

Aldehyde Dehydrogenase 1 Is a Marker for Normal and Malignant Human Colonic Stem Cells (SC) and Tracks SC Overpopulation during Colon Tumorigenesis

Emina H. Huang,^{1,2,3} Mark J. Hynes,² Tao Zhang,^{4,6} Christophe Ginestier,³ Gabriela Dontu,³ Henry Appelman,³ Jeremy Z. Fields,⁷ Max S. Wicha,³ and Bruce M. Boman^{3,4,5,6}

¹Department of Surgery, University of Florida, Gainesville, Florida; ²Department of Surgery, University of Michigan; ³Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan; ⁴Department of Biologic Sciences, University of Delaware; ⁵Helen F. Graham Cancer Center, Christiana Care Health System, Newark, Delaware; ⁶Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania; and ⁷CA²TX Biotechnology, Inc., Gladwyne, Pennsylvania

Abstract

Although the concept that cancers originate from stem cells (SC) is becoming scientifically accepted, mechanisms by which SC contribute to tumor initiation and progression are largely unknown. For colorectal cancer (CRC), investigation of this problem has been hindered by a paucity of specific markers for identification and isolation of SC from normal and malignant colon. Accordingly, aldehyde dehydrogenase 1 (ALDH1) was investigated as a possible marker for identifying colonic SC and for tracking them during cancer progression. Immunostaining showed that ALDH1⁺ cells are sparse and limited to the normal crypt bottom, where SCs reside. During progression from normal epithelium to mutant (*APC*) epithelium to adenoma, ALDH1⁺ cells increased in number and became distributed farther up the crypt. CD133⁺ and CD44⁺ cells, which are more numerous and broadly distributed in normal crypts, showed similar changes during tumorigenesis. Flow cytometric isolation of cancer cells based on enzymatic activity of ALDH (Aldefluor assay) and implantation of these cells in nonobese diabetic–severe combined immunodeficient mice (*a*) generated xenograft tumors (Aldefluor[−] cells did not), (*b*) generated them after implanting as few as 25 cells, and (*c*) generated them dose dependently. Further isolation of cancer cells using a second marker (CD44⁺ or CD133⁺ serially) only modestly increased enrichment based on tumor-initiating ability. Thus, ALDH1 seems to be a specific marker for identifying, isolating, and tracking human colonic SC during CRC development. These findings also support our original hypothesis, derived previously from mathematical modeling of crypt dynamics, that progressive colonic SC overpopulation occurs during colon tumorigenesis and drives CRC development. [Cancer Res 2009;69(8):3382–9]

Introduction

In the cancer stem cell (SC) paradigm, cancers originate from uncommon cells—SC—that show pluripotency and self-renewal (1). Cancer SC were first identified in leukemias and more recently in solid tumors. The latter includes brain cancers where the

transmembrane protein CD133 (Prominin-1 or AC133) was used to identify and isolate SC. As few as 100 CD133⁺ cells implanted in the forebrain of a nonobese diabetic–severe combined immunodeficient (NOD-SCID) mouse could reconstitute the tumor, and isolation of SC from this new tumor could be serially passed to other NOD-SCID mice (2, 3). Subsequently, CD133 was used to isolate SC from a host of other normal and cancerous tissues. Another transmembrane protein, CD44 (hyaluronate receptor or P-glycoprotein 1), has also been used as a marker for SC in solid tumors. For example, breast cancer cells isolated using CD44 in combination with other markers generated xenograft tumor growth (4).

Mounting evidence suggests that the cancer SC concept is also relevant to colorectal cancer (CRC; ref. 5). However, a long-standing problem has been the paucity of specific markers with which to identify and isolate both normal and malignant colonic SC and to investigate their involvement in tumorigenesis. Both CD133 and CD44 have been used to isolate colon cancer SC, but the specificity of these two markers for colonic SC is uncertain. For example, Ricci-Vitiani and colleagues (6) and O'Brien and colleagues (7) reported that the CD133⁺ subpopulation of cells in CRCs could initiate xenograft tumors. However, it was subsequently reported that CD133 expression is not specific to SC in the colon, and both CD133⁺ and CD133[−] metastatic colon cancer cells can initiate xenograft tumors (8). Another recent study by one of us (E.H.) used CD44 in combination with the epithelial marker epidermal surface antigen (ESA) to identify and isolate cells from colon cancers that initiated xenograft tumors (9). However, immunohistochemical analysis of normal colonic epithelium shows that CD44 expression occurs not only in cells at the crypt bottom, where SC reside, but also in cells within the proliferative compartment, where rapidly proliferating cells reside (10–14). Thus, the specificity of CD44 and CD133 for colonic SC remains to be determined and additional SC markers specific for colonic SC need to be discovered.

A promising new marker for cancer SC is aldehyde dehydrogenase 1 (ALDH1). ALDH is a detoxifying enzyme that oxidizes intracellular aldehydes and thereby confers resistance to alkylating agents (15–18). In fact, the detoxification capacity of ALDH, by protecting SC against oxidative insult, might underlie the well-recognized longevity of SC. ALDH also converts retinol to retinoic acid, a modulator of cell proliferation, which may also modulate SC proliferation.

The enzymatic activity of ALDH has been used to isolate SC subpopulations. Jones and colleagues (19) described this method by measuring ALDH activity in cells using dansyl aminoacetaldehyde and used ALDH activity to isolate hematopoietic precursors. Subsequently, it was shown that as few as 10 ALDH⁺ hematopoietic

Requests for reprints: Bruce M. Boman, Cancer Genetics and Stem Cell Biology, Center for Translational Cancer Research, Helen F. Graham Cancer Center, University of Delaware, Christiana Care Health System, 4701 Ogletown-Stanton Road, Suite 1205B, Newark, DE 19713. Phone: 302-623-4517; Fax: 302-623-4554; E-mail: brboman@christianacare.org.

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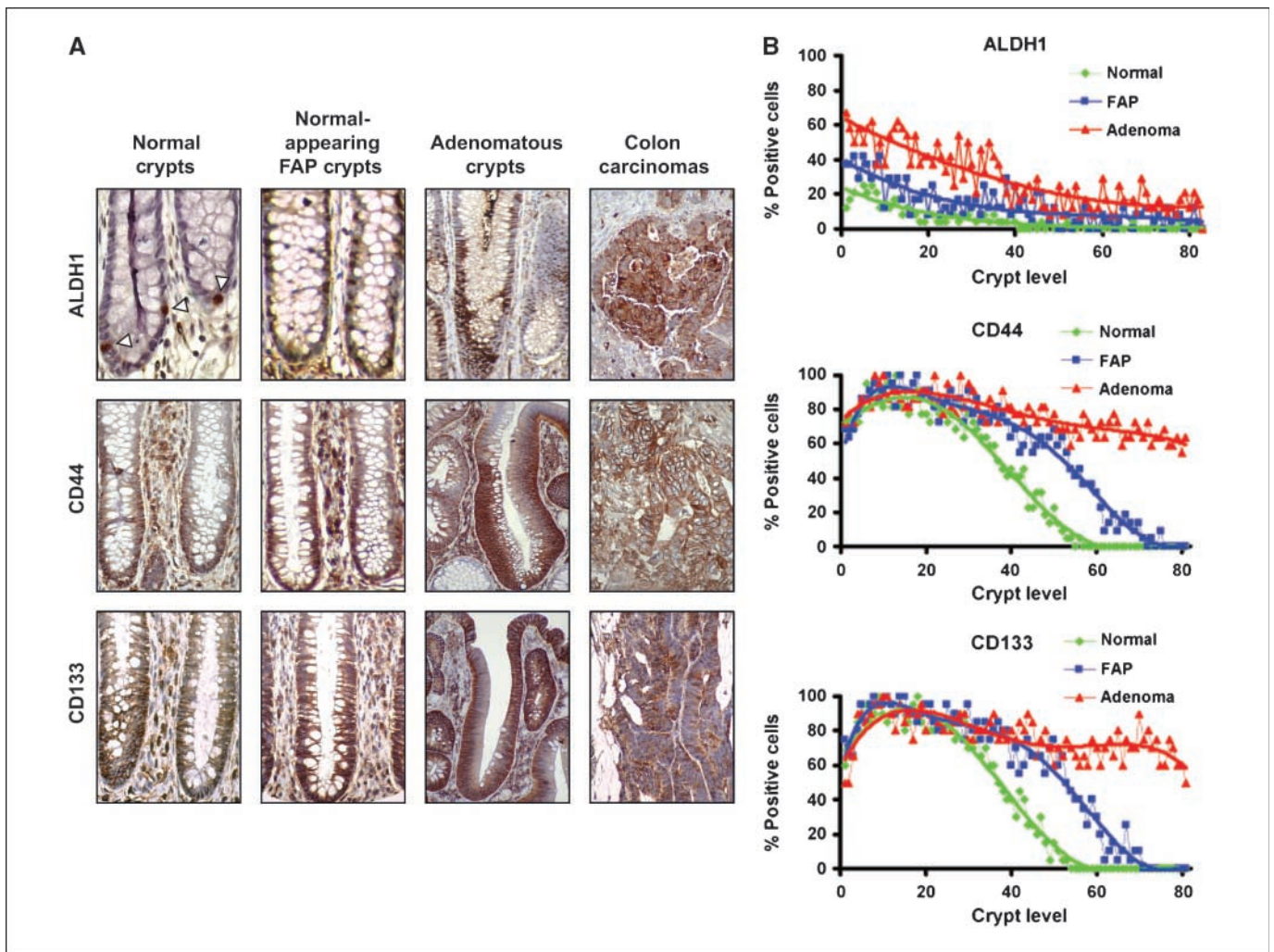


Figure 1. ALDH1, CD44, and CD133 expression in colonic tissues. *A*, immunohistochemical analysis of normal colonic mucosa, normal-appearing colonic mucosa from FAP patients, adenomas, and colon carcinomas. *B*, staining indices for ALDH1, CD44, and CD133. Although CD44 and CD133 profiles were similar, they were considerably different than ALDH1. AUCs for CD44, CD133, and ALDH1 profiles were as follows: normal crypts, 40.3%, 41%, and 5.6%; normal-appearing FAP crypts, 55.7%, 56.7%, and 14%; and adenomatous crypts, 76.9%, 75.9%, and 29.3%.

cells were capable of reconstituting bone marrow in sublethal-irradiated rodents (20). Corti and colleagues (21) showed that ALDH⁺ cells isolated from murine brain were capable of self-renewal and of differentiating into multiple lineages. Further studies by members of our research team (C.G., G.D., and M.S.W.) showed that ALDH1 is a specific marker for breast cancer SC (22).

Accordingly, ALDH was investigated as a specific marker for identifying and isolating normal and malignant human colonic SC and as a way to quantify the number of SC cells over the course of colon cancer development. Moreover, showing an increased number of SC in colonic tumors would validate our original SC overpopulation hypothesis (23–27). This hypothesis was originally developed from our mathematical modeling of colon tumorigenesis (26), in which SC overpopulation was found to be the key event at the cellular level that links the initiating molecular event (typically an *APC* mutation) to the earliest tissue abnormality, a proliferative change in mutant colonic crypts of familial adenomatous polyposis (FAP) patients. SC overpopulation not only initiates colon tumorigenesis but also drives tumor growth (5, 27). Our earlier report (28) using markers for crypt base cells (a SC-enriched subpopulation) provided biological evidence

in support of our SC overpopulation hypothesis. The current study extends that work by studying ALDH as a possible specific marker for colonic SC and for tracking these cells during tumor development.

Materials and Methods

Experimental design. This study used (a) immunostaining to identify putative colonic SC within normal colonic epithelium, normal-appearing colonic epithelium of FAP patients, colonic adenomas, and colon carcinomas; (b) flow cytometry to enumerate and isolate SC from fresh colonic tissues; and (c) xenograft tumor formation in NOD-SCID mice to functionally validate ALDH1, CD44, and CD133 as SC markers.

Isolation of epithelial cells from fresh human tissue and sorting by flow cytometry. Approval for using human tissues was obtained from the University of Michigan Institutional Review Board. Tissue (~1 cm³) was provided by the Tissue Procurement Core pathologist staff. Tissues were minced and dissociated with collagenase (1 mg/mL; type 4; Worthington Biochemical). Flow cytometry used a fluorescence-activated cell sorter [(FACS) FACSria, BD Immunocytometry Systems]. Side-scatter and forward-scatter profiles were used to eliminate cell doublets. Nonviable cells were eliminated using the viability dye 4,6-diamidino-2-phenylindole

(DAPI). ESA was used to obtain carcinoma cell-enriched preparations by eliminating stromal cells (29). For CD44, CD133, and ESA identification, allophycocyanin-coupled CD44 antibody (BD Pharmingen), allophycocyanin-coupled CD133 antibody (Miltenyi Biotec), and phycoerythrin-coupled ESA antibody (Biomedica) were used (1:40 dilution). Nonspecific binding was blocked by Sandoglobulin (1 mg/mL; Sandoz). Controls included the secondary antibody alone. For identification of ALDH⁺ cells, Aldefluor kits (Stem Cell Technologies), which report ALDH enzymatic activity, were used.

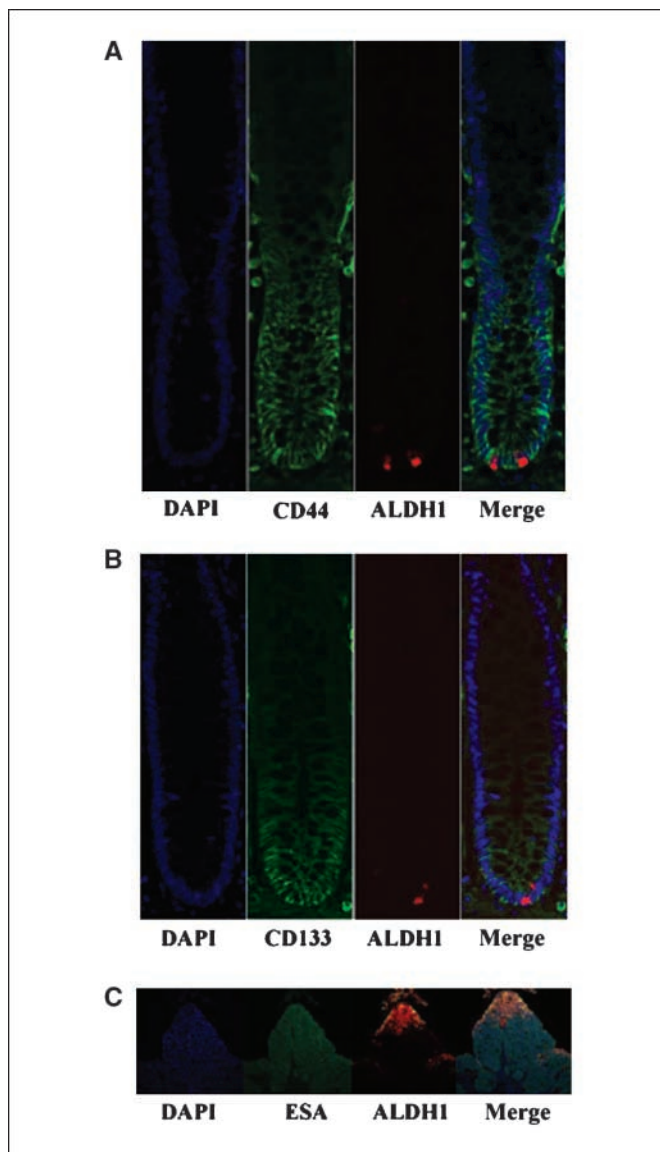


Figure 2. Coexpression of SC markers in normal and malignant colonic tissues. *A*, immunofluorescence staining of normal colonic epithelium shows nuclear staining (DAPI), membranous CD44 expression (green, FITC), and cytoplasmic ALDH1 expression (red, phycoerythrin). The merged image shows a pink-orange color from the overlap of membranous CD44 and cytoplasmic ALDH1 staining, showing that the ALDH1⁺ population is a subset of the CD44⁺ population. *B*, staining of normal colonic epithelium shows nuclear staining (DAPI), membranous CD133 expression (green, FITC), and cytoplasmic ALDH1 expression (red, phycoerythrin). The merged image reveals a pink-orange color showing overlap of membranous CD133 and cytoplasmic ALDH1 staining and showing that the ALDH1⁺ population is a subset of the CD133⁺ population. *C*, staining of an invasive colon carcinoma shows nuclear staining (DAPI), ESA expression (green, FITC), and ALDH1 expression (red, phycoerythrin). The merged image reveals an orange-yellow color that shows overlap of ESA and ALDH1 staining. Doubly positive ESA and ALDH1 cells tended to be present at the invasive front of colonic malignancies.

Methodologic details about these kits are in our previous report (22). Specific ALDH activity is based on the difference between the presence/absence of the Aldefluor inhibitor diethylaminobenzaldehyde (DEAB). With DEAB, Aldefluor expression was <0.01%. On double sorting, cell preparations showed >97% purity.

Immunohistochemistry/immunofluorescence. Immunostaining and antibodies against CD133 (1:25; Miltenyi Biotec), CD44 (1:100; BD Pharmingen), or ALDH1 (1:50; BD Pharmingen) were used. Immunohistochemistry was done as we previously reported (25, 28). For immunofluorescence, slides were incubated with primary antibody and stained with a fluorescently conjugated IgG (1 h; 24°C). Slides were washed and coverslipped (antifade mounting medium for confocal microscopy).

Indexes. The longitudinal distribution of staining along the crypt axis for any given marker—the index—was quantified by counting at each crypt level the number of cells that showed a positive signal and plotting the proportion of cells staining positively versus crypt level. For each marker, 24 to 40 crypt hemiaxes for each of five or more stained slides were counted. Only full-length crypts (~82 crypt levels) from normal and normal-appearing FAP crypts were selected. Because adenomatous crypts have variable length, only ones that had lengths (~82 crypt levels) similar to normal crypts were selected. Graphics and curve fitting (using sixth-order polynomial analysis) used Microsoft Excel (v. 2002). Area under the curve (AUC) for plots was calculated using Prism (GraphPad, Inc.) to determine the proportion of total cells per crypt staining positively.

Xenografting. Cells isolated from human colonic tissues were implanted into the flanks of NOD-SCID mice to analyze their ability to initiate tumor xenografts. Cells that generated xenografts were deemed SC. Xenograft assays were done as we previously described (9). Cell viability was confirmed by trypan blue exclusion. Cells were implanted s.c. with Matrigel (BD Biosciences) at a 1:1 ratio in a total volume of 100 μ L. Animals were euthanized when the tumors reached ~1.0 cm to avoid central tumor necrosis and undue distress. Most studies were done using initial xenografts formed in NOD-SCID mice (rather than xenografts from serial transplantations).

Proportions of cell subpopulations in tissues. To determine marker overlap, cells that were Aldefluor⁺ or Aldefluor⁻ were analyzed for CD44 or CD133. Gates were set to account for background expression, and then proportions of CD44⁺ and CD133⁺ populations were evaluated.

Results

Expression patterns for CD44⁺, CD133⁺, and ALDH1⁺ cells in normal colonic epithelium. Immunohistochemical analysis showed that most CD44⁺ cells reside in the lower half of the normal crypt (Fig. 1A). A similar pattern was seen for CD133. In contrast, ALDH1 positivity was much more limited. Typically, only a few ALDH1⁺ cells (≤ 5) were visualized for each longitudinal crypt section, usually at or near the base of the crypt (Fig. 1A).

Staining indices (Fig. 1B) showed that although CD44⁺ and CD133⁺ profiles were similar to each other, they were considerably different from the profile for ALDH1⁺. In normal crypts, there were ~7-fold more cells that stained positively for CD44 or CD133 than stained for ALDH1 (based on AUC). In addition, CD44⁺ and CD133⁺ cells distributed farther up the crypt into the region known to contain rapidly proliferating cells.

Because, in normal colon, the proportion of ALDH1⁺ cells was smallest among the three markers, two-color immunofluorescence experiments were done to determine if the ALDH1⁺ cell population is a subset of the CD44⁺ and/or the CD133⁺ cell populations. As shown in Fig. 2A, CD44 expression was membranous and limited to the lower half of the crypt. In contrast, ALDH1⁺ cells were few and limited to the base of the crypt. Merged confocal imaging showed ALDH1⁺ cells to be a subset of the CD44⁺ cell population. The ALDH1⁺ population was also a subset of the CD133⁺ population (Fig. 2B).

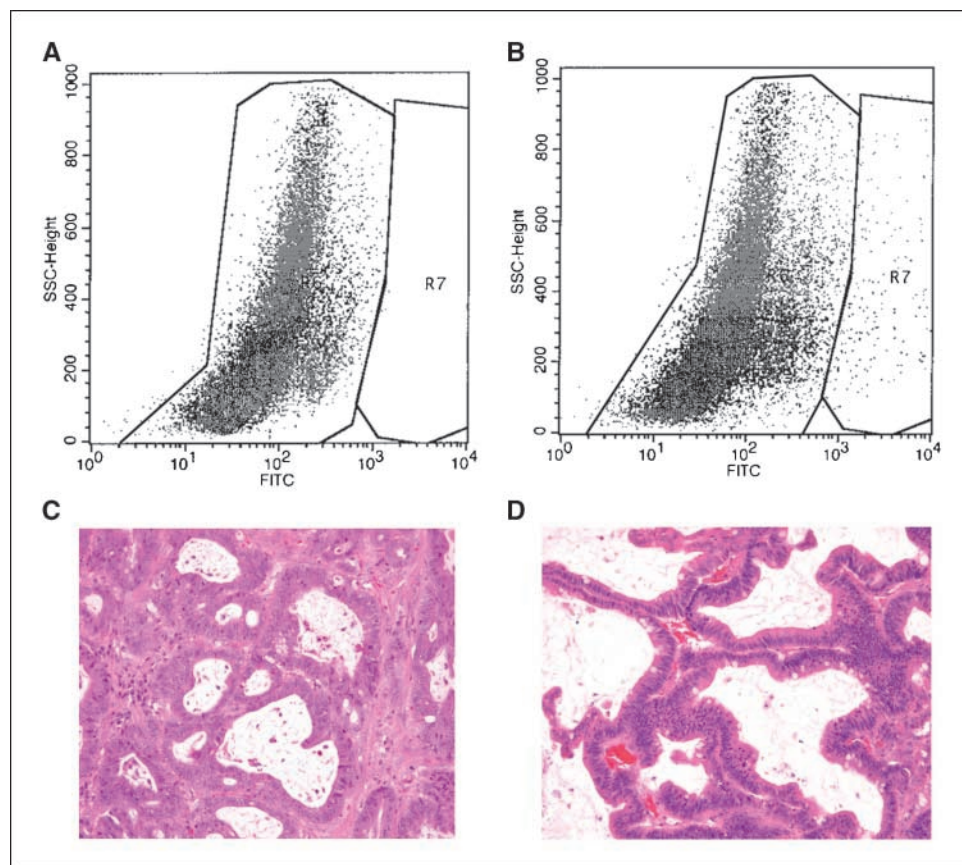


Figure 3. Flow cytometric isolation of cancer-initiating cells based on ALDH enzymatic activity. *A*, FACS histogram showing Aldefluor assay done on human CRC cells in the presence (*A*) and absence (*B*) of ALDH1 enzyme inhibitor. The pattern of background ALDH activity (R7-gated population) was used to select ALDH⁺ cells. *B*, ALDH⁺ cells represent 3.5% (outside R7-gated population of ESA⁺ cells). ESA⁺ selection limited the ALDH⁺ population to carcinoma cells. *C*, H&E stains of primary human CRC (*C*) and the resulting xenograft (*D*) revealing similar histopathology.

Analysis of ALDH1⁺, CD44⁺, and CD133⁺ cells during progression to colon carcinoma. To evaluate changes in SC populations during the stepwise progression to colon cancer, subpopulations of cells expressing these markers was examined, using immunohistochemistry, for normal crypts, normal-appearing FAP crypts, and adenomatous crypts. Both the number of ALDH1⁺ cells and the extent of their distribution along the crypt axis increased with tumor progression (Fig. 1*A* and *B*). In comparison with normal crypts (Fig. 1*B*, *bottom curve*), the number of ALDH1⁺ cells was higher and cells were distributed farther up the crypt axis in normal-appearing crypts from FAP patients (Fig. 1*B*, *middle curve*). These changes were even more pronounced in adenomas (Fig. 1*B*, *top curve*). The percentage of ALDH1⁺ cells in normal crypts was 5.6% (based on AUC); within FAP crypts, 14%; and within adenomatous crypts, 29.3%. Thus, the number of ALDH1⁺ cells increased 5.2-fold with tumor progression.

Changes in CD44⁺ and CD133⁺ subpopulations were also examined. The change in CD44⁺ and CD133⁺ profiles with tumor progression was similar to that for ALDH1⁺, in that for each marker the proportion of positively staining cells increased during tumor progression (normal < FAP < adenoma) and positively staining cells were distributed even farther up the crypt. The percentage of CD133⁺ or CD44⁺ cells in normal crypts was ~40% (based on AUC); in FAP crypts, ~56%; and in adenomatous crypts, ~76%. The number of CD44⁺ or CD133⁺ cells increased 1.9-fold with tumor progression. As for normal crypts, there was a greater number of CD44⁺ and CD133⁺ cells in neoplastic crypts compared with ALDH1⁺. In FAP crypts, there were ~4-fold more CD44⁺ or CD133⁺ cells; in adenomatous crypts, 2.6-fold more.

Staining of colon carcinomas for ALDH1, CD44, and CD133 showed diffuse staining with strongly staining malignant cells being present throughout the tumor (Fig. 1*A*). Because stromal cell populations also express these markers, dual-color immunofluorescence staining was done for each SC marker, coupled with ESA staining to show which cells staining positively for any given SC marker are colon carcinoma cells. Results showed that ALDH1⁺ cells are a subpopulation of the ESA⁺ population (Fig. 2*C*). Similar results were obtained for ESA⁺/CD44⁺ and ESA⁺/CD133⁺ staining (data not shown). These data provide a rationale for using ESA coupled with each SC marker and FACS to isolate preparations of cells from CRCs that are enriched in cancer SC.

Although staining indices for carcinomas were not plotted, as they do not contain recognizable crypt structures, it was found that cells that were positive for both ESA and ALDH1 tended to be present at the invasive front of colonic malignancies (Fig. 2*C*).

Flow cytometric analysis of cells from normal and malignant colonic epithelium. Flow cytometry data for normal colon were consistent with our immunohistochemical data. Within the ESA⁺ population from normal colonic epithelium, the proportion of cells that were ALDH⁺ (i.e., Aldefluor⁺) was $5.4 \pm 3.8\%$. This proportion was considerably lower (4- to 10-fold) than the proportion for CD44 ($49.0 \pm 5.3\%$) or CD133 ($23.1 \pm 6.3\%$). These data for ALDH, CD44, and CD133 were similar to the immunohistochemical data for normal crypts for these same markers (5.6%, 40%, and 40%; Fig. 1). Within the ESA⁺ population, $2.5 \pm 0.3\%$ of normal cells were ALDH⁺/CD44⁺ and $1.7 \pm 0.4\%$ were ALDH⁺/CD133⁺. In other words, 46% of Aldefluor⁺ cells are CD44⁺ and 31% are CD133⁺. Overall, these data indicate that, in normal

Table 1. Description of colon cancer cases used for flow cytometry sorting of ALDH⁺ cells and the number of serial passages of xenograft lines

Case	Gender	Age	Location of tumor	Stage	No. passages*
CCXL 1	Female	52	Ascending	T3N0M0	7/8
CCXL 2	Female	70	Sigmoid	T3N1M0	7/9
CCXL 3	Male	79	Cecum	T3N1M0	9/10
CCXL 4 [†]	Male	75	Sigmoid	T2N0M0	12/12
CCXL 5	Male	41	Descending	T3N1M0	4/5
CCXL 6	Female	67	Ascending	T4N0M0	4/6
CCXL 7 [†]	Female	65	Ascending	T3N1M0	5/5

* Numerator gives the number of passages generated using Aldefluor⁺ cells and the denominator gives the total number of passages to date. All xenograft lines still continue to be passaged.

[†] In these two cases, xenograft tumors were first generated by implanting Aldefluor⁺ cells isolated directly from the primary tumor. In the other five cases, xenograft tumors were initially generated by implanting cells from the primary tumor in a NOD-SCID mouse and then subsequent xenografts were generated serially using Aldefluor⁺ cells isolated from a xenograft tumor.

colonic epithelium, many fewer cells express ALDH than CD44 or CD133.

In colon cancers, the proportions of ALDH⁺, CD44⁺, and CD133⁺ cells were similar to proportions for normal colonic epithelium. Within the ESA⁺ population from colon cancers, the proportion of cells that were ALDH⁺ was $3.5 \pm 1.0\%$. Again, this proportion was considerably lower (5- to 7-fold) than that for CD44 ($19.5 \pm 6.9\%$) or CD133 ($24.8 \pm 6.0\%$).

Ability of cells isolated using SC markers individually to form tumor xenografts. Malignant cells from colon carcinomas were isolated by FACS using ESA and Aldefluor and evaluated for tumor-initiating ability. Figure 3 shows a typical histogram obtained from flow cytometric sorting of Aldefluor⁺ cells and the histopathology of a xenograft tumor that was generated from implantation of Aldefluor⁺ cells. Xenograft tumors were successfully generated using Aldefluor⁺ cells from seven different colon cancer patients (Table 1). Aldefluor⁻ cells did not generate xenografts. In two cases, xenograft tumors were generated by implanting Aldefluor⁺ cells isolated from the primary tumor. Implantation of as few as 100 Aldefluor⁺ cells was capable of tumor initiation. In the other five cases, xenograft tumors were generated using Aldefluor⁺ cells isolated from a xenograft tumor that had

previously been established from a primary colon cancer. In all cases, it was possible to dissociate the xenograft tumor and resort the cells to obtain an Aldefluor⁺ cell population that could be serially passaged as another xenograft tumor, ultimately leading to development of a xenograft tumor line. All xenograft lines were passaged multiple times using Aldefluor⁺ cells (Table 1). Implantation of as few as 25 cells from these serially passaged xenografts was capable of generating another xenograft. All xenograft tumors were adenocarcinomas that typically recapitulated the histologic phenotype of the original primary colon tumor (see Fig. 3). In addition, ALDH⁺ cells from these xenografts produced a flow cytometric profile similar to that for the primary tumor, including a small subpopulation of Aldefluor⁺ cells. Moreover, xenograft tumors were generated in a dose-dependent manner; a larger number of cells tended to decrease the time required for the first appearance of a tumor (Fig. 4A) and increased the proportion of implants forming tumors (Fig. 4B).

CD44 and CD133 were also used individually as markers to isolate cells from the ESA⁺ population from colon carcinomas and these cells were evaluated for tumor-initiation ability. Both CD44⁺ and CD133⁺ subpopulations generated xenograft tumors. CD44⁻ and CD133⁻ cells did not generate xenograft tumors during

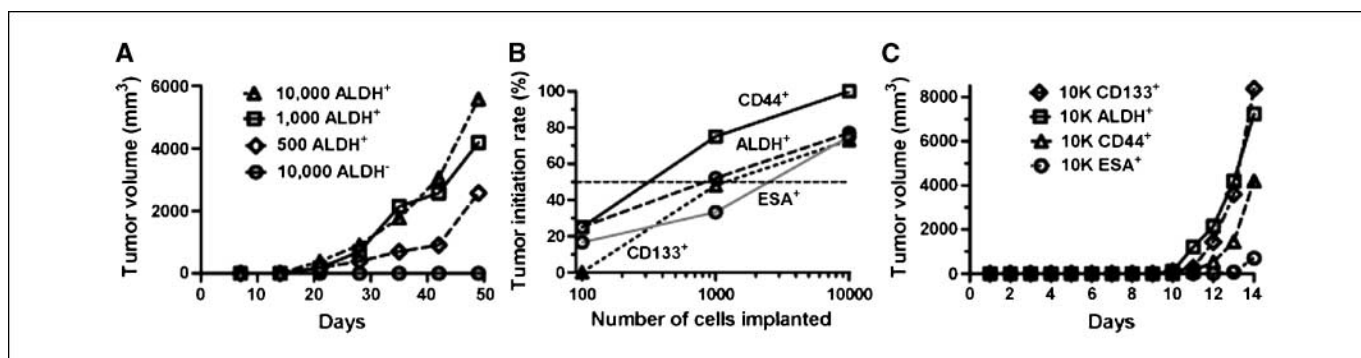


Figure 4. Tumorigenicity of cells isolated using individual SC markers. ESA was used to select carcinoma cells from colon cancers before selection using other markers. Further sorting generated the following additional subpopulations: ALDH⁺ and ALDH⁻ cells, CD44⁺ and CD44⁻ cells, and CD133⁺ and CD133⁻ cells. A, representative experiment showing latency of tumor development after implanting three different doses (500, 1,000, or 10,000) of ALDH⁺ cells or 10,000 ALDH⁻ cells. B, log dose-response curve for generating xenograft tumors. Xenograft tumors were generated by cancer cells obtained from all patient cases ($n = 4$). C, representative experiment showing latency of tumor development after implantation of 10,000 ALDH⁺, CD44⁺, or CD133⁺ cells. All cells, including ESA⁺ alone, were sorted from a single colon cancer.

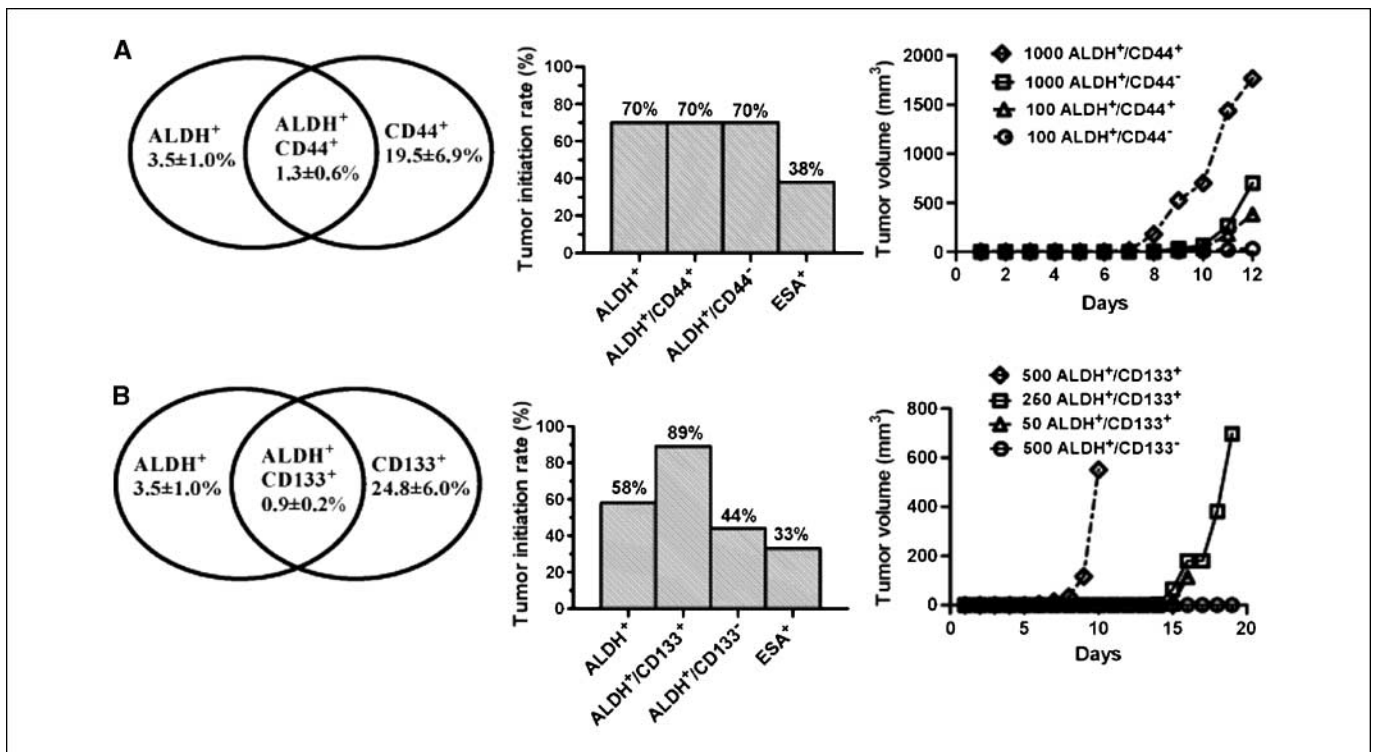


Figure 5. Tumorigenicity of cells isolated using multiple SC markers. ESA was used to select carcinoma cells from colon cancers before selection using other markers. **A**, isolation of cells using ALDH and CD44. Left image gives proportions of ALDH⁺ and CD44⁺ cells in colon carcinomas. Only a minority (3.5%) were ALDH⁺. A larger proportion (19.5%) were CD44⁺. The proportion positive for both was 1.3%. Percentages (mean ± SE) are averages of four experiments. The middle plot shows percentage of implants that produced a xenograft tumor after sorting using ALDH and CD44 markers. Percentages represent a summation of data across xenograft tumor assays using cells isolated from four different cancers involving 10 implants per cell subpopulation category. The same range of implant doses (50–1,000 cells) was used for each category. Percentage for ALDH⁺ alone was deduced from data on percentage tumor formation and proportions of cells for ALDH⁺/CD44⁺ and ALDH⁺/CD44⁻. Tumors generally did not result from ALDH⁻/CD44⁺ or ALDH⁻/CD44⁻ cells. Right plot depicts a representative experiment showing latency of tumor development after implantation of two different doses (1,000 and 100) of ALDH⁺/CD44⁺ or ALDH⁺/CD44⁻ cells. No tumors were generated from ALDH⁻/CD44⁺, ALDH⁻/CD44⁻, or ESA⁺ cells. **B**, isolation of cells using ALDH and CD133. Left image gives proportions of ALDH⁺ and CD133⁺ cells in colon carcinomas. Only a minority (3.5%) were ALDH⁺. A larger proportion (24.8%) were CD133⁺. The proportion positive for both was 0.9%. Percentages (mean ± SE) are averages of four experiments. The middle plot shows percentage of implants that produced a xenograft tumor after sorting using ALDH and CD133. Percentages represent a summation of data across xenograft tumor assays using cells isolated from three different cancers involving nine implants per cell subpopulation category. The same range of implant doses (50–500 cells) was used for each category. Percentage for ALDH⁺ alone was deduced from data on percentage tumor formation and proportions of cells for ALDH⁺/CD133⁺ and ALDH⁺/CD133⁻. Tumors did not form from ALDH⁻/CD133⁺ or ALDH⁻/CD133⁻ cells. Right plot depicts a representative experiment showing latency of tumor development after implantation of different doses (50, 250, and 500) of ALDH⁺/CD133⁺ or ALDH⁺/CD133⁻ cells. No tumors were generated from ALDH⁻/CD133⁺, ALDH⁻/CD133⁻, ALDH⁺/CD133⁻, or ESA⁺ cells.

5 months of observation. ALDH⁺, CD44⁺, CD133⁺, and ESA⁺ cells formed xenograft tumors more frequently with increasing number of cells injected (Fig. 4B). Compared with ESA⁺ alone, the other cell subpopulations seemed to have somewhat greater tumor-initiating abilities. The other cell subpopulations generally elicited larger tumors and elicited them more rapidly than cells isolated using ESA⁺ alone (Fig. 4C).

Ability of cells isolated using ALDH and CD44 together to form tumor xenografts. Malignant cells were isolated from colon carcinomas using ESA followed by Aldefluor and then CD44. The proportion of CRC cells positive for both ALDH and CD44 was 1.3% (Fig. 5A); that is, 37% of Aldefluor⁺ cells were also CD44⁺. ALDH⁺/CD44⁺ cells did not show an increased ability to generate tumor xenografts (Fig. 5A) compared with ALDH⁺/CD44⁻ or ALDH⁺ alone. However, ALDH⁺/CD44⁺ cells tended to elicit larger tumors and elicited them more rapidly compared with ALDH⁺/CD44⁻ cells (Fig. 5A). Taken together, our data on the proportion of tumors that grow, their size, and their speed of development indicate that CD44, when used serially with ALDH to further sort ESA⁺ cells, results in only minimal enrichment of tumor-initiating cells over ALDH⁺ alone.

Ability of cells isolated using ALDH and CD133 together to form tumor xenografts. Malignant cells were isolated from colon carcinomas using ESA followed by Aldefluor and then CD133. The proportion of CRC cells positive for both ALDH and CD133 was 0.9% (Fig. 5B); that is, 27% of Aldefluor⁺ cells were CD133⁺. ALDH⁺/CD133⁺ cells showed a somewhat increased ability to generate tumor xenografts (Fig. 5B) compared with ALDH⁺/CD133⁻ or ALDH⁺ alone. ALDH⁺/CD133⁺ cells tended to elicit larger tumors and elicited them more rapidly compared with ALDH⁺/CD133⁻ cells (Fig. 5B). Taken together, our data on the proportion of tumors that grow, their size, and their speed of development indicate that CD133, when used serially with ALDH to further sort ESA⁺ cells, results in a modest enrichment of tumor-initiating cells over ALDH⁺ alone.

Discussion

The study of colon cancer SC would be greatly enhanced by availability of specific markers to identify and isolate these cells. One key finding of this study was that ALDH identifies colonic cells that exhibit known SC properties: (a) immunohistochemistry identifies a small subpopulation of ALDH1⁺ cells (<5%) localized

to the bottom of normal crypts (where SC reside) and (b) the Aldefluor assay can be used to isolate a subpopulation of malignant colonic cells that generates xenograft tumors (functionally showing the ability for self-renewal). Aldefluor⁻ cells from CRCs did not form xenograft tumors. Thus, our findings substantiate that ALDH1 and Aldefluor are specific markers for SC in normal and malignant colon.

In comparison, CD133 and CD44 positivity on immunohistochemistry and flow cytometry identified a considerably larger subpopulation within normal colonic tissues, subpopulations that were 7-fold the size of the ALDH⁺ population. These results are consistent with previously reported data (10–14). Nevertheless, CD44⁺ or CD133⁺ cells from colon cancers were still capable of generating tumor xenografts and could do so with potency similar to that of ALDH⁺ cells. These data suggest that CD44 and CD133 label the proliferative cell population that contains both rapidly proliferating cells as well as the much smaller population of colonic SC. That Aldefluor⁻ cells, even ones that are CD44⁺ or CD133⁺, do not form xenograft tumors suggests that many of the SCs within the CD44⁺ and CD133⁺ populations are eliminated by removal of Aldefluor⁺ cells during sorting. Thus, we conclude that ALDH1 is more specific for SC in the colon than are CD44 or CD133.

Cells that were isolated using multiple SC markers (particularly Aldefluor⁺/CD133⁺) had slightly better efficiency in generating tumors than did Aldefluor⁺ cells. In addition, cells that were positive not only for ALDH but also for CD44 or CD133 tended to generate tumor xenografts more rapidly than did ESA⁺ cells. This suggests that, in cell selection, the addition of CD133 positivity or CD44 positivity to Aldefluor positivity causes somewhat enhanced SC enrichment.

An apparent discrepancy is that no tumors were generated using cells that were isolated as CD44⁻ when CD44 was used as the sole marker for sorting cells, yet there were tumors generated when cells were implanted from populations that were CD44⁻ if they were isolated as ALDH⁺/CD44⁻ (using these two SC markers in sequence). The same apparent discrepancy applies to CD133⁻ cells. One explanation is that ALDH⁺/CD44⁻ cells and ALDH⁺/CD133⁻ cells have tumor-initiating ability because they are ALDH⁺, but these cells only constitute a small fraction of the total CD44⁻ population and CD133⁻ population. Consequently, implantation of CD44⁻ or CD133⁻ cells (isolated using CD44 or CD133 markers individually) might not generate xenograft tumors because the ALDH⁺/CD44⁻ and ALDH⁺/CD133⁻ cells are in such low concentration in the implanted sample. In contrast, when Aldefluor is used to select ALDH⁺ cells from the ESA⁺ cell population, and then CD44 or CD133 is used serially for cell selection, the enrichment of ALDH⁺/CD44⁻ or ALDH⁺/CD133⁻ cells then becomes high enough to generate tumors.

It is uncertain how the overlap of the CD44⁺ or CD133⁺ populations with the ALDH⁺ population relates to the ability of these populations to initiate tumors. Based on our data, this issue is complex, and our results (Fig. 5, Venn diagrams) suggest that CD44 positivity and Aldefluor positivity are independent variables, as are CD133 and Aldefluor positivity. Although it seems, based on tumorigenic potential, that ALDH is more specific for stemness than CD44 (CD44⁺/ALDH⁻ cells are nontumorigenic), the overall high tumorigenic potential of the CD44⁺ population (Fig. 4) cannot be completely explained by a relatively small subset of cells that are also ALDH⁺ (<10% contained within the CD44⁺ population). One possible explanation is that CD44⁺/ALDH⁻ cells are not by themselves tumorigenic but somehow support the tumorigenic growth of CD44⁺/ALDH⁺ cells. This might occur by the implantation site becoming humanized due to CD44⁺/ALDH⁻ cells

contained in the implanted sample. This humanization effect has been observed when immortalized human fibroblasts are transplanted along with breast cancer cells (22, 30).

A second key finding of this study is that ALDH-based markers can be used to track colonic SC during colon tumorigenesis. ALDH1 positivity on immunohistochemistry indicated an increasing number of colonic SC over the course of colon cancer progression, from normal to FAP to adenoma. If 29% of adenoma cells are ALDH1⁺, and given that an adenoma contains 10⁶ to 10⁸ cells, one can calculate that there is an absolute increase in the number of SC in an adenoma relative to a normal crypt. This trend toward SC overpopulation continued for carcinomas as well. This was deduced from the fact that colon carcinomas typically have 10⁸ to 10¹³ cells and that our flow cytometric data on Aldefluor indicate that 3.5% of these carcinoma cells are SC.

A third key finding is that this progressive increase in SC number strongly supports our SC overpopulation hypothesis (23–27), developed from our mathematical modeling of colon tumorigenesis. In this hypothesis, SC overpopulation is the key event at the cellular level that links the initiating event at the molecular level (typically *APC* mutation) to the earliest event at the tissue level (a crypt proliferative abnormality). Our earlier study (28) using markers for crypt base cells provided biological evidence in support of our SC overpopulation hypothesis. Two other lines of biological evidence support this hypothesis: (a) a study using methylation patterns as epigenetic markers for colonic cell fate showed that there is enhanced SC survival with CRC initiation in FAP (31–33) and (b) the murine intestinal SC marker, *Lgr5*, has been reported to be overexpressed in advanced human CRCs (34–36). Taken together, these studies suggested that an increased number of colonic SC occur during the initiation and progression of CRC. The current study provides even more compelling biological evidence for our SC overpopulation hypothesis.

Our mathematical modeling also predicted that the overall proliferative cell population (both SC and rapidly proliferating cells) increases during colon tumorigenesis (23, 27). The increase, with tumor progression, in the proportion of cells along the crypt axis that stain positively for CD44 and CD133 supports that prediction and is our fourth key finding. Comparing results on ALDH1 (that labels SC) with results on CD44 and CD133 (which seem to label SC and proliferating cells) provides an explanation for how colonic SC contribute to tumor initiation and progression (i.e., that expansion of the SC population leads to expansion of the proliferating cell population), and together, they constitute an overall increase in the proliferative cell population during the stepwise progression to colon carcinoma.

Disclosure of Potential Conflicts of Interest

B. M. Boman has ownership in CA*TX. The other authors disclosed no potential conflicts of interest.

Acknowledgments

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