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Identification and Characterization of Tumorigenic Liver Cancer Stem/Progenitor Cells

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Background & Aims: **Recent efforts in stem cell biology suggest that tumors are organized in a hierarchy of heterogeneous cell populations and that the capability to maintain tumor formation/growth specifically resides in a small population of cells called *cancer stem cells* (CSCs). The aim of this study is to identify, isolate, and characterize the CSC population that drives and maintains hepatocellular carcinoma (HCC) growth and metastasis.** ***Methods:*** **Normal stem cells involved in liver regeneration were identified using a severe partial hepatectomy model. Purified HCC cells, with or without expression of the identified normal stem cell phenotype, were evaluated, based on their tumorigenic potential and exhibition of defined stem/progenitor cell-like properties, to determine whether liver CSCs can be or partly be identified by this surface marker.** ***Results:*** **We report the identification and isolation of a population of CSCs expressing a CD133 surface phenotype from human liver cell lines. CD133$^+$ cells possess a greater colony-forming efficiency, higher proliferative output, and greater ability to form tumor in vivo. These cells are endowed with characteristics similar to those of progenitor cells including the expression of "stemness" genes, the ability to self-renew, and the ability to differentiate into nonhepatocyte-like lineages. Furthermore, CD133 is found to represent only a minority of the tumor cell population in human HCC specimens.** ***Conclusions:*** **We report the identification of a CSC population in HCC characterized by their CD133 phenotype. The identification of tumorigenic liver CSCs could provide new insight into the HCC tumorigenic process and possibly bear great therapeutic implications.**

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide affecting 1 million individuals annually.[1] Intensive research efforts have been directed toward the identification of novel treatment strategies and markers associated with the initiation and progression of HCC.[2] However, despite advances in the detection and treatment of the disease, mortality rate remains high because current therapies are limited by the advanced stage in which the disease is usually diagnosed, when most potentially curative therapies such as resection and transplantation are of limited efficacy. Furthermore, the emergence of chemotherapy-resistant cancer cells leaves this disease with no effective therapeutic options and a very poor prognosis.

It is believed that HCC, like many other cancers, develops from the accumulation of mutations in genes critical to processes such as self-renewal, cell growth, and other functions. It has been estimated that approximately 3 to 6 genetic events are necessary to transform a normal cell into a cancer cell.[3,4] If this hypothesis holds true, the long-term residents of the liver, like stem cells, who have the highest potential for proliferation and a much longer life span compared with their progeny, are possibly the only cells that have the ability to accumulate the requisite number of mutations that are necessary to perturb intrinsic mechanisms regulating normal cell proliferation and differentiation while remaining viable.

In the last few years, a growing body of evidence has been reported supporting the notion that tumors are organized in a hierarchy of heterogeneous cell populations with different biologic properties and that the capability to sustain tumor formation and growth exclusively resides in a small proportion of cells called *cancer stem cells* (CSCs). Studies have shown that these cells are not only responsible for tumor formation and progression but are also endowed with stem/progenitor cell properties, including the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular lineage through differentiation. To date, the existence of CSCs has been proven in the context of leukemia,[5,6] breast cancer,[7,8] glioblastoma,[9–11] and more recently, prostate,[12–14] gastric,[15] lung,[16] and colon cancer.[17,18] The stem cell-like phenotype of these tumor-initiating cells and their limited number within the bulk of the tumor are believed to account for their capability

Abbreviations used in this paper: AFP, α-fetoprotein; ALB, albumin; CSCs, cancer stem cells; CK18, cytokeratin 18; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; TTR, transthyretin.

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to escape conventional therapies, thus leading to disease relapse even when the primary lesion is eradicated.

It has been demonstrated recently that malignant cells and normal stem cells share many common properties.[19–21] Identification of normal liver stem cells may lend insight into the understanding of the events that regulate cellular differentiation of the liver and the consequences of their subversion. The unique ability of the mammalian liver to regenerate itself after massive injury serves as a useful model to study the role of stem cells in tissue reconstitution and possibly cancer formation. Using this model, we identified Prominin-1, the mouse homologue of human CD133, to be highly up-regulated during early liver regeneration. CD133 was found to be expressed at a lesser degree when mass restoration of the liver in the mouse is believed to be complete. To investigate further the role of $CD133^+$ cells in the development of HCC, we isolated these cells from HCC cell lines Huh7 and PLC8024 and hepatoblastoma cell line HepG2. Freshly isolated $CD133^+$ cells were demonstrated to possess characteristics similar to those of stem/progenitor cells, including greater colony-forming efficiency, higher proliferative output, greater ability to form tumor in vivo, ability to self-renew, and ability to differentiate into nonhepatocyte-like, angiomyogenic-like lineages.

Our findings provide a previously uncharacterized model of liver tumor biology in which a defined subset of cells drives tumorigenesis, as well as generating tumor cell heterogeneity. The identification of $CD133^+$ cells as HCC-initiating cells establishes a previously unidentified cellular target for the establishment of more effective therapies.

Materials and Methods

Animals

The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the University of Hong Kong. Nude or SCID mice aged between 4 and 8 weeks were used for partial hepatectomy (PH) experiments and to test the tumorigenicity potential of sorted cells from liver cell lines.

Mouse PH

For liver regeneration studies, nude mice were anesthetized and subjected to PH operations with a 70% liver resection. Animals were killed at days 0, 3, and 7 after surgery. All time points were performed on 2 animals each. Upon death, livers of all animals were harvested for total RNA extraction, total protein extraction, and paraffin embedding.

RNA Isolation, Complementary DNA Synthesis, and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) was synthesized using an Advantage RT-for-PCR kit (Clontech Laboratories, Mountain View, CA) according to manufacturer's instructions. cDNA was subject to quantitative reverse-transcription polymerase chain reaction (qPCR) with a SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) and Prominin-1 or 18S primers (see Supplementary Table 1 online at www.gastrojournal.org) using an ABI PRISM 7700 Sequence Detector and SDS 1.9.1 software (Applied Biosystems).

Collection of Human Tissue Specimen

Liver tumor and adjacent nontumor tissue specimens were obtained with informed consent from 5 patients who underwent hepatectomy for HCC from 1999 to 2001 in the Department of Surgery, Queen Mary Hospital, Hong Kong.

Cell Culture

The Hep3B cell line was obtained from the American Type Culture Collection, Manassas, VA. The Huh7 cell line was provided by Dr H. Nakabayashi, Hokkaido University School of Medicine, Japan.[22] HepG2 and PLC8024 cell lines were obtained from the Institute of Virology, Chinese Academy of Medical Sciences, Beijing, China. H2P, H2M, and H4M cell lines were previously established in our laboratory.[23,24] The MiHA cell line was provided by Dr J.R. Chowdhury, Albert Einstein College of Medicine, New York (Table 1).[25]

Table 1. CD133 Expression in Cultured Human Liver Cell Lines

Cells	Characteristics	CD133 *(%)*[a]	Ability to form subcutaneous tumor	Reference
MiHA	Immortalized, normal	0	No	Brown et al[25]
H2P	Primary HCC	4	No	Hu et al[23]
H2M	Metastatic HCC	6	No	Hu et al[23]
H4M	Metastatic HCC	0	No	Wen et al[24]
HepG2	Hepatoblastoma	8	No	ATCC, HB-8065
Huh7	HCC	65	Yes	Nakabayashi et al[22]
PLC8024	HCC	60	Yes	ATCC, CRL-8024
Hep3B	HCC	90	Yes	ATCC, HB-8064

[a]The numbers represent the mean value of at least 3 independent flow cytometry analyses.

Flow Cytometry

Antibodies used include phycoerythrin (PE)-conjugated CD133/1 (Miltenyi Biotec, Aubum, CA), fluorescein isothiocyanate (FITC)-conjugated CD29, CD117/c-kit (eBioscience, San Diego, CA), CD34, CD44, CD49f, and CD90 (BD PharMingen, San Jose, CA). Cells were incubated in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 0.1% sodium azide with either fluorescence-conjugated primary antibody or primary antibody followed by a PE- or FITC-conjugated secondary antibody. Isotype-matched mouse immunoglobulins served as controls. Samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

Isolation of CD133$^+$ and CD133$^-$ Populations by Flow Cytometry or Magnetic Cell Sorting

For magnetic cell sorting, cells were labeled with CD133/1 microbeads and sorted using the Miltenyi Biotec CD133 Cell Isolation Kit, according to manufacturer's instructions. Magnetic separation was performed twice to obtain greater than 95% pure CD133$^+$ and greater than 95% pure CD133$^-$ populations. Aliquots of CD133$^+$ and CD133$^-$ sorted cells were evaluated for purity with a FACSCalibur machine and CellQuest software (BD Biosciences), using PE-conjugated anti-human CD133/2 antibody (Miltenyi Biotec). For cell sorting using flow cytometry, cells were stained with PE-conjugated anti-human CD133/1 (Miltenyi Biotec). Isotype-matched mouse immunoglobulins served as controls. Samples were analyzed and sorted on a BD FACSVantage SE (BD Biosciences). For the positive and negative population, only the top 25% most brightly stained cells or the bottom 20% most dimly stained cells were selected, respectively. Specifically, magnetic cell sorting was used for the in vivo tumorigenic experiments as listed in Tables 2 and 3. All other experiments were performed using cells purified by flow cytometry cell sorting.

Table 2. In Vivo Tumor Development Experiments of CD133$^+$ and CD133$^-$ Cells Sorted From HCC Cell Lines Huh7 and PLC8024 in Nude Mice

Cell type[a]	Cell numbers injected	Tumor incidence[b]	Latency (days)[c]
Huh7 unsorted	10,000	0/3	—
	50,000	2/3 *(2)	90
	100,000	3/3	70
	300,000	3/3 *(2)	70
	2,000,000	1/1	30
Huh7–CD133$^+$	10,000	0/6	—
	50,000	7/9 *(2)	56
	100,000	3/4	50
	300,000	3/3 *(2)	35
	2,000,000	3/3	29
Huh7–CD133$^-$	10,000	0/4	—
	50,000	0/9 *(2)	—
	100,000	0/5	—
	300,000	0/2 *(2)	—
	500,000	0/4	—
	3,000,000	2/4	45
PLC8024 unsorted	10,000	0/3	—
	50,000	2/3	65
	200,000	4/4	45
	1,000,000	4/4	30
PLC8024–CD133$^+$	10,000	0/4	—
	50,000	3/4	65
	200,000	3/3	34
	500,000	4/4	28
PLC8024–CD133$^-$	50,000	0/3	—
	200,000	0/4	—
	500,000	0/4	—
	1,000,000	1/4	65

[a]CD133$^+$ and CD133$^-$ cells freshly isolated from HCC cell lines Huh7 or PLC8024 were maintained subcutaneously or intrahepatically (where indicated by *[number of injections]) in nude mice.
[b]The number of tumors detected/number of injections.
[c]Approximate number of days from tumor cell injection to appearance of a tumor.

Table 3. In Vivo Tumor Development Experiments of CD133$^+$ and CD133$^-$ Cells Sorted From HCC Cell Line PLC8024 in SCID Mice

Cell type[a]	Cell numbers injected	Tumor incidence[b]	Latency (days)[c]
PLC8024 unsorted	50,000	2/2	45
PLC8024–CD133$^+$	1000	2/2	90
	3000	2/2	55
PLC8024–CD133$^-$	3000	0/2	—
	50,000	2/2	65

[a]CD133$^+$ and CD133$^-$ cells freshly isolated from HCC cell line PLC8024 were maintained subcutaneously in SCID mice.
[b]The number of tumors detected/number of injections.
[c]Approximate number of days from tumor cell injection to appearance of a tumor.

Proliferation Assay

Cells were seeded at a density of 2000 cells per well and allowed to grow for 5 to 7 days. Cell proliferation was assessed by a colorimetric assay using crystal violet (Sigma Chemical Co., St. Louis, MO), a cytochemical stain that binds to chromatin.

Anchorage-Independent Growth Assay

Cells were suspended in soft agar and growth medium in 6-well plates at a density of 5000 cells per well. After 2–3 weeks, colonies (≥10 cells) were counted under the microscope in 10 fields per well and photographed.

In Vivo Tumorigenicity Experiments

Various numbers of cells (Tables 2–4) were either injected subcutaneously or intrahepatically into nude or SCID mice. Mice were killed between 3 and 6 months postinjection, at which time tumors were harvested for further examination. Those animals injected with tumor

Table 4. Subcutaneous In Vivo Tumor Development Experiments of Serially Transplanted CD133$^+$ and CD133$^-$ Cells Resorted From CD133$^+$ HCC Cell-Derived Xenograft Tumors in Nude Mice

Cell type	Cell numbers injected	Tumor incidence[a]	Latency (days)[b]
Serial transplant unsorted	10,000	0/3	—
	50,000	2/3	75
	100,000	3/3	65
	500,000	3/3	40
Serial transplant CD133$^+$-CD133$^+$	10,000	0/4	—
	50,000	2/3	62
	150,000	3/4	60
	500,000	4/4	55
Serial transplant CD133$^+$-CD133$^-$	10,000	0/4	—
	50,000	0/3	—
	150,000	0/6	—
	500,000	1/4	82

[a]The number of tumors detected/number of injections.
[b]Approximate number of days from tumor cell injection to appearance of a tumor.

cells but with no sign of tumor burden were generally terminated 5 months after tumor cell inoculation, and animals were opened up at the injection sites to confirm that there was no tumor development.

Xenograft Tumor Processing

Xenograft tumors were minced into ~1-mm^3 pieces and incubated with 1X Accumax (Innovative Cell Technologies, San Diego, CA) for 20 minutes at 37°C under constant rotating conditions. A single cell suspension was obtained by filtering the supernatant through a 100-μm and 40-μm cell strainer (BD Biosciences).

Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Slides were processed for antigen retrieval by a standard microwave heating technique. Specimens were incubated with mouse anti-human CD133/1 (Miltenyi Biotec) or with goat anti-mouse CD133 (Santa Cruz Biotechnology, Santa Cruz, CA) (for regeneration studies) in a dilution of 1:10. For specimens incubated with mouse anti-human CD133/1, subsequent immunodetection was performed using the standard rapid EnVision technique. For specimens incubated with goat anti-mouse CD133, immunodetection was performed using a standard avidin-biotin peroxidase technique. The reaction was then developed by DAKO Liquid DAB+ Substrate-Chromogen System (DAKO, Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin. Stained sections were reviewed by a histopathologist (K.W.C.). Positive staining for CD133 was observed predominantly in the membrane of both cancerous and noncancerous liver cells. More than 1000 cells expressed in 3–4 different high-power fields (200×) were analyzed for each section.

Differentiation of CD133$^+$ Cells Into Nonhepatocyte-Like, Angiomyogenic-Like Cells

CD133$^+$ and CD133$^-$ cells were grown in growth medium containing M199 medium (Invitrogen), 20% FBS, 10 ng/mL vascular endothelial growth factor $(VEGF)_{165}$, 10 ng/mL brain-derived neurotrophic factor (BDNF), 5 ng/mL basic fibroblast growth factor (bFGF), and 1 U/mL heparin sulfate (Peprotech, Rocky Hill, NJ) in 6-well plates. Medium replenished with new growth medium was changed every 3 days to ensure continuous cytokine delivery. Cultures were followed for 2–3 weeks until nonhepatocyte-like morphology was detected by phase-contrast microscopy.

RT-PCR

cDNA products were used for PCR reactions using primers and conditions listed in Supplementary Table 1 (see Supplementary Table 1 online at www.gastrojournal.org).

Immunofluorescence

Cells were fixed in ice-cold acetone/methanol (1:1) and stained with rabbit anti-human AFP (DAKO) or mouse anti-human CK18 (Santa Cruz Biotechnology), followed by a PE- or FITC-conjugated secondary antibody, respectively. Cells were counterstained with DAPI and visualized by confocal microscopy.

Statistical Analysis

Statistical analysis was performed by applying the independent *t* test using Microsoft Office Excel software (Microsoft Corp., Redmond, WA). Statistical significance was declared if $P < .05$.

Results

CD133$^+$ Cells Are Increased in Regenerated Liver

To determine what normal stem cell markers are involved in liver regeneration, severe PH was performed on mice, and liver was harvested at time point day 0 (control), day 3 (early liver regeneration), and day 7 (late liver regeneration) followed by messenger RNA (mRNA) expression profile comparison using a cDNA microarray containing 34,000 genes (data not shown, unpublished data). Prominin-1, the mouse structural homologue of human CD133, was found to be up-regulated 93-fold in day 3 compared with day 0 post-PH. To validate this observation, we further studied the expression of CD133 by qPCR and immunohistochemistry (IHC). Results were consistent with the findings from the cDNA microarray data. CD133 was found to be dramatically up-regulated 3 days post-PH ($P < .001$) with a significant decrease in expression in later stages of regeneration (day 7) when mass restoration of the liver is believed to be complete ($P < .001$; Figure 1*A*). Similarly, IHC studies also showed

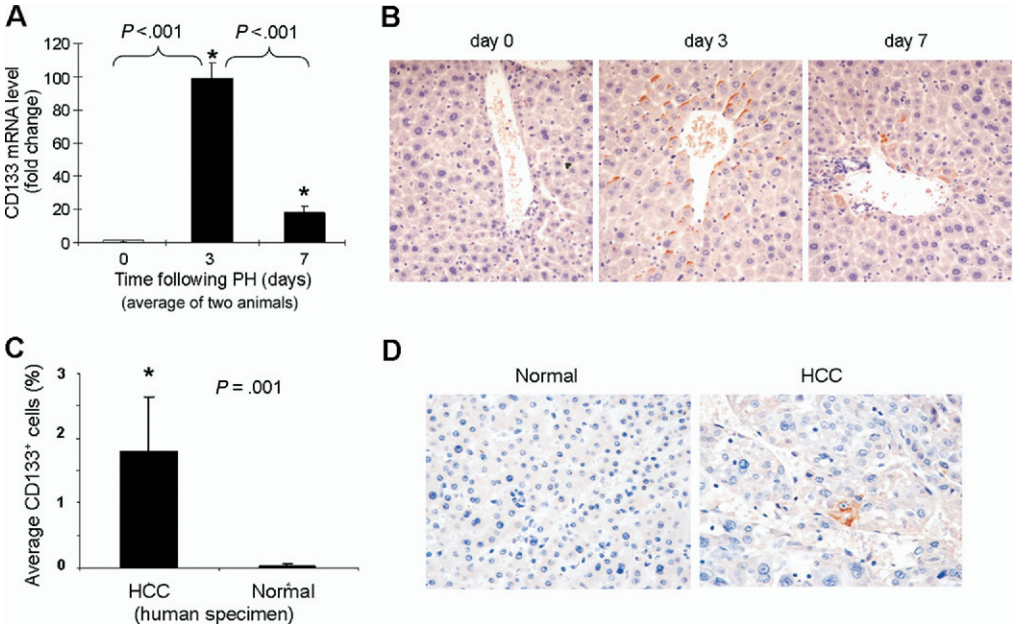

Figure 1. (*A*) Relative CD133 (Prominin-1) mRNA levels were determined by qPCR from RNA preparation obtained from mouse liver that had undergone 0, 3, and 7 days severe partial hepatectomy (PH), with over 70% of the liver mass removed. Two animals were used for each time point, and the data presented here represent the average of the 2 animals. The nearly undetectable amount of CD133 present in liver that had undergone 0 days of PH is defined as 1, and the increase in CD133 seen after PH is expressed as the fold increase over the normal liver baseline level. All samples were normalized to 18S expression. (*B*) Immunohistochemistry analysis of CD133 expression in mouse liver that had undergone 0, 3, and 7 days PH. (*C* and *D*) Immunohistochemical staining of CD133 proteins in human normal liver tissue and their corresponding HCC specimen. On average, CD133 was found to be expressed in less than 2% of the 5 human HCC specimens screened and almost absent (~0.05%) in normal liver specimens. All pictures were taken at 400× magnification.

that CD133$^+$ cells were dramatically increased 3 days post-PH with a significant decrease on day 7 (Figure 1*B*).

CD133 Is Expressed Sporadically in Human HCC Specimens

To determine whether CD133$^+$ cells are present in human HCC patient specimens, we performed IHC staining of CD133 on 5 human HCC tissue samples with their matched nontumor counterparts. The CD133$^+$ fraction in the HCC specimens ranged from 1% to 3%, whereas expression in their nontumor counterparts ranged from 0.025% to 0.1% (P = .001). In all cases, only a small percentage of CD133-expressing cells (average, <2%) could be identified in HCC samples, whereas CD133-expressing cells were almost absent in their nontumor counterparts. Stained CD133$^+$ cells identified in both the normal and the tumor samples looked like large hepatocytes but were generally indistinguishable from the rest of the other normal or tumor cells (Figure 1*C* and 1*D*).

CD133$^+$ Cells in HCC Cell Lines Correlate With Tumorigenicity

To determine whether CD133 marks more tumorigenic liver cells, we examined its expression in a panel of liver cell lines including the nontumorigenic, immortalized cell line MiHA; hepatoblastoma cell line HepG2; and HCC cell lines H2P, H2M, H4M, Huh7, PLC8024, and Hep3B. High CD133 expression was detected in cell lines that were able to form tumor in vivo, whereas, in contrast, the immortalized, nontumorigenic cell line MiHA and cell lines that were incapable of forming tumors in vivo showed no or very low CD133 expression (Table 1). These results suggest that CD133 expression in cultured liver cells seems to correlate with the ability of the cells to develop tumor in vivo.

CD133$^+$ Cells Are More Tumorigenic Than CD133$^-$ Cells In Vitro

To determine whether CD133$^+$ cells are more tumorigenic than their CD133$^-$ counterparts in vitro, we purified CD133$^+$ and CD133$^-$ cells from HCC cell lines Huh7 and PLC8024 and compared their proliferative and clonal ability using proliferation and soft agar colony formation assay, respectively. We began by purifying CD133$^+$ cells from Huh7 cells, which expressed CD133 in 65% of the cells (Figure 2*A*, Table 1). The purity of the CD133$^+$ and CD133$^-$ cell populations was generally greater than 95% as revealed by postsorting analysis (Figure 2*B* and *C*). CD133$^+$ cells sorted from the Huh7 cell line were able to proliferate significantly faster than CD133$^-$ cells (P < .001) (Figure 2*D* and *E*). In addition, they were able to induce bigger and greater numbers of tumor colonies than CD133$^-$ cells (P = .003) (Figure 2*F* and *G*). We similarly purified CD133$^+$ and CD133$^-$ cells from the PLC8024 cell line, which expressed CD133 in 60% of the cells (Figure 3*A*–*D*, Table 1), and the

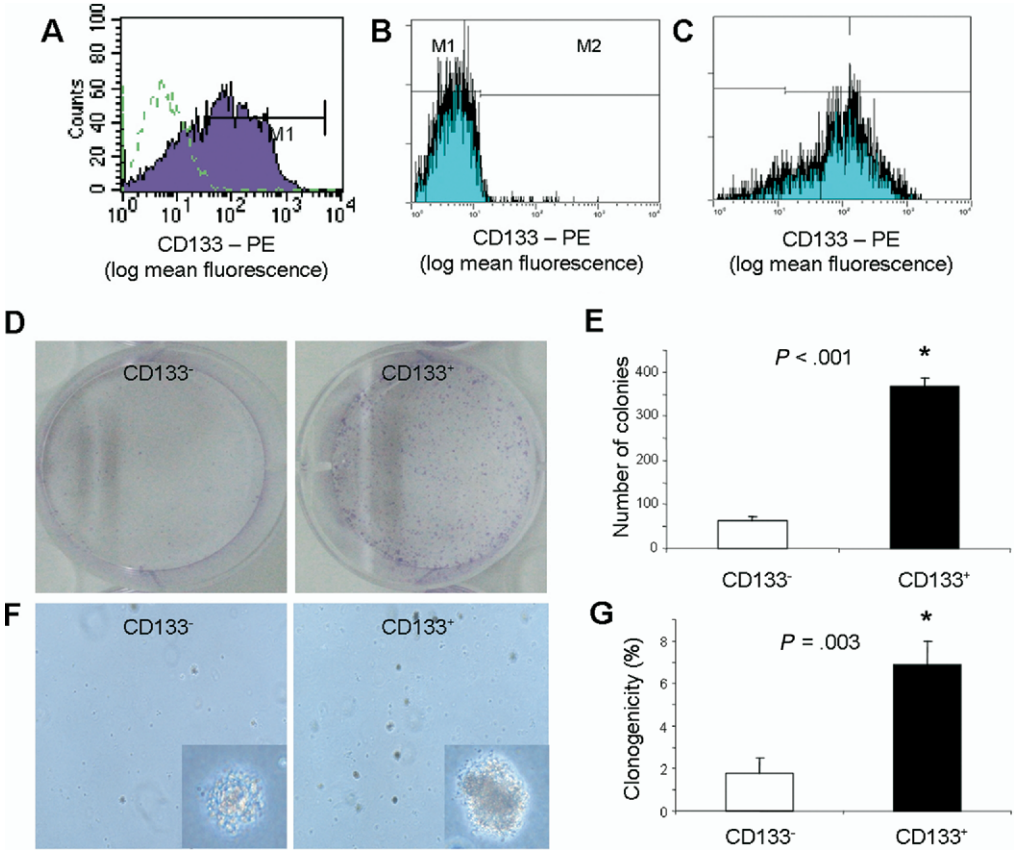

Figure 2. CD133$^+$ cells isolated from the Huh7 cell line possess higher proliferative and clonogenic potential in vitro. (*A*) *Flow cytometry histogram* showing expression of CD133$^+$ cells (65%) in the Huh7 HCC cell line (*dotted line*, isotype control; *solid line*, Huh7 cells). (*B* and *C*) Example of analysis for purity of CD133$^-$ cells (*B*; gate M1) and CD133$^+$ cells (*C*; gate M2) following cell sorting. Purities ranged from 90% to 99.5% (median, 99%) for CD133$^-$ cells and 85% to 95% for CD133$^+$ cells (median, 90%). (*D* and *E*) Freshly isolated CD133$^+$ and CD133$^-$ Huh7 cells were plated at a density of 2000 cells/well in 6-well culture plates and cultured for 1 week. At the end, cells were stained with crystal violet, photographed, and analyzed for their proliferation efficiency. Each experiment was performed 3 times, and representative examples are shown. (*F* and *G*) Purified CD133$^+$ and CD133$^-$ Huh7 cells were plated at a density of 5000 cells/well on soft agar for clonogenicity experiments. The results 20 days following initial plating are shown. The *smaller panels* in (*F*) show representative examples of clonogenic assays magnified at high power. Data represent the mean ± SD derived from 2 separate experiments with triplicate wells per condition.

HepG2 cell line, which expressed CD133 in only 8% of the cell population (Figure 3*E–H*, Table 1), and obtained similar observations. No difference in adhesion and viability between the CD133$^+$ and CD133$^-$ cells was observed. For instance, cells that were plated adhered and were alive but were unable to form sizable colonies.

CD133$^+$ Cells Possess Higher Tumorigenicity Ability In Vivo Than CD133$^-$ Cells

To determine whether CD133$^+$ HCC cells are more tumorigenic than their CD133$^-$ counterparts in vivo, we carried out tumor development experiments using CD133$^+$ and CD133$^-$ cells purified from Huh7 and PLC8024 cell lines. Purified or unsorted cells were maintained either subcutaneously or intrahepatically in nude (Table 2) or SCID mice (Table 3). A significant difference in tumor incidence was observed between the CD133$^+$ and CD133$^-$ populations sorted from both Huh7 and PLC8024 HCC cell lines. As few as 1×10^3 or 5×10^4 CD133$^+$ cells were sufficient for consistent tumor development in SCID or nude mice, respectively, whereas at least 50 to 60 times as many CD133$^-$ cells were necessary to generate consistently a tumor model in the same model. Injection of fewer CD133$^-$ cells either resulted in no tumor formation or formation of tumor with much lower efficiency. Unsorted cell lines showed a cell number dependent on an increase in tumor incidence. Interestingly, no visible lesion was found when fewer than 50,000 cells of any type were injected in nude mice. On average, it took longer for the CD133$^-$ cells to form a tumor than CD133$^+$ cells. Tumor nodules that formed on the liver surface within the liver or subcutaneously in the mice were confirmed by pathologic study. Representative examples of tumor nodule formed by injection of purified CD133$^+$ and CD133$^-$ cells are shown in Figure 4.

CD133$^+$ Cells Preferentially Express "Stemness" Genes

To determine whether CD133$^+$ HCC cells might possess certain stem cell-like properties, we determined

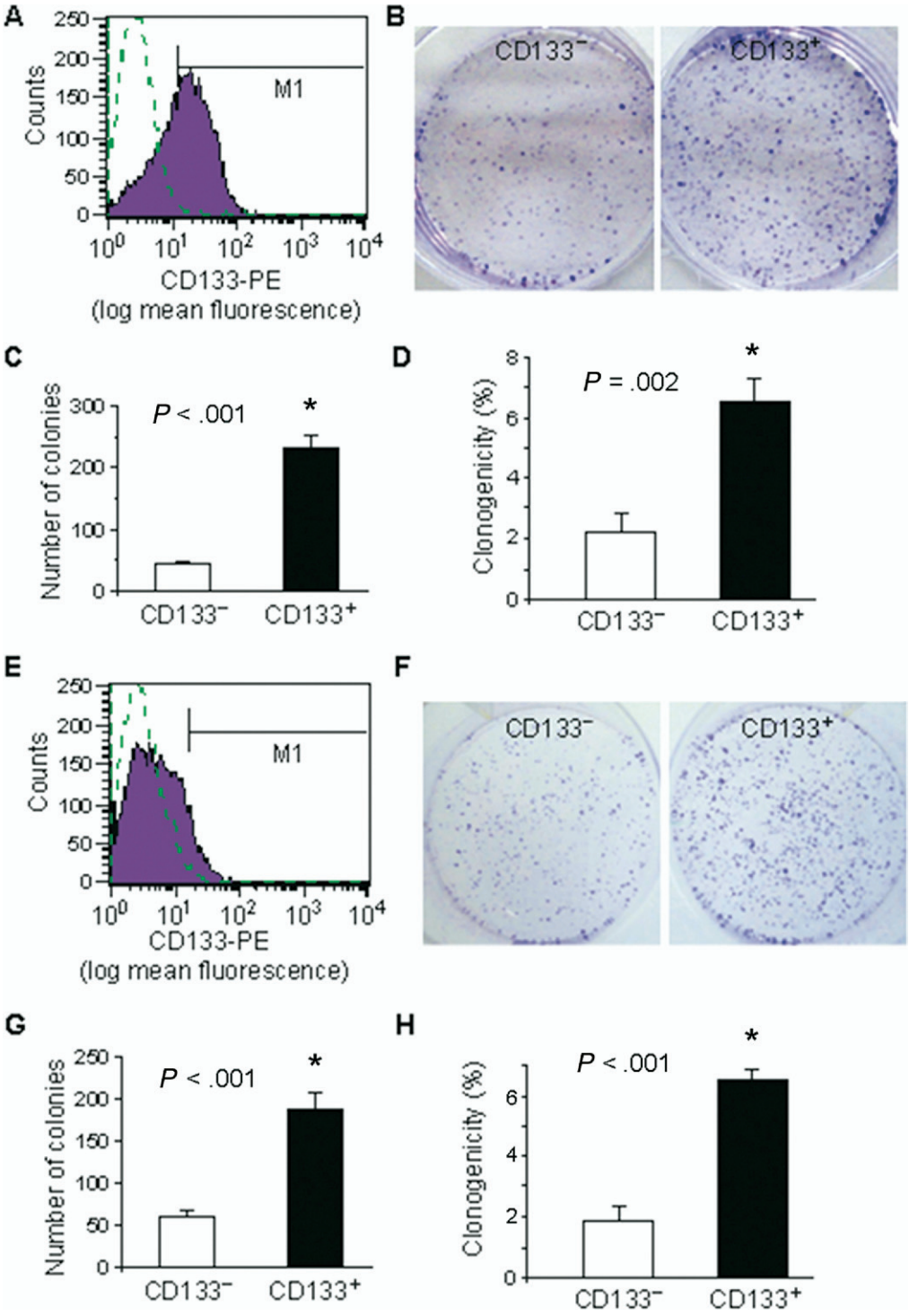

Figure 3. CD133⁺ cells isolated from PLC8024 and HepG2 cell lines possess higher proliferative and clonogenic potential in vitro. (*A*) *Flow cytometry histogram* showing expression of CD133⁺ cells (60%) in PLC8024 HCC cell line (*dotted line*, isotype control; *solid line*, PLC8024 cells). CD133⁺ and CD133⁻ cells purified from PLC8024 cell lines were plated at either 2000 cells/well in 6-well culture plates for proliferation assays (*B* and *C*) or at 5000 cells/well on soft agar for clonogenicity experiments (*D*). The results at 1 week for the proliferation assay or 3 weeks for the clonogenicity assay, after initial plating, are shown. Data represent the mean ± SD derived from 2 separate experiments with triplicate wells per condition. (*E*) *Flow cytometry histogram* showing expression of CD133⁺ cells (8%) in the HepG2 hepatoblastoma cell line (*dotted line*, isotype control; *solid line*, HepG2 cells). CD133⁺ and CD133⁻ cells purified from the HepG2 cell line were plated at either 2000 cells/well in 6-well culture plates for proliferation assays (*F* and *G*) or at 5000 cells/well on soft agar for clonogenicity experiments (*H*). The results at 1 week for the proliferation assay or 3 weeks for the clonogenicity assay, after initial plating, are shown.

whether CD133⁺ HCC cells might preferentially express certain genes important for the proliferation, self-renewal, and differentiation of stem cells, including those involved in the Wnt/β-catenin, Notch, Hedgehog/SMO, Bmi, and Oct3/4 pathways.[26–29] Using semiquantitative RT-PCR analyses, we found that all CD133⁺ HCC cells purified from Huh7 and PLC8024 cell lines or primary culture established from CD133⁺ HCC induced xenograft tumor consistently expressed higher mRNA levels of β-catenin, Oct-3/4, Bmi, SMO, and Notch-1 (Figure 5*A*).

CD133⁺ Cells Exhibit Increased Potential for Self-Renewal

A key property of all normal and cancer stem cells is their unique ability to self-renew. One method to determine whether CD133⁺ cells have self-renewal capac-

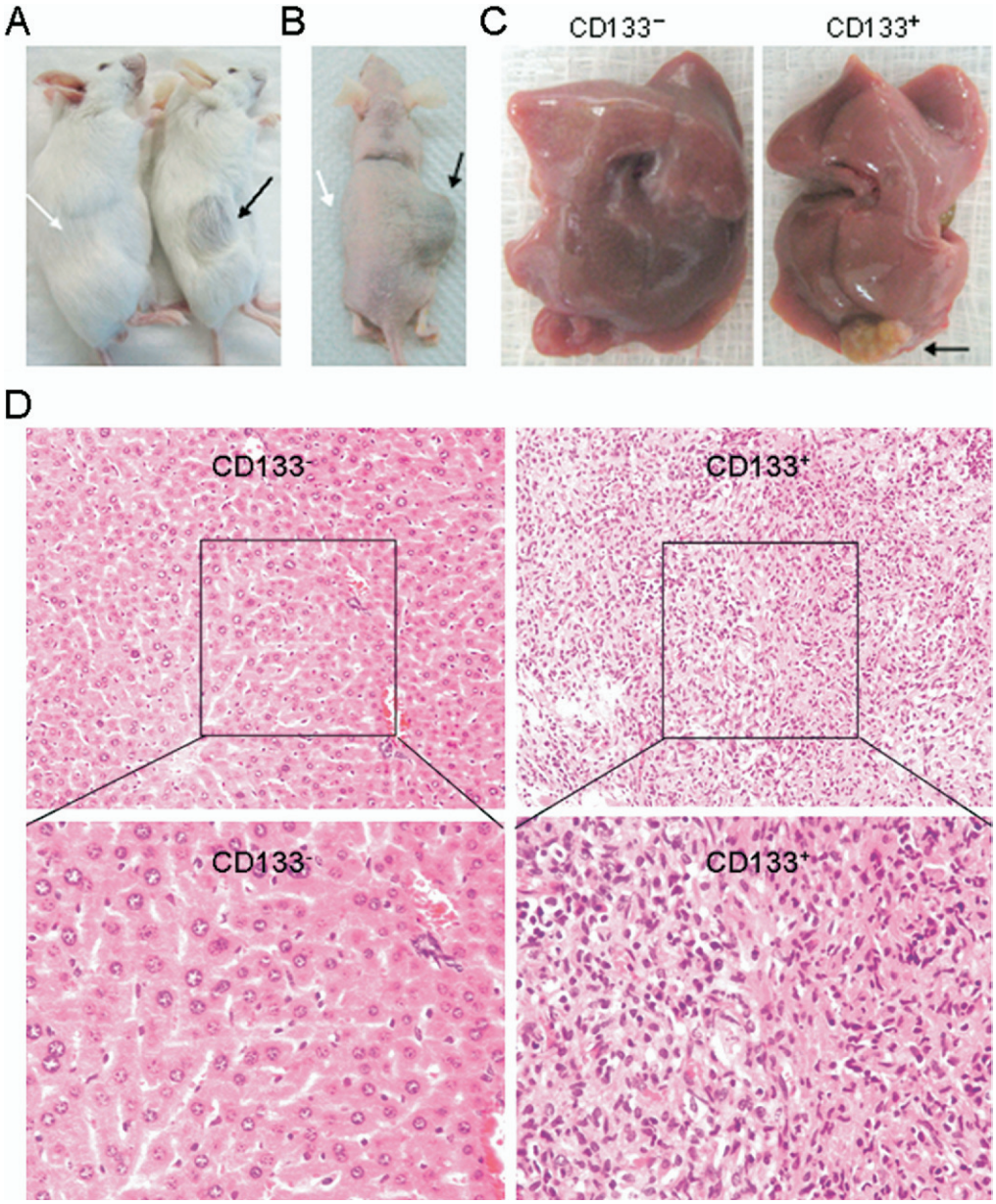

Figure 4. Representative examples of SCID mice (*A*) and nude mice (*B*) injected subcutaneously and intrahepatically (*C*) with CD133$^+$ and CD133$^-$ cells freshly isolated from PLC8024 or Huh7. Tumor nodules, indicated by *black arrows* in *A–C*, were only observed in mice injected with CD133$^+$ cells. Injection site of CD133$^-$ cells, indicated by *white arrows* in *A* and *B*, revealed no tumor. (*D*) H&E sections of liver injected with CD133$^-$ cells revealed normal tissue only, whereas CD133$^+$ injection site contained malignant cells (original magnification, 200× and 400× magnification).

ity is to test their capability of serial passage. We performed serial retransplantation experiments from CD133$^+$ HCC cell-derived tumors that grew from an initial injection of 50,000 CD133$^+$ cells. After 12 weeks, the xenograft tumor was excised from the primary mouse recipient, dissociated into single cell suspension, grown in culture for approximately 4 weeks, resorted for CD133$^+$ and CD133$^-$ cells using an antibody specific to human CD133, and then reinjected into secondary mouse recipients. Mouse cells were excluded from this experiment because the antibody used for cell sorting is specific to human CD133. Control experiments by IHC with the antibody on day 3 mouse regenerating liver sections, known to express high levels of mouse CD133 (Prominin-1), revealed no signal, ensuring that the antibody used did not cross-react with mouse CD133 and that no mouse cells were included in the retransplantation experiments (data not shown). Furthermore, monitoring of the cells dissociated from the xenograft tumor by phase contrast microscopy during the 4 week in vitro culture period did not reveal any morphology atypical of the cell line from which the xenograft tumor initially grew. The majority of CD133$^+$ secondary xenografted mice formed tumors that resembled the phenotype of the primary xenograft, providing direct evidence for the self-renewal capability of the population. Interestingly, as few as 50,000 CD133$^+$ cells were sufficient for tumor formation, whereas at least 10 times as many CD133$^-$ cells were necessary to generate a tumor in the same model (Table 4). However, even at 10 times the number, CD133$^-$ cells

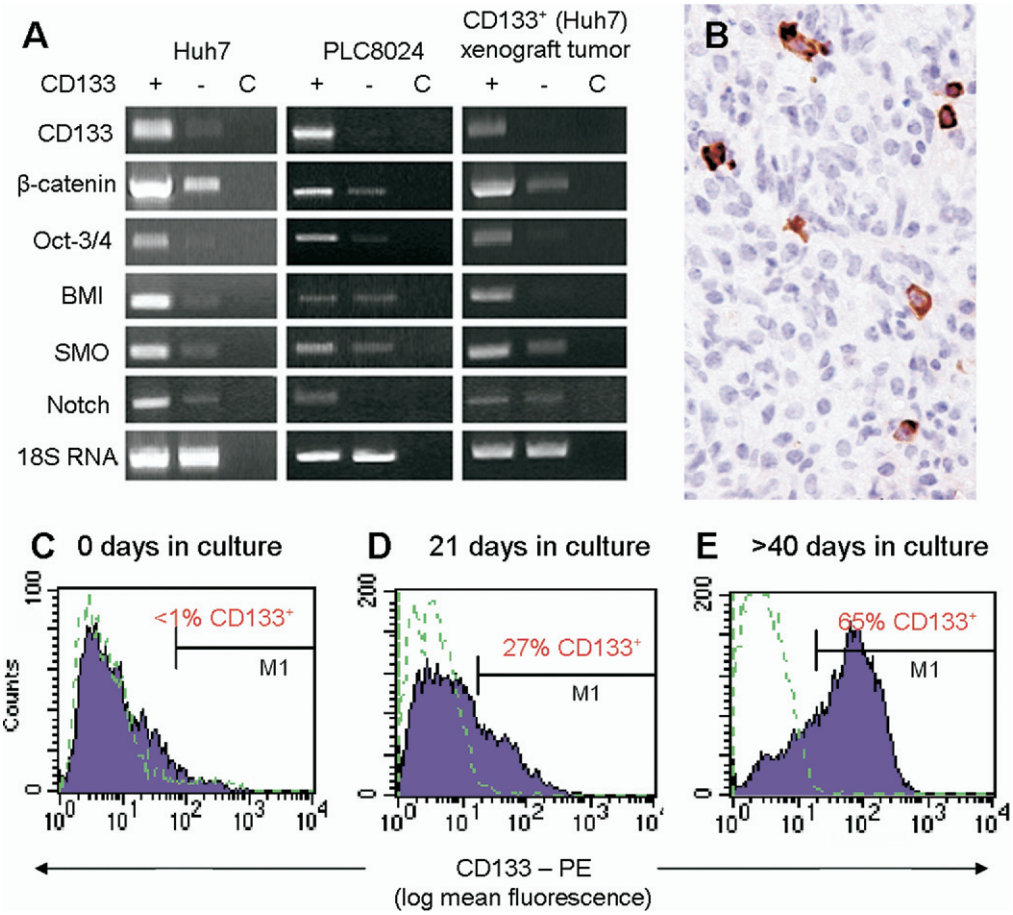

Figure 5. CD133$^+$ HCC cells show marked stem cell characteristics including the ability to self-renew and to differentiate. (*A*) Semiquantitative RT-PCR analysis of "stemness" genes using CD133$^+$ and CD133$^-$ cells purified from Huh7 and PLC8024 cell lines or cells derived from CD133$^+$ (purified from Huh7)-induced xenograft tumor. 18S was used as an internal control. C: water, negative PCR control. (*B*) Immunohistochemical staining of CD133 proteins in CD133$^+$ HCC cell-derived tumor nodules revealed single positive cells, amid large groups of negative cells. Pictures were taken at 400× magnification. (*C–E*) Flow cytometry analysis showed increasing CD133 expression in xenograft tumors induced by CD133$^+$ cells following 0, 21, and >40 days in culture (*dotted line*, isotype control; *solid line*, primary cell line derived from xenograft tumor induced by CD133$^+$ cells).

were only able to generate tumor in approximately 25% of the mice injected and required a longer latency time in those experiments that did form a tumor. Injection of fewer CD133$^-$ cells either resulted in no tumor formation or formation of tumor with much lower efficiency. No visible lesion was found when fewer than 50,000 cells of any type were injected.

To further provide evidence for the self-renewal capacity of CD133$^+$ HCC cells, we also characterized the tumor phenotype of the CD133$^+$ HCC xenografts induced by injection of CD133$^+$ cells sorted from HCC cell line Huh7 or PLC8024 by immunohistochemical and flow cytometry analyses. We purified tumor cells from Huh7 and PLC8024 tumors derived from either intrahepatic or subcutaneous-injected CD133$^+$ tumor cells and used flow cytometry and/or IHC to analyze the percentages of CD133$^+$ cells in these tumors. In every case, we observed that the CD133$^+$ HCC cell-derived tumor nodules revealed single positive cells, among large groups of negative cells, indicating that not every cell in the xenograft is CD133$^+$ despite the fact that the tumor nodule was formed by freshly isolated CD133$^+$ cells alone (Figure 5*B*). Flow cytometry analyses of cells harvested from CD133$^+$ HCC xenograft tumor also revealed low or no CD133 expression (Figure 5*C*). The fact that transplanted CD133$^+$ cells result in a heterogeneous primary xenograft consisting of a minority of CD133$^+$ cells (<1%) and a majority of CD133$^-$ cells (99%) suggest that a tumor hierarchy exists in which the CD133$^+$ cells may generate CD133$^-$ tumor cells, in turn lending support to the fact that only the CD133$^+$ fraction is proliferating and demonstrating self-renewal in vitro.

To confirm the ability of CD133$^+$ HCC cells to generate CD133$^-$ cells in vitro, we carried out flow cytometric analyses of CD133 expression on short-term passages of CD133$^+$ and CD133$^-$ cells purified from Huh7 and PLC8024 cell lines or primary culture established from CD133$^+$ HCC induced xenograft tumor. Equal numbers of cells from both subpopulations were cultured in vitro, and the cells were restained with CD133 over a 6-week culture period, with time points at 0, ≤10, 10–20, 20–30, and ≥40 days, and then reanalyzed by flow cytometry. The CD133$^+$ cells underwent discerning asymmetric division, which generated an increasing CD133$^+$ as well as a decreasing CD133$^-$ subpopulation in a time-dependent manner. The ratio of positive to negative cells reached a steady state within 4 weeks and returned to the CD133 expression level of the original cell line from which it was sorted originally (Figure 5*C–E*, Table 5). Note that the viability of all cells was identical after sorting and remained so throughout the study period.

Table 5. Flow Cytometry Analysis of CD133 Expression on Short-Term Passages of $CD133^+$ and $CD133^-$ Cells Purified From HCC Cell Lines Huh7 and PLC8024 and Xenograft Tumor Formed by $CD133^+$ Tumor Cells Over a 6-Week In Vitro Culture Period

		Percentage of CD133 expression as measured by Flow Cytometry				
		Days in culture				
$CD133^{+/-}$ cells sorted from	CD133	0	<10	10–20	20–30	>40
Huh7	+	95%	75%	68%	65%	63%
	−	<2%	15%	21%	57%	65%
PLC8024	+	95%	82%	73%	65%	58%
	−	<2%	14%	22%	38%	47%
Xenograft tumors derived from $CD133^+$ HCC cells	+	95%	81%	72%	65%	65%
	−	<1%	22%	34%	47%	56%

$CD133^+$ Cells Differentiate to Produce Nonhepatocyte-Like, Angiomyogenic-Like Cells In Vitro

Another key property of stem cells is their ability to differentiate into different lineages. To assess whether freshly isolated $CD133^+$ HCC cells were able to differentiate into nonhepatic lineages, we cocultured purified $CD133^-$ and $CD133^+$ HCC cells in appropriate growth factors for 2 weeks in attempt to differentiate the cells into angiomyogenic-like tissue.[30] Phase-contrast microscopy analysis revealed changes in the cell morphology of the 2 subgroups following cell-directed differentiation (Figure 6*A*). $CD133^+$, but not $CD133^-$, cells were able to differentiate from dense cellular colonies into flat adherent, longitudinal stretched cells (nonhepatocyte-like cells). We then further characterized purified $CD133^-$ and $CD133^+$ cells before differentiation and $CD133^+$ cells after differentiation by RT-PCR for mRNA expression of hepatocyte expressing genes including α-fetoprotein (AFP), cytokeratin 18 (CK18), transthyretin (TTR), and albumin (ALB) (Figure 6*B*). Freshly isolated $CD133^+$ cells prior to differentiation were found to express higher levels of each marker as compared with $CD133^-$ cells. Interestingly, expression of the markers tested was found to be down-regulated after differentiation of $CD133^+$ cells into nonhepatocyte-like cells. $CD133^+$ and $CD133^-$ cells prior to and following differentiation were also characterized by RT-PCR for the presence of muscle- and cardiac-specific marker MEF2C, skeletal muscle-specific marker MYOD1, and cardiac-specific marker GATA4 (Figure 6*C*).[30] Following cell-directed differentiation of $CD133^+$ cells into nonhepatocyte-like, angiomyogenic-like cells, a dramatic increase in expression of both MEF2C and MYOD1 was detected in $CD133^+$ cells, suggesting that $CD133^+$ cells have the capacity to differentiate into lineages with skeletal and cardiac features. In addition, immunofluorescence experiments were also performed to confirm the observation where expression of both AFP and CK18 was decreased following differentiation of $CD133^+$ cells into nonhepatocyte-like cells, as compared with $CD133^+$ cells prior to differentiation or $CD133^-$ cells postdifferentiation (Figure 7*A* and *B*). Taken together, these data suggest that subsets of $CD133^+$ HCC cells have the capacity to differentiate into nonhepatocyte-like cells.

Expression of Stem/Progenitor Markers CD29, CD34, CD44, CD49f, CD90, and CD117 in Freshly Isolated $CD133^+$ and $CD133^-$ Subpopulations

To better characterize the liver CSC population, we further analyzed the expression of several stem/progenitor cell surface markers by flow cytometry in

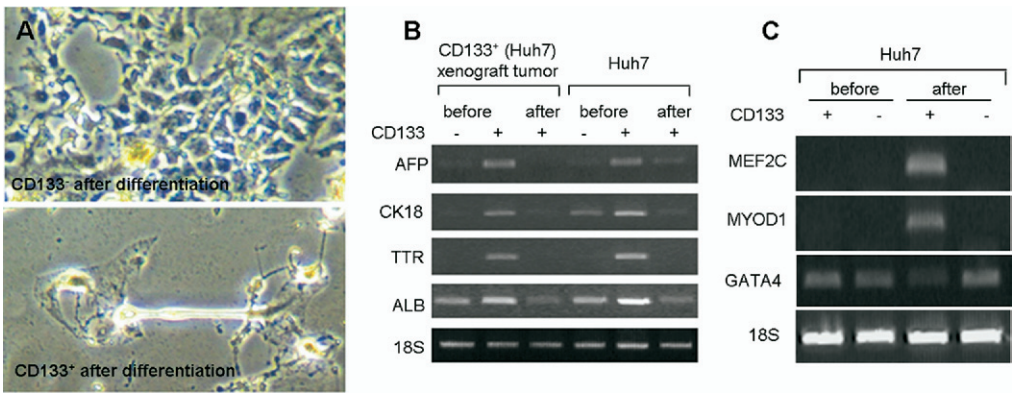

Figure 6. (*A*) Differentiation of freshly isolated $CD133^+$ HCC cells, but not $CD133^-$ cells, into longitudinal stretched, nonhepatocyte-like cells was observed following 2 weeks of induced differentiation, as visualized by phase contrast microscopy. (*B*) Semiquantitative RT-PCR analysis of freshly isolated $CD133^-$ and $CD133^+$ cells prior to differentiation (before) and $CD133^+$ cells postdifferentiation (after) for expression of endodermal and liver-specific markers including AFP, CK18, TTR, and ALB. (*C*) Semiquantitative RT-PCR analysis of freshly isolated $CD133^+$ and $CD133^-$ cells before and after differentiation for expression of muscle-specific marker MEF2C, skeletal muscle-specific marker MYOD1, and cardiac-specific marker GATA4. 18S was used as an internal control.

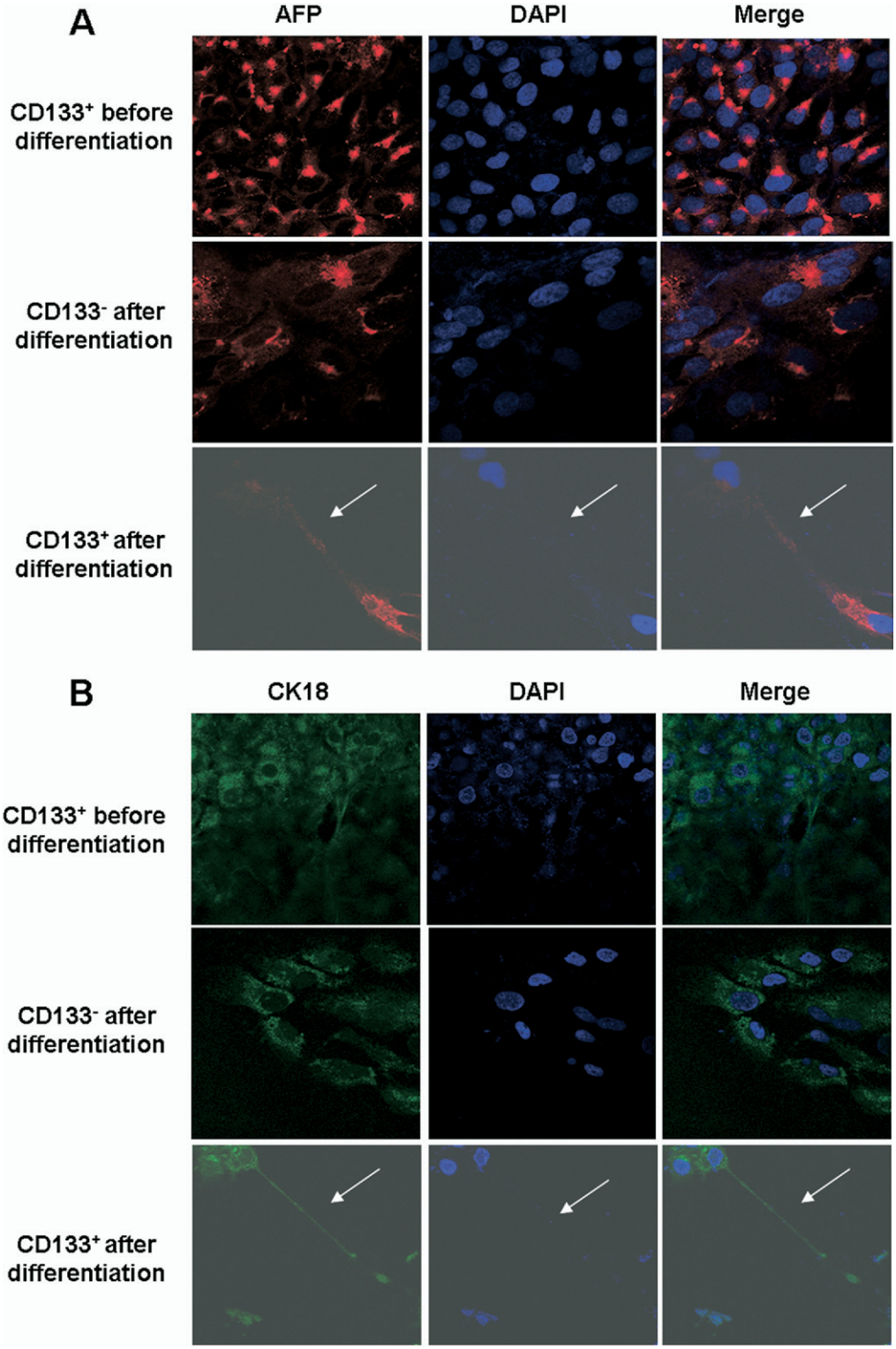

Figure 7. Expression of AFP (*A*) and CK18 (*B*) in freshly isolated CD133$^+$ HCC cells prior to differentiation and CD133$^+$ or CD133$^-$ HCC cells postdifferentiation was examined by immunofluorescence staining. AFP and CK18 were significantly decreased following differentiation of freshly isolated CD133$^+$ HCC cells into nonhepatocyte-like cells. Morphologic change of differentiated CD133$^+$ cells into longitudinal stretched, nonhepatocyte-like cells is indicated by *white arrows*. Nuclei (*blue*) are labeled with DAPI stain.

CD133$^+$ or CD133$^-$ subpopulations isolated from HCC cell lines Huh7 and PLC8024 following cell sorting. Markers studied include stem cell factor receptor CD117/c-kit; integrin β_1 CD29; hematopoietic stem cell markers CD34, CD90, integrin α_6 CD49f; and cell adhesion molecule CD44. CD133$^+$ subpopulation isolated from both HCC cell lines Huh7 and PLC8024 expressed higher levels of CD34 and CD44 expression when compared with their CD133$^-$ counterparts. CD29, CD49f, CD90, and CD117 were not preferentially expressed by either of the CD133 subpopulations (Table 6).

Table 6. Flow Cytometry Analysis of Cell Surface Markers CD29, CD34, CD44, CD49f, CD90, and CD117/c-kit in Freshly Isolated CD133$^+$ and CD133$^-$ Cells From HCC Cell Lines Huh7 and PLC8024

CD133$^{+/-}$ cells sorted from	Percentages of expression as measured by FCM[a]																	
	CD29			CD34			CD44			CD49f			CD90			CD117		
	U	+	−	U	+	−	U	+	−	U	+	−	U	+	−	U	+	−
Huh7	99	10	64	3.5	1.2	0	8	1.5	0.1	75	27	27	0	0	0	1.5	0	0
PLC8024	90	88	93	2	0.7	0	4	2.9	1	7	2	2.5	0	0	0	0	0	0

*U, unsorted; +, CD133$^+$; − CD133$^-$.
[a]The numbers represent the mean value of at least 3 independent flow cytometry analyses.

Discussion

Stem cells are believed to sit at the top of the developmental hierarchy, possessing the unique ability to self-renew and to generate mature cells of all lineages through differentiation. They have the highest potential for proliferation and possess a longer life span compared with their progeny.[19] It has been suggested that stem cells have 2 unique properties that make them likely to be involved in cancer development. First, they are often the only long-lived cells in a tissue that have the ability to replicate. Because typically, multiple mutations, occurring over many years, are necessary before a cell becomes cancerous, it has been suggested that these long-lived stem cells have the greatest opportunity to accumulate such cancer-inducing mutations while remaining viable. Second, through a process called *self-renewal*, stem cells generate new stem cells with similar proliferation and differentiation capacities. However, by the same argument, and because it is believed that normal cells and cancer cells share the same self-renewal mechanism, it has been suggested that cancers arise either from normal stem cells or from progenitor cells in which self-renewal pathways have become deranged. In other words, cancer can be regarded as a disease of unregulated self-renewal.[19,31,32]

In fact, there has been accumulating evidence supporting the fact that progenitor cells are the cell of origin in many cancer types, including the liver. It is observed that 55% of small cell dysplastic foci, the earliest premalignant lesions in human HCC, consist of progenitor cells and intermediate hepatocytes. In addition, in hepatoblastomas, the most common liver tumor in childhood, cells resembling progenitor cells have also been noted.[33] Both of these observations provide a strong argument in favor of the progenitor cell origin of liver tumors.

Because it is commonly believed that regeneration depends on a subset of progenitor cells that resist terminal differentiation and retain their potency for proliferative capacity, we began by studying which normal stem cells are involved in the liver regeneration process using a severe partial hepatectomy model. Using this model, we identified Prominin-1, the mouse homologue of CD133, to be highly expressed during early liver regeneration, making it the second most highly up-regulated gene after AFP in this data set. CD133, a 5 transmembrane domain cell-surface glycoprotein, is regarded as an important marker for the identification and isolation of primitive stem/progenitor cells in both hematopoietic and nonhematopoietic tissues. It was originally found on hematopoietic stem cells and hematopoietic progenitor cells deriving from human fetal liver, bone marrow, and peripheral blood. Recently, it has been suggested that stem/progenitor cells expressing CD133 isolated from the bone marrow are able to repopulate up to 10% of the normal liver when transplanted.[34,35] Most importantly, CD133 has been recently identified as part of the CSC population responsible for tumor formation in brain, prostate, and colon cancer.[9–11,14,17,18]

In light of the correlation between normal stem cells and CSCs, we went on to study whether CD133 is involved in the development of HCC. We report here the isolation and characterization of the CSC population from human liver HCC and hepatoblastoma cell lines based on their cell surface expression of CD133. We began our studies with cell lines because they are able to provide us with a renewable source of genetically stable tumor material. Our findings reveal that the characteristics of the CD133$^+$ subpopulation were consistent with the predicted behavior of both primitive stem and cancer cells.

First, CD133$^+$ cells are capable of extensive proliferation, generating progeny several orders of magnitude higher than that of the CD133$^-$ population. Recent studies in the CSC field have demonstrated that as few as 100 cells with the CSC phenotype are able to generate tumor in vivo. For example, studies in prostate and breast cancer have shown that as few as 100 CD44$^+$ or CD44$^+$CD24$^{-/low}$ cells are able to generate tumor in NOD-SCID mice, respectively, whereas at least approximately 10 to 100 times as many of their nontumorigenic counterparts were necessary to generate a tumor in the same model.[8,12] On the other hand, the data reported in our study demonstrate that a minimum of 5×10^4 CD133$^+$ cells were necessary for consistent tumor development in nude mice, whereas at least 60 times as many CD133$^-$ cells were needed to generate the same effect. We attribute this difference to

the fact that NOD-SCID mice are significantly more immunocompromised than nude mice, thus allowing an easier formation of tumor. Nonetheless, the data presented in this study are consistent with previously published data in the relative fold difference needed for tumorigenic and nontumorigenic cell populations to form tumor in vivo. We believe that if NOD-SCID mice were used in replacement of nude mice in this study, the number of tumorigenic cells necessary to give rise to a tumor in vivo should decrease significantly. Although we acknowledge that more work will have to be done to understand better the minimum number of cells necessary for the generation of a tumor in vivo using NOD-SCID mice models, we believe that we have provided compelling evidence showing that $CD133^+$ cells are indeed more tumorigenic in nude mice and have also provided preliminary data suggesting that as few as 3×10^3 $CD133^+$ cells are able to generate consistently a tumor in SCID mice.

Second, $CD133^+$ cells are expressed infrequently in human HCC specimens. This observation is consistent with past reports showing that the CSC population is often found to represent less than 5% of the total tumor mass. It is interesting to note that CD133-expressing cells are also found to be maintained only in very few numbers in $CD133^+$ HCC-induced xenograft tumors, resembling their low abundance in the original human HCC tumors.

Third, $CD133^+$ cells express genes important for the self-renewal and proliferative and fate-determining properties of stem/progenitor cells including those of β-catenin, Notch, SMO, Bmi, and Oct3/4. Many of these genes or associated pathways have been previously implicated to be involved in HCC. β-catenin is involved in the Wnt-signaling pathway, best known to govern cell proliferation, migration, and differentiation during embryogenesis. The SMO receptor is involved in the Hedgehog signaling pathway, which is important in the regulation of stem cell self-renewal. The Notch-signaling pathway has been shown to be an important inhibitor of differentiation. Bmi is important for the self-renewal and proliferation of neural, hematopoietic, and leukemia stem cells. Oct3/4 is a transcription factor involved in the self-renewal of teratocarcinoma and embryonic stem cells.

Fourth, $CD133^+$ cells exhibit an increased potential for self-renewal. $CD133^+$ HCC cell-derived xenograft tumors could be serially transplanted to secondary mice, recapitulating the phenotype of the original tumor. Moreover, $CD133^+$ HCC cells resulted in a heterogeneous primary xenograft consisting of a minority of $CD133^+$ cells (<5%) and a majority of $CD133^-$ cells, suggesting that a tumor hierarchy exists in which the $CD133^+$ cells may generate $CD133^-$ tumor cells. Furthermore, short-term in vitro cultures of $CD133^+$ HCC cells were capable of generating $CD133^-$ cells in a time-dependent manner, over a 6-week culture period, and eventually reached a steady state and returned to the CD133 expression level of the cell line from which it was originally sorted. This observation suggests that most of the $CD133^+$ HCC cells may be stem/progenitor cells lacking self-renewal capacities and therefore rapidly develop into $CD133^-$ cells when cultured. However, a small fraction of this $CD133^+$ population has persisted in culture, as well as in tumors, suggesting that some $CD133^+$ cells possess certain self-renewal properties and perhaps represent relatively more primitive tumor progenitors or CSCs. Interestingly, it was also observed that $CD133^-$ cells were also able to give rise to $CD133^+$ cells when cultured. It is speculated that, because the purity of the sorted cells is only 95% pure, contaminated $CD133^+$ cells in the $CD133^-$ population may be sufficient to give rise to the heterogeneous population.

Last, $CD133^+$ HCC cells exhibit a marked ability to differentiate. Freshly isolated $CD133^+$ cells, which presumably are already differentiated, were able to dedifferentiate into nonhepatocyte-like, angiomyogenic-like lineages when induced. Mature hepatocyte markers such as AFP, TTR, CK18, and ALB detected at high levels in purified $CD133^+$ subpopulations significantly decreased following cell-directed differentiation into nonhepatocyte-like cells. AFP is produced in response to stimulus of liver diseases and is only expressed in liver cancers. TTR represents endodermal differentiation and is expressed throughout liver maturation.[36] ALB is a protein synthesized by mature hepatocytes only.[37] CK18, although not specific to the liver, is also commonly expressed in mature hepatocytes. In addition, a dramatic increase in expression of muscle- and cardiac-specific marker MEF2C and skeletal muscle-specific marker MYOD1 was detected in $CD133^+$ HCC cells following cell-directed differentiation of cells to angiomyogenic-like lineages, providing direct evidence suggesting that $CD133^+$ cells are able to dedifferentiate into nonhepatocyte-like lineages with skeletal and cardiac features.

During the preparation of this manuscript, Chiba et al demonstrated that a small, uncharacterized (<1%) side population (SP), defined by Hoechst dye efflux properties, exists in 2 of 4 human liver cell lines screened and that transplantation of as few as 1000 of these cells was able to generate xenograft tumors in NOD-SCID mice.[38] Although there are increasing reports showing that there exists an SP in various primary tumors and cell lines[39,40] and that this SP possesses unique defense mechanisms like the increased expression of membrane transporters thus making them more resistant to chemotherapeutic drugs, it has been suggested that one cannot solely rely on SP sorts for stem cell identification because (1) it has been suggested that possession of an SP phenotype is not a universal property of CSCs because there may exist other defense mechanisms other than ATP-binding cassette drug transporters that CSCs use to evade drug therapies that are not identified by Hoechst dye staining,[41] and (2) non-SP cells may suffer from Hoechst dye toxicity and thus may lose any potential "stemness" prop-

erties.[42,43] Therefore, one cannot ensure that the SP cells identified by Hoechst dye staining fully contain all CSCs. This may also rationalize why there is a discrepancy in the percentage of CSC population identified by Chiba et al (0.25%–0.8% in Huh7 and PLC/PRF/5) and in our present study. However, on the other hand, we also acknowledge the fact that not all (60%–65%) of $CD133^+$ cells in the Huh7 and PLC8024 cell lines identified in our study are representative of CSCs and that, in reality, CSCs in other cancer types have also been characterized by more than one surface marker, for example, $CD44^+/CD24^{-/low}$ in breast cancer,[8] $CD34^+/CD38^-$ in acute myeloid leukemia,[6] and $CD44^+/\alpha2\beta1^{hi}/CD133^+$ in prostate cancer,[13] and, for this reason, identification of other surface markers, expressed along with CD133, as a goal to better characterize liver CSCs is worthwhile. Preliminary data included in this study suggest that the $CD133^+$ subpopulation preferentially expresses CD34 and CD44 cell surface markers when compared with their $CD133^-$ counterparts, and such work is currently under further investigation in our laboratory. From a different perspective, it is also worthwhile to note that the results in our study match well with those findings of Chiba et al. In agreement with our findings in which we found that 60%–65% of Huh7 and PLC8024 cell lines are $CD133^+$ cells as opposed to only 8% in the HepG2 cell line, Chiba et al also identified an uncharacterized SP in the 2 former cell lines but not the latter HepG2 cell line. It will be interesting to investigate whether CD133 identified in our study can characterize this SP fraction.

It is also interesting to note that following submission of this revised manuscript, Yin et al showed that $CD133^+$ cells isolated from the SMMC-7721 cell line manifested high tumorigenicity and clonogenicity as compared with $CD133^-$ HCC cells and that $CD133^+$ cells represented only a very small subpopulation (0.1%–1%) of the total cancer cells.[44] Their findings are in line with our present studies in which $CD133^+$ cells were found in human HCC tissue samples but not in normal liver tissues.

Existing therapies against HCC and most cancerous diseases are usually developed largely against the bulk population of the tumor mass, and, although these therapies are able to shrink initially the size of the primary tumor, they ultimately fail to eradicate consistently the lesion and appreciably extend the life of patients because of disease relapse. The identification of molecules expressed in the small subpopulation of cells that are at risk of becoming cancerous and the selective targeting of these cells that are pivotal for the growth of the entire HCC tumor mass should not only lead to the more efficient elimination of this crucial population of cancer cells but also open new avenues to the development of more effective cancer therapies.

Appendix

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.04.025.

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