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Human Fetal Hepatic Progenitor Cells are Distinct from, but Closely Related to, Hematopoietic Stem/Progenitor Cells

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ABSTRACT

Much controversy surrounds the identity and origin of human hepatic stem and progenitor cells in part because of a lack of small animal models in which the developmental potential of isolated candidate cell populations can be functionally evaluated. We show here that adoptive transfer of $CD34^+$ cells from human fetal liver into sublethally irradiated NOD-SCID $Il2rg^{-/-}$ (NSG) mice leads to an efficient development of not only human hematopoietic cells but also human hepatocyte-like cells in the liver of the recipient mice. Using this simple in vivo assay in combination with cell fractionation, we show that $CD34^+$ fetal liver cells can be separated into three distinct subpopulations: $CD34^{hi}CD133^{hi}$, $CD34^{lo}CD133^{lo}$, and $CD34^{hi}CD133^{neg}$. The $CD34^{hi}CD133^{hi}$ population contains hematopoietic stem/progenitor cells (HSPCs) as they give rise to T cells, B cells, NK cells, dendritic cells, and monocytes/macrophages in NSG mice and colony-forming unit (CFU)-GEMM cells in vitro. The $CD34^{lo}CD133^{lo}$ population does not give rise to hematopoietic cells, but reproducibly generates hepatocyte-like cells in NSG mice and in vitro. The $CD34^{hi}CD133^{neg}$ population only gives rise to CFU-GM and burst-forming unit-erythroid in vitro. Furthermore, we show that the $CD34^{lo}CD133^{lo}$ cells express hematopoietic, hepatic, and mesenchymal markers, including CD34, CD133, CD117, epithelial cell adhesion molecule, CD73, albumin, α-fetal protein, and vimentin and transcriptionally are more closely related to HSPCs than to mature hepatocytes. These results show that $CD34^{lo}CD133^{lo}$ fetal liver cells possess the hepatic progenitor cell properties and that human hepatic and hematopoietic progenitor cells are distinct, although they may originate from the same precursors in the fetal liver. STEM CELLS *2013;31:1160–1169*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Hepatic stem cells and progenitor cells have attracted considerable research interests because of their potential clinical applications. During early ontogeny, fetal liver is rich in hepatic stem cells and progenitor cells as well as in hematopoietic stem cells and progenitor cells. CD34 and CD133 are the most widely used markers for selectively isolating hematopoietic stem/progenitor cells (HSPCs) [1–3]. In contrast, the phenotype of hepatic stem and progenitor cells in the fetal liver is still being debated. In rodents, some studies show that hepatic stem/progenitor cells share surface markers associated with HSPCs; these markers include CD90 [4, 5], CD117 [6–8], and CD34 [8–10]. Based on expression of these and other hematopoietic cell markers, some investigators suggest that hepatic stem/progenitor cells are derived from HSPCs. Other studies report that hepatic stem/progenitor cells can be distinguished from HSPCs owing to their expression of hepatic and epithelial cell markers such as albumin, α-fetoprotein (AFP), cytokeratin (CK) 18, and CK19 [11–14]. Such conflicting phenotypes have also been reported for human hepatic stem/progenitor cells [15–20]. The lack of a unique surface marker, or a combination of markers, has hampered the isolation of hepatic stem/progenitor cells and undermined efforts at determining the origin of hepatic stem/progenitor cells. The nature

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of the relationship between hepatic and HSPCs in the fetal liver and the issue of whether they are distinct and can be isolated separately have yet to be resolved.

Identification of human hepatic stem/progenitor cells is further hampered by a lack of small animal models in which developmental potential of putative cell populations can be evaluated. While adoptive transfer into syngeneic recipients can be used to evaluate the stem/progenitor property of cell populations from the fetal livers of mice and rats, the induction of graft rejection makes it difficult to use immunocompetent mice or rats to assess the developmental potential of putative human hepatic stem/progenitor cells. Even with use of immunodeficient mice, such as SCID [19], NOD/SCID [16], Pfp/Rag2$^{-/-}$ [17], or Rag2$^{-/-}\gamma^{-/-}$ mice [20], the widely used protocol of surgical intrasplenic injection is cumbersome and the level of human cell reconstitution is generally very low (<1%). Most studies on human hepatic stem/progenitor cells have resorted to clongenic and in vitro differentiation assays, with the use of long-term cultures. Despite their potential for yielding useful information, in vitro clongenic assay cannot replace an in vivo assay only in which the appropriate microenvironment and physiological condition can be provided. There is an urgent need for an easy and robust animal model that can be used to evaluate the developmental potential of putative human hepatic stem/progenitor cells.

We have constructed humanized mice by adoptive transfer of CD34$^+$ cells from midgestation human fetal liver into sublethally irradiated NOD-SCID Il2rg$^{-/-}$ (NSG) mice. We found that recipient mice efficiently reconstitute human hematopoietic cells as well as human hepatocyte-like cells in their livers. This technical breakthrough has enabled us to unequivocally identify and isolate human fetal hepatic progenitor cells (HPCs), which can differentiate into hepatic lineage cells through successive transfer, from HSPCs that give rise to various blood lineage cells. The isolated HPCs express hepatic and mesenchymal markers as well as many hematopoietic markers and are transcriptionally more closely related to HSPCs than to mature hepatocytes. Our findings clarify the current controversies about the phenotype and origin of hepatic stem/progenitor cells. The identification of human fetal HPCs, along with the development of a robust mouse model to functionally evaluate these cells, provides a platform for basic and applied research on human hepatic stem/progenitor cells in health and disease.

Materials and Methods

Isolation of CD34$^+$ Cells from Human Fetal Liver and Cord Blood

Human fetal livers ($n > 8$) were obtained from aborted fetuses at 15–23 weeks of gestation, in accordance with the institutional ethical guidelines of the National University Hospital of Singapore. All women gave written informed consent for the donation of their fetal tissue for research. Fetuses were collected within 2 hour of the termination of pregnancy. Fetal liver tissue was initially cut into small pieces and digested with collagenase VI (2 mg/ml in Dulbecco's modified Eagle's medium [DMEM]) for 15 minutes at 37°C with periodic mixing. Single-cell suspension was prepared by passing the digested tissue through a 100 μm cell strainer (BD Biosciences, Franklin Lakes, NJ. http://www.bdbiosciences.com). Umbilical cord blood was obtained from the national disease research interchange or the Singapore Cord Blood Bank. CD34$^+$ cells from both fetal liver and cord blood were purified with the use of a CD34-positive selection kit (Stem Cell Technologies, Vancouver, BC. http://www.stemcell.com); the purity of CD34$^+$ cells was 90%–99%. Viable cells were counted by trypan blue exclusion of dead cells. All cells were isolated under sterile conditions. To purify CD34^{hi}CD133^{hi}, CD34^{lo}CD133^{lo}, and CD34^{hi}CD133^{neg} cells, cells isolated with the CD34-positive selection kit were stained with anti-CD34 and anti-CD133 antibodies and purified by sorting on an Aria cell sorter (Beckton Dickinson, Franklin Lakes, NJ). The purity of sorted cells was between 90% and 99%.

Mice and the Injection of Cells

NSG mice were obtained from the Jackson Laboratory and bred in the animal facilities at the National University of Singapore (NUS). Pups were sublethally irradiated (100 rads) within 24 hours of birth, and engrafted with purified fetal liver cells or cord blood cells, by intracardiac or intrahepatic injection: 2×10^5 CD34$^+$ cells, or 1×10^5 CD34^{hi}CD133^{hi} cells, CD34^{lo}CD133^{lo} cells, or CD34^{hi}CD133^{neg} cells were injected into each recipient. All research with human samples and mice were performed in compliance with guidelines of NUS and Massachusetts Institute of Technology.

Serial Transfer

Purified CD34^{lo}CD133^{lo} cells (1×10^5) were injected intrahepatically into sublethally irradiated newborn NSG pups. After 9 weeks, mouse livers were first perfused with prewarmed liver perfusion medium (Life Technologies, Grand Island, NY. http://www.invitrogen.com) at 0.7 ml/minute for 10 minutes, then with prewarmed liver digestion medium (Life Technologies, Grand Island, NY) for 10 minutes. Single-cell suspensions were prepared and washed with ice-cold DMEM. Post-treatment cell viability exceeded 90% as assessed by trypan blue dye exclusion. CD34$^+$ cells were purified from the cell suspensions with the CD34-positive selection kit (Stem Cell Technologies, Vancouver, BC) and injected into sublethally irradiated newborn NSG pups (1×10^5 per mouse). After another 8 weeks, livers of the secondary recipient mice were harvested for histology and sera were analyzed for human albumin by ELISA.

Flow Cytometry

Conjugated antibodies specific for epithelial cell adhesion molecule (EpCAM) (9C4), CD44 (BJ18), CD34 (561), CD166 (3A6), CD105 (43A3), CD29 (TS2/16), CD24 (ML5), CD90 (5E10), CD73 (AD2), CD117 (104D2), CD97 (VIM3b), CD45 (HI30), or mouse H-2D^b (KH95) were obtained from BioLegend (Biolegend, San Diego, CA. http://www.biolegend.com/); antibodies against CD133 (EMK08) and mouse CD45.1 (A20) were from eBioscience (eBioscience, Inc. San Diego, CA. http://www.ebioscience.com/). Cells were stained with appropriate antibodies in 100 μl Phosphate buffered saline (PBS) containing 0.2% Bovine serum albumin (BSA) and 0.05% sodium azide for 30 minutes on ice. Flow cytometry was performed on an LSRII flow cytometer using the FACSDiva software (BD, Franklin Lakes, NJ): 10,000–1,000,000 events were collected per sample and analyzed using the Flowjo software.

Immunostaining

Livers were embedded in paraffin and sectioned at a thickness of 3-μm. Deparaffinized sections were blocked and stained with optimal dilutions of rabbit anti-human albumin (Abcam, Cambridge, MA. http://www.abcam.com); sections were developed with SuperPicture third Gen IHC Detection Kit (Life Technologies, Grand Island, NY). For immunofluorescence staining, deparaffinized sections or cells were blocked with 3% BSA/0.2% Triton X-100/PBS and stained with rabbit anti-human albumin (Abcam, Cambridge, MA), mouse anti-human E-cadherin (Abcam, Cambridge, MA), mouse anti-

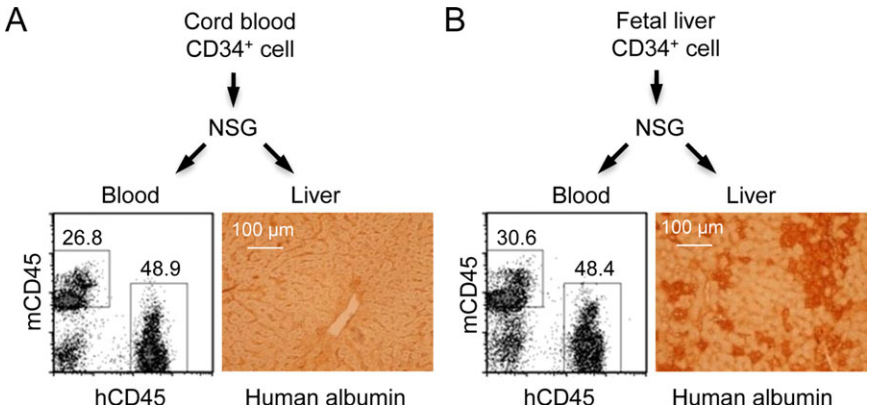

Figure 1. $CD34^+$ cells from human fetal liver generate hematopoietic and hepatic cells in NSG recipient mice. $CD34^+$ cells isolated from cord blood **(A)** and fetal liver **(B)** were engrafted separately into sublethally irradiated NSG newborn pups by intracardiac injection. Eight weeks later, peripheral blood mononuclear cells were stained with antibodies for human CD45 (hCD45) and mouse CD45 (mCD45), and analyzed by flow cytometry. Representative hCD45 versus mCD45 staining profiles are shown on the left; the numbers indicate percentages of human and mouse $CD45^+$ cells among total live cells. Paraffin sections of the livers of the same mice were stained with antibody specific for human albumin. Representative stains are shown on the right; scale bar applies to both panels. Shown are representative results from over 20 mice reconstituted with $CD34^+$ cells from three different cord blood samples and over 20 mice reconstituted with $CD34^+$ cells from three different fetal livers. Abbreviation: NSG, NOD-SCID $Il2rg^{-/-}$.

human CD29 (Abcam, Cambridge, MA), rabbit anti-human AFP (Sigma, St. Louis, MO. http://www.sigmaaldrich.com), rabbit anti-human cytokeratin7 (CK7) (Sigma, St. Louis, MO), or rabbit anti-human CK19 antibodies (Sigma, St. Louis, MO). Rhodamine-conjugated goat anti-rabbit IgG (Life Technologies, Grand Island, NY), Rhodamine-conjugated goat anti-mouse IgG, or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies, Grand Island, NY) were used as the secondary antibodies. Slides were mounted with Prolong Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY) and examined with a MIRAX MIDI Fluorescence microscope (Zeiss Oberkochen, Germany. http://corporate.zeiss.com). Additional experimental procedures are provided in Supporting Information Materials.

Results

$CD34^+$ Cells from Human Fetal Liver Generate Both Hematopoietic and Hepatic Cells Following Engraftment into NSG Mice

To construct humanized mice, we used $CD34^+$ cells isolated from both cord blood and fetal liver as sources of HSPCs. Typically, 2×10^5 $CD34^+$ cells were engrafted into a sublethally irradiated newborn pup of NSG mice by intracardiac injection. As expected, 8 weeks following reconstitution, human $CD45^+$ leukocytes were detected in the peripheral blood of recipient mice, regardless the source of the engrafted $C34^+$ cells (Fig. 1). When liver sections of the recipient mice were stained for human albumin, no positive signal was detected in recipients that were engrafted with cord blood $CD34^+$ cells (Fig. 1A); in contrast, a significant fraction of liver cells was stained positive for human albumin if the recipient mice were engrafted with $CD34^+$ fetal liver cells (Fig. 1B). Generation of human albumin-expressing liver cells, referred to as hepatocyte-like cells, was consistently observed using purified $CD34^+$ cells from livers of fetuses ranging from 15 to 23 weeks of gestation. The frequency of human hepatocyte-like cells was slightly higher when $CD34^+$ fetal liver cells were engrafted into recipient pups by direct intrahepatic injection (data not shown). For this reason, intrahepatic injection was used in most of the subsequent experiments unless specified otherwise. These results suggest that, in addition to HSPCs, $CD34^+$ cells from human fetal liver also contain HPCs that give rise to albumin-expressing human hepatocyte-like cells when engrafted into the mouse liver.

$CD34^{lo}CD133^{lo}$ Cells Give Rise to Human Hepatocyte-Like Cells, Whereas $CD34^{hi}CD133^{hi}$ Cells Give Rise to Hematopoietic Cells

$CD34^+$ cells from fetal liver could be separated into three distinct subpopulations, characterized by high, low, or negative expression of CD133 when costained with an anti-CD133 antibody (Fig. 2A), whereas $CD34^+$ cells from cord blood displayed uniformly high levels of CD133. Based on forward scatter and Giemsa staining, $CD34^{lo}CD133^{lo}$ cells were much larger in size than $CD34^{hi}CD133^{hi}$ and $CD34^{hi}CD133^{neg}$ cells (Supporting Information Fig. S1A). Depending on the gestation stage of the donor fetal livers, the proportion of the $CD34^{hi}CD133^{hi}$ cells ranged from 10% to 25%, whereas the proportion of the $CD34^{lo}CD133^{lo}$ cells ranged from 5% to 18% (data not shown).

To assess the developmental potential of each subpopulation of $CD34^+$ fetal liver cells, $CD34^{hi}CD133^{hi}$, $CD34^{lo}CD133^{lo}$, and $CD34^{hi}CD133^{neg}$ cells were purified by cell sorting, and engrafted separately into sublethally irradiated newborn NSG pups (1×10^5 cells per recipient). Ten weeks later, peripheral blood mononuclear cells (PBMCs) from recipient mice were analyzed for the presence of human $CD45^+$ leukocytes, and sera were assayed for the level of human albumin by ELISA. When recipient pups were injected with $CD34^{hi}CD133^{hi}$ cells, human $CD45^+$ cells were readily detected in PBMCs, but no human albumin was detected in the sera (Fig. 2C, 2D). In contrast, when recipient pups were injected with $CD34^{lo}CD133^{lo}$ cells, a significant level of human albumin was detected in the sera of recipient mice, but no human $CD45^+$ cells were detected in PBMCs. When recipient pups were injected with $CD34^{hi}CD133^{neg}$ cells, neither human $CD45^+$ cells nor human albumin was detected in the recipient mice. Consistently, only liver sections from mice engrafted with $CD34^{lo}CD133^{lo}$ cells, but not those from mice engrafted with $CD34^{hi}CD133^{hi}$ or $CD34^{hi}CD133^{neg}$ cells, were stained positive for human albumin (Fig. 2E) and for a human pan-centromere probe (Supporting Information

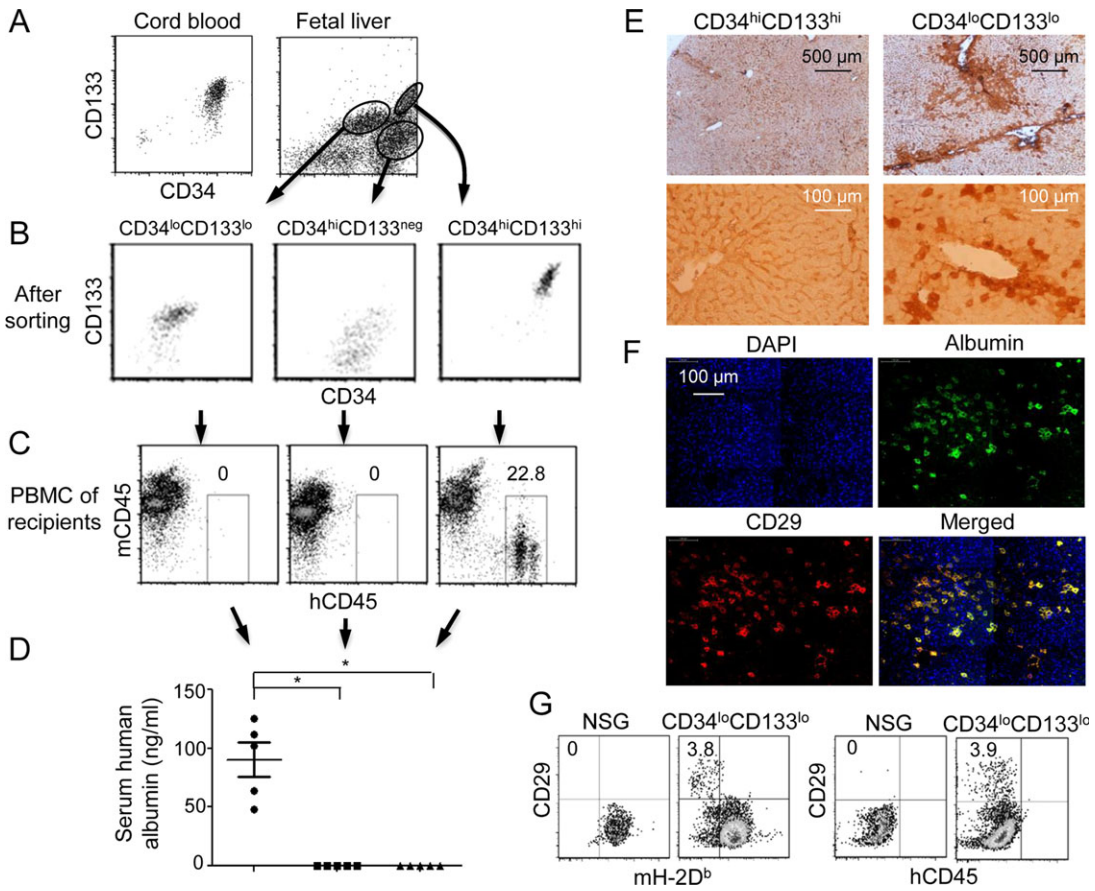

Figure 2. Identification of hematopoietic stem/progenitor cells and hepatic progenitor cells in $CD34^+$ fetal liver cells. **(A, B):** Comparison of $CD34^+$ cells from human cord blood (left; $n = 5$) and fetal liver (right; $n = 3$) for CD133 expression. Purified $CD34^+$ cells were stained for CD133 and analyzed by flow cytometry (A). Subsets of $CD34^{hi}CD133^{hi}$, $CD34^{lo}CD133^{lo}$, and $CD34^{hi}CD133^{neg}$ cells were isolated from $CD34^+$ fetal liver cells by cell sorting and reanalyzed (B). Representative staining profiles for CD34 versus CD133 are shown. **(C, D):** The sorted subpopulations were injected separately into three groups of NSG pups ($n = 5$ per group). Ten weeks later, PBMCs of recipient mice were stained for human CD45 and mouse CD45 and analyzed by flow cytometry. Shown are representative staining profiles for one mouse per group (C). The number indicates percentage of $hCD45^+$ cells. Human albumin in the sera of recipient mice was measured by ELISA (D); each symbol represents one mouse. *, $p < .001$. The experiment was repeated once with cells from a different fetal liver sample. **(E):** Livers were harvested from mice that had been injected with $CD34^{hi}CD133^{hi}$ or $CD34^{lo}CD133^{lo}$ cells. Paraffin sections were stained with an antibody specific for human albumin. Shown are representative data from over 10 mice per group reconstituted with cells from at least three fetal liver samples. **(F):** Liver sections from mice that had been injected with $CD34^{lo}CD133^{lo}$ cells were stained with antibodies specific for human albumin and CD29. Shown are the same liver sections stained and imaged for DAPI, albumin, CD29, or all three stains together (merged). Shown are representative data from over six mice reconstituted with cells from three fetal liver samples. **(G):** Quantification of human hepatocytes in mouse liver. Nine weeks after engraftment with $CD34^{lo}CD133^{lo}$ fetal liver cells, single-cell suspensions were prepared from the livers, stained for human CD45 and CD29 and mouse H-$2D^b$ ($n = 8$), and analyzed via flow cytometry. Livers from nonreconstituted NSG mice were used as negative controls ($n = 4$). Shown are representative staining profiles of hCD29 versus mouse H-$2D^b$ and hCD45 versus hCD29. The numbers indicate the average percentage of $CD29^+$H-$2D^{b-}$ or $CD29^+CD45^-$ cells ($n = 8$). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; NSG, NOD-SCID $Il2rg^{-/-}$; PBMC, peripheral blood mononuclear cell.

Fig. S1B). Furthermore, human albumin-positive cells were stained positive for human hepatocyte surface protein CD29 [21, 22] (Fig. 2F) but negative for AFP (data not shown).

To quantify the number and frequency of human hepatocyte-like cells in the mouse liver, recipient mice were perfused 9 weeks after injection of $CD34^{lo}CD133^{lo}$ cells, and single-cell suspensions of hepatocytes were prepared; the cells were stained for human CD45, CD29, and mouse H-$2D^b$ followed by flow cytometry. Anti-CD29 staining identified a cell population that was negative for hCD45 and mH-$2D^b$ (Fig. 2G). On average, $4\% \pm 1.2\%$ ($n = 8$) of the cells in the liver of recipient mice was $CD29^+CD45^-$mH-$2D^{b-}$ human hepatocyte-like cells (Fig. 2G). Based on the frequency of human hepatocyte-like cells, and the total cells (5×10^7) in the liver of a 9-week-old mouse, the total number of human hepatocyte-like cells was found to be approximately 2×10^6 per recipient mouse. Since only 1×10^5 $CD34^{lo}CD133^{lo}$ cells were injected per mouse initially, this indicates a 20-fold expansion as $CD34^{lo}CD133^{lo}$ cells differentiate into human hepatocyte-like cells in the recipient mice.

Together, these results show that, based on CD133 expression, $CD34^+$ human fetal liver cells can be separated into three subpopulations, each of which has a unique developmental potential: $CD34^{hi}CD133^{hi}$ cells likely contain HSPCs since they give rise to hematopoietic cells in the recipient mice. In contrast, $CD34^{lo}CD133^{lo}$ cells expand and give rise to hepatocyte-like cells in recipient mice, indicating that they likely contain HPCs.

$CD34^{Lo}CD133^{Lo}$ Cells Can Differentiate into Human Hepatocyte-like Cells In Vitro

When grown on collagen plates with hepatocyte differentiation medium, $CD34^{lo}CD133^{lo}$ cells adhered to the collagen substratum, formed a monolayer, and gradually developed a

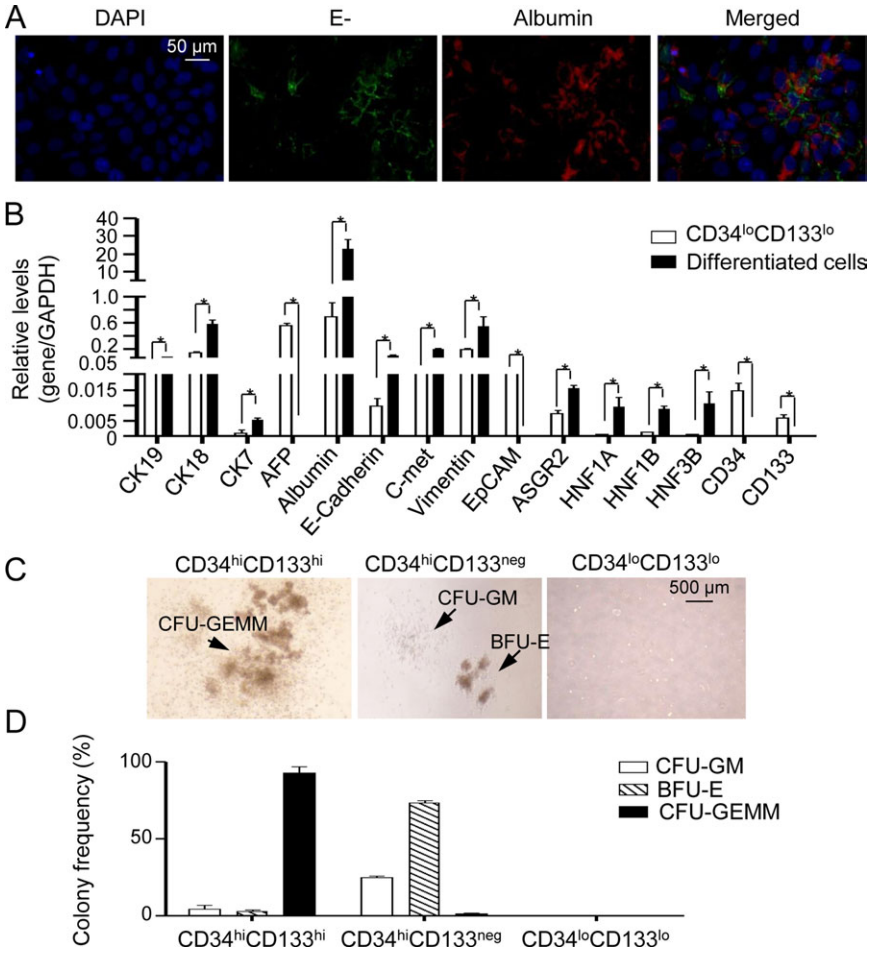

Figure 3. In vitro differentiation of $CD34^{hi}CD133^{hi}$, $CD34^{hi}CD133^{neg}$, and $CD34^{lo}CD133^{lo}$ fetal liver cells. **(A):** Representative images of cultured $CD34^{lo}CD133^{lo}$ cells immunofluorescently stained on day 28 for human albumin (red), human E-cadherin (green), and DAPI (blue) from two separate experiments **(B):** Comparison of relative transcript levels of selected genes in $CD34^{lo}CD133^{lo}$ fetal liver cells and their differentiated cells on day 28. Total RNA was isolated from cells and assayed by quantitative RT-PCR and normalized to GAPDH. Data shown are average ± SEM from two different cultures, each with triplicate PCR reactions. *, $p < .001$. **(C, D):** Colony-forming assays: $CD34^{hi}CD133^{hi}$, $CD34^{hi}CD133^{neg}$, and $CD34^{lo}CD133^{lo}$ cells were plated under conditions of colony formation. Representative phase-contrast micrographs, taken 9 days after culture, are shown (C). Arrows point to the CFU-GEMM, CFU-GM, and BFU-E colonies. The frequencies of the different colonies at day 9 of culture are shown (D). Data shown are average ± SEM from two different fetal liver samples, each with duplicate culture wells. Abbreviations: AFP, α-fetoprotein; BFU-E, burst-forming unit-erythroid; CFU, colony-forming unit; DAPI, 4′,6-diamidino-2-phenylindole; EpCAM, epithelial cell adhesion molecule.

typical polygonal morphology (Supporting Information Fig. S2A). The total cell number increased steadily over the course of the culture (Supporting Information Fig. S2B), achieving an approximately 70-fold increase by day 28. The differentiation of $CD34^{lo}CD133^{lo}$ cells into hepatocyte-like cells was further confirmed by positive staining for both human albumin and E-cadherin on day 28 (Fig. 3A). The percentage of albumin-positive cells in the in vitro differentiation was around 60%. We also compared the gene expression profile before and after culture (Fig. 3B). Compared to $CD34^{lo}CD133^{lo}$ cells, the differentiated cells upregulated the expression of CK18, albumin, E-cadherin, c-met, asialoglycoprotein receptor 2, and hepatocyte-specific transcription factors HNF1A, HNF1B, and HNF3B, but lost expression of AFP, EpCAM, CD34, and CD133. An increase in the expression of CK19, CK7, and vimentin was also observed, indicating differentiation of biliary epithelial cells and mesenchymal cells in the same culture. In contrast, $CD34^{hi}CD133^{hi}$ and $CD34^{hi}CD133^{neg}$ cells did not proliferate in the hepatocyte differentiation medium. These findings indicate that $CD34^{lo}CD133^{lo}$ fetal liver cells are also capable of differentiating into hepatocytes-like cells in vitro.

To investigate the hematopoietic potential of $CD34^{hi}CD133^{hi}$, $CD34^{lo}CD133^{lo}$, and $CD34^{hi}CD133^{neg}$ cells, each subpopulation was analyzed by standard colony-forming assays. $CD34^{hi}CD133^{hi}$ cells proliferated rapidly and gave rise to mixed hematopoietic colonies containing granulocytic, erythroid, monocyte-macrophage, and megakaryocytic elements (colony-forming unit [CFU]-granulocyte, erythrocyte, monocyte, megakaryocyte (GEMM), >95%), while $CD34^{hi}CD133^{neg}$ cells formed single colonies of granulocyte-macrophage progenitors (CFU-GM, ~25%) and erythroid precursor cells (burst-forming unit-erythroid, >75%) (Fig. 3C, 3D). In contrast, $CD34^{lo}CD133^{lo}$ cells did not generate any hematopoietic colonies; instead they are attached to the bottom of the plates as a single-cell layer. Consistent with these in vitro differentiation assays, human $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, $CD14^+$ monocyte/macrophages, $CD11c^+$ BDCA-1^+ myeloid dendritic cells (DCs), and $ILT7^+$ $CD303^+$ plasmacytoid DCs were detected in the spleen of

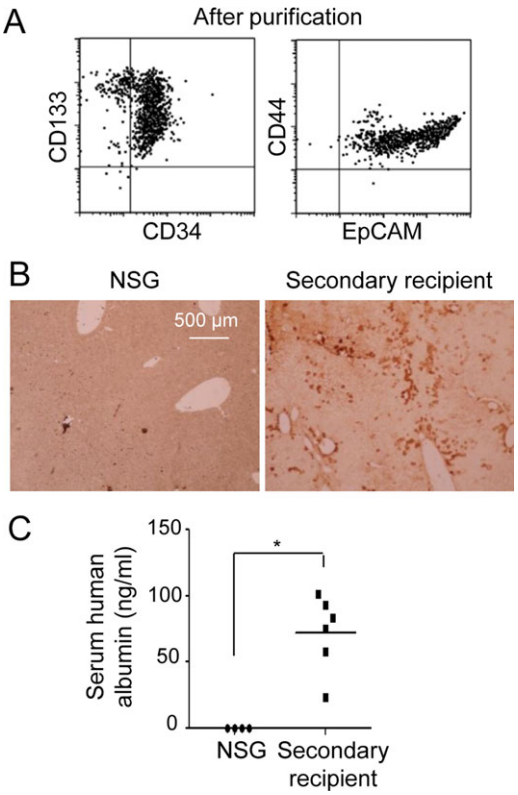

Figure 4. $CD34^{lo}CD133^{lo}$ fetal liver cells support secondary transfer. Purified $CD34^{lo}CD133^{lo}$ fetal liver cells were engrafted into newborn NSG recipients. Nine weeks later, human $CD34^{+}$ cells were purified from the livers of 11 primary mice by magnetic cell sorting. **(A):** Purified cells were stained for human CD34, CD133, CD44, and EpCAM. Staining profiles for CD34 versus CD133, and EpCAM versus CD44, are shown. **(B, C):** Purified $CD34^{+}$ cells were injected into sublethally irradiated secondary newborn NSG pups (1×10^5 cells per recipient); 8 weeks later, liver sections were stained for human albumin and the level of human albumin in the sera was quantified. Adult nonreconstituted NSG mice were used as negative controls. Shown are representative human albumin staining of one liver section of one of the six mice (B) and serum levels of human albumin of individual mice (C). One symbol represents one mouse. *, $p < .001$. Abbreviations: EpCAM, epithelial cell adhesion molecule; NSG, NOD-SCID $Il2rg^{-/-}$.

recipient mice 10 weeks after the engraftment with $CD34^{hi}CD133^{hi}$ fetal liver cells (Supporting Information Fig. S3). These results further support the conclusion that the $CD34^{lo}CD133^{lo}$ population contains HPCs, while the $CD34^{hi}CD133^{hi}$ population contains the multipotent HSPCs. The $CD34^{hi}CD133^{neg}$ population likely comprises hematopoietic progenitors that do not stably reconstitute blood lineage cells in mice.

$CD34^{Lo}CD133^{Lo}$ Fetal Liver Cells Support Secondary Transfer

The progenitor property of $CD34^{lo}CD133^{lo}$ fetal liver cells was further investigated through serial transfer. Purified $CD34^{lo}CD133^{lo}$ fetal liver cells were engrafted into sublethally irradiated newborn NSG pups; 9 weeks later, we confirmed reconstitution of human hepatocyte-like cells by assaying for the presence of human albumin in the serum of recipient mice. Livers from the primary recipient mice were perfused and single-cell suspensions were prepared; we were able to purify 6×10^5 human $CD34^{+}$ cells from 11 mice. Flow cytometry analysis showed that the purified $CD34^{+}$ human cells were also positive for human CD133, CD44, and EpCAM (Fig. 4A). These cells were injected into six irradiated newborn NSG pups (1×10^5 cells per recipient). Eight weeks after the secondary transfer, human hepatocyte-like cells were detected in the liver sections by human albumin staining, and human albumin was also detected in the sera of all six recipient mice (Fig. 4B, 4C). Thus, despite the significant expansion and differentiation of the $CD34^{lo}CD133^{lo}$ cells in the primary recipient mice, some of the transferred cells maintain the ability to reconstitute human hepatocytes in secondary recipient mice. We thus refer to the $CD34^{lo}CD133^{lo}$ fetal liver cells as fetal HPCs.

$CD34^{Lo}CD133^{Lo}$ HPCs Are Transcriptionally More Similar to HSPCs than to Mature Hepatocytes

The cell surface phenotype of HPCs was characterized and compared to the phenotypes of $CD34^{hi}CD133^{hi}$ HSPCs from fetal liver (FL HSPCs) and from cord blood (CB HSPCs). As shown in Supporting Information Figure S4 and Table 1, HSPCs from both sources displayed a very similar surface phenotype, including high levels of CD45, CD44, CD117, and CD166 expression, medium levels of CD90, CD105, CD24, and CD97 expression, and negative for EpCAM and CD73. In contrast, the staining pattern exhibited by fetal HPCs was quite distinct: these cells expressed significant levels of CD73, EpCAM, CD166, and CD45, low levels of CD44, CD117, and CD24, but were negative for CD90, CD105, and CD97. Thus, fetal HPCs are positive for epithelial cell markers such as EpCAM and CD73, and also for some hematopoietic cell markers such as CD34, CD117, and even CD45.

Quantitative-PCR (qPCR) analyses revealed that both FL HSPCs and HPCs expressed hepatocyte and epithelial cell-related transcripts, including CK18, AFP, albumin, c-met, E-cadherin, and CD29 (Fig. 5A). HPCs also expressed biliary cell markers such as CK19 and CK7 as well as mesenchymal marker vimentin and CD31. Mature human hepatocytes expressed a much higher level of albumin and were positive for CK18, c-met, E-cadherin, and CD29, but negative for AFP, CD31, or vimentin. The qPCR results were confirmed by immunofluorescence staining, which further showed that all HPCs were positive for albumin, AFP, CK7, CK19, and E-cadherin (Fig. 5B), while FL HSPCs were negative for these five markers. The uniform expression of hepatic markers (albumin, AFP, and E-cadherin) as well as of biliary markers (CK7 and CK19) by HPCs further verifies that they are a relatively homogenous cell population.

The developmental relationship between HPCs and FL HSPCs was explored by undertaking analyses of genome-wide transcriptional profiling of purified HPCs, FL HSPCs, CB HSPCs, $CD34^{hi}CD133^{neg}$ fetal liver cells, and mature human hepatocytes (Fig. 6A). Comparison of data from surface staining, PCR analyses, and microarray analyses revealed good correlation among the three assays. For example, CD34, CD45, CD133, EpCAM, AFP, and CK19 were detected in HPCs via all three methods. Hierarchical clustering (Fig. 6A) and principal component analysis (Supporting Information Fig. S5A) uncovered that the transcriptional profile of HPCs was much more similar to that of FL HSPCs and of CB HSPCs than to that of mature hepatocytes. Taking mature hepatocytes and CB HSPC as standards for hepatic and hematopoietic lineages, we found that HPCs and FL HSPCs shared 4,577 genes that were upregulated more than fivefold as compared to hepatocytes; however, they shared only 468 genes that were upregulated fivefold as compared to CB HSPCs (Supporting Information Fig. S5B). Among the differentially

Table 1. Comparison of phenotypes among FL HPCs, FL HSPCs, and CB HSPCs reported in this report as well as other reported human hepatic stem/progenitor cells

Gene	FL HPC	FL HSPC	CB HSPC	Schmelzer et al. [16]	Weiss et al. [17]	Dan et al. [20]
CD34	Low	+	+	−	+	+
CD133	Low	+	+	+	N.R	−
CD45	+	+	+	−	+/−	−
CD44	Low	+	+	+	N.R	+
CD117	Low	+	+	+	+	+
EpCAM	+	−	−	+	N.R	+
CD90	−	+	+	−	+	+
CD105	−	+	+	N.R	N.R	N.R
CD24	Low	+	+	N.R	N.R	N.R
CD97	−	+	+	N.R	N.R	N.R
CD73	+	−	−	N.R	N.R	N.R
Vimentin	+	−	−	N.R	N.R	+
CD166	+	+	+	N.R	N.R	N.R
Albumin	+	−	−	Low	+	−
AFP	+	−	−	−	−	−
CK7	+	−	−	N.R	−	N.R
CK19	+	−	−	+	+	+
E-cadherin	Low	−	−	+	N.R	N.R

Abbreviations: AFP, α-fetoprotein; CB, cord blood; EpCAM, epithelial cell adhesion molecule; FL, fetal liver; HPC, hepatic progenitor cell; HSPC, hematopoietic stem/progenitor cell; N.R, not reported.

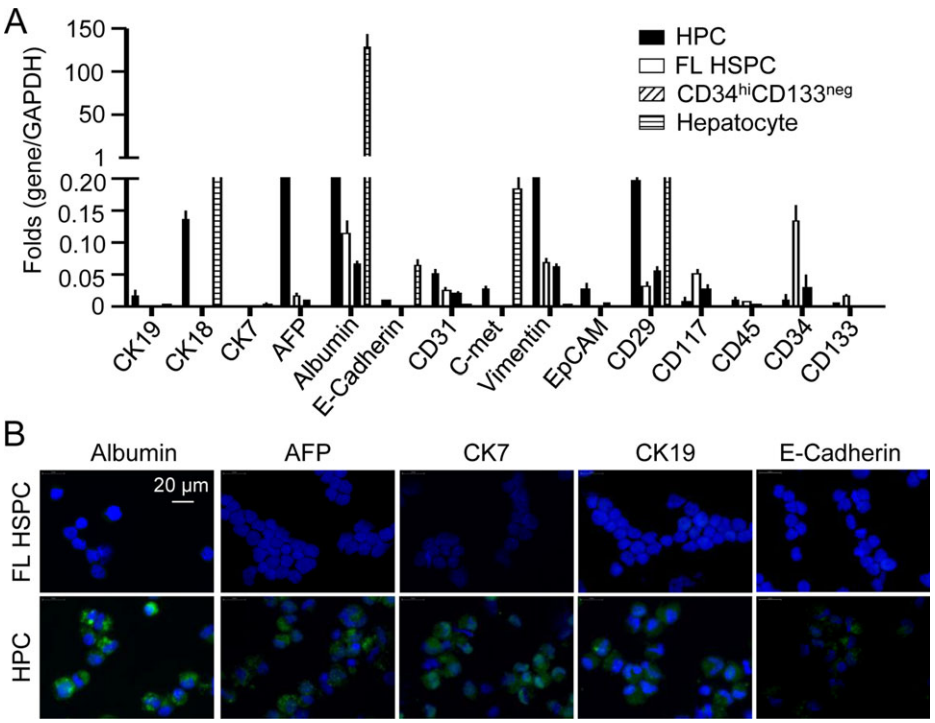

Figure 5. $CD34^{lo}CD133^{lo}$ HPCs express both hematopoietic and epithelial cell markers. **(A):** Comparison of relative transcript levels of selected genes in purified human HPCs, FL HSPCs, $CD34^{hi}CD133^{neg}$ fetal liver cells, and mature hepatocytes. Total RNA was isolated from purified cell subsets and assayed by quantitative RT-PCR. Data shown are average ± SEM from two different fetal liver samples in two separate experiments. **(B):** Cytospins were prepared with purified FL HSPCs and HPCs, then stained with antibodies specific for human albumin, AFP, CK7, CK19, and E-cadherin (green) and DAPI (blue). Representative stains are shown from one of the two different fetal liver samples. Scale bar applies to all panels. Abbreviations: AFP, α-fetoprotein; HPC, hepatic progenitor cell; DAPI, 4′,6-diamidino-2-phenylindole; HSPC, hematopoietic stem/progenitor cell.

expressed genes, we focused on validation of known lineage-specific transcriptional factors by quantitative RT-PCR. Consistent with the microarray data, expression levels of the HSPC-specific transcription factors PBX1, SOX4, MEIS1, and HOXA9 [23] were high in both FL HSPCs and CB HSPCs. However, a lower level expression of these transcription factors was also detected in HPCs, but not in mature hepatocytes (Fig. 6B). Conversely, HPCs and mature hepatocytes, but not FL HSPCs and CB HSPCs, expressed endoderm-specific transcriptional factors such as SOX17, SOX7, GATA4 [24],

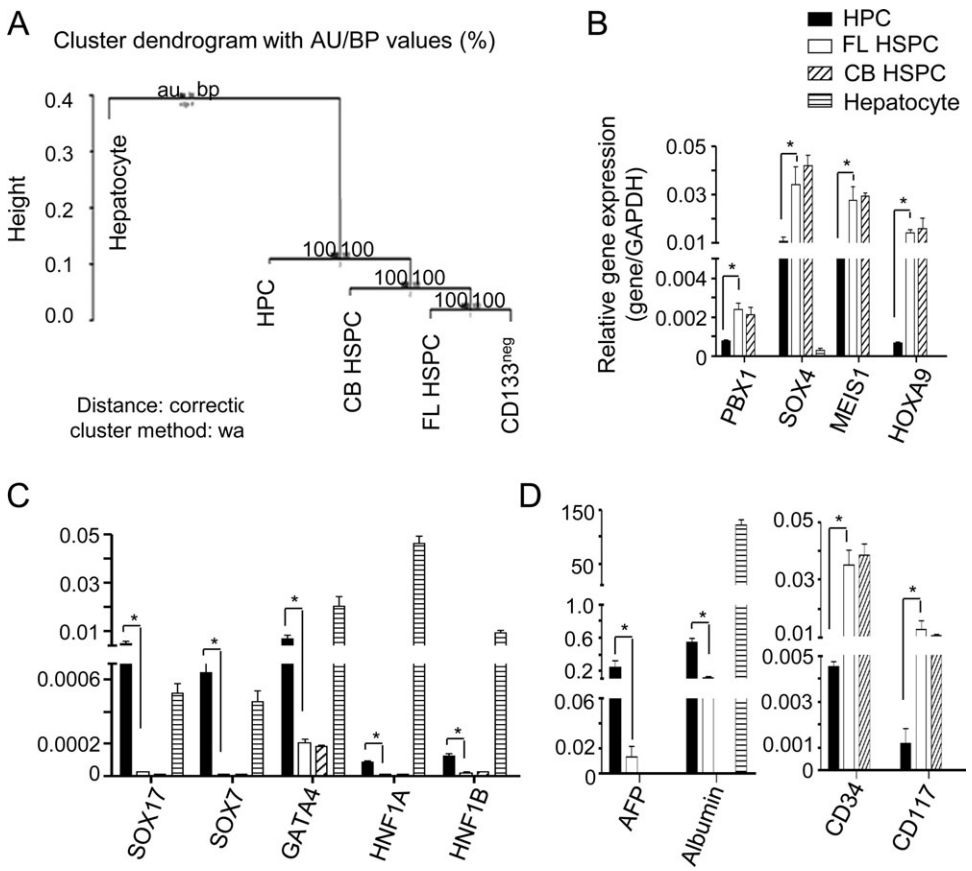

Figure 6. Transcription profiles of HPCs are more closely related to those of HSPCs from fetal liver and cord blood than to those from mature hepatocytes. **(A):** Transcriptional analysis was performed with purified $CD34^{hi}CD133^{hi}$ (FL HSPCs), $CD34^{lo}CD133^{lo}$ (HPCs), $CD34^{hi}CD133^{neg}$ fetal liver cells, as well as $CD34^{hi}CD133^{hi}$ cord blood cells (CB HSPCs), and mature hepatocytes. Hierarchical cluster analysis of transcriptomes of the six cell types is shown. Values at branches are AU *p*-values (left), BP values (right), and cluster labels (bottom). The height scale reflects a distance measure that is scaled between 0 and 1. Data on HPCs, FL HSPCs, and $CD34^{hi}CD133^{neg}$ cells were from three analyses of three different biological samples. **(B--D):** Comparison of transcript levels for selected HSPC-specific transcription factors (B), endoderm signature genes (C), and hepatic and hematopoietic signature genes (D) in HPCs, FL HSPCs, CB HSPCs, and mature hepatocytes. CD34 and CD117 were used as positive controls for HSPCs. Data shown are average ± SEM of three biological samples. *, $p < .001$. Abbreviations: AFP, α-fetoprotein; AU, approximately unbiased *p*-value; BP, bootstrap probability value; HPC, hepatic progenitor cell; HSPC, hematopoietic stem/progenitor cell.

HNF1A, and HNF1B (Fig. 6C). In addition, significant levels of hepatic progenitor-specific transcripts such as albumin and AFP were detected in FL HSPCs but not in CB HSPCs (Fig. 6D), although no protein was detected (Fig. 5B). We conclude that fetal HPCs and FL HSPCs are closely related in gene expression, suggesting a possible common origin.

Discussion

The controversy in the literature over the identity and origin of human hepatic stem/progenitor cells in part results from a lack of surface markers that can be used to identify and isolate the cells prospectively, and the absence of a simple, robust assay for evaluating their developmental potential. Here, we show that the simple adoptive transfer of $CD34^{+}$ cells from human fetal liver into sublethally irradiated newborn pups of NSG mice leads to an efficient development of human hematopoietic cells and hepatic cells in the livers of the recipient mice. By testing a panel of surface markers, we found that CD133 staining can further separate $CD34^{+}$ fetal liver cells into three distinct subpopulations: $CD34^{hi}CD133^{hi}$, $CD34^{lo}CD133^{lo}$, and $CD34^{hi}CD133^{neg}$. The $CD34^{hi}CD133^{hi}$ population contains HSPCs as they give rise to T cells, B cells, NK cells, DCs, and monocytes/macrophages in NSG recipient mice and CFU-GEMM in vitro. Consistently, cord blood HSPCs, which give rise to only hematopoietic cells in NSG mice, uniformly express high levels of CD133. In contrast, the $CD34^{lo}CD133^{lo}$ population does not give rise to hematopoietic cells, but reproducibly generate hepatocyte-like cells in recipient mice and in vitro. Based on their large size, the expression of hepatic markers, such as albumin, AFP, CK17, CK19, and E-cadherin, and their ability to expand and differentiate into hepatocyte-like cells both in vivo and in vitro, the $CD34^{lo}CD133^{lo}$ fetal liver cells fulfills the criteria as HPCs. Furthermore, following their engraftment in the primary recipients some of the cells persist as $CD34^{lo}CD133^{lo}$, can be reisolated, and readily give rise to hepatocyte-like cells when transferred into secondary recipient mice, suggesting that the $CD34^{lo}CD133^{lo}$ population may contain hepatic stem cells. These results show that hepatic and HSPCs are distinct in the fetal liver and they can be isolated separately.

Following engraftment of $CD34^{lo}CD133^{lo}$ fetal liver cells into NSG mice, the human hepatocyte-like cells that we detected in the recipient liver are not derived from fusion between human cells and mouse hepatocytes. In contrast to low frequencies reported for fusion between mouse hematopoietic cells and hepatocytes or between human hematopoietic cells and mouse hepatocytes (<0.05%) [25–27], we detected

an average of 4% of human hepatocyte-like cells in the liver of the recipient mice (Fig. 2G). These human cells express hepatic markers CD29 and albumin (Fig. 2F), but not hematopoietic marker CD45 (Fig. 2G), excluding their being hematopoietic lineage cells. Furthermore, these human hepatocyte-like cells are negative for the ubiquitously expressed mouse major histocompatibility complex molecule H-2D (Fig. 2G), demonstrating that they are not generated from fusion between human cells and mouse hepatocytes.

The phenotype of human HPCs that we have identified is similar to, but also significantly different from, previously reported human hepatic progenitor or stem cells (Table 1). Besides CD34 and CD133, our identified HPCs express additional hematopoietic markers including CD117, CD44, and CD45. They also express mesenchymal markers CD73 and vimentin and hepatic markers EpCAM, AFP, and albumin. In comparison, Schmelzer et al. reported that human hepatic stem/progenitor cells express CK19, CD133, CD44, and albumin but not CD34 and CD45 [16]; Weiss et al. reported that human HPCs are positive for CK19, CD90, CD34, c-kit (CD117), and the hepatic marker Hep-Par1 [17]; and Dan et al. reported a human fetal liver multipotent progenitor cell with a phenotype of $CD34^+$, $CD90^+$, c-kit^+, $EpCAM^+$, $CK19^+$, and $vimentin^+$, but negative for albumin and AFP [20]. As we showed that virtually all purified HPCs express CD34, AFP, albumin, CK7, CK19, and E-cadherin (Figs. 2, 5), the reported differences would suggest that either only a small fraction of the cells are true HPCs or hepatic progenitor/stem cells are naturally heterogeneous. Nevertheless, it is interesting to note that hepatic cells with a phenotype of $CD133^+EpCAM^+$ or $CD34^+c\text{-}kit^+$ have been shown to contribute to liver homeostasis and regeneration in carbon tetrachloride induced liver injury models [16, 20]. Similarly, some human hepatic cellular carcinomas (HCC) express hematopoietic as well as hepatic markers such as CD34, CD133, CD117, CK19, and albumin [28–30]. We have also found that some human HCC lines are positive for CD34, CD133, CD117, CK19, and AFP (Chen et al. unpublished data). Thus, our identified HPCs may be normally involved in both physiological and pathological processes.

The physical and functional separation of HPCs from fetal HSPCs in human fetal liver prompted us to use genome-wide transcriptional analysis to systematically investigate the relationship between HPCs and HSPCs in more detail. Compared to mature human hepatocytes, the transcriptional profile of HPCs is much closer to that of FL and CB HSPCs. In fact, HPCs transcribe many genes, including the HSPC-specific transcription factors PBX1, SOX4, MEIS1, and HOXA9, which are traditionally associated with hematopoiesis [23]. Because virtually all purified HPCs express CD34, AFP, albumin, CK7, CK19, and E-cadherin, the expression of HSPC-specific genes is unlikely due to contamination of HPCs with HSPCs. Conversely, FL HSPCs, but not CB HSPCs, also express a significant level of albumin and AFP transcripts, but not of the corresponding proteins. Since the purity of the isolated HSPCs that we used for transcriptional profiling analysis was high (99% $CD34^{hi}CD133^{hi}$), and since HPCs express CK7 and CK19, while hepatocytes express apolipoprotein E (data not shown), which were not detected in HSPCs, it is unlikely that the transcripts for albumin and AFP in FL HSPCs are due to a contamination by HPCs or hepatocytes. Despite the similarity in the transcriptome between HPCs and HSPCs, $CD34^{hi}D133^{hi}$ HSPCs do not give rise to hepatocytes and conversely $CD34^{lo}CD133^{lo}$ HPCs do not give rise to hematopoietic cells in NSG recipient mice or in vitro. These findings suggest that HPCs and HSPCs may share a common origin in the fetal liver, but once they are differentiated, they are committed to separate lineages.

A major challenge for the study of human hepatic stem and progenitor cells has been the lack of a simple and robust in vivo assay for assessing the developmental potential of cell populations from fetal liver, adult liver, or other sources. Here, we have overcome this challenge using adoptive transfer of putative cell populations into sublethally irradiated newborn NSG pups, then detecting the development of human hepatocytes in the recipient mouse liver by assaying for human albumin in the serum, staining for human albumin-expressing cells in liver sections, and/or analyzing human hepatocyte-like cells by flow cytometry. Compared to the existing mouse models of human hepatocyte reconstitution, our method is simple, without a need for special genetic engineering, chemical treatment, or surgical manipulation of recipient mice. A much higher degree of human liver chimerism can be achieved by transplanting mature human hepatocytes into immunodeficient mice such as urokinase-type plasminogen activator transgenic mice [31, 32], fumarylacetoacetate hydrolase knockout mice [33], and more recently BALB/c $Rag2^{-/-}$ γc^{null} mice that express an inducible suicide fusion gene of FK506 binding protein and caspase 8 [34]. However, these recipient mice are specially engineered, and the endogenous mouse hepatocytes have to be ablated when human hepatocytes are transferred—thus, despite their potential utility in a variety of applications, these mice have not been used to assay the developmental potential of human hepatic stem/progenitor cells. Recently, a number of researchers have used intrasplenic injections to assay the developmental potential of human HPCs in adult immunodeficient mice [16, 17, 19, 20]. Besides the need for technically challenging surgical procedures and chemical treatments of the recipient mice, this technique resulted in only a very low level (~0.1%) of reconstitution of human hepatocyte-like cells in the liver of these mice. In contrast, our simple methodology of directly injecting human HPCs into sublethally irradiated NSG pups leads to ~4% of human hepatocyte-like cells in the mouse liver. It is notable that although reconstitution of human hepatocyte-like cells was ~4% based on flow cytometry quantification with anti-human CD29 staining in our study, the level of human albumin in the serum was much lower than expected from the ~4% of hepatocyte reconstitution. This discrepancy between percentages of human hepatocyte reconstitution and serum levels of human albumin is consistent with previous reports [20]. One possibility is that the human hepatocytes generated in the mouse liver may not have fully matured in terms of albumin secretion. As we show that the human hepatocyte-like cells can be costained positive for human albumin and CD29 but negative for AFP, the development of human hepatocyte in the mouse liver should have passed the hepatoblast stage.

Conclusion

In summary, our study identifies a novel cell population in the midgestation human fetal liver that exhibits the properties of HPCs, and that shares a close relationship with HSPCs. These findings resolve many controversies with regard to the identity and origin of hepatic stem/progenitor cells, and will likely usher a new area of hepatic stem cell research within the context of health and disease.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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