

## CD44 is of Functional Importance for Colorectal Cancer Stem Cells

Lei Du,<sup>1,2</sup> Hongyi Wang,<sup>3</sup> Leya He,<sup>4</sup> Jingyu Zhang,<sup>3</sup> Biyun Ni,<sup>1,2</sup> Xiaohui Wang,<sup>1</sup> Haijing Jin,<sup>1</sup> Nathalie Cahuzac,<sup>1</sup> Maryam Mehrpour,<sup>1,5</sup> Youyong Lu,<sup>3</sup> and Quan Chen<sup>1</sup>

**Abstract Purpose:** Both CD44 and CD133 were reported as putative markers for isolating colorectal cancer stem cells (CSC). It remains to be resolved if both of these markers are of functional importance for colorectal CSC.

**Experimental Design:** The expression of CD44 and CD133 in normal colonic tissues and primary colorectal cancer was assessed by immunohistochemistry in a series of 60 patients on tissue microarray sections. Both *in vitro* clonogenic and *in vivo* tumorigenic assay were applied to measure CSC activities from the cells isolated from patients. Lentiviral RNA interference was used to stably knock down CD44 or CD133 in colorectal cancer cells from patients.

**Results:** We found that CD44<sup>+</sup> cells displayed clustered growth and they did not colocalize with CD133<sup>+</sup> cells within colorectal cancer. As few as 100 CD44<sup>+</sup> cells from a patients' tumor initiated a xenograft tumor *in vivo*. A single CD44<sup>+</sup> cell from a tumor could form a sphere *in vitro* which has characteristic stem cell properties and was able to generate a xenograft tumor resembling the properties of the primary tumor. Knockdown of CD44, but not CD133, strongly prevented clonal formation and inhibited tumorigenicity in xenograft model.

**Conclusions:** These results indicate that CD44 is a robust marker and is of functional importance for colorectal CSC for cancer initiation.

Increasing evidence suggests that there is a small subset of cells, called cancer stem cells (CSC), that are responsible for cancer initiation and development (1). The concept of CSC emerged decades ago, and the best evidence came from acute myeloid leukemia in which the rare CD34<sup>+</sup>CD38<sup>-</sup> population was capable of propagating acute myeloid leukemia in a xenograft transplant system (2, 3). It is believed that CSC has the characteristics of normal adult stem cells including the self-renewal property and multipotency for differentiation into distinct types of cells (4, 5). It is a technical challenge to identify and to characterize the cancer-initiating cells with CSC proper-

ties due to the rarity of CSC in the tissue of origin and the lack of specific markers. Recently, CSC has been described in several solid tumors (6). These studies used putative stem cell markers or side populations to isolate unique subsets of cancer cells from different types of tumors. Indeed, these cells had a higher capacity to form clones *in vitro* and to generate xenograft tumors in immunodeficient mice (7). These markers included CD44, CD133, CD24, and CD166 that are also expressed in other types of normal cells. Among these markers, both CD44 and CD133 were widely used for isolating CSC from solid tumors.

CD44 is a transmembrane glycoprotein which participates in many cellular processes, including growth, survival, differentiation, and motility (8–10). It is a unique adhesion molecule and plays a role in cancer cell migration and matrix adhesion in response to a cellular microenvironment, thus enhancing cellular aggregation and tumor cell growth (11). CD44<sup>+</sup> cells were breast CSC which possessed higher tumorigenicity and metastatic potential (12). CD44 marker was also used to isolate prostate CSC (13, 14), pancreatic CSC (15), and colorectal CSC (16). CD133 is also a cell surface transmembrane glycoprotein which exists in the cholesterol-rich domain of lipid rafts. Although its cellular function is not clear, CD133 is an important marker for a number of different CSC lineages (17). CD133 was used to isolate CSC from brain (18, 19), prostate (14), liver carcinoma (20), and colon carcinoma (21, 22). In several independent reports, both CD44 and CD133 were used as markers for isolating colorectal CSC. This raises the question of whether both markers are applicable to colorectal CSC. It also remains to be resolved if these markers are of functional importance for colorectal CSC. Clarification of this question and a better understanding of the properties of CSC may be useful to develop better therapeutic approaches to fight cancer. Here, we provide evidence showing that

**Authors' Affiliations:** <sup>1</sup>Joint Laboratory of Apoptosis and Cancer Biology, the State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, and College of Life Sciences, Nankai University, <sup>2</sup>Graduate University of the Chinese Academy of Sciences, <sup>3</sup>Laboratory of Molecular Oncology, Peking University School of Oncology, Beijing Cancer Hospital & Institute, Beijing, China, <sup>4</sup>Cancer Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, and <sup>5</sup>INSERM U756, Faculté de Pharmacie, Université Paris-Sud, Chatenay-Malabry, France

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**Requests for reprints:** Quan Chen, The Laboratory of Apoptosis and Cancer Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P.R. China. Phone: 86-10-6480-7321; Fax: 86-10-6480-7321; E-mail: chenq@ioz.ac.cn or Youyong Lu, Laboratory of Molecular Oncology, Peking University School of Oncology, Beijing Cancer Hospital & Institute, Beijing 100142, P.R. China. Phone: 86-10-8819-6765; E-mail: yongylu@public.bta.net.cn.

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## Translational Relevance

Cancer stem cells (CSC) represent an exciting avenue for cancer study and novel target for drug discovery. In this study, we showed that CD44 was a robust colorectal CSC marker and it is of functional importance for colorectal CSCs using clinic samples and xenograft models. The expression of CD44 and CD133 was assessed in primary tumors and matched normal tissues of patients with colorectal cancer (CRC) by using immunohistochemistry and flow cytometric analysis. We found that CD133 and CD44 did not appear on the same region of tumor tissue and it is infrequent to detect that both markers coexisted in the same cell. Knockdown of CD44 expression by RNA interference or inhibition of its function by specific antibodies could significantly inhibit tumor initiation and development in nude mice. These results indicate that CD44 and its related signaling pathway could be a critical diagnostic and therapeutic target for CRC.

CD44-positive and CD133-positive cells do not colocalize in the same region of colorectal cancer tissues. A single cell can form a sphere *in vitro* which can initiate a xenograft tumor resembling the properties of primary tumors. Knockdown of CD44, but not CD133, strongly prevents the initiation of a tumor *in vivo*.

## Materials and Methods

**Patients, reagents, and animals.** Sixty fresh colorectal cancer and matched normal colon samples were collected from the tumor bank of Beijing Cancer Hospital (Beijing, China) and Tongji Hospital (Wuhan, China), as approved by the Research Ethics Board at the Beijing Institute for Cancer Research. The patients were fully informed and in agreement with the collection of clinical samples.

All antibodies used included CD44 (DF1485, Santa Cruz, with 0.1% sodium azide), CD44 (2C5, R&D), CD44-FITC (G44-26, BD PharMingen), CD133 (AC133, Miltenyi, with 0.1% sodium azide), CK20 (Ks20.8, DAKO), CDX2 (AMT28, ZSBio), bromodeoxyuridine (BrdUrd, 3D4, BD PharMingen), and Oct3/4 (C-10, Santa Cruz). Trizol and the reverse transcriptase kit were purchased from Invitrogen, other reagents were purchased from Sigma unless specified otherwise. Four-week-old female nude mice (BALB/c-*nu/nu*) were purchased from the Chinese Academy of Military Medical Sciences and maintained in standard conditions according to institutional guidelines.

**Tissue microarray construction and immunohistochemistry.** For each patient (60 in total), we sampled five tissues including three tumors and two matched normal colonic tissues by cylinders of 0.6 mm in diameter to construct the tissue microarray. Four-micrometer-thick sections of these tissue microarray blocks were transferred to aminopropylthoxysilane-precoated glass slides.

Formalin-fixed paraffin-embedded tissue sections were dewaxed in xylene, rehydrated with distilled water and boiled in EDTA antigen retrieval buffer (pH8.0). Then the sections were incubated with antibodies and the staining was completed by using avidin-biotin complex systems (DAKO) and 3,3'-diaminobenzidine substrate chromogen (ZSBio) and followed by hematoxylin counterstaining.

**Flow cytometric cell sorting.** Fresh normal colonic tissue and colorectal cancer were rinsed in DMEM (Life Technologies) supplemented with 200 units/mL of penicillin, 200 µg/mL of streptomycin, and 4 units/mL of amphotericin B and minced, followed by incubation

with 300 units/mL of collagenase at 37°C for 3 h. A single cell suspension was obtained by filtration through a 40 µm filter. After discarding lymphocytes by gradient centrifuge, the cell suspension was incubated with a fluorochrome-conjugated antibody on ice for 20 min and then sorted by FACSDiVa Option (BD PharMingen). The sorted cells were re-run through the flow cytometer and purity was determined using quality controls.

**BrdUrd labeling and immunofluorescent staining.** Cells were treated with 10 µg/mL of BrdUrd for 12 to 24 h before being fixed by 3.7% paraformaldehyde. After being permeabilized in 0.2% Triton, cells were treated with 1 mol/L of HCl on ice for 10 min and then 2 mol/L of HCl at 37°C for 20 min.

Immunofluorescent staining was done on paraformaldehyde-fixed cells and clones as mentioned on the above. Usually, permeabilized cells were subsequently incubated with specific antibodies. We used 4',6-diamidino-2-phenylindole to stain nuclei before mounting the slice using a Mowiol mounting solution.

**Clonal formation and clonogenic assays.** For clonal formation assays, cells were diluted and seeded at a low density (100-1,000 cells per well) in six-well tissue culture dishes or (0.5 cell per well) in 96-well plates, and cultured in DMEM containing 5% of heat-inactivated FBS (fetal bovine serum, Hyclone). After plating, each well was examined under a phase contrast microscope. A single cell from 96-well plates was checked daily and maintained in DMEM/5% fetal bovine serum. The clones were stained with crystal violet to calculate clonal formation *in vitro*.

For clonogenic assays, cells were plated at 1,000 cells per well in six-well culture dishes precoated with a thin layer of 1.2% agar. Spheres or spheroids that arose within 2 weeks were presented as clonogenicity. Triplicate samples were performed for each cell type and at least two independent experiments were done as the above clonal formation assay.

**In vivo tumorigenicity assay.** BALB/c-*nu/nu* 4.5-week-old athymic mice (nude mice, 5-10 per group) were injected s.c. with 100 to 2,000,000 cells, monoclonal, or single cell-derived spheres in 100 µL of a mixture of DMEM/Matrigel (1:1). Tumors were measured by a slide gauge every 3 to 4 days and tumor volume was calculated as length × width × width / 2. Tumor latency and incidence were also recorded till 90 days after transplantation.

**Adhesion assay.** The assay was done in 48-well plates precoated with 5 µg/mL of hyaluronan overnight. After an additional incubation with 0.2% bovine serum albumin for 2 h,  $2 \times 10^5$  cells were seeded onto the coated substrate and incubated at 37°C for 1 h. Wells were washed twice and the adherent cells were quantified by counting the number of cells in four random visual fields under a microscope (10×), cells on hyaluronan-free wells were used as negative controls.

**RNA interference.** Target sites were designed on the Invitrogen web site. For each gene, we designed three pairs of oligos to choose the effective one. CD44 small interfering RNA (siRNA) sequences were AGCTCTGAGCATCGGATTT, TGGCTGATCATCTTGGCAT, and CACCTCCCAGTATGACACA; sequences targeting CD133 were GGA-CAAGGCGTTCACAGAT, GCATTGGCATCTTCTATGG and GCTAG-GAGGCGGAATTCTT. The recombinant lentivirus pseudotyped particles were generated using a triple plasmid system provided by Professor Guangxia Gao (Institute of Biophysics, Chinese Academy of Sciences, Beijing, P.R. China). Briefly, the constructed vector was cotransfected with the vesicular stomatitis virus envelope glycoprotein and the murine leukemia virus gag/pol expression plasmid (pHIT60) in 293T cells. The virus-containing culture medium was harvested and cells were infected directly. The RNA interference efficiency was verified by Western blotting and reverse transcription-PCR.

**Western blotting.** Minced frozen tissues or cells were fractionated in lysis buffer (150 mmol/L NaCl, 25 mmol/L HEPES, 1% NP40, 50 µmol/L phenylmethylsulfonyl fluoride, 50 µg/mL trypsin inhibitor, 3 mmol/L EDTA, 8 mmol/L EGTA, and 1 mmol/L DTT) on ice. After gentle homogenization, lysates were centrifuged at 1,000 × g for 5 min to remove unbroken cells and nuclei. Proteins (20-80 µg) were loaded onto SDS-PAGE gels then transferred onto nitrocellulose membranes as described previously (23), and probed with the indicated antibodies

followed by the appropriate secondary antibodies and enhanced chemiluminescence visualization.

**Statistical analysis.** Data are expressed as the mean  $\pm$  SE. Statistically significant differences were determined by Student's *t* test and  $\chi^2$  analysis, where appropriate, and defined as  $P < 0.05$ .

## Results

**CD44<sup>+</sup> cells display clustered growth in colorectal cancers and do not colocalize with CD133<sup>+</sup> cells in the same region.** We first analyzed the expression of CD44 and CD133 in fresh colorectal cancer samples and normal colonic tissues from the same patients. The characteristics of the eight patients enrolled in the study are listed in Table 1. Reverse transcription-PCR analysis showed that CD44 expression was up-regulated in colorectal cancer compared with the adjacent normal colonic tissues (Supplementary Fig. S1). Western blotting analysis confirmed the differential expression identified by reverse transcription-PCR and showed that, in most of the cancer tissues, CD44 was overexpressed. CD44 isoforms of v9, v6, and v6-10 were increased in most, but not all, tumors as detected by reverse transcription-PCR and Western blotting (Supplementary Fig. S1). In contrast, the expression of CD133 did not show up-regulation in colorectal cancer compared with that in normal colonic tissues (Fig. 1A).

Because the protein levels of these markers from the extracts of tissues may not necessarily equate to the expression in a rare subpopulation, we next determined anatomical distribution and examined CD44 and CD133 expressions in normal colonic tissues and paired colorectal cancers by immunohistochemistry on tissue microarray sections. In normal tissue, besides lymphocytes in colon, we detected some CD44<sup>+</sup> cells located at the bottom of the crypt where the normal stem cells existed as reported by Gorham et al. (ref. 24; Fig. 1B). In contrast, CD133<sup>+</sup> cells were located at the luminal surface of the gut, indicating the differentiated status of colonic cells. Both CDX2 (caudal type homeobox transcription factor 2), a specific marker for differentiated colorectal epithelium, and CK20 (cytokeratin 20), which is essentially restricted to differentiated intestinal epithelium allowed us to distinguish the differentiated epithelium.

In colorectal cancer specimens, we detected no more than 5% CD44<sup>+</sup> cancer cells that showed the clustering phenotype in most specimens examined. Each cluster contains approximately 10 to 30 cells in the section amid CD44<sup>+</sup> cells (Fig. 1C). However, we detected weak CD133 signals in most of the colorectal cancers and these CD133<sup>+</sup> cells were scattered, whereas in a few samples (2/60), there were >40% of CD133<sup>+</sup> cells. Serial section followed by immunohistochemical analysis clearly revealed that CD44<sup>+</sup> and CD133<sup>+</sup> cells did not colocalize in the same region (see comparison in Fig. 1D).

To further determine if both markers coexist in the same cancer-initiating cells, we analyzed the existence of CD44 and CD133 subpopulations from the same patient samples using flow cytometry. The percentage of CD44<sup>+</sup> cancer cells ranged from 0.2% to 5.4% depending on individual patients, whereas the CD133<sup>+</sup> cancer cells ranged from 0.06% to 43.08% (Table 1; Fig. 2A). Double-positive cells were extremely rare in most colorectal cancer samples except in patient 3 in which 43.08% of CD133<sup>+</sup> cells and only 3.2% CD44<sup>+</sup>/CD133<sup>+</sup> cancer cells existed.

**CD44<sup>+</sup> cells have higher clonal formation capacities in vitro and robust tumorigenicity in xenograft model.** To examine which of these markers are more important for CSC, we next compared the clonal potential of the tumor-derived cells from patients 1 to 6 listed in Table 1 at the presence of CD44 or CD133. The purities of the cells sorted by fluorescence-activated cell sorting (BD FACSDiVa option) were >95% as analyzed by flow cytometry and immunofluorescence (data not shown). Clonal formation assay showed that 500 CD44<sup>+</sup> colorectal cancer cells from patient 6 formed  $32 \pm 5$  clones, whereas CD44<sup>-</sup> cancer cells from the same sample formed only  $4 \pm 2$  clones (Fig. 2B). These data showed that CD44<sup>+</sup> colorectal cancer cells had much greater clonal formation capacities than that of CD44<sup>-</sup> cancer cells.

To determine the efficacy of tumor initiation from cells with or without CD44 markers, we performed limiting dilution experiments. A variable number of human cells, ranging from 100 to 2,000,000, were injected into nude mice to test their xenotumor abilities. As few as 100 CD44<sup>+</sup> cells from three out of six patients were sufficient to generate a tumor within 28 days after implantation, whereas the number of CD44<sup>-</sup> cells that had the similar capacity was more than 10,000 (Table 2). At the 65th day, the average volume of xenograft tumors from 100 CD44<sup>+</sup> cells was  $14 \pm 5.7$  mm<sup>3</sup>, whereas from 2,000,000 CD44<sup>-</sup> cells of the same patient, the tumor volume was only  $6.4 \pm 2.2$  mm<sup>3</sup> at the 90th day. In contrast, cells from normal colons failed to generate any tumors in nude mice. We also isolated CD44<sup>+</sup>/CD133<sup>+</sup> cancer cells from patient 3 and found that tumorigenicity did not increase compared with that of CD44<sup>+</sup>-only cancer cells from the same patient. These data showed that the tumorigenicity of CD44<sup>+</sup> colorectal cancer cells was 100 times higher than CD44<sup>-</sup> cells and that CD133 did not increase tumorigenicity *in vivo*.

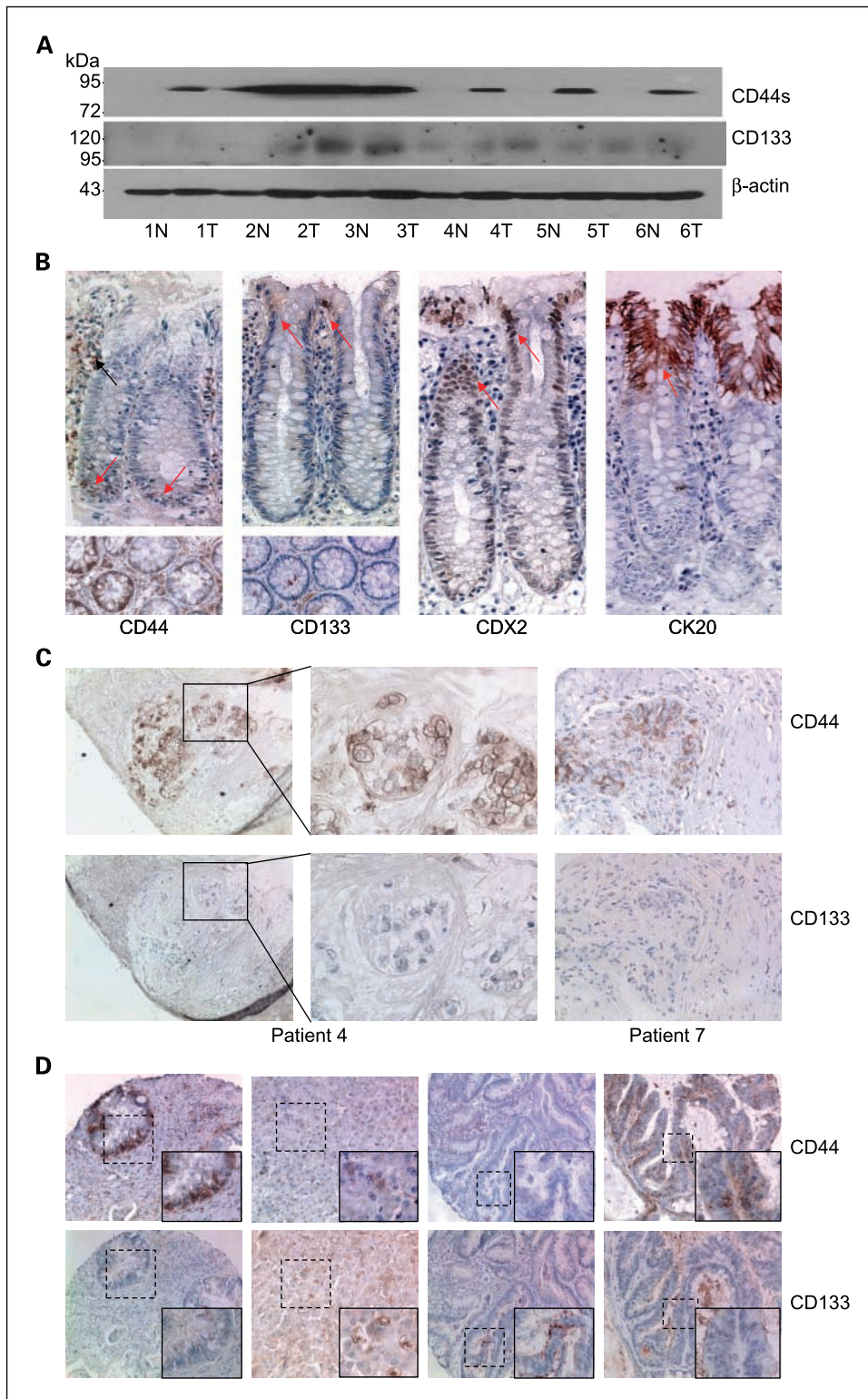
The percentage of CD44<sup>+</sup> cells and CD44<sup>+</sup> clusters increased significantly in xenograft tumors, as measured by flow cytometric assay (Supplementary Fig. S2) and immunohistochemical analysis (Fig. 2C). Interestingly, we found that CD44<sup>+</sup> cells also resided in clusters in xenograft tumors, resembling some of the primary colorectal cancers (Fig. 1C). Immunohistochemical assay also revealed that CD133<sup>+</sup> cells have scattered distribution. Furthermore, the similarity of morphologic characters of the primary cancer and their xenografts generated from CD44<sup>+</sup> cells were shown by H&E staining (Fig. 2D). In xenograft tumors, we noticed fewer CDX2 and CK20 expressions which

**Table 1. Patients and tumor characteristics**

Case no.	Gender	Age (y)	Site	Stage	CD44 <sup>+</sup> (%)	CD133 <sup>+</sup> (%)
1	Male	70	Sigmoid	T <sub>3</sub> N <sub>2</sub> M <sub>0</sub>	0.5	UD
2	Male	49	Left	T <sub>2</sub> N <sub>2</sub> M <sub>0</sub>	5.7	0.15
3	Male	62	Rectal	T <sub>2</sub> N <sub>1</sub> M <sub>0</sub>	4.0	43.08
4	Male	82	Right	T <sub>3</sub> N <sub>2</sub> M <sub>1</sub>	3.7	0.06
5	Female	60	Rectal	T <sub>4</sub> N <sub>1</sub> M <sub>1</sub>	5.4	UD
6	Male	53	Sigmoid	T <sub>4</sub> N <sub>2</sub> M <sub>1</sub>	0.2	1.16
7	Male	48	Sigmoid	IV	UD	UD
8	Male	59	Rectal	III	UD	UD

NOTE: For the eight primary colorectal cancers, histologic analysis by the Department of Pathology revealed the stages of the tumors. CD44 and CD133 in the first six patients were determined by flow cytometry.

Abbreviation: UD, undetected.



**Fig. 1.** Expression of CD44 and CD133 in normal colonic tissues and colorectal cancer. *A*, the expression level of *CD44* and *CD133* in normal colon (*N*) and colorectal cancer (*T*) determined by Western blotting.  $\beta$ -Actin was used as the loading control. *B*, representative immunohistochemical analysis of normal colon stained with the indicated antibodies.  $CD44^+$  lymphocytes (*black arrow*) in submucosal and epithelial cells (*red arrow*) located at the bottom of the crypt.  $CD133^+$  colonic cells (*red arrow*) are at the luminal surface of colonic guts (magnification,  $\times 20$ ). Bottom left, transsections of colonic tissue (magnification,  $\times 40$ ). Two markers, CDX2 and CK20, are used to determine differentiated cells (magnification,  $\times 20$ ). *C*, colorectal cancer stained with the indicated antibodies (magnification,  $\times 20$ ). Insets for patient 4 shown at higher magnification (magnification,  $\times 40$ ). *D*, serial sections of tissue microarray show that  $CD44^+$  and  $CD133^+$  cancer cells do not colocalize in the same region in patients 2, 6, 8 and the enriched  $CD133^+$  cells in patient 3 (magnification,  $\times 10$ ).

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revealed a less differentiated status compared with primary colorectal cancer. The primary xenografts and tumors passaged into secondary and tertiary recipients have similar or even higher capacities to form tumors (Supplementary Table S1). These data showed that  $CD44^+$  cells were colorectal cancer-initiating cells and that these cells could develop into a xenograft

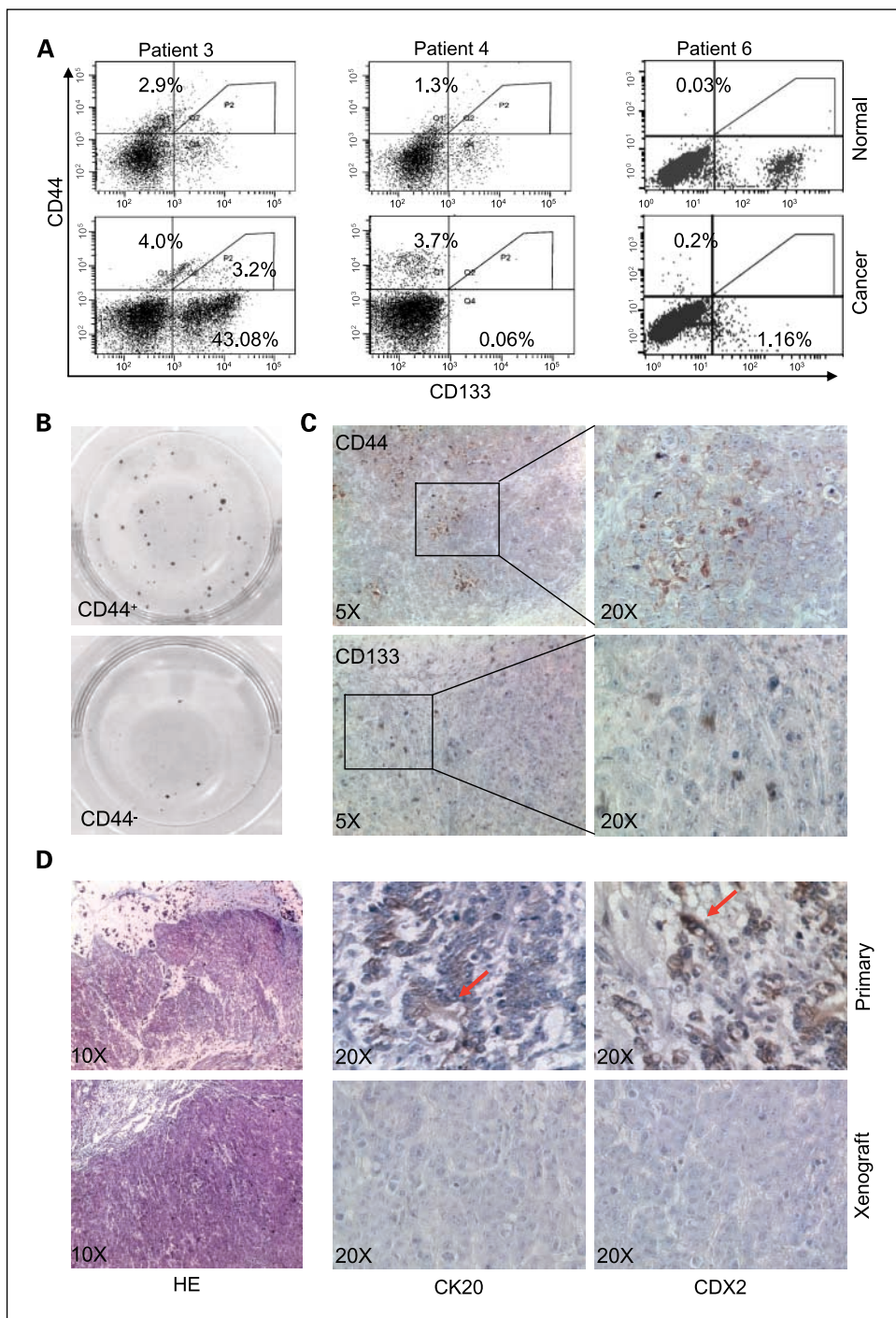
tumor, which had a distinct morphologic similarity and more undifferentiated characteristics compared with primary cancer from patients.

*Single  $CD44^+$  colorectal cancer cells can form a sphere which has certain stem properties.* The ultimate test for stem cell properties of the tumor-initiating cells is if these cells can

form a tumor from a single cell. To this end, we seeded the sorted CD44<sup>+</sup> or CD133<sup>+</sup> colorectal cancer cells into a 96-well plate. Cultured cancer cells have shown distinct morphologies called holoclones, meroclones, and paraclones (25). Holoclones consist of tightly packed small cells, whereas paraclones contain larger and fewer cells. Indeed, we observed that 13.5% (21/156) of CD44<sup>+</sup>/CD133<sup>-</sup> and 15.8% (18/114) of CD44<sup>-</sup>/CD133<sup>+</sup> colorectal cancer cells formed paraclones, whereas 1.28% (2/156) of CD44<sup>-</sup>/CD133<sup>-</sup> and 1.8% (2/114) of CD44<sup>+</sup>/CD133<sup>+</sup> cells generated holoclones in 9 days

(Fig. 3A). For CD44<sup>+</sup> cells, 24% of them divided and formed holoclones and further generated sphere-like clones from a single cell (Fig. 3B).

To further identify if the CD44<sup>+</sup> clones have true stem cell property, we used BrdUrd to label proliferating cells in the sphere-like clones. Interestingly, we found that the great majority of BrdUrd<sup>+</sup> proliferative cells were at the edge of the clones, and all the BrdUrd<sup>+</sup> cells were CD44<sup>+</sup>. In contrast, the vast majority (82.5%) of CD44<sup>-</sup> were in the center of the clone where only 6.8% of BrdUrd<sup>+</sup> cells were detected (Fig. 3C).



**Fig. 2.** CD44<sup>+</sup> colorectal cancer cells are more tumorigenic than CD44<sup>-</sup> and CD133<sup>+</sup> cells. *A*, flow cytometric assay of CD44<sup>+</sup> CD133<sup>+</sup> cells in normal colon and paired colorectal cancer in patients 3, 4, and 6. *B*, 500 sorted CD44<sup>+</sup> and CD44<sup>-</sup> colorectal cancer cells from patient 6 were seeded on six-well plates, clones were visualized by crystal violet staining. *C*, enrichment of CD44<sup>+</sup> cells in xenograft tumors by immunohistochemistry. *D*, H&E staining of the tumors generated from CD44<sup>+</sup> (patient 6) shows similar histologic features to the corresponding patient's primary colorectal tumor (magnification,  $\times 10$ ; *left*). Representative immunohistochemistry with the indicated antibodies. Arrows, positive signals (magnification,  $\times 20$ ; *middle and right*).

**Table 2.** Limiting dilution analysis of the human colorectal cancer initiation cells

Case	No.	Incidence	Latency (d)	No.	Incidence	Latency (d)
		CD44 <sup>+</sup>			CD44 <sup>-</sup>	
Patient 2	100	1 of 5	28	100	0 of 5	
	1,000	2 of 5	23	1,000	0 of 5	
				10,000	1 of 5	82
				2,000,000	1 of 5	71
Patient 4	100	2 of 5	32	100	0 of 5	
	1,000	2 of 5	28	1,000	0 of 5	
				10,000	0 of 5	
				2,000,000	1 of 5	58
Patient 6	100	1 of 5	49	100	0 of 5	
	1,000	2 of 5	34	1,000	0 of 5	
				10,000	0 of 5	
				2,000,000	2 of 5	65
		CD44 <sup>+</sup> /CD133 <sup>-</sup>		CD44 <sup>-</sup> /CD133 <sup>+</sup>		
Patient 3	1,000	0 of 5		1,000	0 of 5	
	10,000	1 of 5	48	10,000	0 of 5	
	100,000	2 of 5	43	100,000	1 of 5	69
		CD44 <sup>+</sup> /CD133 <sup>+</sup>		CD44 <sup>-</sup> /CD133 <sup>-</sup>		
	1,000	0 of 5		1,000	0 of 5	
	10,000	1 of 5	45	10,000	0 of 5	
	100,000	2 of 5	38	100,000	0 of 5	

NOTE: Freshly isolated subpopulations of colorectal cancer cells were s.c. injected in Matrigel into female nude mice to generate tumors in mouse xenografts. The success rate is shown by tumor incidence (described as the number of tumors developed/number of injections) and latency (time in days when animals generated the first xenograft tumor).

Transplanting these clones subcutaneously into nude mice showed that all of them (eight of eight) were tumorigenic.

In order to achieve optimized conditions for sphere formation, we precoated dishes with 1.2% agarose which could reduce the attachment of the cells to the plate. The sorted CD44<sup>+</sup> cancer cells proliferated in spheres in DMEM supplemented with 5% fetal bovine serum (Fig. 3D). Although CD44<sup>-</sup> cells also proliferated to some extent *in vitro*, most of them could not form large spheres on the agarose. Single CD44<sup>-</sup>/CD133<sup>+</sup> cancer cells failed to grow and form spheres as previously reported (21). Immunofluorescent labeling showed that in CD44<sup>+</sup> spheres, there were Oct3/4<sup>+</sup> cells (data not shown). In these spheres, we detected 98.82% CD44<sup>+</sup> and 56.13% CD133<sup>+</sup> cells. Based on the differentiation markers such as CDX2 and CK20, there were 20.97% and 18.21% positive cells in spheres, respectively.

We then randomly picked spheres carefully with a needle and transplanted them subcutaneously into nude mice. We found that all these CD44<sup>+</sup> spheres (10 of 10) generated xenograft tumors in nude mice within 36 days. Collectively, these data suggested that CD44<sup>+</sup> cells have certain stem cell properties.

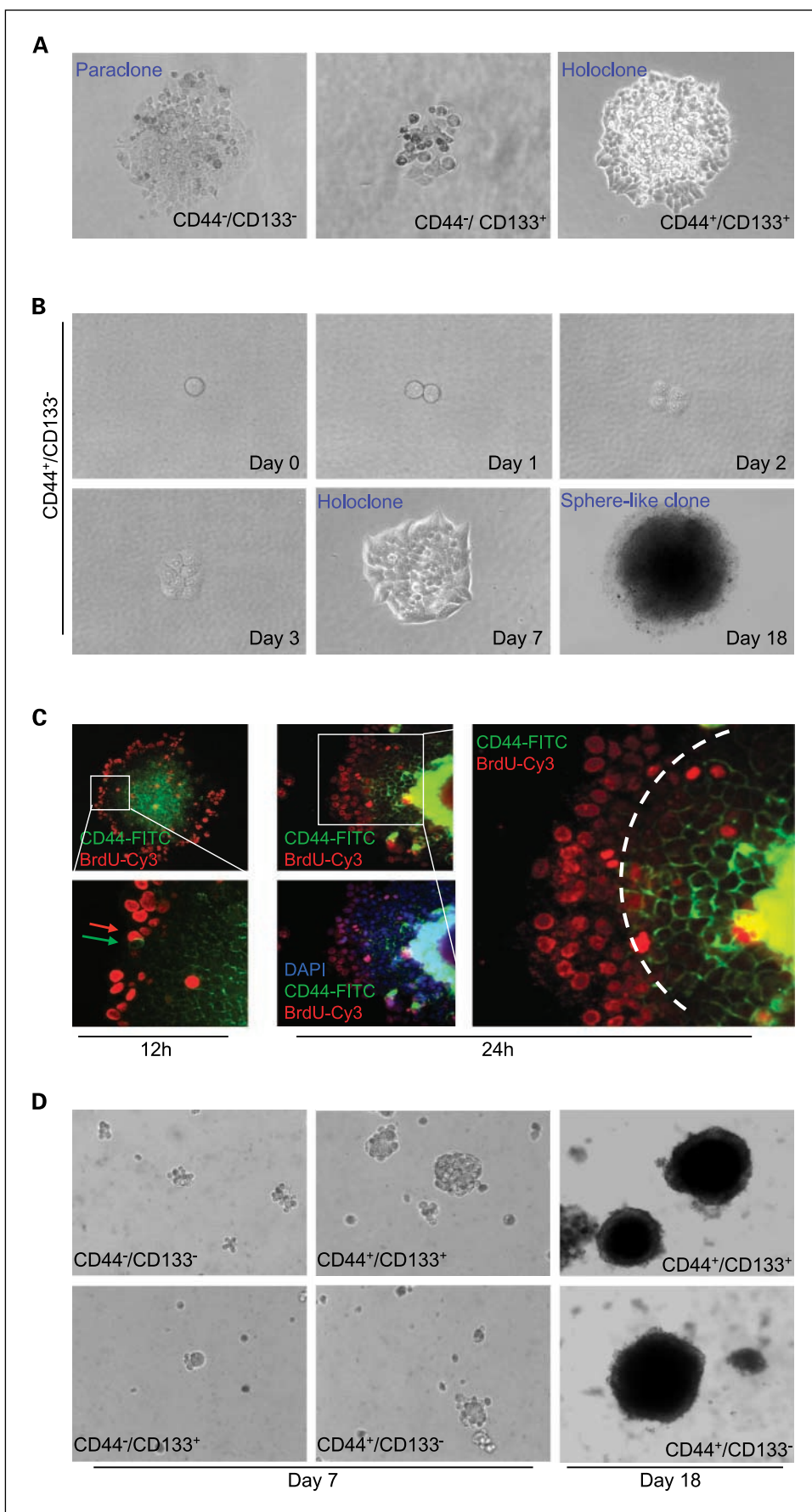
**Knockdown of CD44 reduces clonal formation in vitro and tumorigenicity in vivo.** To understand if CD44 or CD133 are of functional importance for tumor initiation, we used lentiviral RNA interference to stably knock down CD44 or CD133 in cancer cells from patient 6. Western blotting showed that ~80% of CD44 cells and 93% of CD133 cells were blocked, respectively (Fig. 4A). We then found that knockdown of CD44 significantly reduced clonal formation, whereas CD133 siRNA had little effect compared with that of the scrambled siRNA (Fig. 4B).

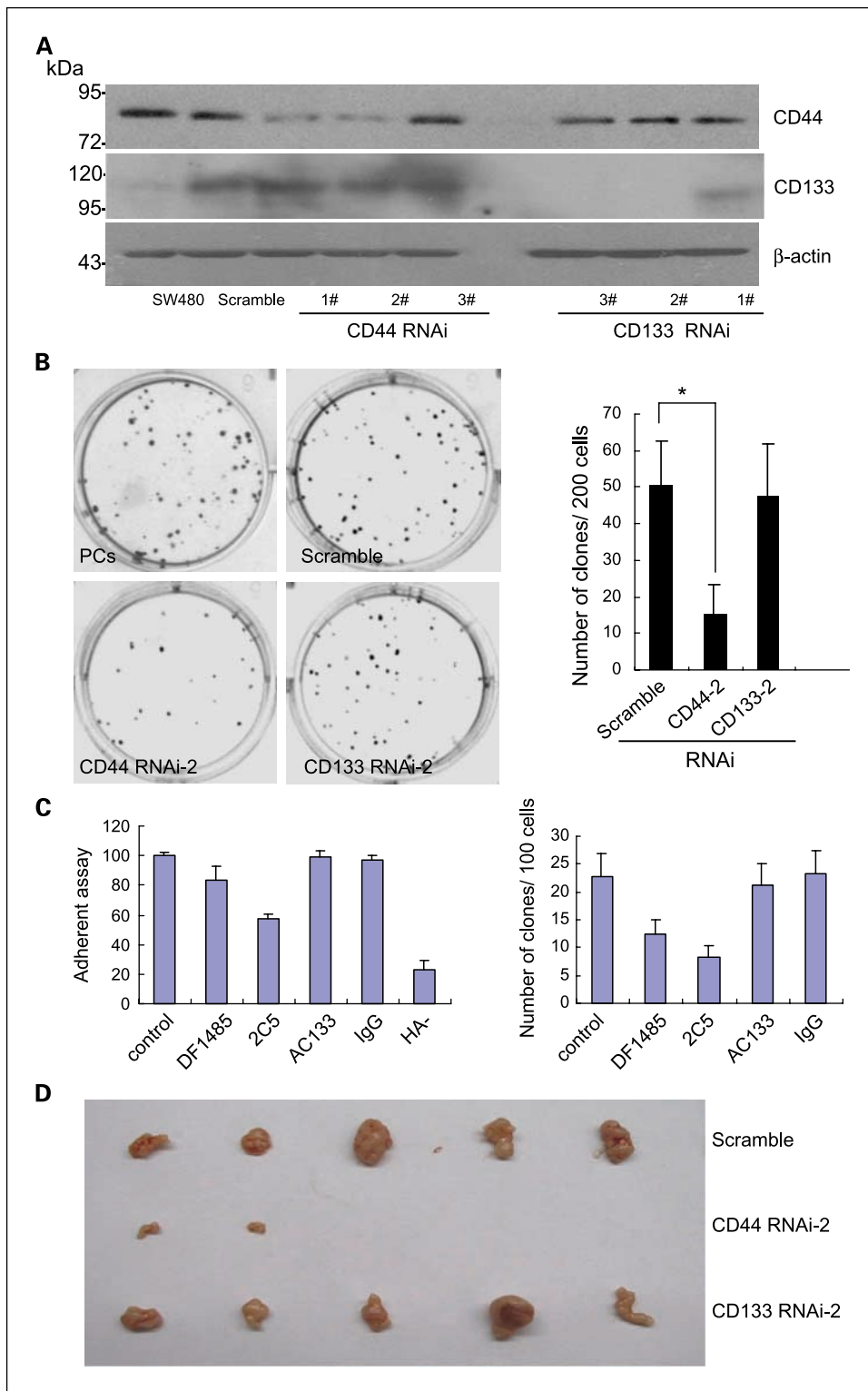
To further substantiate our finding that CD44 is of functional importance for CSC, we also tested if specific antibodies against CD44 or CD133 had any effects on clonal formation. DF1485 and 2C5 were two clones of the CD44 antibody which targeted different sites of CD44. Adherent cells following hyaluronan analysis showed that 2C5 decreased CD44 function by  $42.77 \pm 3.27\%$ . This antibody also significantly inhibited clonal formation from  $22.67 \pm 4.33\%$  to  $8.33 \pm 2.12\%$  ( $P < 0.01$ ) when 100 cells were seeded in six-well plates (Fig. 4C). Specific antibodies against CD133 had no effect under similar cultural conditions.

We then s.c. injected 50,000 colorectal cancer cells stably transfected with CD44 siRNA, CD133 siRNA, or scramble siRNA into nude mice and found that knockdown of CD44 could dramatically inhibit tumorigenesis both in tumor incidence and tumor volume. Only two out of six mice grew a small tumor 38 days after injection of cells with CD44 siRNA transfection, whereas six out of six mice generated tumors within 18 days when transplanted with CD133 siRNA or scramble siRNA transfected cells (Fig. 4D). A considerable reduction of tumor size was also observed in CD44 siRNA-transfected cells as compared with the scramble siRNA.

To further understand the mechanism by which CD44 siRNA inhibits tumor growth, we detected the transcriptional level of stemness genes such as *Oct3/4*, *β-catenin*, *Nanog*, and *Bmi* in cells transfected with siRNA. Interestingly, we found that *β-catenin*, *Oct3/4*, and *Bmi* were inhibited and *Nanog* was less affected when CD44 was knocked down (Supplementary Fig. S3). These data indicate that CD44 might be involved in the stem cell signaling pathway, which plays a role in tumor initiation and development.

**Fig. 3.** A single CD44<sup>+</sup> colorectal cancer cell could initiate a tumor. *A*, paraclone or holoclone of indicated colorectal cancer cells after 7 to 9 days proliferation from patients 3 and 6 (*left and middle*). *B*, holoclones that arose within 7 days traced from a single CD44<sup>+</sup> cells and further sphere-like clones. *C*, CD44<sup>+</sup> holoclones labeled with BrdUrd for 12 h (*left*) and 24 h (*middle and right*) before BrdUrd and CD44 double staining. *D*, CD44<sup>-</sup>/CD133<sup>-</sup>, CD44<sup>-</sup>/CD133<sup>+</sup>, CD44<sup>+</sup>/CD133<sup>-</sup>, and CD44<sup>+</sup>/CD133<sup>+</sup> cells cultured in sphere conditions (magnification, ×10).





**Fig. 4.** CD44, but not CD133, RNA interference inhibits tumorigenesis. *A*, inhibition of CD44 or CD133 expression in primary colorectal cancer cells from patient 6 by siRNA transfection using Western blotting analysis. *B*, clonal formation of primary colorectal cancer cells from patient 6 transfected CD44 or CD133 siRNA in six-well plates (*left*); statistical analysis (*right*). *C*, adhesive capacity is decreased in all anti-CD44 clones (*left*) and clonal formation was reduced in the presence of CD44 antibodies (*right*). *D*, representative image of xenograft tumors in mice obtained following the injection of colorectal cancer cells transfected with CD44 or CD133 siRNA.

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## Discussion

Both CD44 and CD133 were reported as putative markers for isolating colorectal cancer-initiating cells or CSC. Here, we provide evidence showing that CD44 is a robust marker for colorectal CSC and is of functional importance for tumorigenesis. Following tissue microarray analysis of 60 patients, we did

not find colocalization of CD133 with CD44-positive cells in the same region. This observation was further substantiated by flow cytometric analysis because it was infrequent to find that both markers coexisted in the same cell. Our results do not seem to support CD133 as a marker for colorectal CSC, although we could not rule out its role for cancer development. It has been reported that CD133 was a colorectal CSC marker



based on its high tumorigenicity in immunodeficient mice (21, 22), however, it should be noted that some colorectal cancers lack the expression of CD133 (this study; ref. 16) and that CD133<sup>+</sup> cells may be enriched in metastatic cancer. Several reports showed that CD133 is expressed in hepatocarcinoma and glioblastoma cell lines which have greater tumorigenic capacity, and it is regarded as an important cancer-initiating or stem cell marker (26–28).

Despite CD44 being widely used for isolating CSC, the functional aspects of maintaining CSC have yet to be determined. An important notion of the current study is that CD44 is of functional importance for cancer initiation and progression. Applying standard techniques in measuring CSC activities, we clearly showed that CD44<sup>+</sup> cells possess higher capacities in clonal formation *in vitro* and tumorigenicity *in vivo*. Several isoforms of CD44 were detected in colorectal cancer both by Western blotting and reverse transcription-PCR. Notably, we showed that CD44 is important for maintaining the properties of CSC and cancer initiation by RNA interference experiments. Also, we found that the stemness genes such as *Oct3/4*, *Bmi*, and  $\beta$ -*catenin* were down-regulated when CD44 was knocked down, suggesting a crosstalk between CD44 signaling and stemness gene expression. It is worth noting that the *Nanog* gene is less affected when CD44 was knocked down, suggesting that these stemness genes are differentially regulated by CD44. Our data are in line with studies that support a CSC model in which these stemness genes play important roles in regulating the self-renewal of normal and tumorigenic human mammary stem cells (29). Further studies are required to examine how CD44 affects stemness gene expression or if stemness genes could regulate CD44 expression. We also found that antibodies against CD44, but not CD133, block the clonal formation of cancer cells from patient tumors. Collectively, these results clearly show that CD44 is of functional importance for maintaining the CSC phenotype and for supporting cancer cell expansion. It has been previously shown that knockdown of CD44 or its specific variant isoforms could inhibit prostate cancer invasion (30), cancer formation from cancer cell lines (31), and sensitize the cancer cell towards drug

toxicity (32). These results further indicate that targeting CD44 and signaling pathways are useful for colorectal cancer therapy. Indeed, antibodies against CD44 could eradicate acute myeloid leukemia (33).

Although CSCs represent an exciting avenue for cancer study and a novel target for drug discovery, the extent to which CSC shares the characteristics of normal stem cells, including the properties of self-renewal and multipotency, remains unclear. A stringent test requires a single transplanted CSC to generate a xenograft tumors *in vivo* (34). It is technically a challenge to inject a single cell and monitor its growth into a tumor *in vivo*. An elegant study showed that a single cell from the rat mammary gland LA7 cell line can form a mammosphere which has the differentiation potential (35). We took the approach to allow single CD44<sup>+</sup> cells clonally expanded *in vitro* to form a clone/sphere which grew into a tumor *in vivo*. Intriguingly, we found that CD44<sup>+</sup> cells, which were negative for BrdUrd labeling and were surrounded by CD44<sup>-</sup>/BrdUrd<sup>+</sup> cells in a clone, were more stable in cell division and these CD44<sup>+</sup> cells could retain the sphere formation capacity with stem cell properties. We conclude that CD44<sup>+</sup> cells have certain stem cell properties for self-renewal and differentiation into distinct cell types. Further work is under way to investigate the molecular details of the maintenance of CSC and their differentiation.

In conclusion, data showed that CD44 is a robust marker and is of functional importance for colorectal CSC for cancer initiation. A better understanding these issues will bring an exciting opportunity for targeted therapy to eradicate cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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