Cell Cycle–Dependent Variation of a CD133 Epitope in Human Embryonic Stem Cell, Colon Cancer, and Melanoma Cell Lines

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Abstract

CD133 (Prominin1) is a pentaspan transmembrane glycoprotein expressed in several stem cell populations and cancers. Reactivity with an antibody (AC133) to a glycoslyated form of CD133 has been widely used for the enrichment of cells with tumor-initiating activity in xenograph transplantation assays. We have found by fluorescence-activated cell sorting that increased AC133 reactivity in human embryonic stem cells, colon cancer, and melanoma cells is correlated with increased DNA content and, reciprocally, that the least reactive cells are in the G_1-G_0 portion of the cell cycle. Continued cultivation of cells sorted on the basis of high and low AC133 reactivity results in a normalization of the cell reactivity profiles, indicating that cells with low AC133 reactivity can generate highly reactive cells as they resume proliferation. The association of AC133 with actively cycling cells may contribute to the basis for enrichment for tumor-initiating activity. [Cancer Res 2008;68(19):7882–6]

Introduction

Tumors may be composed of a hierarchy of cells in which only a subset is responsible for self renewal, while the remainder may not be tumorgenic. Putative cancer stem cells (CSC) have been identified in multiple types of human cancers by their ability to initiate tumors in immune compromised mice (1). However, some tumor cells that do not express CD133 are capable of self-renewal and are tumorigenic (2–4), and not all human tumor cell lines that are capable of generating tumors, at low cell numbers, are AC133 positive. Nevertheless, markers that allow enrichment for CSCs from whole tumor tissues are essential for the purification, characterization, and eventual targeting of CSCs. A very specific antibody designated AC133 (5) against a glycosylated form of the cell surface protein CD133 (Prominin1) has been widely used to enrich for CSC (6). Reaction with the AC133 antibody (Miltenyi Biotech) is not identical with CD133 protein detection but rather seems to be due to a glycosylated form of membrane-associated CD133 (6). The AC133 epitope is expressed on some human stem and progenitor cells but is not present on mouse cells (6). Cells that react with AC133 are reported to be more likely to form tumors in transplantation tests than cells that are negative (7–10). AC133 reaction has been used to enrich for cells with tumor-initiating activity from human brain tumors, colon cancers, and prostate cancer (7–9).

We have found that in culture, AC133 reactivity is correlated with the cell cycle DNA profile of colon cancer, melanoma, and human embryonic stem cells. In some cell types, differential AC133 expression may more accurately reflect cycling cells rather than a differentially expressed stable stem cell lineage marker.

Materials and Methods

Cell culture. The human colon epithelial cancer cell line Caco2 was obtained from American Type Culture Collection. Caco2 cells at passage 10 were infected with lentivirus reporter vectors that contain the mouse maternal embryonic leucine zipper kinase (MELK) promoter driving enhancer green fluorescent protein (MELK-GFP; ref. 11) or a control PGK promoter-driven H2B-GFP vector. Individual clones were isolated, and two of the clones were used for further experiments. Caco2 cells were cultured as suggested by the supplier. Caco2 cells were cultured 2 d (subconfluent), 3 d (confluent), and 14 d (postconfluent) to generate differentiated cells. The melanoma cell line WM115, provided by Boris Fichtman and Zeev Ronai (Burnham Institute for Medical Research), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum. The H9 hES cells were provided by Brandon Nelson and Mark Mercola (Burnham Institute for Medical Research). They were cultivated with mouse embryo fibroblast feeder cell conditioned medium supplemented with basic fibroblast growth factor, as described (12). The undifferentiated state of the hES cells was routinely monitored by staining for Oct4 and other hES markers.

Immunocytochemistry. Single-cell suspensions were applied to glass slides with a Shandon Cytospin 3 centrifuge at 500 rpm for 5 min. The cells were fixed for 5 min in 2% paraformaldehyde (Sigma-Aldrich) at room temperature, washed twice in PBS, and then blocked with 1.5% normal goat serum for 1 h at room temperature. The cells were incubated with a 1:10 dilution of the primary antibody (anti–CD133-PE, AC133, Miltenyi Biotec) at 37°C for 1 h and subsequently washed twice with 0.1% Tween 20 (Sigma-Aldrich) in PBS and twice with PBS. The primary antibody was detected with Alexa 568–conjugated goat anti-mouse IgG 1:100 (Invitrogen), and nuclei were stained with 4′,6-diamidino-2-phenylindole. The stained cells were mounted in Vectashield mounting medium (VECTOR).

Flow cytometry analysis. AC133 reaction was identified by direct immunofluorescent staining using the AC133 mouse monoclonal antibody directly conjugated with phycoerythrin. All cells were stained according to manufacturer's recommendations. In brief, 2×10^5 live cells were suspended in 100 μL of buffer (0.5% FCS and 2 mmol/L EDTA) and stained for 10 min at 4°C with 10 μL of the AC133 antibody (1:11). Cells were analyzed for phycoerythrin and GFP expression by flow cytometry on a FACSort cytometer (Becton Dickinson). Ten thousand events were acquired and analyzed using FlowJo software.

Cell cycle analysis. DNA in MELK-GFP expressing Caco2 cells was stained using Hoechst 33342 (Invitrogen), whereas WM115 and hES cells were stained with Draq5 (Biostatus Ltd.). For the Hoechst 33342 staining, 2×10^5 to 3×10^5 cells, previously stained for AC133, were washed 1× in wash buffer (0.5% FCS and 2 mmol/L EDTA in PBS). Cells were resuspended in 250 μL of culture media (DMEM), and Hoechst 33342 was added to a final concentration of 15 μg/mL. Cells were incubated at 37°C for 90 min. For the Draq5 staining, 1×10^5 cells, previously stained for AC133, were washed 1× in wash buffer. The cells were resuspended in DMEM and Draq5 at a final concentration of 10 μmol/L. Cells stained with Hoechst 33342 were analyzed on a FACSDiVa flow cytometer (Becton Dickinson), and cells

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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stained with Draq5 were analyzed on a FACSort cytometer (Becton Dickinson). All fluorescence-activated cell sorting (FACS) data were analyzed using FlowJo software.

FACSort. For sorting cells expressing AC133, the cells were removed from the culture dish with 0.05% trypsin and 0.02% EDTA (Invitrogen), washed in PBS containing 1% FCS, stained as described above, and resuspended at 10^6 cells/mL in the same buffer. The cells were filtered through a 35-μm nylon filter before FACSort. Sorting was performed on a FACSDiVa flow cytometer (Becton Dickinson). Side and forward scatter profiles and propidium iodide staining were used to eliminate cell doublets and dead cells. The top 10% of the AC133-reactive cells and the AC133-negative cells were collected. An aliquot was removed at the end of the sort and reanalyzed to evaluate purity.

Colony formation assay. Caco2 cells sorted on AC133 reactivity were cultured in 24-well plates at concentrations of 100, 300, 1,000, and 5,000 cells per well in triplicates. After 7 d, the cells were fixed in methanol, stained with giemsa stain, and counted with a dissection microscope at 10× magnification.

Proliferation assay. AC133 high-sorted and negative-sorted cells were plated at a concentration of 50, 500, and 5,000 cells per well in duplicate. Cells were cultured for 1, 2, or 3 d. The cells were washed 2× in PBS before they were frozen at −70°C in the 96-well plate. The CyQUANT Cell Proliferation Assay kit (Invitrogen) was used according to manufacturer's instruction.

Microarray analysis. Total RNA from AC133 high-sorted and negative-sorted Caco2 and WM115 cells was extracted using the TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. Two samples each of two cell lines were analyzed as biological duplicates. Labeled cRNA was prepared from 500 ng RNA using the Illumina RNA Amplification kit from Ambion. The biotin-labeled cRNA (750 ng) was hybridized 18 h at 58°C to the HumanRef-8 v2 Expression BeadChip (>22,000 gene transcripts; Illumina) according to the manufacturer's instructions. BeadChips were scanned with an Illumina BeadArray Reader, and hybridization efficiency was monitored using BeadStudio software (Illumina). BeadStudio software was used for the normalization and quality control of the data. To identify statistically significant changes, the data were evaluated by GeneSpring software. A volcano plot was used to identify genes with changed at least 2-fold and had reproducibility P values of 0.05 or less. The list of genes passing these thresholds was compared with publicly available data using the Nextbio search engine. Complete primary data are available through the Gene Expression Omnibus support by the National Center for Biotechnology Information as GEO accession number GSE11757.

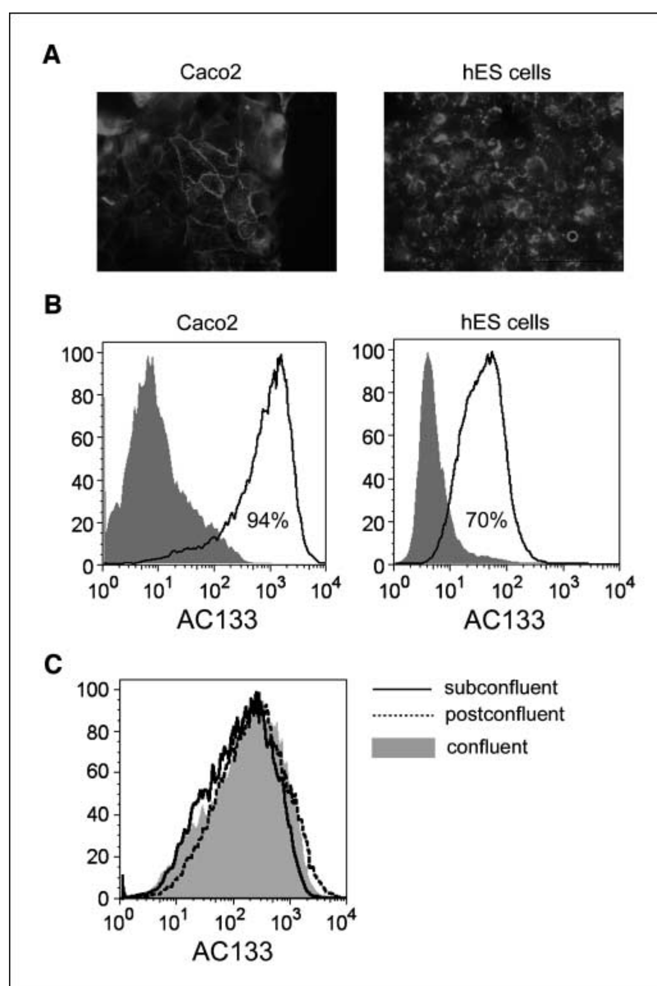

Figure 1. AC133 expression in Caco2 and hES cells. *A,* immunofluorescent analysis of AC133 staining in Caco2 colon cancer cells and cytospin preparation of H9 hES cells (40× magnification). *Scale bar,* 100 μm. *B,* flow cytometry analysis of AC133 staining on live cells (*gray,* negative controls). *C,* AC133 expression on confluent, subconfluent, and postconfluent Caco2 cells.

Results and Discussion

AC133 expression in Caco2 and hES cells. A screen of seven human cell lines for AC133 expression revealed three that were positive. We did not detect reaction with MDA-MB231, MCF7, Du145, or U87 cells. FACS analysis and immunofluorescence staining of Caco2 and H9 hES cells detected high levels of the AC133 epitope (Fig. 1). Ninety-four percent and 70% of Caco2 and hES cells, respectively, showed positive staining. These results confirm previous reports of AC133 reactivity on Caco2 (13) and hES cells (14). The human melanoma cell line WM115 was also positive (15). Interestingly, immunoreactivity for the AC133 antigen, but not CD133 mRNA level, is reported to be down-regulated upon differentiation of Caco2 cells for 40 days (13). In our study we did not see a significant difference in AC133 expression in subconfluent, confluent, and 14-day postconfluent cells (Fig. 1*C*).

AC133 expression and DNA profile in subpopulations. FACS analysis of the DNA contents of AC133-reactive cells revealed that cells with greatest AC133 reaction were enriched for cells with DNA content of 4N or even greater, in the case of hES cells (Fig. 2). When equal subpopulations of $AC133^+$ cells (Fig. 2*A*) were analyzed for DNA content, the fraction of cells with 4N DNA content or greater was found to correlate with increasing AC133 reactivity, whereas the fraction of cells with 2N DNA (G_1 and G_0 portion of the cell cycle) was inversely correlated with AC133 reaction (Fig. 2*B*). In cells with the least AC133 binding, almost 70% of the cells contained 2N DNA content compared with only 20% of the cells with highest levels of AC133 (Fig. 2*C, left*). Similar results were found for subcloned populations of Caco2 cells. Eighty percent of the cells that expressed the lowest levels of AC133 showed a DNA profile similar to cells with 2N DNA content compared with only 14% of the cells expressing the highest levels of AC133. These results were also confirmed by the AC133 expression in the melanoma cell line WM115. Figure 2*D* summarizes the results from all three cell lines. Cell cycle profiles on cells gated on the highest 10% and the lowest 10% of AC133 expression are shown.

Expression of MELK protein is cell cycle–dependent (16). We compared the expression of a MELK-GFP reporter gene with its corresponding DNA content profile. The expression of the GFP protein from a MELK-GFP reporter gene in cloned Caco2 cells has the same correlation with DNA content as the AC133 antigen (Fig. 2*C, right*). These data are consistent with previous reports that

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CD133+ cells have a much higher expression of MELK mRNA compared with autologous CD133-negative tumor cells from glioblastoma patients. Grskovic and colleagues documented that CD133+ cells are more mitotically active than CD133− cells after the first week of cultivation (17, 18).

Gene expression in AC133 high-sorted and negative-sorted cells. The gene expression profiles for AC133 high versus negative-sorted Caco2 and WM115 cells were compared. Figure 3*A* and *B* shows scatter plots from the obtained microarray data with a fold difference of 2 or more. The r^2 values (r^2 = 0.9859 and r^2 = 0.9842) indicate high similarity in gene expression between cells sorted on extremes of AC133. An unsupervised cluster analysis of individual-gene expression data sets did not distinguish AC133-reactive cells (Fig. 3*C*). Noteworthy, the most striking difference was the up-regulated expression of Prominin1 in AC133 high expressing cells, 15× and 9× for Caco2 and WM115, respectively. This was the only gene that was differentially expressed in both cell lines. This indicates that AC133 reaction correlates well with Prominin1 RNA. A total of only 39 Caco2 RNAs and 7 WM115 RNAs (Supplementary Table S1) were significantly different (≥2-fold and reproducibility; P < 0.05) between AC133 high and negative cell fractions.

Cultivation of high-sorted and negative-sorted cells. To determine the stability of AC133 antigen expression, high and negative AC133 sorted Caco2 cells were cultivated. A colony formation assay showed a significant difference in colony formation frequency between the two sorted cell populations (P < 0.05; Fig. 4*A*). However, although the AC133-negative cells were less likely to grow from single cells, the proliferation rate for the two cell populations were not significantly different (P > 0.05; Fig. 4*B*). After a few passages, AC133-negative cells expressed similar levels of AC133 as the starting population (Fig. 4*C*). Similarly, cells sorted on high expression of AC133 generated cells with less expression (Fig. 4*C*). Hence, continued culturing of cells with extremes of AC133 reactivity leads to redistribution of the degree of reaction. Also, the morphology of the colonies formed after cultivation is very similar between the two different subpopulations (Fig. 4*D*). Chang and colleagues (19) have recently showed similar results with another stem cell marker, Sca-1. In clonal populations of mouse hematopoietic progenitor cells, they found that spontaneous "outlier" cells with either extremely high or low expression levels of Sca-1 reconstitute the parental distribution of Sca-1 after 1 week. These extremes of Sca-1 expression were associated with differential gene expression consistent with increased probability of differentiation along two different lineages. However, in our study, the high and negative expressing sorted cells do not seem to belong to two distinct cell populations based on gene expression profiles. The AC133 epitope seems not to be a stable marker for a particular population of cells but rather may reflect mitotic activity. Cell cycle dependence might be one of the metastable variables contributing to transcriptome variation (15).

The cell cycle–associated reactivity of AC133 is similar to the cell cycle–dependent expression of CD34, a hematopoietic

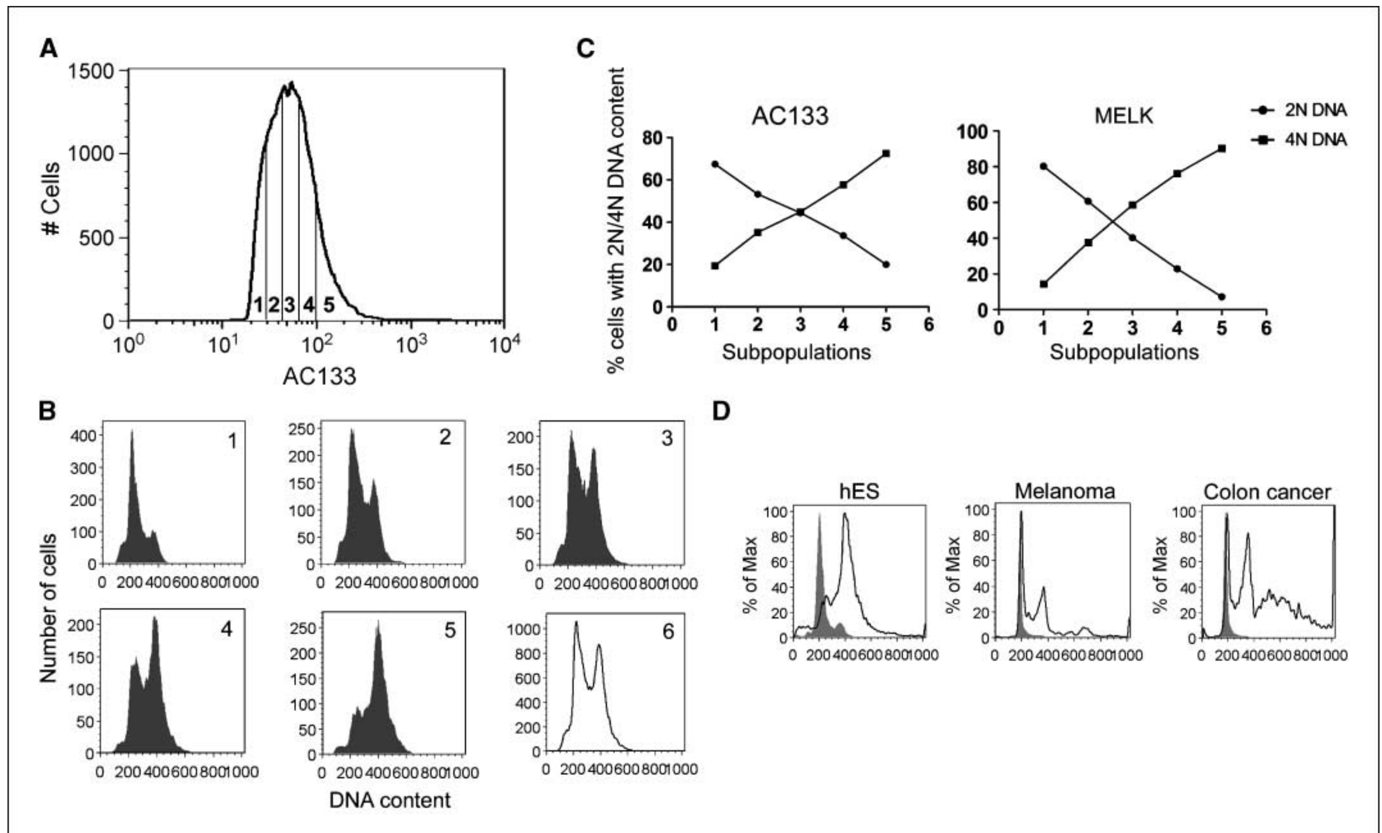

Figure 2. Correlation of AC133 reaction and DNA content. *A,* the AC133-reactive populations of hES cells were divided into five groups (1–5), each representing 20% of the total population. *B,* DNA contents for the individual fractions of AC133-reactive cells. Graph 6 shows the cell cycle profile for all AC133-positive cells. *C,* graphs show the percentage of cells with 2N DNA content and cells with 4N DNA content for each subpopulation of cells with AC133 reactivity and cells with MELK-GFP expression. *D,* DNA content profiles for cells gated on the highest 10% (*black line, no fill*) and the lowest 10% (*gray fill*) of AC133 expression in three different cell lines, hES cells, melanoma cells WM115, and colon cancer Caco2 cells.

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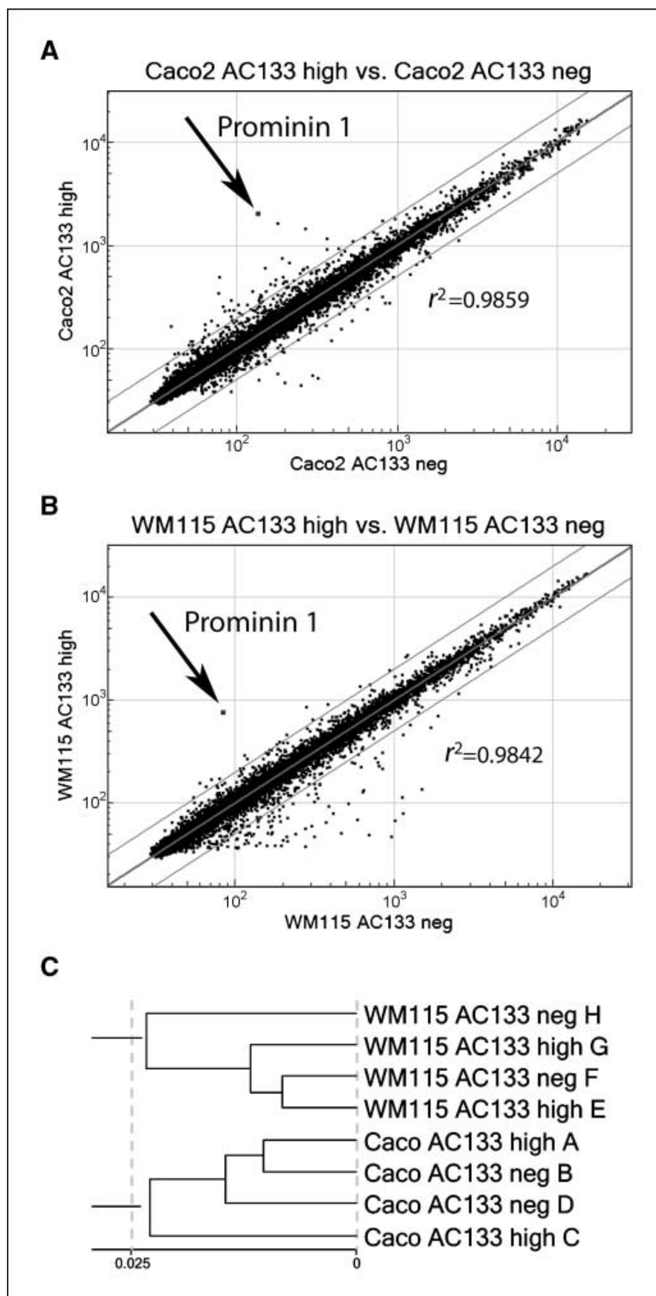

Figure 3. Gene expression in AC133 high versus negative cells. *A* and *B,* scatter plots of AC133 high versus negative cells for Caco2 and WM115 cells, respectively. Fold changes of ≥2 are indicated above and below the lines parallel to the diagonal. Not all 2× changes are statistically significant. *C,* cluster analysis of microarray data. Gene expression in AC133 high-sorted and negative-sorted Caco2 (sample A-D) and WM115 (sample E-H). The Pearson correlation using a 1 − *r* distance measure was applied.

progenitor marker that is also commonly detected by a glycosylation-dependent epitope. CD34 expression has been a valuable tool for identification and purification of human hematopoietic stem cells. However, CD34 expression in mice is not required for survival, and murine hematopoiesis could be reconstituted by $CD34^-$ cells (20). Recently, Dooley and colleagues (21) showed that CD34 expression increased as $CD34^-$ cells shifted from quiescence to proliferation. Cultured $CD34^-$ cells up-regulate CD34 antigen expression in as little as 42 hours, and $CD34^+$ precursors lost expression in culture if they remained in G_0 for >2 days.

Tumor-initiating cells with stem-like characteristics might have differential resistance to chemotherapy or radiotherapy. $CD133^+$ glioblastoma cells are significantly more resistant to conventional chemotherapeutic agents, and resistance is correlated with higher expression of survivin, an antiapoptotic acting protein in $CD133^+$ cells (18, 22). However, survivin expression is cell cycle–dependent, increasing in the G_2-M phase of the cell cycle followed by a rapid

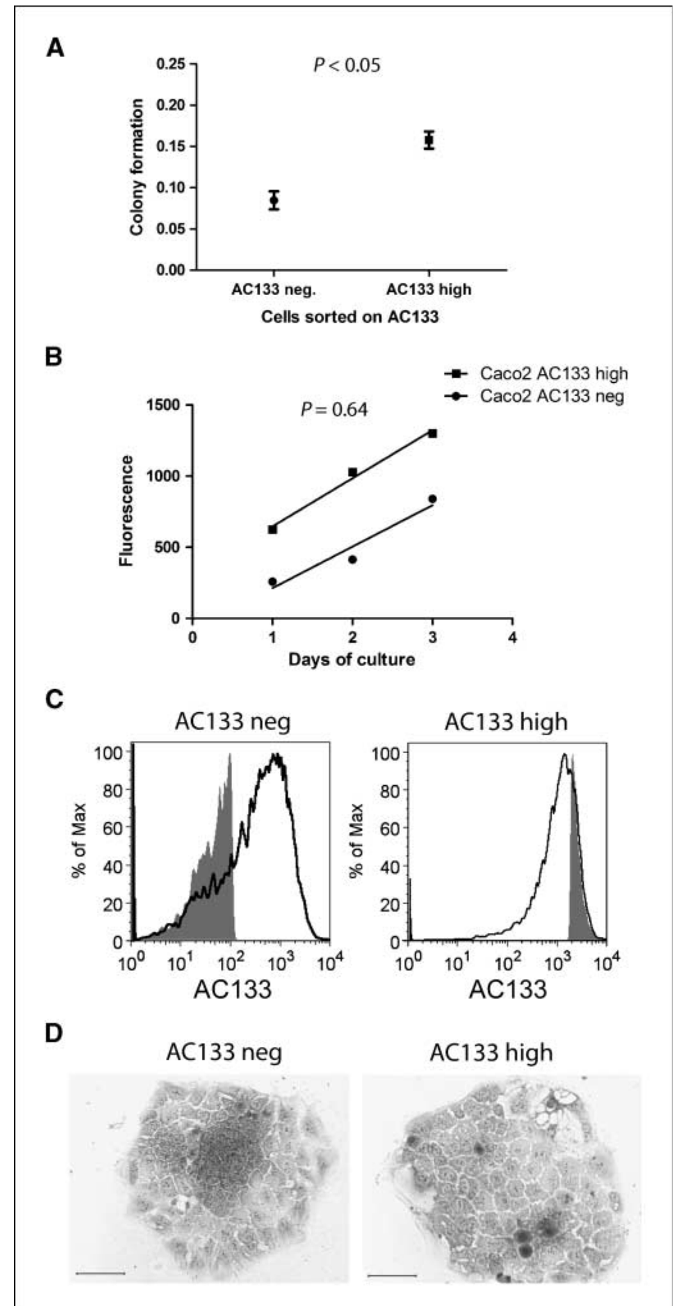

Figure 4. Colony formation, proliferation, and AC133 expression in AC133 high-sorted and negative-sorted cells. *A,* colony formation of AC133 high-sorted and negative-sorted Caco2 cells [colony forming units (*cfu*)/seeded cells]. *B,* proliferation rate in AC133 high-sorted and negative-sorted Caco2 cells. *C,* AC133 expression in sorted Caco2 cells before and after cultivation of negative-sorted and high-sorted cells, respectively. *Gray fill,* AC133 expression of the sorted cells; *black lines,* AC133 expression after three passages of cultivation. *D,* morphology of AC133 high-sorted and negative-sorted Caco2 cells after 7 d of cultivation (20× magnification). *Scale bar,* 100 μm.

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decline in the G_1 phase. The selection of AC133-reactive cells in this system might also be expected to enrich for cells with high survivin expression and, thus, increased resistance to apoptotic agents.

Primary human cancers are commonly heterogeneous with both host and tumor-related components. The proportion of actively proliferating cancer cells varies greatly, depending on the tumor and its progression. The general strategy of targeting proliferative cells of cancers is being challenged by the CSC model that may include extrapolations from the behavior of slow cycling normal stem cells. However, the essential defining characteristic of a CSC, supported by the biology of teratocarcinoma and certain leukemias, is the directional, moderating influence of differentiation to a stable benign state. AC133 reactivity may be used for enrichment for tumor-initiating activity without necessarily supporting a CSC theory.

To summarize, our study indicates that within three different cell types that express AC133, the antigen expression is highest in cells with 4N DNA content and lowest in cells with 2N DNA. This is consistent with higher expression in actively proliferating cells and low expression in cells in G_1 or G_0. We show that cultivation of cells with the extremes of AC133 reactivity resulted in a redistribution of the antigen expression, which suggests that high and low expressing cells do not belong to stable distinct populations. AC133 reactivity may be valuable to identify cells with increased tumorgenicity. However, the basis of this utility may be due to the distinction between proliferative and quiescent cells and should be used cautiously as a putative marker of a stable, distinct stem cell–like population.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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