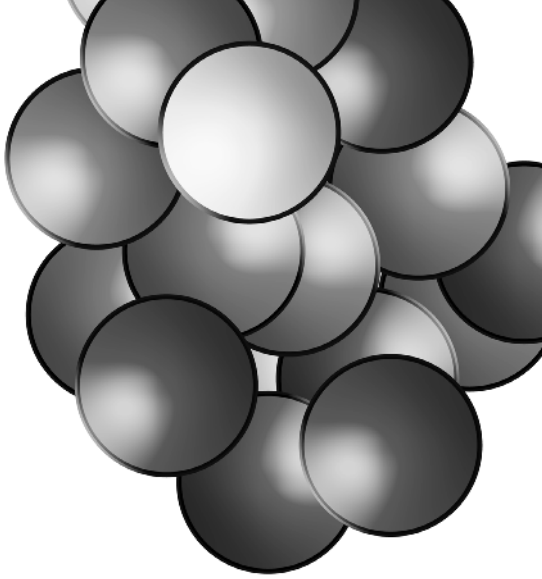
Molecular identification and targeting of colorectal cancer stem cells

Kemper, K.

Publication date
2012



Chapter 4

The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation

Kristel Kemper, Martin R. Sprick, Martijn de Bree, Alessandro Scopelliti, Louis Vermeulen, Maarten Hoek, Jurrit Zeilstra, Steven T. Pals, Huseyin Mehmet, Giorgio Stassi and Jan Paul Medema

Cancer Research 2010, 70(2); 719–729

Abstract

Colon cancer stem cells (CSC) can be identified with AC133, an antibody that detects an epitope on CD133. However, recent evidence suggests that expression of CD133 is not restricted to CSCs, but is also expressed on differentiated tumor cells. Intriguingly, we observed that detection of the AC133 epitope on the cell surface decreased upon differentiation of CSC in a manner that correlated with loss of clonogenicity. However, this event did not coincide with a change in CD133 promoter activity, mRNA, splice variant, protein expression, or even cell surface expression of CD133. In contrast, we noted that with CSC differentiation, a change occured in CD133 glycosylation. Thus, AC133 may detect a glycosylated epitope, or differential glycosylation may cause CD133 to be retained inside the cell. We found that AC133 could effectively detect CD133 glycosylation mutants or bacterially expressed unglycosylated CD133. Moreover, cell surface biotinylation experiments revealed that differentially glycosylated CD133 could be detected on the membrane of differentiated tumor cells. Taken together, our results argue that CD133 is a cell surface molecule that is expressed on both CSC and differentiated tumor cells, but is probably differentially folded as a result of differential glycosylation to mask specific epitopes. In summary, we conclude that AC133 can be used to detect cancer stem cells, but that results from the use of this antibody should be interpreted with caution.

Introduction

Cancer stem cells (CSC) are thought to be responsible for tumor growth. These CSCs can drive tumor growth and possess multilineage differentiation potential[1,2], allowing them to reform the original malignancy on transplantation in mice. In several cancers, CSCs have been identified using one or multiple markers, like CD24[3,4], CD29[4], CD44[5-7], CD133[8-12], ALDH1[13,14], or Hoechst exclusion[15-17]. However, the appropriateness of these markers is an ongoing discussion.

The pentaspan membrane protein CD133, originally identified as a marker for $CD34^+$ hematopoietic stem and progenitor cells[18,19], has been used for CSC identification in several types of cancer, such as glioblastoma[11,20], melanoma[8], liver cancer[21], osteosarcoma[22], and colon cancer[9,10,12]. Several monoclonal antibodies have been developed against CD133. The most commonly used are AC133 (CD133/1) and 293C/AC141 (CD133/2), which are reported to recognize distinct epitopes[23]. AC133 is frequently used to isolate CSCs and suggested to recognize a glycosylated epitope on CD133[18], which contains eight putative N-linked glycosylation sites. However, the use of CD133 as a marker for identifying and isolating CSCs is controversial because its expression pattern is debated. Several groups have shown that $AC133^+$, but not $AC133^-$, cells sorted from primary colon carcinomas can form tumors in immunodeficient mice that recapitulate the morphology of the original tumor [9,10,12]. In addition, expression of mouse CD133 (prominin-1) has been shown to mark stem cells in the small intestine[24,25]. In contrast, CD133 mRNA expression was found in cells other than the (cancer) stem cell fraction[25-28]. For example, insertion of a LacZ reporter directly after the ATG start site of mouse CD133 (prominin-1), or between exons 3 and 8, showed CD133 expression throughout the mouse colon[25,28], including differentiated goblet cells[25], and also in mouse colon adenocarcinoma[28]. Moreover, Shmelkov and colleagues[28] described that CD133 does not uniquely mark CSCs from human colon carcinomas but is also expressed on differentiated tumor cells.

The above-mentioned data clearly show that there is a contradiction between AC133 as a CSC marker and the broad CD133 mRNA and protein expression found in colon (cancer). The functionality of CD133 as a CSC marker is therefore unclear and should be clarified. In this study, we addressed this question by analyzing CD133 promoter activity and mRNA, splice variant (Sv), protein, and AC133 epitope expression between stem cells and differentiated cells in normal colon epithelium and colon cancer. In addition, we addressed the possibility that posttranslational modification, such as glycosylation, might play a role in recognition of CD133 by the AC133 antibody. We show that CD133 is expressed on the cell surface of CSCs and differentiated tumor cells but is differentially glycosylated. Nevertheless, AC133 does

not recognize a glycosylated epitope or a specific Sv. Instead, we show that the failure to recognize CD133 is due to the inaccessibility of the epitope, which we hypothesize to depend on the tertiary structure of CD133 on differentiated cells.

Materials and Methods

Tissue collection, CSC isolation, culture and xenografting

Samples of human colon carcinomas were obtained according to standard medical ethical procedures of the Academic Medical Center or the University of Palermo. CSC cultures were derived and cultured as described previously[16]. C001 and C002 were derived from the primary cancer, whereas LMIV and LMV were obtained from liver metastases. All lines were from different patients. Cells were differentiated on adherent plates (Corning) by withdrawal of epidermal growth factor and fibroblast growth factor and addition of non–heat-inactivated 2% FCS. Primary colon tumor pieces of 1 mm3 were implanted s.c. in nonobese diabetic/severe combined immunodeficient mice. Before tumors reached 1 cm3, the mice were sacrificed and tumors were digested as described in ref. 12.

Antibodies

The antibodies used were FITC-anti-ESA (Biomeda), anti-AC133, anti-CD133/2 (AC141), anti-CD133 (W6B3C1), anti-CD133/2 (293C; Miltenyi), anti–extracellular signal-regulated kinase (ERK; a kind gift from B. Burgering, Physiological Chemistry, University of Utrecht, Utrecht, the Netherlands), anti-actin (Santa Cruz Biotechnology), anti- Epcam (Abcam), IRDye 680 anti-mouse and IRDye 800 anti-rabbit (LI-COR Biosciences), horseradish peroxidase–labeled anti-mouse IgG (Southern Biotechnologies), anti–cytokeratin-20 and anti–intestinal alkaline phosphatase (Genetex), anti–Muc-2 (Abcam), mouse IgG1 (DakoCytomation), rhodamine red–conjugated anti-mouse antibodies (Molecular Probes), Alexa546-anti-mouse IgG and Alexa546-anti-rabbit IgG (Invitrogen).

Flow cytometry

Cells were stained with directly labeled antibodies for 30 min at 4˚C in PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide. Intracellular fluorescence-activated cell sorting (FACS) staining was done with Cytofix/cytoperm

(BD Biosciences) according to the manufacturer's protocol. Analysis was done on a FACSCalibur, and sorting on a FACSAria.

Laser-aided microdissection of villus and crypt regions

Sections of snap-frozen colonic tissue of familial adenomatous polyposis (FAP) patients were stained with hematoxylin and digitally scanned with a Veritas Microdissection System (Molecular Devices Corporation). Epithelial cells from the base and the upper part of normal crypts distant from adenomatous tissue were cut and collected into Capsure Macro LCM caps (Molecular Devices).

Protein isolation and immunoblotting

Immuoblotting was done as described in ref. 29. For deglycosylation, lysates were treated overnight with 500 units/μL of peptide-N-glycosidase F (PNGase F) at 37°C.

RNA isolation and PCR analysis of CD133 mRNA and CD133 promoter activity

Total RNA was isolated by TRizol extraction (Invitrogen). RNA quality and quantity were assessed using Nanodrop technologies, and cDNA was prepared with reverse transcriptase III (Invitrogen). The following intron-spanning primers were used: CD133, 5′-TTC TAT GCT GTG TCC TGG GGC-3′ and 5′-TTG TTG GTG CAA GCT CTT CAA GGT-′3; actin, 5′-ATG GAA GAA GAG ATC GCC GC-3′ and 5′-TCG TAG ATG GGC ACC GTG TG-3′; exon 3, 5-ATA TCT TTC TCT ATG TGG TAC AGC C-3 and 5′-AAT AAA CAG CAG CCC CAG GA-3′ (30); exons 25–29, 5′-AAA ACT GGC TAA GTA CTA TCG-3′ and 5′-AGA CCC AGA AAC TAC CAA AA-3′. The CD133 promoter activity was determined as described in ref. 31.

Cell surface protein isolation

C002 cells were cultured under cancer stem cell conditions and plated adherently (Corning) for the last 20 h or differentiated for 10 d. Cell surface protein isolation was done with the Pierce Cell Surface Protein Isolation Kit, except that columns were washed twice with radioimmunoprecipitation assay (RIPA) buffer (Thermoscientific) and the biotinylated protein was eluted with RIPA buffer containing 50 mmol/L DTT.

Bacterial expression of CD133

DNA encoding CD133 from start to end of the NH2-terminal part and the first and second extracellular loops was acquired by PCR performed with the following reverse primers: NH2 terminus, 5′-TTA CTA CTC GAG CTA CCC TGC TTC ATA GTA GAC-3; half first loop, 5′-TTA CTA CTC GAG CTA GTT CAT GTT CTC CAA CGC C-3′; first loop, 5′-TTA CTA CTC GAG CTA GTA TGA ATC ATA CTC TTC-3′; second loop, 5′-ATC TTG GAT CCG GAG GGC AGC CTT CAT CCA CAG ATG-3′, and with the forward primer, 5′-TTA CTA CTC GAG CTAA TTC AAG GGG TCG ATA AT-3′. PCR product was cloned into a pET-28 vector with BamH1 and Xho1 restriction sites and transformed into BL21 (DE3) bacteria (Stratagene). Bacterial expression of CD133 was done according to the manufacturer's protocol.

Immunofluorescence staining of colon cancer specimens

Human colon carcinoma samples were obtained from 15 patients. Immunofluorescence was done on fresh frozen tissues post-fixed in 2% paraformaldehyde for 20 min at 37°C (Shmelkov's procedure) or on fresh tissue specimens embedded in liquid optimum cutting temperature compound, solidified gradually in liquid nitrogen vapor, and fixed in acetone. Sections of 5 μm were blocked with TBS containing 3% AB human serum for 10 min. Primary antibody was incubated overnight at 4°C (1:5) and secondary antibody for 1 h at room temperature (1:300 in 1% BSA/PBS). Counterstaining was done using Toto-3.

Results

Promoter regulation of CD133 in normal human colon

Human CD133 is a highly complex gene that contains five promoters, differing in the 5′ untranslated region (ref. 31; Figure 1A), and seven Svs, which differ in coding exons and generate distinct protein isoforms (Figure 2A). In previous studies, LacZ was inserted into a part of CD133 that was unaffected by differential splicing, thereby generating a setting that allows for detection of all CD133 promoter activities and Svs[25,28]. However, this approach is not adequate to detect distinct CD133 promoter activity or isoform expression within separate regions of colon epithelium and carcinoma. Promoter regulation exists for human CD133[31] and could result in differential Sv expression[32-34]. Moreover, several mouse CD133 Svs reportedly cannot reach the cell surface[32]. We therefore hypothesized that differential Sv expression

influences CD133 recognition, and we analyzed promoter activity and Sv expression to gain more insight into the regulation and detection of CD133.

We detected CD133 mRNA expression in the upper and lower parts of the crypt in normal human colon derived from FAP patients (Figure 1B), in agreement with earlier findings that CD133 mRNA was expressed in all epithelial cells of mouse colon[25,28].

All five CD133 promoter activities could be detected using nested PCR (ref. 31; Supplementary Figure S1A–C), and all promoters, except P4, showed activity in colon cancer cell lines. Sequencing of the products revealed all reported as well as two additional alternatively spliced noncoding exons for promoter 3 (sequences to be submitted), which are consistent with the splice acceptor-donor site rules[31], and additional alternative Svs for exons transcribed from promoter 5 (Supplementary Figure S1D).

Next, we analyzed the differential CD133 promoter usage on microdissected crypt surface and base and showed that promoters 1, 2, and 3 were active in the human normal colon, whereas promoter 4 and 5 activities were not detected (Figure 1B). Besides a small variation in splicing of exons transcribed from promoter 3, we observed no major changes in promoter usage. Although this nested PCR only allows for semiquantitative measurements of promoter activity, we conclude that differential CD133 promoter activity is not a dominant effect in differential CD133 protein expression in normal colon.

CD133 promoter regulation in cancer stem cells

Because CD133 is applied as a marker for CSCs in colon cancer, we analyzed CD133 promoter activity in colon CSCs versus differentiated cancer cells (DCC). Therefore, CSCs derived from primary colon carcinomas were cultured as spheroids or adherent under differentiation-inducing conditions to generate DCCs[4,12]. CSCs had high expression of surface CD133 as detected with the AC133 antibody, whereas differentiation decreased this detection significantly (Figure 1C). Previously, we have shown that these high AC133-expressing spheroid cultures retain the capacity to induce colon adenocarcinomas on xenotransplantation, whereas DCCs lose this capacity[12]. In addition, differentiation markers such as cytokeratin-20, Muc-2, and intestinal alkaline phosphatase are only expressed in DCCs (Supplementary Figure S2A). More importantly, high AC133 expression selected for the clonogenic population as shown by xenotransplantation[10,12,35] as well as clonogenic growth (ref. 4; Supplementary Figure S2B), confirming that AC133 expression can identify CSCs in these cultures.

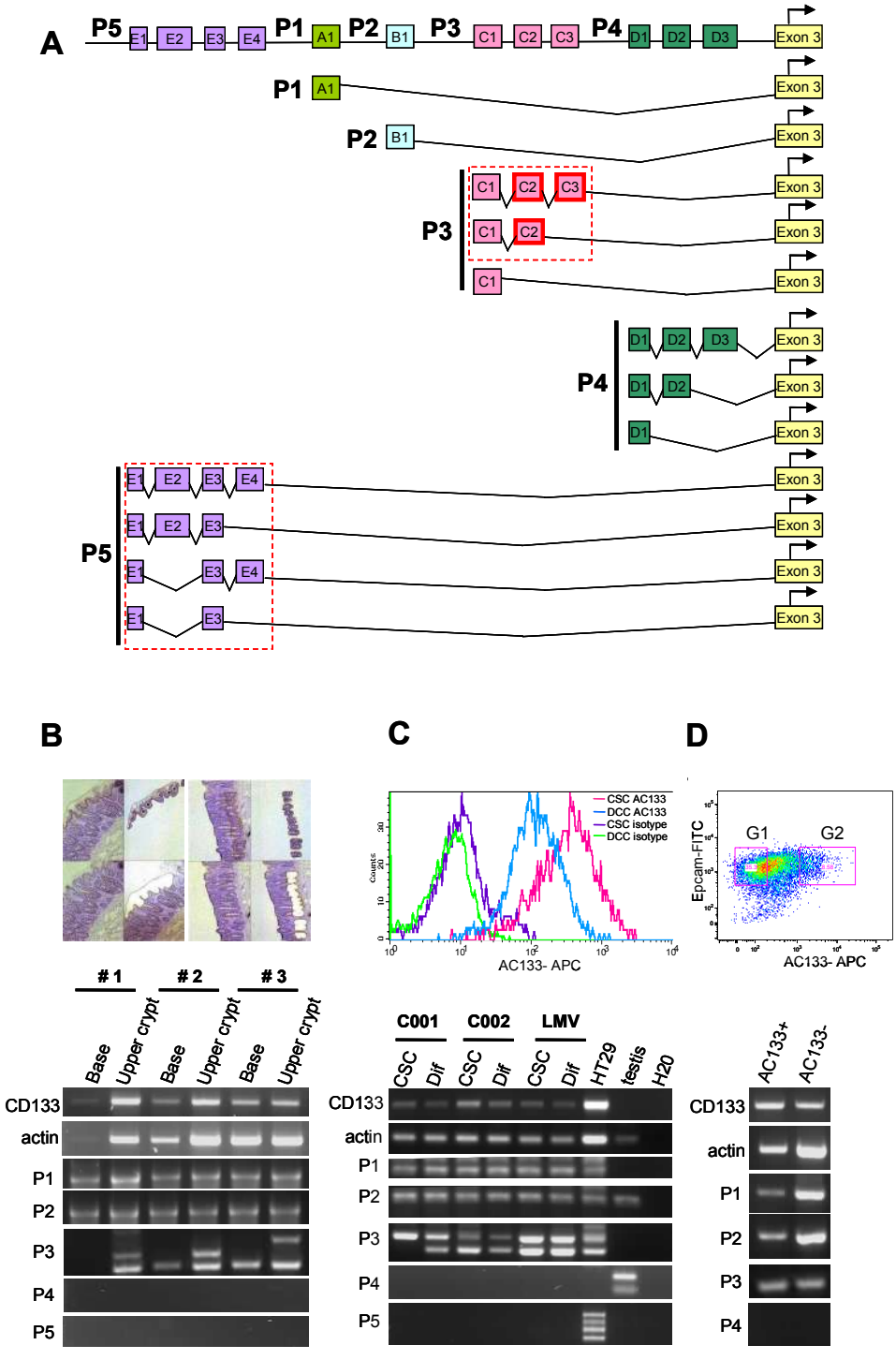

A
P5
E1
E2
E3
E4
P1
A1
P2
B1
P3
C1
C2
C3
P4
D1
D2
D3
Exon 3
B
1
2
3
Base
Upper crypt
CD133
actin
P1
P2
P3
P4
P5
C
CSC AC133
DCC AC133
CSC isotype
DCC isotype
Counts
AC133- APC
D
G1
G2
Epcam-FITC
AC133- APC
C001
C002
LMV
CSC
Dif
HT29
testis
H20
AC133+
AC133-

Figure 1: CD133 mRNA expression and promoter activity in colon and colon cancer. A. Schematic representation of the 5′ region of CD133, based on ref. 34. CD133 has five alternative promoters, each containing one or multiple corresponding exons. Additionally found exons and Svs are boxed. **B.** Reverse transcription-PCR (RT-PCR) analysis of CD133 mRNA expression and promoter activity was done on microdissected base and upper crypt regions of normal colon of three different FAP patients. CD133 primers were developed against a region of CD133 unaffected by splicing. Actin was used as an input control. Promoter PCR assay was done as described in ref. 34. The three different bands of promoter 3 correspond to three different Svs (Supplementary Fig. S2). **C.** Decrease in AC133 recognition during CSC differentiation shown by FACS analysis. RT-PCR analysis of CD133 mRNA and promoter activity on CSCs and differentiated progeny in four CSC lines. RT-PCR for promoter-3 activity gave two bands, representing Sv C1 and C1 + C2. **D.** FACS profile of dissociated tumor cells from a human colon carcinoma xenograft. G1 and G2 represent $AC133^{-}Epcam^{+}$ and $AC133^{+}Epcam^{+}$ sorted populations, respectively, used for RT-PCR analysis of CD133 mRNA and promoter activity.

CSCs as well as DCCs showed promoter 1, 2, and 3 activities, whereas promoter 4 was not active in either population (Figure 1C). Although promoter 5 was active in colon CSCs, its activity was very low and not changed upon differentiation. Combined, we conclude that CD133 promoter activity does not differ substantially between CSCs and DCCs *in vitro* and thus cannot explain differential recognition of AC133 during differentiation. Surprisingly, CD133 mRNA expression was not decreased upon *in vitro* differentiation (Figure 1C), which indicates that protein detection by FACS, which is decreased by almost 10-fold in DCCs, and mRNA expression do not correlate.

In vitro culture of CSCs is a powerful method, but could influence expression of many genes including CD133. To exclude culture artifacts, we studied CD133 promoter and mRNA expression of a colon cancer xenograft, grown by direct implantation of a primary human colon carcinoma. The tumor-derived cells were FACS sorted into $Epcam^{+}AC133^{+}$ and $Epcam^{+}AC133^{-}$ fractions, while nonepithelial cells were discarded (Figure 1D). Importantly, promoter activities were not significantly different in *ex vivo* sorted $AC133^{+}$ and $AC133^{-}$ fractions (Figure 1D). Moreover, despite a more than 50-fold difference in AC133 reactivity, CD133 mRNA levels were only slightly lower in the $AC133^{-}$ fraction (Figure 1D). This clearly shows that AC133 staining is not at all correlated to CD133 mRNA expression.

CD133 Svs in cancer stem cells

Although CD133 mRNA levels are fairly comparable in CSCs and DCCs, the PCR used does not discriminate between alternative Svs in the coding sequence. In

addition, regulation of differential CD133 splicing by distinct promoters has not been shown yet. The seven reported Svs of CD133 (Figure 2A; Table 1) generate different CD133 protein isoforms and could potentially encode proteins that lack the AC133 epitope.

The Svs differ mainly in the presence of exon 5[30] or in splicing of exon 26a, 26b, and/or 27. Analysis of the different Svs using PCRs directed at the spliced regions (Figure 2A) indicated that several CD133 Svs could be detected in both the base and the upper surface of the crypt. However, it was also quite evident that Sv2 was by far the most prominent form present. Importantly, differential expression of Svs between the base and the upper part of the crypt was not found (Figure 2B). Similarly, CSCs as well as DCCs almost only displayed expression of one Sv that lacked exon 5 and contained exons 26b and 27, consistent with the expression of Sv2 (Figure 2C). This was confirmed by sequencing, which revealed only one major sequence (data not shown). This is not unique to *in vitro* cultured cancer cells, but also freshly isolated $AC133^-$ and $AC133^+$ cancer cells from xenografts mainly expressed Sv2 at the mRNA level (Figure 2D). Although the function of the different CD133 Svs remains enigmatic, it is clear that several of these are expressed at the mRNA level in colon cancer samples. Nevertheless, the vast majority of the expressed CD133 mRNAs encode for Sv2. More importantly, no change is observed during CSC differentiation.

Table 1: Splice variants of CD133

Splice variants	NCBI nomenclature	Fargeas et al.[33] nomenclature	Amino acids	Accession numbers
Sv1	Isoform 1, transcript variant 1	s2	865	AF0272208 NM_006017
Sv2	Isoform 2, transcript variant 2, transcript variant 2	s1	856	AF507034, NM_001145847 NM_001145848
Sv3	Isoform 3	s9	830	AY449689
Sv4	Isoform 4, transcript variant 4	s7	825	AY449690 NM_001145852
Sv5	Isoform 5, transcript variant 5	s10	833	AY449691 NM_001145851
Sv6	Isoform 6, transcript variant 6	s11	834	AY449692, NM_001145850
Sv7	Isoform 7, transcript variant 7	s12	842	AY449693 NM_001145849

NOTE: This table includes the nomenclature of the CD133 Svs used in the literature and by the National Center for Biotechnology Information (NCBI) database, as well as refers to the sequences.

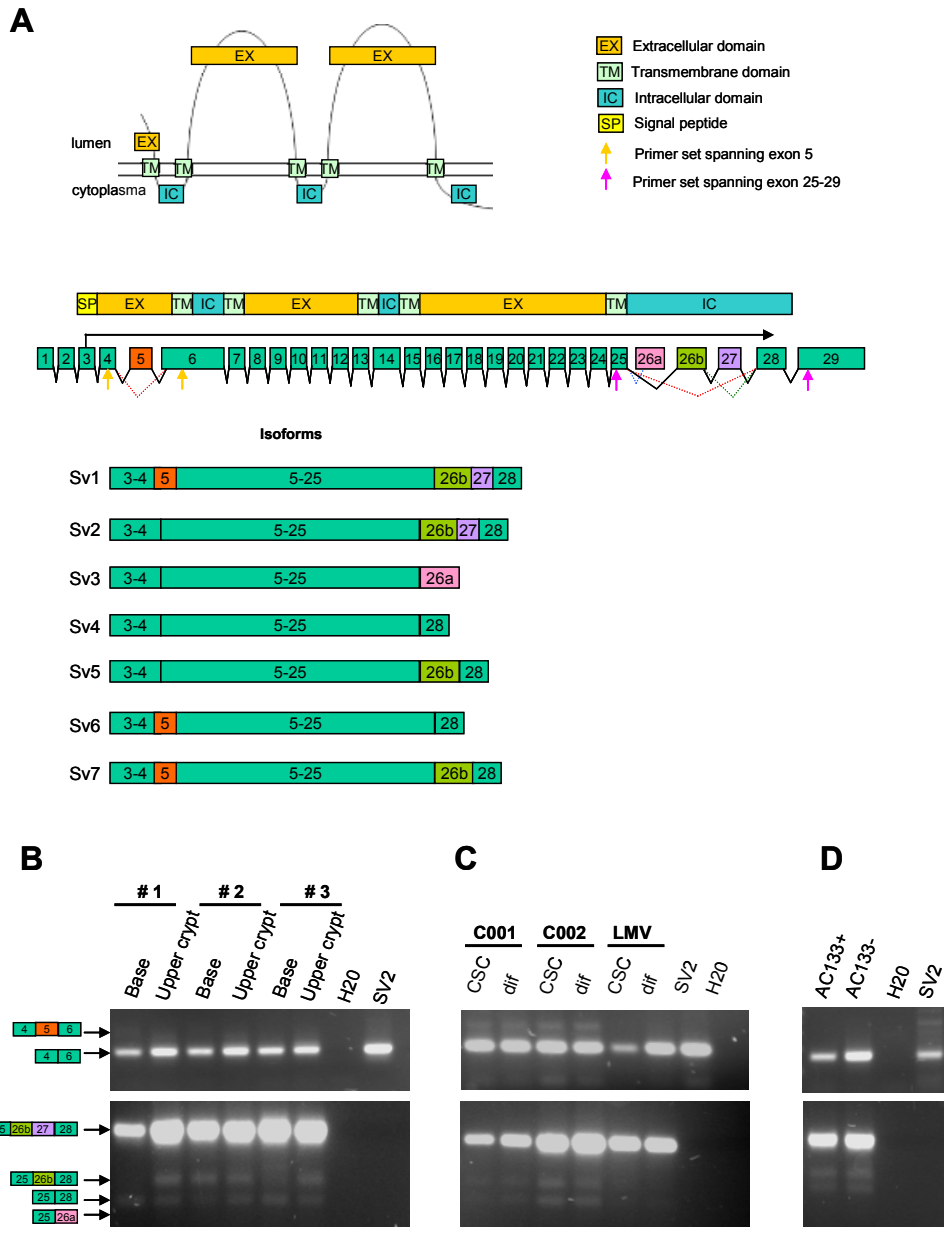


Figure 2. CD133 Sv expression in colon and colon cancer. A. CD133 has seven different Svs, varying in expression of exon 5 and exon 26a, 26b, and/or 27. **B.** Sv expression in the base and the upper part of the colon crypt. Positive control is pcDNA3.1 vector containing Sv2. **C.** CD133 Sv expression in CSCs and in 8-d differentiated DCCs. **D.** Sv expression in AC133^{-}Epcam^{+} and AC133^{+}Epcam^{+} sorted colon xenograft.

We therefore conclude that the presence of the AC133 epitope can define colon CSCs, as determined by *in vitro* clonogenicity and *in vivo* tumor growth. However, the promoter activity and mRNA and Sv expression did not vary between CSCs and DCCs, indicating that expression of the AC133 epitope is likely regulated at the translational or posttranslational level.

CD133 protein is not downregulated upon differentiation, but its glycosylation is changed

Downregulation of the AC133 epitope during differentiation of CSCs could be caused by a reduction in translation and thus total CD133 protein. To monitor this, the effect of differentiation on the recognition of CD133 by other antibodies was studied. The antibodies AC141 and 293C (CD133/2) have been reported to detect overlapping epitopes distinct from the AC133 epitope, which was confirmed by cross-blocking studies (Supplementary Figure S3). Nevertheless, they display similar downregulation as AC133 on CSC differentiation (Figure 3A). For W6B3C1, an antibody normally used for immunoblotting, we also observed epitope downregulation on CSC differentiation (Figure 3A). In contrast, analyzing CD133 expression by immunoblotting revealed no decrease of total CD133 protein during differentiation (Figure 3B), confirming our mRNA data and indicating that CD133 protein levels are not modified upon CSC differentiation but that either CD133 is retained inside the cell or specific epitopes are shielded. Interestingly, we observed an enhanced mobility of CD133 derived from DCCs on immunoblot, which is likely caused by a change in posttranslational modification and which could determine protein folding or trafficking.

CD133 is a highly glycosylated protein (Supplementary Figure S4*A*; refs. 18,36), and several groups have suggested that the AC133 epitope is a glycosylated epitope[18,37,38], which could be lost upon differentiation. We therefore analyzed whether the observed change in mobility was due to glycosylation differences. Lysates of CSCs and DCCs were treated with PNGase F to remove N-linked glycans, which resulted in a mobility shift equaling around 30 kDa, confirming that CD133 is heavily glycosylated (Figure 3C). Intriguingly, we also observed that deglycosylated CD133 from CSCs and DCCs comigrate at the exact same height, whereas the non–PNGase-F-treated samples displayed a clear migration shift upon differentiation (Figure 3C). Therefore, we conclude that the change in molecular weight of CD133 induced by differentiation reflects a change in glycosylation.

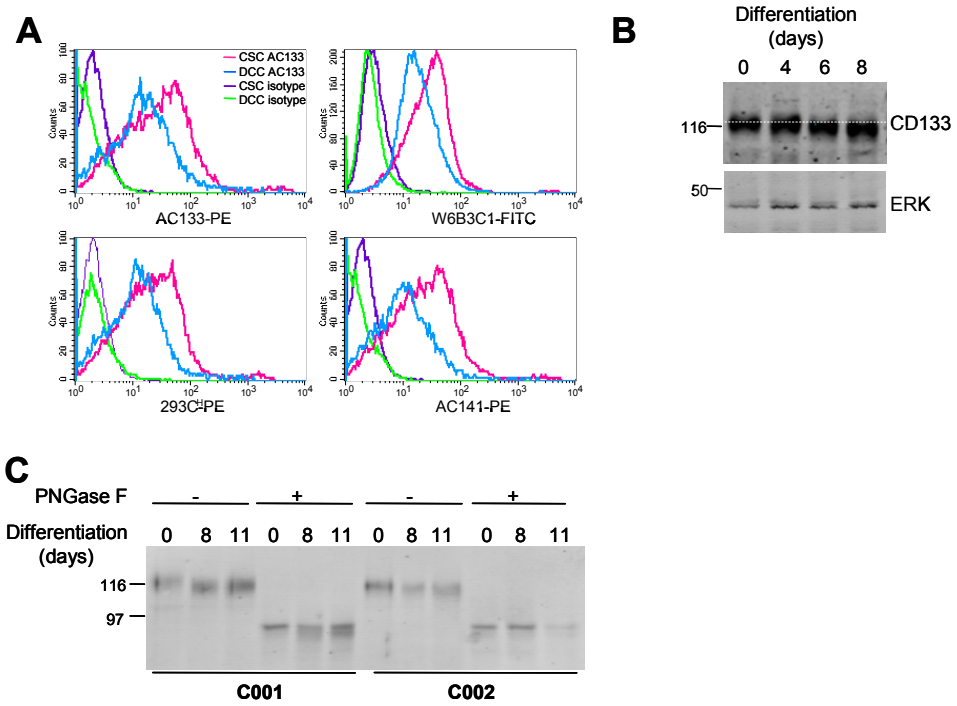


Figure 3. Differentiation-induced downregulation of the AC133 epitope correlated with differential glycosylation, but not with CD133 protein downregulation. A. FACS staining done on CSCs and DCCs for four different CD133 antibodies. **B.** Immunoblot for CD133 with W6B3C1 antibody. Lysates were taken from C002 CSC cultures and at several differentiation time points. Protein loading was controlled by ERK1/2 detection. **C.** Lysates of CSCs and DCCs were either left untreated or treated with PNGase F and immunoblotted for CD133 (W6B3C1).

AC133 does not recognize a glycosylated epitope

The previous experiments indicated that altered glycosylation of the CD133 protein during differentiation coincided with decreased recognition of at least two different epitopes of CD133 (CD133/1 and CD133/2), hinting that these epitopes are glycosylated, as has been suggested for AC133[18,37,38]. To study this possibility and to largely map the epitopes, we bacterially expressed CD133 because eukaryotic proteins are not glycosylated in bacteria. DNAs encoding different C-terminal truncated forms of His-tagged CD133 were cloned into a pET vector and expressed in bacteria (Figure 4A). Immunoblotting with an antibody against the His tag confirmed the expression and predicted size of the recombinant proteins. However, all CD133 antibodies tested only recognized the recombinant protein that contained the second extracellular loop (Figure 4A). First of all, these findings show that AC133 does not recognize a

glycosylated epitope, in contrast to previous suggestions[18,37,38]. Second, because the epitopes were mapped to the second extracellular loop (Figure 4A), which is present in all known CD133 Svs, these data confirm that differential splicing is not the cause of differential AC133 recognition.

Intracellular retention is not the cause of differential epitope expression

Previously, CD133 was shown to reside mainly in microvilli of epithelial cells[18,39], which seemed to depend on cholesterol[40]. Although our observations indicate that glycosylation is not directly influencing epitope expression, it could orchestrate differential trafficking of protein, causing intracellular retention or localization to subdomains of the cell membrane. To test this hypothesis, we performed cell surface biotinylation and subsequent isolation of proteins present on the cell membrane. Using this approach, we observed that CSCs and DCCs display clear expression of CD133 on the cell surface (Figure 4B). Moreover, these data showed that differentially glycosylated forms of CD133 can reach the cell surface (Figure 4B) and therefore make intracellular retention an unlikely explanation for epitope loss. In agreement, intracellular FACS analysis confirmed that AC133 and 293C did not show enhanced detection of CD133 when DCCs were permeabilized, whereas detection in CSCs increased (Figure 4C). Combined, this indicates that CD133 is not retained intracellularly in DCCs but is transported to the cell surface, in spite of differential glycosylation.

AC133 epitope is masked on differentiation

Our results indicate that differentiation decreased AC133 detection by FACS, coinciding with a change in glycosylation, but that this is not due to the loss of total membrane CD133 protein or of a glycosylated epitope. Differential glycosylation therefore seems to result in a distinct overall tertiary structure or localization of CD133 on the membrane that disallows the antibodies to access their epitopes. We hypothesized that AC133 epitope recognition could be enhanced by unfolding the CD133 protein chemically. In agreement, CD133 derived from DCCs or bacteria is recognized by AC133 on immunoblot, suggesting that this is indeed feasible. To directly analyze this possibility, we compared the immunofluorescence protocol used by Shmelkov and colleagues, who found CD133 staining on all epithelial tumor cells[28], to our procedure that showed isolated $CD133^{+}$ cells within colon carcinomas[4,12]. Treatment of the same colon carcinoma specimen with these two different procedures revealed that only a small percentage of tumor cells stain positive for AC133 when the

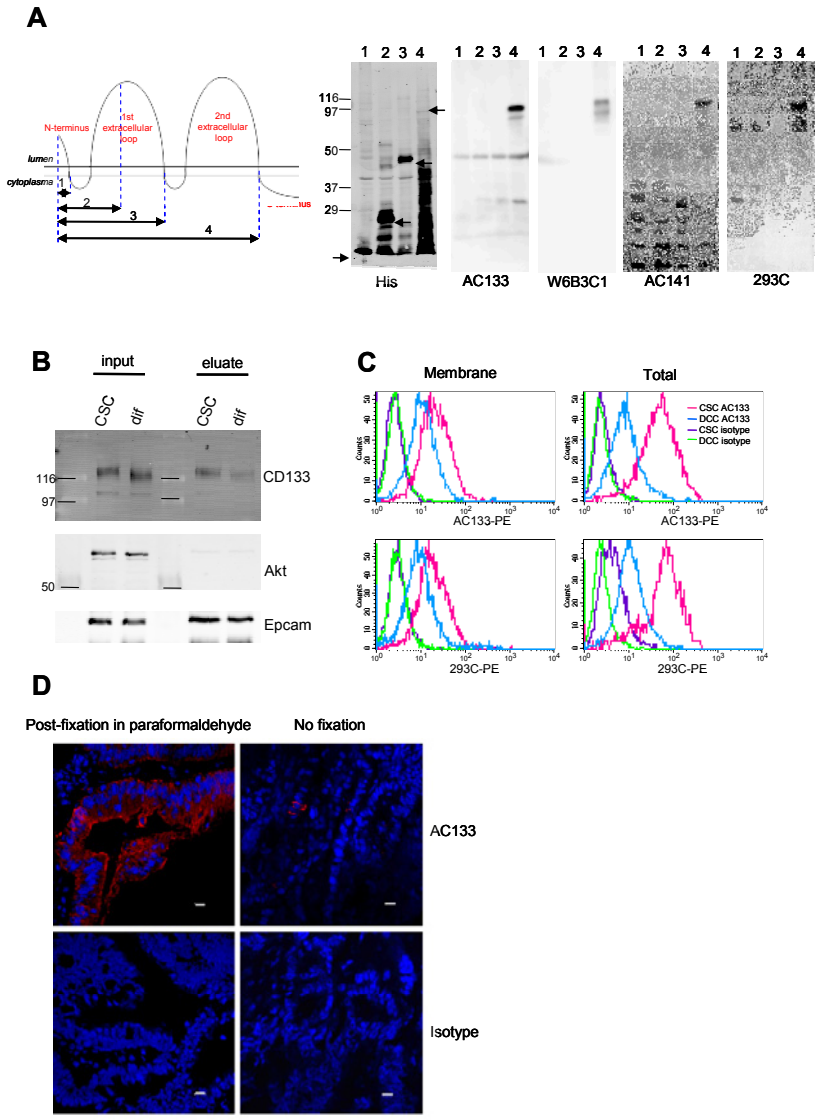


Figure 4. CD133 glycosylation did not affect epitope recognition or intracellular retention. A. Bacterially expressed C-terminal truncations of the CD133 protein, as depicted in the figure, were immunoblotted with anti-His or all CD133 antibodies. **B.** Cell surface protein isolation of CSCs and DCCs. Immunoblot for CD133 with W6B3C1 antibody on the lysates before (Input) and after isolation of the membrane proteins (Eluate). The efficiency of the isolation was checked by Akt, whereas the input was controlled by Epcam. **C.** Intracellular FACS staining for AC133 and 293C antibodies comparing CSCs and DCCs. **D.** Confocal microscopy analysis of AC133 (red) on cryostat sections of a representative colon adenocarcinoma. Right, standard procedure. Left, Shmelkov's method, including post-fixation of tissue in paraformaldehyde. Nuclei were counterstained with Toto-3 (blue). Bar, 20 μm.

samples were treated more harshly (Figure 4D). Although these data do not formally prove our model, they lend further support to the idea that CD133 mRNA and protein are not decreased when CSCs differentiate and lose their stemness, but that the epitope for AC133 is lost due to shielding.

Discussion

Several recent publications have addressed the expression pattern of the CSC marker CD133 in different tissues and discovered that CD133 could also be detected in more differentiated cell types, questioning its function as a marker for (cancer) stem cells[25-28,36]. In contrast, the AC133 epitope has been convincingly used to sort CSCs from primary colon carcinomas[9,10,12]. In this article, we addressed these conflicting data by studying possible regulation mechanisms for expression of the AC133 epitope. We showed that the AC133 and 293C/AC141 epitopes were downregulated during CSC differentiation, whereas total CD133 protein expression remained equal. The loss of these epitopes was not induced by a switch in activation of the different CD133 promoters or by differential Sv expression that could influence antibody detection. Previously, two mouse models using CD133 promoter–driven LacZ showed that CD133 mRNA is expressed throughout the colon[25,28], supporting our data. However, mouse CD133-LacZ expression marked the stem cell and progenitor population in the small intestine[24,25], indicating that CD133 regulation differs between the small intestine and colon in mouse. Additionally, mouse CD133 was shown to be a CSC marker for tumors induced in the small intestine[25] and could not be found in differentiated tumor cells. Although this points to CD133 as a direct CSC marker in mouse small intestinal tumors, care should be taken when translating these data to human because human colorectal cancer tumors obviously do not emanate from the small intestinal cells and mouse colon does not seem to display the same expression pattern.

Next to unchanged CD133 mRNA and protein expression, our data reveal that differential splicing could not explain the disappearance of the epitope. On one hand, differential splicing was, if at all present, not very prominently observed upon differentiation of CSCs. On the other hand, epitope mapping indicated that the AC133 antibody recognizes extracellular loop 2, which does not contain alternatively spliced exons (Figure 5). Moreover, our data indicate that the CD133 protein was also not retained intracellularly upon differentiation but remained expressed on the membrane. In contrast, differentiation was associated with reduced glycosylation of CD133, marked by a small loss in molecular weight. Interestingly, a microarray study

comparing spheroid cultured glioblastoma cells with serum-cultured differentiated glioblastoma cells revealed that expression of glycosylation enzymes differed significantly between these two populations[41], confirming that differentiation of CSCs is associated with a change in glycosylation enzymes. Alternatively, the change in mobility could be caused by a change in sialylation of CD133, which has been reported for malignant colon tissue[42,43].

Although decreased glycosylation correlated with reduced detection of the AC133 and 293C/AC141 epitopes after differentiation, we found that this was not due to loss of glycosylated epitopes by immunoblotting of CD133 from CSCs and DCCs or by using completely unglycosylated bacterially expressed CD133 protein. In addition, mutation of each of the eight glycosylation sites of CD133 did not prevent recognition of the protein on an immunoblot (Supplementary Figure S4B). Combined with the fact that the CD133 protein (*a*) did not decrease upon differentiation, (*b*) is still present on the cell surface, and (*c*) is also not detected when DCCs are permeabilized, this indicates that the epitope is not deleted upon differentiation but is more likely shielded. Epitope masking could be caused by differential folding of the protein or, for instance, be the result of a CD133 binding partner that masks the AC133 epitope. Although our data would suggest that epitope masking is a result of differential glycosylation, alternative explanations are possible. For instance, cholesterol plays a role in localization of CD133 on the membrane because mild depletion of cholesterol induced redistribution of CD133 from the microvilli to the entire apical plasma membrane[40]. Differential localization could also determine protein folding and, therefore, recognition of CD133 by AC133. In addition, Taïeb and colleagues showed that a glycosylation-independent epitope on the N-terminal part of CD133 was also lost during differentiation of Caco-2 colon cancer cells. The N-terminal epitope was found to be masked by ganglioside binding during differentiation[44]. Although this seems to be similar to our observations, the AC133 epitope resides in the second extracellular loop and therefore is not related to the N-terminus. However, disruption of lipid rafts with β-methyl-cyclodextrin recovered the N-terminal epitope, but not the AC133 epitope[44], which confirms that CD133 is also still present on differentiated Caco-2 cells but cannot be detected with AC133.

Importantly, our observations can explain the contradictions found in literature between AC133 as a CSC marker and the broad expression of CD133 in differentiated cell types. For example, Caco-2 cells, expressing high levels of AC133, showed reduced staining with the AC133 antibody when cells were differentiated[45]. However, a homemade antiserum against CD133 (αhE2) detected CD133 on differentiated Caco-2 cells. Moreover, this antibody detected CD133 at the apical

membrane in a range of primary tissues, whereas AC133 did not. The hypothesis that CD133 is present on DCCs but that the AC133 epitope is lost after differentiation is further substantiated by the observation that CD133 is detected on the cell surface of DCCs using cell surface biotinylation and can be unmasked by immunofluorescence using differential fixing procedures.

Although our data provide an explanation on why AC133 can be used as a bona fide CSC marker when using FACS (or magnetic activated cell sorting)-based isolation, they also call for caution when using this marker. For instance, our data help explain the vastly different, sometimes uniform, stainings of CD133 seen in tissues[26-28]. Because CD133 mRNA seems to be constant, its usage as a CSC marker when analyzing microarray is clearly flawed. Multiple studies have now reported on the clinical significance of CD133 mRNA based on such analyses. Although these associations are undeniable, we believe they do not reflect a difference in CSC incidence but point to a different feature of these subsets of tumors. In this light, it is important to note that the CD133 promoter region can also be inactivated by hypermethylation[46] and that knockdown of CD133 is apparently not affecting CSC features[47], potentially explaining why Shmelkov and colleagues[28] observed that $CD133^-$ colon cancer cells derived from liver metastasis can initiate tumor growth.

To conclude, we have shown that the colon CSC marker CD133 should be used with caution because it is widely expressed throughout the colon and colon carcinomas when analyzed for mRNA expression, by immunoblotting, or by immunohistochemical staining. However, our data also show that the differential accessibility of the AC133 epitope makes CD133 a bona fide CSC marker when using the right conditions.

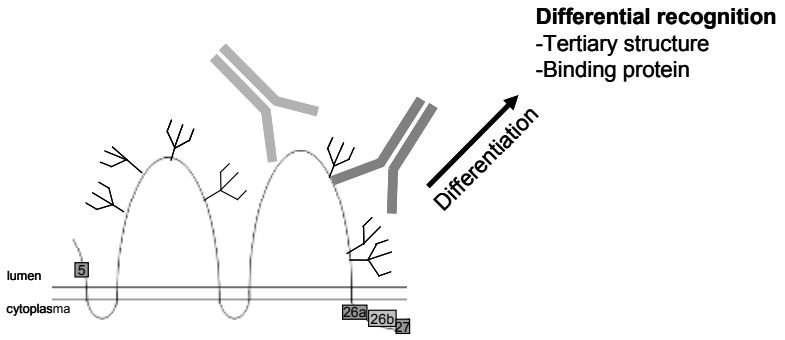


Figure 5. Schematic drawing of CD133 protein, including regions of splicing and glycosylation sites. CD133/1 and CD133/2 epitopes are located on the second extracellular loop. CSCs have highly glycosylated CD133, whereas differentiation reduces glycosylation of CD133 thereby probably shielding the CD133 epitopes, possibly caused by tertiary changes or binding of another protein.

References

1. Borovski T, Vermeulen L, Sprick MR, Medema JP. One renegade cancer stem cell? Cell Cycle 2009.

2. Vermeulen L, Sprick MR, Kemper K, Stassi G, Medema JP. Cancer stem cells - old concepts, new insights. Cell Death and Differentiation 2008; 15: 947-958.

3. Vassilopoulos A, Wang RH, Petrovas C, Ambrozak D, Koup R, Deng CX. Identification and characterization of cancer initiating cells from BRCA1 related mammary tumors using markers for normal mammary stem cells. International Journal of Biological Sciences 2008; 4: 133-142.

4. Vermeulen L, Todaro M, de Sousa MF, Sprick MR, Kemper K, Perez AM, Richel DJ, Stassi G, Medema JP. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc.Natl.Acad.Sci.U.S.A 2008; 105: 13427-13432.

5. Al Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America 2003; 100: 3983-3988.

6. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Research 2005; 65: 5506-5511.

7. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proceedings of the National Academy of Sciences of the United States of America 2007; 104: 973-978.

8. Monzani E, Facchetti F, Galmozzi E, Corsini E, Benetti A, Cavazzin C, Gritti A, Piccinini A, Porro D, Santinami M, Invernici G, Parati E, Alessandri G, La Porta CAM. Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. European Journal of Cancer 2007; 43: 935-946.

9. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007; 445: 106-110.

10. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. Nature 2007; 445: 111-115.

11. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. Neurosurgery 2003; 53: 487-488.

12. Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP, Stassi G. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 2007; 1: 389-402.

13. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS. Breast Cancer Cell Lines Contain Functional Cancer Stem Cells with Metastatic Capacity and a Distinct Molecular Signature. Cancer Research 2009; 69: 1302-1313.

14. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu SL, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of

normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007; 1: 555-567.

15. Addla SK, Brown MD, Hart CA, Ramani VAC, Clarke NW. Characterization of the Hoechst 33342 side population from normal and malignant human renal epithelial cells. American Journal of Physiology-Renal Physiology 2008; 295: F680-F687.

16. Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, Goodell MA, Brenner MK. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proceedings of the National Academy of Sciences of the United States of America 2004; 101: 14228-14233.

17. Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Research 2007; 67: 4827-4833.

18. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK, Buck DW. A novel five-transmembrane hematopoietic stem cell antigen: Isolation, characterization, and molecular cloning. Blood 1997; 90: 5013-5021.

19. Yin A, Miraglia S, Magee K, Zanjani E, Ogawa M, Buck DW. AC133, a novel monoclonal antibody to human hematopoietic stem and progenitor cells. Experimental Hematology 1997; 25: 523.

20. Singh SK, Hawkins C, Clarke ID, Squire J, Bayani J, Hide T, Cusimano MD, Dirks PB. Adult human glioma growth is exclusively maintained in vitro and in vivo by CD133+cancer stem cells. Neuro-Oncology 2004; 6: 348.

21. Ma S, Chan KW, Hu L, Lee TKW, Wo JYH, Ng IL, Zheng BJ, Guan XY. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007; 132: 2542-2556.

22. Tirino V, Desiderio V, Graziano A, d'Aquino R, De Francesco F, D'Anna M, Pirozzi G. Cd133(+) stem cells in a cell line of human osteosarcoma (SAOS2). Cytometry Part A 2008; 73A: 108.

23. Green CL, Loken M, Buck D, Deeg HJ. Discordant expression of AC133 and AC141 in patients with myelodysplastic syndrome (MDS) and acute myelogeneous leukemia (AML). Leukemia 2000; 14: 770-772.

24. Snippert H, van Es J, van den Born M, Begthel H, Stange D, Barker N, Clevers H. Prominin-1/CD133 marks stem cells and early progenitors in mouse small intestine. Gastroenterology 2009.

25. Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, Poppleton H, Zakharenko S, Ellison DW, Gilbertson RJ. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 2008.

26. Immervoll H, Hoem D, Sakariassen PO, Steffensen OJ, Molven A. Expression of the "stem cell marker" CD133 in pancreas and pancreatic ductal adenocarcinomas. Bmc Cancer 2008; 8.

27. Karbanova J, Missol-Kolka E, Fonseca AV, Lorra C, Janich P, Hollerova H, Jaszai J, Ehrmann J, Kolar Z, Liebers C, Arl S, Subrtova D, Freund D, Mokry J, Huttner WB, Corbeil D. The Stem Cell Marker CD133 (Prominin-1) Is Expressed in Various Human Glandular Epithelia. Journal of Histochemistry & Cytochemistry 2008; 56: 977-993.

28. Shmelkov SV, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T, St Clair R, Baljevic M, White I, Jin DK, Chadburn A, Murphy AJ, Valenzuela DM, Gale NW, Thurston G, Yancopoulos GD, D'Angelica M, Kemeny N, Lyden D, Rafii S. CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. J.Clin.Invest 2008; 118: 2111-2120.

29. Mullauer FB, Kessler JH, Medema JP. Betulin is a potent anti-tumor agent that is enhanced by cholesterol. Plos one 2009; 4.

30. Yu Y, Flint A, Dvorin EL, Bischoff J. AC133-2, a novel isoform of human AC133 stem cell antigen. Journal of Biological Chemistry 2002; 277: 20711-20716.

31. Shmelkov SV, Jun L, St Clair R, McGarrigle D, Derderian CA, Usenko JK, Costa C, Zhang F, Guo X, Rafii S. Alternative promoters regulate transcription of the gene that encodes stem cell surface protein AC133. Blood 2004; 103: 2055-2061.

32. Fargeas CA, Joester A, Missol-Kolka E, Hellwig A, Huttner WB, Corbeil D. Identification of novel prominin-1/CD133 splice variants with alternative C-termini and their expression in epididymis and testis. Journal of Cell Science 2004; 117: 4301-4311.

33. Fargeas CA, Huttner WB, Corbeil D. Nomenclature of prominin-1 (CD133) splice variants - an update. Tissue Antigens 2007; 69: 602-606.

34. Xin D, Hu L, Kong X. Alternative Promoters Influence Alternative Splicing at the Genomic Level. Plos one 2008; 3: e2377.

35. Lardon J, Corbeil D, Huttner WB, Ling ZD, Bouwens L. Stem cell marker prominin-1/AC133 is expressed in duct cells of the adult human pancreas. Pancreas 2008; 36: E1-E6.

36. Corbeil D, Roper K, Weigmann A, Huttner WB. AC133 hematopoietic stem cell antigen: Human homologue of mouse kidney prominin or distinct member of a novel protein family? Blood 1998; 91: 2625-2626.

37. Bidlingmaier S, Zhu XD, Liu B. The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells. Journal of Molecular Medicine-Jmm 2008; 86: 1025-1032.

38. Mizrak D, Brittan M, Alison MR. CD133: molecule of the moment. Journal of Pathology 2008; 214: 3-9.

39. Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM, Simmons PJ, Peault B, Buck DW, Huttner WB. The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. Journal of Biological Chemistry 2000; 275: 5512-5520.

40. Roper K, Corbeil D, Huttner WB. Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. Nature Cell Biology 2000; 2: 582-592.

41. Lee J, Kotliarova S, Kotliarov Y, Li AG, Su Q, Donin NM, Pastorino S, Purow BW, Christopher N, Zhang W, Park JK, Fine HA. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006; 9: 391-403.

42. Hutchins JT, Reading CL. Characterization of Mono-O-Acetylated, Di-O-Acetylated, and Tri-O-Acetylated Sialic Acids on Human-Cells. Journal of Cellular Biochemistry 1988; 37: 37-48.

43. Hutchins JT, Reading CL, Giavazzi R, Hoaglund J, Jessup JM. Distribution of Mono-O-Acetylated, Di-O-Acetylated, and Tri-O-Acetylated Sialic Acids in Normal and Neoplastic Colon. Cancer Research 1988; 48: 483-489.

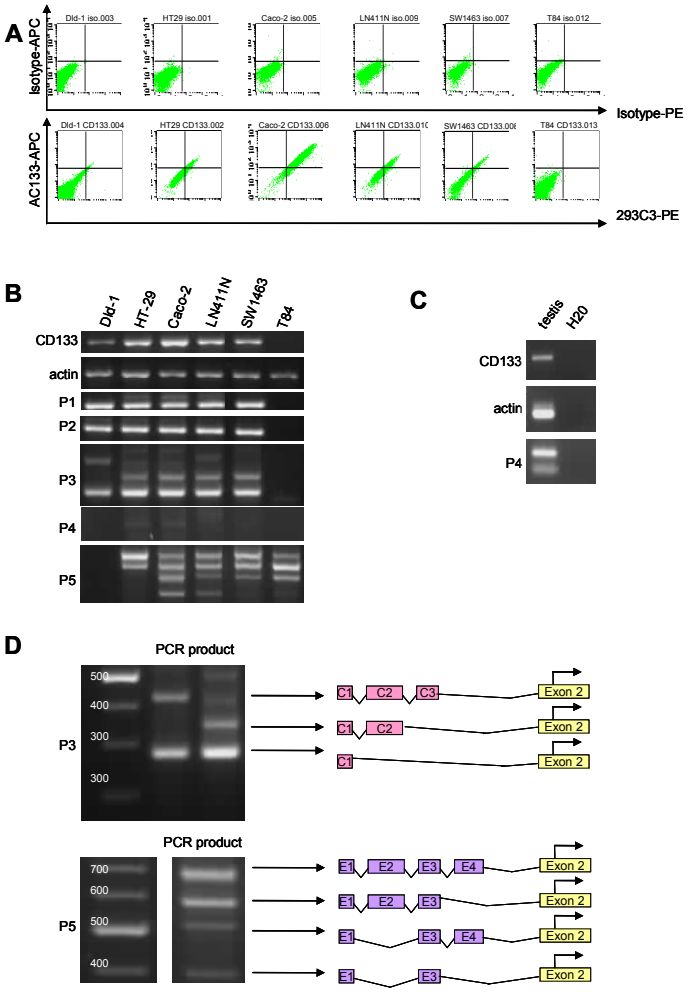
44. Taieb N, Maresca M, Guo XJ, Garmy N, Fantini J, Yahi N. The first extracellular domain of the tumour stem cell marker CD133 contains an antigenic ganglioside-binding motif. Cancer Lett. 2009; 278: 164-173.

45. Florek M, Haase M, Marzesco AM, Freund D, Ehninger G, Huttner WB, Corbeil D. Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer. Cell and Tissue Research 2005; 319: 15-26.

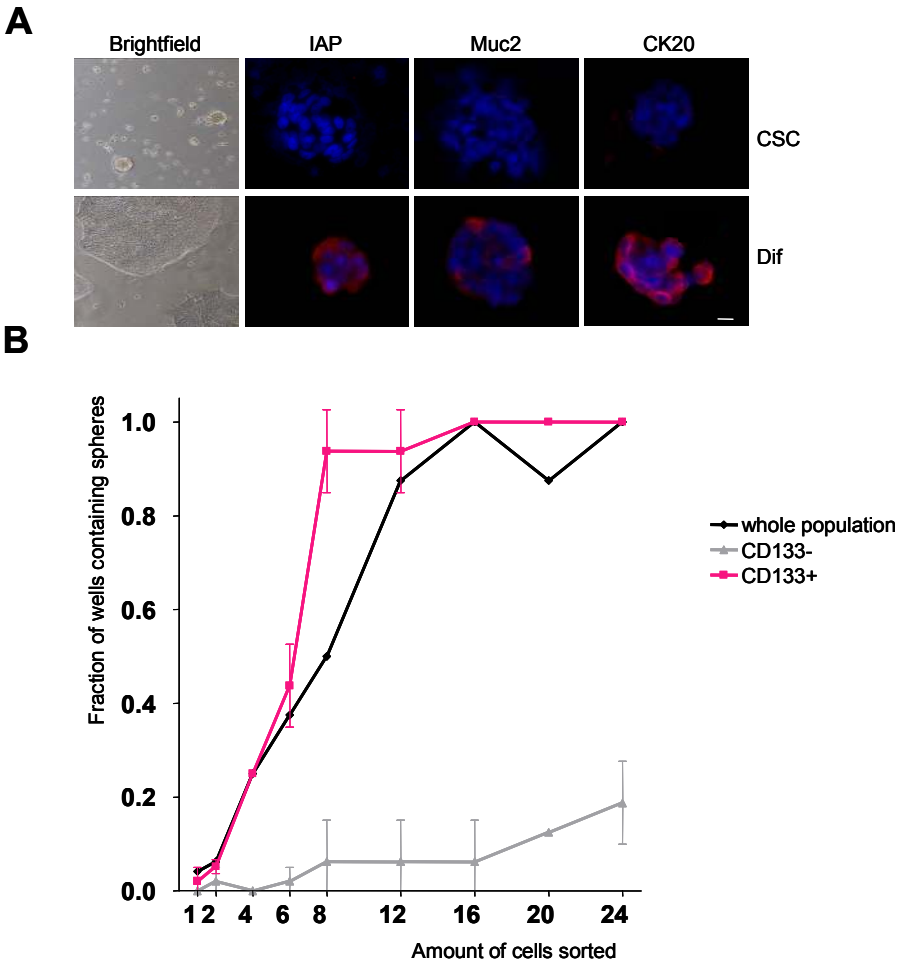
46. Yi JM, Tsai HC, Glockner SC, Lin S, Ohm JE, Easwaran H, James CD, Costello JF, Riggins G, Eberhart CG, Laterra J, Vescovi AL, Ahuja N, Herman JG, Schuebel KE, Baylin SB. Abnormal DNA methylation of CD133 in colorectal and glioblastoma tumors. Cancer Research 2008; 68: 8094-8103.

47. Horst D, Scheel SK, Liebmann S, Neumann J, Maatz S, Kirchner T, Jung A. The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer. Journal of Pathology 2009; 219: 427-434.

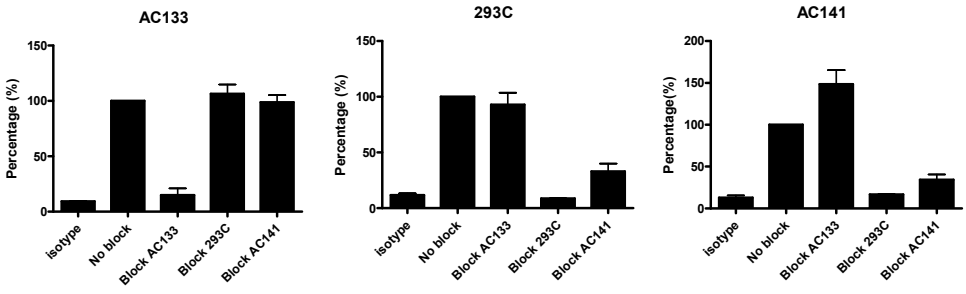
Supplementary Figures



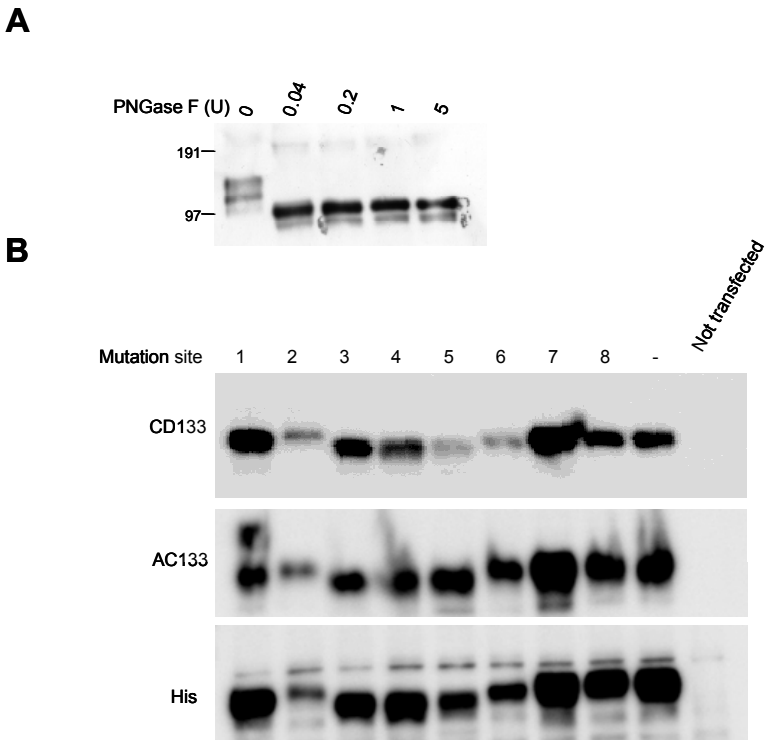
Supplementary figure 1. Promoter PCR assay in colon cancer cell lines. A. FACS staining of cancer cell lines for the AC133 and 239C epitopes. All colon cancer cell lines were found positive for CD133 protein expression, except for T84. **B.** PCR analysis of CD133 mRNA and promoter region of CD133 in colon cancer cell lines. All lines were found positive for CD133 mRNA, except for T84. All promoters, excluding promoter 4, were active in these cell lines. Promoter 5 was also active in the CD133- cell line T84, questioning its functional role. For promoter 3 and 5, all bands were cut out of the gel and sequenced. **C.** Testis was used as a positive control for the PCR on the corresponding exons of promoter 4. To detect CD133 mRNA in the testis, the PCR required 40 cycles of amplification. **D.** Corresponding splice variants of the PCR products of promoter 3 and 5. The three different bands correspond to three different splice variants; the highest band containing exons C1, C2 and C3, the middle band containing exons C1 and C2 and the lowest band representing only exon C1. For promoter 5, four different bands were found that correspond to four different splice variants.



Supplementary figure 2. Clonogenic assay for CD133 sorted cells. A. CSCs acquired from primary colon carcinomas, were cultured in vitro as spheroids or differentiated for 14 days. Cells were adhered to a 24-well plate by drying overnight at 37°C, fixed in 2% paraformaldehyde for 20min at 37°C, permeabilized with PBS-0.1% Triton-X-100 for 10min and blocked with 10% FCS in PBS for 1h. First and second antibody were incubated in blocking buffer for 1h at RT. Stainings were kept at 4°C in PBS containing 5ng/ml DAPI (Invitrogen). Pictures were taken on an Axiovert 200-M microscope (Zeiss). Differentiation was confirmed by immunofluorescence staining for differentiation markers CK-20 and Muc-2 and IAP (red). Nuclei were counterstained with DAPI (blue). Scale bar represents 100 µm. Brightfield pictures are taken under 10x magnification. **B.** C001 cells were sorted into 96-well plate based on either low or high AC133 detection. Either 1, 2, 4, 8, 16 or 32 cells were plated per well. After two weeks, the clonogenic outgrowth of these cells was monitored. The graph represents the fraction of plated wells per cell number that contain spheres.



Supplementary figure 3. Several antibodies clones, like AC133, 293C, AC141 have been developed to detect extracellular localized epitopes of CD133. It has been suggested, but never formally shown that AC133 (also referred to as CD133/1) recognizes a distinct epitope compared to 293C and AC141 (also referred to as CD133/2) which are thought to recognize the same epitope on the CD133 protein. To confirm this, we performed cross blocking studies with the AC133, 293C and AC141 antibodies. CSCs were pre-incubated with for 30min with 500ng/ml unlabeled antibodies to block their specific epitope, then stained with fluorescent labeled AC133, 293C or AC141 antibody and analyzed by FACS. Binding of AC133–PE wasblocked by pre-incubation with the AC133 antibody, but not by 293C and AC141 showingthat the AC133 epitope is located on a different part of the protein than the 293C and AC141epitope. On the other hand, 293C and AC141 could cross block each other, confirming that they recognize the same or closely linked epitope(s).



Supplementary figure 4. Glycosylation of CD133. A. The CD133 protein has eight predicted N-glycosylation sites on its two extracellular loops, five sites on the first extracellular loop, three on the second loop. The complete open reading frame of human CD133 (accession number AY449689) was amplified by PCR from a colon cancer cell line and cloned into pcDNA3.1-V5-His-TOPO vector (Invitrogen) introducing a stop-codon to exclude the vector-encoded C-terminal tag. To show that CD133 is normally glycosylated, 293T cells were transfected with pcDNA3.1-CD133-V5-HIS-TOPO and lysates of these cells were treated with PNGaseF to remove N-linked glycans from the protein. Treatment with PNGaseF reduced the apparent molecular weight of the CD133 approximately 15-20 kD, confirming that CD133 is normally highly glycosylated, as has been published earlier[21, 23]. **B** All 8 predicted N-glycosoylation sites of the mature protein (accession number O43490) were mutated into alanine. Mutations were performed using the Quick-Change II site-directed mutagenesis kit (Stratagene) according to manufacturers' instructions. All final constructs were verified by sequencing. Mutated CD133 was transfected into 293T cells and lysates were loaded on a SDS-PAGE. Detection was performed with AC133, W6B3C1 and a His antibody. Mutation of single site affected mobility in the gel, but not binding of the CD133 antibodies, indicating that antibody recognition does not depend on glycosylation.