CD133 + colon cancer cells are more interactive with the tumor microenvironment than CD133− cells

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Experimental data indicate that colorectal cancer cells with CD133 expression exhibit enhanced tumorigenicity over CD133-negative (CD133−) cells. We hypothesized that CD133-positive (CD133 +) cells, compared with CD133−, are more tumorigenic because they are more interactive with and responsive to their stromal microenvironment. Freshly dissected and dissociated cells from a primary colon cancer were separated into carcinoma-associated fibroblasts (CAF) and the epithelial cells; the latter were further separated into CD133 + and CD133− cells using fluorescence-activated cell sorter. The CD133 + cells formed large tumors in non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice, demonstrating the phenotypic cellular diversity of the original tumor, whereas CD133− cells were unable to sustain significant growth. Affymetrix gene array analyses using t-test, fold-change and multiple test correction identified candidate genes that were differentially expressed between the CD133 + vs CD133− cells. RT-PCR verified differences in expression for 30 of the 46 genes selected. Genes upregulated (+ vs − cells) included CD133 (9.3-fold) and CXCR4 (4-fold), integrin β8 and fibroblast growth factor receptor 2. The CAF highly express the respective ligands: stromal-derived factor-1 (SDF-1), vitronectin and FGF family members, suggesting a reciprocal relationship between the CD133 + and CAF cells. SDF-1 caused an increase in intracellular calcium in cells expressing both CD133 and CXCR4, confirming functional CXCR4. The CD133 +/CXCR4 + phenotype is increased to 32% when the cells are grown in suspension compared with only 9% when the cells were allowed to attach. In Matrigel 3-D culture, the CD133 +/CXCR4 + group treated with SDF-1 grew more colonies compared with vehicle, as well as significantly larger colony sizes of tumor spheres. These data demonstrate proof of principle that the enhanced tumorigenic potential of CD133 +, compared with CD133−, cells is due to their increased ability to interact with their neighboring CAF.

Human solid tumors are characterized by phenotypically heterogeneous populations of malignant cells with varying degrees of differentiation and tumor-initiating potential. The trans-membrane glycoprotein CD133, originally characterized as a cell surface marker for hematopoietic stem cells,[1,2] identifies a subset of malignant cells with enhanced tumorigenic activity in cancers from a variety of tissues, including prostate, brain, breast, pancreas, liver, uterus and colon.[3–12] Similar to the other cancers, CD133-positive (CD133 +) cells isolated from colorectal tumors grow in suspension culture as anchorage-independent epithelial spheroids (colospheres) and efficiently initiate new tumor formation when xeno-grafted into non-obese diabetic-severe combined immuno-deficient (NOD-SCID) mice.[7,8] Ricci-Vitiani et al[8] showed that subcutaneous injection of as few as 3×10^3 CD133 + human colon cancer cells, suspended in Matrigel, generated visible tumors in mice between 4 and 5 weeks post-trans-plant, whereas injection of as many as 10^5 CD133-negative (CD133−) cells isolated from the same patient failed to produce tumors over the same time course. Subpopulations of CD133 + cells isolated from established colon cancer-derived cell lines such as HT29 and LoVo also exhibit increased proliferation, invasion through extracellular matrix (ECM) and colony formation in culture,[13] and clinically, the

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level of CD133 expression in tumor tissue negatively correlates with both disease-free and overall survival of colorectal cancer patients.[14–16]

Although the preponderance of data indicates that expression of CD133 identifies a subpopulation of cancer cells with enhanced tumorigenic potential and prognostic value, CD133 does not appear to functionally regulate the aggressive phenotype. This point is illustrated by the observations that siRNA-mediated suppression of CD133 neither compromised the tumorigenic potential of primary human colon cancers cells xenografted in nude mice[17] nor the proliferation, migration, invasion and anchorage-independent growth of CD133+ LoVo and Caco-2 cells in culture.[18] Thus, a mechanistic explanation for the enhanced tumorigenicity of CD133+ colon cancer cells remains enigmatic.

In addition to cell autonomous properties, the tumorigenicity of cancer cells is also influenced by their interactions with the tumor microenvironment. Solid tumor tissue contains a multitude of non-malignant cells, collectively known as tumor stroma, which directly through cell-to-cell contacts, and indirectly through paracrine signaling networks, mediate regulation of protease activity and modulation of ECM proteins to promote tumorigenesis, angiogenesis and metastatic spread.[19,20] The most prevalent cells found in the tumor stroma are the cancer-associated fibroblasts (CAFs),[21] which in multiple studies have been shown to promote and/or enhance the tumorigenic potential of both adenoma and adenocarcinoma cells. An example of the former is a study by Olumni et al,[22] in which CAFs isolated from malignant human prostate tissue promoted robust tumor formation when co-injected subcutaneously with an SV40 T-antigen immortalized, but non-tumorigenic, human prostate epithelial cell line called Tag-HPE into immune-deficient mice,[22] whereas neither the prostate CAFs nor Tag-HPE cell line alone produced tumors when injected into the mice. Similarly, Orimo et al[23] showed that CAFs isolated from breast cancers, but not normal breast fibroblasts, significantly enhanced the tumorigenicity of human MCF-7-ras breast cancer cells when co-injected into immune-deficient mice, and Hwang et al[24] demonstrated that tumor-associated human pancreatic stellate cells (an activated myofibroblast-like cell) enhanced the tumorigenicity and metastasis of human BxPC-3 pancreatic cancer cells in mice. Taken together, these studies demonstrate that CAF can enhance tumor growth and spread. Thus, we hypothesized that the increased tumorigenicity of CD133+ colon cancer cells may be due, in part, to its enhanced interactions with CAFs of the tumor microenvironment.

To begin testing this hypothesis, we performed a comprehensive molecular profiling of CD133+ and CD133− carcinoma cells, as well as of the CAFs isolated from the same patient specimen, to reveal phenotypic differences between these cell populations. Herein, we report that, compared with CD133− colon cancer cells, CD133+ cells have reduced transcript levels for cell adhesion and matrix proteins, but increased transcripts for proteases, and cell surface receptors such as the cytokine receptor CXCR4, which mediates tumor cell proliferation and metastasis.[25] In addition, CAFs express the ligands for the receptors overexpressed by the CD133+ cells, including the CXCR4 receptor ligand CXCL12 (also known as stromal-derived factor-1 or SDF-1), indicating the potential for enhanced paracrine signaling between these cells. Functional studies confirmed that CD133+ colon cancer cells exhibit enhanced proliferation in response to SDF-1 compared with CD133− cells, supporting the hypothesis that the enhanced tumorigenic potential of CD133+ cells is due, in part, to enhanced paracrine signaling with CAFs of the tumor microenvironment.

MATERIALS AND METHODS
Cell Isolation and Culture
Cell cultures were established from a freshly dissected colon tumor, which was obtained under an IRB-approved protocol from a 68-year-old woman with Stage II (pT3 N0) disease. The tissue was rinsed twice in Dakin's solution (4% boric acid and 0.5% bleach), minced and digested as described previously.[26] Dissociated cells were cultured in DMEM supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 0.25 μg/ml amphotercin B and 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum and 1% insulin–transferrin–selenium (ITS) (Invitrogen). Using a tumor outgrowth procedure, we isolated and separately cultured both the carcinoma cells and CAF from the same tumor. Suspension epithelial cell cultures were grown in ultra-low-adhesion flasks (Costar; Corning, Lowell, MA, USA). The isolated CAFs were grown in standard tissue culture flasks (Cellstar; Grenier Bio-One; Monroe, NC, USA). Both the carcinoma cells and CAF were maintained in the DMEM, 10% FCS, 1% ITS, 1% non-essential amino acids (Invitrogen) and 1% penicillin–streptomycin.

Immunocytochemistry
Verification of epithelial and mesenchymal cell types was performed by immunocytochemistry as described previously.[26] Briefly, cells were fixed in 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS) and then permeabilized in absolute methanol (ice-cold) for 10 min. The coverslips were then placed in 2% milk for blocking and then rinsed in PBS containing 1% BSA. Primary antibody incubation was applied for 1 h at room temperature (RT) and rinsed, and then secondary antibody was applied for 1 h RT. Antibodies used were pan-cytokeratin AE1/3 (1:200 dilution; Santa Cruz), α-smooth muscle actin (α-SMA) (A5228; 1:200; Sigma) and vimentin (V6630; 1:40; Sigma). Secondary antibodies (1:200) used were either goat anti-rabbit IgG or goat anti-mouse IgG labeled with Alexa 546 and 488 (Molecular Probes, Eugene, OR, USA), respectively.

Immunohistochemistry

Spheroids were fixed in 10% neutral formalin for 24 h, immobilized in 2% agar solution and embedded in paraffin blocks and sectioned (5 μm). Before staining, the sections were deparaffinized with xylene, dehydrated with ethanol and subjected to antigen retrieval using citrate buffer (10 nM (pH 6) at 98°C for 20 min) to optimize antigen immunoreactivity. Antibodies for immunohistochemistry included CD133 (AC133/1, 1:50 dilution; Miltenyi Biotec, Auburn, CA), CD44 (1:50; Cell Signaling), MUC4 (clone 1G8, 1:100; Invitrogen), carcinoembryonic antigen (CEA) (1:800; Biocare Medical, Concord, CA, USA), CD34 (1:100; Biocare Medical), CD166 (1:400; GeneTex, Irvine, CA, USA), α-SMA (ab5694; 1:400; Abcam) and Mayer's mucicarmine (Poly Scientific, Bay Shore, NY, USA). In addition, CK7 (1:200), CK20 (1:100), Ki67 (1:100) and the universal negative control for anti-rabbit and anti-mouse IgG were all purchased from Dako (Carpinteria, CA, USA). The ApopTag Peroxidase *In Situ* Apoptosis Detection Kit was used to detect apoptotic cells by labeling and detecting DNA strand breaks by the TdT-mediated dUTP nick-end labeling (TUNEL) method (Millipore, Billerica, MA, USA). The streptavidin–biotin technique was used to visualize the immunostain. The slides in Figures 8 and 9 underwent a tertiary step with streptavidin alkaline phosphatase at 1:200 for 15 min (SA-5100; Vector Labs) and the chromagen was fast red for 5 min.

Cell Sorting

Cells were dissociated in Accumax (Innovative Cell Technologies, San Diego, CA, USA) and resuspended in PBS containing 0.5% BSA and 2 mM EDTA. Human FcR blocking reagent was used to block nonspecific Fc receptor-mediated antibody labeling. Fluorescence-activated cell sorting (FACS) with a FACSAria (Becton Dickinson Biosciences, San Jose, CA, USA) analysis was performed using monoclonal antibodies for CXCR4 (clone 12G5; BD Biosciences, San Jose, CA, USA), CD133/1 (AC133) conjugated to R-phycoerythrin (PE) or allophycocyanin (APC), and isotype-matched IgG controls (Miltenyi Biotec).

In Vivo Tumorigenicity Assay

CD133+ and CD133– cells were purified by FACS sorting. Serial limiting dilution of equivalent numbers of both CD133+ and CD133– cells mixed 1:1 in growth factor-reduced Matrigel (BD Biosciences), and PBS were injected subcutaneously into a 10-week-old male NOD-SCID mice under an IACUC-approved protocol. Tumor sizes were measured transcutaneously over time. Tumor weight and tumor volumes ($V = (\pi/6)hd^2$) were obtained at 6 weeks. Specimens were fixed with 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Gene Expression Analysis

Total cellular RNA was extracted using RNAqueous (Ambion, Austin, TX, USA) according to the manufacturer's recommendations from three pairs of samples (CD133+ and CD133−) that were sorted on three separate days. Total RNA was also prepared from three separate CAF cultures in the same manner. RNA was quantitated using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was assessed by visualization of 18S and 28S RNA bands using an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Total RNA extracted from the samples was processed using the RNA labeling protocol described by Ambion (MessageAmp aRNA Kit Instruction Manual) and hybridized to Affymetrix Gene Chips (HGU133 Plus 2.0 arrays). Data quality was assessed by applying the quality matrix generated by the Affymetrix GeneChip Command Console (AGCC) software. The resulting data were analyzed with Partek Genomics Suite (Partek, St Louis, MO, USA). Principal component analysis as a quality assurance measure was performed. The raw data were normalized through robust multichip averaging upon import to Partek Genomics Suite. To identify differentially expressed genes, an ANOVA was applied to the extracted gene expression measures. To reduce the occurrence of false-positives, multiple test corrections (Benjamani–Hochberg and Bonferroni) were applied. The data set was filtered for a P-value of <0.05 and <0.01, resulting in the final list of differentially expressed genes.

Real-Time Quantitative Polymerase Chain Reaction

Real-Time SYBR Arrays were utilized to validate a subset of the genes generated by the analysis of the Affymetrix gene expression data. This approach combines the quantitative performance of SYBR Green-based real-time quantitative polymerase chain reaction (qPCR) with the multiple gene profiling capabilities of a microarray. The real-time array is a 96-well plate containing qPCR primer assays for 45 genes of interest plus 3 housekeeping genes (*GAPDH*, *Rpl19* and *Bpol*) to serve as normalizers. The 48 assays were duplicated on the same plate to facilitate comparison of CD133+ and CD133− samples and eliminate plate to plate variance. Biological replicate sets (test, control) were assayed on three separate plates for proper statistical analysis. A melt-curve analysis was carried out at the end of each PCR run protocol to identify multiple PCR products that would confound the data. The list of primers used is shown in Table 2 of Supplementary Materials.

Total RNA (1 μg) was used in the Affymetrix gene expression analysis, which was used in a single reverse transcription reaction to generate cDNA. The resulting product was distributed equally among the 48 wells containing the assay primers. The assays were carried out under standard real-time run parameters specific to the *Taq* polymerase (Applied Biosystems, Foster City, CA, USA) being used. Delta CT values were calculated and used to determine fold changes. Student's *t*-test was carried out on the biological replicates to determine the degree of confidence at which differential expression can be discerned.

Intracellular Calcium Imaging

Single-cell recordings of agonist-mediated increases in intracellular calcium ($[Ca^{2+}]_i$) was performed as described previously.[27] Briefly, cells were washed in KRH physiological medium and then loaded with 2.5 μm of the calcium sensing dye Fura-2 AM ester (Molecular Probes) with 0.055 Pluronic F-127 (wt/vol) for 50 min at 25 °C. Changes in $[Ca^{2+}]_i$ were monitored with a Nikon Diaphot inverted microscope (Nikon Instrument Group, Garden City, NY, USA) equipped with a Nikon × 40 (1.3 NA) Neofluor objective. The fluorescent light source was a PTI deltaScan RD-1 ratio fluorescence spectrometer system, equipped with a light-path chopper and dual-excitation monochrometers. The Fura-2 was alternately excited at 340 and 380 nm, and two fluorescence emissions were monitored through a 510 nm bandpass filter (Omega) with a Photometrics Coolsnap EZ camera (Tucson, AZ, USA). Increases in $[Ca^{2+}]_i$ were recorded in response to 400 ng/ml of the ligand SDF-1α (Peprotech, Rocky Hill, NJ, USA) at room temperature to verify the presence of functional CXCR4.

Matrigel Culture

Cells were embedded in Matrigel (BD Biosciences) in the top of a transwell insert (8 μm pore; Costar) in duplicate. A single treatment of SDF-1α (400 ng/ml) or vehicle (0.2% BSA) was placed in the bottom well in DMEM and cells were allowed to grow. After 30 days, the number of cell clusters was quantified by visual counting using a 25 mm^2 reticle (Upstate Technical Equipment Company, East Syracuse, NY, USA). The size of the cell clusters were measured with a micrometer. Student's t-test was used to assess significance between the paired groups, defined at $P < 0.05$.

RESULTS

Isolation and Characterization of Human Colon Cancer Cells and CAFs

Colon cancer cells and CAFs were isolated from a freshly resected Stage II, moderately differentiated, right colon tumor in accordance with an institutional-approved IRB protocol as described in Materials and Methods section. The primary cell culture, named CT03, consisted of two morphologically distinct cell populations: non-adherent clusters of cells and adherent cells that spread on the bottom of the tissue culture flask (Figure 1a). Immunocytochemical analysis identified the spheroid-cell clusters (colospheres) as cytokeratin-positive (Figure 1b), CD34− (data not shown) cells, whereas the adherent cells stained positive for vimentin and were negative for cytokeratin expression (Figure 1c), consistent with a fibroblast phenotype. A subpopulation of
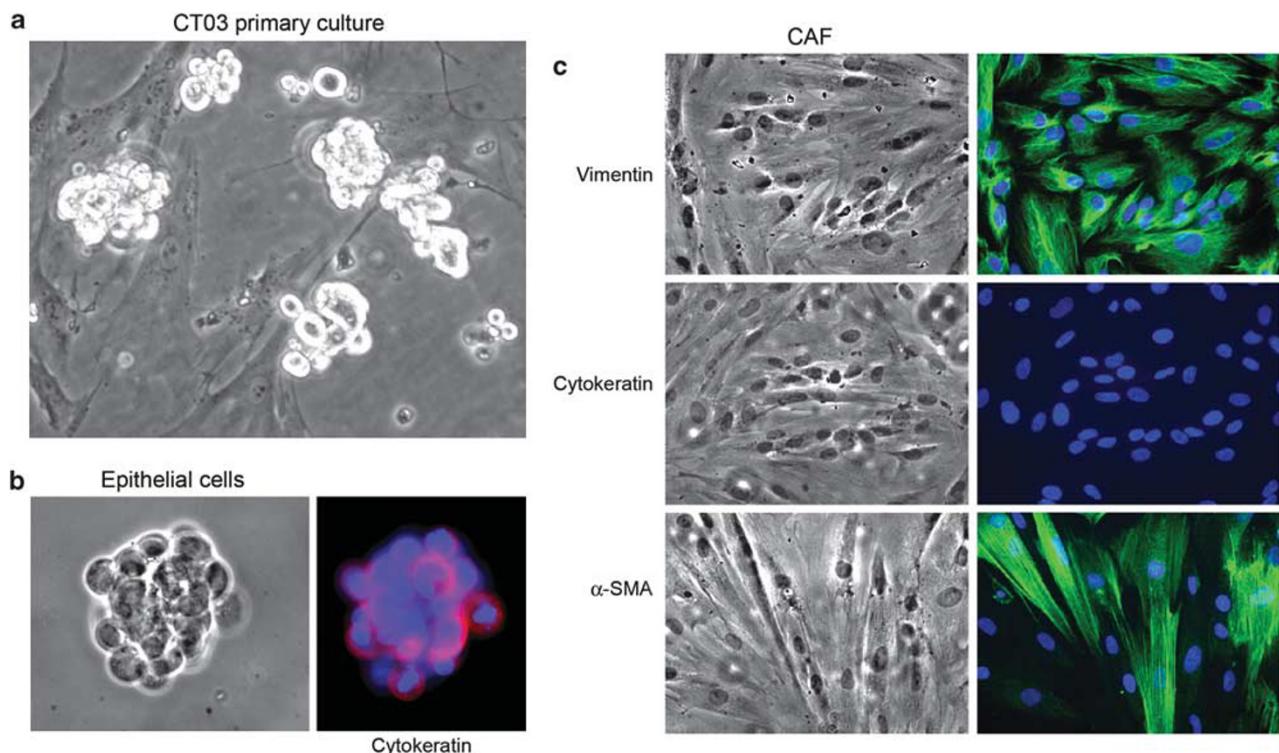

Figure 1 Phase-contrast image of characteristic features of primary colon cancer cells and carcinoma-associated fibroblast (CAF) isolated from a patient with Stage II disease maintained. (**a**) Mixed-cell population with two phenotypes in cell culture, consisting of (**b**) a non-adherent colon cancer cell line that form spheres in culture (phase-contrast microscopy) that is cytokeratin-positive (pan-cytokeratin AE1/3) and (**c**) carcinoma-CAFs that stain for vimentin, but not cytokeratin, with a subpopulation that are α-smooth muscle actin (α-SMA)-positive. Original magnification, × 400.

the vimentin-positive adherent cells also expressed SMA (Figure 1c), suggestive of myofibroblasts.[28]

Isolated Colospheres Exhibit Phenotypic Heterogeneity and are Similar to the Surgical Specimen

Cells within the colon epithelial spheroids exhibit phenotypic heterogeneity and varying degrees of differentiation. Similar to the patient's original tumor (Figure 2), a subpopulation of cells express CD133. Glycosylated CD133 localizes to the apical microvilli of primitive epithelial cells[29] and on the luminal surface of the glands;[15] this pattern of expression is recapitulated in the colon spheroids (Figure 2). Cross-sections of the colospheres demonstrate that the central core do not express CD33, but are apoptotic, as evidenced by the stain for TUNEL. Cells peripheral to the core, however, are highly proliferative, with $59.9 \pm 8.8\%$ (mean $\pm$ s.e.m.) of the cells showing strong immunostaining for nuclear Ki67. Some cells within the spheroids exhibited glandular organization, with positive mucicarmine staining within acellular lumens (Figure 2, pink areas indicate the presence of mucin). The original tumor stained positive for mucicarmine and also a specific mucin protein, MUC4. Both the patient tumor and
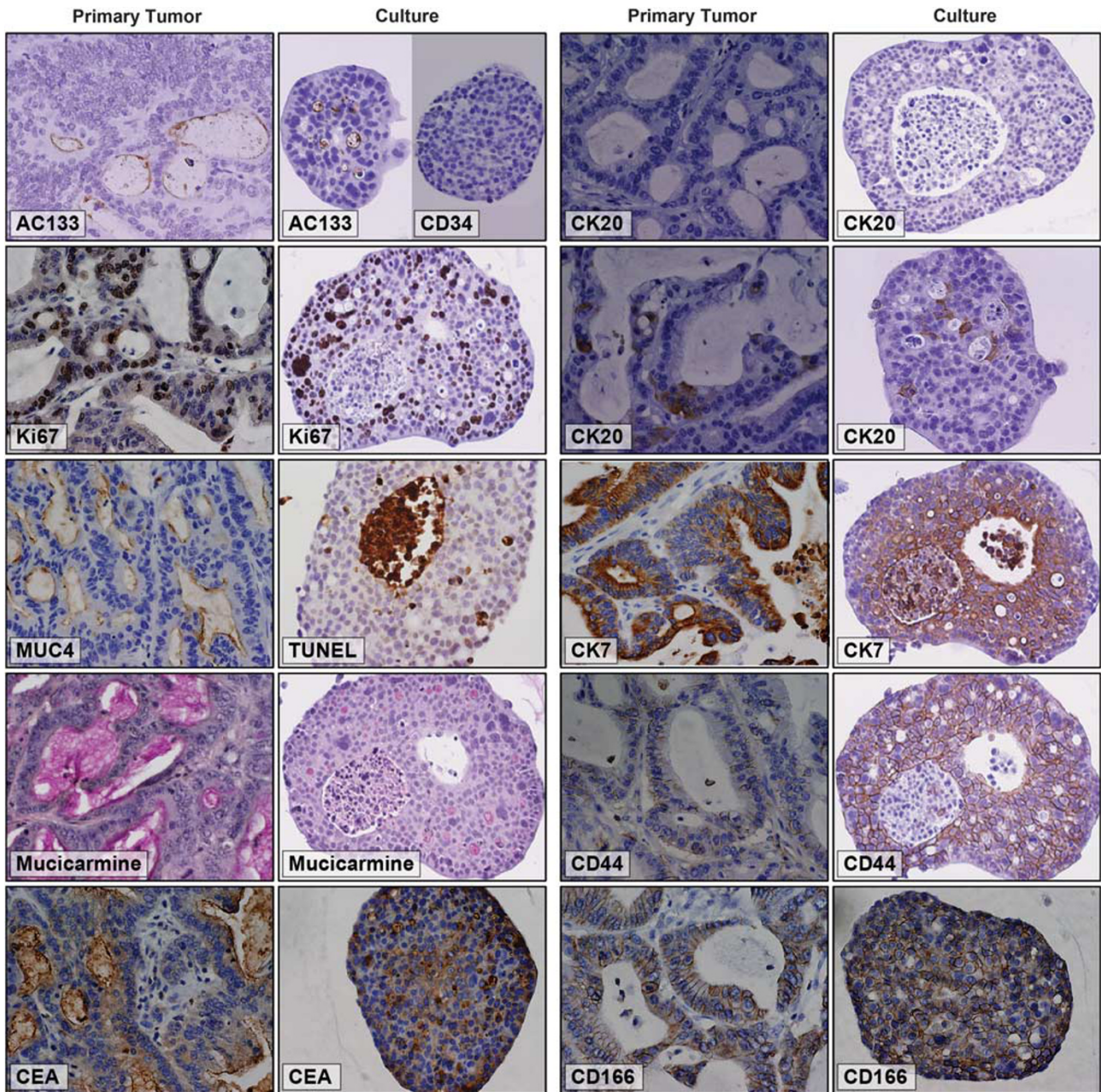

Figure 2 Immunohistochemical characterization of the original patient tumor sample (original magnification, $\times 400$) compared with the colospheres maintained in vitro (original magnification, $\times 200$).

the colospheres stained positive for the cell surface glyco-protein CEA (Figure 2). Although previous reports indicate that CD133+ colon cancers do not express CK20,[13,30] the spheroid cells did rarely express CK20, consistent with the original tumor that was predominately negative for CK20, with only a small proportion of the tumor sampled positive for CK20. CK20 is a marker for the differentiated phenotype of colorectal cancers, expressed in up to 92% of colon cancers.[31] CK7+ tumors characterizes only 16% of all colorectal cancers, while colorectal cancers that express both CK20 and CK7, such as this patient's tumor, represent only 12%.[31] This phenotype is recapitulated in the cultured colospheres (Figure 2). Finally, most cells within the spheroids as well as the original tumor sample express both CD44 and CD166, both putative colon cancer stem cell surface protein markers.[32,33]

CD133+ Cells Exhibit Enhanced Tumorigenicity when Compared with CD133− Cells

To assess the relative tumorigenicity of the subpopulation of CD133+ cells, the epithelial spheroids were dissociated into single cells using Accumax (Innovative Cell Technologies), labeled with fluorescently tagged antibody to CD133 (AC133) and separated by FACS (Figure 3a). Cells exhibiting the highest level of CD133 expression (CD133+ cells) were separated from the cells expressing very low levels of detectable CD133 (Figure 3b; similar expression levels as cells were labeled with nonspecific IgG control antibody). To evaluate tumorigenicity, the sorted cells were serially diluted and resuspended in growth factor-reduced Matrigel. CD133+ cells transplanted subcutaneously at 10^3 were able to form tumors in NOD-SCID mice, whereas CD133− cells were either unable to sustain growth or formed very small tumor masses (Figure 3c and d). Tumor volumes at 6 weeks were 11 ± 4 and $420 \pm 114 \, mm^3$ (CD133− *vs* CD133+, respectively, $P = 0.01$, *t*-test) (Figure 3d). The tumors harvested from the xenograft demonstrate phenotypic cellular heterogeneity similar to the primary tumor tissue isolated from the patient (Figure 3e and h, respectively). Regions of the xenograft tumor displayed both moderately differentiated (Figure 3f) and well-differentiated cancer (Figure 3g), recapitulating the histopathology of the patient's tumor (Figure 3i and j, respectively).

Gene Expression Analysis: Comparison of CD133+, CD133− and CAF Cells
Overview
To explain the different *in vivo* biological behaviors, we hypothesized that the increased tumorigenicity of CD133+ colon cancer cells may be due, in part, to enhanced interactions with CAFs of the tumor microenvironment. Using the Affymetrix Gene Chip platform (HGU133 Plus 2.0 array), we (1) compared the differential gene expression between CD133+ and CD133− cells, and (2) examined the reciprocal receptor–ligand relationships between the epithelial cells (CD133+ or CD133−) and CAF isolated

from the same tumor. As expected, unsupervised hierarchical clustering showed that the CAF and the epithelial cells clustered at opposite ends of the dendrogram, confirming that CD133+ cells were more similar to CD133− cells, than they were to the CAF (Figure 4).

ANOVA analysis of the CD133+ *vs* CD133− carcinoma cells revealed 1410 and 318 differentially expressed genes significant at the $P \leq 0.05$ and 0.01 level, respectively. To reduce the false discovery rate, two multiple test corrections were applied to the 1410 genes list: The method of Benjamani and Hochberg identified 1155 gene transcripts, whereas the very stringent Bonferroni correction only identified 35 significant genes. We selected 45 of the 1155 genes for real-time PCR validation (qPCR). Of the 45 genes selected, all 14 gene transcripts evaluated as significant by Bonferroni correction were also verified by qPCR (data not shown). However, many true-positive results would have been excluded with this level of stringency. Of the 45 genes selected that were significant by the Benjamani–Hochberg analysis ($P < 0.05$), 27 of 45 genes (60%) were concordant by qPCR (Table 1).

Similarities between CD133+ and CD133− Cells
Because this isogenic cancer cell line was segregated by CD133 expression levels, as expected, gene array results verified that *PROM1* (CD133) was 9.3-fold enriched in the CD133+ cells compared with the CD133− cells (Table 1). Many investigators have suggested that other stem cell markers such as CD44 (a subset of CD133+ cells), EpCAM and CD166[30,32] select for *in vivo* tumorigenesis in immuno-deficient mice. In our microarray analysis, there were no appreciable differences in the co-expression of other putative colon cancer stem cell markers CD44, EpCAM, Lgr5, CD166, CD49b and Bmi1 between the CD133+ and CD133− cells (Supplementary Table 1). To determine whether key genetic determinants of the adenoma-to-carcinoma sequence are differentially expressed by CD133+ *vs* CD133− colon cancer cells, we compared the gene expression levels of oncogenes (*Src*, *Raf-1*, *Met*, *K-ras*), tumor suppressor genes (*APC*, *DCC*, *TP53*, *PTEN*), DNA mismatch repair genes (*MLH1*, *MSH2*, *MSH*, *MGMT*) and other genes specific for colon carcinogenesis (*CHK2*, *TGFBR2*). No statistically significant differences were identified between CD133+ and CD133– cells, indicating that the cells share the same genetic background for colon cancer-specific oncogenes and tumor suppressor genes (Supplementary Table 1).

Differences between CD133+ and CD133− Cells
Our analysis revealed gene expression differences between CD133+ and CD133– cells in three major categories: cell adhesion/matrix proteins, matrix-digesting proteases and cell surface receptors involved in proliferation/metastasis (full gene list available at GEOarchive at http: //www.ncbi.nlm.-nih.gov/geo). Specifically, genes downregulated in CD133+ relative to CD133− cells included the ECM proteins: *COL1A1* (collagen type 1 α1; 3-fold), *COL1A2* (collagen 1α2;
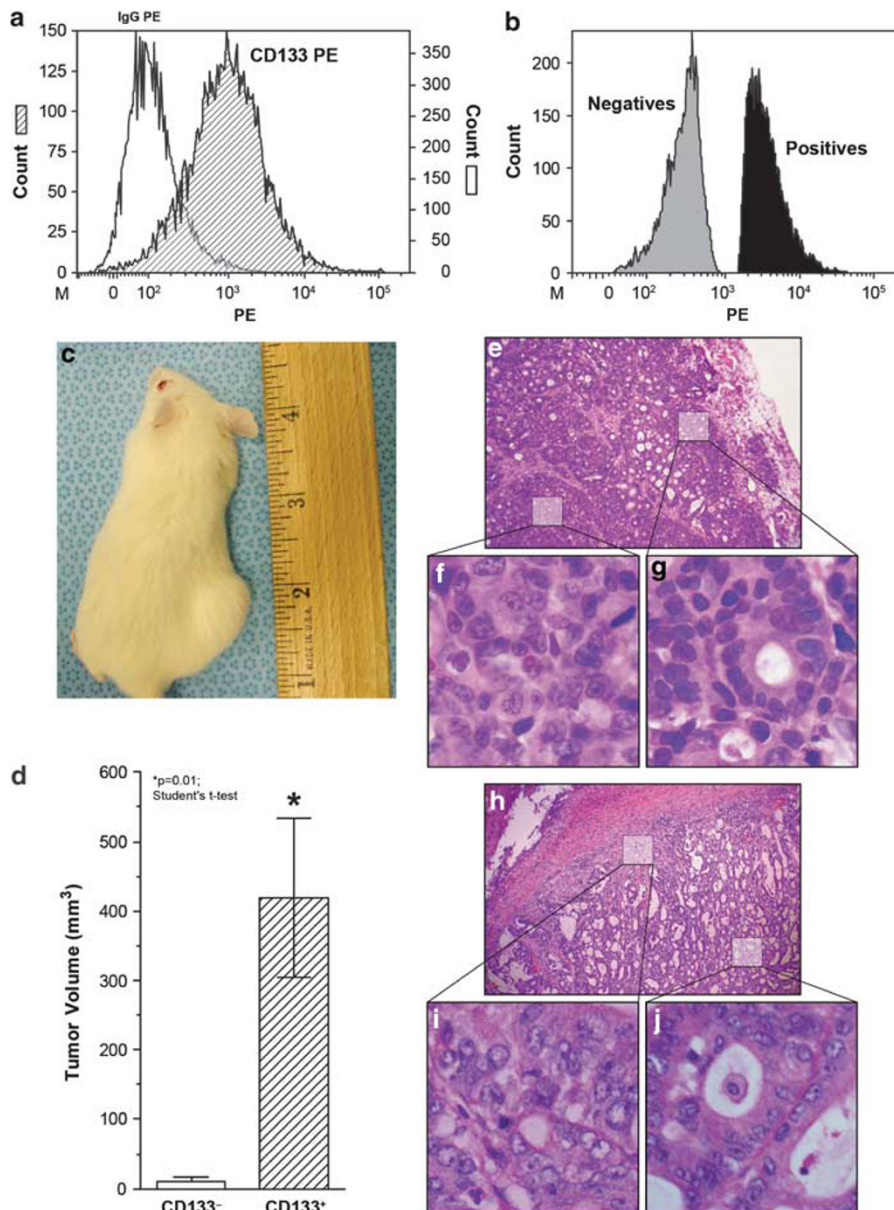
Figure 3 (a) Comparison of fluorescence by colon cancer cells labeled with AC133, an antibody to CD133 conjugated to R-phycoerythrin (PE) and isotype-matched immunoglobulin G (IgG) controls labeled with PE (Miltenyi Biotec). (b) Fractions of colon cancer cells with high expression levels of CD133 (positives) and relatively low expression levels (negatives) collected by fluorescence-activated cell sorting. (c) CD133+ and CD133− cells were serially diluted, resuspended in Matrigel and injected subcutaneously in non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice to assay for tumorigenic potential; representative mouse injected with CD133+ cells at the time of being killed is shown. (d) Summary of tumor volumes measured at 6 weeks comparing CD133− vs CD133+ tumors. (e) Representative hematoxylin and eosin stain (H&E) of the tumor xenograft (×40). (f, g) Inset from xenograft tumor demonstrates a moderately differentiated tumor and well-differentiated tumor forming glandular structures (×400), respectively. (h) Representative H&E of the tumor from the patient's tumor block (×40). (i, j) H&E (×400) from patient's original tumor block showing similar cellular features from the mouse xenograft, moderately and well-differentiated features.

5-fold), fibronectin (*FN1*; 3.8-fold), chondroitin sulfate proteoglycan 2 (*CSPG2*; 2.5-fold), and osteonectin (*SPARC*; 7-fold). CD133+ also exhibited increased expression, relative to CD133− cells, of proteases that may mediate degradation of ECM proteins and facilitate cell migration, including cathepsin C (*CTSS*; 1.65-fold), transmembrane protease-serine 3 (*TMPRSS3*; 2.15-fold) and sulfatase-2 (*SULF2*; 2.21-fold). CD133+ cells, compared with CD133− cells (Table 2a), overexpressed transcripts for cell surface receptors that may mediate cell surface-linked inter-cellular signal transduction, such as *CXCR4* (4-fold), a chemokine receptor vital to stem cell growth and homing, integrin-β8 (*ITGβ8*, 2.1-fold increase), CD74 molecule (*CD74*; 3.1-fold) and fibroblast growth factor receptor-2 (*FGFR2*, 1.2-fold).
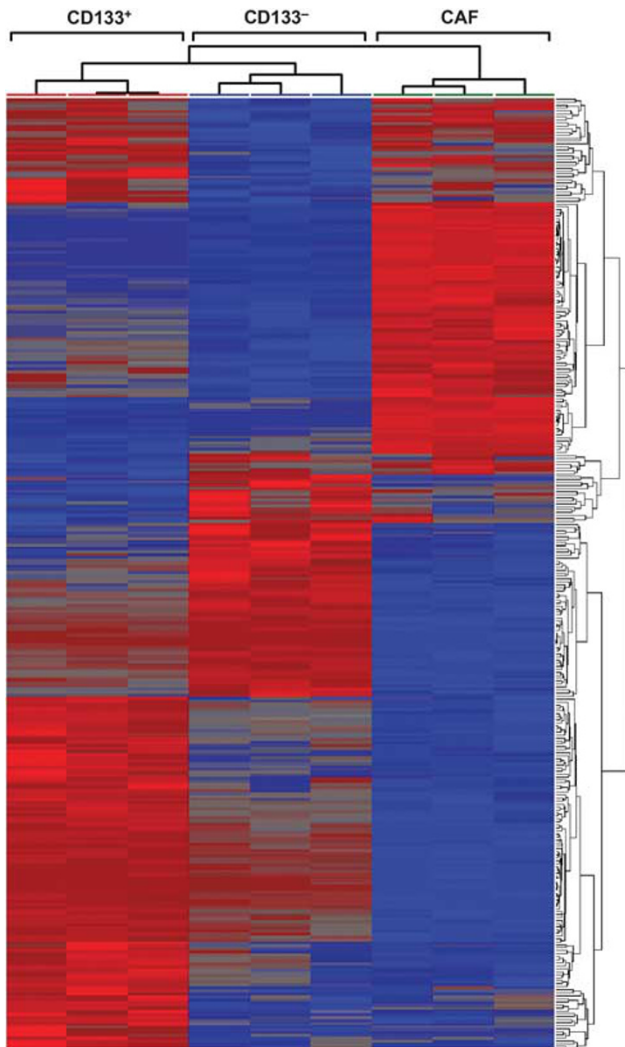
Figure 4 Heat map comparing CD133+ and CD133− colon cancer cells with the accompanying carcinoma-associated fibroblast (CAF) cells. Unsupervised hierarchical clustering comparing the CD133+, CD133− and CAF cells.

Table 1 Significant genes identified by gene array validated by quantitative real-time PCR

Gene symbol	*P*-value	Gene array fold change	RT-PCR fold change
IGFBP3	7.56E-06	1.77362	1.2545
PLECKHB1	1.01E-05	3.22459	1.1764
CP	1.47E-05	2.86229	0.8721
SULF2	7.45E-05	2.20567	1.4212
IFIH1	0.000106	1.36982	0.285
PROM1	0.000168	9.36934	1.774
CEACAM5	0.000305	1.37876	0.1536
FZD2	0.000635	1.39114	0.3026
ALDH1A3	0.00088	2.33778	1.2188
SMAD7	0.001114	1.34044	0.4519
PTPRN2	0.001715	2.32938	0.7781
RRM2B	0.002429	1.20816	0.4979
RARRES1	0.002608	2.59494	1.2729
MUC4	0.002968	1.79323	2.9041
CTSS	0.003889	1.6457	1.2689
PRKAR2B	0.004508	2.239	0.6976
ITGB8	0.006254	2.18013	2.1667
PPIC	0.012036	1.24324	0.4395
UBD	0.012722	2.24373	1.1454
CXCR4	0.014884	4.0673	1.3265
TNFAIP2	0.017107	1.327	1.2429
FGFR2	0.017493	1.21569	1.3451
PLCG2	0.020757	1.16869	0.9214
MUC1	0.021106	1.38278	0.6375
SCD5	0.024365	1.72136	0.3941
CD74	0.038686	3.12507	1.3607
MS12	0.040851	1.18797	0.1003

Statistical significance demonstrated at *P*-value ≤0.05.

The transcripts for the cognate ligands, *CXCL12* (also known as SDF-1, the ligand for CXCR4), macrophage inhibitory factor (*MIF*, the ligand for CD74), vitronectin (*VTN*, a ligand for ITGβ8) and fibroblast growth factor family members (*FGF*, ligands for FGFR2), are all expressed equally by both CD133+ and CD133− cells (Table 2a). Compared with the CD133+ or CD133− carcinoma cells, the CAF cells, isolated from the original patient cancer specimen, demonstrate high expression levels for *CXCL12, MIF, VTN* or the *FGF* ligands (Table 2b). However, only the CD133+ cells significantly overexpressed the corresponding cell surface receptors for the ligands: CXCR4, CD74, ITGβ8 and FGFR2 (Table 2c), indicating that only the CD133+ cells, but not CD133− cells, have a strong reciprocal relationship with the CAF. Taken together, these results are consistent with the hypothesis that the CD133+ cells may be better positioned to engage in paracrine signaling with CAF in the micro-environment.

The CD133+/CXCR4+ Phenotype can be Maintained in Anchorage-Independent Culture Conditions

To test if cells that are enriched for CD133+ can propagate and maintain CD133+/CXCR4+ cell surface expression in culture, we sorted the colospheres into CD133+ and CD133− single cells and maintained each cell population in low-adhesion flasks. After 9 weeks, repeat FACS analysis demonstrated that the CD133+ group continued to remain relatively enriched for CD133 cell surface expression (Figure 5a), with a proportion of CD133+ cells also co-expressing CXCR4

Table 2 Comparison of reciprocal relationships between CAF and CD133+ or CD133− colon cancer cells: (a) CD133+ *vs* CD133− cells, (b) CAF *vs* CD133+ or CD133− cells and (c) CD133+ or CD133− cells *vs* CAF

Receptor (gene symbol)		*P*-value	Fold change
(a) CD133+ vs CD133− cells			
CXCR4	Chemokine (C–X–C motif) receptor 4	0.02*	4.07
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	0.04*	3.12
ITGβ8	Integrin β8	0.01*	2.18
FGFR2	Fibroblast growth factor 2	0.03*	1.21
Ligand			
CXCL12	Chemokine (C–X–C motif) ligand 12 (stromal cell-derived factor 1)	0.89	1.02
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	0.88	1.01
VTN	Vitronectin	0.82	−1.04
FGF ligands			
1, 2, 7, 5, 13	Fibroblast growth factor ligands	NS	

Ligand		CAF *vs* CD133+ *P*-value CAF *vs* CD133− *P*-value	CAF *vs* CD133+ Fold change CAF *vs* CD133− Fold change
(b) CAF vs CD133+ or CD133− cells			
CXCL12	Chemokine (C–X–C motif) ligand 12 (stromal cell-derived factor 1)	0.0003*	3.73
		0.0002*	3.8
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	3.71E-05*	1.87
		4.01E-5*	1.85
VTN	Vitronectin	1.54E-05*	7.29
		1.72E-05*	7.03
FGF2	Fibroblast growth factor 2 (basic)	1.49E-09*	28.32
		1.48E-09*	28.51
FGF7	Fibroblast growth factor 7 (keratinocyte growth factor)	3.52E-07*	15.35
		3.52E-07*	15.36
FGF13	Fibroblast growth factor 13	3.26E-07*	8.10
		5.22E-07*	7.9
FGF5	Fibroblast growth factor 5	2.83E-05*	2.57
		3.50E-05*	2.48
FGF1	Fibroblast growth factor 1	7.48E-05*	2.12
		3.53E-05*	2.35

Receptor (gene symbol)		CD133+ *vs* CAF *P*-value CD133− *vs* CAF *P*-value	CD133+ *vs* CAF Fold change CD133− *vs* CAF Fold change
(c) CD133+ or CD133− cells vs CAF			
CXCR4	Chemokine (C–X–C motif) receptor 4	0.007*	5.26
		0.56	1.29
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	0.03*	3.32
		0.89	1.06
ITGβ8	Integrin β8	0.009*	2.04
		0.73	−0.107
FGFR2	Fibroblast growth factor 2	0.005*	1.48
		0.015*	1.22

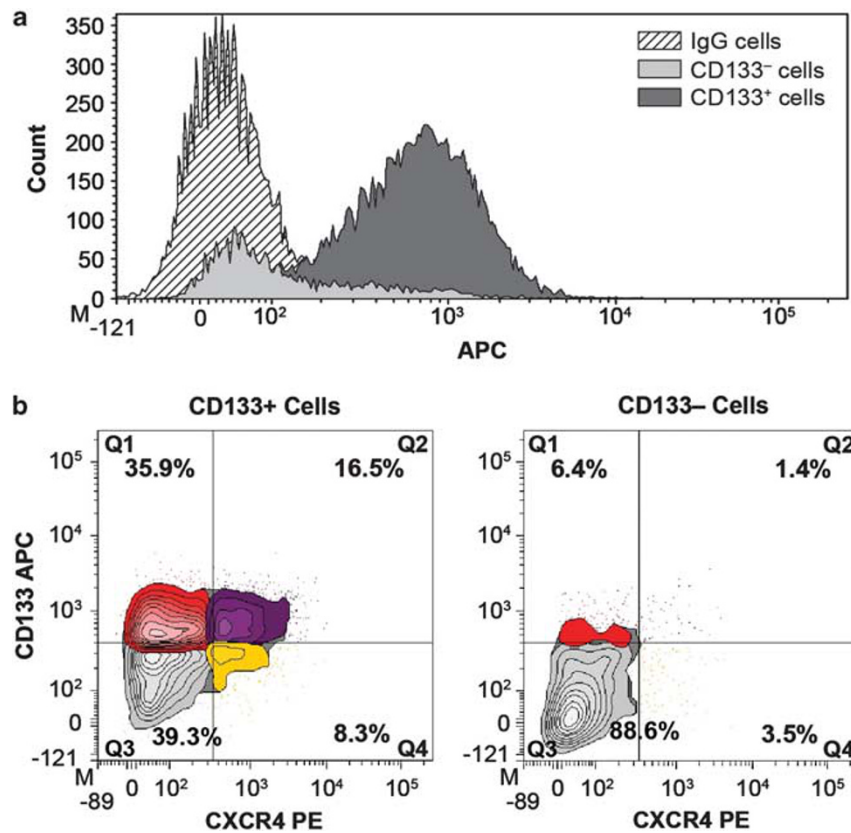*Statistical significance demonstrated at *P*-value ≤0.05.

Figure 5 Colospheres, enriched for CD133+ or relative lack of CD133 (CD133−), sorted by flow cytometry, were maintained in culture for up to 8 weeks. (**a**) Overlay of histograms (from left to right) comparing fluorescence of CD133+ cells labeled with immunoglobulin G-allophycocyanin (IgG-APC) (negative control), AC133-labeled CD133− cells and AC133-labeled CD133+ (enriched) cells. (**b**) After 8 weeks, the enriched cells each maintained their respective CD133 phenotype. Co-expression of cell surface CD133 and CXCR4 in CD133+ cells and CD133− cells.

(Figure 5b). The CD133− group maintained minimal CD133 and CXCR4 expression (Figure 5a and b).

To evaluate whether cell culture conditions affected CD133/CXCR4 expression, the cells were grown either in a suspension culture using low-adhesion flasks or allowed to adhere onto tissue-culture plastic. If the cells were grown in low-adhesion flasks, the cells grew in suspension as spheres, as expected (Figure 6a). However, if the cells were grown in standard tissue-culture plastic, after approximately 2 weeks, most of the cells settled to the bottom of the flask and grow as a monolayer in an anchorage-dependent manner (Figure 6b). If the colon cancer cells were allowed to grow as a suspension culture, there was marked enrichment of the double-positive (CD133+/CXCR4+) phenotype (32% of the total cell population) compared with the same cells grown as a monolayer (9% of the total cell population) (Figure 6c and d, respectively). Conversely, the CD133−/CXCR4− phenotype was increased to 46.7% of the total monolayer cell population if grown in adherent flasks compared with 29.8% if grown in suspension. Therefore, these results indicate that the anchorage-independent growth condition (suspension culture) preferentially enriched for the CD133+/CXCR4+ cell type,

whereas the anchorage-dependent growth condition selected for the CD133−/CXCR4− phenotype.

CD133+/CXCR4+ Cells have Functional CXCR4
As the gene array analysis revealed that CXCR4 was over-expressed fourfold in the CD133+ over the CD133− cells, we validated functional CXCR4 in CD133+ cells using two-color FACS and selecting for the relative presence or absence of CD133, as well as the relative presence or absence of CXCR4. CXCR4 is a G-protein-coupled receptor activated by the chemokine SDF-1α, initiating a signal-transduction cascade involving the generation of inositol triphosphate, release of intracellular calcium and activation of downstream kinases that affect cellular proliferation, migration and invasion.[23,34,35] An increase in intracellular calcium was measured in 75% of the double-positive (CD133+/CXCR4+) cells in response to ligand SDF-1α (400 ng/ml) (Figure 7a), confirming the presence of functional receptor. We used carbachol (10 μM) as a positive control to assure that the cells were adequately loaded with the calcium indicator dye Fura-2. The M_3 muscarinic receptor has been shown to be expressed in most human normal and colon cancer tissue tested,[36]
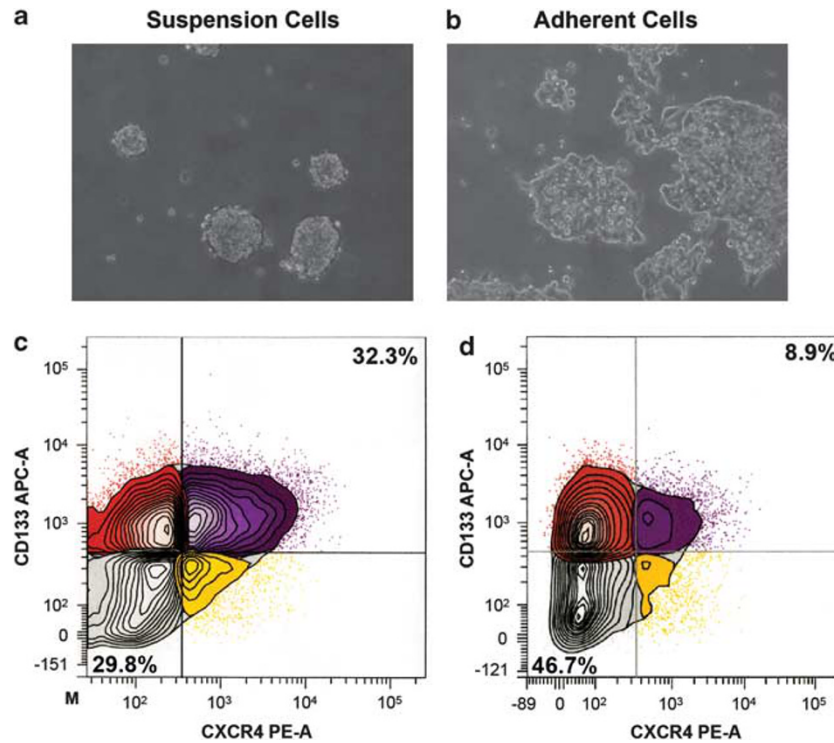
Figure 6 Effects of growth conditions (suspension *vs* attached) on co-expression of CD133 and CXCR4. (**a**) Photomicrograph ($\times$ 400) of colon cancer cells grown in suspension culture (low-adhesion flasks). The cells maintain anchorage-independent growth after 9 days. (**b**) Photomicrograph ($\times$ 400) of same cells grown in standard tissue culture flasks. After 9–14 days, the cells adhere to the bottom of the flask and exhibit a spread phenotype, consistent with anchorage dependence. (**c**) Fluorescence-activated cell sorting (FACS) analysis demonstrating that when grown in suspension culture, 32% of the cells are double-positive for cell surface CD133 and CXCR4, and that 30% of cells are double-negative for both receptors. (**d**) When the cells are allowed to adhere and spread, the double-positive cells decrease to 8.9%, while the double-negative cells increase to 46.7%.

and thus the acetylcholine agonist carbachol should elicit an increase in intracellular calcium on the cells (Figure 7a–c). As expected, SDF-1α did not stimulate a calcium response in the CD133 + /CXCR4− cell fractions nor in the CD133−/ CXCR4− cell fractions (Figure 7b and c, respectively), demonstrating the lack of functional CXCR4 in these cell sub-populations. This assay was not performed on the CD133−/ CXCR4 + cells because this fraction represented a small minority (<10% total cell population) and the amount of healthy live cells retrievable for this assay was insufficient for analysis.

SDF-1α Stimulates Growth in CD133 + /CXCR4 + Cells

CAF-derived SDF-1α has been shown to stimulate carcinoma cell growth directly through the CXCR4 receptor on the cancer cell.[23] Co-culture of CAF with the epithelial cells will not directly test whether SDF-1α can stimulate CD133 + / CXCR4 + cell growth because CAF secrete multiple growth factors, which may stimulate other CD133 + cell surface receptors. To eliminate potential confounding conditions, we performed *in vitro* growth assays by adding SDF-1α directly to the colospheres embedded in Matrigel. Cells were sorted with dual-color FACS for CD133 and CXCR4 and allowed to grow either in the presence of SDF-1α (400 ng/ml) or vehicle (0.2% BSA). After 30 days, both the total number of cell colonies and the mean diameter of each colony were assessed.

The CD133 + /CXCR4 + group treated with SDF-1α sustained the growth of more colonies compared with vehicle (80 ± 1.5 *vs* 67 ± 3.5, respectively; Figure 7d), as well as significantly larger colony sizes of tumor spheres (25 ± 0.01 *vs* $17 \pm 0.008 \, \mu m$ diameter, Student's *t*-test, $P < 0.0002$) (Figure 7g). The CD133 + /CXCR4− (Figure 7e) cells formed fewer tumor colonies (68 ± 2 colonies in the presence of SDF-1 *vs* 62 ± 4 colonies with vehicle) compared with the double-positive cells. However, unexpectedly, the CD133 + / CXCR4− cells treated with SDF-1α grew larger colonies compared with the same cells treated with vehicle (21 ± 0.009 and $16 \pm 0.006 \, \mu m$, respectively, *t*-test, $P < 0.0001$; Figure 7g). This result may be explained by the fact that the sorted CXCR4− cell fraction express relatively less CXCR4 compared with the CXCR4 + fraction, but is not completely depleted of all CXCR4 + cells. Finally, cell colonies characterized by CD133−/CXCR4− (Figure 7f) formed only 48 ± 3 and 26 ± 9.5 colonies in the presence of ligand and vehicle, respectively, with average size per colony of 16 ± 0.005 *vs* $14 \pm 0.01 \, \mu m$. CD133−/CXCR4 + cells were relatively rare and those that were retrieved after cell sorting did not survive in culture. In summary, cells co-expressing both CD133 and CXCR4, when treated with SDF-1α, sustained the greatest number of spheroid colonies and the largest diameter per colony of spheroid colonies.
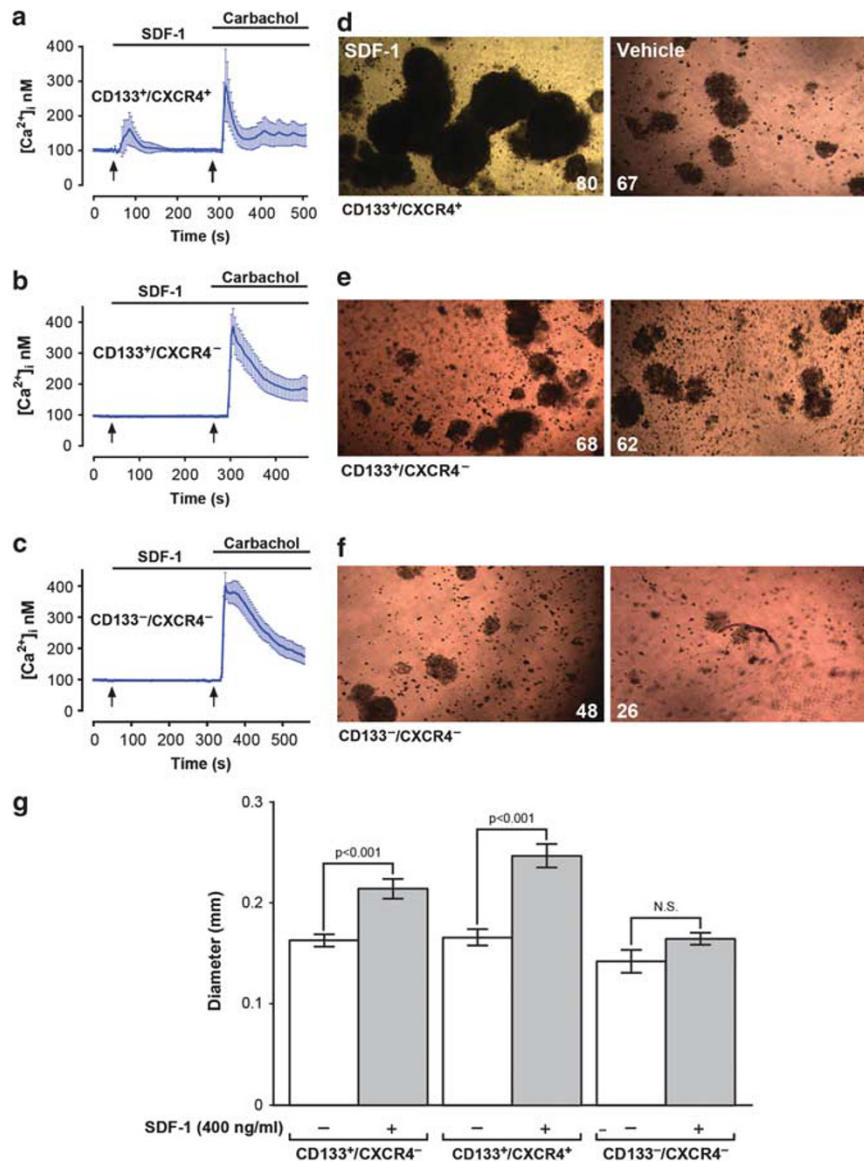
Figure 7 Characterization of CD133 + /CXCR4 + cells. (**a**) CD133 + /CXCR4 + cells exhibit an increase in intracellular calcium in response to 400 ng/ml of stromal-derived factor-1 (SDF-1) (CXCL12). Subsequent treatment with 10 μM carbachol ensures that the cells were loaded with Fura-2. (**b**) CD133 + /CXCR4 − cells do not respond to SDF-1; however, these cells do have muscarinic receptors, as verified by their calcium response to carbachol, indicating that the calcium indicator dye Fura-2 was loaded in the cells. (**c**) CD133 − /CXCR4 − cells fail to increase intracellular calcium with SDF-1, but does respond to carbachol. (**d–f**) Effects of cell growth in Matrigel in the presence of SDF-1 (400 ng/ml) or vehicle (0.2% bovine serum albumin (BSA)). The average number of cell colonies per well are denoted by the number in the corner of the photo. Photomicrographs (original magnification, × 400) of representative areas of CD133 + /CXCR4 − (**d**), CD133 + /CXCR4 + (**e**) and CD133 − /CXCR4 − (**f**) cell colonies are shown. (**g**) Bar graph depicting average diameter of the cell colonies, corresponding to figures shown in panels **d–f**.

CD133 + Cells are Spatially Located Adjacent to a Relative Abundance of CAF in Colorectal Cancer

To show that CD133 + cells are spatially in close proximity to CAF in the original tumor sample, we co-stained a formalin-fixed paraffin-embedded block for CD133 (shown in red) and α-SMA (brown) (Figure 8). CD133 − cells are not surrounded by α-SMA-positive CAF (Figure 8a), whereas, in contrast, the CD133 + cells are surrounded by CAF (Figure 8b). Using tumor samples from another 11 CD133 +

colon cancer specimens, we demonstrate that CD133 + cells have a greater propensity to be situated adjacent to CAF (α-SMA-positive cells) compared with CD133 − cells (Figure 9). Under an IRB-approved protocol, we performed dual staining of CD133 and α-SMA. Figure 8c demonstrates in another patient that the normal margin colonic villi are surrounded by some α-SMA-positive fibroblasts. The carcinoma from the same patient (as Figure 8c) is characterized by CD133 + surrounded by a relative abundance of CAF (left) compared
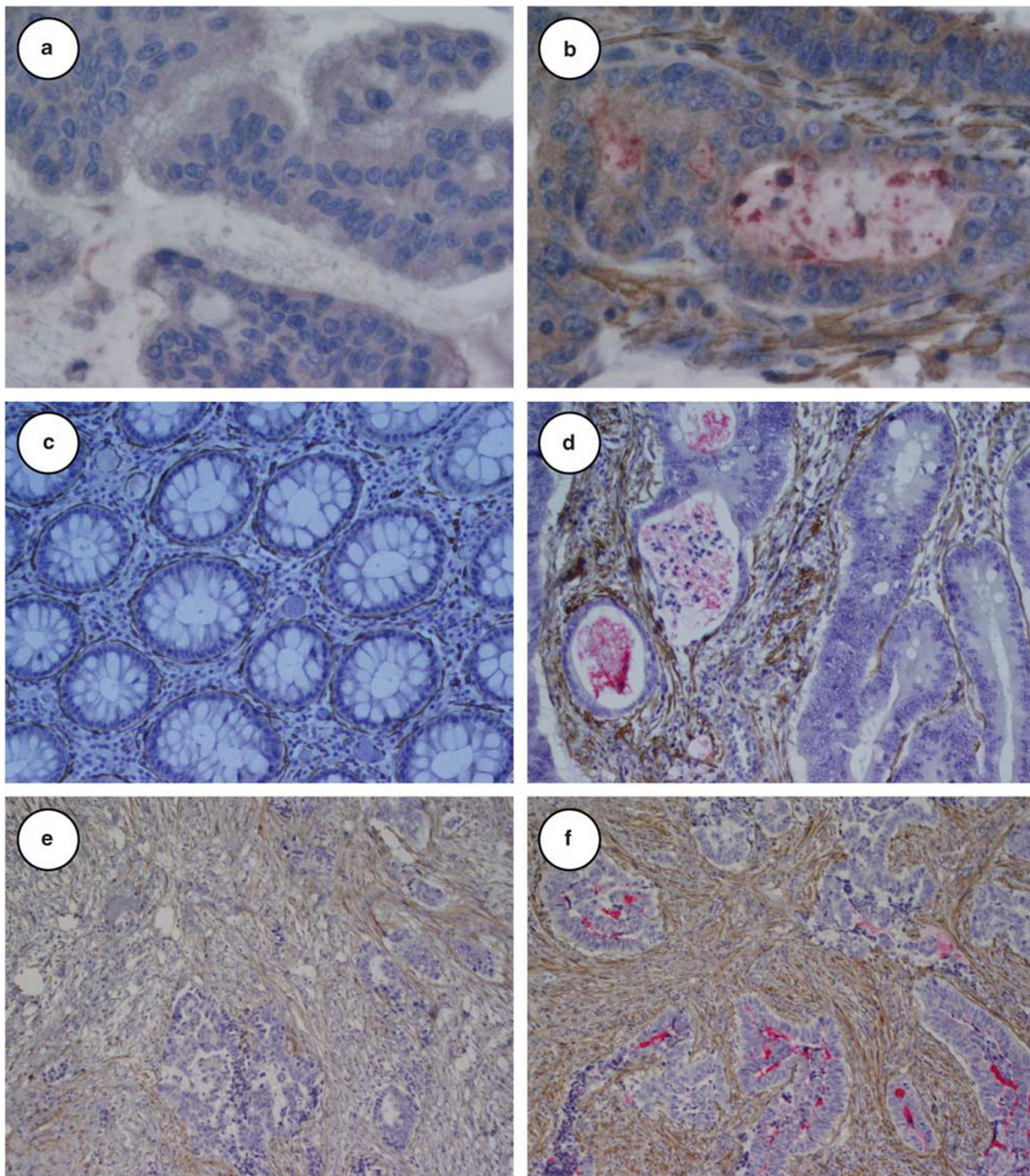
Figure 8 Co-staining of CD133 and α-smooth muscle actin (α-SMA) showing the spatial relationship between CD133 + cells and carcinoma-associated fibroblast (CAF), respectively. (**a**, **b**) Immunohistochemical characterization of the original patient tumor sample (original magnification, × 600) of a representative area with (**a**) CD133− colon cancer cells and paucity of CAF and (**b**) CD133 + colon cancer cells (red stain) surrounded by relative abundance of CAF (brown stain). (**c**, **d**) Representative area from another patient in the area of (**c**) normal colonic villi surrounded by α-SMA-positive (brown stain) and (**d**) malignant tumor with CD133 + (red) and CD133− cells, × 200. (**e**, **f**) Colon cancer from third patient in areas with (**e**) CD133− colon cancer cells and (**f**) CD133 + colon cancer cells (original magnification, × 100).
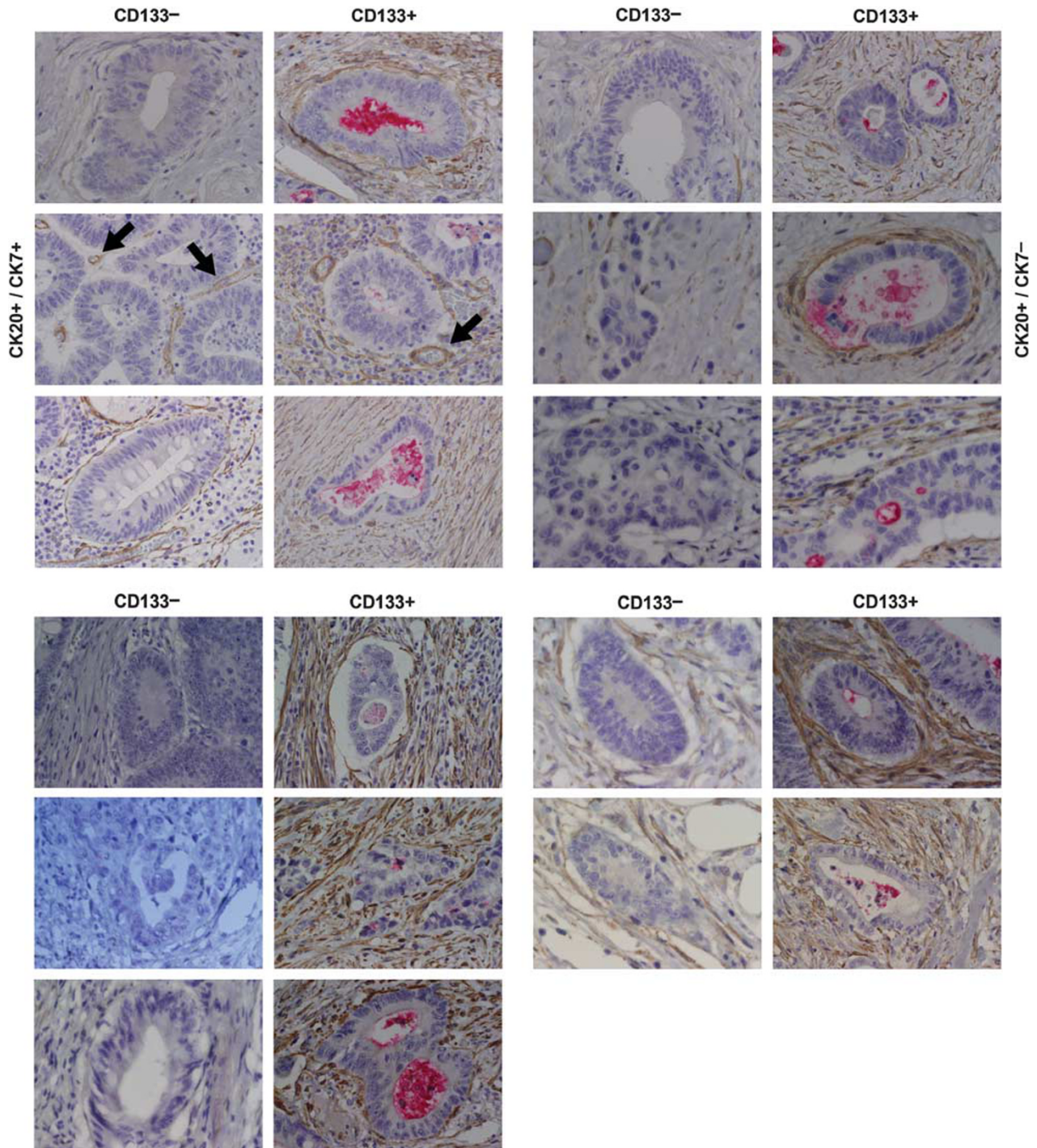
Figure 9 Paired samples of CD133− and CD133+ colon cancer cells from 11 colon cancers. The slides are co-stained for CD133 (red, apical) colon cancer cells and α-smooth muscle actin (α-SMA) (original magnifications, ×400 and ×600). The arrow demonstrates α-SMA immunopositivity in the walls of small blood vessels (internal positive control).

with CD133− cells (right, Figure 8d). In a third patient tumor sample, the CD133− cells are in close proximity to both α-SMA+ and α-SMA− CAF (Figure 8e); however, the CD133+ cells are surrounded by predominately α-SMA+ CAF. Close-up representative paired CD133− and CD133+

cells from the 11 additional patients are shown in Figure 9. In six of these patient samples, CK20 and CK7 staining were also performed. Three of the six colon cancer specimens were CK20+/CK7−, similar to the majority of colon cancers,[31] and the other three were double positive for CK20 and CK7

(data not shown). The close proximity of CD133 + cells to CAF support our hypothesis that CD133 + cells better poised to interact in a paracrine manner to promote tumor progression compared with CD133 − cells.

DISCUSSION

Experimental studies show that the CD133 + cells have a higher tumorigenic potential among the other malignant cells that comprise a solid tumor. Yet, increased cell proliferation, migration, invasion and tumorigenicity of colorectal cancer cells in nude mice cannot be attributable to a direct function of CD133 because siRNA-mediated knockdown of CD133 has demonstrated no significant differences in these measurable functions compared with non-targeted siRNA control cells.[17,18] Although the specific function of CD133 is unknown, the CD133 phenotype is clearly associated with more aggressive tumor characteristics such as chemotherapy resistance[37,38] and poor clinical outcome.[15,39] Previously, our understanding of the CD133 phenotype has been limited by the lack of a specific model to address the contributions of the other cells in the tumor microenvironment. In this study, we concurrently isolated the carcinoma cells, along with the CAF, and showed by gene microarray analysis that the CD133 + phenotype is characterized by increased co-expression of cell surface receptors to chemokines and growth factors, increased expression of proteases that can break down matrix proteins and decreased expression of matrix proteins. We have shown that the CD133 + cell is more tumorigenic in NOD-SCID mice (Figure 3c and d), and molecular profiling (Table 2) suggests that the CD133 + cells may have an advantage over CD133 − cells that allows them to cooperate with other cells in the stromal microenvironment, such as their companion CAF, to promote tumor growth, invasion and metastasis. We demonstrate marked overexpression of functional CXCR4 in a population of CD133 + cells compared with the CD133 − cells, and show that the paracrine CXCR4/SDF-1 signaling axis contributes to the growth advantage that CD133 + cells have over the CD133 − cells.

Zeelenberg et al[40] previously reported that CXCR4-deficient colon cancer cells can colonize the lung parenchyma to the same extent as CXCR4 + cells, but only CXCR4 + cells can grow in this metastatic niche. In this primary colorectal cancer, the CD133 +/CXCR4 + population is a candidate cell type that may be likely to become the metastatic cell type, by interacting with CAF and/or other cell types that secrete SDF-1α. Furthermore, our analysis revealed that CD133 + cells also overexpress other cell surface receptors that mediate proliferation and metastasis, all of which may contribute to the overall aggressive behavior of CD133 + cells in the tumor microenvironment: FGFR2, macrophage inhibitory factor receptor (CD74) and ITGβ8. The companion CAF derived from the primary colon cancer overexpress the gene transcripts for the activating ligands: the FGF family members, MIF and ECM protein vitronectin, respectively (Table 2b and c).

ITGβ8 exclusively pairs with αv (ITGAV) and has been shown to promote neurosphere proliferation and survival of in vitro neural progenitor cells.[41] ITGβ8 binds vitronectin, a matrix protein found only in the stroma of colorectal cancers and not normal colon mucosa,[42] implicating a potential role for β8 in the microenvironment of colon cancer. MIF is a cytokine secreted by immune cells, CAF and tumor cells upon inflammation and stress, and has been shown to be a non-cognate ligand of the chemokine receptor CXCR4.[43] CD133 + cells also have relatively increased gene transcript expression for proteases (SULF2, TMPRSS2, PRSS3, CTSO, CTSS) and marked decrease expression for ECM proteins (COL1A1, COL1A2, COL3A1, SPARC, FN1, CSPG2) (http://www.ncbi.nlm.nih.gov/geo). By breaking down the surrounding ECM components, proteases facilitate the invasion and metastatic spread of tumor cells, as well as release and activate growth and angiogenic factors. Future studies on CD133 + colon cancer and their CAF involving other receptor–ligand pairs will reveal potentially important paracrine signaling systems.

Other investigators have shown that engraftment of human tumors in mice is dependent on the specific mouse strain, which harbor the critical microenvironment necessary for supporting tumor cell growth. Kelly and co-workers[44] demonstrated that while only a few human acute myeloid leukemia cells can survive in irradiated NOD-SCID mice, a large proportion of murine lymphoid or myeloid malignant cells engrafted into non-radiated NOD-SCID mice, and these mice developed disseminated disease with very high frequency. Quintana et al[45] also confirmed that the tumorigenesis is very dependent on a permissive microenvironment. They found that human melanoma cells injected into NOD-SCID mice were significantly less tumorigenic (1:46 700 cells engrafted) compared with the same cells injected into the more immunocompromised NOD-SCID-IL2Rγ−/− mice (1:9 cells engrafted), which lack the interleukin-2γ receptor responsible for natural killer cell activity. Recently, Vermulen et al[46] demonstrated that CAF-derived hepatocyte growth factor activated c-met on colon cancer stem cells to promote cell growth via β-catenin-dependent transcriptional activation. Taken together, these recent studies underscore the importance of studying the colon cancer CD133 + cell phenotype in the context of its relevant microenvironment (eg, in association with the accompanying CAF). After the establishment of distant metastasis, CD133 expression is downregulated, and is no longer essential for the initiation of tumors in xenografts.[47] Shmelkov et al[47] showed that metastatic CD133 + colorectal cancer cells can propagate into both CD133 + and CD133 − cells, and both subpopulations subsequently have the ability to initiate tumorigenesis in NOD-SCID mice, suggesting that the metastatic cancer cells may have acquired alternative ways to sustain tumor growth that does not involve the CD133 cell surface marker. Future studies are needed to address the possibility that CD133 is important for the initiation and

progression of primary tumors, but not necessary after the tumor has successfully spread distantly.

Our data support the contention that the pro-tumorigenic potential of the CD133+ cancer cell type is dependent on specific microenvironmental determinants. Each primary colorectal cancer not only has a unique cancer genome, but also has specific reciprocal (paracrine) relationships between the epithelial and stromal components, all of which contribute to the biological behavior of that specific tumor, including the CAF, tumor-associated macrophages,[48] other hematopoietic cells and vascular endothelial cells.[49] In this report, we show that (1) CD133+ cells are a heterogeneous cell population and that (2) the co-expression of additional cell surface receptors, for example, CXCR4, mediates a critical paracrine signaling axis, indicating that the enhanced tumorigenic properties attributable to CD133 phenotype relies on the subpopulation of CD133+ cells that can interact with its microenvironment. Future studies, particularly *in vivo* tumorigenesis studies, are needed to verify a paracrine signaling loop between CAF and CD133+ colorectal cancers. In the emerging era of 'personalized medicine' for the treatment of cancers, it will be important to define patient-specific therapies based on the molecular characteristics of the individual's tumor, as well as the specific paracrine signaling axes promoted in the cancer microenvironment.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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