

Hypermethylation of hepatic *Gck* promoter in ageing rats contributes to diabetogenic potential

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Abstract

Aims/hypothesis Hepatic glucokinase (GCK) is a key enzyme in glucose utilisation. Downregulation of its activity is associated with insulin resistance and type 2 diabetes mellitus. However, it is unknown whether hepatic *Gck* expression is influenced by age and is involved in ageing-mediated diabetes, and whether the degree of methylation of the hepatic *Gck* promoter is correlated with the transcription of *Gck*. To address the question, we evaluated hepatic *Gck* transcription and promoter methylation in young (14 weeks), adult (40 weeks) and aged (80 weeks) rats.

Methods Hepatic glycogen, *Gck* expression and the kinase activity of GCK were measured in three age groups. The CpG methylation status was determined by both bisulphite direct sequencing and clone sequencing of the PCR amplicates of *Gck* promoter. The causal relationship between *Gck* methylation and mRNA expression was

confirmed by treating rat primary hepatocytes with 5-aza-2'-deoxycytidine (5-Aza-CdR).

Results We have shown an age-associated decline in hepatic glycogen, *Gck* expression levels and the kinase activity of hepatic GCK. The eleven CpG sites studied displayed age-related progressive methylation changes in hepatic *Gck* promoter, which were confirmed by two methods: direct and clone sequencing. After 5-Aza-CdR treatment of rat primary hepatocytes, there was a fourfold increase in *Gck* expression.

Conclusions/interpretation Our results demonstrate that an age-related increase in methylation is negatively associated with hepatic *Gck* expression, suggesting that DNA methylation could be involved in increasing age-dependent susceptibility to hepatic insulin resistance and diabetes. Thus, the epigenetic modification of the hepatic *Gck* promoter may represent an important marker for diabetogenic potential during the ageing process.

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Keywords Ageing · Diabetes · Glucokinase ·
Insulin resistance · Methylation

Abbreviations

5-Aza-CdR 5-aza-2'-deoxycytidine
GCK glucokinase
nt nucleotide

Introduction

Insulin resistance, glucose intolerance and type 2 diabetes mellitus are becoming progressively more common in older populations [1–3]. These age-related changes may be caused in part by increased adiposity and the decreased

Table 1 Primer sequences for real-time PCR and bisulphite sequencing PCR of hepatic *Gck* promoter

Parameter	Forward primer(5'→3')	Reverse primer(5'→3')
mRNA expression by real-time PCR		
Gck	AGTATGACCGGATGGTGGAT	CCGTGGAACAGAAGGTTCTC
Hprt	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAAGCTTATAGCC
Beta-actin	GATTACTGCCCTGGCTCCTA	TCATCGTACTCCTGCTTGCT
Bisulphite sequencing PCR of <i>Gck</i> promoter		
pMGck 1st	GAATTTTATAGAAGAGTTTAGAATGTTTTGG	CACACCTTATAATATCCATAACCATCTC
pMGck 2nd	GGGTGTTAGGGTAGTTAGAGGATTTG	CCTAACTCCTAAAACCACCTATTAC

physical activity in older people [4, 5]. Although ageing plays a key role in the pathogenesis of insulin resistance, the underlying molecular mechanism is still poorly understood.

The pathophysiology of ageing and ageing-related diabetes is complex. Insulin resistance is a major contributor to development of the disease and to its complications [6–8]. Insulin resistance occurs because of defective insulin-mediated glucose utilisation by the liver and peripheral tissues. The progression of insulin resistance to diabetes is caused by numerous metabolic events spanning a period of many years [9–11]. Glucose phosphorylation by glucokinase (GCK) is the initial event in glucose metabolism in the liver. Thus, glucose phosphorylation by GCK is important in the regulation of glucose utilisation in hepatocytes. The main role of insulin in the activation of glycolytic genes appears to be the stimulation of GCK synthesis, which leads to increased glucose utilisation [12–16]. Regulation of *Gck* activity is mainly due to changes in the transcription of this gene. In fact, decreased GCK enzymatic activity has been reported in patients with type 2 diabetes mellitus [17], and mutations in the *Gck* gene can cause MODY-2 [18, 19]. Accordingly, *Gck* is considered to be as a diabetes-susceptibility gene.

Since type 2 diabetes mellitus incidence increases in older populations and their metabolic situations deteriorate over time, we hypothesised that the effect of age on glucose metabolism is associated with the level of hepatic *Gck* transcription and attempted to investigate mechanisms of the age regulation of *Gck* expression in liver. Ling et al demonstrated that DNA methylation is associated with age-dependent decline in *NDUFB6* expression in human skeletal muscle [20], opening up the possibility that involvement of epigenetic factors like DNA methylation could predispose an individual to insulin resistance and type 2 diabetes mellitus. Accordingly, we specifically examined whether epigenetic modification was involved in the regulation of hepatic *Gck* expression in rats during postnatal life and whether it played a role in the pathogenesis of insulin resistance.

The aim of this study was to test whether the level of *Gck* expression and the activity of hepatic GCK were age-related and whether the degree of DNA methylation in *Gck*

promoter changes with age, from young (14 weeks) to adult (40 weeks) and aged (80 weeks) animals. In addition, to reveal the functional significance of DNA methylation in rat livers, we also investigated whether *Gck* expression can be reactivated after treating rat primary hepatocytes with the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-Aza-CdR).

Methods

Animals Male Wistar rats were obtained from the Animal Developmental Center, Chinese Academy of Sciences, Shanghai. We used three different age groups ($n=7$ rats per group): 14 weeks (young), 40 weeks (adult) and 80 weeks (aged) in this study. Rats were housed under standard light–dark conditions (12/12 h light/dark cycle) and were given free access to commercial laboratory animal chow diet. Experiments were carried out after 16 h fasting. All experimental procedures involving the use of animals were conducted in accordance with NIH Guidelines and were reviewed and approved by the Animal Use and Care Committee of Fudan University.

Glycogen determination The content of hepatic glycogen was measured as described [21, 22]. Briefly, livers were weighed and incubated in four volumes (vol./wt) 1 mol/l KOH for 20 min at 100°C. After cooling, the lysates were diluted tenfold with water then added slowly to the anthrone reagent, which was placed in cold water to prevent excessive heating. After mixing by lateral shaking, the tube was placed in a boiling water bath for exactly 5 min for colour development and subsequently cooled in tap water. Absorbance at 620 nm was measured against a blank control using glucose as a standard. The glycogen content was calculated using the formula: mg of glycogen per g of tissue = $D_U/D_S \times 0.1 \times 40$ (fold total dilution) $\times 0.9$, where D_U is the OD value of the unknown, D_S is the OD value of 0.1 mg of glucose in standard solution and 0.9 is the factor for converting the glucose value into glycogen value. Triplicate samples per liver were analysed and data are presented as mg of glucose units per g of liver.

RNA extraction and real-time PCR Total RNA was isolated from liver tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesised by reverse transcription using ReverTra Ace (Toyobo, Osaka, Japan). The primer sequences of rat *Gck*, housekeeping gene hypoxanthine phosphoribosyl-transferase (*Hprt*) [23] and beta-actin for quantitative RT-PCR are shown in Table 1. A 10 μ l reaction mixture contained 1 μ l of cDNA and 125 nmol/l of primers. The SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) was used for PCR. The amplification and detection of specific products were performed using a sequence detection system (ABI PRISM7900; Applied Biosystems, Foster City, CA, USA). PCR cycle conditions were 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Reactions were performed in triplicate with rat *Hprt* and beta-actin as an internal control. Fluorescent signals were normalised to an internal reference (ΔR_n) and the threshold cycle (C_t) was set within the exponential phase of PCR. The relative gene expression was calculated by comparing cycles for each target PCR. Cycle threshold values were converted to relative gene expression levels using the $2^{-\Delta\Delta C_T}$ method [24].

Determination of GCK activities GCK activity was determined as described [25, 26]. Liver tissue (50 mg) was homogenised and diluted 20 times by buffer containing 50 mmol/l HEPES, 5 mmol/l MgCl₂, 1 mmol/l EDTA, 100 mmol/l KCl and 2.5 mmol/l dithioerythritol at pH 7.4. The homogenate was then passed through a 23-gauge needle syringe (five times) to ensure the proper tissue suspension and later centrifuged at 10,000 $\times g$ and 4°C. The reaction was started with 0.5 mmol/l NAD⁺, 4 U glucose-6-phosphate dehydrogenase and 5 mmol/l ATP at 30°C. The continuous spectrophotometric assay of glucose-phosphorylating activity was performed at two glucose concentrations: 100 mmol/l (measures all hexokinases, including GCK) and 0.5 mmol/l (measures only the low- K_m hexokinases). The difference between the two assays gives GCK activity. One unit is the amount of enzyme that catalyses the formation of 1 μ mol of substrate per min under the conditions of assay. The Bio-Rad DC (Bio-Rad Laboratories, Hercules, CA, USA) protein assay method was used to measure protein concentration.

Bisulphite-sequencing of *Gck* promoter All reagents were purchased from Sigma (St Louis, MO, USA). Genomic DNA was prepared from the livers of three groups using the SDS and proteinase K methods, and then subjected to sodium bisulphite treatment [27]. The *Gck* hepatic promoter sequence (–518 to 83) was amplified with AmpliTaq Gold polymerase (Applied Biosystems, Warrington, UK) by two rounds of PCR. Bisulphite-sequencing primers for rat *Gck*

promoter were synthesised according to the reported sequences [28] (Table 1). PCR of the rat *Gck* promoter was performed under the following conditions: (1st round) 1 min 95°C, 2 min 50°C, 2 min 72°C for five cycles, then 1 min 95°C, 1 min 50°C, 1 min 72°C for 30 cycles. The first round products (2 μ l) were then subjected to a second round PCR under the following conditions: (second round) 1 min 95°C, 2 min 58°C, 1 min 30 s 72°C for 35 cycles. We then sequenced 1 μ l of the second round PCR product using a DNA sequencer (ABI PRISM 3730; Applied Biosystems, Foster City, CA, USA) with dye terminators (Perkin-Elmer, Foster City, CA, USA). The rest of the PCR products were kept and used in the following experiments.

Quantification of methylation levels We used two methods to analyse levels of DNA methylation. First, we directly calculated the methylation level by measuring the heights of cytosine (shown as ‘C’) and thymine (shown as ‘T’) peaks at each CpG [29–33]. A single ‘C’ at the corresponding CpG site was considered to be 100% methylation and a single ‘T’ was considered to be no methylation; overlap of ‘C’ and ‘T’ was ranked as partial methylation. The percentage methylation was calculated as peak height of ‘C’ versus peak height of ‘C’ plus peak height of ‘T’ for each position.

In order to confirm whether the method above was reliable for estimation of methylation levels, we sequenced subclones of PCR products [34–36]. In brief, the residual PCR amplified products after direct sequencing were purified by gel electrophoresis and subcloned into a pGEM-T vector (Promega, Madison, WI, USA). A total of 30 clones per rat liver were isolated using a kit (QiaPrep Spin Plasmid Miniprep; Qiagen, Hilden, Germany) and sequenced. The methylation level for each CpG site was calculated by dividing the number of methylated CpGs of each site by the total number of clones sequenced.

Treatment of 5-Aza-CdR in rat primary hepatocytes Hepatocytes were isolated from a 40-week adult male Wistar rat fasted for 48 h by two-stage collagenase perfusion of the liver as described [37]. The isolated hepatocytes were seeded at a density of 2×10^6 cells per 35 mm plate. After 6 h incubation at 37°C and 5% CO₂, attached cells were treated with freshly prepared 5 μ mol/l 5-Aza-CdR (Sigma) and the medium was changed every 24 h. RNA was isolated from both untreated and treated confluent hepatocytes after 72 h as described above. Then the transcription level of *Gck* was analysed with real-time PCR.

Statistical analyses Results are presented as mean \pm SD. Statistical analysis was conducted using the software package Minitab (version 15.1.0.0; Minitab, State College, PA, USA). Significance was assessed by Student’s *t* test and one-way ANOVA followed by Bonferroni *t* test for the

Table 2 General and biochemical characteristics

Characteristics	Young, 14 weeks	Adult, 40 weeks	Aged, 80 weeks
<i>n</i>	7	7	7
Weight (g)	248.7±25.0	402.0±34.8**	629.9±41.8**
Glucose (mmol/l)	5.04±0.36	5.02±0.22	5.09±0.44
Insulin (pmol/l)	174.5±17.9	215.2±15.7**	233.3±14.1**, †

Values are means±SD

Results were analysed by Bonferroni *t* test

***p*<0.01 vs young rats; †*p*<0.05 vs adult rats

comparisons of multiple means within an experiment, or by Kruskal–Wallis test and subsequent pair-wise comparisons. The statistical method used for each group of data is indicated and a *p* value of less than 0.05 was considered to be statistically significant.

Results

Physical and biochemical characteristics of the experimental rats Body weights, basal plasma glucose, plasma insulin levels and liver glycogen contents from three different age groups (young, adult, aged) are listed in Table 2. The plasma glucose levels were comparable among the three groups, while the plasma insulin levels were elevated progressively in adult and aged rats compared with young rats (*p*<0.01). We also observed a steady increase of body weight from young through to adult and aged rats.

Hepatic glycogen contents in three groups of rats Liver glycogen contents decreased with age, the greatest difference (from 6.36±1.43 mg/g tissue to 4.76±1.11 mg/g tissue) occurring between the young and adult groups (*p*<0.05). Only a moderate decrease of glycogen content was seen between adult and aged rats (*p*>0.05) (Fig. 1a).

***Gck* expression and activity in different age groups** To determine the age-related changes in the *Gck* gene products, we measured hepatic *Gck* mRNA levels and GCK enzymatic activities from three age groups. The *Gck* mRNA levels were comparable in real-time PCR with either rat *Hprt* or beta-actin as an internal control. As shown in Fig. 1b, hepatic *Gck* expression, with rat *Hprt* as an internal control, was 60% (*p*<0.05) lower in adult and 86% (*p*<0.01) lower in aged groups than in the young animal group, respectively. Furthermore, the decrease trend of GCK activities in the three groups was very similar to that of *Gck* mRNA, i.e. about 50 and 80% decreases in adult and aged rats, respectively, compared with young rats (Fig. 1c). The results indicate that the age-related decline of hepatic GCK activity is mainly controlled at the transcription level and is consistent with the tendency of changes seen in hepatic glycogen contents.

Methylation levels of the *Gck* promoter in different age groups To determine whether the expression of *Gck* was correlated with the status of *Gck* promoter methylation, we analysed the methylation levels of *Gck* promoter. The *Gck* promoter structure [13, 28, 38], shown in Fig. 2a, encompassed 11 CpG sites located at nucleotides (nt) –349, –342, –330, –314, –280, –245, –236, –93, –85, –4 and +5. The promoter region of the rat hepatic *Gck* gene was amplified

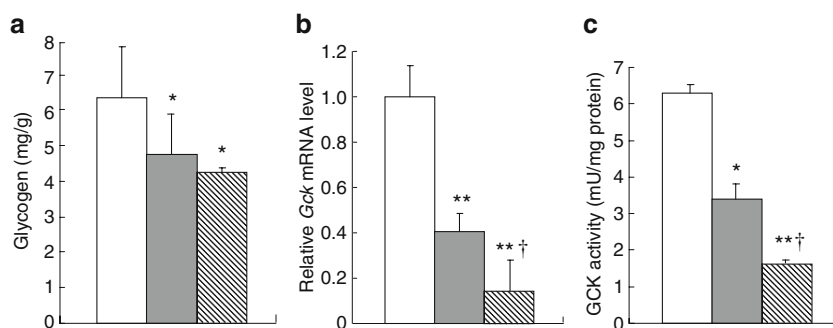


Fig. 1 Hepatic glycogen content (a), transcription of *Gck* using rat *Hprt* as a control (b) and hepatic GCK activities (c) in rat livers after 16 h fasting. White bars, young rats; grey bars, adult rats; hatched

bars, aged rats. Values are means±SD, *n*=7 rats per group. **p*<0.05 and ***p*<0.01 vs young rats; †*p*<0.05 vs adult rats

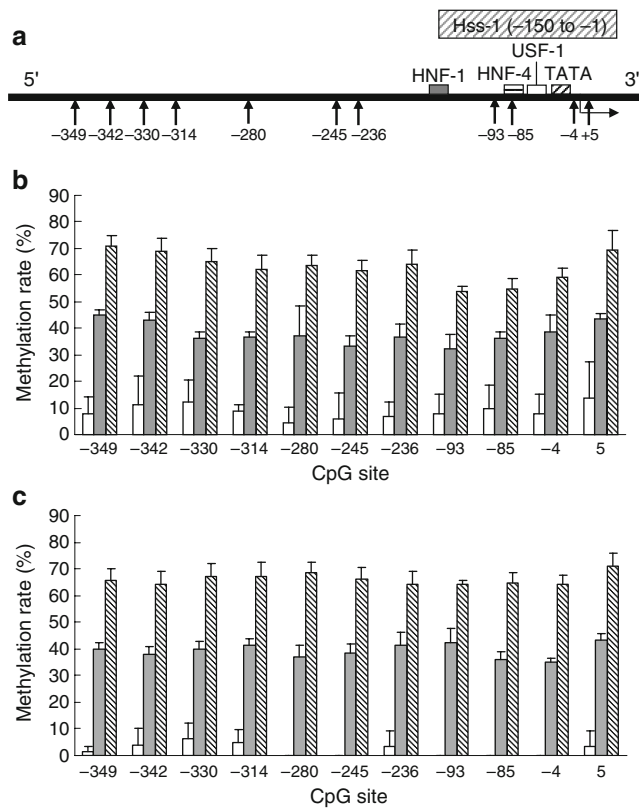


Fig. 2 Schematic diagram of *Gck* gene promoter structure and methylation status of hepatic *Gck* promoter in three age groups. **a** There are 11 CpG sites within the promoter region (indicated by arrows) from nt -518 to nt +83. TATA box, DNase I hypersensitive sites (HSS-1) and binding sites of hepatic nuclear factor 1 (HNF-1), hepatic nuclear factor 4 (HNF-4) and upstream stimulatory factor 1 (USF-1) are also indicated. **b** Genomic DNA was modified by sodium bisulphite and amplified by two rounds of PCR. Then, PCR products were directly sequenced and the methylation percentage of the individual CpG site was calculated by peak height of the methylated residues (C) divided by the sum of methylated and unmethylated residues (C plus T). Methylation rate (%) is represented as the average ratio of methylated cytosine to total cytosine (methylated plus unmethylated) of all samples in each group. **c** PCR products were subcloned into the pGEM-T vector. The levels of methylation were determined in 30 clones for each sample. White bars, young rats; grey bars, adult rats; hatched bars, aged rats. Values are means±SD, $n=4-7$ rats per group. The difference of the overall methylation level between each groups is significant ($p<0.05$)

by nest PCR from nt -518 to nt +83 and sequenced directly. The sequencing results indicated that all non-CpG cytosines were converted to thymines, whereas the status and ratio of C: (C+T) was very different at each CpG site among the three groups. However, the ratios were almost identical in rats of the same age. In order to quantify the methylation levels, we calculated the methylation rates of individual CpG sites from the direct sequencing results. As shown in Fig. 2b, methylation levels of the CpG site in the *Gck* promoter were significantly different ($p<0.05$) among the three age groups. The aged rats exhibited considerably higher methylation (60–80%) of all CpG sites than that

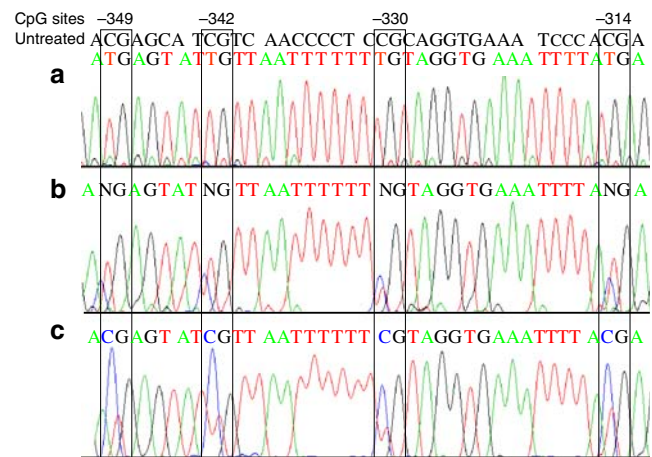


Fig. 3 Representational genomic sequencing chromatograms from nt -349 to nt -314 for each age group. DNA was first treated with sodium bisulphite, and the amplified PCR products were then directly subjected to sequencing. The upper sequence is the untreated *Gck* promoter sequence. Lower sequences are treated sequences in young rats (**a**), adult rats (**b**) and aged rats (**c**). The open boxes indicate the CpG sites

seen in the other two groups. The *Gck* promoter in adult rats showed a lower level of methylation (35–50%), whereas the young rats showed the lowest methylation levels (0–10%) in the CpG sites of the *Gck* promoter. Three representative DNA sequencing chromatograms are shown in Fig. 3. Different methylation levels of hepatic *Gck* promoter derived from the three age groups were shown to contain four CpG sites from nt -349 to nt -314. We sequenced 30 clones of each individual PCR reaction to confirm the level of methylation. The patterns of CpG methylation analysed by these clones were similar to the direct bisulphite-sequencing method above. Only less than 10% differences between the two methods were seen at some of the CpG sites (Fig. 2c).

5-Aza-2'-deoxycytidine treatment of the primary rat hepatocytes upregulated Gck expression In order to establish whether *Gck* expression was influenced by methylation status, we treated primary adult rat hepatocytes with 5-Aza-

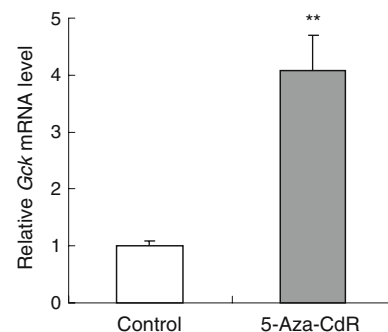


Fig. 4 Effects of 5-Aza-CdR on *Gck* (relative to *Hprt*) expression in primary rat hepatocytes. Values are means±SD of triplicate experiments. ** $p<0.01$ vs untreated cells (control)

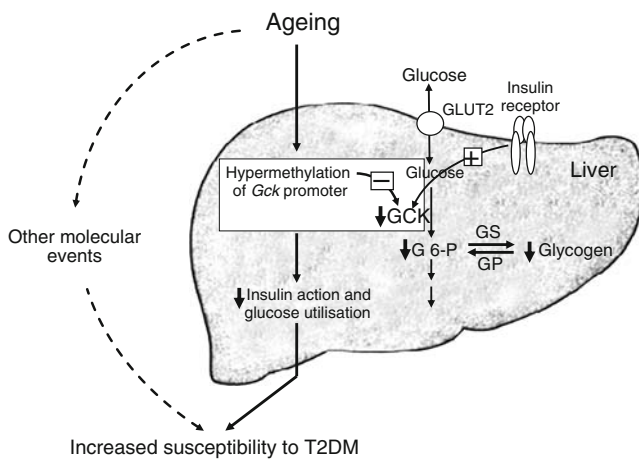


Fig. 5 Ageing-dependent hypermethylation of hepatic *Gck* promoter increases susceptibility to type 2 diabetes mellitus (T2DM) by downregulating *Gck* expression in rat liver. In hepatic glycolysis, glucose is transported through the hepatocyte plasma membrane by GLUT2. Hepatic GCK, induced by insulin, phosphorylates glucose to glucose 6-phosphate (G 6-P), which is an allosteric activator of glycogen synthase (GS). Promoter hypermethylation represses the expression of hepatic *Gck*, resulting in two effects in liver: (1) decreased insulin action and glucose utilisation; and (2) diminished glycogen synthesis. Other complex molecular events are also involved in age-related type 2 diabetes mellitus. Even though compensatory normoglycaemia is maintained, aged rats are highly susceptible to the disease. +, increased *Gck* expression; –, decreased *Gck* expression; downward arrows, significant decrease; solid line, efferent pathway; broken line, unknown pathway

CdR, a demethylating agent, for 3 days and then *Gck* mRNA expression was monitored. In Fig. 4, *Gck* mRNA was upregulated fourfold compared with the untreated hepatocytes. The result suggests that *Gck* expression was significantly reactivated by demethylation.

Discussion

Glucose intolerance may emerge during the human ageing process and is most often associated with normal or elevated plasma insulin concentrations [39]. Ageing promotes a state of insulin resistance primarily by blunting the action of insulin on the uptake of glucose in liver and peripheral tissues [1, 3]. Levels of GCK, a rate-limiting enzyme in glycolysis, are reduced in liver of patients with type 2 diabetes mellitus and in diabetic animals [17, 40]. Nevertheless, it remains unknown whether ageing is associated with diabetes through a gradual downregulation of hepatic *Gck* expression. Furthermore, less is known about DNA methylation changes of the specific genes in ageing. The important finding of the present study is that age-related DNA methylation changes were associated with a decline in the expression of hepatic *Gck* during the ageing process of rats, which could add to the risk of insulin

resistance and type 2 diabetes mellitus (Fig. 5). The epigenetic modification of hepatic *Gck* gene might be an important marker for diabetogenic potential during the ageing process. Recently, Ling C et al. [20] demonstrated that DNA methylation played a role in the pathogenesis of insulin resistance and type 2 diabetes mellitus. Our finding provides another example of how epigenetic factors are involved in an age-dependent increase of susceptibility to insulin resistance.

Insulin resistance in liver is a major contributor to the increased prevalence of type 2 diabetes mellitus with age. Hepatic glucose metabolism is a good predictor of insulin sensitivity. The primary function of insulin in liver is to stimulate the key enzyme of glycolysis, GCK, which enhances hepatic glucose disposal. To determine why insulin sensitivity decreases with age, we analysed the *Gck* expression and the activity of GCK in livers from young (14 weeks), adult (40 weeks) and aged (80 weeks) rats. The adult and the aged rats showed a sharp decrease in the transcription of *Gck* compared with the young group. These declines were of the same magnitude as that observed in GCK activities of adult and aged rats. We deduce from this that the downregulation of hepatic *Gck* with ageing would severely attenuate the effect of insulin in liver and diminish glucose utilisation (Fig. 5).

In our study, *Gck* expression levels in liver were positively associated with hepatic glycogen. Consistent with the trend of *Gck* expression, glycogen levels progressively decline with age. GCK plays a crucial role in glycogen synthesis, and small changes in its total activity resulted in much larger changes in glycogen synthesis [41, 42]. The potential mechanism of GCK-mediated glycogen synthesis might be through catalysation of glucose 6-phosphate synthesis and promotion of glycogen synthase activation in hepatocytes [43–46] (Fig. 5). Furthermore, we determined the glycogen content in rat muscles, a tissue that does not express *Gck*, and found no difference among the three age groups (Electronic supplementary material [ESM] Fig. 1).

Considering the significant role of *Gck* expression in hepatic insulin resistance and glycogen synthesis, we explored the potential mechanisms of *Gck* expression with ageing. Here, we have presented data to support the notion that differential DNA methylation results in the age-dependent reduction of *Gck* expression. The *Gck* promoter structure [13, 28, 38] is shown in Fig. 2a. The same region of this gene was hypomethylated in the young livers, with less than 10% methylation, while in adult livers the percentage methylation increased significantly (about 50%) and was highest (above 70%) in aged liver in the region investigated (Fig. 2b, c). These results demonstrate that hepatic *Gck* promoter methylation increased gradually with ageing (Fig. 5).

We quantified the methylation levels of *Gck* promoter in different age groups using bisulphite direct sequencing and clone sequencing of the PCR products derived from *Gck* promoter. In the present study, ‘T’ and ‘C’ overlapped at almost all of the CpG sites in the *Gck* promoter region, but there were significant differences in the three age groups (Fig. 3). From the sequencing chromatograms we directly calculated the degree of methylation of 11 CpG sites in the promoter region (Fig. 2b). Furthermore, we also sequenced the subclones of bisulphite-treated *Gck* promoter to confirm the methylation levels (Fig. 2c). The methylation levels calculated by two methods were quite similar, with fewer than 10% differences at some of the CpG sites. The minor differences can probably be attributed to the background noise affecting the exact peak heights of ‘C’ or ‘T’ in the sequencing chromatogram, as well as to the number of clones selected in this study. In spite of the minor differences, these two methods generated consistent results for calculating DNA methylation levels. Especially the direct sequencing of the PCR product is relatively straightforward.

To confirm the links between methylation and gene expression, a demethylation agent, 5-Aza-CdR, was used to treat primary adult rat hepatocytes. 5-Aza-CdR is a cytosine analogue that acts as a suicide substrate for DNA methyltransferase and hence is an inhibitor of DNA methyltransferase when incorporated into DNA at CpG dinucleotides of the target site for DNA methylation [47]. As expected, 5-Aza-CdR significantly increased *Gck* mRNA transcription.

Gck gene has two promoters, one active in pancreas and the other active in liver [48]. We determined pancreatic *Gck* expression using real-time PCR. There were no significant differences in the three age groups, which was consistent with earlier studies [49]. Thus, we did not further study the methylation status of the *Gck* promoter specific to islets. We also quantified methylation of the liver-specific *Gck* promoter in rat skeletal muscles, a tissue that does not express *Gck*. Almost 100% methylation was found at the same region of the *Gck* promoter in all age groups (ESM Fig. 2). We were not sure whether the hypermethylation of *Gck* promoter was the major factor in silencing *Gck* mRNA expression in muscle or other *Gck* non-expressing tissues. However, according to our findings, the degree of CpG methylation in the *Gck* promoter increased in an age-dependent manner as a specific biological feature in rat livers, which should be the critical factor for suppression of *Gck* mRNA expression.

Ageing is associated with marked basal hyperinsulinaemia despite normal blood glucose. As shown in Table 2, the basal insulin levels were higher in the adult and aged rats than in young animals. Basal hyperinsulinaemia is suggestive of an insulin-resistant state, so both adult and aged rats

exhibited marked whole-body insulin resistance. Furthermore, body weight increase (Table 2) and fat accumulation of healthy old rats might aggravate insulin resistance in liver and other insulin-targeting organs, so the combined impact of ageing and obesity on insulin sensitivity contributes greatly to insulin resistance in elderly people [1, 4, 50]. Accordingly, we cannot rule out the potential role of age-related obesity in hypermethylation of the *Gck* promoter, since, under normal conditions, rats increase weight as they age. We have now begun a new study of *Gck* methylation in either ageing or obese rats. Our preliminary findings indicate that obesity could increase susceptibility to age-induced insulin resistance and the level of hepatic *Gck* methylation in aged rats (M.H. Jiang, J. Fei, M.S. Lan, Z.P. Lu, M. Liu, W.W. Fan, X. Gao and D.R. Lu, unpublished results).

In summary, we have demonstrated that hypermethylation of hepatic *Gck* promoter is associated with the downregulation of hepatic *Gck* transcription in aged rats and contributes to diabetogenic potential (Fig. 5). However, the detailed mechanisms that link ageing and hypermethylation of *Gck* promoter still merit further investigation. Also, a systematic profiling of DNA methylation changes in the ageing process would yield valuable insights into the epigenetic mechanism of ageing, insulin resistance and other ageing-related diseases.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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