MS runs. The use of this rolling PIE method increased the number of proteins identified by 55%, compared to that without PIE. Combining the above strategies, 1204 unique proteins were identified from zebrafish liver cytosolic fraction and 5945 unique proteins were identified from the membrane fraction. A total of 14407 peptides from 3749 unique proteins were identified from the human breast cancer tissue.

Innovative aspects

- Sequential solubilization of the tissue sample lysates simplified the protein contents for downstream analysis;
- Maximized sample loading and precursor ion exclusion strategies enhanced the peptide identification efficiency for LC MS/MS analysis;
- >6000 unique proteins were identified from zebrafish liver tissue and 3749 proteins were identified from human breast tumour tissue.

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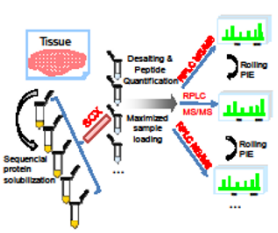

Figure 1. Workflow for comprehensive tissue proteome analysis.

T108

Interrogating the Proteome of Formalin-Fixed Paraffin-Embedded Tissues

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While proteomic technology has proven to be extremely powerful in basic research, its impact has not been as great in the clinical laboratory. Meeting this challenge will require the development of methods that utilize proteomic techniques, in particular mass spectrometry (MS), to analyze clinically relevant samples such as tissues. Beyond the analytical challenges, large numbers of fresh biopsied tissue samples from human patients are difficult to obtain. One of the more exciting recent developments in MS-based proteomics is the ability to analyze formalin-fixed paraffin-embedded (FFPE) tissues. Once thought intractable to MS analysis, there are now a number of studies that have shown the ability to obtain decent proteomic coverage from FFPE tissues. The almost inexhaustible supply of these tissues will enable proteomic laboratories access to clinical important specimen that will undoubtedly lead to their increased use in the future. In this presentation, I will discuss strategies for extracting useful proteomic information and show applications where potentially useful biomarkers have been discovered from FFPE tissues.

T109

Pancreatic Cancer Serum Detection Using A Lectin/Glyco-Antibody Array Method

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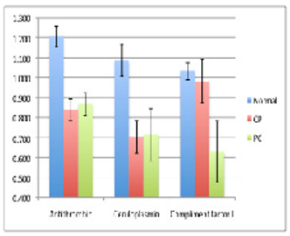
Introduction: Pancreatic cancer (PC) is the fourth leading cause of cancer-related mortality in the US. Patients with PC commonly present with advanced disease. Serologic markers with high specificity and sensitivity can be clinically useful in screening high-risk PC populations to identify early resectable lesions. Alteration in glycosylation patterns of serum glycoproteins is known to be associated with malignant transformation. Identifying unique glycosylation patterns in PC may serve as a unique biomarker of the disease.

Methods: Nine monoclonal antibodies targeting previously identified serum glycoprotein biomarkers (*List)* were diluted to 50ug/mL in PBS and spotted on ultra-thin nitrocellulose coated slides (PATH slides, GenTel Bioscience) with a piezoelectric non-contact printer (Nano plotter; GESIM). Each antibody was printed in triplicate. The slides were inserted into the SIMplex (Gentel) 16 Multi-Array device which separates the blocks and prevents cross contamination when different samples are applied on neighboring wells. Four serum samples for *normal, chronic pancreatitis, pancreatic cancer, esophageal cancer, lung cancer* and *renal cancer* groups were used. Serum was diluted 10 times and hybridized with the slides for 1 hour to extract the target glycoproteins. Lectins (ConA, AAL, SNA and MAL) were subsequently hybridized with captured glycoproteins in a sandwich assay format. Chemical derivatization of the glycans on the spotted antibodies prevented lectin binding to those glycans. The arrays were then treated with different detection biotinylated lectins (Vector Laboratory) to determine lectin response and streptavidinylated fluorescent dye (Alexa555; Invitrogen Biotechnology) was used for detection. After a final wash, the slides were dried and scanned with a microarray scanner (Genepix 4000A; Axon). The program Genepix Pro 6.0 was used to extract the numerical data. Two control blocks on each slide were used to standardize the signal between slides.

Results: The microarray data shows excellent reproducibility (<15% for three of the four lectins). Statistically significant diminution of mannosylation *(ConA)* on Antithrombin and Ceruloplasmin was observed in serum of patients with pancreatic disease compared to normal. Diminished serum mannosylation of ceruloplasmin in PC patients was also significantly ($p < 0.001$) less then in patients with lung cancer, esophageal cancer, and renal cell carcinoma, but not chronic pancreatitis ($p = 0.807$). Compliment Factor I showed >35% reduction of mannosylation in PC patients compared to both normals and chronic pancreatitis ($p = 0.007$).

Innovative Aspects: PC can be distinguished from normals, chronic pancreatitis, esophageal cancer, lung cancer, and renal cell cancer by determining mannosylation level of serum Antithrombin, Ceruloplasmin, and Compliment Factor I. Larger studies will be needed to validate these results.



T110

Quantitative Proteomics Analysis of Alcohol-induced Cardiomyopathy Using Label Free LC-MS Approach

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Introduction
Long-term heavy alcohol consumption causes alcoholic cardiomyopathy (1). Several genes, proteins and signaling pathways related to cardiac structure, Ca^{2+} homeostasis and metabolism have been shown to be involved in the disease process of alcoholic cardiomyopathy (2, 3). However, there are not any biomarkers emerged that could be used for disease diagnosis at an early reversible stage. Using multidimensional label-free LC-MS, we carried out differential proteomic analysis on the left ventricle of animals fed alcohol chronically and age matched controls to identify alcohol mediated proteins.

Methods
Adult male Sprague-Dawley rats were fed a 5%-alcohol containing or an isocaloric liquid diet without ethanol for a year. Cardiac function was assessed in vivo by echocardiography and hemodynamic performance. Hearts were isolated from rats with alcoholic cardiomyopathy and age-matched controls (n=8). Left ventricles were minced, lysed and first fractionated into subcellular fractions. The organelle and membrane fraction was further fractionated using either SDS-PAGE followed by in gel-digestion with trypsin or directly digested with endoproteinase Lys-C followed by strong cation exchange fractionation. Fractions were analyzed using a label-free LC/MS strategy on a FT-ICR mass spectrometer. Instrument reproducibility was evaluated with spiked enolase peptides standard. Data processed for quantification and statistical analysis using Elucidator software.

Preliminary results
Animals fed alcohol chronically present compensated cardiac function with lowered left ventricular ejection fraction and altered hemodynamic parameters consistent with early stages of dilated cardiomyopathy. A coefficient of variation less than or equal to 12% for a set of the spiked-in peptides, in a total of 60 LC-MS acquisitions was maintained. A subsequent differential analysis on these data sets using the Elucidator system identified a total of 70 differentially expressed proteins. A large percentage of the proteins are down regulated in alcoholic cardiomyopathy. Alcohol has been shown to have a negative effect on net protein synthesis. Our results indicate changes in specific proteins consistent with this hypothesis. To understand the biological relevance of the differentially expressed proteins and the direction of their fold change implications in the context of alcoholic cardiomyopathy, the list of proteins with their expression data were visualized on MetaCore™ pre-existing pathway maps. The result of this mapping revealed predominant proteome themes involved in oxidative phosphorylation ($p = 6.54E-16$). Subsequently, some of our targets from label free and pathway analyses will be validated with targeted MRM analysis and/or immunoblotting analysis to confirm the magnitude and the direction of the fold changes.

Innovative aspects
Identification of molecular signatures for alcohol-induced cardiomyopathy in rat model

Acknowledgments
This research was supported in part by NIAAA Grant R01 AA016210

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T111

Apis mellifera Proteomics of Innate reSistance (APIS): Quantitative proteomic analysis of honey bee populations within Canada.

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Introduction

The honey bee (*Apis mellifera*) is the most economically valuable pollinator of agricultural crops in the world today. Bees can suffer from a variety of diseases that can significantly reduce the health of the hive. In Canada the current bee colony losses are consistently 2-fold the normal long term trends. Complex host/pathogen relationships exist within the hive, and mechanisms have evolved allowing bees to tolerate infestation [1]. We have selected diverse populations of bees in Canada and are using proteomics to investigate these mechanisms.

Methods

Our aim is to identify and quantitate protein biomarkers that are expressed in bee populations exhibiting desirable physiological and behavioral characteristics. In order to carry out the global analysis of the bee proteome, we have implemented a triplex method based on the labeling of primary amines by dimethylation with distinct isotopic forms of formaldehyde and deuterated proton donors [2]. Complex peptide samples isolated from key bee tissues are separated by strong cation exchange chromatography and reversed phase HPLC with online ESI mass spectra acquired on an LTQ-FT or LTQ-Orbitrap MS. Peptide ratios are calculated using the software MS Quant and in house scripts are used to generate quantified protein data. Data is then analyzed for statistical significance.

Results

Initial results focus on development of the workflow for generating data in large population based biomarker studies. Results are also given demonstrating the suitability and accuracy of the methods chosen. Preliminary data regarding the distribution of protein expression within bee populations is presented. Here analysis of protein expression data is correlated with the distribution of phenotypes within the bee populations. Conclusions will be drawn from functional annotation of these proteins and novel hypotheses for molecular mechanisms of disease resistance discussed.

Innovative aspects

- Honey bee population proteomics
- Triplex protein quantification

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T112

Detection of Novel Biomarkers of Liver Cirrhosis by Proteomic Analysis

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Introduction
Hepatic cirrhosis is a life-threatening disease arising from different chronic liver disorders. One major cause for hepatic cirrhosis is chronic hepatitis C. Chronic hepatitis C is characterized by a highly variable clinical course, with ~20% developing liver cirrhosis within 40 years. Since so far only liver biopsy allows a reliable evaluation of the course of hepatitis C by grading inflammation and staging fibrosis, serum biomarkers for hepatic fibrosis with high sensitivity and specificity are needed.

Methods
In order to identify new candidate biomarkers for hepatic cirrhosis, we microdissected cirrhotic septa and liver parenchyma cells from 7 patients with chronic hepatitis C virus infection for proteomic analysis. Using fluorescence dye protein saturation labelling we were able to analyse 2,500 – 3,500 microdissected cells. After image acquisition DeCyder™ image analysis software revealed 49 differentially abundant proteins ($p < 0.05$ and fold change > 3.0). The protein identification was performed using LC-ESI mass spectrometry. Potential biomarkers for hepatic cirrhosis were validated by immunohistochemistry, followed by Western blot and ELISA analysis in sera of healthy controls and diseased patients.

Results
In cirrhotic tissue we detected an increasing expression of cell structure associated proteins including actin, prolyl 4-hydroxylase, tropomyosin, calponin, transgelin and human microfibril associated glycoprotein 4 (MFAP-4). Tropomyosin, calponin and transgelin reflect a contribution of activated stellate cells / myofibroblasts to chronic liver injury. The expression of tropomyosin, transgelin and MFAP-4, an extracellular matrix associated protein, were further evaluated by immunohistochemistry. Tropomyosin and microfibril associated glycoprotein 4 demonstrated high serum levels in patients with hepatic cirrhosis of different etiologies. A quantitative analysis of MFAP-4 serum levels in a large number of patients revealed MFAP-4 as novel candidate biomarker with high diagnostic accuracy for the prediction of non-diseased liver vs. cirrhosis (area under receiver operating characteristic curve (AUC) = 0.97, $p < 0.0001$) as well as stage 0 vs. stage 4 fibrosis (AUC = 0.84, $p < 0.0001$), and stages 0-3 vs. stage 4 fibrosis (AUC = 0.76, $p < 0.0001$).

Innovative aspects
- Identification and verification of the first candidate biomarkers for liver cirrhosis
- MFAP-4 allow the discrimination between patients with hepatic cirrhosis and non diseased controls by sera analysis
- MFAP ELISA results show high potential for the use as a liver fibrosis biomarker for improved diagnosis and therapy

T113

The Effect of Inflammation on the Human Visceral Adipose Tissue Secretome

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Introduction
Chronic inflammation of adipose tissue is involved in the development of insulin resistance and type 2 diabetes, probably via altered secretion of adipokines. To determine how inflammation changes the adipose tissue secretome, visceral adipose tissue was challenged with LPS, a potent immune activator. Secretome changes were analyzed using Comparison of Isotope Labeled Amino acid Incorporation Rates (CILAIR). CILAIR compares incorporation rates of 13C-labeled lysine in secreted proteins between conditions. As shown previously (1), this provides a quantitative method to asses secretome changes.

Methods
Human visceral adipose tissue obtained from a single patient was divided over six dishes (1 g/plate). After subsequent washing steps, to remove contaminating serum proteins, 13C-Lys containing medium was added (1). To three dishes also LPS (100 µg/ml) was added and all dishes were incubated for 24 hrs. Subsequently, media were collected and separately processed, involving concentration by ultra-filtration and fractionation by SDS-PAGE followed by in-gel digestion of excised bands and LC-MS/MS analyses. The obtained spectra were used for database searching (Uniprot) with ProteinPilot 2.0 (Applied Biosystems), which provides C13/C12 ratios for identified proteins.

Results
276 and 249 labeled proteins were identified present in one or more dishes for the control and LPS condition, respectively. 40 proteins were found to be significantly changed ($p<0.05$) in expression between the two conditions while 107 remained unchanged. Of the proteins changed in expression, 21 were up-regulated by the LPS treatment and 19 were down-regulated. Examples of up-regulated proteins are: Plasminogen activator inhibitor 2, Growth-regulated alpha protein, CD44 antigen and Interleukin-6.
Examples of down-regulated proteins are: alpha-2-macroglobulin and several collagen types.
In addition, a number of proteins were only detected in LPS-treated cultures and with relatively high synthesis rates (high label incorporation), indicating they are only expressed in the presence of LPS. Examples are: Granulocyte colony-stimulating factor, Macrophage inflammatory protein 2-alpha, Tumor necrosis factor and C-C motif chemokine 2 and 20.
These results indicate that protein secretion by inflamed adipose tissue is profoundly changed, which may have implications for the regulation of whole body energy metabolism and regulation of insulin sensitivity in particular.

Innovative aspects
- The study provides new insights in how inflammation regulates adipokine/cytokine secretion by adipose tissue.
- Application of the CILAIR method allows quantitative assessment of secretome changes in differentiated tissues were SILAC can not be used.

References
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T114

Optimisation of protein extraction from tissue

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Introduction:
Extraction of proteins and peptides from body fluids and tissues is a crucial step during sample preparation for proteomic profiling. Body fluids such as serum or plasma are pre-fractionated to break down sample complexity e.g. by liquid chromatography or magnetic beads prior to mass spectrometry. Preparing proteins from tissue or cell culture prior to MALDI-TOF mass spectrometry is a much more demanding step regarding buffer composition and extraction conditions compared to the fractionation of serum or plasma.
Here, we present the development of optimised conditions of protein extraction from rat tissue and cell cultures. Several different extraction buffers and disruption methods were tested in order to obtain MALDI-TOF MS compatible protein solutions. Also, we tested the impact of pre-fractionation with magnetic beads compared to direct deposit onto MALDI targets.

Methods:
Rat tissues and lymphoblastoid cell lines were used to extract proteins and peptides. Buffers containing detergents, salts and chaotropes were tested as well as mechanical disruption with glass beads (Qiagen TissueLyser), with a protein extraction kit (Allprep DNA/RNA/Protein kit, Qiagen) and targeted ultrasound (KBiosience, Covaris). The extracts were tested for protein concentration (BCA assay), for fragment composition (SDS PAGE) and were either applied to magnetic beads for fractionation (Bruker Daltonic) or directly applied to MALDI-TOF MS (Ultraflex II, Bruker Daltonics) after mixing with alpha-Cyano-4-hydroxycinnamic acid.

Results:
We tested several buffer systems on rat liver, fat and muscle. The best buffer composition regarding protein yield and MS peak number consisted of 50 mM TrisHCl (pH 7.5), 50 mM ammonium phosphate, 1 % CHAPS, 7 M urea and 2 M thiourea.
Also, we tested the efficiency of three different protein extraction methods on lymphoblastoid cell lines. The most efficient method was the cell disruption with targeted ultrasound which yielded a 500 % higher protein quantity than the protein extraction kit, and 65 % more than the mechanical disruption (fig 1).
Furthermore, the extract obtained by the extraction kit was not suitable for MALDI-TOF MS due to the high amount of SDS in the extraction buffer.

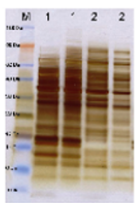

Fig 1: SDS PAGE of protein extracts from lymphoblastoid cells. 2 µg per lane. M – marker, 1 - ultrasonic cell disruption (Covaris), 2 – mechanical disruption (TissueLyser).

On cell lines we tested two different buffer systems for protein extraction: TrisHCl (50mM) with either ammonium phosphate (150mM) or NaCl (150mM). The NaCl buffer extract showed higher protein quantities than the phosphate buffer but less species in the MS spectrum (fig 2).

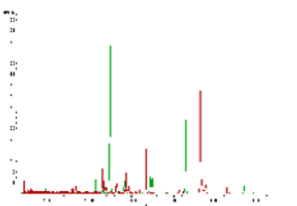

Fig 2: MS spectra of two protein extracts obtained with different buffers. Green – NaCl buffer, Red – phosphate buffer

Innovative aspects
- Application of targeted ultrasound improves the efficiency of cell disruption
- Ammonium phosphate buffers improve peptide read-out with MALDI-TOF MS
- Fast and reliable protein extraction due to fewer preparations steps

T115

Biological Significance of a Novel Mechanism of Protein Modification

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Introduction
The molecular structure of sporidesmin is characterized by an internal disulfide bridge that is thought to be responsible for toxicity by either (a) redox cycling with generation of superoxide, or (b) a mixed disulfide mechanism. While investigating the cellular effects of this disease-causing fungal toxin we found that the redox cycling mechanism is not the primary process of its *in vitro* cellular pathogenicity, as formerly suspected. We also discovered that this compound chemically modifies a restricted group of proteins by reacting with the side chains of cysteine residues, in which toxin sulfurs covalently bind to thiol groups of specific proteins and then modify protein function (1). Here we describe the use of minimal labeling and a modification of the previously published 2-D DIGE saturation labeling method to detect altered protein thiols (2). Together, these approaches have given more insight into the molecular toxicity of sporidesmin and a novel mechanism of protein modification.

Methods
Initially, the protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with and without hydrogen peroxide (H_2O_2) treatment was used to optimize both minimal and saturation labelling 2-D DIGE.. Minimal labeling was performed as per the Amersham CyDye DIGE Fluors user manual; modified saturation labeling was performed as described by Hurd *et al.* (3). Then, an experiment using HepG2 cell lysates treated with sporidesmin or a positive control (500 µM H_2O_2) was conducted to assess the similarities/differences of these two treatments compared with control non-treated groups. Analysis of multiple gels to determine statistically significant redox changes on proteins was carried out. Spot excisions from 2-D gels and protein identification by mass spectrometry were then performed.

Results
Our current work indicates that sporidesmin modifies only a small number of proteins in cells, less than expected from oxidative change but consistent with specific interaction with proteins to form mixed disulfides. Proteins generally associated with oxidative stress were seen in H_2O_2-treated samples and not in sporidesmin-treated samples. Both minimal and saturation 2-D DIGE labeling are complementary methods to understand the molecular pathogenicity of sporidesmin action and allowed us to confirm that the mechanism is likely to be specific protein modification via modification of cysteine residues.

Innovative aspects
- Minimal labelling of lysine can be used to assess oxidative stress in cell systems
- Saturation DIGE can be used to characterize protein modification caused by the disulfide toxin sporidesmin that modifies cysteine residues

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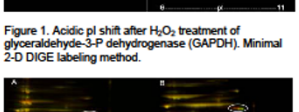

Figure 1. Acidic pI shift after H_2O_2 treatment of glyceraldehyde-3-P dehydrogenase (GAPDH). Minimal 2-D DIGE labeling method.

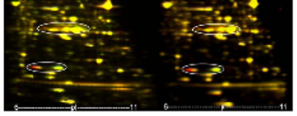

Figure 2. pI shifts due to protein oxidation in H_2O_2-treated (-) group. Similar changes were not observed in controls or sporidesmin (-)-treated samples. Minimal 2-D DIGE labeling method.

T116

Analysis of Infected Tissues by Laser Microdissection and LC-MS/MS

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Introduction
Infection of human by bacterial pathogens is studied thoroughly by proteomics. Much experimental work has focused on the analysis of host-pathogen interaction. So far, the major goal of bacterial proteomics has been the investigation of virulence factors of the studied organisms usually by exposing the bacteria to conditions that mimic the host environment. The results can be misleading since these conditions do not reflect the bacterial infection *in vivo*. In order to overcome this limitation we have employed a model of infection in mice to our study that leads to progression of tissue granulomas. This model was used to capture bacterial proteins within the tissue by mass spectrometry analysis following laser microdissection (LMD).

Methods
Laser microdissection (LMD Olympus IX71) of skin granulomas of mice infected by *Francisella tularensis* LVS (live vaccine strain) expressing GFP protein was performed. The tissue sections (minim. 5mm^2) were lysed and digested by trypsin in order to identify *Francisella tularensis* proteins that are present at the site of infection. The peptides were analysed by nanoLC ESI QqTOF (QTOF Ultima API) and identified by Phenyx 2.5.

Results
We optimized conditions for successful extraction of proteins from dissected infected tissue by LMD. Despite the mouse proteins were more prevalent in the mixture; we have identified several *Francisella* proteins. Among these proteins, type IV pili and related proteins were found that support the idea of pili playing an important role in *Francisella* virulence especially in case of skin route of infection (1). Other proteins, including several hypothetical proteins were found. The role of these proteins is not clear in the current knowledge of the virulence mechanism of *Francisella tularensis*.

Conclusion
Bacterial proteins directly involved in skin infection of *Francisella tularensis* were captured by LMD and analysed by LC-MS/MS. Several proteins were confirmed that have already been known to be related to the pathogenic process of *Francisella* as well as new proteins that were not linked to the process of infection before.

Innovative aspects
- Laser Microdissection of infected tissue in order to identify bacterial proteins

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Acknowledgements
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T117

Comprehensive Analysis of Wild and AQP 8 Knockout Mouse Colon by Nanoflow Lc-Ms/Ms

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Introduction
Although the molecular nature of aquaporin 8 (AQP8) is well known as a water-transporting protein in the mammalian gastrointestinal tract including pancreas, colon and liver, its physiological role(s) remains to be elucidated. We have conducted an in-depth, comparative proteomic analysis of colon from wild and AQP8 knockout mice to disclose differences and characteristics in proteome profile between wild and AQP8 knockout mice colon. To our knowledge, this is the first proteomic attempt to disclose phenotypic changes of AQP8 null mice as well.

Methods
Protein extracts from colons of 3 wild and 3 AQP8 knockout mice were fractionated on SDS-PAGE gels, cut into 14 slices, and subjected to in-gel trypsin digestion. Each of trypsin digests were analyzed by nanoflow-linear iontrap-TOF MS. Proteins were identified using Mascot search engine against IPI_mouse protein database (total LC runs, 84). Proteins significantly identified with two or more peptide matches using MudPit scoring algorithm of Mascot with false positive rate of less than 5 % were compared between both strains. Triplicate Mascot output data files from each group were pooled and subjected to 3 different strategies for proteome profiling; (proteins integrated by IPI accession #, proteins integrated by gene symbol, and proteins removed of possible redundant hits that shared common matched peptides) to reduce bioinformatics redundancy and increase confidence of comparison. By the aid of our developed searching methodology to target protein hits uniquely expressed in 3 analyses of one group and not shown in corresponding group, the most reliable protein candidates were quantitatively measured thereafter using Progenesis Lc-Ms.

Results
More than 2,000 proteins were successfully identified with high-confidence in each of wild and AQP8 knockout mouse. Our extensive comparison between colon proteomes of wild and AQP8 knockout mice unambiguously revealed alteration in expression of 11 proteins: 6 down-regulated and 5 up-regulated in AQP8 knockout mouse (Fig. 1). The down-regulated proteins are categorized as transfer/carrier proteins, lyase and oxidoreductase, while the up-regulated proteins are mainly categorized as ion channel, isomerase, transporters and hydrolase.

Innovative aspects
- Although little phenotypic information of AQP8 knockout mouse have been reported (1, 2), our extensive proteomic analysis revealed proteins that markedly different in expression in the colon of AQP8 knockout mouse.
- The proteomic analysis of liver proteome employing the same strategy for AQP8 Knockout mice is now under progress. The findings in this study coupled with the ongoing study on liver will provide insights into the physiological role of AQP8.

References
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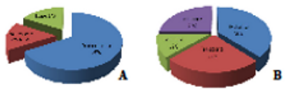

Figure (1): Venn diagram illustration of the molecular function for down-regulated proteins **(A)** *[ATP1a2, ATP1a3, Amylase1, Amylase2, prolyl-4 hydroxilaze and hemoglobin subunit epsilon y2]*, and up-regulated proteins **(B)** *[Lacrimal androgen binding protein delta, pyrroline 5 carboxylate reductase3, pyrroline 5 carboxylate reductase 2© KD, mitochondrial carrier homolouge2,and carbonic anhydrase3]*.

T118

Overexpression of Prothymosin alpha in Oral Premalignant Lesions

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Introduction
Leukoplakia, the most common heterogeneous oral precancerous lesion, with a global prevalence of 2-3% in the general population, is associated with increased risk of cancer. Recently using multidimensional LC-MS/MS with iTRAQ-tagging, we identified a panel of potential biomarkers for oral premalignant lesions (OPLs) (1). Prothymosin alpha (PTHA) was one of the promising candidate among differentially expressed proteins in OPLs. Here, we report the verification of PTHA in an independent set of clinical samples by immunohistochemistry, and investigate its potential as a biomarker for OPLs.

Methods
Fifty-three archived OPLs tissues were retrieved from the research tissue bank to determine the expression of PTHA using immunohistochemistry. Each slide was evaluated for PTHA immunoreactivity using a semiquantitative scoring system for both staining intensity and the percentage of positive epithelial cells (2). For PTHA protein expression, sections were scored as positive if epithelial cells showed immunopositivity in the nucleus. The immunohistochemical data were subjected to statistical analyses using the SPSS 13.0 software (Chicago). Sensitivity and specificity were calculated and quantified using receiver operating characteristic (ROC) analyses. Two-sided p values were calculated and $p < 0.05$ was considered to be significant.

Results
Of the 40 normal tissues analyzed, 38 cases (95%) showed faint or no detectable PTHA immunostaining in the nucleus of the epithelial cells. Chi square analysis showed significant increase in nuclear staining of PTHA in leukoplakia as compared to normal oral tissues. Of the 53 leukoplakias analyzed, 37 cases (70%) showed significant increase in nuclear PTHA immunostaining in comparison with the normal tissues ($p < 0.001$, Odds ratio (OR) = 43.93, 95% CI = 9.44 – 204.56). Receiver Operating Characteristic (ROC) analysis was used to determine the potential of PTHA as a biomarker for diagnosis of leukoplakia. The area-under-the-curve (AUC) value was 0.841 (Fig.1) and sensitivity and specificity were 70% and 95% respectively. The overexpression of PTHA in oral lesions was further validated by immunoblotting and RT-PCR analyses in the same tissue samples as used for immunohistochemical analysis. Immunoblot analysis showed a single intense band of 12.5 kDa and RT-PCR analysis demonstrated increased levels of PTHA transcripts in the same tissue confirming the increased expression of PTHA in oral leukoplakias with as compared to the normal tissues, thus supporting the immunohistochemical findings.

Innovative aspects
- Prothymosin alpha is a novel potential marker for oral premalignant lesions.

References
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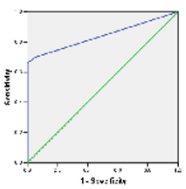

Figure 1. Receiver operating characteristic curves of PTHA in normal vs. leukoplakia. Blue line shows ROC analysis for nuclear PTHA and green line is the reference line.

T119

Nano LC-MS/MS for oocyte and early embryo proteome profiling with minute sample amounts

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Introduction

Assisted reproduction techniques (ARTs) become more and more important both in veterinary and in human medicine. Since *in vitro* generation of early embryos is a key step of ART (1), it is necessary to deepen the understanding of underlying biochemical processes. The generation of comprehensive proteomic datasets is indispensable, because of the fact that a number of biochemical and physiological processes cannot be deduced from mRNA data. However, the analysis of proteomes from mammalian oocytes and early stage embryos is challenging due to the very small sample amounts available.

Methods

Preparation of bovine oocytes/morulae (5 day old embryos):
From bovine ovaries Cumulus-oocyte complexes (COCs) were obtained by aspirating follicles. To prepare oocyte samples the COCs were denuded and washed in PBS (2). To produce morulae, COCs were *in vitro* maturated, fertilized and cultivated.
LC-MS/MS analysis: For a sensitivity study 10 and 1 oocytes were prepared and analyzed by LC-MS/MS. In addition, protein amounts corresponding to 400 oocytes/morulae were prefractionated by 1D-SDS-PAGE. Each of the 26 gel slices was subjected to tryptic in-gel digestion and analyzed independently by LC-MS/MS. The MS/MS data was subjected to stringent statistical validation by Scaffold and by the trans-proteomic-pipeline in order to avoid false positive identifications.

Results

Despite very limited sample amounts a comprehensive set of identified peptides, derived from bovine oocytes and morulae, could be obtained. All identified proteins were ontologically classified in clusters concerning function and cellular localization followed by comparison. The dataset presented here consists of more than 1200 identified oocyte proteins, representing one of the largest proteomic data sets of bovine oocytes, generated until today. In addition we demonstrated that from even a single oocyte (90ng Protein) 44 proteins and from 10 oocytes 133 proteins can be identified. This reveals that the sensitivity of the applied nano-LC-MS/MS approach should be high enough to perform future proteome analysis with *in vivo* generated early stage embryos (< day 10) of which the generation is associated with high costs and efforts.

Innovative aspects

- Nano LC-MS/MS is a valuable tool to perform proteome profiling of minute sample amounts from oocytes and early embryos.
- The results presented here are one of the largest proteomic datasets from bovine oocytes and early embryos generated until today.
- The applied nano-LC-MS/MS approach should not only be suitable to analyze *in vitro* but also *in vivo* generated embryos.

References

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T120

Molecular dissection of spinal cord injury using a refined proteomic approach

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Introduction

Acute spinal cord injury has a range of potentially devastating outcomes and is a significant long-term health care burden. Although several promising intervention strategies are being investigated, a more thorough molecular level understanding of the mechanisms inherent to injury and post-injury responses is of significant importance to focus and fine-tune these strategies. Previously, we systematically established optimal approaches for the analysis of a clinically relevant rat spinal injury model using gel-based proteomic analyses; this also fully enabled quantitative transcriptomic analyses from the same samples [1]. Here we begin to apply these refined proteomic methods to analyze the extent of injury itself. In lieu of a traditional cataloguing approach, we have sought to implement strategies to identify what are likely to be the least known molecular changes resulting from spinal injury.

Methods

Male Sprague-Dawley rats were anesthetized and underwent a contusion injury of the spinal cord at level of the T9 vertebra using a displacement controlled impactor (OSU-device). Sham animals underwent all surgical steps, but no impact. At 24h post-surgery, the animals were sacrificed and a 5 mm segment of the spinal cord, with the injury at its centre, was excised (segment 0). Four adjacent 5 mm segments were excised in each direction from segment 0 (in the rostral direction, segments -1 through -4 and in the caudal direction segments +1 through +4). Protein from each segment was resolved and detected using a refined two dimensional gel electrophoresis (2DE) protocol [1]. Protein profiles of sham vs. injury, for each segment, were quantitatively compared using automated digital image analysis. Only spots detected with 100% reproducibility in each condition were considered.

Results

A large number of proteins, (e.g. over 16% of all proteins detected in segment 0, Figure 1) met the inclusion criteria and differed significantly in abundance between sham and injury. Interestingly, significant differences were detected up to four segments from the injury epicentre, in both directions (Figure 2). Clearly the effects of injury are not restricted to the injury epicentre.

This observation raises an important question whether the changes to the proteome outside the injury epicentre related to anterograde and retrograde degeneration of ascending and descending neural tracts? Alternatively they could be linked to neural responses to axotomy and early regenerative events.

Twelve protein spots differed significantly between sham and injury in 3 or more sequential spinal cord segments; 5 occurred in ≥ 5 sequential segments. For the time being these proteins stand out as the most promising first targets for sequence identification. At the time of this writing mass spectrometric analysis is pending.

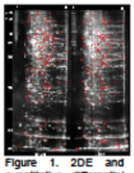

Figure 1. 2DE and quantitative differential image analysis indicate 196 significant differences (marked in RED) between sham and injured spinal cords at the injury epicenter, Segment 0. (T-test, $p < 0.05$, 100% reproducible spots; n =4).

At the very least, the spatial continuum of proteomic data generated in this study will serve as a reference map for subsequent investigations using pharmacological or other interventions as experimental tools separating the effects of the different physiological aspects of injury (e.g. inflammation). It may additionally be interesting (and important) to asses expression patterns in the spinal cord even farther from the injury epicentre to determine the physical extent of the resulting molecular effects.

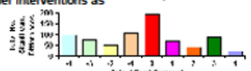

Figure 2. The spatial distribution of significant differences in the abundance of specific proteins between injury and sham across a 45 mm length of the spinal cord centered around the injury site (Segment 0).

Literature Cited

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Support: Canadian Institute for Health Research and Michael Smith Foundation for Health Research, Canada.

T121

A Label-free Quantitation Strategy for Personalized Membrane Proteomics Signature in Colorectal Cancer

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Introduction (maximum 600 characters, including spaces)
Among various cellular components, dysregulation of membrane proteins has been linked to human diseases, including cancer. The limited quantity of clinical specimens and time-consuming proteomics workflow presents challenges for in-depth characterization of individual profiles of large cohort of patients. In this study, we developed a simple label-free strategy for multiplexed quantitation of membrane proteomics and applied the new platform to analyze the personalized membrane proteome of paired normal and tumor tissues from 29 patients at different stages of colorectal cancer (CRC).

Methods (maximum 800 characters, including spaces)
Following hospital human ethics committee approval, fresh paired tumor and adjacent nonmalignant tissues were obtained from the same patient after surgery. Tissues were washed by 0.9% NaCl and then homogenized in hypotonic buffer. Nucleus was removed by centrifugation with 260 x g for 5 minutes and the supernatant was adjusted to contain 0.25 M sucrose. After centrifugation for 13000 rpm for 1 hr, the membrane pellet was washed by 0.1 M Na_2CO_3. For label-free quantitation, internal standard protein (BSA) was spiked into the purified membrane proteins and subjected to gel-assisted digestion (1). Peptides were collected by sequential extraction. After desalting by C_{18} Ziptip, the peptides were analyzed by triplicate LC-MS/MS and quantified by a home-built bioinformatics software, IDEAL-Q.

Results (maximum 1200 characters, including spaces)
The new label-free strategy provided accurate (6% error) and reproducible (34% RSD) quantitation for membrane proteomics. Without peptide fractionation, SEMI algorithm using alignment on m/z, retention time, and confident identification result identified a total of 1482 proteins; 73% of which were annotated as membrane proteins or membrane-associated proteins. On contrast to most previous studies using pooled samples, the simple strategy offers advantage for providing individual proteomics pattern analysis of 29 patients. The preliminary analysis revealed that 25, 37, 53, and 31 proteins show specifically differential expression level in tissues in Dukes' A, B, C, and D patients, respectively. Carcinoembryonic antigen-related cell adhesion molecule 5 and 6, the known biomarkers of CRC, show significantly increased levels in tumor tissues, which is consistent with previous study in CRC and other cancers. Further validation of selected proteins on large cohort of patients is still ongoing and we expect that the altered individual membrane proteomics profiles may promise to discover new biomarker candidates for more accurate diagnosis and efficient treatment for CRC patients.

Innovative aspects (maximum of 3 bullet points)
1. high-throughput proteomics for clinical samples
2. Individual membrane proteomics profiles for patients

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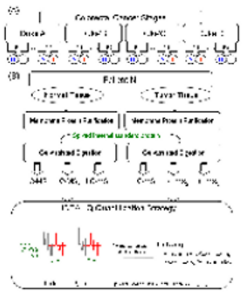

Figure 1 Workflow of the label-free quantitation strategy for personalized membrane proteomics.

T122

Multicenter Analysis of Proteome and Transcriptome of Liver Cancer Showing Dysregulated Catabolism

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Abstract

Hepatocellular carcinoma is the major type of primary liver cancer. By using a unique multicenter design, we attempted to identify pathways dysregulated in hepatitis B virus-associated hepatocellular carcinoma (HCC) through identification of interstudy-validated genes that were dysregulated at both mRNA and protein expressions. Using two-dimensional gel electrophoresis, we identified 58 proteins differentially expressed in HCC (FDR < 5%). Hierarchical clustering analysis indicated a progressive change of expression patterns from normal liver, adjacent non-tumor liver tissues, HCC tissues, finally to HCC cell lines, suggesting their importance in hepatocarcinogenesis. The gene expression data corresponding to the differential proteins were retrieved from 5 independent gene expression microarray studies in HBV-associated HCC, and subjected to meta-analysis to examine whether mRNA levels of these genes were significantly altered. Meta-analysis showed that the overall protein expression ratios significantly correlated ($r = 0.48$, $p < 0.0005$) with the mRNA ratios between HCC and non-tumorous liver tissues, and identified 38 interstudy-validated genes that were dysregulated at both mRNA and protein expressions. Gene ontology and KEGG pathway analyses showed a number of specific catabolic pathways were down-regulated. Some of them have not been reported before. These findings provide new insights in carcinogenesis and designing biomolecule analogs as novel drugs for cancer therapy. (This work was carried out within the Hepatocellular Carcinoma Research Group supported by the Central Allocation Grant CUHK 2/02C from the University Grants Committee of Hong Kong, and was supported by the Li Ka Shing Foundation.)

T123

Comparative analysis of sialome and salivary gland proteome from vectors of tropical diseases

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Abstract:

Exposure to vectors of infectious diseases has been associated with antibody responses against salivary antigens of arthropods among people living in endemic areas. The existence of such antibody responses in travelers briefly exposed to malaria or arbovirus vectors in tropical areas has been little investigated. This immune response has been proposed as a surrogate marker of exposure to vectors for evaluating the protective efficacy of antivectorial devices. In this aim, three strategies were developped: (i) analysis of IgG antibody responses of French soldiers against *Anopheles* and *Aedes* saliva, (ii) consequences on saliva and salivary gland protein repertoires, of inbred *Ae. aegypti* reared in laboratory from three colonies, (iii) characterisation of species and/or genus specific markers for travellers exposure to mosquito bites. For travellers, we succeded to describe an immune response which is specific to mosquito genus. Moerover, an immunoproteomic approach allowed the identification of antigenic saliva proteins. Additionally, we described limited variations in protein expression between three *Ae. aegypti* colonies (*Rockefeller, PAEA,* and *Formosus*), at saliva and salivary glands levels, indicating that these colonies conserved their own species characteristics. These results are major for the identification of antigenic marker species specific.. Finally, the comparison of salivary glands proteome of 6 species from two mosquito genus was performed using two complementary approaches: (i) 1D SDS-PAGE coupled to MS, (ii) iTRAQ technology. Based on these MS data, several mosquito salivary proteins specific of species were selected and will be further evaluated as potential species and/or genus candidates for the estimation of travellers exposure to mosquito bites.

T124

Proteomic Analysis to Identify Proliferation-Specific Factors using Cultured Amniocytes

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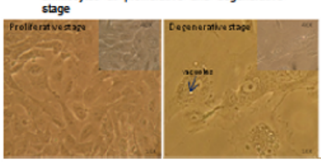
Introduction
Amniocytes, the cells of the fetus released in amniotic fluid, are excellent source to develop primary cell culture and explore proliferation factors. Comparative protein profiling of the proliferative and degenerative stages, have been carried out using two-dimensional gel electrophoresis and mass spectrometry to identify potential proliferation-specific proteins.

Methods
Amniotic fluid collected from 12-14 week pregnant woman with normal karyotype was cultured *in vitro* and the cultured adherent amniocytes were collected at two stages - proliferative (cells with high proliferative index -initial stage) and degenerative stage (cells with low proliferative index - late stage) (Fig 1). Total protein extracted from each of the the above two stages were resolved in first dimension with pH gradient of 5-8 and second dimension on 10-15% gradient SDS-PAGE gel. Gel images were acquired and image analysis was performed using PD Quest software version 8.0 (Bio Rad). Differentially expressed proteins were then identified by MALDI ToF mass spectrometry (MS and MS/MS).

Results
Peptide mass fingerprint search after mass spectrometric analysis led to the identification of 99 differential proteins with high confidence - 60 up-regulated and 39 down-regulated proteins. The differentially expressed proteins include a range of proteins from cytoskeletal proteins (keratin, vimentin, dsemin, tubulin, fascin, TRIO and F-actin binding protein, spectrin) and cell stress proteins (HSP70, Thioredoxin, peroxiredoxin, superoxide dismutase) to cell signaling (Mitogen-activated protein kinase 4, Adenylate kinase isoenzyme 5, Serine/threonine-protein kinase 9, Tyrosine-protein kinase, Protein inhibitor of activated STAT2) and nuclear proteins (Nucleoprotein TPR, DNA repair protein RAD50, UV excision repair protein RAD23 homolog B , EF-hand domain-containing protein 2, GTP-binding nuclear protein Ran) as well as transport proteins (Mannose-6-phosphate receptor binding protein 1, Chloride intracellular channel protein 1, Rho-GTPase-activating protein 7).

Innovative aspects
- The differentially expressed proteins with regulatory function may be important in the context of maintenance of the proliferation capacity of the amniocytes and even other cell types and may have clinical implications.

Fig 1. Microscopic image of the cultured adherent amniocytes at proliferative and degenerative stage



T125

Optimum method designed for 2D-DIGE of arterial intima and media isolated by laser microdissection

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4. Department of Cardiology. Hospital Virgen de la Salud, Toledo, Spain.
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Introduction
Tissue proteomic studies on atherosclerosis have traditionally focused on whole artery extracts from biopsy or necropsy origin. Arterial intima and media layers are both involved in atherosclerotic development. In the present work, we describe an optimum method which employs the combination of Laser Microdissection and Pressure Catapulting (LMPC) and 2D-DIGE saturation labelling to investigate the human intima and media sub-proteomes isolated from atherosclerotic (coronary and aorta) or non-atherosclerotic (preatherosclerotic coronary) arteries.

Methods
Coronary biopsies from patients undergoing bypass surgery and coronary and aorta necropsies were immediately washed in saline and freezed embedded with OCT. Intima and media were isolated by LMPC with a Microbeam System (PALM Microlaser). Several methods for staining (hematoxilin, cresyl violet), protein extraction and DIGE labelling were tested in order to achieve the better protocol for 2-DE analysis of human arterial layers. First of all, a spin column chromatographic step with Protein Desalting Spin Columns (Pierce) was assayed in order to minimize negative effects from remaining stain. In addition, different lysis buffers were tested to assure efficient extraction of arterial layers proteome, which is problematic due to highly acidic myofilament proteins and presence of calcium.

Results
Concerning staining methods, both hematoxilin and cresyl violet were found to negatively affect protein extraction and IEF. A chromatographic spin column step prior to saturation DIGE labelling improved substantially 2-DE gels, permitting the use of both stains for human arterial layers proteomic analysis. Ethanol solved cresyl violet minimizes proteases action and facilitates visualization of arterial tissue. For this reasons this stain was chosen for further analysis. Optimal DIGE labelling conditions were set at 1nmol TCEP and 2nmol Dye, so that free dye deleterious effects were minimized. The addition of SDS, and in a higher extend DTT, on lysis buffer significantly increased protein solubilization, particularly on high molecular weight proteins as visualized on 2-DE gels. Finally, LMPC method was checked by LC-MS/MS to assure correct layers isolation. 22 and 38 proteins were identified from in-solution digested preatherosclerotic coronary intima and media extracts, respectively. Although some proteins are common between layers, due to similar cell types present; differential proteins are specific of contractile phenotype of VSMCs in the media and proliferative in the intima.

Innovative aspects
- Removal of remaining stain and free dye interferences for saturation labelling DIGE
- 2-DE gels of human arterial intima and media layers for further differential expression proteomic analysis
- Protein lists of preatherosclerotic human coronary intima and media layers

T126

Characterization of Zebrafish Gill Proteome Changes After Naphthenic Acid Exposure

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Introduction
Fish are a valuable model system for investigating the impact of toxins on aquatic environments. They require a large surface area for gas and ion exchange with their environment. These functions are largely accomplished by the gills, which provide a direct route for the uptake of contaminants. Naphthenic acids (NAs) are saturated aliphatic and alicyclic carboxylic acids found in petroleum, oil sands bitumen, and crude oils. NAs are environmentally significant because they are acutely toxic to aquatic organisms. Our goal is to study the effects of NA exposure on fish biology and toxicology using zebrafish as a model.

Methods
Adult zebrafish were kept in a controlled environment where they were exposed to NAs. Following exposure for a specified period of time, zebrafish were sacrificed and their gill proteins extracted. Following in-solution tryptic digestion, the sample was aliquoted into two sets for labeling with CH_2O and $^{13}CD_2O$, respectively. Zebrafish from a control group were similarly treated. The labeled digests were mixed such that a CH_2O-labeled sample and a $^{13}CD_2O$-labelled control constituted the "forward" case, and a "heavy" labeled sample and a "light" labeled control constituted the "reverse" case. The "forward" and "reverse" labeled mixtures were subjected to 2D LC-ESI MS/MS for relative peptide quantification and protein identification.

Results
Our strategy to analyze the biological effects of zebrafish exposure to NAs involved a stable isotope labeling method that uses dimethylation after guanidination (2MEGA) for differential protein expression analysis. The zebrafish gill proteome exhibited excellent coverage of important biochemical pathways relevant to the function of the gill in respiration, ion and acid-base homeostasis, and energy metabolism.[1] In this study, we used both "forward" and "reverse" labeling to corroborate our quantification results. Peptides were considered differentially expressed if their ratios in the "forward" and "reverse" labeling cases were inversely proportional. Peptide identification and quantification were performed using the NCBInr database and MASCOT Distiller. Preliminary analysis of the data shows extensive variations of protein abundance for a great number of proteins including those previously identified as stress biomarkers. We are currently analyzing these results based on known protein interaction networks to determine putative biomarkers in the gill indicative of NA exposure. Further studies are also ongoing to ascertain the effects of NA exposure on gill function.

Innovative aspects
Discovery of potential biomarkers for naphthenic acid exposure in zebrafish gill based on differential protein expression analysis.

References
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T127

Quantitation of Her2 in FFPE Breast Cancer Tissue by Mass Spectrometry MRM Analysis

Marlene Darfler, Michael Ford, Richard Jones, Albert Kovatich, Brian Hood, Jeff Zhang, Timoth Veenstra, Thomas Conrads, David Krizman

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Introduction
Almost one third of invasive breast carcinomas over-express the Her2 protein and the use of anti-Her2 therapies that block this protein have become important in the management of and in prolonging the survival of patients with aggressive breast cancer. The effectiveness of this therapy is dependent on accurately evaluating Her2 status in the tumor, which is currently done by immunohistochemistry (IHC) fluoresecent in situ hybridization (FISH). Variability in this approach occurs due to the general drawbacks of these methodologies which rely on subjective interpretation of tissue staining.

Methods:
To quantitatively measure and better understand the changes in Her2 protein expression in breast cancer we have developed a mass spectrometry-based Multiple Reaction Monitoring (MRM) assay designed to provide absolute quantitation of a single Her2 peptide in any biological sample including formalin fixed paraffin embedded (FFPE) tissue. This assay was performed on ten FFPE breast cancer samples expressing a range of Her2 protein levels as determined by IHC, and demonstrating a range of Her2 gene amplification by FISH. Proteins were extracted from microdissected cancerous epithelium using Liquid Tissue® reagents and protocols followed by analysis of the soluble lysates by the MRM assay.

Results:
Quantitative measurement of the specific Her2 peptide was determined in these FFPE tissue samples as a function of amount (in fmol)/ug protein analyzed. As shown in Figure 1, the Her2 MRM assay provides quantitative measurement of protein expression that compares directly to IHC and FISH scoring, the current standard methods of Her2 analysis, while demonstrating a broader dynamic range than either the IHC or FISH analysis methods.

Innovative Aspects:
- Her2 was quantitated by MRM analysis of Liquid Tissue® lysates prepared from FFPE breast cancer tissue
- Her2 MRM data correlated precisely with IHC and FISH data
- Her2 MRM data provides absolute quantitation with a very broad dynamic range of quantitation and detection

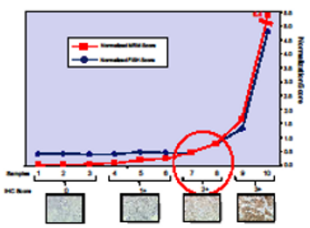

Figure 1: Comparing Normalized Her2 MRM Assay and FISH Analysis
Her2 MRM and FISH results can be more effectively compared after normalizing the data. The score for each data point for each sample was normalized by dividing the value for that sample by the average of all the values in the assay. The circle indicates precise correlation of values from the MRM assay and the FISH analysis in the most clinically-pertinent breast cancer samples (IHC 2+).

T128

Digital pathology: Quantifying Barretts' markers in tissue samples using LCM-MS

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Introduction
The incidence of esophageal adenocarcinoma (EAC) arising from Barrett's esophagus (BE) has increased by 350% since 1970, and at the time of presentation, 50% of patients will have advanced disease with virtually no chance for cure. The potency of BE as a risk factor for EAC has been seriously called into question. With potentially 1% of the general population having BE in the face of a very low incidence of EAC, there is clearly a need to define patients that are at the highest risk for cancer development. Currently, risk for esophageal cancer is determined by the histologic appearance of BE, which has large degree of intra- and inter-observer variability among pathologists. The ability to identify biomarkers of disease progression from BE to EAC when treatment outcome is still favorable, could result in a reduction in mortality, by aiding in the identification of new diagnostic and treatment options for this disease.

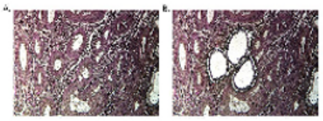
In this report, we present a novel workflow where we have utilized laser capture microdissection (LCM) to procure cells from defined regions of BE metaplasia, dysplasia and EAC for a differential proteomic analysis using high-resolution mass spectrometry (MS) to yield objective qualitative and quantitative information on known as well as novel biomarkers. This information can be used to complement traditional immunohistochemistry analysis.

Methods
Cells from formalin-fixed paraffin embedded (FFPE) tissue sections showing metaplasia, dysplasia and carcinoma of the esophagus were procured by LCMIysed, digested and analyzed on an LTQ-Orbitrap. Label-free differential analysis was accomplished by SIEVE to generate a list of putative biomarkers of progression. The SIEVE results were mined by Pinpoint to generate targeted SRM assays on a triple-quadruople MS. A novel software algorithm was developed to link SRM assay results from LCM tissue section images.

Results
Several known Barrett's disease biomarkers were identified in the LC-MS/MS discovery experiment and localized within the tissue microenvironment. This information was used to perform the SIEVE label-free differential expression analysis yielding many new putative biomarkers predicting progression of BE to EAC.

The MS data derived from these discovery experiments (peptides, product ions) was then mined by Pinpoint SRM software to generate optimized SRM assays. A novel software algorithm was developed to link the LCM tissue images with the sample analysis information. We hypothesize that coupling of objective SRM-based assay information with subjective histology-based information will increase the sensitivity and specificity for risk stratification of BE patients. In addition, the candidate biomarkers of progression discovered in this work are currently being verified prospectively bySRM assays on prospective LCM tissue sections and serum from BE patients.

Figure 1: Example of LCM capture from Barrett's metaplasia. Panel A, before LCM; panel B, after LCM.



Innovative aspects
- Coupling laser microdissection of tissue samples with mass spectrometry to enable quantitative and qualitative analysis of tissue biomarkers.

References
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T129

Enrichment of Low Abundance Proteins in Tissue and Cell Line Samples Using ProteoMiner™ Protein Enrichment Technology

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Introduction
Proteomic methods are now widely used to address basic biological questions and to find biomarkers in clinical research, among other fields. The protein concentration in biological samples can range from 6 to 12 orders of magnitude, depending on the nature of the sample. Despite recent success to cover a higher number of proteins in a single proteomics experiment, no analytical method covers the concentration range of biological tissue samples. In order to limit the number of proteins in a sample, protein fractionation is the only hope to separate and detect medium to low abundant proteins. For serum and plasma samples, which are dominated by a relatively small number of known proteins, immunodepletion methods have been very successful. Recently, we introduced a depletion method based on bead-bound hexapeptides, ProteoMiner™ protein enrichment technology. The hexapeptides are synthesized as a combinatorial library and represent binders to all protein binding sites. The use of ProteoMiner for serum and plasma samples has been successfully demonstrated. In contrast to the immunodepletion methods, which use sample specific antibodies, ProteoMiner™ is sample-independent and therefore can be used for all biological samples. We will discuss here the application of ProteoMiner™ to heart muscle and liver tissue as well as HeLa cell extracts.

Methods
Heart muscle, liver tissue and HeLa cell culture samples were dounced prior to protein extraction. Different buffer combinations, compatible with binding to the ProteoMiner™ beads were tested (see Results section). PBS and buffer systems containing trifluorethanol (TFE) were found to result in a 5 -10% protein yield and used in combination with ProteoMiner™ spin columns to generate enriched samples for 1D and 2D electrophoresis. Criterion-sized 1D and 2D gels from Bio-Rad were stained with Flamingo stain or Coomassie Blue, imaged with a fluorescent or UV-Vis imager. A set of spots were excised with an EXQuest spot cutter, and in-gel digested with trypsin. The resulting peptides were analyzed with either a Bruker Autoflex MALDI TOF mass spectrometer or a nanoLC coupled to a Thermo-Fischer LTQ mass spectrometer to get protein identification. Western blots were achieved with commercial antibodies against actin, tubulin and myosin.

Results
ProteoMiner™ relies on binding a complex protein sample to beads covered with hexapeptides. Successful binding depends on the proteins retaining some 3D structure, therefore high concentrations of detergents and chaotropic agents should be avoided. We found that the use of fluorinated alcohols increases protein solubility without interfering with the binding step. We see in the presented 1D and 2D data after processing with ProteoMiner, a pronounced decrease of the high abundance proteins, for example actin, tubulin and myosin. The decrease in the presence of these proteins was also demonstrated with Western blots when 1D gels of samples with and without ProteoMiner™ processing was compared. Using 2D gels, a number of novel spots were excised and subjected to trypsin digestion, followed by mass spec identification.

Innovative aspects
Our study demonstrates that the ProteoMiner™ technology is applicable to non-serum and non-plasma sample and allows for fractionation of the protein content, thereby enabling detection of medium- to low-abundance proteins in tissue and cell line samples.

T130

Identification of Periostin as an Invasion Promoter Protein in the Stroma of Nasopharyngeal Carcinoma by Proteomic Analysis

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Introduction
The importance of stromal cells and the proteins that they expressed during cancer initiation and progression have been highlighted by recent literature. Metastasis is a final and eventually fatal step in the progression of nasopharyngeal carcinoma (NPC). To identify the stromal proteins associated with the pathogenesis of NPC, we assessed differences in protein expression of the stroma cells and cancer cells in NPC using a comparative proteomic approach combined with laser capture microdissection (LCM).

Methods
LCM was employed to purify stromal cells and cancer cells from NPC respectively. Proteins between the pooled microdissected stromal cells and cancer cells were analyzed by two-dimensional difference gel electrophoresis (2-DE) and differential proteins were identified by mass spectrometry (MS). One differential protein (periostin) was further confirmed by Western blotting and immunohistochemical analysis. Furthermore, nonmetastatic 6-10B cells with high periostin expression was stably transfected with plasmids that expressed periostin cDNA, or with empty vector and investigated for its role in the metastasis of NPC. At the same time, the expression and location of integrin-$\alpha_v\beta_3$ was detected by immunohistochemical analysis in primary NPC and noncancerous nasopharyngeal mucous. The effects of periostin expression on in vitro cell invasion, and the mechanism of periostin were analyzed in the transfected cells.

Results
As a result, twenty-five differential proteins were identified. Periostin was significantly up-regulated in the stroma of NPC compared with cancer cells. Overexpressed periostin in 6-10B cells could increase its in vitro cell invasion and improve the activity of MMPs. Integrin was significantly up-regulated in the membrane of NPC cells compared with noncancerous nasopharyngeal epithelial cells, Spearman ananlysis showed that the expression of periostin is significantly correlated with the expression of integrin (r=0.0.682, p=0.000). Taken together, our results suggest that periostin may be a NPC metastasis promoter, and increased periostin expression is associated with the increased invasive capability of NPC cells possibly through improve the activity of MMPs by combining with integrin.

Innovative aspects
- LCM was employed to purify stromal cells and cancer cells from NPC respectively.
- Stromal protein were analyzed by 2-DE and differential proteins were identified by mass spectrometry (MS).

References
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T131

3D Liver Co-Culture Proteome as a Model for Functional Tissues

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Introduction:
Mammalian cells quickly lose their structure and tissue-specific functions when grown in classic 2D mono-cultures. However, many functions are retained when multiple cell types are grown in 3D co-culture systems.[1] These 3D co-cultures may prove useful in chronic *in vitro* studies and to reduce animal testing. Here we compare the proteome of perfused mouse liver tissue with 3D liver co-cultures over an 8 day period using PAGE-LC-MS/MS. The detectable presence of a panel of known liver-specific proteins is used as evidence of maintenance or changes in liver functions.

Methods:
A C57BL/6 mouse liver was perfused *in situ* with buffered saline prior to excision and a section was homogenized in RIPA buffer to isolate soluble proteins. 3D C57BL/6 mouse liver co-cultures (RegeneMed Inc), which contain both liver stromal and parenchymal cells, grown on nylon mesh in 24 well plates were maintained in supplemented Williams Medium E. Soluble proteins were isolated in T-PER buffer, separated by 1D PAGE into 24 bands and digested with trypsin in-gel. Peptide extracts from each band were analyzed by LC-MS/MS using a nanoAcquity UPLC coupled to a ThermoFinnigan LTQ FT Ultra. Tandem MS data from all 24 bands were combined and searched using Mascot and XTandem! software. Lists of identified proteins from each sample were compared using Scaffold software.

Results
We have analysed the proteome from the soluble fraction of perfused mouse liver and identified 916 proteins. Liver perfusion *in situ* reduced the inclusion of blood and serum proteins compared to published reports,[2] thereby providing a more tissue-specific proteome.

The proteome from two separate 3D mouse liver co-cultures had 834 proteins of which 60% coincided with the whole liver proteome. Many of these were liver-associated proteins including albumin, AST, RBP4, transferrin, carboxylesterases, alcohol dehydrogenase, GST, and UGT.

When another two 3D liver co-cultures were collected 8 days later and analyzed, 764 and 265 proteins were identified with 96% and 94% homology with the starting cultures, respectively. The lower number of identified proteins reflects the lower total protein concentration. These data lend preliminary support to studies on the biological stability of 3D liver co-cultures and are being compared to functional and genomic assays to assess the potential for using human engineered livers in toxicology and pathology research.

Innovative aspects
- The use of *in situ* organ perfusion prior to protein isolation.
- Comparison of whole liver proteome to that of engineered liver cultures
- Time course evaluation of proteome from engineered liver cultures

References:
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Development of protein extraction methods for deer antler proteome research

T132

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Introduction
Deer antler is a kind of famous TCMs, with the obvious regulation effects on diseases of nervous system, immunologic system, cardiovascular system and genital system. There are several kinds of components in deer antler (1), including polypeptides, amines, vitamins, hormones, enzymes, microelements, unsaturated fatty acids, phospholipids, prostaglandins, steroidals, cortisols and so on. However, the bioactivity of deer antler has been considered to be related with the active components, such as peptides and proteins. In order to study antler proteome, various protein extraction methods were developed, and by five-step in parallel extraction, 497 proteins were identified, which was of great significance to perform comparative proteome study of antlers from different growing stages.

Methods
The red deer antlers were frozen by liquid N_2 and ground into small particles. Then proteins were extracted by HCl, NaOH, Tris+guanidine-HCl, Tris+guanidine-HCl+EDTA, Tris+guanidine-HCl+CHAPS+DTT, at 4°C for 24 hours, respectively. After acetone precipitation, the extracted proteins were redissolved, reduced, alkylated and digested, followed by further separation by μRPLC with a coupled C_{18} column(5+10+15cm), and identified by ESI-MS/MS system. Each sample was analyzed in triple runs.

Results
Antlers are composed of various structures, such as perichondrium, chondroprogenitor, cartilage, non-mineralized and mineralized bones. Therefore, several lysis buffers with different chemical properties were used for protein extraction. In our experiments, 5 buffers, including HCl, NaOH, Tris+guanidine-HCl, Tris+guanidine-HCl+EDTA, and Tris+guanidine-HCl+CHAPS+DTT were applied for proteins extraction from antler, and the base peak chromatograms of protein digests extracted by different method were shown in Figure 1. With a stringent threshold of Xcorr(+/-1,2,3) = 1.90, 2.20, 3.75 for the unambiguous identification of digests, 207, 22, 182, 169, and 140 proteins were respectively identified by each method, and in total 497 proteins were identified, much more than that reported by 2D-gel method (2). Among these identified proteins, only 3 (0.6%) proteins were found in all five extracts, while 131 (26.36%), 13 (2.62%), 75 (15.09%), 72 (14.49%), and 72 (14.49%) proteins were uniquely observed by each method. The low overlapping ration of identified proteins proves the complementarity of such five extraction methods, by which more proteins could be identified from antler with further combination with μRPLC-ESI-MS/MS.

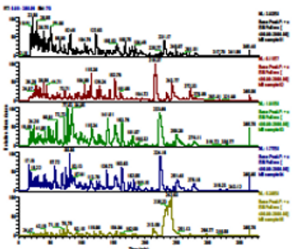

Figure 1. Base peak chromatogram of protein digests extracted by HCl, NaOH, Tris+guanidine-HCl, Tris+guanidine-HCl+EDTA and Tris+ guanidine-HCl+CHAPS+DTT (from up to bottom)

Innovative aspects
- 5 parallel protein extraction methods for antler proteome study
- In-depth understanding of proteomes of animal medinces in TCMs

References
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(2) H.J. Park et al, Proteome analysis of red deer antlers. Proteomics 2004, 4, 3642-3653.

Local Snakebite Pathophysiology Induced By Jararhagin Injection: A Tissue Proteomics Approach

T133

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Introduction
Snake venom metalloproteinases (SVMPs) are the main toxins involved in the development of the complex pathophysiology of viperid snake envenomations. Jararhagin is a SVMP able to cause hemorrhage and provoke an inflammatory response. Although some of these protein activities have been extensively described, the precise molecular mechanism by which this and other SVMPs cause tissue damage are not fully understood. Therefore, here we describe, for the first time, a tissue proteomic approach to illustrate the local effects of snakebite envenomations, caused by Jararhagin intradermal injection.

Methods
Mice envenomation was performed by intradermal injection of Jararhagin or saline under the presence or absence of blood flow in the tissue, as described (1). Skin tissue was collected 15 min after treatment and either lyophilized for mass spec analysis or paraffin embedded for histology. Protein samples were loaded in a 12% SDS-PAGE. Gel lanes were cut in 10 equal-sized slices and LC/MS/MS was performed in a LTQ mass spectrometer. Spectra were extracted and analyzed utilizing Bioworks Sequest and validated with Scaffold software. Searches were performed against an IPI Mouse database. Ratios between samples were calculated using Quantitative values. Quantitative value lower than 5 were not considered; an arbitrary ratio cutoff of 2 was used to determine significantly changed proteins.

Results
Histological observation of envenomated tissue showed evidence of edema, hemorrhage and epidermis detachment. Hemorrhage was also noted, under blood flow condition, by higher abundance of plasmatic and erythrocytes proteins. In addition to the expected infiltrate, two major proteins groups were found in greater abundance in the treated tissue: acute phase proteins (e.g. C3, Inter α-trypsin inhibitor, Ceruloplasmin) and oxidative stress proteins (e.g. catalase, glutathione peroxidase 1). Moreover, degradation and destabilization of extracellular matrix was observed by the decrease of collagen I and coagulation factor XIII. Adjacent muscle tissue damage was also observed by the presence of Troponin I. Under the absence of blood flow, local effects of envenomation were subtle, as previously observed (1). Altogether, these results indicate a rapid, direct action of this toxin in promoting hemorrhage, inflammation and oxidative stress. Moreover, the identification of decreased Factor XIII is the first indication that matrix degradation and destabilization is not only by direct proteolysis, but by the decrease of this protein that is involved in collagen cross-link and tissue repair.

Innovative aspects
- First application of tissue proteomics in vivo as a tool to the study of envenomation conditions pathophysiology
- Identification of decrease in coagulation factor XIII as a possible important aspect in extracellular matrix destabilization and tissue damage

References:
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Identification of Lipids in Placentas from Control and Preeclamptic Pregnancies

T134

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Introduction
Preeclampsia is a pregnancy-associated disorder characterized by maternal hypertension and proteinuria, and is accompanied by dyslipidemia in the maternal bloodstream. To determine if the placenta is also affected by dyslipidemia, it is necessary to develop a method to profile the major placental lipid species. As a precursor to quantitative analysis of the placental lipid profile in preeclampsia, we have qualitatively analyzed lipids from control and preeclamptic placentas by FT-MS (Fourier transform mass spectrometry).

Methods
Samples of placental chorionic villus were taken immediately after delivery from two control and two preeclamptic pregnancies and lipids were extracted by the Bligh-Dyer method. The organic phase extracts were then ionized by direct infusion nano-electrospray and analyzed in a Varian QFT 12 Tesla FT-MS.

Results
In a typical analysis, 78 peaks of m/z between 400 and 900 were detected in negative ion mode and 40 in positive. The LIPID MAPS database was searched using these m/z ratios with a mass tolerance of 0.01 mass units. 57 (48%) of all of the detected peaks could be unambiguously assigned to a unique lipid class and elemental composition, accounting for 69% of the total peak intensity. The identified peaks predominantly represented glycerophospholipids including phosphatidyl cholines, phosphatidyl ethanolamines, phosphatidyl serines, phosphatidyl inositols, and phosphatidic acid. 55 species of lipid were identified as common between all 4 placental samples analyzed.

Qualitative analysis has identified lipid species that can be targeted for further quantitative analysis of placental lipids in preeclampsia. Identifying changes in placental lipid composition associated with preeclampsia will facilitate greater understanding of this disease.

Innovative aspects
- Lipid profiling of the placenta
- Lipidomic analysis by high-field FT-MS

References
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Detection and quantification of potential markers in FFPE tissue and plasma samples.

T135

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Introduction

Targeted analysis by Multiple Reaction Monitoring (MRM) in combination with mass-tagging provides an ideal means of detecting and quantifying potential cancer markers particularly when studying limited amounts of proteins. This technique was applied to verify differential expression of a number of proteins of interest in extracts of cells that were laser microdissected from formalin fixed paraffin embedded (FFPE) tissue samples. The technique was also successfully applied to plasma samples that had been immunodepleted of the 20 most abundant proteins.

Methods

MRM transitions of peptides from multiple potential markers were developed after an in-depth inspection of archived iTRAQ data that had been generated from tissue homogenates previously during the discovery phase of the project. Three transitions were chosen for each peptide and typically 2 peptides were targeted for each protein of interest.
Pathologist directed areas of interest in sections of FFPE tissue were captured using laser microdissection and solubilized using a proprietary reagent as previously described.[1] Each solubilized sample was trypsinized and labeled with mTRAQ reagents (Applied Biosystems Inc). Each sample was mixed with the oppositely labeled control and 2D-LC MS analysis was performed. A similar analysis was performed on the immunodepleted plasma.

Results

MRM analysis of mTRAQ labeled FFPE tissue samples successfully detected multiple potential markers, proving that FFPE samples can be utilized as a resource for the biomarker detection by mass spectrometry-based analysis. The mTRAQ labeling enabled quantification, which verified differential expression levels of some of those proteins in endometrial samples relative to the normal control pool. Others showed no differential expression, suggesting that the differential expression previously observed in the study of the tissue homogenates might be attributed to tumour environment rather than the actual tumour cells themselves. More importantly the results represent intermediate, if not an altogether alternative, means of interrogating FFPE samples, to ELISA analysis. As such, this study successfully demonstrates that FFPE samples can now be used in efforts to validate potential markers prior to any investment in the development of an ELISA assay. In an extension of this powerful technique, we successfully detected some potential markers in plasma. This further demonstrates that the approach can be used as a means of screening in plasma, minimizing the need for any invasive procedures.

Innovative aspects

- Detection and quantification of potential markers from FFPE tissue samples.
- Detection and quantification of potential markers from plasma.
- Demonstration that the tissue-to-plasma approach is feasible for developing novel biomarkers for cancer.

References:

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T136

An Integrated Platform For MALDI Imaging

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Introduction

Matrix-assisted laser desorption/ionization (MALDI) imaging is a technique that allows for 2-dimensional spatial resolution of proteins, peptides and small molecules in tissue sections. To fully understand the biological functions of different cellular regions of tissue, it is crucial to identify the location and abundance of the proteins contained within them.

Here we have combined the technologies of three different mass spectrometers for an integrated platform of MALDI imaging. Using this platform we have discovered, identified, and quantitated proteins of interest from mouse brain tissue.

Methods

A Microm HM 500 cryostat was used to slice 12 µm serial sections from snap frozen mouse brains. A Bruker ImagePrep station was used to apply CHCA and DHB matrices to the tissue sections. Data was acquired on the following mass spectrometers: a Bruker Ultraflex III TOF/TOF equipped with a smart beam laser, a Bruker Apex-Qe 12-Tesla UltraShield Plus FTICR equipped with a MALDI source, and an Applied Biosystems QTrap 4000 fitted with a prototype vacuum MALDI source. For the quantitation experiments, isotope coded standards were applied to the thin sections at appropriate concentrations. *In situ* trypsin digestion was performed on the tissue samples analyzed by the FTICR and QTrap. Data was analyzed using the Bruker fleximaging software as well as custom built software.

Results

Using the data acquired on the MALDI-TOF/TOF we made an ion image of the mouse brain tissue. This image showed several areas of compartmentalized *m/z* values within different regions of the brain. These areas were further investigated with trypsin digested serial sections using the MALDI-FTICR. MALDI FTICR MS combines high resolving power, high mass accuracy and the possibility of performing multistage MS/MS experiments. The FTICR MS/MS data gave us the sequences of several peptides. We combined the sequences with the parent mass data acquired from the TOF/TOF to identify the compartmentalized proteins. We quantitated the proteins we had discovered and identified by performing MRM experiments on trypsin digested tissue sections using the MALDI-QTrap, as described by Parker C.E. *et al.*

With this technology, 2D images can be created from the organs of animal models in different stages of disease and drug administration. These images can be combined to produce 4 dimensional spatial and temporal models of disease progression. The results presented here, using our comprehensive integrated platform of MALDI imaging, represents an important first step towards this 4D modeling.

Innovative aspects

Data acquisition for MALDI imaging using the combination of three technologies.

References

Parker C.E., Smith D., Suckau D., Borchers C.H. Mass Spectrometry-Based Tissue Imaging. Advanced Imaging in Biology and Medicine. 2009, 131-146.

T137

Quantitative Proteomics of Glutathione S-Transfereases in Mouse Liver Mitochondria

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Introduction

The glutathione S-transferases (GSTs) are one of the major enzymes involved in detoxified and antioxidant reactions. The GST family contains eight classes, in which the members play different physiological roles in response to various stress (1). Although mitochondria are believed to have their own special distribution of GSTs as to deal with high oxidative stress, we still lack an overall knowledge of which kinds of GSTs are localized in mitochondria and what are their relative quantities. Herein we report a systematic investigation to the GST proteome in the mouse liver mitochondria.

Methods

Three mouse tissue mitochondria, heart, kidney and liver, were isolated with Nycodenz gradient centrifugation and their purity and integrity were examined through Western blot and transmission electron microscopy. The proteins were separated by SDS-PAGE or enriched by GSH affinity chromatography. The separated or enriched proteins were tryptic digested, and the digestions were delivered to LC-MS/MS for protein identification. The quantitative GSTs were estimated based upon spectrum counts (2).

Results

Based upon SDS-PAGE for protein separation and LC-MS/MS for protein identification, five GSTs classes (pi, mu, kappa, alpha, zeta) were identified in mouse liver mitochondria, while only three GSTs classes (pi, mu, alpha) were recognized in the samples enriched by GSH affinity chromatography. The strategy of SDS-PAGE/LC MS/MS, thus, was selected to profile the relative abundance of GSTs in the mitochondrial samples.

As compared of the GST proteomes in the three mouse tissue mitochondria, three GSTs, pi, kappa, and alpha, were generally identified in these organelles. The GST profile of liver mitochondria is similar to kidney, in which GSTmu and GSTzeta were located in liver and kidney, but not in heart (Table 1).

The relative abundance of the GSTs in mouse liver mitochondria was estimated with label-free approach based upon the counts of MS/MS spectra. As depicted in Figure 1, the distribution patterns of GSTs in mouse liver cytoplasm and mitochondria was quantitatively illustrated, indicating that the relative abundances of GSTpi, mu, alpha and zeta in mitochondria are quite close to that of in cytoplasm, nevertheless, GSTtheta was only found in liver cytoplasm and GSTkappa was specifically located in mitochondria (Figure 1). In spite of undetectable for some GSTs, their presence of mitochondria, even at very low abundance, could be excluded. Our study provides the first hand information that the GST proteomes are variable in different tissue mitochondria and organelles, and the relative abundances of some GSTs in the mitochondria are distinctive from the cytoplasm's ones.

Innovative aspects

- Identification of GSTs in mitochondria by multiple strategies.
- Estimation of the relative abundances of the mitochondrial GSTs.

References

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Table1 Classes of GST in three mouse tissue mitochondria

Mitochondria of tissue	classes of GST
heart	alpha, kappa, pi
liver	alpha, kappa, pi, mu, zeta
kidney	alpha, kappa, pi, mu, zeta, theta

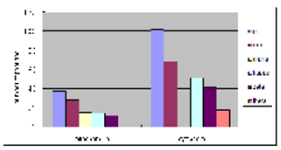

Figure 1 Ratios of GSTs in mouse liver mitochondria and cytoplasm

T138

MRM ASSAY TO IDENTIFY BIOMARKERS FOR LUNG CARCINOMA OF LARGE-CELL NEUROENDOCRINE (LCNEC)

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Introduction

The retrospective proteomic analyses were carried out using the formalin-fixed paraffin-embedded (FFPE) tissues (1,3) of lung large-cell carcinoma (LCC), small-cell lung carcinoma (SCLC), and large bcell carcinoma neuroendocrine (LCNEC). LCNEC has a poor prognosis similarly as small-cell lung carcinoma (SCLC), and survival rate is just 18% in IA-stage only by resection(2). Therefore, accurate differentiation of LCNEC from other types of NSCLC is important because it identifies those patients at highest risk for developing recurrent disease(2).

Methods

FFPE tissues (LCC 5, SCLC 5, and LCNEC 4 cases) were obtained and used for this study from Tokyo Medical University Hospital under the approval of the Ethical Committee, and informed consent was obtained from individual patients. FFPE proteome analyses have been carried out by the high resolution LC-MS (LTQ-Orbitrap® MS) in triplicate runs per sample following laser microdissection, Liquid Tissue® solubilization, and tryptic digestion. Candidate marker proteins have been selected by both semi-quantitative comparison based on spectral counting method and statistical evaluation (U-, and G- tests). We have developed multiple-reaction monitoring (MRM) assays (3) of candidate proteins and have verified their specificities prior to further large scale validation study.

Results

The exploratory studies on FFPE tissues of LCC, SCLC and LCNEC revealed more than 1,000 proteins identified ($p < 0.05$), respectively. Their semi-quantitative comparison by both spectral counting analyses and statistical tests resulted in more than 100 proteins which would significantly characterize LCC, SCLC and LCNEC. Those candidate proteins have been subjected to quantitative verification by MRM assays for individual cases. Significant proteins include markers known to be recommended for cancers of Neuroendocrine (NE) such as secretagogin (SEGN), NSE (neuron specific enolase), NCAM (neural cell adhesion molecule), and chromogranin A (CgA). We have carried out the quantitative LC-MRM mass spectrometric measurements (in triplicate) for about 40 proteins of interest, and in which MRM assays were comprised of their 182 MRM transitions. It has been revealed among them that 3 proteins would be specific to lung tumors of NE- type, 8 proteins to LCC, and 1protein to SCLC. SEGN as one of the known markers were found to be consistently specific to both SCLC and LCNEC as expected (Figure 1).

Innovative aspects

- More than 20 novel proteins promising markers able to differentiate LCNEC, LCC and SCLC.
- Each type of lung cancer tissue could be identified by quantifying the expressions of specific combination of 3 protein markers.

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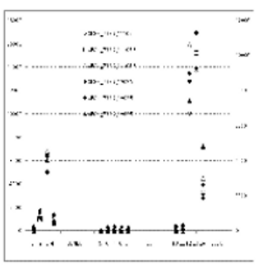

Figure 1. MRM measurements for SEGN.

T139

Comparatively proteomic analysis to drosophilatreated with/without hypoxia

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Introduction

Drosophila strain was tolerant to hypoxia through long-term selection. Zhou's group found that several metabolic gene expressions were down-regulated, whereas the transcriptional suppressor, hairy, was up-regulated. Hence, the hairy-mediated metabolic suppression was proposed to be a critical mechanism for hypoxia tolerance (1). Do the transcriptional changes directly reach to the translational level during the adaptive process? We adopted the same drosophila model, and analyzed the protein abundant responses in the thorax muscle and its mitochondria between normal and hypoxia drosophila.

Methods

The drosophila thorax muscle were carefully isolated and divided into two fractions, one directly for protein extraction and the other for mitochondrial preparation. The drosophila muscle mitochondria were prepared through density-gradient centrifuge with sucrose. The proteins in the drosophila muscle or mitochondria were extracted in lysis buffer containing 7M urea, 2M thio-urea 4% CHAPS and 40mM Tris-HCl, pH 8.0. The extracted proteins were separated through 2DE, then the differential spots were excised and tryptic digested for protein identification by MALDI-TOF/TOF MS.

Results

1. Comparison of 2DE images of the drosophila thorax muscle mitochondria

Three pools were collected from control or hypoxia drosophila thorax muscle. Each pool was run through 2DE with pH 3-10 in parallel. On average, there were 480±12 (n=6) 2DE spots in control, 478±13 (n=6) in hypoxia. The image analysis revealed that a total of 9 spots were found in significant changes of spot volume (>3) (Figure 1). Furthermore, 7 unique proteins were identified including some typical mitochondrial proteins such as ATP synthetase β, aconitase and prohibitin.

2. Comparison of 2DE images of the drosophila thorax muscle

As similar most muscle proteins in insect or mammal tissues, two abundant proteins, actin and CamK, interfere to 2DE image analysis. Thus, a 2DE with narrow pH range 5-8 was employed to separate the drosophila muscle proteins. There were 778±1 (n=2) spots in control, 780±1 (n=2) in hypoxia (Figure 2). Through image analysis, only one spot was defined as a differential one, which was SODII.

In summary, the preliminary analysis to proteomes obtained from the thorax muscle suggested that no globally proteomic response was found in the tolerant drosophila after long adaptation of hypoxia.

Innovative aspects

- Select a successful hypoxia drosophila model for proteomic investigation
- Systematic analysis to drosophila thorax muscle proteomic changes in response to long-term hypoxia.

Reference

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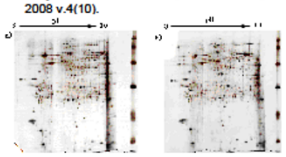

Figure1. The 2DE images of mitochondrial proteins.
a)Control b)Hypoxia tolerant

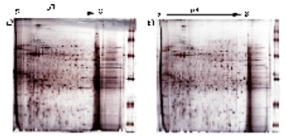

Figure2. The 2DE images of muscle proteins.
a)Control b)Hypoxia tolerant

T140

Collection and Characterization of Human Liver Cell Secretome Using a Hollow Fiber Bioreactor Culture System Coupled with Tangential Flow Filtration Enrichment

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Introduction

Secretome, the special proteins that are secreted into the extracellular environment of cells, participates in biological processes such as cell growth, differentiation, immune response, blood coagulation, and transportation. A commercially available hollow fiber bioreactor (HFB) culture system was used to develop an analytical platform for the efficient collection of HepG2-secreted proteins and subsequent proteomics analysis. The proposed secretome analytical platform has two special features. First, HFB system can support a high cell-density culture to efficiently collect secreted proteins. Second, a tangential flow filtration (TFF) device was used to remove the unwanted low molecular weight molecules in the conditioned medium and to enrich the secreted proteins.

Methods

HepG2 was used in this study to demonstrate the investigation of a cellular secretome. Cells were adapted to serum-free medium and inoculated into the HFB culture system. The conditioned medium (CM) containing secretome was collected at the stationary phase in the HFB culture system. Subsequently, the CM was processed by TFF device to remove the unwanted low molecular weight molecules and to enrich the secreted proteins. The secreted proteins in the CM were analyzed by using liquid chromatography tandem mass spectrometry (LC-MS/MS). All the identified proteins were predicted their existence of signal peptide sequence and transmembrane domain by using bioinformatics tools. In addition, all the proteins were classified by their reported subcellular locations in the human protein reference database (HPRD).

Results

It was calculated that only a small fraction of cells (<0.01%) were lysed in the HFB culture system, in contrast to the 5% using conventional dish culturing. Ninety-seven secreted proteins from HepG2 cells were identified using this secretome analytical platform. In addition, 59% of the identified proteins in HFB culture system posses signal peptide sequences and/or transmembrane domains, whereas only 34% of the identified proteins collected from the conventional dish culture medium do (Fig. 1). The subcellular location categories of identified secreted proteins in Fig. 2 indicate 51% of the identified proteins from HFB culture system that were classified as extracellular proteins. In contrast, only 30% of the identified proteins using the conventional dish culturing were classified as extracellular proteins. We suggest that the combination of the HFB culture system, TFF device and LC–MS/MS analysis may be an efficient procedure for the collection and characterization of cell-secreted proteins.

Innovative aspects

- Compared with the conventional dish culturing, the proposed HFB secretome analytical platform reduced greatly the lysis rate of cells from ~5% to <0.01%.
- The HFB culture system can enrich more secreted proteins than conventional dish culturing could.

References

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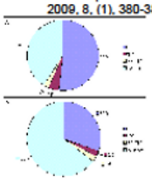

Figure 1. A pie chart shows distribution of identified proteins possessing signal peptide sequence and/or a transmembrane domain. (A) The CM containing secreted proteins collected from HFB culture system (B) The CM containing secreted protein collected from conventional dish culture.

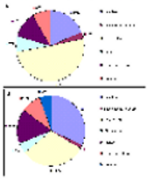

Figure 2. The subcellular location categories of identified secreted proteins. (A) The CM containing secreted proteins collected from HFB culture system (B) The CM containing secreted protein collected from conventional dish culture.

T141

Thermal treatment stabilizes the brain proteome during sample preparation

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Introduction

Sample degradation is frequently a problematic factor in proteomic studies, leading to the generation of protein fragments which create difficulties in quantitative proteomic studies. Typically snap freezing in liquid nitrogen is considered the optimum method for maintaining animal and human specimens to as close as their in vivo state as possible. Here, we assessed the efficiency of thermal treatment to preserve the intact proteome of mouse heart and brain tissue and compared it to standard snap-freezing (1).

Methods

Cerebral cortex and whole heart tissue were removed from nine male CD-1 mice. Three samples of each tissue type were snap frozen immediately in liquid nitrogen (LN). A further group of three tissue samples were subjected to thermal treatment using the "Stabilizor T1" device (Denator) (TT). A final group of three tissue samples were snap frozen, removed and immediately thermally treated using the "Stabilizor" device (LNTT). Samples were analysed by 2DE and silver stained. Proteins with statistically significant changes in protein abundance ($p<0.05$) between the three treatments were grouped according to their expression profile. Approximately 10-15 proteins were selected from each expression profile group for LC-MS/MS analysis, based on p-value and also on spot intensity on 2D gels.

Results

A total of 189 and 77 spots were significantly altered from post-mortem brain and heart tissue respectively when compared to LN treatment alone. Strikingly, in the case of brain tissue, TT resulted in a significant increase in abundance of high molecular weight (MW) proteins, with a concurrent decrease in the number of lower MW proteins. MS analysis of these differentially expressed lower MW proteins revealed some of these as fragments of higher MW proteins. Protein disulfide isomerase A3, was a specific example of a protein which is stabilised by TT with increased abundance of the intact protein and decreased abundance of a fragment identified. The majority of proteins identified have roles in metabolism and cytoskeletal reorganisation. Overall TT appears to preserve intact proteins from post-mortem brain tissue.

Mouse heart tissue did not show such conclusive results. A number of fragments were identified which were increased by both TT and LN treatments. Thus, certain proteins are preferentially preserved with LN treatment while others with TT. Heart tissue is thought to be relatively robust and resistant to protein degradation and this study here would agree with these findings.

Innovative aspects

- Novel technology of thermal treatment with the "Stabilizor T1" which preserves protein integrity of mouse post-mortem tissue
- Thermal treatment is equally as efficient a liquid nitrogen at preserving post-mortem heart tissue

References

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Fig. 1a

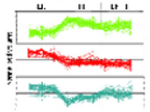

Fig. 1b

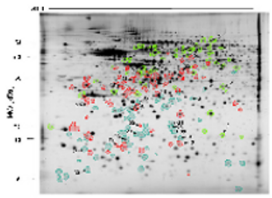

Figure 1: 2DE analysis of mouse post-mortem brain tissue following thermal treatment. Samples were either snap frozen in liquid nitrogen (LN), thermally treated (TT) or snap frozen and TT (LNTT), separated by 2DE and silver stained. Significantly altered spots ($p<0.05$) were separated according to their protein expression profiles (A) and their positions on the gel can be visualised (B).

T142

Proteomic analysis of a transgenic mouse model of heart failure resulting from cardiac hypertrophy

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Introduction

Heart failure represents a leading cause of mortality worldwide (1). Commonly seen in the progression of heart disease is cardiac hypertrophy. Thus, analysis of mechanisms involved in the establishment of this disease state could potentially enhance our capacity for effective treatment interventions. Proteomic profiling allows for the quantification of protein expression during the disease state, and allows for the elucidation of disease pathways. Here we use a transgenic mouse model in conjunction with proteomics analyses to assess protein expression alteration in hypertophed hearts.

Methods

Transgenic mice expressing a constitutively active form of Calcineurin (CnA) were used in the present study (2). Ventricular samples were subjected to a multi-dimensional protein identification technology (MudPIT) analysis, via gel-free shotgun liquid chromatography and tandem mass spectrometry (3). Spectra were cross matched against a mouse Uniprot database using the Sequest search algorithm and matches were assessed statistically using the Statquest algorithm. Significantly altered proteins were subjected to FDR correction using the Benjamini-Hochberg calculation. Finally proteins with significant corrected P-values were examined by immunoblotting as well as RT-PCR.

Results

Our proteomic approach identified 5398 proteins in the hypertrophic and wild-type littermate samples. Following normalization and statistical filtering, we identified 477 proteins that were differentially expressed between CNA transgenic mice and their wild-type littermates. We then implemented the Benjamini-Hochberg calculation to eliminate false positives. This resulted in 53 proteins with significantly altered expression in CNA transgenic cardiac ventricles compared to their wild-type littermates. An extensive series of RT-PCR and immunoblotting analyses coincided well with proteomics data for the top 53 proteins.

Innovative Aspects

- Identification of novel proteins affected during the development of hypertrophy when using a proteomic approach

References

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Wildtype Hypertrophic

Figure 1: Myocardial cross-sections reveal severe hypertrophic cardiomyopathy.

T143

Role of Corneal Epithelial Proteins in Re-epitheliazation

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Introduction

The expression of extracellular matrix proteins laminin, fibronectin (1), integrin (2), F-actin and vinculin have been known to play a vital role during eye development and wound repair. As yet, the cytoskeleton related proteins that regulate cell division, and wound healing are not fully characterized. Hence, identification of various proteins that could associate with healing process is of great significance.

Methods

Migrating (wounded) and nonmigrating (unwounded) rabbit corneal epithelia were prepared by organ culture. Proteins expressions were analyzed by 2-D electrophoresis and proteins spots identified by MALDI-TOF-MS and confirmed by western blotting.

To determine the inhibitory and promotory effects of identified proteins by topical applications of their respective antibodies on cell migration in healing corneal epithelium of organ-cultured rabbit eyes, Corneas were allowed to heal in vitro for 0 to 120 hours in serum free medium in the presence and absence of antibodies. Immunofluorescent staining was used to detect albumin precursor and hsp70 proteins in corneal epithelium at various time intervals following an epithelial defect.

Results

Many proteins expression (either up-or down-regulation) was observed when proteome of migrating and non-migrating epithelium was assessed using 2-DE. From the upregulated set, we have identified a 69 kDa albumin precursor protein with four peptides sequences and 70 kDa hsp70 with one peptide in the active phase of cell migration (48 hours) during the healing process for the first time. Western blot analysis was used to further characterize these proteins at different phases (24, 48 and 72 hours) of healing (3). Delay in wound healing ($p<0.005$) was observed with 10µg/ml anti-albumin antibody labelling. A similar pattern was observed when anti-fibronectin antibody (5µg/ml) alone and in combination with anti-albumin (10µg/ml) was ectopically added with wound closure occurring at 120 hours. Whereas with anti-Hsp70 antibody (5µg/ml) slightly delayed ($p<0.005$) wound closure was observed at 96 hours and considerable retardation >120 hours with 10µg/ml. Additionally, immunofluoresence showed a strong co-localization of Hsp70 and albumin precursor during the active phase of wound healing.

Innovative aspects

- Increased expression of 69 and 70 kDa proteins was found only in migrating corneal epithelium and identified as albumin precursor and Hsp70 my mass spectrometry.
- Exogenous 10ug/ml anti-albumin antibody and anti-Hsp70 antibody were found to delay wound closure in serum free media.
- Albumin and Hsp70 strongly localized at superficial layer and Basal cells of the ocular surface epithelium as linear staining.

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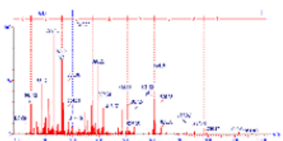

Figure 1. ESI-Q-TOF Tandem MS spectrum of tryptic peptide KQTALVELLK of protein albumin precursor spot 2 (P2) obtained from representative silver stained 2-D PAGE gel of migrating corneal epithelia harvested at 48 hours of post wounding.

T144

Skin Pathology Induced by Snake Venom Metalloproteinases: Analysis of the Hemorrhageome

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Introduction
The hemorrhage induced by snake venom metalloproteinases (SVMPs) is due to their synergic effect on plasma proteins, platelets and capillary vessels. Here we analyzed the interaction and degradation profile of plasma and ECM proteins with different SVMPs: HF3 (P-III) is highly glycosylated and is 80 times more hemorrhagic than bothropasin (P-III) which has a minor carbohydrate moiety, BJ-PI (P-I) is a non-hemorrhagic enzyme, and the DC protein, is composed of the disintegrin-like and cysteine-rich domains. We also used proteomic approaches to evaluate the effects of HF3 on the mouse skin.

Methods
Plasma and ECM proteins (fibrinogen, fibronectin, vitronectin, vWF, collagens I, IV and VI, laminin and Matrigel™ were incubated *in vitro* with the SVMPs and the degradation profile was analyzed by SDS-PAGE. Interaction studies were carried out using solid-phase-binding- and surface-plasmon-resonance assays. The target proteins for proteolysis by HF3 *in vivo* were analyzed by injecting mice with 1 µg HF3. After 2h the dorsal skin was sectioned and the proteins were submitted to 2D electrophoresis (2D-E), in gel trypsin digestion and LC/MS/MS analysis of differential spots. The skin peptide fraction was analyzed by LC/MS/MS. HF3 was also injected in the thigh muscle of mice and after 4h plasma proteins were analyzed by trypsin digestion and LC/MS/MS.

Results
HF3, bothropasin and BJ-PI showed different degradation profiles of fibrinogen, fibronectin, vitronectin, von Willebrand factor, collagens IV and VI, laminin and Matrigel™, however only bothropasin degraded collagen I. In solid-phase binding assays HF3 and bothropasin interacted with fibrinogen, fibronectin, laminin, collagens I and VI; the DC protein bound only to collagens I and VI, and to fibrinogen, however, no binding of BJ-PI to these proteins was detected. *N*-deglycosylation caused loss of structural stability of bothropasin and BJ-PI and consequently they lost their proteolytic activity. *N*-deglycosylated HF3 was stable, however, its hemorrhagic and fibrinogenolytic activities were partially impaired. Interestingly, *N*-deglycosylated HF3 bound with higher affinity to collagens I and VI while *N*-deglycosylated bothropasin lost its binding ability to collagens, as demonstrated by surface plasmon resonance. These findings suggest that the ability of SVMPs to cause hemorrhage is not only related to the function of their catalytic and non-catalytic domains but also to their carbohydrate moieties which play a role in their stability and interaction with key substrates. As expected, some of the results of the hemorrhageome analysis corroborate previous data generated using *in vitro* assays, however, new extracellular, intracellular and plasma proteins have been found to be cleaved *in vivo* by the directly and/or indirectly effect of HF3. On the other hand, some plasma proteinase inhibitors showed higher abundance compared to control. Some proteins affected *in vivo* by HF3 were confirmed to be directly cleaved *in vitro* by HF3. Gelatin-zymography of skin proteins showed *in vivo* activation of pro-metalloproteinases by HF3. Taken together, these findings underscore new mechanisms by which the SVMPs participate in the development of the skin pathology observed with viperid envenomation.

Financial support: FAPESP

Yeast proteomics, Sparks for new biology

(Y101 – Y106)

Y101

Optimized Protein Extraction from Thermotolerant Probiotic Yeast (*S. Cerevisiae* OBV-9) for 2-D Gel Electrophoresis

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Introduction
Probiotics are defined as non-pathogenic live microorganisms that may beneficially affect the host upon ingestion by improving the balance of the intestinal micro flora and exert a positive influence on host health or physiology. Our experiment involves use of thermotolerant yeast as probiotic to tolerate the conditions of the rumen in animals (1). As a part of this research, to differentiate the proteins expressed in mesophilic and thermotolerant yeast, experiments were carried out using 2D gel electrophoresis for which there is a need of optimization of protein extraction method (2). The present paper focuses optimization of the protein extraction method from fungi, by using yeast as model system. It is clearly known that protein extraction from fungi is very difficult due to the thickness and composition of its cell wall. The present existing protocols either use enzyme based method (Zymolyase) for disruption of cell wall or use of mechanical methods (glass beads). Here, we describe a method which we have optimized by using liquid nitrogen and other simple components of the lab.

Methods
In the present study, proteins were extracted from yeast by using four different methods i.e. A) Proteins were extracted by using glass bead method coupled with TCA precipitation, B) by using enzyme based method followed by TCA precipitation, C) protein extraction by standardized lysis buffer along with liquid nitrogen and D) Protein extraction by standardized lysis buffer along with liquid nitrogen followed by TCA precipitation. The last method (D) uses addition of liquid nitrogen to the yeast pellet followed by immediate addition of standardized buffer solution containing a buffer of optimum pH, a protease inhibitor to inhibit the protease action, a detergent to increase the solubility of proteins and a reducing agent to separate the protein subunits (2). This was followed by homogenization. The homogenate was centrifuged, the supernatant extract was collected and TCA precipitated. The pellet after precipitation was dissolved in the buffer for 2DE and gel electrophoresis was performed. The images of 2D gels run by all the methods were compared by image analysis software.

Results
The gels were compared for the number of spots and quality of proteins. The number of protein spots obtained in the four methods were 234, 110, 173 and 349 respectively. It indicates that the last method (D) was a very effective, quick and simple method of protein extraction from yeast. Also, more number of spots with fine quality was observed in this procedure. Therefore, protein extraction by standardized lysis buffer along with liquid nitrogen followed by TCA precipitation method (D) was selected for the 2D gel preparations and proteomic characterization of the thermotolerant yeast (Fig 1).

Innovative aspects
- Quick, simple and reliable method of protein extraction from yeast for 2D gel preparations.
- Can be extended to other complex systems such as filamentous fungi and plants.
- 2D gels are qualitatively and quantitatively comparable with existing protein extraction methods.

References
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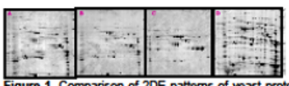

Figure 1. Comparison of 2DE patterns of yeast proteome extracted by different methods (A) glass bead method, (B) enzyme based method, (C) by using standardized protein extraction buffer and (D) by using standardized protein extraction buffer followed by TCA precipitation.

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Y102

Heat-Shock Induced Misfolded Cytoplasmic Proteins Are Ubiquitinated by A Network of Ubiquitin E3 Ligases in *Saccharomyces cerevisiae*

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Introduction
The Ubiquitin Proteasome System (UPS) is a major pathway to eliminate cellular misfolded proteins targeted by the Protein Quality Control (PQC) machinery. Previous studies have shown that different E3 ubiquitin ligases ubiquitinate PQC targets according to their localization. E3s functioning in the ER-associated protein degradation (ERAD) pathway and in the nucleus have been identified. However, the main components of UPS ubiquitinating cytoplasmic PQC targets in yeast have not yet been identified.

Methods
1. $^{14}N/^{15}N$ Quantitative MS analysis: Yeast cells that stably expressed His_6-ubiquitin were used. Cells grown in ^{14}N or ^{15}N containing media were mixed in 1:1 ratio before protein lysis. Ubiquitinated proteins were purified by nickel chromatography following by trypsin digest for LC-MS/MS analysis. Different quantitative analyses were done for identifying HS-induced PQC targets and E3s specific HS substrates.
2. Heat-Shock (HS) screening: Wild type (WT) and E3Δ stains were treated with 15min HS at 45°C and compare to no treatment. The HS response deficiency was quantified by Q-dot fluorescent blotting assay.
3. HS viability assay: Viabilities of WT and E3Δ strains were assessed after HS treatment (30min at 45°C).

Result
We first determined that heat-shock (HS) induced a large increase of polyubiquitination in yeast cells. We performed quantitative MS on purified ubiquitin-conjugates and determined that HS mainly affected cytoplasmic proteins (Figure1A). This indicated that the HS stress is ideal for studying the cytoplasmic PQC. After screening all known and putative yeast E3s, we identified 12 individual E3 deletions that impaired the increase of ubiquitination after HS. Two of these E3 candidates showed decrease viability after HS and two others displayed growth delay. Because none of the tested individual E3 deletion fully abolished HS-induced ubiquitination, there is most likely a network of several E3s ubiquitinating cytoplasmic PQC targets. Using the same quantitative MS approach, we were able to identify several proteins that required *HUL5* (one of the identified E3) to sustain ubiquitination after HS. When we compared these proteins to the ubiquitinated proteins enriched after HS, we determined that a fifth of the HS-affected proteins required *HUL5* for ubiquitination (Figure 1B).

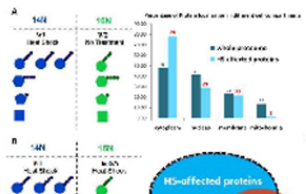

Figure 1. $^{14}N/^{15}N$ quantitative MS analysis
A. Diagram of the $^{14}N/^{15}N$ comparison for identifying HS-affated proteins (left). The localization of identified proteins were then compared to global GFP-protein localization database (right).
B. Diagram of the $^{14}N/^{15}N$ comparison for identifying HUL5 substrates(left). The comparison of the proteins further ubiquitinated after HS and HUL5 substrates identified by MS.

Innovative aspects
1. Develop a proteomic approach to identify ubiquitinated proteins.
2. Screen for E3 ubiquitin ligases affecting global response after heat shock.
3. Gain insight of the cytoplasmic PQC machinery.

Y103

Proteomic Analysis of *Aspergillus fumigatus* to Identify Molecular Targets of Artemisinin

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Introduction
Aspergillus fumigatus (*A. fumigatus*) is a saprophytic, ubiquitous, opportunistic fungus, which leads to allergic or invasive aspergillosis in humans depending on the immune status of the host (1). Proteome analysis may lead to further understanding on the proteins and molecular pathways functional in *A. fumigatus*. Artemisinin is commonly used as an antimalarial drug, however, it is also reported to have antifungal activity against *Candida albicans*, *Caenorhabditis neoformans* and *Aspergillus species* but not yet against *A. fumigatus* (2). In the present study, we have attempted to understand its molecular targets in *A. fumigatus*.

Methods
Proteome analysis: Total protein extracted from *A. fumigatus* Af293 strain cultured for 48 h was resolved on two-dimensional gel electrophoresis (2-DE) and proteins were identified by mass spectrometric (MS) analysis, matrix-assisted laser desorption/ionization-time of flight, MALDI-ToF and ToF/ToF. **Effect of artemisinin on *A. fumigatus***: Minimum inhibitory concentration, MIC_{50}, of artemisinin against *A. fumigatus* was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For proteomic analysis, *A. fumigatus* was cultured for 48 h and treated with artemisinin at MIC_{50} concentration for 3 h. Comparative analysis of the proteins extracted from treated cultures and the controls was carried out by 2-DE and MS approach.

Results
Proteome analysis led to the identification of 205 proteins from 487 protein spots belonging to various functional classes or pathways. MIC_{50} of artemisinin against *A. fumigatus* was determined to be 125 µg/ml. Proteomic profiles of *A. fumigatus* treated with artemisinin showed differential expression of 175 proteins- 66 up-regulated and 109 down-regulated as compared to the control. Peptide mass fingerprint search analysis led to the identification of 96 proteins- 28 up-regulated and 68 down-regulated proteins with high confidence. The differentially expressed proteins belong to cell stress (Thioredoxin reductase, HSP70 chaperone, mitochondrial peroxiredoxin), amino acid metabolism (choline oxidase, glutamate/leucine/ phenylalanine/ valine dehydrogenase), energy metabolism (succinyl CoA synthetase alpha subunit, electron transport flavoprotein alpha subunit, vacuolar ATP synthase catalytic subunit A), glyoxylate pathway (isocitrate lyase), carbohydrate metabolism (GNAT family acetyltransferase, UTP-glucose-1-phosphate uridylyltransferase), translation (translation elongation factor EF-2 subunit, cofactor for methionyl- and glutamyl-tRNA synthetases), cell wall proteins (cell wall protein PhiA, conidial hydrophobin B) and others including hypothetical proteins.

Innovative aspects
- 2-DE reference map of *A. fumigatus*, a human pathogen
- Some of the proteins and pathways targeted by artemisinin may serve as important leads for development of new antifungal drugs.

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Y104

Building a global map of Rub1 function in *S. cerevisiae*

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Introduction

Rub1p is the *S. cerevisiae* ortholog of mammalian NEDD8, a ubiquitin-like protein that is covalently conjugated to lysine residues on target proteins (1). Similar to ubiquitylation, rubylation is facilitated by a cascade of E1 (Ula1/Uba3), E2 (Ubc12) and E3 (e.g. Dcn1) enzymes. Rub1p/NEDD8 target many different types of proteins, including ubiquitin E3 ligases, ribosomes and critical cell cycle regulators. The Rub1/NEDD8 system thus appears to be important for many different cellular functions, but how it regulates these processes is poorly understood.

Methods

To identify biochemical pathways that compensate for rubylation deficiency, we conducted synthetic genetic array (SGA) analysis (2). A *ubc12* temperature sensitive yeast mutant and a *rub1* knockout strain were screened against an ordered array of ~ 4700 viable deletion mutants, and ~500 temperature-sensitive mutants of essential genes, for synthetic lethal or slow growing (sick) genetic interactions. All mapped interactions were confirmed by random spore analysis (RSA) to eliminate high-throughput false positives. We also used affinity purification-mass spectrometry (AP-MS) to identify Rub1p binding proteins in a yeast whole cell lysate.

Results

Using this systematic functional genomics approach we mapped a network of more than 110 synthetic genetic interactions. Over 320 Rub1p physical interactions were identified in the AP-MS screen. By combining data from the SGA, the AP-MS screen, and curated literature data, we have thus obtained a global rubylation network, highlighting a role for this important PTM in several major biological processes. For example, genes found in these two very different experimental approaches clustered together in phosphorylation signaling, invasive growth, mitosis, chromatin remodeling and transcription (Fig.1). Our Rub1 functional map represents a valuable resource that can be mined to identify the roles of this important regulatory system in the regulation of individual proteins, protein modules, and signaling pathways.

Figure 1. Percentage of total hits for each of two different Rub1 datasets (SGA and protein interactors) falling within seven selected biological processes.

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Y105

Proteome Analysis of *Aspergillus ochraceus*

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Introduction

Aspergillus ochraceus is a filamentous fungus which produces toxic and carcinogenic secondary metabolite Ochratoxin A (OTA). OTA, an isocoumarine compound linked to L-β-phenylalanine, is a potent nephrotoxic and one of the important mycotoxin due to its high toxicity. Genome sequencing for many important fungi have been attempted during recent years, however there is still deficiency in proteome profiling of aspergilli. Proteomic analysis has proven to be the most powerful method for identification of proteins and their expression under different physiological conditions. To obtain a comprehensive overview about the expression of proteins which are concerned with OTA production a proteomic approach based on 2D Gel electrophoresis and MALDI-TOF/TOF mass spectrometry has been applied.

Methods

Two different strains of *Aspergillus ochraceus*, NRRL 5175 and NRRL 5221 were used for proteome analysis. *A. ochraceus* was cultivated in malt extract broth and Yeast extract sucrose medium at 30°C with shaking at 200 rpm for 15 days, 20 days and 30 days. Ochratoxin A was measured by HPLC. Cell disruption was performed by grinding the mycelium with liquid nitrogen. Proteins were extracted using lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 20 mM EDTA, 10% v/v glycerol, 30 mM DTT, 1 mM PMSF, 1% v/v protease inhibitor cocktail; 5ml of lysis buffer per gram of mycelia), precipitated with TCA-acetone and then cleaned. The concentration of extracted protein was estimated by Bradford method. The whole protein extract was separated in a 2D-gel electrophoresis system (figure 1). Proteins were identified using MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) with the help of standard database search engines (e.g., MASCOT). The proteins with low confidence score from database search engines were *de novo* sequenced and were subjected for Homology search to identify proteins.

Results

A total of 30 proteins were identified from *A. ochraceus*. These proteins are reported first time in *A. ochraceus*. Out of 30 proteins 16 proteins were identified by MASCOT search engine and 14 proteins were identified by *de novo* sequencing and MS homology search. Most of identified proteins belong to metabolic pathways. Following identified proteins are involved in energy production: NADH-quinone oxidoreductase, nucleoside diphosphate kinase and glyceraldehyde-3-phosphate dehydrogenase. Oryzin/Alkaline proteinase acts as virulence factor in invasive aspergillosis. Autophagic serine protease Alp2 is concerned with regulation of sexual differentiation and autophagy. HSP60 are ATPases and have affinity for unfolded proteins and involved in osmoadaptation. Nevertheless, the present results provide the first proteomic profile from *A. ochraceus* and will be helpful in triggering factors for OTA production.

Innovative aspects

- First time identification of 30 proteins from *Aspergillus ochraceus*.
- Triggering proteins which are involved in the production of OTA.
- Proteins stress response.

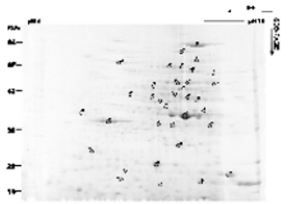

Figure 1: 2D-PAGE separation of whole protein extract from *A. ochraceus*. Protein extract (350µg) were subjected to IEF followed by SDS-PAGE and coomassie blue staining.

Y106

Preserving the Yeast Proteome from Sample Degradation

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Introduction

Sample degradation is a frequent problem in all types of proteomic analyses as it generates protein and peptide fragments that can interfere with analytical results. An important step in preventing such artefacts is to preserve the native proteome as early as possible during sample preparation. During our initial 2D-PAGE analysis of samples prepared using published methods, the resulting gels produced a pattern that appeared to be due to protein degradation. We therefore designed a study to investigate sample pre-treatment methods to compare their efficacy for protein stabilisation.

Methods

Using *S.cerevisiae* as the test system, 6 sample pre-treatment methods were compared to an untreated control. We evaluated the effects of TCA and thermal (Stabilizor T1 device, Denator AB, Sweden) treatments prior to protein extraction as a means to minimise proteolysis. The efficacy of these methods was compared to protease inhibitors and lyophilisation as well as combinations of TCA and thermal stabilisation methods. 2-DE (pH 4-7 gradient) followed by silver staining was used to visualise the resulting protein expression patterns. All treatment groups were analysed in replicate and image analysis and statistical comparison were performed. Protein identification of selected differentially-abundant spots was carried out by peptide mass fingerprinting and LC-MS/MS.

Results

Gel image analysis revealed > 400 protein spot differences between untreated controls and TCA- and thermal-treated samples. The gel patterns from thermally-treated samples were visually similar to those of TCA-treated cells and did not show the apparent proteolysis cloud seen in untreated samples. Multi-variate analysis used to cluster individual spots revealed 3 main clusters: one group represented proteins that were stabilised by thermal treatment with and without TCA treatment; a second group represented all samples that were not TCA- or thermal-treated; and the third cluster represented spots that were selectively affected by TCA treatment alone. The results suggested a high degree of protein degradation in untreated yeast cell samples and for a selected number of spots this was confirmed by MS; these spots included enolase and alcohol dehygrogenase fragments. The analysis showed that TCA or thermal treatment significantly reduced the degree of degradation and that these pre-treatment protocols were more effective than treatment with either protease inhibitors or lyophilisation.

Innovative Aspects

- First comparative study of pre-treatment on protein stabilisation in yeast
- TCA precipitation and thermal denaturation showed a stabilising effect on yeast proteome
- Established standardised sample preparation methods for reproducible 2D-PAGE in yeast

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Grassl J *et al.* Preserving the yeast proteome from sample degradation. Proteomics 2009, in press.

Fig. 1A

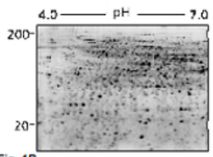

Fig. 1B .

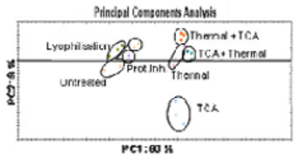

Figure 1. (A) 2D-PAGE image showing proteolysis cloud in untreated yeast cell samples (B)Unsupervised multivariate analysis shows the distribution of untreated and treated samples.

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- Bill Jordan
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- HUPO Initiatives Committee
- Nomination & Elections Committee
- New Technology & Resources Committee
- Education and Training Committee
- Ethics Committee
- Meetings Committee
- Membership Committee
- Awards Committee
- Finance Committee
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- Human Liver Proteome Project (HLPP)
- Human Brain Proteome Project (HBPP)
- Proteomic Standards Initiative (PSI)
- Human Antibody Initiative (HAI)
- Plasma Proteome Project (PPP)
- Human Disease Glycomics/Proteome Initiative(HGPI)
- HUPO Cardiovascular Initiative (HUPO CVI)
- Proteome Biology of Stem Cells Initiative
- Disease Biomarkers Initiatives (DBI)
- Mouse Models of Human Disease (MMHD)
- Kidney and Urine Initiative (HKUPP)

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Member at Large	Ole Jensen (2009-2010)

HUPO Industry Advisory Board 2009 Members

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Toronto Local Organizing Committee

Peter Liu (co-chair), Michael Siu (co-chair), Peter Lewis (co-chair), Eleftherios Diamandis, Kenneth Evans, Thomas Kislinger, Michael Moran, Anthony Gramolini, Anne-Claude Gingras, Andrew Emili, Igor Jurisica, John Marshall, Brian Raught, Bob Phillips, and Gerold Schmitt-Ulms.

Peter Lewis
University of Toronto

Pierre Legrain
Secretary General
HUPO

Daniel Chan
Editor-in-Chief
Clinical Proteomics

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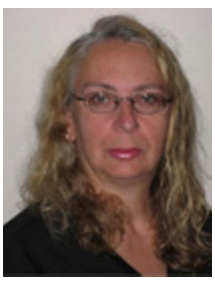

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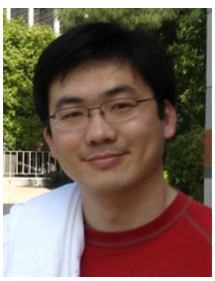

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