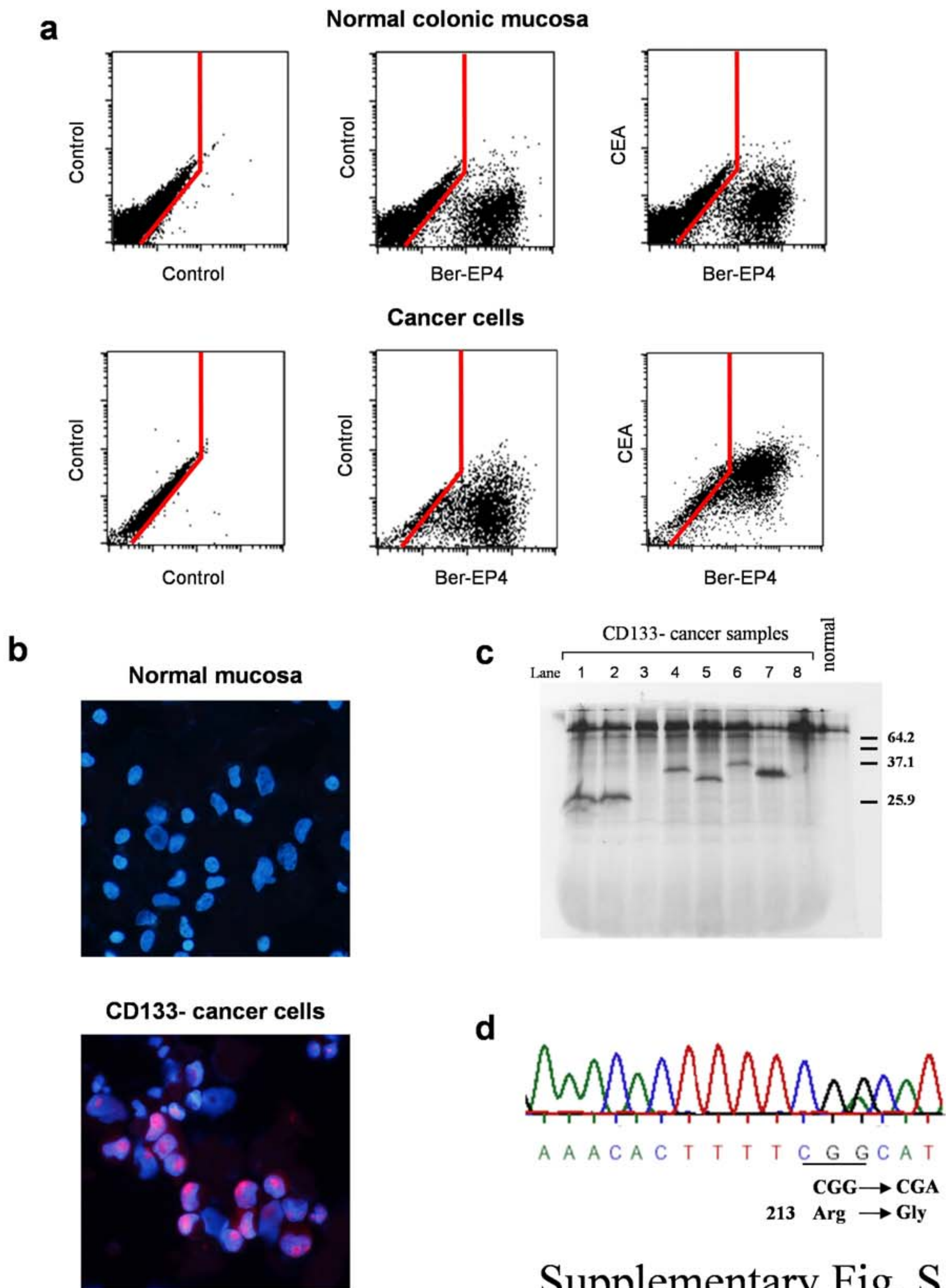
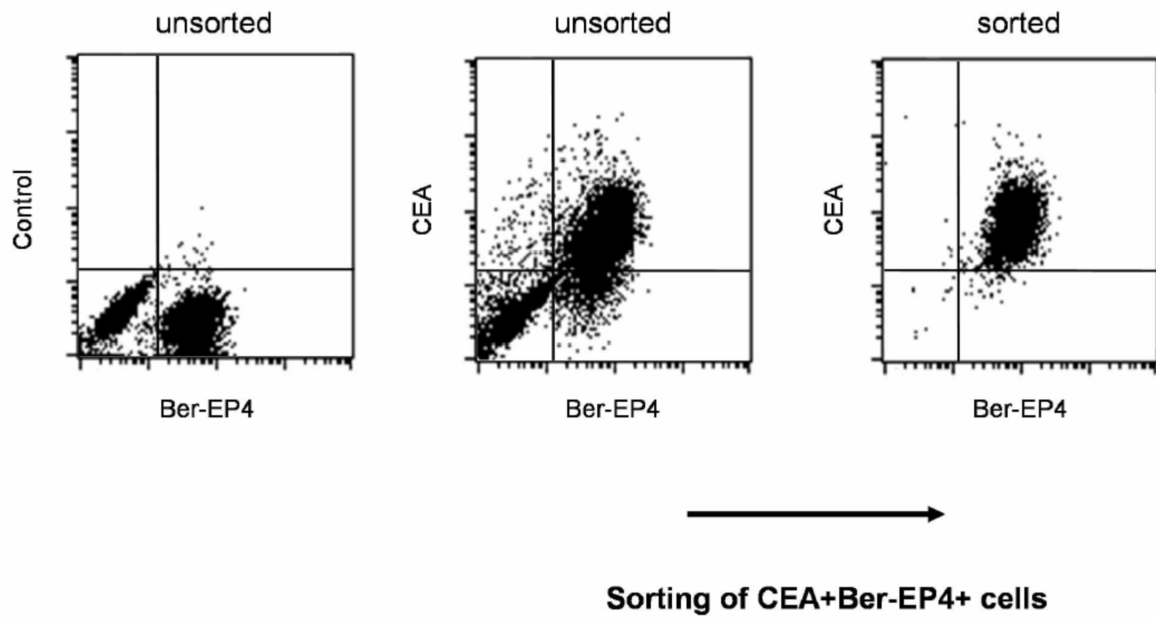
Supplementary Figures and Legends



Supplementary Fig. S1

Supplementary Figure 1

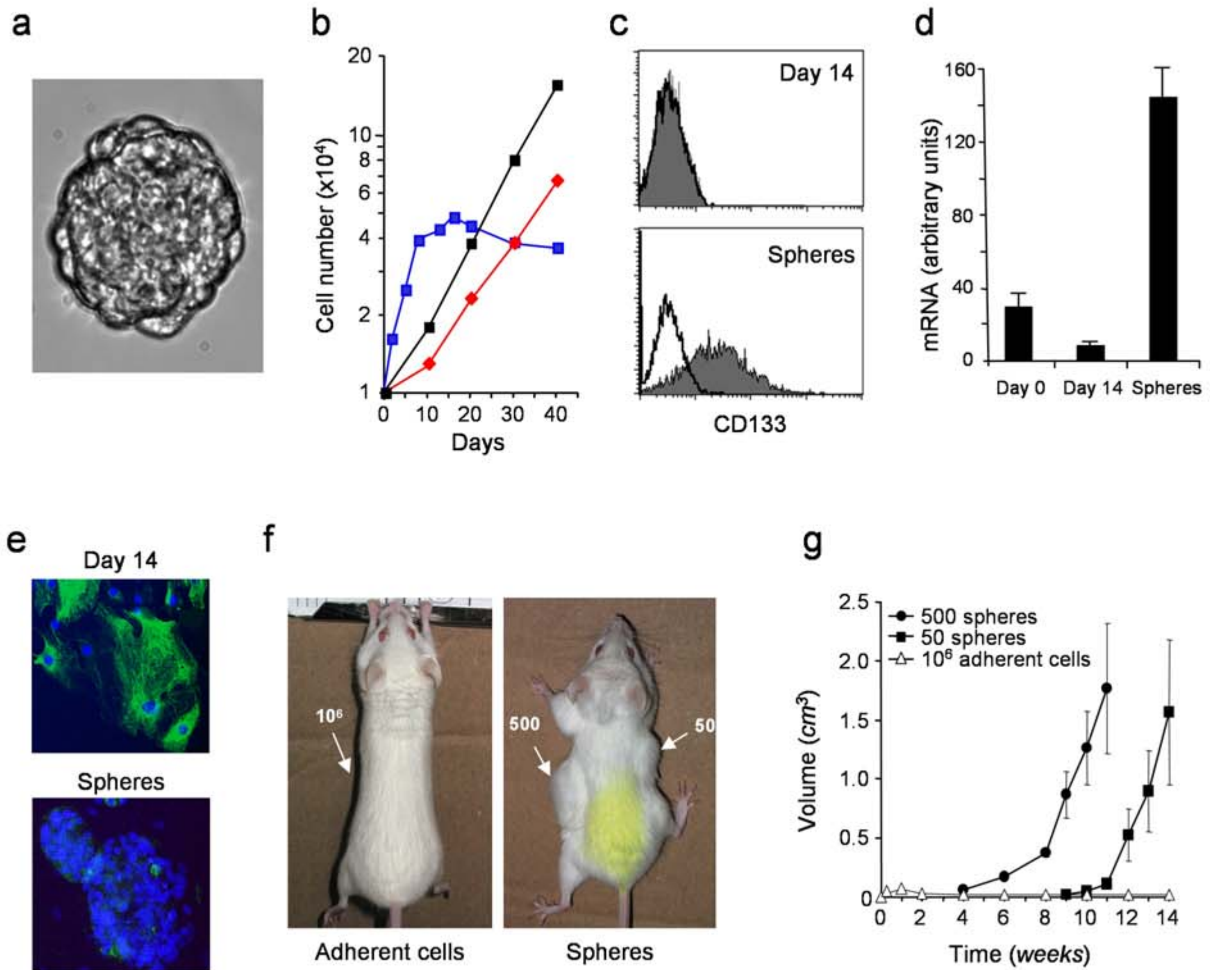
The CD133- population purified from colon cancer contains a considerable number of cancer cells. **a**, Flow cytometry analysis of freshly dissociated colon adenocarcinoma cells. Ber-EP4 expression as single staining or in combination with CEA is shown. **b**, Immunofluorescence analysis of mutant p53 (red) in cytospin preparations of CD133- colon cancer cells and control colonic mucosa cells. Nuclei are stained with Hoechst 33342 (blue). **c**, SDS-polyacrylamide gel electrophoresis analysis of protein truncation test products from APC fragment 15-2. Lanes 1-8 show DNA samples extracted from CD133- cancer cells. Samples 1, 2, 4, 5, 6 and 7 contain truncating mutations located at different positions along the fragment. The last lane shows a normal mucosa cell DNA sample (70 kDa). **d**, Genomic DNA from samples 3 and 8, which did not show mutations in APC, was used for PCR amplification and sequencing of exons 5 to 8 of p53 gene. Exon 6 sequencing analysis of sample 3 is shown. Detected point mutation consists in G/A substitution at codon 213.



Supplementary Fig. S2

Supplementary Figure 2

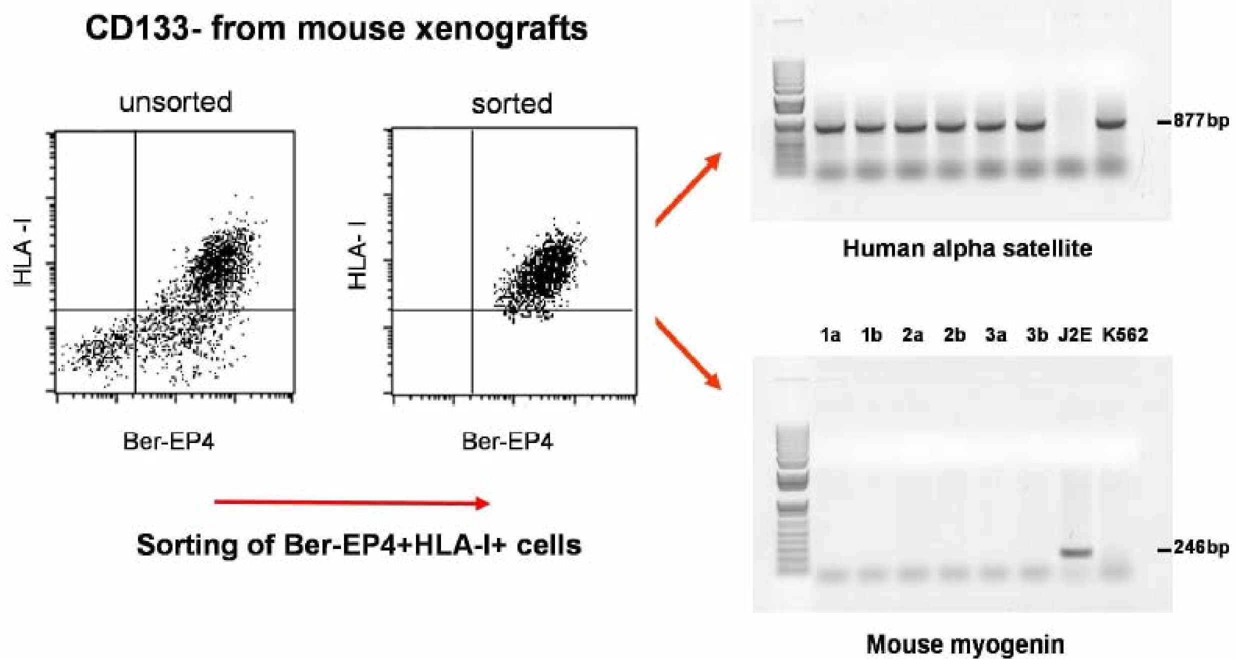
Flow cytometry analysis of BerEp4 expression as single staining or in combination with CEA in freshly dissociated human colon cancer before (left and middle) and after sorting of double positive cells (right).



Supplementary Fig. S3

Supplementary Figure 3

Tumorigenic CD133+ colon cancer cells can be expanded *in vitro* as undifferentiated spheres. **a**, A typical colon cancer sphere obtained in serum-free medium containing EGF and FGF-2. **b**, Growth curve of colon sphere cells before passage 10 (red) or after passage 30 (black), as compared with the growth of primary colon cancer cells in standard medium with 10% serum (blue). **c, d**, Expression of CD133 by flow cytometry analysis (**c**) and real time PCR (**d**) in standard adherent primary colon cancer cultures at day 14 (Day 14), as compared with colon spheres (spheres) and freshly isolated colon cancer cells (Day 0). **e,** Immunofluorescence analysis of CK20 (green) in day 14 primary colon cancer cells and in colon cancer spheres. Nuclei are counterstained with Hoechst 33342 (blue). **f**, **g**, Tumorigenic potential of *in vitro* expanded CD133+ colon cancer spheres (Spheres) as compared with day 14 primary colon cancer cells from standard serum-driven cultures (Adherent cells). Injection sites of indicated number of cells and spheres in SCID mice are shown by arrows (**f**). Tumor volumes of mice injected with 10^6 adherent cells from standard primary cultures, 50 spheres or 500 spheres (**g**). Data are mean ± s.d. of 4 independent experiments in triplicate.



Supplementary Figure 4

The CD133- population purified from mouse xenografts derived from CD133+ cells contains a considerable number of human cells. Flow cytometry analysis of HLA-I and Ber-EP4 in CD133- cells isolated from freshly dissociated xenografts before (unsorted) and after sorting of double positive cells (sorted). Genomic DNA was extracted in duplicate from three different sorted samples and amplified for human alpha satellite (right, top) and mouse myogenin (right, bottom). Mouse (J2E) and human (K562) cell lines were used as negative and positive controls.

Supplementary Methods

Cell culture. Colon adenocarcinoma samples were obtained from Sant'Andrea Hospital (Rome) upon patients' informed consent and approval by the local ethical committee. Surgical specimens were washed several times with phosphate buffered saline (PBS) and incubated overnight in DMEM-F12 containing 25 units/ml of penicillin, 25 µg/ml streptomycin and 10 µg/ml amphotericin B. Samples were subsequently subjected to mechanical and enzymatic dissociation. The resulting cancer cells were cultured in a NS-A basal serum-free medium (Euroclone, Irvine, UK), containing 2 mM L-glutamine, 0.6% glucose, 9.6 µg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin sodium salt (Sigma, St Louis, MO), and supplemented with 20 ng/ml EGF and 10 ng/ml FGF-2. To obtain primary tumor cell cultures, after enzymatic dissociation cells were plated on collagen-coated dishes in DMEM medium containing 10% FCS. Cultures of differentiated tumor cells were obtained from tumor spheres after growth factors removal and addition of 5% FCS.

Antibodies. To characterize colon cancer stem cells, the following antibodies were used: anti-CD31-PE (clone WM-59, mouse IgG_1, BD Pharmingen); anti-CD45-PE (clone T29/33, mouse IgG_1, DakoCytomation); anti-Epithelial Antigen-FITC (clone Ber-EP4, mouse IgG_1, DakoCytomation); anti-Carcinoembryonic Antigen, CEA (polyclonal rabbit, DakoCytomation), anti-Cytokeratin 20 (K_s20.8, mouse IgG_{2A}, DakoCytomation) or isotytpe-matched control antibodies. FITC-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories) were used where needed. To isolate CD133- human cells from mouse xenografts anti-HLA-ABC (clone W6/32, mouse IgG_{2a}, DakoCytomation) antibody was used. Samples were analyzed with FACSCan or LSR II flow cytometers (Becton Dickinson) and data were analyzed with CELLQuest or Diva software (Becton Dickinson). Immunofluorescence for p53 was performed on cytospin preparations of freshly purified CD133- cells, with anti-p53 (Pab240, mouse IgG_1, Calbiochem, Darmstadt, Germany) antibody specific for the conformational mutant form[23].

Transplantation of cancer cells. After magnetic or cytofluorimetric cell sorting, $CD133^+$ and $CD133^-$ purified populations were resuspended in 100 µl of PBS and, before the injection, cell aliquots were diluted 1:1 with Growth Factor Reduced MATRIGEL Matrix (BD Biosciences). The injection was performed subcutaneously into the flank without anaesthesia. After 8-10 weeks mice

were sacrificed by cervical dislocation, tumors were removed and analyzed by histology or flow cytometry as for the original tumor.

Immunohistochemistry. Immunohistochemistry was performed on formalin fixed paraffin embedded tissue, cell blocks or frozen tissue. Five µm sections of paraffin embedded tissue and cell blocks were dewaxed in xylene and rehydrated with distilled water. Sections were then processed with the heat-induced epitope retrieval technique using a citrate buffer (0.01M pH6) followed by incubation with 3% hydrogen peroxide. The slides were subsequently incubated with the following antibodies for 1 hour at room temperature: CDX2 (BioGenex, clone CDX2-88, 1:100), CK20 (Dako, clone Ks20.8, 1:50), β-catenin (BD Transduction Laboratories, clone 14, 1:50). Five µm cryostat sections were acetone-fixed and incubated at room temperature with anti-human CD133/1 1:10 (Miltenyi Biotec, clone AC133). The reaction was performed using Elite Vector Stain ABC systems (Vector Laboratories) and DAB Substrate Chromogen (DakoCytomation) followed by haematoxylin counterstaining.

Real-Time PCR. The relative quantification of CDX2 mRNA was performed by *Taq*Man technology, using the ABI PRISM 7900 DNA sequence detection system (Applied Biosystems). Commercial ready-to-use primers/probe mixes were used (Assay-on-Demand Gene Expression products, Hs00230919_m1; Applied Biosystems). CD133 mRNA relative quantification was performed by using SYBR® Green technology. CD133 specific primers were selected on the sequence NM_006017 (Gene Bank). The forward primer was GCGTGATTTCCCAGAAGATA and the reverse primer was CCCCAGGACACAGCATAGAA, which produced an amplicon of 145 base pairs. Amplification was performed with 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Human GAPDH was used as a housekeeping gene in both amplifications. Original input RNA amounts were calculated with a relative standard curve for CDX2 or CD133 and GAPDH RNA. Gene expression values were reported as the normalized percentage obtained by dividing the copy numbers of specific genes by GAPDH.

Detection of p53 mutations. The analysis was performed on genomic DNA from CD133- tumor samples, which was extracted with PureLink™ Genomic DNA Purification Kit (Invitrogen S.R.L., Milan Italy) according to the manufacturer's protocol. The sequences analyzed corresponded to exons 5, 6, 7, and 8. Primer sequences were as follows: exon 5 forward, 5'-GCTGCCGTGTTCCAGTTGC-3', and reverse, 5'-CCAGCCCTGTCGTCTCTCCA-3'; exon 6 forward, 5'-GGCCTCTGATTCCTCACTGA-3' and reverse, 5'-GCCACTGACAACCACCCTTA-

3'; exon 7 forward, 5'-TGCCACAGGTCTCCCCAAGG-3' and reverse, 5'-AGTGTGCAGGGTGGCAAGTG-3'; exon 8 forward, 5'-CCTTACTGCCTCTTGCTTCT-3' and reverse, 5'-ATAACTGCACCCTTGGTCTC-3'. These primer sets defined PCR products of 293, 209,195, and 224 bp, respectively, which were used as template for sequencing reactions.

Protein truncation test (PTT) of APC. Genomic DNA from normal and CD133- tumor samples was extracted with PureLink™ Genomic DNA Purification Kit (Invitrogen S.R.L., Milan Italy) according to the manufacturer's protocol. *APC* exon 15 was investigated by four overlapping PCR fragments[24]. PCR amplification was used to introduce the 17 bp consensus T7 promoter sequence and a mammalian initiation sequence in-frame with a unique *APC* sequence. Genomic DNA was amplified by standard PCR conditions as previously described[25]. The PCR products were used in a TnT T7-coupled reticolocyte lysate system (Promega, Madison, WI, USA) incorporating [^{35}S]-methionine in accordance with the manufacturer's instructions. The translation products were separated on 15% SDS-polyacrylamide gel in a vertical minigel apparatus (BioRad, Hercules, CA., USA). Electrophoresis was performed until the bromophenol blue dye had run off the bottom of the gel. Gel was fixed, soaked in 10% glycerol, dried on a vacuum slab gel dryer and exposed to Kodak X-OMAT AR film overnight at –70°C.

Detection of murine cells in mouse xenografts. To verify the absence of contaminant mouse cells into the CD133- population of tumor cells isolated from mouse xenografts, genomic DNA was extracted from HLA-I+BerEP4+ cells. Human alpha satellite and mouse myogenin expression was evaluated by PCR amplification. The following primers were used: human alpha satellite forward, 5'-GGATAATTTCAGCTGACTAAACAGA-3', and reverse, 5'-TTCCGTTTAGTTAGGTGCAGTTATC-3'; mouse myogenin forward, 5'-TTACGTCCATCGTGGACAGGA-3', and reverse, 5'-TGGGCTGGGTGTTAGCCTTA-3'. PCR was performed with 20 cycles for 30 seconds at 95 °C, 30 seconds at 62 °C, and 30 seconds at 72 °C.

Supplementary Table S1

Case	Long term cultures			Xenografts					
					Number of cells injected		Number of spheres injected		
	Total	CD133^{+}	CD133^{-}	Number of spheres injected	Undifferentiated	Differentiated	Secondary	Tertiary	Quaternary
1	Yes	–	–	50 (3/3); 500 (3/3)	$5x10^5$ (2/2) $5x10^5$ (2/2)	$5x10^5$ (0/2) $5x10^5$ (0/2)	50 (2/2) 50 (2/2)	50 (2/2) 50 (2/2)	50 (2/2) 50 (2/2)
2	No	No	No						
3	Yes	Yes	No	50 (3/3); 500 (3/3)	$5x10^5$ (2/2) $5x10^5$ (2/2)	$5x10^5$ (0/2) $5x10^5$ (0/2)			
4	No	No	No						
5	No	No	No						
6	No	No	No						
7	Yes	Yes	No	50 (2/3); 500 (3/3)	$5x10^5$ (2/2) $5x10^5$ (2/2)	$5x10^5$ (0/2) $5x10^5$ (0/2)	50 (2/2)	50 (2/2)	50 (2/2)
8	No	No	No						
9	No	–	–						
10	Yes	–	–	50 (3/3); 500 (2/3)					
11	No	–	–						
12	No	–	–						
17	No	–	–						
18	No	–	–						
19	Yes	–	–	50 (2/3); 500 (3/3)					

Generation of long term cultures and tumorigenic activity of colon cancer cells. Successful engrafment rate is indicated in brackets

Supplementary Notes

23. Gannon, J.V. et al. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J* **9**, 1595-602 (1990).
24. Prosser, J. et al. APC mutation analysis by chemical cleavage of mismatch and a protein truncation assay in familial adenomatous polyposis. *Br J Cancer* **70**, 841-6 (1994).
25. Norheim Andersen, S. et al. Germline and somatic mutations in exon 15 of the APC gene and 25 K-ras mutations in duodenal adenomas in patients with familial adenomatous polyposis. *Scand J Gastroenterol* **34**, 611-7 (1999).