Expansion of CD133-Expressing Liver Cancer Stem Cells in Liver-Specific Phosphatase and Tensin Homolog Deleted on Chromosome 10-Deleted Mice

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ABSTRACT

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a lipid phosphatase that regulates mitogenic signaling pathways, and deficiency of PTEN results in cell proliferation, survival, and malignancy. Murine liver-specific Pten deletion models develop liver malignancy by 12 months of age. Using this model, we describe a population of CD133+ liver cancer stem cells isolated during the chronic injury phase of disease progression and before primary carcinoma formation. We performed immunohistochemistry and flow cytometry isolation using livers from 3- and 6-month-old Ptencre/+ AlbCre+ mice (mutants) and controls. CD133+CD45− nonparenchymal (NP) cells were analyzed for gene expression profile and protein levels. Single CD133+CD45− oval cells were isolated for clonal expansion and tumor analysis. Cultured and freshly isolated liver CD133+CD45− and CD133−CD45− NP cells were injected into immune-deficient and immune-competent mice. In mutant mice, the NP fraction increased in CD133+CD45− cells in 3- and 6-month-old Pten-deleted animals compared with controls. Clone lines expanded from single CD133+CD45− cells demonstrated consistent liver progenitor cell phenotype, with bilineage gene expression of hepatocyte and cholangiocyte markers. CD133+ cells from expanded clone lines formed robust tumors in immune-deficient and immune-competent mice. Furthermore, freshly isolated CD133+CD45− NP liver cells from 6-month-old mutants formed tumors in vivo, and CD133−CD45− NP cells did not. Consistent with a cancer stem cell phenotype, CD133+ cells demonstrate resistance to chemotherapy agents compared with CD133− cells. CD133+CD45− nonparenchymal cells from chronic injury Ptenloxp/loxp AlbCre+ mice represent a bipotent liver progenitor cell population with cancer stem cell phenotype.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common solid tumors in the world, ranked fifth in incidence and third in mortality [1]. As much as 50% of HCC can be defined as having a progenitor cell phenotype, with markers of both hepatocytes and cholangiocytes [2]. Although these progenitor-based HCCs have a more aggressive phenotype, the exact cellular origin of these progenitor-based tumors is unknown [3].

During chronic liver injury and cirrhosis, two models of carcinoma progression have been proposed: HCC and cholangiocarcinoma (CC) arise from mature hepatocytes and cholangiocytes, respectively, or HCC and CC arise from a common progenitor cell [2–4]. Current research describes the liver progenitor cell, or oval cell (OC), as a small cell, within the nonparenchymal (NP) fraction of the liver, that resides near the terminal bile ducts, at the hepatocyte-cholangiocyte interface [5, 6]. Although the exact role of oval cells in liver regeneration is unknown, oval cells proliferate during chronic injury [7].

In HCC and CC, deregulation of the epithelial growth factor receptor (EGFR)/phosphoinositide 3’-kinase (PI3K)/AKT signal pathway has been clearly demonstrated as a cause of cell cycle progression and cancer formation [8–11]. Human HCC demonstrates increased expression of EGFR ligands in HCC tissue compared with normal hepatocytes in surrounding liver tissue [12, 13]. Recently, chronic hepatitis B virus and hepatitis C virus infection-associated HCC was linked to upregulated EGFR/PI3K/AKT signaling [9]. Furthermore, downregulation...
of PTEN (phosphatase and tensin homolog deleted on chromosome 10), the primary negative regulator for the EGFR/PI3K/AKT signaling pathway, is associated with many human HCC patients [11, 14].

In human disease, PTEN is a tumor suppressor mutated in a wide range of cancers [15]. PTEN is a phosphoprotein/phospholipid dual-specificity phosphatase that antagonized the activity of PI3K [16]. PTEN acts as a tumor suppressor in most cells, and loss of PTEN leads to constitutive activation of AKT and resistance to apoptosis [17, 18]. Specific downregulation of the tumor suppressor PTEN has been demonstrated in human patients and validated with murine models of HCC [14, 15]. In mice, liver-specific loss of PTEN (PtenloxP/loxP; Alb–Cre+) leads to the development of both HCC and CC by 1 year, indicating the potential expansion of a malignant stem cell population during disease progression [19, 20]. In human patients, PTEN downregulation is associated with a poor prognosis [14].

Using the murine model of liver-specific PTEN loss, we intend to define a population of liver cancer stem cells isolated during chronic liver injury and before primary carcinoma formation. On the basis of prior studies, the PtenloxP/loxP; Alb–Cre+ mouse model develops chronic steatosis and injury prior to the onset of liver carcinoma. In a recent study, we demonstrated that CD133+ oval cells isolated from premalignant methionine adenosyltransferase 1A (Matla)-deficient mice contributed to a tumorigenic phenotype when grafted [21]. These liver cancer stem cells from Matla−/− were identified using CD133 expression and isolated before primary hepatocellular carcinoma development. Using human HCC cell lines, other groups have demonstrated that CD133 expression defines an aggressive cancer stem cell phenotype [22, 23]. Many tumors can be attributed in part to cancer stem cells, which are resistant to chemotherapy and can account for chemotherapy failure [24].

Here, we tested the hypothesis that deletion of Pten leads to the proliferation and enrichment of liver progenitor cells. Fluorescence-activated cell sorting (FACS) isolation of CD133+CD45− liver nonparenchymal cells from premalignant 3- and 6-month-old PtenloxP/loxP; Alb–Cre+ mice identified cells with an oval cell phenotype. After clonal expansion of single CD133+CD45− cells, 100% maintained expression of hepatoblast/oval cell-associated genes, confirming the stem cell nature of these CD133+ cells. Consistent with cancer stem cells, CD133+CD45− cells demonstrated resistance to chemotherapy compared with CD133−CD45− cells. Furthermore, CD133 expression was highly correlated with tumor formation in both cultured and freshly isolated cells.

**FACS Analysis of the Oval Cell-Enriched NP Fraction**

One million freshly isolated liver NP cells, RBC- and CD45-depleted, were resuspended in PBS. Alternatively, cells in culture were trypsinized for 3 minutes and washed once in culture medium and a second time in PBS. Following Fe blocking, the following combinations of FACS antibodies were added: CD45 fluorescein isothiocyanate (FITC), phycoerythrin (PE), and allophycocyanin (APC); CD34 FITC and PE; Thy 1.2 FITC and PE; c-Ki FITC, Sca-1 FITC, and PE; CD49f PE (BD Pharmingen); and CD133 FITC and PE (eBioscience Inc., San Diego, http://www.ebioscience.com). Cells were then incubated at 4°C for 30 minutes. Cells were washed with PBS prior to analysis using a FACS Calibur (BD Biosciences). Cell isolation was conducted on a FACS Vantage (BD Biosciences). Compensation for FITC, PE, and APC was performed using compensation beads (BD Pharmingen). Analysis was done using the Flow-Jo program (Tree Star, Ashland, OR, http://www.flojo.com). Positive and negative gates were determined using IgG-stained and unstained controls.

**Real-Time Polymerase Chain Reaction**

Cells were pelleted at 200g for 5 minutes, and total RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Valencia, CA, http://www1.qiagen.com) per the manufacturer’s protocol. RNA was quantified using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, http://www.nanodrop.com). Two hundred nanograms of purified mRNA per 20-μl reaction volume was used to construct first-strand cdNA using an oligo(dT) reverse transcriptase kit at 37°C for 60 minutes (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Real-time experiments were conducted by use of an ABI Prism 7700 Thermal Cycler and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, http://www. appliedbiosystems.com). Housekeeping genes included β-Actin, Hypoxanthine Phosphoribosyltransferase 1, and Ubiquitin C, and a geometric mean of housekeeping Ct values was used for all ΔΔCt calculations [27]. The relative level of expression was calculated for the genes Albumin, Ck19, Hnf4α, C-Met, Epithelial growth factor receptor (Egfr), Cyclin D1, Kras, Nras, Survivin, c-My, Nanog, Oct4, and Abcg2. These genes were assessed using real-time polymerase chain reaction (PCR) primer/probe sets (Applied Biosystems). Amplification efficiency was determined by the ΔΔCt method of the amplification plots [28].

**Cell Culture**

CD45− cells were isolated from the NP fraction using magnetic bead separation as described above. This CD45− NP cell fraction was labeled with CD133 magnetic bead antibodies (Miltenyi Biotech), and CD133+CD45− cells were isolated using positive cell selection per the manufacturer’s protocol. This enriched population of CD133+CD45− cells was plated in six-well BioCoat lamin-coated culture plates (BD Biosciences) at a density of 1 × 10⁶ cells per cm². Culture medium was modified from Suzuki et al. [29]. Medium contained Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium at 1:1 (Sigma-Aldrich) with 10% heat-inactivated fetal calf serum (Omega Scientific), and the following additives: insulin (1μg/ml), dexamethasone (1 × 10⁻⁷ mol/l), nicotinamide (10 mmol/l), Hepes (5 mmol/l), and penicillin/streptomycin (1% vol/vol) (all Sigma-Aldrich). Recombinant hepatocyte growth factor

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**Materials and Methods**

**PtenloxP/loxP; Alb–Cre+ Mice and Nude Mice**

Mice were fed ad libitum a standard diet (Harlan Teklad irradiated mouse diet 7912; Harlan Laboratories, Madison, WI, http://www.harlan.com) and housed in a temperature-controlled animal facility with 12-hour light-dark cycles. Animals were treated humanely, and all procedures were in compliance with our institutions guidelines for the use of laboratory animals and approved by the Institutional Animal Care and Use Committee. Three- and 6-month-old PtenloxP/loxP; Alb–Cre+ mice (mutants) and wild-type (WT) littermates were used for all experiments, as described [19]. Six-week-old nude mice (Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) and WT mice were used for tumor formation analysis.

**Parenchymal and Nonparenchymal Cell Separation**

Protocol for the digestion and centrifugation of the liver cells was modified from Shimano et al. [25] as described [26]. Liver was minced and digested with collagenase, pronase, and DNase (Sigma-Aldrich, St. Louis, http://www.sigmaldrich.com) and filtered using a 70-μm pore filter (BD Biosciences, Falcon Lakes, NJ, http://www.bdbiosciences.com). After suspension in 10% fetal calf serum (Omega Scientific, Tarzana, CA, http://www.omicscientific.com) in phosphate-buffered saline (PBS) (Mediatech, Herndon, VA, http://www.cellgro.com), cells were centrifuged [26]. The final NP cell pellet was resuspended in 1 x ammonium chloride red blood cell (RBC) lysis buffer (PharM Lyse; BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml) and washed, and the NP cells were counted and subjected to CD45 depletion using Miltenyi magnetic bead depletion using the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA, http://www.milenxybiotec.com).

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(50 ng/ml) and epithelial growth factor (20 ng/ml) (both Sigma-Aldrich) were added on day 1. Medium was changed every 3 days. For alkaline phosphatase analysis, cells were fixed in 90% methanol/10% formalin (Sigma-Aldrich) for 1 minute and stained using the Alkaline Phosphatase Detection Kit (Chemicon, Temecula, CA, http://www.chemicon.com).

Gene Expression of CD133+CD45− Oval Cells
RNA was extracted directly from the culture well using an RNeasy Kit (Qiagen). Per 20-μl reaction volume, 200 ng of purified RNA was used in the synthesis of first-strand cDNA as described. All primers were selected in two separate exons to distinguish cDNA from possible contaminating genomic DNA, and PCR conditions were used as described [26]. (Primer sequences are given in supporting information Table 1).

Western Blot Analysis
For Western blot analysis, cell lysates were harvested by the addition of lysis buffer (40 mM Tris [pH 7.4], 150 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM glyceraldehyde 3-phosphate, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride), supplemented with protease inhibitor cocktail (Roche, Indianapolis, http://www.roche.com). Whole-cell lysates were centrifuged at 10,000g for 15 minutes at 4°C to remove the cell debris. Protein concentration was quantified using the BCA assay (Pierce, Rockford, IL, http://www.piercenet.com). Forty nanograms of protein per lane were separated on a NuPage 4%–12% Bis-Tris Gel (Invitrogen). The blot was blocked with Western blot buffer (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20) containing 5% nonfat milk (Bio-Rad, Hercules, CA, http://www.bio-rad.com) and transferred to a polyvinylidene difluoride membrane (Invitrogen). The blot was incubated with horseradish peroxidase-conjugated primary antibody according to the manufacturer's recommendation. After washing with Western blot buffer, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Pittsburgh, http://www.amersham.com) for 1 hour at room temperature. Signals were detected using enhanced chemiluminescence solutions (Amersham Pharmacia Biotech, Piscataway, NJ, http://www.amershambiosciences.com). The full list of Western blot antibodies is provided in supporting information Table 2.

Single-Cell Analysis
For single-cell experiments, 4,6-diamidino-2-phenylindole (DAPI) was used as a marker of cell viability. Single CD133+CD45−DC1−OCs (n = 192) were isolated using a FACSVantage set for single cell purity, and single cells were robotically plated directly into a flat-bottomed, 96-well laminin-coated plate (BD set for single cell purity, and single cells were robotically plated CD133 transplant at a concentration of 1

Cell Viability Assay
Doxorubicin and 5-fluorouracil (5-FU) (both Sigma-Aldrich) were used for the chemotherapeutic assay. Cell viability was tested using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) kit (catalog no. 4891-025-K; Trevigen Inc., Gaithersburg, MD, http://www.trevigen.com), per the manufacturer’s protocol. In each well of a 96-well plate, 1 × 106 cells were seeded. Twenty-four hours after doxorubicin or 5-FU treatment at defined dosages, cells were analyzed using the Trevigen XTT protocol.

Statistical Analyses
A paired, two-tailed Student’s t test was used when comparing two groups. A p value of less than .05 was considered significant. Analysis of variance was used for comparison of multiple groups, followed by pairwise multiple comparison procedures (Systat Software Inc., Richmond, CA, http://www.systat.com).

RESULTS

Expansion of Oval Cells with Age in PtenloxPloxP; Alb−Cre+ Mice
Three- and 6-month-old PtenloxPloxP; Alb−Cre− (control WT) and PtenloxPloxP; Alb−Cre+ (mutant) mice were analyzed for degree of injury and OC proliferation using hematoxylin and eosin (H&E) staining and pan-CK FIHC as an OC/bile duct marker [33]. In the control animals, H&E staining revealed no injury, steatosis, or OC proliferation (Fig. 1A, 1B). In the PtenloxPloxP; Alb−Cre+ mice, H&E staining revealed significant injury and steatosis without evidence of gross or microscopic tumor [19, 20]. Further analysis demonstrated increased duct and oval cell proliferation associated with chronic injury (Fig. 1D–1H). Immunohistochemistry for pan-Ck, a duct and
oval cell marker, revealed increased staining in both 3- and 6-month-old mutant mice compared with control mice, consistent with the overall injury (Fig. 1C, 1F, 1I).

Whole liver tissue lysate from 3- and 6-month-old PtenloxP/loxP; Alb/H11002 Cre/H11001 mice demonstrated loss of PTEN protein and constitutive phosphorylation of AKT compared with age-matched controls (Fig. 1J). PCR amplification of mouse tails from PtenloxP/loxP; Alb/H11002 Cre/H11001 mice confirmed Pten deletion, as indicated by excision of the Pten locus (Pten55) (data not shown).

To obtain CD133-expressing liver progenitor cells from the Pten mutant animals, we depleted CD45+ hematopoietic cells and analyzed this CD45− NP fraction, as previously described in isolating CD133+CD45− OCs [7]. Using FACS analysis, we demonstrated that Pten mutant livers are enriched in CD133+CD45− NP OCs (6-month-old control WT 4.2% ± 2%; 3-month-old mutant 8.5% ± 3%, 6-month-old mutant 12.5% ± 4%; n = 3; p < .05 for 6-month-old mutant group compared with WT; Fig. 2A).

Enrichment of CD133+ Oval Cells
To further characterize their lineage potential, CD133+CD45− OCs were isolated from 6-month-old PtenloxP/loxP; Alb−Cre+ mice and plated in six-well, laminin-coated tissue culture plates using a fetal hepatoblast medium [29]. Laminin-coated plates allow for further selection of liver stem cells [21]. On day 1, small round cells were observed adhering to the plate. After 1 week in culture, clusters of cells were observed growing, with the majority of the cells having the morphology of cuboidal cells in clusters/sheets (Fig. 2B, 2C). After 1 week in culture, the cells were 90% confluent, and they were trypsinized, replated 1:3, and passaged every 3 days thereafter.
After 4 months of replating (passage 25), the basic morphology of these bulk culture cells remained unchanged. Protein cell lysate from CD133+/H11001CD45+/H11002oval cell bulk culture demonstrated complete loss of PTEN, phosphorylation of AKT, and strong expression of α-fetoprotein (αFP) (Fig. 2D). We also observed single cells or small clusters of cells with alkaline phosphatase expression, a potential marker of progenitor cells (supporting information Fig. 1) [34, 35]. Costaining of hepatocyte (αFP) and cholangiocyte (biliary cytokeratin [CK]) markers was conducted to demonstrate an oval cell phenotype within these bulk cultures. Of the 1,000 cells counted, 72 cells were positive for αFP, 63 cells were positive for CK, and 59 cells (6% of the total counted) were positive for both markers. (Fig. 2E–2G) This analysis indicates maintenance of a population of bipotent oval cells in later-passage cultures (passage 25).

**Single-Cell Isolation Defines Bipotency in CD133+/CD45− OCs**

To isolate this potential progenitor cell population, single cells were isolated by FACS using automated plating robotics. CD133+/CD45−DAPI− cells were selected from early passage CD133+ bulk cultures (passage 6). Absence of DAPI is a marker of cell viability, as DAPI stains the nuclei of dead cells. Of 192 individual cells plated, 26 (13.5%) demonstrated colonies growing over 50% of the well bottom after 2 weeks. These colonies were replated into six-well plates, and the basic cell morphology matched that of the original culture, with flat, cuboidal cells. After 2 weeks, 100% of analyzed colonies (n = 5) demonstrated gene expression of both hepatocyte (Albumin) and cholangiocyte (Ck19) markers, using RT-PCR (Fig. 3A). All CD133+ derived clone lines demonstrated expression of oval cell/hepatoblast-associated genes (αFP, Hnf1α, Hnf3β, and Abcg2) (Fig. 3A) [36] and expression of growth factor receptors c-Met and Egfr. Lastly, no expression of hematopoietic (Cd45) or satellite cell (α-Smooth muscle actin) markers were demonstrated in any CD133+ derived clone line (Fig. 3A).

Protein lysate from each CD133+ derived clone line was analyzed for PTEN, phospho-AKT, pan-AKT, and β-Actin. This functional analysis demonstrated complete loss of PTEN protein in all CD133+ derived clone lines with constitutive phosphorylation of AKT (Fig. 3B). Pten deletion was confirmed with PCR amplification, as indicated by excision of the PTEN locus (PtenloxP/loxP; Alb−Cre+ mice (Mut), (n = 3; p < .05 for 6-month-old Mut mice compared with age-matched Con; mean + SD). Phase-contrast images of CD133+/CD45− cells in vitro from 6-month-old Mut mice animals at 1 week (B) and 2 weeks (C) demonstrate a relatively homogeneous population of polygonal/cuboidal flat cells. Western blot analysis of these cells at 2 weeks demonstrated complete loss of PTEN protein, constitutive phosphorylation of AKT, and expression of αFP (D) in cells from Mut mice compared with a CD133+/CD45− oval cell fraction from con animals. AFP fluorescent immunohistochemistry (green) identified cells with maintenance of hepatocyte lineage after 25 passages (E); staining of biliary CK (red) (F) indicated cells with maintenance of cholangiocyte lineage. Merge image (G) shows the cells with bilineage expression, indicating maintenance of oval cell phenotype (6%; n = 1,000 cells counted). Abbreviations: αFP, α-fetoprotein; AFP, α-fetoprotein; CK, cytokeratin; Con, control; DAPI, 4,6-diamidino-2-phenylindole; Mut, mutant; NP, nonparenchymal; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

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**Figure 2.** Flow cytometry (FACS) analysis of CD133 expression. (A): FACS analysis demonstrated expansion of the CD133+/CD45− liver NP cells with aging in PtenloxP/loxP; Alb−Cre+ mice (Mut), (n = 3; p < .05 for 6-month-old Mut mice compared with age-matched Con; mean + SD). Phase-contrast images of CD133+/CD45− cells in vitro from 6-month-old Mut mice animals at 1 week (B) and 2 weeks (C) demonstrate a relatively homogeneous population of polygonal/cuboidal flat cells. Western blot analysis of these cells at 2 weeks demonstrated complete loss of PTEN protein, constitutive phosphorylation of AKT, and expression of αFP (D) in cells from Mut mice compared with a CD133+/CD45− oval cell fraction from con animals. AFP fluorescent immunohistochemistry (green) identified cells with maintenance of hepatocyte lineage after 25 passages (E); staining of biliary CK (red) (F) indicated cells with maintenance of cholangiocyte lineage. Merge image (G) shows the cells with bilineage expression, indicating maintenance of oval cell phenotype (6%; n = 1,000 cells counted). Abbreviations: αFP, α-fetoprotein; AFP, α-fetoprotein; CK, cytokeratin; Con, control; DAPI, 4,6-diamidino-2-phenylindole; Mut, mutant; NP, nonparenchymal; PTEN, phosphatase and tensin homolog deleted on chromosome 10.
FIHC) represented up to 33.3% of cells in CD133+/H11001-derived clone lines, a significant increase compared with the 6% in the original, nonpurified culture (Fig. 3C).

Tumor Formation from CD133+ Derived Clone Lines

To assess the tumor forming ability of CD133+/H11001-derived clone lines, a tumor model using immune-deficient mice was initially used. Two million cells isolated from each CD133+/H11001-derived clone line (lines A–E; passage 6 after single-cell isolation) were injected SQ into immune-deficient mice (n = 6 per cell line; five lines total). Four of the five oval cell lines expanded from single CD133+ cells formed tumors to various degrees (clone line A, three of six; clone line B, two of six; clone line C, one of six; clone line E, three of six; Fig. 4A–4C). Tumor histology revealed mixed populations with dysplastic columnar and cuboidal epithelial cells, with similar histology in all tumors (Fig. 4D–4I). Clone line D did not contribute to tumor formation in this xenograft model. FACS analysis of tumor cells demonstrated a high level of CD133 expression (Fig. 5A, 5B). PCR analysis of tumor cells demonstrated strong expression of liver-specific genes Albumin and Ck19 (Fig. 5C), an indication of bipotential differentiation from the grafted CD133+/CD45− cells. Western blot of tumor cells confirmed consistent loss of PTEN protein, constitutive phosphorylation of AKT, and high levels of CD133 protein (Fig. 5D).

We further compared the expression of different progenitor and liver cell markers between the tumorigenic clones (clones A, B, C, and E) and the nontumorigenic clone, clone D. Real-time PCR analysis demonstrated that the tumorigenic lines had increased expression for the progenitor cell markers Survivin, cMyc, Egfr, and CD133 and lower expression of the differentiated cell markers CK19 and Hnf4α, compared with the nontumorigenic clone line (Fig. 6).

CD133+ Cancer Stem Cells

To further confirm that the CD133+ cells led to the tumorigenic phenotype, we isolated CD133+ and CD133− cells prior to SQ injection. We injected 1 million CD133+ and CD133− cells from each of the tumorigenic clone line (passage 10 from single-cell isolation) into nude mice. We demonstrated that CD133+ cells gave rise to robust tumors (six of six tumors; 41 mm3/week growth rate; Fig. 7A), and CD133− cells only formed one small tumor (one of six; 1.2 mm3/week growth rate; Fig. 7A). Using 100,000 cells in the identical assay resulted in 50% tumor formation with CD133+ cells and 0% tumor formation using
CD133+/H11002 cells (CD133+/H11001, three of six tumors; CD133+/H11002, zero of six tumors; supporting information Table 3). Furthermore, 1 million cultured CD133+/H11001 cells formed tumors in immune-competent WT mice (two of six; passage 14; Fig. 7B, 7C; supporting information Table 3), and CD133− cells did not (zero of six; supporting information Table 3).

Figure 4. Clone line bulk culture xenograft assay. Nude mice were injected subcutaneously with 2 million cells from each CD133+ derived clone line (passage 6). (A–C): Mice with tumors (arrows), after 4 months, from clone lines A (A), B (B), and C (C). (D–I): Corresponding low-magnification (×4 objective) H&E stains of each tumor are shown in (D–F); boxes indicate the high-magnification (×20 objective) areas shown in (G–I). High-magnification images show dysplastic epithelial tumors with mixed morphology.

Figure 5. Tumor analysis. Fluorescence-activated cell sorting (FACS) analysis of xenograft tumors demonstrated high CD133 expression. (A, B): Representative FACS plots from tumors derived from clone line A (A), also shown with a summary graph of all tumors from clone lines A, B, C, and E with SE bars (B). (C): Gene expression (reverse transcription-polymerase chain reaction) analysis of representative tumors from clone lines A (tumor A), B (tumor B), and C (tumor C) and negative control water demonstrated coexpression of hepatocyte marker Albumin and cholangiocyte marker Ck-19 in each tumor. (D): Western blot analysis confirmed loss of PTEN in tumor cells from clone lines A (tumor A) and C (tumor C), as well as constitutive phosphorylation of AKT compared with positive control (+) CD133+ cells from wild-type animals. A high level of CD133 protein expression was demonstrated in tumor cells. Abbreviations: Ck-19, Cytokeratin-19; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PTEN, phosphatase and tensin homolog deleted on chromosome 10.
Figure 6. Gene expression of tumorigenic clone lines. Summary of real-time polymerase chain reaction (PCR) analysis of all five clone lines from 6-month-old Ptenfl/fl; Alb-Cre+ mice. Tumorigenic CD133+ derived clone lines (A, B, C, and E) demonstrated higher expression of stem cell-associated genes CD133, c-Myc, Egfr, and Survivin compared with nontumorigenic CD133+ derived clone line D. Two tumorigenic CD133+ derived clone lines had lower expression of genes of differentiation Ck19 (cholangiocyte marker) and Hnf4a (hepatocyte marker) compared with nontumorigenic CD133+ clone line D (n = 4 real-time PCR experiments per line; each primer/probe series in triplicate; ± SD). Abbreviations: Alde-Dehyd, aldehyde dehydrogenase; CK-19, Cytokeratin 19; EGFr, epithelial growth factor receptor; Hnf4a, hepatocyte nuclear factor 4a.

Given that cells in vitro may acquire additional transformation events with passage, we isolated CD133+CD45− and CD133−CD45− NP liver cells from 3- and 6-month-old Ptenfl/fl; Alb-Cre+ mice and injected 1 million cells directly (without culture) into nude mice. Only the CD133+ fraction from 6-month-old mutants gave rise to tumors (two of six; Fig. 7D, 7E; supporting information Table 3). The CD133− fraction from the same set of mutant livers did not contribute to tumor formation (zero of eight; supporting information Table 3). None of the CD133+ and CD133− cells from 3-month-old mutants gave rise to tumors (CD133+, zero of four; CD133−, zero of four; supporting information Table 3). The tumors that arose from cultured and freshly isolated CD133+ cells demonstrated both hepatocyte-like and duct-like structures, indicating a potential stem cell-initiated tumor (Fig. 7C, 7E; supporting information Fig. 2).

To determine whether the CD133+ cells from each clone line have additional CSC characteristics, we analyzed cell viability after chemotherapy treatment. CD133+ and CD133− cells from the tumorigenic clone lines were exposed to IC90, IC75, and IC50 concentrations of doxorubicin and 5-FU. As demonstrated, CD133+ cells have significant survival at each concentration for these two chemotherapy drugs compared with CD133− cells (supporting information Figs. 3, 4).

**DISCUSSION**

The ability to identify and isolate adult stem cells during premalignant liver injury is an essential first step in studying liver cancer stem cells [37]. In models of liver damage, with hepatic infiltration of inflammatory cells, separation of hematopoietic cells from liver cells is critical prior to OC analysis [26]. In terms of defining a reliable OC immunophenotype, the surface markers previously attributed to OCs, such as c-kit and CD34, are traditionally assigned to hematopoietic stem cells [38]. Recently, we defined a population of cells that express CD133 but not the hematopoietic marker CD45. The cell surface marker CD133 has been associated with both pluripotent stem cells and cancer stem cells of epithelial origin [39]. Upreregulated CD133 expression was detected in rat OCs, isolated by size, as part of a microarray screen [40]. Our group used single-cell gene expression analysis to confirm the bilineage hepatic potential of CD133+CD45− OCs isolated from murine liver injury models [7].

In this study, we used CD133+CD45− as a marker to identify a population of cells that are proliferating during premalignant liver injury in the liver Pten deletion mouse model. In this model, we and other have documented chronic liver injury and steatosis prior to the development of primary liver carcinomas [19, 20]. Interestingly, analysis of this murine model of Pten deletion reveals a bilineage origin of tumors, with the development of both HCC and CC [41]. Three possible cellular origins of HCC and CC have been proposed previously: (a) the mature hepatocyte causing HCC; (b) the mature cholangiocyte causing CC; and (c) the bipotent cancer stem cell causing HCC, CC, and mixed tumors [3]. Tumors with progenitor or OC phenotypes represent 25%–50% of HCC and have a more aggressive phenotype [2]. Despite the effort of multiple international investigators, a defined, specific population of liver cancer stem cells remains elusive [24, 42].

The cell surface marker CD133 has been used to identify populations of liver cancer stem cells. Using HCC cell lines, CD133 expression identifies an aggressive phenotype of cancer stem cell [22, 23]. Furthermore, in multiple human gastrointestinal cancer cell lines, CD133+ cells are associated with the side-population cancer stem cell phenotype, which has ABCG2 membrane-pump expression and resistance to chemotherapy. Our group has recently described CD133+ liver cancer stem cells isolated during premalignant liver injury in methionine adenosyltransferase 1A-deficient mice [21]. Using the same basic techniques described, we isolated the CD133+CD45− oval cells from the liver Pten deletion model. This CD133+CD45− oval cell population is more resistant to doxorubicin and 5-FU treatment in culture than the CD133− cell population, supporting the concept that CD133 expression identifies a cancer stem cell population. We demonstrated that these cells are bipotential, with markers of both hepatocytes and cholangiocytes. To further determine whether deletion of Pten in this specific CD133+CD45− cell population is sufficient to convert OCs to liver cancer stem cells, we performed multiple cell transplant studies. The results from these experiments demonstrate that expression of CD133 is associated with the tumorigenic cell fraction. The clone line with the lowest CD133 expression (clone D) was unable to form tumors in nude mice, further supporting the notion that CD133 cell surface expression is a cancer stem cell marker in liver.

In liver cancer, which is the third cause of cancer mortality worldwide, upregulation of the EGFR/Pi3K/AKT signaling plays a key role in cell growth regulation [43]. The molecular interaction of this signaling pathway with the most common etiologial agents of HCC, hepatitis B and C viruses, is also established [44, 45]. As the negative regulator of the EGFR/Pi3K/AKT signaling pathway, PTEN loss is unequivocally correlated with human liver cancer.

**SUMMARY**

In summary, using the liver Pten deletion mouse model, we demonstrated that deletion of Pten leads to the proliferation of the liver OC population defined as CD133+CD45− NP cells. Given that tumors that are formed from CD133+ cells demonstrate both hepatocyte-like and duct-like structures, the
CD133+ progenitor population is a potential cellular origin of the bilineage tumor phenotype (HCC and CC) observed in this Pten deletion mouse model. This data suggest that the association between high malignant potential/poor prognosis and PTEN mutation observed with human liver cancer may have a progenitor cell mechanism. Future studies focusing on ablation of this liver cancer cell population are needed to test the therapeutic potential of targeting this cell population.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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