Human hepatic stem cells from fetal and postnatal donors

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Human hepatic stem cells (hHpSCs), which are pluripotent precursors of hepatoblasts and thence of hepatocytic and biliary epithelia, are located in ductal plates in fetal livers and in Canals of Hering in adult livers. They can be isolated by immunoselection for epithelial cell adhesion molecule–positive (EpCAM+) cells, and they constitute ~0.5–2.5% of liver parenchyma of all donor ages. The self-renewal capacity of hHpSCs is indicated by phenotypic stability after expansion for >150 population doublings in a serum-free, defined medium and with a doubling time of ~36 h. Survival and proliferation of hHpSCs require paracrine signaling by hepatic stellate cells and/or angioblasts that coisolate with them. The hHpSCs are ~9 μm in diameter, express cytokeratins 8, 18, and 19, CD133/1, telomerase, CD44H, claudin 3, and albumin (weakly). They are negative for α-fetoprotein (AFP), intercellular adhesion molecule (ICAM) 1, and for markers of adult liver cells (cytochrome P450s), hemopoietic cells (CD45), and mesenchymal cells (vascular endothelial growth factor receptor and desmin). If transferred to STO feeders, hHpSCs give rise to hepatoblasts, which are recognizable by cordlike colony morphology and up-regulation of AFP, P4503A7, and ICAM1. Transplantation of freshly isolated EpCAM+ cells or of hHpSCs expanded in culture into NOD/SCID mice results in mature liver tissue expressing human-specific proteins. The hHpSCs are candidates for liver cell therapies.

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Abbreviations used: AFP, α-fetoprotein; CK, cytokeratin; EpCAM, epithelial cell adhesion molecule; hHpSC, human hepatic stem cell; ICAM, intercellular adhesion molecule; KM, Kubota's Medium; NCAM, neural cell adhesion molecule; VEGFr, vascular endothelial growth factor receptor.

The role of human hepatic stem cells (hHpSCs), particularly in the maintenance and regeneration of the adult liver, has been a subject of debate without a clear consensus (1–11). During embryonic development, endodermal cells in the mid-region of the embryo bulge into the cardiac mesenchyme, are affected by critical signaling from endothelia forming vasculature, and form the liver bud (6, 7). The cells within the liver bud are recognized as hepatoblasts because of the expression of a signature marker, α-fetoprotein (AFP), and are bipotent, giving rise to hepatocytes and bile-duct epithelial cells, which are called cholangiocytes (11). We and others have described the isolation and expansion in culture of AFP+ cells from fetal and adult livers of several species (8–10). Clonogenic expansion assays of rodent hepatoblasts under wholly defined conditions have demonstrated that hepatoblasts are capable of extensive expansion ex vivo, as well as differentiation to both hepatocytic and biliary lineages (8). The findings from investigations of liver organogenesis, as well as the ex vivo studies of hepatoblasts, have led to a long-standing assumption that hHpSCs correspond to hepatoblasts, and that hHpSCs would express AFP. However, AFP+ cells are rare in normal adult livers (<0.01%), except in livers with severe injury or disease (11–13). In addition, the renowned replicative capacity of hepatocytes in vivo (14) has led to the opinion that adult livers do not have hHpScs and that all regenerative responses are from mature parenchymal cells, except in certain disease states (1).

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We define a novel class of AFP-negative cells in fetal and adult human livers that are precursors to hepatoblasts and have properties consistent with hHpSCs. The hHpSCs are negative for AFP, but positive for epithelial cell adhesion molecule (EpCAM; CD326, C017-1A antigen, and GA733-2). This protein, encoded by the tumor-associated calcium signal transducer 1 gene, is expressed by many carcinomas and serves a regulatory function in certain normal epithelia, including all of those derived from endoderm (liver, lung, pancreas, and intestine) (15, 16). By immunohistochemistry, Balzar et al. observed that hepatoblasts in embryonic human liver are EpCAM+, whereas mature hepatocytes are EpCAM− (15). In adult livers, most, but not all, bile duct epithelia are EpCAM+. Also, expanded ductular structures, seen in cases of focal nodular hyperplasia or biliary cirrhosis, contain numerous EpCAM+ cells (15).

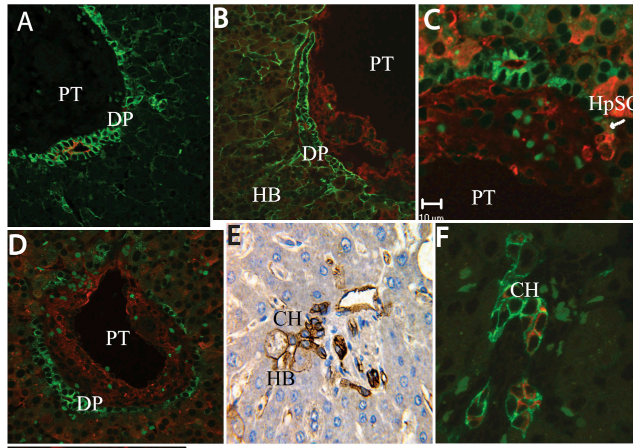
We have previously reported that EpCAM+, AFP− cells from human livers are hHpSCs, and we have compared their pattern of gene expression with that of hepatoblasts and mature liver parenchyma (17). We now show that the hHpSCs are located in ductal plates in fetal and neonatal livers and in the proximal branches of the intrahepatic biliary tree, the Canals of Hering, in pediatric and adult livers of all donor ages, with the frequency of hHpSCs remaining relatively constant throughout life. We further document the immunoselection of these cells using monoclonal antibodies to EpCAM and test whether they meet the defining criteria for stem cells, i.e., self-renewal and pluripotency.

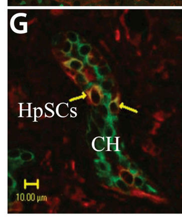
RESULTS

In vivo localization of EpCAM+ hHpSCs

Sections of fetal and adult livers were stained for EpCAM and for liver-specific markers (albumin, AFP, and CK19; Fig. 1). We found that ductal plates, bands of tissue encircling each of the portal triads in fetal and neonatal livers, have small cells (7–10 μm) with a paucity of cytoplasm, and stained intensely both cytoplasmically and at the surface for CK19 and EpCAM, and weakly for albumin, but are negative for AFP. In nondiseased, postnatal (pediatric and adult) livers, cells staining positively for EpCAM by immunohistochemistry appear exclusively in the Canals of Hering in the vicinity of the portal triads of acini. Theise et al. (18) reported that cells lining these ductules express cytokeratin (CK) 19, which is present in biliary epithelia, but not in hepatocytes. Our data confirm their study and demonstrate that the CK19+ cells within the Canals of Hering express EpCAM, and that subpopulations of them also express albumin (Fig. 1; yellow color is caused by overlap of CK19 and albumin expression). The coexpression of CK19 and albumin is consistent with hHpSCs and corroborates the hypothesis of others that the Canals of Hering comprise a niche for hHpSCs (19). Shown are data from ex vivo studies of EpCAM+ cells supportive of the interpretation that they include hHpSCs.

Most parenchymal cells of fetal and neonatal livers consisted of hepatoblasts, slightly larger cells (10–12 μm in diameter) that stained positively for albumin, AFP, and CK19. In hepatoblasts, the distribution of CK19 is more particulate and less intense than that in ductal plate cells. EpCAM expression in AFP+ cells, both in fetal and postnatal livers, occurred at the membrane only. In pediatric and adult livers, hepatoblasts were found as individual cells or small clusters of cells tethered to the ends of the Canals of Hering (Fig. 1). The hepatoblasts in nondiseased, postnatal livers constitute <0.01% of cells and express AFP weakly.





Antigenic Profile of hHpSCs vs Hepatoblasts (*in vivo*)

Marker	hHpSCs	Hepatoblasts
AFP	Negative	+++
Albumin	Negative or ±	++
CK19	+++	++
EpCAM	+++	++
NCAM	Fetal/Neonatal: ++ Pediatric/Adult: ~40% ++	Negative
CD133/1	++	++
ICAM1	Fetal/Neonatal: Negative Pediatric/Adult: - or ±	++
CD45	Negative	Negative

Figure 1. Immunohistochemical studies on human fetal livers. Confocal microscopic images on 5-μm liver sections. The antigenic profiles are given in the table (bottom left). In human fetal livers, sections were stained for: EpCAM (green) and CK19 (red) (A); EpCAM (green) and AFP (red) (B); CK19 (green) and albumin (red) (C); CK19 (green) and AFP (red) (D). In adult livers, sections were stained for: EpCAM (E). (F) Canals of Hering around portal triad with EpCAM (green) and CK19 (red). (G) A Canal of Hering showing EpCAM+ cells (green), some of which also express albumin (red).

Flow cytometry of EpCAM+ cells

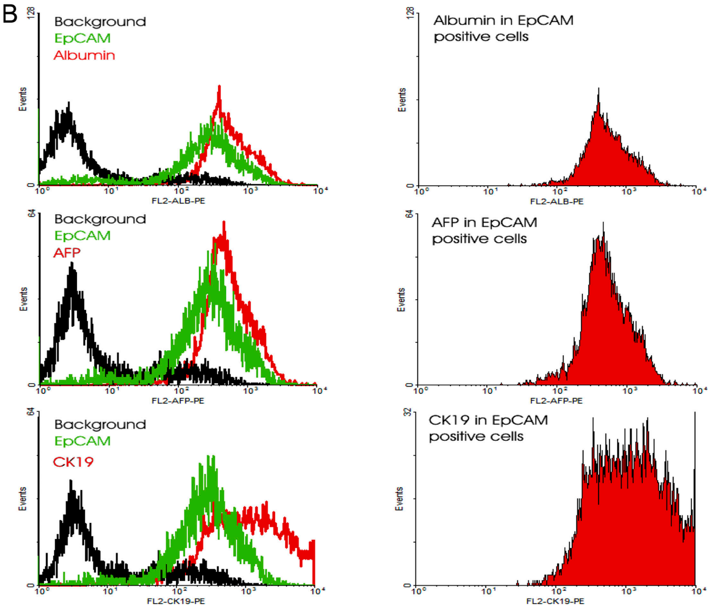
Using flow cytometry, we observed EpCAM+ cells in human liver cell suspensions of all donor ages (Fig. 2 A). Suspensions from fetal livers from which hemopoietic cells had been purged contained, on average, 12% EpCAM+ cells. However, the percentage could be as high as 20% depending on the gestational age of the fetus. Most of the EpCAM− cells in fetal livers were of nonhepatic lineage and were predominantly hemopoietic. The vast majority (>90%) of the EpCAM+ cells in fetal livers coexpress AFP, albumin, and CK19 (Fig. 2). They could be subdivided into two subpopulations: (a) hepatoblasts showing expression of ICAM1, AFP, albumin, CK19, CD133/1, P450A7, and CD44H (hyaluronan receptor), and (b) hHpSCs, constituting ~5% of EpCAM+ cells from fetal

A Percentage of EpCAM+ Cells in Livers from Donors of Varying Age

Age Group	# samples	% EpCAM+ Cells	% EpCAM+ Cells that are hHpSCs	% EpCAM+ Cells that are Hepatoblasts
Fetuses (16-20 wks)	10	12.1 ± 2.3	~5%	~95%
Neonates (0-1 yr)	7	2.7 ± 2.4	~50%	~50%
Pediatric (2-13 yrs)	17	2.1 ± 1.6	>99%	<0.01%
Adult (19-81 yrs)	38	1.3 ± 1.0	>99%	<0.01%

hHpSCs =positive for EpCAM, NCAM and/or claudin 3 but not AFP
Hepatoblasts=positive for EpCAM, ICAM, AFP but not NCAM or claudin 3

B



C Gene Expression in EpCAM+ versus EpCAM- Cells in fetal vs Postnatal Livers

Gene	Fetal Livers	Postnatal Livers	hHpSC Colonies
	Ratio of # of Transcripts in EpCAM+ Cells/EpCAM- Cells		# Transcripts
AFP	2.0	0/0	0.0 ± 0.0
Albumin	2.9	0.14	4.0±1.6
CK19	1.7	433.3	97.2± 18.0
CD117 (ckit)	0.01/0.0	23.3	0.1±0.03
P450 3A4	0/0	0.045	0.0±0.0

Figure 2. Flow cytometric characterization of EpCAM+ cells. (A) The percentage of EpCAM+ cells in livers of varying donor ages. The numbers for fetal livers have been previously reported (28), but are presented here for comparison to findings in livers from older donors. (B) Analyses of EpCAM+ cells from fetal livers (similar findings occur with EpCAM+ cells from adult livers, except that few cells express AFP). (C) Quantitative RT-PCR assays on freshly isolated EpCAM+ versus EpCAM− cells from fetal versus postnatal livers. These data are compared with the findings from colonies of hHpSC grown on plastic and in serum-free KM for 30–60 d.

livers, had an overlapping, but distinct, profile; they were positive for albumin (weak), CK19, CD44H, CD133/1+, neural cell adhesion molecule–positive (NCAM+), and claudin 3, but negative for AFP and P450A7.

Cell suspensions from adult human livers averaged 1.3% EpCAM+ cells, and those from neonatal and pediatric donors two- to threefold higher. Preparations having EpCAM+ cell populations at levels >1.5% were typically obtained from livers subjected to ischemia (cold or warm) before organ procurement and/or during transportation. This suggests that the EpCAM+ liver cells are more resistant to ischemia than mature liver cells. The postnatal EpCAM+ liver cells also coexpress CK19 and albumin (unpublished data), but have no detectable AFP+ cells (by flow cytometry), except in rare cases of overt hepatic disease (e.g., cirrhosis; unpublished data). Neonatal livers, including some from premature births, showed rapidly decreasing levels of AFP+ cells as a function of age, falling below detection level by a few months after birth. Based on considerations detailed in our study and from our previously published work (17), we identify the EpCAM+ cells from pediatric and adult livers as almost exclusively hHpSCs, not hepatoblasts.

Culture selection on plastic and in serum-free Kubota's medium (KM) isolates hHpSCs, but not hepatoblasts

Suspensions of liver cells plated in KM, which is a serum-free medium optimized for ex vivo expansion of hepatic progenitors (8), either on tissue culture plastic or on embryonic stromal cell feeders, yielded parenchymal cell colonies with two distinct morphologies. Type I colonies consisted of cells forming a cordlike morphology interspersed with clear channels and expressing EpCAM, albumin, CK19, ICAM, and AFP, but not NCAM (Figs. S1–S4, available at http://www.jem.org/cgi/content/full/jem.20061603/DC1). Type 2 colonies consisted of densely packed, morphologically uniform cells, strongly expressing EpCAM, NCAM, CD44H, and claudin 3; weakly expressing or negative for albumin; and negative for AFP and ICAM-1 (Fig. 3 and Fig. S3). We interpret the type 1 colonies as corresponding to hepatoblasts and the type 2 colonies as corresponding to hHpSCs (Table I).

In cultures on plastic, by 5–7 d (mean 5.2 ± 1.6 d; maximum number of days), the hepatoblast colonies disappeared. However, if cultured on STO feeders, hepatoblasts survived for up to 2 mo, continuing to show coexpression of albumin, AFP, and CK19 (Figs. S1 and S2). The hepatoblast colonies typically contained fewer than ~100 cells.

In contrast to hepatoblasts, hHpSC colonies on plastic continued to expand. A time-lapse sequence of a growing hHpSC colony (Fig. 3, A–E, and Video 1, available at http://www.jem.org/cgi/content/full/jem.20061603/DC1), in which the expansion of hHpScs seeded at very low density is shown on day 1, 3, and 8. The hHpSCs can be subcultured after mechanical disaggregation and continue to multiply extensively. Their doubling time on plastic is ~36 h. That doubling time decreased to <24 h if they are plated on specific extracellular matrix substrata (unpublished data). By 2–3 wk, hHpSC colonies typically contained many thousands of cells.

The hHpSC colonies were assessed for expression of lineage markers by immunofluorescent staining. The expression pattern closely resembled that of ductal plate cells in vivo. They were positive for CK19, NCAM, EpCAM, and CD44H (Figs. 3, F–L). In addition, they were positive for albumin (weak), E-cadherin, N-cadherin, CK8 and 18, CD133/1, integrin β-1 (CD29), claudin 3, and telomerase (unpublished data). They were negative for AFP, any form of cytochrome P450, hemopoietic markers (CD34, CD45, CD38, CD14, CD90, and glycophorin A), endothelial cell markers (vascular endothelial growth factor receptor [VEGFr], von Willebrand factor, and platelet/endothelial cell adhesion molecule or CD31), and mesenchymal markers, such as those for hepatic stellate cells (CD146, desmin, and α-smooth muscle actin). The expression of NCAM by the hHpSCs is important because previous studies have shown that this marker is present on the ductal plate in fetal livers and evident on liver cell populations proliferating under various disease states (20–23).

Immunoselection using EpCAM isolates hepatoblasts from fetal and neonatal livers; immunoselection using EpCAM or NCAM isolates hHpSCs from livers of all donor ages

To enrich for hepatic progenitors from liver cell suspensions, we explored several fractionation strategies, including

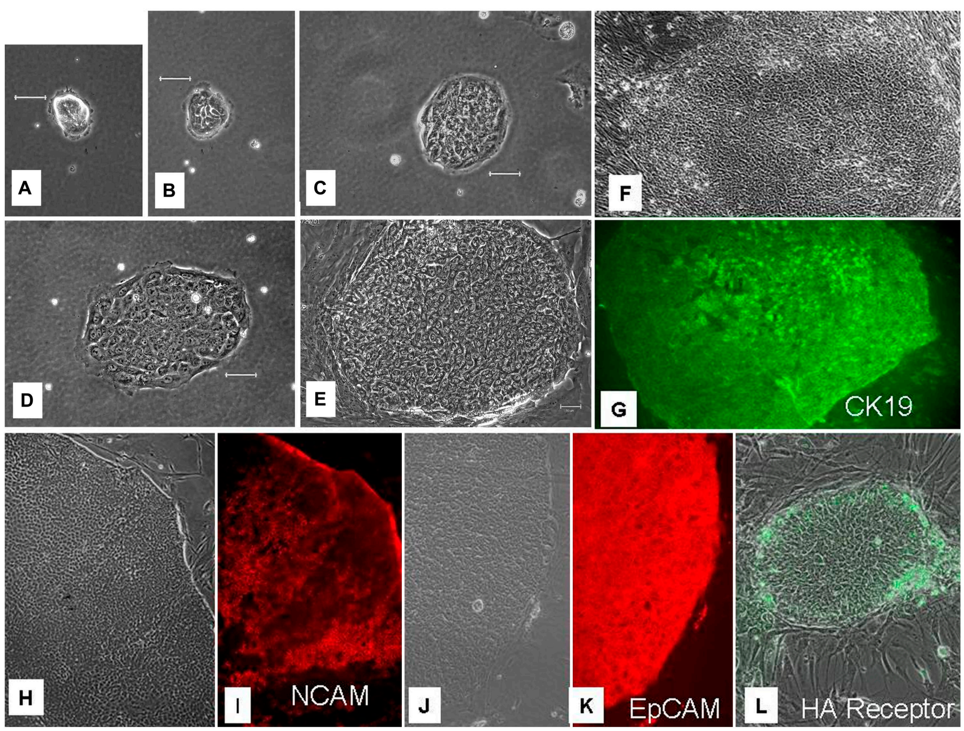


Figure 3. hHpSCs in culture. A–D show a stem cell colony forming at 2 (A), 4 (B), 7 (C), 10 (D), and 14 d (14) in culture on plastic and in KM. Phase contrast coupled with image of cells with staining for NCAM (E and F), CK19 (G and H), EpCAM (I and J), and CD44H (L). F and G are the phase and immunohistochemistry (IHC) for CK19; H and I are the phase and IHC for NCAM; J and K are the phase and IHC for EpCAM; and L is the IHC for CD44, the hyaluronan receptor. Bar, 20 μm.

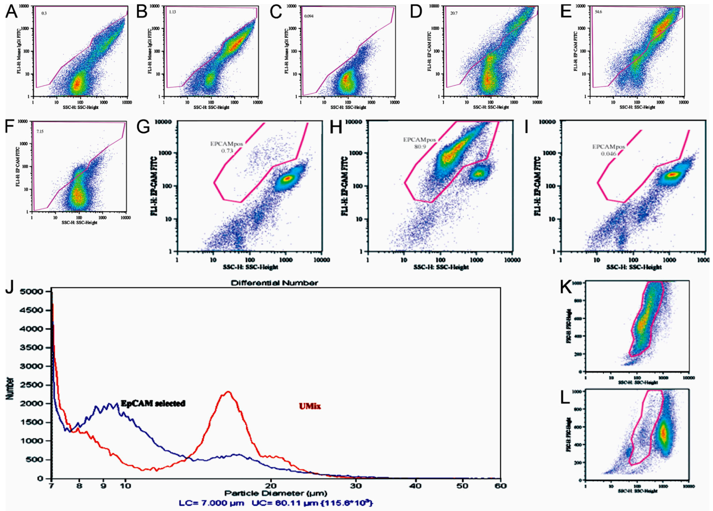
Table I. Antigenic profiles of hHpSC and hepatoblasts

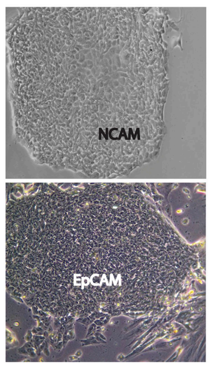
Markers	hHpSCs on plastic and in KM[1]	hHpSCs on STO feeders and in KM[1]	Hepatoblasts
AFP[2]	–		+++ in those from fetal liver; ± in those from postnatal livers
Albumin[2]	– or ±		++
P450-3A4[2]	–		–
P450-A7[2]	–		+
CK 8/18, CD29, CAM 5.2	+++		+++
CK19[2]	+++		+ particulate staining in cytoplasm
EpCAM[2]	+++ Cytoplasm and membrane		++ at membrane surface but not cytoplasm
NCAM[2]	+++		–
Indian Hedgehog	+++; in center of cells		++
Sonic Hedgehog	+++ at periphery of cells		++
ICAM-1[2]	–		+ (Fig. 6)
Claudin 3[2]	+++		–
CD44H [4]	+++		+++
CD133/1[2]	+++		+++
Telomerase[5]	+++ +++		
CD117[2]	Debatable[9]		–
Mesenchymal[6] cell markers	–		–
Endothelial cell markers[7]	–		
Hemopoietic markers[8]	–		

+, weakly expressed; ++, expressed strongly; +++, expressed very strongly.
[1]Description of KM development (9) and a review for its details of its preparation have been previously provided (56).
[2]Phenotypic characterization of >20 genes by RT-PCR and Western blot analyses was done on hHpSCs, hepatoblasts, and hepatocytes from livers from donors of varying age (16).
[3]More extensive studies on hedgehog signaling are presented elsewhere (39).
[4]More extensive studies on hyaluronan receptors and their relevance to use of hyaluronan hydrogels for ex vivo maintenance of hHpSCs are given elsewhere (57).
[5]Telomerase activity has been measured in hHpSCs, hepatoblasts versus in mature liver cells (unpublished data).
[6]Mesenchymal markers: CD146, desmin, and α-smooth muscle actin.
[7]Endothelial cell markers: VEGFr (also called KDR), Von Willebrand factor, and CD31.
[8]Hemopoietic cell markers: CD45, CD34, CD14, CD38, Thy 1 (CD90), and Glycophorin A.
[9]It is not detected on freshly isolated EpCAM+ cells from fetal or postnatal livers. RNA for it is enriched in EpCAM+ cells (16). It is variably found in hHpSC colonies from fetal but not adult livers and, when found, it is always near or overlapping with the companion cells. Immunoselection for it does not yield hHpSCs. We suspect it is on angioblasts, but not the hHpSCs (and for certain is not on hepatoblasts).

separation by buoyant density on Ficoll gradients (Table S1, available at http://www.jem.org/cgi/content/full/jem.20061603/DC1) and by immunoselection. The most satisfactory results were obtained using magnetic immunoselection. Although FACS was able to yield highly purified cellular subpopulations, the shear forces and the use of buffers (PBS) that are not optimal resulted in low yields of viable cells. We used magnetic microspheres conjugated with monoclonal antibody to EpCAM (Miltenyi Biotec) to immunoselect EpCAM+ cells from liver cell suspensions and obtained robust, highly viable sorted cells that survived and expanded well when cultured (Fig. 4 and Table S2). From postnatal livers, up to 10 billion viable cells were processed in a single pass using the CliniMACS apparatus (Miltenyi Biotec). This yielded >100 million EpCAM+ cells. Purity of the enriched EpCAM+ cells was typically 75–90%, and recovery usually exceeded 90%. Representative fractionations of a fetal liver and of a postnatal liver are depicted in Fig. 4. A cell suspension from the liver of a 2-yr-old donor was found to contain 0.7% EpCAM+ cells. The immunoselected population contained 81% EpCAM+ cells, whereas the flowthrough fraction was almost entirely depleted of EpCAM+ cells. The majority of hepatic EpCAM+ cells were of 8–10 μm in diameter, as judged by Coulter Counter analysis, in contrast to 18–20 μm for diploid hepatocytes, which is the predominant population in the initial liver cell suspension. A small peak of presumptive tetraploid cells also is evident, measuring ~25 μm in diameter. Light scatter ("side scatter") profiles indicate that the EpCAM+ liver cells are considerably smaller and less granular than the bulk of the parenchymal cell population.

Magnetic immunoselection for NCAM+ cells from fetal livers enriched for cells capable of forming only hHpSC colonies (Fig. 4). The majority of EpCAM+ cells from fetal liver coexpressed NCAM, whereas only ~40% of those from adult liver were also NCAM+. Therefore, sorting for NCAM+ cells proved useful for isolation of hHpSCs from fetal livers, but less so from adult livers. It is unknown at this time whether NCAM and EpCAM coexpression is a





M **Antigenic Profiles of Immunoselected Cells**

Markers	Cells Immunoselected for: EpCAM+	NCAM +	VEGFr (KDR)+	EpCAM-/KDR-
AFP	+++ (fetal/neonatal) Neg (pediatric/adult)	Neg	Neg	Neg
Albumin	++	Weak	Neg	Neg
CK19	+++	+++	Neg	Neg
EpCAM	+++	+++	Neg	Neg
Claudin 3	Mixed + and Neg (fetal/neonatal) +++ (pediatric/adult)	+++	Neg	Neg
CD133/1	+++	+++	+++	Neg
Epithelial	+++	+++	Neg	Neg
Endothelial	Neg	Neg	+++	Neg
Hemopoietic	Neg	Neg	Neg*	Mixed + and Neg
Stellate Cell	Neg	Neg	Neg	+++

Epithelial markers: CK 8 and 18, CD29, CAM 5.2
Endothelial Markers:CD133/1, VEGFr, CD31, Von Willebrand Factor; *some with CD34
Hemopoietic Markers: CD45, CD34, CD38, CD14, CD90, Glycophorin A
Hepatic Stellate Cell Markers: Desmin, CD146, α-smooth muscle actin

Figure 4. Magnetic immunoselection. (A–F) Flow cytometry on human fetal liver cells stained for EpCAM (D; A is the isotype control for D used for setting the gate shown in pink) indicated 20.7% of the cell suspension was positive for EpCAM. The cells were subjected to magnetic bead sorting and yielded a suspension enriched for EpCAM to 54.6% of the cells (B is the isotype control used for the data in E). The flow-through cells (F; C is the isotype control for F) were depleted in EpCAM, yielding 7.15% of the cells. (G–I) Flow cytometry on cells from adult livers. EpCAM expression (y axis) versus side scatter (x axis). In the original, unfractionated cell suspensions were found in 0.73% EpCAM+ cells. (H) A single pass through microbead sorting resulted in enrichment for EpCAM+ cells to 80.9%. (I) Only 0.06% EpCAM+ cells were found in the flowthrough. (J) The EpCAM+ cells were ~9–10 μm in diameter versus 18–22 μm in diameter for mature parenchyma. EpCAM+ cells had low side scatter (K) relative to that found for mature cells in the unfractionated mixture (UMIX) of liver cells (L). The table shows a summary of profiles of cells immunoselected for EpCAM, NCAM, KDR (VEGFr), or for KDR-/EpCAM− cells. Phase micrograph images are of an hHpSC colony from an EpCAM+ and an NCAM+ sort.

definitive property of hHpSCs. An alternative hypothesis, which is currently being tested, is that NCAM is present on angioblasts or other mesenchymal companion cells that are tightly bound to the hHpSCs such that immunoselection for it results in coselection of the two cell types (see later in this paper for more on this theme). Sorts for KDR (VEGFr) resulted mostly in angioblasts (Fig. 4). However, these sorts also yielded an increase in hHpSC colonies caused by, we assume, coselection of hHpSCs and angioblasts.

Proteins and genes expressed by EpCAM+ cells

Immunoselected EpCAM+ cells from fetal and postnatal livers were examined by flow cytometry for expression of lineage markers characteristic of various cell types that reside in the liver (Figs. 2 and 4 and Table S1). As judged by double-label flow cytometry, ~95% of the immunoselected EpCAM+ cells expressed CK19, and comparable percentages expressed albumin and CD133. Evaluations of many preparations indicated that >90% of the EpCAM+ cells are positive for CD133, which was detected with monoclonal antibodies to two distinct epitopes (CD133-1 with monoclonal antibody AC133; CD133-2 with monoclonal antibody AC141). Virtually all CD133-1+ cells in adult liver cell suspensions were found in the EpCAM+-selected fraction, and mature hepatocytes were clearly negative. However, it appeared that ~40% of liver cells with light scatter profiles consistent with mature hepatocytes were positive for CD133-2. Examination by immunofluorescent microscopy showed that staining for CD133-1 clearly outlines cell membranes, whereas that for CD133-2 shows a more diffuse pattern (unpublished data). It is likely that the staining of many more liver cells by CD133-2 results from a known cross-reactivity with CK18 (24) that is expressed by hepatocytes and can reportedly be found on the cell surface (25). Based on the more specific CD133-1 antibody, we conclude that EpCAM and CD133 (prominin) are coexpressed by the vast majority of hHpSCs.

NCAM (CD56), which was previously shown to be expressed by glia, muscle cells, and neurons (26), was found on the majority of hHpSCs derived from fetal and neonatal livers, but only ~40% of the EpCAM+ cells from adult livers. In our prior studies, NCAM mRNA was enriched strongly in EpCAM+ cells from both fetal and postnatal livers, but expression at the protein level was variable (17). NCAM staining was most evident at the borders of the hHpSC colonies (Fig. 3).

Less than 1% of the enriched EpCAM+ cells stained for the hemopoietic marker CD45 (leukocyte common antigen), which is found on Kupffer cells (tissue macrophages) and lymphocytes in the liver. The EpCAM+ cells were negative for expression of other hemopoietic markers assayed (CD34, CD14, CD38, CD4, CD90, and glycophorin A), for endothelial cell markers (CD34, VEGFr or KDR, von Willebrand factor, and CD31), and for mesenchymal markers, especially those associated with hepatic stellate cells (CD146, also called Mel-CAM, desmin, and α-smooth muscle actin; unpublished data). Finally, we found AFP expression at the RNA and protein levels in EpCAM+ cells from fetal and neonatal livers, but not from pediatric or adult livers. As noted, small numbers of cells weakly positive for AFP, as judged by immunohistochemistry, were observed to be tethered to the ends of the Canals of Hering in sections from pediatric and adult livers (Fig. 1 E). In our experience, these cells are too few and express AFP too weakly to permit recognition as a defined subpopulation by flow cytometry.

Assessment by RT-PCR of RNA expression in the EpCAM+ liver cells (Fig. 2) gave results consistent with the flow cytometry data. Further details of these findings are reported elsewhere (17). In brief, EpCAM+ selection from fetal livers more than doubled the expression levels of albumin, AFP, and CK19. Immunoselection for EpCAM+ cells from postnatal livers strongly enriched for transcripts encoding EpCAM, CK19, CD133, and CD117 (c-Kit); these transcripts were barely detectable in the EpCAM− cells. AFP transcripts were not detectable in EpCAM+ or EpCAM− cells from postnatal livers.

Although immunoselected cells are enriched for relative expression of CD117 mRNA, we have not observed the corresponding protein by immunostaining of freshly isolated cells from fetal or postnatal livers, or on cultured cells from postnatal livers. However, we have occasionally observed low levels of CD117 staining on cells at the periphery of hHpSC colonies from fetal livers that are located in regions where hHpSCs overlap with mesenchymal companion cells.

Cytochrome P450 3A4 (CYP3A4), which is a protein expressed by mature hepatocytes, was not found at all in parenchymal cells, either EpCAM+ or EpCAM−, from fetal livers in terms of both mRNA and protein level of it. The level of mRNA for cytochrome P450 in EpCAM+ cells was 20-fold lower relative to EpCAM-negative cells from postnatal livers. The small amount of CYP3A4 RNA in the EpCAM+ cell fraction from postnatal livers could be accounted for by residual hepatocyte contaminants. In contrast, the hepatoblasts, but not the hHpSCs, were found to express P4503A7, which is a protein found in fetal livers (unpublished data). EpCAM+ cells from postnatal livers also showed eightfold lower relative expression of albumin RNA than the flow-through (EpCAM-negative) population. Again, some transcripts can be attributed to incomplete removal of hepatocytes. However, the detection of albumin protein in EpCAM+ cells by flow cytometry, together with the transcript data, demonstrates that these progenitor cells express the albumin gene, albeit at a significantly lower level than differentiated hepatocytes.

Assays for telomerase activity indicate significant levels in freshly isolated EpCAM+ cells from livers of all donor ages

Table II. Evidence for self-renewal

	hHpSCs	Hepatoblasts
Minimum conditions for survival	KM[1] + culture plastic	KM[1] + STO Feeders
Lifespan of cells	Culture plastic: >6 mo STO feeders: indefinite	Plastic: no survival after ~5–8 d STO feeders: ~2 mo
Doubling time on plastic or feeders[2]	Plastic: 1.5–2 d STO feeders: 12–24 d (<24 h on certain matrix substrata[4])	Plastic: no survival STO Feeders: essentially no growth
[2]Cell number/colony after 2 wk ("clonogenic" expansion)	Plastic: $1.4 \times 10^3 + 5.2 \times 10^2$ (derived from a single hHpSC partnered with a single companion cell; Videos 1–3)	Plastic: no survival
Phenotype of hHpSCs after > 150 divisions (>6 mo in culture)	Identical to that of cells after initial plating; characterization summarized in Table I	Within the ~2 mo of survival time on STO feeders, cells retained expression of albumin, AFP, and CK19
Ability to form liver tissue after transplantation [3]	Capable after 1–2 mo in culture on plastic and in KM[1]	Only if transplanted within ~7 d of culture on plastic (not tested with cells on STO feeders)

[1]KM = serum-free RPMI 1640 with no copper, low calcium (0.3 mM), and supplemented with zinc, selenium, insulin, transferrin, HDL, and lipids (12).
[2]See Videos 1–3, which show colony formation at low seeding densities and over days 1-8. Clonogenic expansion occurs, but requires each hHpSC to be partnered with at least one companion cell; the companion cells proved to be angioblasts or hepatic stellate cell precursors (Fig. 5).
[3]In Fig. 8, images from liver sections from animals transplanted with hHpSCs are shown.
[4]Elsewhere, we report that plating the stem cells onto specific forms of extracellular matrix, found in abundance in embryonic or fetal tissues enables them to go for months through rapid divisions with doubling times of <24 h (unpublished data).

and in cultures of colonies of both hHpSCs and hepatoblasts. Full characterization of telomerase activity and its regulation in various fractions of human liver cells from fetal and postnatal donors is presented elsewhere (unpublished data), as are studies on the effects of purified matrix substrata on telomerase activity in cultures of hHpSCs (unpublished data).

Ex vivo clonogenic expansion: evidence for self renewal

Colony formation by committed hepatic progenitors or diploid adult parenchyma involves a limited number of divisions (typically 5–7 divisions) over a relatively short period of time (2–3 wk) (8). In contrast, self-renewal involves clonogenic expansion that can go on for >100 population doublings with phenotypic stability, which are properties associated with stem cells. We previously found that rat hepatoblasts multiply far more extensively in KM with STO feeder cells than on tissue culture plastic (8). However, STO feeders and KM were not permissive for clonogenic expansion of human hepatoblasts. Under these conditions, the hepatoblasts survived for a few months, but demonstrated limited growth. In contrast, hHpSCs from livers of all donor ages could undergo clonogenic expansion for >6 mo (>150 population doublings) in culture on tissue culture plastic and in KM with only the native feeders (the companion cells) (Table II). The cells maintained phenotypic stability as assessed by morphology and by antigenic and biochemical profiles (Tables 1 and 2). hHpSC colonies starting from 1–3 cells (Videos 1–3, available at http://www.jem.org/cgi/content/full/jem.20061603/DC1) grew to cover 4.9 ± 0.3 mm^2 in area and contained an average of 1,400 ± 520 cells (3 independent counts of total cells from 50 dispersed colonies). Thus, the cells in this representative experiment had gone through 10–11 population doublings in 2 wk, corresponding to an average doubling time of 31–34 h (Table S2).

Mesenchymal companion cells provide critical paracrine signaling for hHpSCs

The tightly packed colonies of hHpSCs have a prominent ridge at the perimeter (Figs. 3, 4, and 6) at which we have identified mesenchymal companion cells (Fig. 5). As the colonies grow, the companion cells penetrate the colonies and are found throughout them. Time-lapse movies (Videos 1–3) reveal a boundary zone between the companion cells and the hHpSCS in which the companion cells fluctuate back and forth, touching the edge of the hHpSC colony, or traversing it and moving below the colony. When removed from a culture

Antigenic Profiles of Mesenchymal "Companion" Cells to hHpSCs

Markers	Angioblasts	Hepatic Stellate Cell Precursors
Parenchymal cell markers*	Neg	Neg
CD133/1, CD31 (PECAM), VEGFr (KDR)	+++	Neg
CD146	Not tested	+++
CD45, CD38, CD14, Glycophorin A	Neg	Neg
CD34	Variable (some positive)	Neg
CD117	+++	Variable
Desmin, α-smooth muscle actin (ASMA)	Neg	+++

*Parenchymal cell markers: albumin, AFP, EpCAM, CK19, CK8 and 18

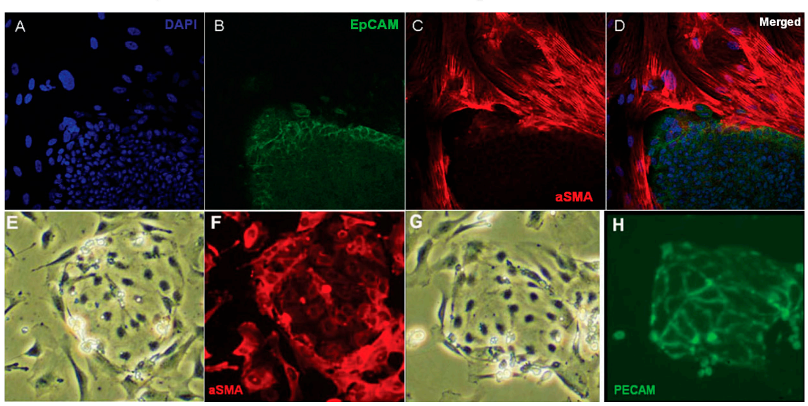


Figure 5. Companion cells to the hHpSC colonies comprise hepatic stellate cells and angioblasts. hHpSCs are associated with mesenchymal companion cells with distinct antigenic profiles (Videos 1–3). Two types of companion cells are evident: angioblasts (positive for VEGF-R, CD133/1, and CD117, and weakly positive for CD31 and von Willebrand's factor) and hepatic stellate cells (positive for desmin and α-smooth muscle actin [aSMA]). Videos 1–3 are available at http://www.jem.org/cgi/content/full/jem.20061603/DC1.

dish, the attachment to the plastic surface is evident only at the edge of the colonies, not in the center. This suggests that attachment to the plastic is mediated either by mesenchymal cells or by cooperative interactions between the hHpSCs and the mesenchymal cells.

Phenotypic analyses of the companion cells indicates at least two distinct populations: angioblasts (VEGFr+ or KDR, CD133/1+, CD117+, Von Willebrand factor, CD31^{weak}); and hepatic stellate cells (desmin+, α-smooth muscle actin+, CD146+) (Fig. 5). A comparison of their morphological and antigenic phenotypes is given in Fig. 5. Cells rigorously purified away from the companion cells (by repeated immunoselection for EpCAM+ cells) did not survive on culture plastic, but only on STO feeders (unpublished data). Immunoselection of CD117+ cells yielded angioblasts, but neither hepatic stellate cells nor hHpSCs (unpublished data). Immunoselection for other markers found on the companion cells (VEGFr) resulted mostly in selection of the companion cells alone, though we did find coselection for hHpSCs to occur at low and variable frequency (Fig. 4). We still cannot rule out that the consistent enrichment of hHpSCs from fetal liver by immunoselection for NCAM could actually result from coselection of the stem cells via tight association with NCAM+ companion cells.

Proof of pluripotency of hHpSCs

Passaging (transfer) of colonies of hHpSCs (whether derived from fetal or adult livers) from culture plastic onto feeder layers of STO cells resulted, within hours, in eruption of hepatoblasts from the periphery of hHpSC colonies (Figs. 6 and 7; Table I). After the transfer, the morphology and antigenic profile of the cells within the hHpSC colonies proper did not change in most of the cells, although there were occasional cells with distinct gene expression within the colony (Fig. 7). Instead, the colonies of hHpSCs gave rise to cordlike eruptions from their edges, yielding cells with morphology, antigenic, and biochemical profiles identical to that of hepatoblasts. The cells in these erupted areas strongly expressed AFP, ICAM-1, and albumin, and were positive for cytochrome P450-A7 (not depicted), but were negative for NCAM (Fig. 7). In addition, committed biliary progenitors were sometimes observed erupting from a colony of hHpSCs, as shown by staining for CK19, but not for albumin (Fig. 7).

In cultures of cells from postnatal livers, in colonies stained by double-label immunofluorescence for CK19 and albumin, we have observed distinct sectors positive for one or the other marker, but not both (Fig. S4). This was found most frequently in colonies of hepatoblast morphology. We interpret such sectors as deriving from unipotent cells, corresponding to committed progenitors for biliary and hepatocytic lineages, respectively. The sectoring could occur if at division a bipotent cell gives rise to a daughter cell that was restricted to the biliary or to the hepatocytic lineage. Occasionally, small colonies showed expression of only one of the lineage markers (CK19 or albumin); these colonies are assumed to have arisen from committed progenitors for the corresponding cell type.

EpCAM+ cells and colonies of hHpSCs give rise to human liver tissue in vivo

Transplantation of freshly isolated EpCAM+ cells or of hHpSC colonies, from either fetal or postnatal livers, into livers of NOD/SCID mice resulted in engraftment and the formation of human liver tissue (Fig. 8). Islands of cells staining positive for human albumin, CK19, and AFP were found within 2 d of transplantation (Fig. 8, A, C, and E), and they persisted within the livers for weeks (Fig. 8 F). The extent of engraftment and expansion of human liver cells in vivo was enhanced by treatment of the mice with carbon tetrachloride (CCL4), which is a poison for the pericentral zone of the liver acinus and is often used to create a cellular vacuum in transplanted hosts (Fig. 8, B, D, and G). Human-specific DNA sequences were found in the liver of animals that received the

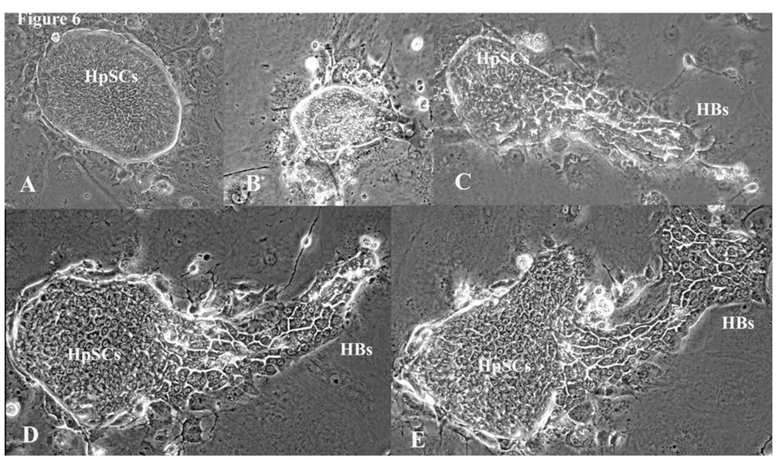


Figure 6. hHpSCs shifted to STO feeders erupt hepatoblasts. Passage of hHpSCs from plastic to STO feeders results in cordlike eruptions that morphologically and antigenically are identical to hepatoblasts. (A) An hHpSC colony shortly after passaging. (B–E) A small group of passaged stem cells appears as a tightly compacted group of cells with cords of hepatoblasts erupting at the periphery of the colonies. Shown is a colony and the steady eruption of hepatoblasts by the end of day 1 (B), 3 (C), 5 (D), and 7 (E) after passaging.

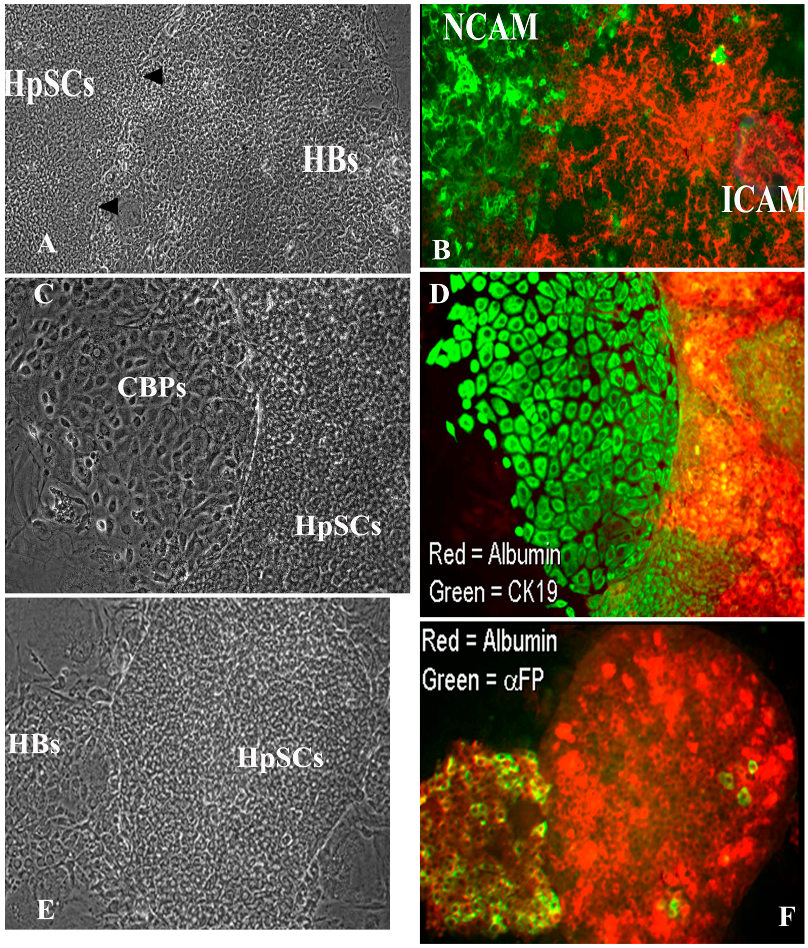


Figure 7. Shift in antigenic profile from hHpSCs to hepatoblasts when on STO feeders. (A and B) The border between the hHpSC colony and hepatoblast outgrowths is marked by arrowheads. (C and D) The antigenic profile of the cords of cells erupting from the parent colony is identical to that of hepatoblasts and includes a shift from expression of NCAM (green) to ICAM (red). Lineage restriction to committed biliary progenitors (CBPs) indicated by staining for CK19 (green) and albumin (red). (E and F) expression of AFP (green) and albumin (red) indicates that erupting cells are hepatoblasts.

human cell transplants, but not in other tissues or in control animals that did not receive human cells (unpublished data). Before transplantation, as expected, the cells were shown to express EpCAM and CK19 strongly and albumin weakly, but were negative for AFP at both the RNA and the protein levels. The liver sections from mice transplanted with hHpSCs contained cells strongly expressing human-specific forms of albumin, CK19, and AFP, but were negative for EpCAM. However, human cytochrome P450 3A was not detectable. Therefore, it appears that after transplantation and expansion in recipient livers, the human cells lost expression of a marker (EpCAM) found only on stem and progenitor cells, and, acquired some, but not yet all, of the functions specific to mature hepatocytes. We think it logical that transferrin, but not P450 3A, is expressed in transplanted cells given that in mature liver, transferrin is expressed by zone 2 parenchymal cells, whereas P450 3A by zone 3 parenchymal cells within the acinus. Thus, P450 3A is a late gene produced by cells at the end of the liver's maturational lineage.

As an independent test of engraftment, we assessed expression of the human transferrin gene, encoding a protein characteristic of mature hepatocytes. We found by quantitative RT-PCR analysis with human-specific primers that the livers of mice sampled 1 wk after injection of the EpCAM+ cells derived from postnatal human livers contained significant levels (2,100 ± 1,140 strands/100 ng) of human transferrin RNA. Such sequences were undetectable in RNA from livers of control mice (<100 strands/100 ng RNA). Although before transplantation >80% of cells in the test cell population expressed EpCAM and CK19, cells in recipient animals were positive for human albumin and were negative for both of the progenitor cell markers. Collectively, the data suggest that within 7 d in vivo, the engrafted hHpSCs gave rise to mature human liver cells.

DISCUSSION

Cells in the ductal plates in fetal and neonatal livers and in the Canals of Hering in pediatric and adult livers are hHpSCs.

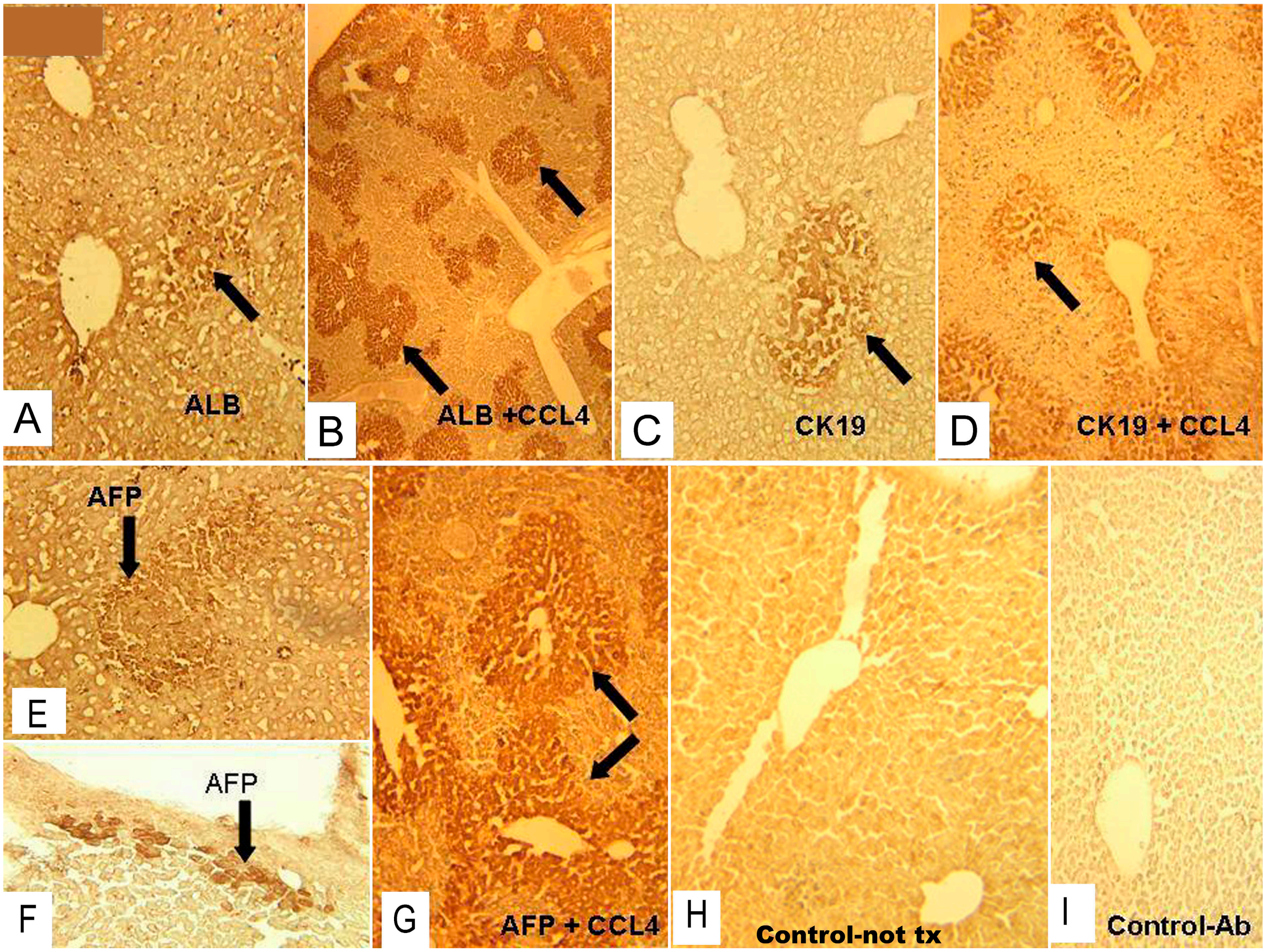


Figure 8. Transplantation of EpCAM+ cells (or colonies of stem cells in culture) results in engrafted liver tissue in NOD/scid mice. NOD/scid mice were transplanted with 10^6 cells of either freshly isolated and immunoselected EpCAM+ cells or colonies of hHpSCs from culture on plastic for 30–60 d (more than ~40 population doublings). Similar results were obtained with both populations. After transplantation, half the mice were treated with CCL4 (representative results shown in B, D, and F; representative results from transplanted mice not treated with CCL4 are shown in A, C, E, and F. A, C, and E show sections of murine livers stained for human-specific proteins 2 (A, C, and E), 8 (F), or 8 d after transplantation and 7 d after CCL4 treatment (B, D, and G). Controls included sections of mice not transplanted (H) or sections stained with only the secondary antibody (I). All antibodies, except for the antibody to albumin, gave little or no background; the control for albumin is shown. The arrows denote the location of human liver tissue within the section of murine liver tissue.

They can be isolated efficiently by selective culture conditions and by immunoselection for EpCAM (CD326) and/or NCAM (CD56). The hHpSCs have features typical of stem cells including Sonic and Indian Hedgehog signaling (27) and high telomerase activity (unpublished data). They are capable of self-renewal, as shown by clonogenic expansion for >150 population doublings, and are pluripotent, with the ability to give rise directly to committed biliary progenitors and hepatoblasts, and thence to hepatocytic and biliary lineages, as well as to other endodermal cell types (our unpublished data). The hHpSCs express certain markers of both hepatocytic and biliary lineages, but lack expression of mature liver functions (17). They yield human liver tissue when transplanted intrahepatically in immune-deficient mice. Hepatoblasts, expressing AFP, albumin, and CK19, and emerging in newly forming liver tissue, have long been considered hepatic stem cells (28). However, AFP− hHpSCs are actually precursors to hepatoblasts.

Recognition of the ductal plate as the liver's stem cell niche provides a new insight into organogenesis. The specification of foregut cells to a hepatic fate is associated with expansion of endoderm into the surrounding cardiac mesenchyme, a process leading to ductal plate formation (27). Our data suggests that ductal plates are directly antecedent to the Canals of Hering, which have been identified as the reservoir of stem cells in postnatal livers (18, 29)

At all ages, the liver displays a remarkable capacity to regenerate after physical or toxic injury (1). Two forms of regenerative response are known. The first is a hypertrophic cellular response by mature hepatocytes that undergo DNA synthesis with minimal cytokinesis, which is the predominant mechanism of regeneration after partial hepatectomy in postnatal livers (30, 31). The other is a hyperplastic response by both progenitor cells and diploid hepatocytes after significant loss of liver cells in zones 2 and 3, as previously detailed (2, 32, 33). Contributions of progenitors to regeneration after partial hepatectomy have been presumed negligible based on assumptions that they should be AFP+ (1). Because hHpSCs are AFP-, their role in liver regeneration in adults requires reevaluation by tracking the involvement of EpCAM+ and NCAM+ cells. The frequency of EpCAM+ cells in suspensions prepared from postnatal human livers is consistent at all ages beyond a few months, in the range of 0.5–2.5%. We infer that a substantial pool of hHpSCs is maintained throughout life. The number of hHpSCs is much higher than estimates based on the frequency of AFP+ cells (<0.01%). Some authors have argued that the mature liver contains only "facultative" stem cells, which are activated in response to pathological states and injuries that invoke a hyperplastic response (3, 18, 33, 34). We have previously raised the alternative hypothesis that hHpSCs function routinely to replenish the liver as mature cells are lost slowly through terminal differentiation (17, 35, 36). The presence of a much larger pool of hHpSCs than previously suspected in normal adult livers provides a further rationale to examine this possibility more carefully.

During development, a limited number of hHpSCs are associated with developing portal tracts and steadily give rise to hepatoblasts that we hypothesize are the liver's transit-amplifying cells. The hepatoblasts, in turn, are precursors to committed hepatocytic and biliary progenitors. Further evidence for hepatoblasts in normal adult livers is given elsewhere (unpublished data). In the findings reported in this work, we show the generation of hepatoblasts and unipotent progenitors from hHpSC colonies in culture. This occurs spontaneously from discrete regions at the periphery of hHpSC colonies and may correspond to a localized signal that triggers a rapid burst of expansion from one or more cells. As noted below, cell–cell interactions are key to both maintenance and differentiation of hHpSCs. STO feeder cells promote differentiation of hHpSCs, whereas they contribute primarily to survival and expansion of rodent hepatoblasts, and may offer a tool to identify some of the differentiation-promoting signals for hHsPCs.

Functions of cell surface markers of hHpSCs

Characteristic cell surface antigens of hHpSCs and hepatoblasts overlap extensively, with both populations expressing EpCAM, E-cadherin, integrin β-1 (CD29), and CD133. The hHpSCs and hepatoblasts are negative for markers of hematopoietic (CD34, CD45, CD38, CD14, CD90, and glycophorin A), endothelial (VEGFr, von Willebrand's factor, and CD31), and mesenchymal (CD146, desmin, and α-smooth muscle actin) cell lineages.

EpCAM is present on proliferating epithelial cells in most, if not all, organs derived from endoderm (liver, lung, pancreas, and intestine). The extracellular domain of EpCAM is thought to generate a relatively weak homotypic bond between adjacent cells (15). Conversely, the cytosolic domain of EpCAM is believed to diminish the effectiveness of E-cadherin binding through impairment of the interaction between β-catenin and the actin cytoskeleton (16). This role may be physiologically relevant for hHpSCs and hepatoblasts, as E-cadherin was expressed with the same distribution as EpCAM.

The role of NCAM in the biology of hHpSCs requires further elucidation. We found that the hHpSCs from fetal and neonatal liver consistently show strong NCAM expression, and that immunoselection for NCAM enriches for hHpSCs. However, only ~40% of hHpSCs from adult livers are positive for this antigen. In cultures of hHpSCs, NCAM expression is observed in a characteristic scalloped pattern located most prominently at the borders of the colonies. Thus far, we have not been able to ascertain unequivocally whether NCAM is expressed by hHpSCs, by tightly associated mesenchymal companion cells, or both. Ultrastructural studies by electron microscopy are needed to resolve this point. NCAM is a member of the Ig superfamily, with >20 alternatively spliced mRNAs encoding multiple protein isoforms (22, 37). It is the only sialated cell adhesion molecule, and it forms homotypic cell–cell attachments that are inversely proportional to the degree of sialation; an increase in sialation results in muted cell–cell adhesion and consequent increase in migration and invasion (22). Several groups have reported that NCAM is expressed by ductal plate cells within the fetal liver and, interestingly, also by proliferative ductular cells that characterize pathologies collectively termed ductal plate malformations, such as primary biliary atresia (20, 38–41). Thus, positive staining for NCAM, in addition to albumin, CK19, and CK8/18, supports the interpretation of these cells as ones derived from the ductal plate.

We found consistent expression of CD133 (prominin-1) by hHpSCs cultured on both plastic and on STO substrata, and by >90% of EpCAM+ cells immunoselected from adult livers. Although angioblasts also are CD133+, the staining in hHpSC colonies was associated with most or all cells, indicating that CD133 is expressed by the hHpSCs and not only by companion cells. This pentaspan transmembrane glycoprotein was first identified on hematopoietic stem cells, and its expression also has been observed on stem/progenitor cells of a variety of lineages, including endothelia, muscle, neural, prostatic, epidermal, and others (42, 43). The role, if any, of CD133 in the self-renewal and differentiative capacity of hHpSCs is not yet understood. However, it may be significant that an isoform of CD133 specifically associated with stem cells was found in cells of the basal layer of human neonatal epidermis, and co-expressed there with integrin β-1, which is also expressed by hHpSCs and hepatoblasts. Furthermore, CD133 expression was lost as the epidermal cells stopped proliferating and acquired a differentiated phenotype in culture (44).

Association of mesenchymal "companion" cells with hHpSCs

The specific association of multiple adhesion molecules with hHpSCs and hepatoblasts suggests that they play important regulatory functions in modulating interactions with cells that comprise local inductive environments and/or stem cell niches. A critical, enabling event, required for formation of the liver, is that angioblasts from the septum transversum induce the hepatic bud to form (7). Such key interactions are now amenable to study in vitro using hHpSCs. We observed that colony expansion and cell outgrowth of hHpSCs depends on the mesenchymal companion cells that are prominent at the periphery of hHpSC colonies and identified by antigenic profiles as angioblasts (positive for VEGFr, CD31 or platelet/endothelial cell adhesion molecule, CD117, and CD133) or hepatic stellate cell precursors (positive for CD146 [MEL-CAM and MCAM], desmin, and α-smooth muscle actin). These findings parallel our prior work defining hepatic stellate cell precursors as supportive of rodent hepatic progenitors and in which we find that they express not only vitamin A, desmin, and α-smooth muscle actin but also various markers associated with endothelia, such as VCAM (45).

CD146 forms homotypic cell–cell connections that were first localized on melanoma cells and, subsequently, at cell junctions within endothelial cell layers (46). CD146 has now been identified on many different cell types, including keratinocytes and hair follicle epithelia, stromal cells in adipose tissue and bone marrow, and most cell types in the thymus (47, 48). The expression of CD146 on cells at the periphery of colonies of hHpSCs, and extending to the innermost cells of aging colonies on plastic, is consistent with the known anticohesive activity of CD146. In this capacity, CD146 functions as an outside-in transducer that suppresses gap junction connections, inhibits β-1–mediated integrin binding, and disturbs E-cadherin–based adherens junctions (46, 49). Furthermore, the strong expression of ICAM-1 by more differentiated cells emerging from hHpSC colonies may also reflect modulation of cell–cell interactions. ICAM has been shown to act in conjunction with CD146 to disturb E-cadherin–based cell junctions (50). The dramatic increase in expression of CD146, in association with differentiation of the hHpSCs into hepatoblasts, is assumed to model angiogenesis in the forming liver, an interpretation that is supported by the findings in a study of Sonic and Indian Hedgehog signaling and the Patched receptor in the ridge formed by the angioblasts and the hepatic stellate cells (27).

The possibility of coisolation of hHpSCs with mesenchymal cells may account for some apparent differences between the hHpSCs described in this work and candidate hHpSCs reported by others (51). For example, liver-derived, stemlike cells have been reported to express markers shared with hematopoietic progenitors, including CD45 (leukocyte common antigen), CD34, and CD117, and/or to be capable of giving rise to both hepatic and endothelial cells (52). Another study describes candidate hepatic stem cells found in regeneration after massive hepatic necrosis as "lymphoid blastlike" cells that express CD133 and CD117, but not CD45 (53). We suspect that the multipotent stem cells found in fetal liver and reported to give rise to liver and also mesenchymal lineages (cartilage) and that coexpress EpCAM and various mesenchymal cell markers are also an example of coselection (51).

The hHpSC populations from both fetal and adult livers appear essentially negative for CD45, CD34, and CD117. However, CD34 and CD117 are expressed by angioblasts, which we have found in companion cells associated with hHpSCs. Based on immunofluorescent staining, CD117 was variably present in the border zone between the companion cells and some (but not all) hHpSC colonies cultured on plastic. Also, transcript analysis revealed a slight enrichment in CD117 mRNA in EpCAM+ cell populations from fetal livers and a significantly greater enrichment in EpCAM+ cells from postnatal livers (17). Nonetheless, immunoselection for CD117 or CD34 yielded angioblasts, not hHpSCs. In summary, our data remain inconclusive; we cannot rule out that a minor subpopulation of hHpSCs cells express CD117.

It is conceivable that phenotypic differences between hHpSCs obtained by different isolation procedures reflect varying stages within a common lineage and/or subtle effects of in vitro selection protocols. However, it is clearly important to be aware of the physical and functional interaction of hepatic (endodermal lineage) and mesenchymal lineage cells both in the developing liver and the adult organ, and the possibility of ascribing properties to a single cell type that actually correspond to a mixed population. In any case, we argue that the relative frequency and anatomical location of EpCAM+, CD133+, and CD34− cells, and the growth and differentiation capacity of these cells, provide strong evidence that the hHpSCs described here are authentic stem cells in fetal and postnatal livers.

Purified EpCAM+ cells from fetal or postnatal livers are able to engraft the livers of immunodeficient adult mice, with or without prior injury, yielding mature human liver tissue. The engrafted cells lose expression of stem cell markers (EpCAM, CD133, and CK19) and show enhanced expression of mature human proteins and mRNAs characteristic of hepatocytes (albumin and transferrin). The use of human-specific antibodies and sequence probes confirmed that these were made by donor origin cells. The extent of humanization of the murine livers was greatly enhanced by treatment of mice with CCl4, which is known to selectively kill mature parenchymal cells, and thereby, to create a cellular vacuum in the host.

The efficiency with which EpCAM+ cells can be isolated from human livers, their ability to clonogenically expand ex vivo, their pluripotency, and the evidence that they yield mature liver tissue after transplantation encourage consideration of their clinical utility. Potential applications include cell-based therapies of liver disease and generation of cells for bioartificial livers.

MATERIALS AND METHODS

Human liver sourcing

Fetal livers. Liver tissue was provided by an accredited agency (Advanced Biological Resources) from fetuses between 18–22 wk gestational age that were obtained by elective terminations of pregnancy. The research protocol was reviewed and approved by the Institutional Review Board for Human Research Studies at the University of North Carolina.

Postnatal livers. Intact livers from cadaveric neonatal, pediatric, and adult donors were obtained through organ donation programs via the United Network for Organ Sharing. Those used for these studies were considered normal, with no evidence of disease processes. Informed consent was obtained from next of kin for use of the livers for research purposes, protocols received Institutional Review Board approval, and processing was compliant with Good Manufacturing Practice.

Liver processing

Fetal livers. All processing and cell enrichment procedures were conducted in a cell wash buffer composed of a basal medium (RPMI 1640) supplemented with 0.1% BSA (BSA Fraction V; Sigma-Aldrich), insulin and iron-saturated transferrin (both at 5 ug/ml; Sigma-Aldrich), trace elements (300 pM selenious acid and 50 pM ZnSO4), and antibiotics (AAS; Invitrogen). Liver tissue was subdivided into 3-ml fragments (total volume ranged from 2–12 ml) for digestion in 25 ml of cell wash buffer containing type IV collagenase and deoxyribonuclease (both at 6 mg per ml; Sigma-Aldrich) at 32°C with frequent agitation for 15–20 min. This resulted in a homogeneous suspension of cell aggregates that were passed through a 40-gauge mesh and spun at 1,200 RPM for 5 min before resuspension in cell wash solution. Erythrocytes were eliminated by either slow-speed centrifugation (54, 55) or by treating suspensions with anti–human red blood cell antibodies (1:5,000 dilution; Rockland) for 15 min, followed by LowTox Guinea Pig complement (1:3,000 dilution; Cedarlane Labs) for 10 min, both at 37°C. Estimated cell viability by Trypan blue exclusion was routinely >95%. See Supplemental materials and methods for further details (available at http://www.jem.org/cgi/content/full/jem.20061603/DC1).

Postnatal livers. The livers were perfused through the portal vein and hepatic artery for 15 min with EGTA-containing buffer, and then with 600 mg/liter collagenase (Sigma-Aldrich) for 30 min at 34°C. The organ was mechanically dissociated in either collection buffer; the cell suspension passed through filters of pore size 1,000, 500, and 150 μm; the single cells were collected, and live cells were fractionated from dead cells and debris using density gradient centrifugation (500 *g* for 15 min at room temperature) in OptiPrep-supplemented buffer in a Cobe 2991 cell washer. The resulting hepatic cell band residing at the interface between the OptiPrep/cell solution and the RPMI 1640 without Phenol red was collected.

Magnetic immunoselection

Isolation of cells expressing EpCAM from human liver cell suspensions was performed using the monoclonal antibody HEA-125 coupled to magnetic microbeads, and separated using a miniMACS, a MidiMACS, an AutoMACS, or a CliniMACS magnetic column separation system from Miltenyi Biotec, following the manufacturer's recommended procedures. Similar protocols were used for sorts for NCAM+, CD117+, VEGFr (KDR)+, CD34+, and CD146+ cells.

Colony formation

Cells were plated in serum-free, hormonally defined medium (KM) in 6-well tissue culture dishes seeded with monolayers of Mitomycin-treated STO feeder cells, as described by Kubota and Reid (8). Medium was changed every 2–4 d. Colonies were observed within 7–10 d and were followed for up to 6 mo using an inverted microscope (1X-FLAIII; Olympus).

Online supplemental material

We present details for liver processing, histology, flow cytometry and immunohistochemistry in the supplemental materials and methods available online. In addition, results of the antigenic profile can be found in supplemental table 1. In supplemental table 2, we present the number and type of colonies obtained after fractionating and plating cells from postnatal human livers of various ages. Supplemental figures 1 and 2 show hepatoblast morphology, antigenic profile, and immunostaining results when cultured on plastic or feeder cells. Microscopy images of hepatic progenitors with specific immunohistochemistry markers can be seen in supplemental figures 3 and 4. Clonogenic expansion of hepatic stem cells from days 1, 3, and 8 can be seen in supplemental movies 1-3. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20061603/DC1.

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Patents have been filed on the intellectual property, are owned by University of North Carolina, and are licensed to Vesta Therapeutics.

The authors have no conflicts of interest.

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