The American Journal of Pathology, Vol. 173, No. 6, December 2008

DOI: 10.2353/ajpath.2008.080411

Stem Cells, Tissue Engineering and Hematopoietic Element

Hematopoietic Progenitors from Early Murine Fetal Liver Possess Hepatic Differentiation Potential

Satish Khurana and Asok Mukhopadhyay

From the Stem Cell Biology Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India

Bipotential hepatoblasts differentiate into hepatocytes and cholangiocytes during liver development. It is believed that hepatoblasts originate from endodermal tissue. Here, we provide evidence for the presence of hepatic progenitor cells in the hematopoietic compartment at an early stage of liver development. Flow cytometric analysis showed that at early stages of liver development, approximately 13% of CD45$^+$ cells express Δ-like protein-1, a marker of hepatoblasts. Furthermore, reverse transcriptase-PCR data suggest that many hepatic genes are expressed in these cells. Cell culture experiments confirmed the hepatic differentiation potential of these cells with the loss of the CD45 marker. We observed that both hematopoietic activity in Δ-like protein-1$^+$ cells and hepatic activity in CD45$^+$ cells were high at embryonic day 10.5 and declined thereafter. Clonal analysis revealed that the hematopoietic fraction of fetal liver cells at embryonic day 10.5 gave rise to both hepatic and hematopoietic colonies. The above results suggest a common source of these two functionally distinct cell lineages. *In utero* transplantation experiments confirmed these results, as green fluorescent protein-expressing CD45$^+$ cells at the same stage of development yielded functional hepatocytes and hematopoietic reconstitution. Since these cells were unable to differentiate into cytokeratin-19-expressing cholangiocytes, we distinguished them from hepatoblasts. This preliminary study provides hope to correct many liver diseases during prenatal development via transplantation of fetal liver hematopoietic cells. ***(Am J Pathol 2008, 173:1818–1827; DOI: 10.2353/ajpath.2008.080411)***

Mouse liver development is induced by cardiac mesoderm at the 7 to 8 somite stage when the hepatic diverticulum emerges from the foregut endoderm.[1,2] The liver rudiment begins as an evagination of the gut endodermal cells in the hepatic bud at late embryonic day 9 (E9), where they proliferate and invade the septum transversum mesenchyme.[3,4] Following this inductive interaction, hepatic progenitors emerge from the foregut endodermal tissue, where they mature. Fetal liver, being an important transient hematopoietic site, allows simultaneous development of hematopoietic and hepatic cells. Once the liver bud emerges from the developing gut, hematopoietic cells migrate (E9 to E10) to the fetal liver from the aorta-gonad-mesonephros, proliferate, and apparently produce liver morphogenic and growth factors.[5–7] Subsequently, the fetal liver acts as a major site of hematopoiesis till E16. Hence, there exists a close relationship between endodermal and mesodermal tissue components in fetal liver, which contributes to the development of hepatoblasts, the common progenitor for hepatic and biliary epithelial cells in mammals.[8–10] In mouse, during mid-gestation the hepatoblasts are colocalized with hematopoietic cells in the fetal liver.[11,12]

Lipp[13] first proposed the contribution of mesoderm in the development of liver in guinea pig. Later in turtle, it was shown that about 80% of the liver parenchyma originates from mesoderm tissue.[14] The argument for the involvement of hematopoietic cells in liver development was further strengthened after discovering phenotypic similarities between hematopoietic stem cells (HSCs) and the liver stem cells, also known as oval cells.[15] Moreover, recent reports have shown that HSCs can directly differentiate into hepatocytes in a mouse model of liver injury.[16,17] Human HSCs have also been shown to differentiate into hepatocytes in pre-immune fetal sheep by *in utero* transplantation.[18] The above studies[15–18] suggest a contribution of adult hematopoietic cells in the regeneration of liver.

In the present study, we have examined the possible contribution of donor fetal liver hematopoietic cells in prenatal development of mouse liver. We have analyzed hematopoietic and non-hematopoietic cellular compartments of mice from early stages of liver development. *In utero* transplantation experiments suggest that a subset

Supported in part by grants from Department of Biotechnology, Government of India.

Accepted for publication September 9, 2008.

Address reprint requests to Asok Mukhopadhyay, Ph.D., National Institute of Immunology, New Delhi-110067, India. E-mail: ashok@nii.res.in.

of cells in the hematopoietic compartment, at early stages of liver development, is capable of differentiating into hepatocytes.

Materials and Methods

Animals

Six to ten-week old FVB/NJ and green fluorescence protein (GFP) transgenic mice [FVB.Cg-Tg(GFPU) 5NAGY/J] mice were used in this investigation. Mice were obtained from The Jackson Laboratories (Barharbor, ME) and maintained in the Institute's experimental animal facility. All experiments using mice were conducted as per procedures approved by the Institutional Animal Ethics Committee.

Embryo Generation and Isolation of Fetal Liver Cells

Embryos were generated from timed matings between male and female mice. Detection of the vaginal plug was designated as E0.5. Pregnant females were sacrificed by cervical dislocation at E10.5 to E16.5. The uterine horns were removed; fetuses were separated from maternal tissue and placed in a Petri dish containing PBS. The fetal liver tissues were removed and washed in Iscove's modified Dulbecco's medium supplemented with 3% fetal calf serum. Single cell suspension was prepared by passing fetal liver tissue through a 23-gauge needle.

Cell Sorting

Fetal liver cells (FLCs) were sorted using a MidiMACS system (Miltenyi Biotech, Gladbach, Germany) by a two-step procedure. Ter119$^-$ cells were sorted by negative selection, which was followed by positive selection of hematopoietic (CD45$^+$) or hepatoblastic (delta-like protein-1$^+$, Dlk-1$^+$) cells, and negative selection of non-hematopoietic (CD45$^-$) cells. Antibodies (anti-Ter119 and anti-CD45, biotinylated) were procured from BD Pharmingen (San Jose, CA) and anti-Dlk-1 (MBL Medical & Biological, Naka-Ku Nagoya, Japan).

Semiquantitative and Quantitative Real Time PCR

Total RNA from sorted FLCs was isolated using TRI Reagent (Sigma Aldrich Corporation, St Louis, MO), and cDNA was synthesized from 0.5-μg total RNA using ProtoScript First Strand cDNA Synthesis Kit (NEB, Beverly, MA). The PCR was performed with hot start at 95°C for denaturation, annealed at specific temperatures, and amplified at 72°C. The amplified products were resolved in 1.5% to 2.0% agarose gels and visualized by ethidium bromide staining. Images were recorded using a UVP Bio-imaging System (UVP, Upland, CA). Real-time PCR was performed using SYBR green technology and an ABI PRISM 7000 thermocycler (Applied Biosystems, Foster City, CA). A list of genes studied in this investigation, their spe-

Table 1. Primer Sequences, Amplicon Size, Annealing Temperature, and Cycle Numbers of Different PCR Reactions

Gene	Primer sequence	Amplicon size	Annealing temp (°C)	Cycles
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward: 5′-GAATACGGCTACAGCAACAG-3′ Reverse: 5′-CTAGGCCCCTCCTGTTATTA-3′	209	59.8	30
Albumin	Forward: 5′-GTGCAAGAACTATGCTGAGG-3′ Reverse: 5′-ACTCACTGGGGTCTTCTCAT-3′	465	58.0	30
Cytokeratin-18 (CK-18)	Forward: 5′-CACCACCTTCTCCACCAACT-3′ Reverse: 5′-GCCTCGATTTCTGTCTCCAG-3′	573	57.3	30
Oncostatin M (OSM)	Forward: 5′-GTGTCCCCTACTACCACTCAGG-3′ Reverse: 5′-GTGTCTTGCTCACAGTCACACA-3′	451	56.7	30
OSM receptor (OSMR)β	Forward: 5′-CACTCCACACACTATAGGCACT-3′ Reverse: 5′-GTTTTGAGTAGGTAGGCTCTGC-3′	306	57.4	30
Mesoderm specific transcript (MEST)	Forward: 5′-CTGCACTCATGGAAGACTTCTG-3′ Reverse: 5′-GATGCGTCTCAGGAAAGATACC-3′	439	54.9	30
α-fetoprotein (AFP)	Forward: 5′-CGCTCTCTACCAGACCTTAGG-3′ Reverse: 5′-CTCCTCTGTCAGTTCAGGCTTT-3′	451	59.0	30
Hepatocyte nuclear factor (HNF)-1α	Forward: 5′-GCCTCCTCTTCCCAGTAACC-3′ Reverse: 5′-GGAGCAGCAGTGTCAATGA-3′	187	58.6	30
HNF-4α	Forward: 5′-ACAGGAGAGGTGCAGAAGCA-3′ Reverse: 5′-GATGTTTGCACAACCACAGG-3′	180	59.2	30
GATA-1	Forward: 5′-AGTGTGTGAACTGTGGAGCAAC-3′ Reverse: 5′-GTGGTCGTTTGACAGTTAGTGC-3′	187	59.8	30
CD45	Forward: 5′-TCTCCCAGGAGTATGAGTCCAT-3′ Reverse: 5′-GGCCAATACTGATCACACTTCA-3′	339	59.8	30
β-actin	Forward: 5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′ Reverse: 5′-TTGATGGCTACGTACATGGCTGGG-3′	437	56.9	30
For qPCR				
Dlk-1	Forward: 5′-ATCGTAGCCGCAACCAACA-3′ Reverse: 5′-CAGGTCTTGTCGACGAATC-3′	116	60.0	
β-actin	Forward: 5′-AGAGGGAAATCGTGCGTGA-3′ Reverse: 5′-CAATAGTGATGACCTGGCCGT-3′	137	60.0	

cific primers, product size, annealing temperature, and number of amplification cycles are mentioned in Table 1.

Cell Culture

Two million E16.5 fetal liver cells (FVB/NJ mice), used as a feeder layer, were cultured for 48 hours in a 6-well plate in the presence of Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum. Two hundred fifty thousand $GFP^+Ter119^-CD45^+$ or $GFP^+Ter119^-CD45^-$ cells (E10.5) were cultured on a feeder layer for 5 days in the same medium. The GFP^+ cells were analyzed by flow cytometry for the expression of CD45 and albumin.

Clonal Analysis and Colony Assays

$Ter119^-CD45^+$ and $Ter119^-CD45^-$ cells were plated at different densities (1 cell/well, 2 cells/well, and 5 cells/well) in 96-well plates in the presence of either 100 μl hepatic or methylcellulose colony assay medium. Hepatic and hematopoietic colony assays were essentially performed as previously described.[19,20] Specifically, colony assay protocols have been described in our prior publication.[21] Twenty four hours after plating, wells were examined under a light microscope to identify wells that received 1 cell each. Only these wells were considered for clonal analysis. Hepatic colonies were identified on the basis of morphology of the colony and the expression of albumin by immunostaining. Myeloid colonies were identified by examining the morphology of the colony.

In Utero Transplantation and Partial Hepatectomy

In utero transplantation was done as described in the previous report.[22] Pregnant FVB/NJ mice of E10.5 were anesthetized by intraperitoneal injection consisting of a mixture of ketamine (100 mg/kg) and xylazine (15 mg/kg). The uterus was exposed through a vertical laparotomy incision. A 28-gauze needle attached to a microsyringe was used for injecting 10 μl cell suspension in PBS. The tip of the needle was inserted transplacentally through the uterine wall into the fetuses to deliver the cells. Two groups of mice received either 1×10^5 $GFP^+Ter119^-CD45^+$ or $GFP^+Ter119^-CD45^-$ cells of E10.5. The incision portion was stitched with silk sutures. The pregnant mice were allowed to give birth, and the pups were nurtured for 8 weeks before tissue analysis.

Partial hepatectomy was performed on anesthetized mice, as described in the literature.[23] In brief, median and lateral hepatic lobes were ligated with silk suture and excised through a 1-cm longitudinal abdominal incision. The resected tissue was used for immunohistochemical and flow cytometric analyses. Two hours before sacrifice, mice were given an injection of 100 mg/kg of bromodeoxyuridine (BrdU). Liver tissue was collected 24 hours postpartial hepatectomy for subsequent analysis. The chimerism of bone marrow (BM) with donor cells and the number of donor-derived hepatocytes incorporating BrdU were determined by flow cytometry.

Flow Cytometry

The antibodies and conjugates used in this study were biotinylated anti-albumin (Nordic Immunological Laboratories, The Netherlands), anti-BrdU (ebioscence, Inc., San Diego, CA), anti-Ter119 (BD Pharmingen), anti-Dlk-1 (MBL), and anti-CD45/phycoerythrin (PE)/PerCP. Before staining for intracellular proteins, cells were fixed with 4% paraformaldehyde and permeabilized by treatment with 0.1% saponin in 1% bovine serum albumin-PBS for 15 minutes at 4°C. The secondary conjugates and antibodies used were streptavidin-fluorescein isothiocyanate (FITC)/PE/PerCP (BD Pharmingen) and anti-rat I_gG-PE (Jackson Immunologicals). Antibody-labeled cells were analyzed with a flow cytometer (BD-LSR, BD Biosciences, San Jose, CA).

Immunohistochemistry

Liver tissue was fixed in 4% paraformaldehyde; tissue blocks were made in optimal cutting temperature (OCT) medium. Five-micron cryosections were used for staining with antibodies. Before staining with BrdU-specific antibody, sections were treated with 2N HCl for 20 minutes, followed by neutralization with borate buffer. The washed sections were incubated with rabbit anti-GFP, goat anti-albumin (Bethyl Laboratory Inc., Montgomery, TX), mouse anti-CK-18 and goat anti-CK-19 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), and mouse anti-BrdU antibodies for 2 hours at room temperature. The washed sections were further treated with anti-rabbit IgG/Alexa fluor488 and anti-goat IgG/Alexa fluor594 or anti-mouse IgG/Alexa fluor594 (Molecular Probes Inc., Eugene, OR). The stained sections were examined using a fluorescence microscope (Olympus, Japan).

Statistical Values

Results of multiple experiments were reported as the mean ± SEM. One-way analysis of variance was followed to calculate the significance between two means.

Results

Dlk-1 Is Expressed in E10.5 Fetal Liver Hematopoietic Cells

Dlk-1, expressed in many tissues during early development,[24] has been considered as a specific marker for hepatoblasts.[25] We compared hematopoietic ($CD45^+$) and non-hematopoietic ($CD45^-$) FLCs for the expression of Dlk-1. Flow cytometric analysis revealed that at E10.5, Dlk-1 expression was associated with the $CD45^+$ fraction of FLCs, and not with the $CD45^-$ fraction (Figure 1A, lower left). Subsequently at E12.5, Dlk-1 was also ex-

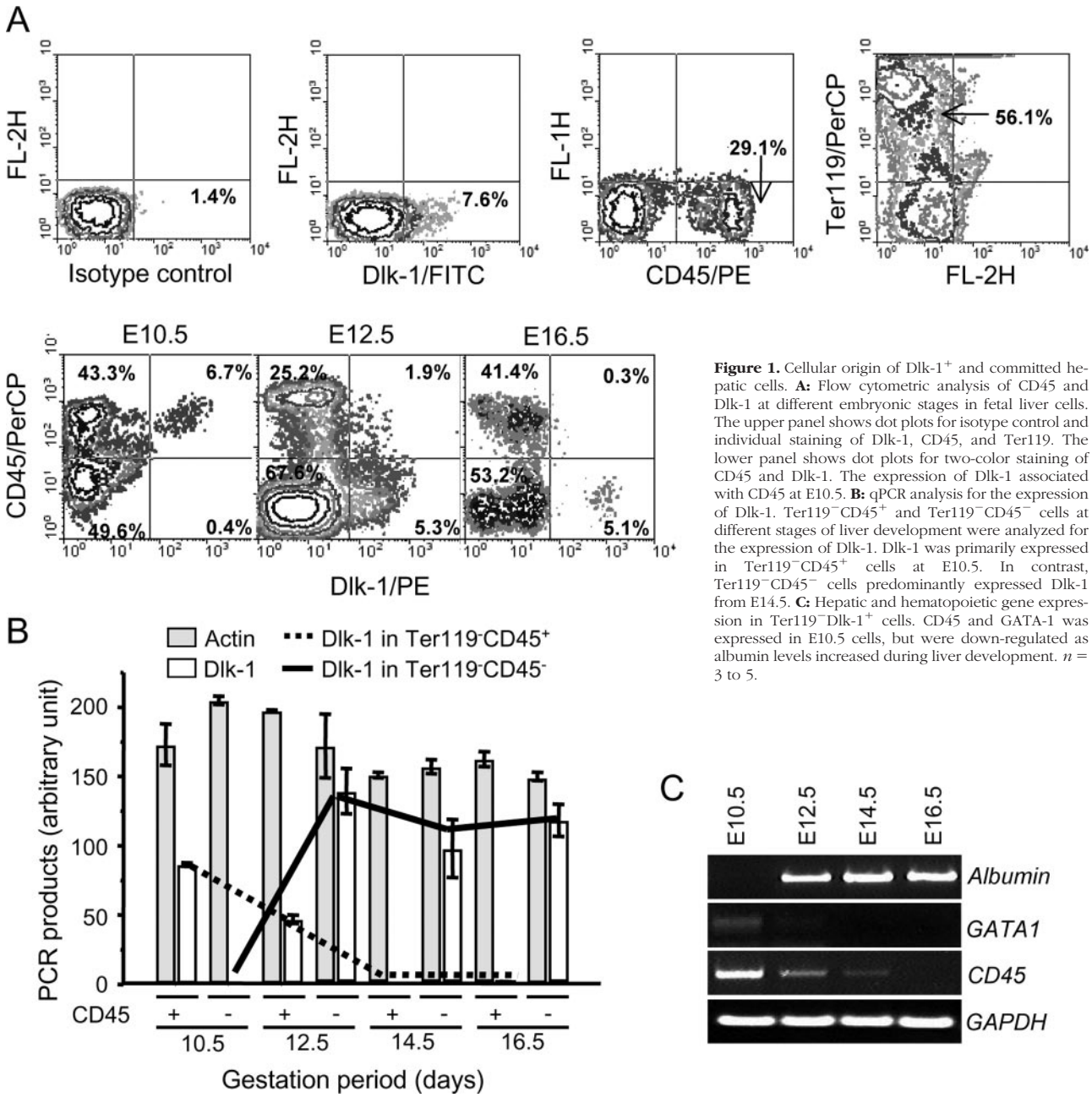

Figure 1. Cellular origin of Dlk-1$^+$ and committed hepatic cells. **A:** Flow cytometric analysis of CD45 and Dlk-1 at different embryonic stages in fetal liver cells. The upper panel shows dot plots for isotype control and individual staining of Dlk-1, CD45, and Ter119. The lower panel shows dot plots for two-color staining of CD45 and Dlk-1. The expression of Dlk-1 associated with CD45 at E10.5. **B:** qPCR analysis for the expression of Dlk-1. Ter119$^-$CD45$^+$ and Ter119$^-$CD45$^-$ cells at different stages of liver development were analyzed for the expression of Dlk-1. Dlk-1 was primarily expressed in Ter119$^-$CD45$^+$ cells at E10.5. In contrast, Ter119$^-$CD45$^-$ cells predominantly expressed Dlk-1 from E14.5. **C:** Hepatic and hematopoietic gene expression in Ter119$^-$Dlk-1$^+$ cells. CD45 and GATA-1 was expressed in E10.5 cells, but were down-regulated as albumin levels increased during liver development. n = 3 to 5.

pressed in non-hematopoietic (CD45$^-$) FLCs (Figure 1A, lower middle), and finally at E16.5 expression was primarily restricted to non-hematopoietic cells. We observed that irrespective of the gestation period, 6.41 ± 0.6% (n = 13) of FLCs expressed Dlk-1. The shifting of Dlk-1 expression from hematopoietic to non-hematopoietic cells could be either due to down-regulation of CD45 expression on these cells or expression of Dlk-1 in CD45$^-$ cells with simultaneous decline in CD45$^+$ cells.

To confirm the flow cytometry results, we performed quantitative RT-PCR for Dlk-1 using sorted hematopoietic and non-hematopoietic FLCs at different stages of development (Figure 1B). Ter119$^-$CD45$^+$ and Ter119$^-$CD45$^-$ FLCs from different stages of gestation were sorted and the purity of the cells was determined to be greater than 97% by flow cytometry. At E10.5, Dlk-1 was found to be exclusively expressed in Ter119$^-$CD45$^+$ FLCs. Expression in Ter119$^-$CD45$^-$ FLCs was first observed at E12.5, with expression levels 2.5-fold higher than in Ter119$^-$CD45$^+$ FLCs of the same stage. Confirming the flow cytometry results, we also found that the expression of Dlk-1 was restricted to Ter119$^-$CD45$^-$ cells at E14.5 to E16.5. We therefore speculated that CD45$^-$Dlk-1$^+$ cells might have originated from E10.5 CD45$^+$ FLCs by losing hematopoietic activity.

To further establish the link between the hematopoietic and hepatic lineages, we analyzed gene expression in Ter119$^-$Dlk-1$^+$ cells at different stages of gestation. In this case, we avoided the issue of hematopoietic or non-hematopoietic source of the cells, and asked the ques-

tion "how do hematopoietic and hepatic gene expression change with development?" We selected GATA-1 and CD45 as hematopoietic markers and albumin as a hepatic marker. It was observed that at E10.5, Ter119$^-$Dlk-1$^+$ cells expressed CD45 and GATA-1, but not albumin (Figure 1C). However, over time, the same populations expressed albumin with concomitant down-regulation of both CD45 and GATA-1 transcripts. The expression of Dlk-1 in CD45$^+$ cells and of GATA-1 and CD45 in Dlk-1$^+$ cells from E10.5 fetal liver supported our notion of a developmental relation between these two lineages.

Kinetics of Hepatocytes and Cholangiocytes Specific Gene Expression

Since hepatoblasts are known as biopotential cells that give rise to hepatocytes and cholangiocytes, we compared their gene expression in CD45$^+$ and CD45$^-$ FLCs at different stages of fetal liver development. Our results showed that albumin expression was first observed at E12.5 in Ter119$^-$CD45$^+$ cells (Figure 2A), but later was more strongly expressed in Ter119$^-$CD45$^-$ cells. On the other hand, CK-19 expression was restricted to non-hematopoietic lineage (Figure 2B). Intriguingly, HNF-1α and OSMRβ were also first detected in Ter119$^-$CD45$^+$ cells at E10.5 (Figure 2, A and B). This is noteworthy because HNF-1α and OSMRβ are involved in development of embryonic liver[26] and maturation of hepatocytes,[19] respectively. In agreement with earlier reports,[19,26] we also observed HNF-4α and OSMRβ expression in non-hematopoietic cells at E14.5. From this study, it appears that a fraction of CD45$^+$ FLCs at E10.5 expresses Dlk-1, HNF-1α, and OSMRβ. Later, development of these cells commits to hepatocytes, as evidenced by albumin expression (Figure 2A) and loss of CD45 antigen (Figure 1C).

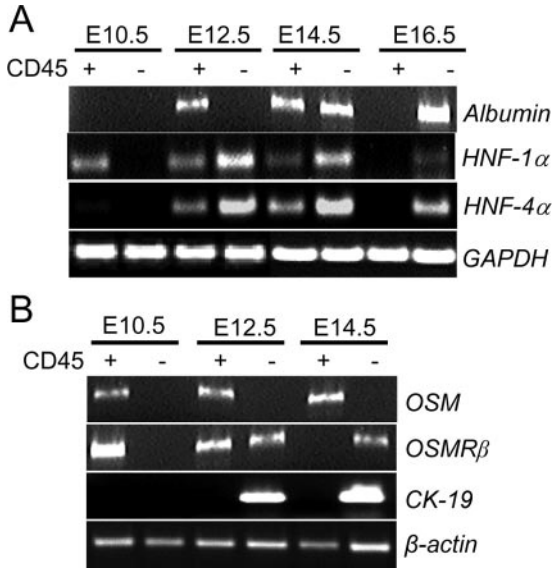

Figure 2. Hepatic gene expression in hematopoietic and non-hematopoietic FLCs. **A** and **B:** HNF-1α and OSMRβ gene expression were observed from E10.5 in the CD45$^+$ fraction of cells. In addition, HNF-4α and albumin were expressed in the CD45$^+$ and CD45$^-$ fractions of cells from E12.5, depending on the stage of the development. CK-19 was expressed in the CD45$^-$ fraction of cells. (n = 3).

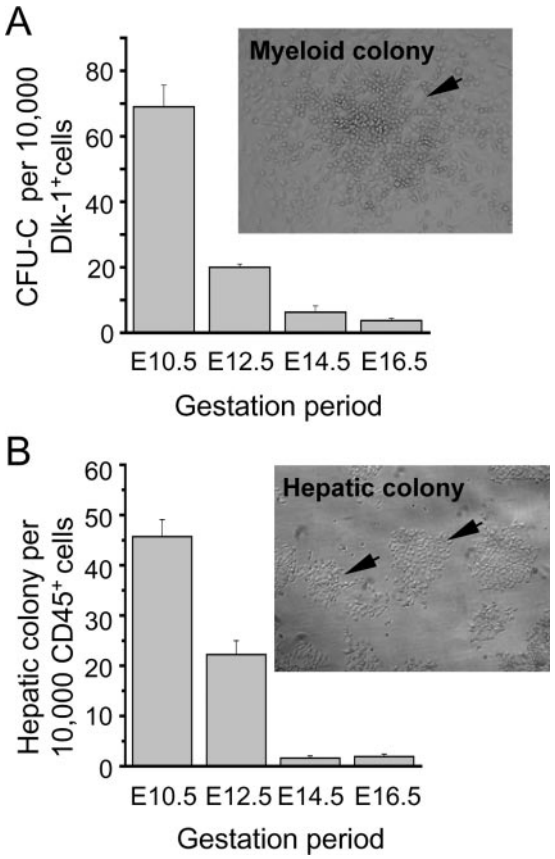

Figure 3. Hematopoietic and hepatic colony forming ability of FLCs. **A:** Total myeloid colony forming potential of Ter119$^-$Dlk-1$^+$ cells. The number of myeloid colonies was reduced with liver development. **B:** Hepatic colony forming potential of Ter119$^-$CD45$^+$ cells. The number of hepatic colonies was reduced with liver development. **Arrows** indicate hematopoietic and hepatic colonies. (n = 3).

Hematopoietic and Hepatic Colony Forming Ability in FLCs

After knowing that at E10.5, CD45$^+$ FLCs express hepatic as well as hematopoietic genes, we examined the hematopoietic and hepatic colony forming potential of Dlk-1$^+$ and CD45$^+$ cells, respectively. The myeloid colony forming potential of Ter119$^-$Dlk-1$^+$ cells was highest (69.0 $\pm$ 6.6 per 10,000 cells) at E10.5, but significantly decreased from E12.5 to E16.5 (Figure 3A). Similarly, we found that Ter119$^-$CD45$^+$ cells yielded the highest number of hepatic colonies (45.7 $\pm$ 3.4 per 10,000 cells) at E10.5, but gradually declined with time (Figure 3B). The appearance of hematopoietic colonies from E10.5 Dlk-1$^+$ cells indicates that they possessed hematopoietic activity, which was later not detected due to commitment of cells toward the hepatic lineage. Since Dlk-1$^+$ cells expressed CD45 at E10.5 and develop hematopoietic colonies; we concluded that these cells have the potential to differentiate to both the hematopoietic and hepatic lin

eages. As liver development progressed, however, the hepatic activity was restricted to CD45$^-$Dlk-1$^+$ cells.

Coculture of E10.5 CD45$^+$ Cells with E16.5 FLCs Causes Down-Regulation of CD45 with Concomitant Expression of Albumin

To examine these phenotypic changes, we conducted *in vitro* culture of hematopoietic cells in the presence of fetal liver cells. Ter119$^-$CD45$^+$ and Ter119$^-$CD45$^-$ cells were isolated from E10.5 fetal livers of GFP transgenic mice. Before initiating coculture experiments, the purity of cells was checked by flow cytometric and RT-PCR analyses. The results of flow cytometric analysis confirmed that both fractions of cells were highly purified (Figure 4A). These results were further validated by analyzing germ layer specific gene expression in the cells. CD45$^+$ cells expressed only mesoderm specific transcript (MEST) and not an endoderm marker (α-fetoprotein). Similarly, CD45$^-$ cells expressed α-fetoprotein and not MEST. Thus, we concluded that both the cellular fractions were reasonably pure with respect to their germ layer specific cells (Figure 4B).

After confirming the purity, we cocultured E10.5 GFP$^+$Ter119$^-$CD45$^+$ and GFP$^+$Ter119$^-$CD45$^-$ cells on E16.5 liver cells (non-GFP) as a feeder layer for 5 days. It was revealed that the expression of CD45 in GFP$^+$ cells was significantly ($P < 0.005$) reduced from 95.8 ± 2.5% to 23.1 ± 2.0% (Figure 4C, left). The decrease in CD45 expression in the test cells was accompanied by an increase in the expression of albumin. The expression of albumin after 5 days of culture was detected in 45.7 ± 7.1% cells (Figure 4C, right). We also observed that Ter119$^-$CD45$^-$ cells express albumin (14.5 ± 3.1%),

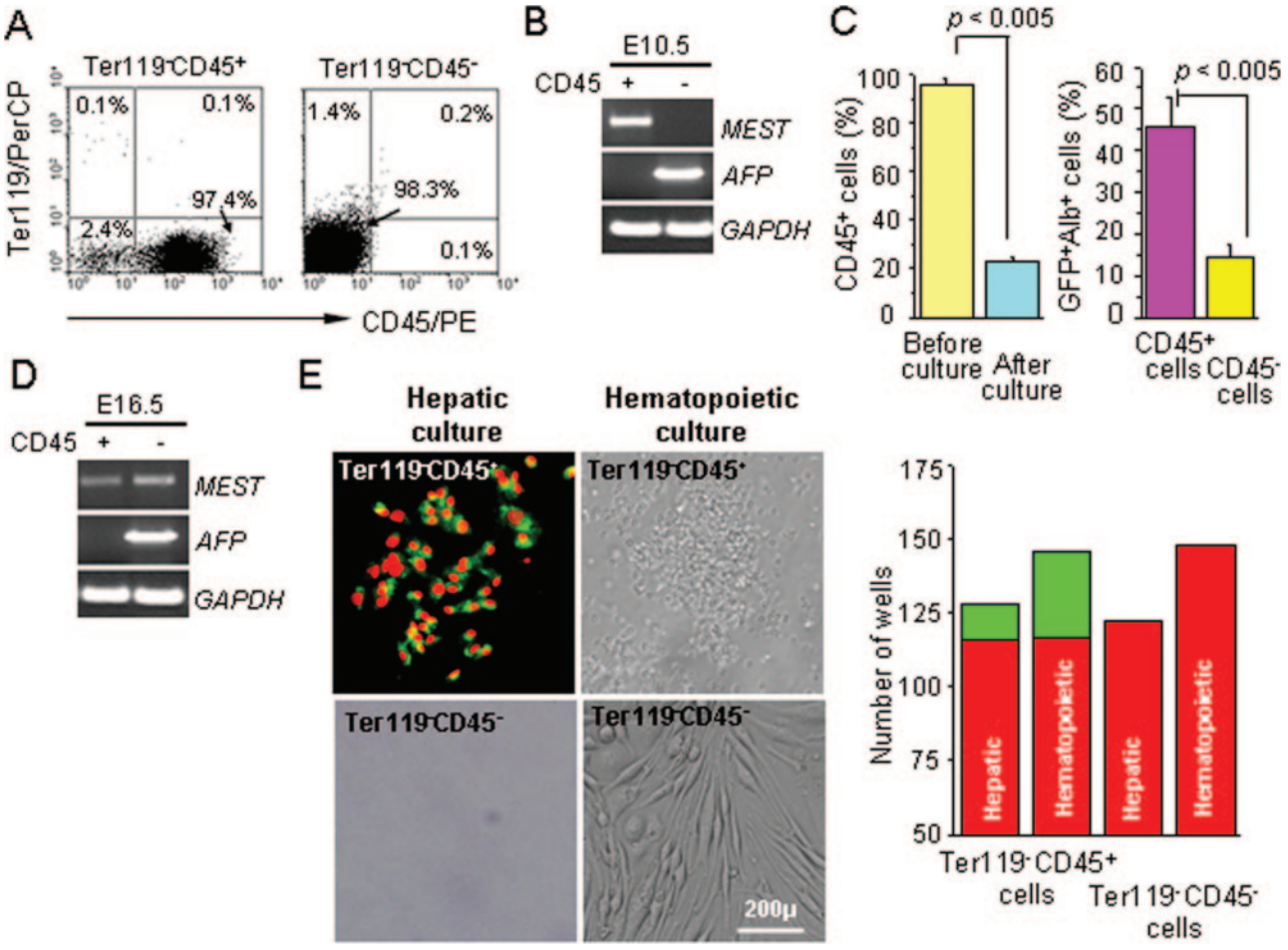

Figure 4. Hepatic differentiation potential of Ter119$^-$CD45$^+$ and Ter119$^-$CD45$^-$ cells of E10.5 in culture. **A:** Flow cytometric analysis of sorted cells. Dot-plots show more than 97% purity. **B:** RT-PCR analysis of germ layer specific genes. Results show that hematopoietic and non-hematopoietic fractions of cells were pure with respect to the expression of germ layer specific signature genes ($n = 3$). **C:** *In vitro* hepatic differentiation potential of hematopoietic and non-hematopoietic cells. The majority of the hematopoietic cells expressed CD45 antigen before culture, but CD45 expression was significantly reduced (left figure) in cultured cells with simultaneous differentiation into albumin expressing cells (right figure). Down-regulation of CD45 antigen was associated with the expression of albumin in CD45$^+$ cells. CD45$^-$ cells also expressed albumin, but at much lower levels as compared to the CD45$^+$ cells ($n = 5$). **D:** RT-PCR analysis of germ layer specific gene at E16.5 FLCs. Results show that non-hematopoietic (CD45$^-$) cells expressed MEST along with an endoderm specific marker (α-fetoprotein) ($n = 3$). **E:** Clonal analysis of E10.5 Ter119$^-$CD45$^+$ and Ter119$^-$CD45$^-$ FLCs. Sorted cells (1 cell/well) were cultured in the presence of either hepatic or hematopoietic specific culture medium. Photomicrographs (left) show representative colonies obtained in hepatic and hematopoietic culture medium. Ter119$^-$CD45$^+$ cells gave rise to both albumin expressing hepatic and myeloid colonies in respective medium (top panel). Ter119$^-$CD45$^-$ cells did not furnish any hepatic colonies, but produced some fibroblastic (non-hematopoietic) colonies (bottom panel). Bar diagram (right) explain the number (red + green) of wells (1 cell/well) tested and the frequency of colonies identified (green).

although at significantly ($P < 0.005$) lower levels than in the hematopoietic fraction of cells. The decline in the expression of the hematopoietic marker with simultaneous expression of albumin in Ter119$^-$CD45$^+$ cells suggested that hematopoietic cells differentiate into hepatocytes. Since E10.5 Ter119$^-$CD45$^-$ cells provided albumin-expressing cells in culture, although at a lower extent, we presumed that these cells became competent for hepatic differentiation. To confirm the contribution of mesoderm tissue to liver development, we examined E16.5 CD45$^+$ and CD45$^-$ FLCs for expression of MEST, as during development MEST is expressed in the mesoderm derived tissues.[27] Our results suggest that a fraction of CD45$^-$ FLCs at E16.5 was indeed derived from mesoderm (Figure 4D).

Next, we performed clonal analysis of E10.5 Ter119$^-$CD45$^+$ and Ter119$^-$CD45$^-$ cells for their ability to form hepatic and hematopoietic colonies in culture. It was revealed that the hematopoietic fraction of FLCs yielded both hepatic and hematopoietic colonies, depending on the conditions of the culture. Clonal growth of a hepatic colony is shown in Figure 4E (left top), with the majority of the cells in the hepatic colony expressed albumin. About 9% of wells tested in this experiment formed hepatic colonies (Figure 4E, right). Conversely, no hepatic colonies were derived from clonal culture of

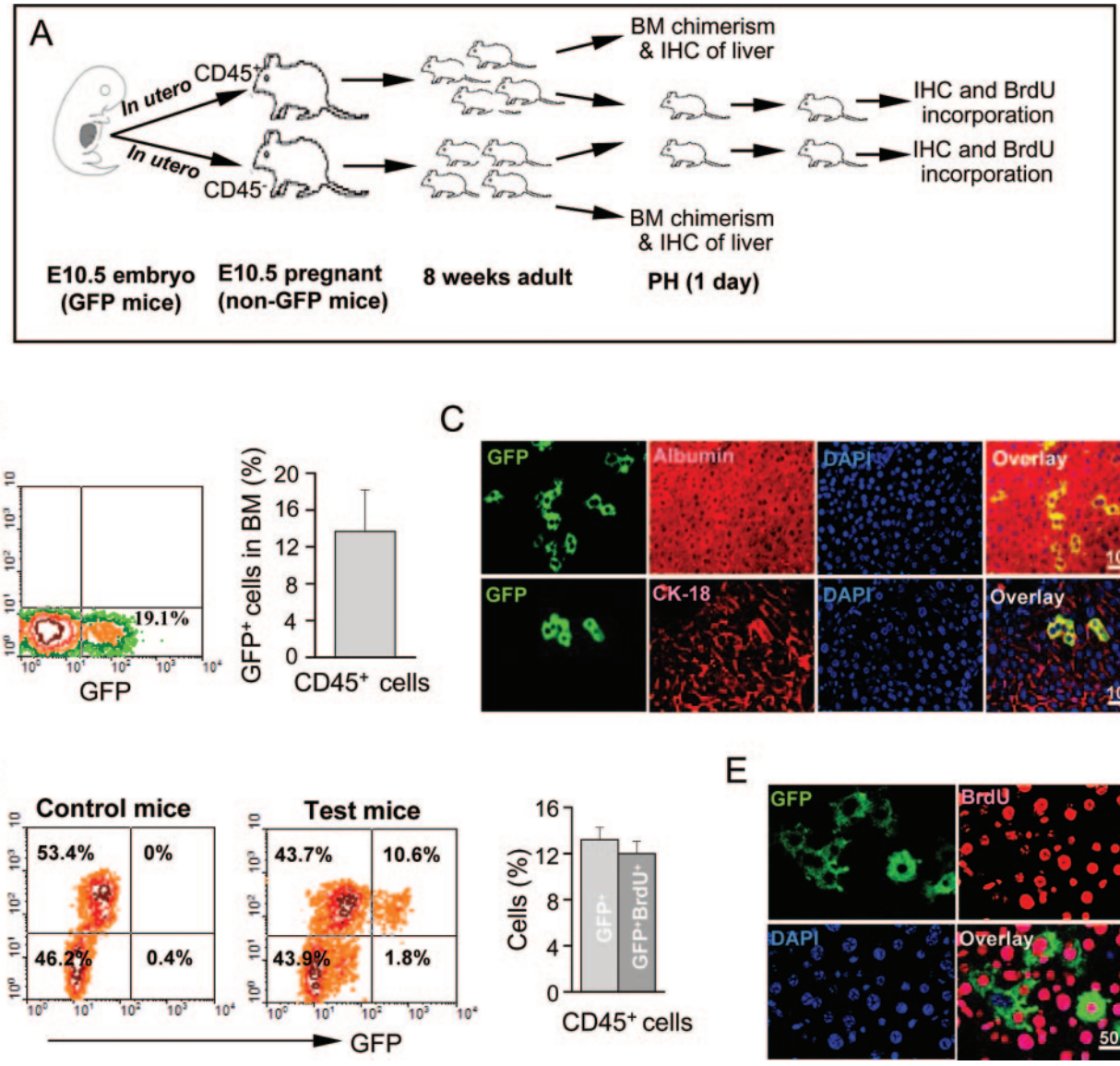

Figure 5. *In utero* transplantation of E10.5 Ter119$^-$CD45$^+$ FLCs. **A:** Experimental procedure for *in utero* transplantation. Pups delivered by *in utero* transplanted mice were analyzed after 8 weeks for the presence of donor-derived albumin expressing cells and bone marrow chimerism. Three mice underwent partial hepatectomy (PH) followed by BrdU pulse for 2 hours before analysis of liver tissue. **B:** Flow cytometric analysis of GFP cell engraftment in BM. Bar diagram shows engraftment of the CD45$^+$ fraction of FLCs in BM (right figure). A representative dot plot of donor cells engraftment is shown in the left figure. **C:** The engrafted GFP$^+$ cells in the liver lobules expressed albumin (upper panel) and CK-18 (lower panel). (Number of mice = 5; magnification = original ×200; scale bar = 100 μm). **D:** Flow cytometric analysis of BrdU incorporation in liver cells. Single cells were prepared by collagenase digestion. Bar diagram (right figure) shows about 85% of engrafted GFP$^+$ cells in liver incorporated BrdU in the nuclei, following partial hepatectomy (Number of mice = 3). Representative dot plots for BrdU incorporation in control (non-transplanted) and test (GFP$^+$ cells transplanted) mice (left and middle figure) are shown. **E.** Immunohistochemical analysis of BrdU incorporated cells. GFP-expressing hepatocytes incorporated BrdU in the nuclei (Number of mice = 3; magnification = original ×400; scale bar = 50 μm).

Ter119$^-$CD45$^-$ FLCs (Figure 4E, left bottom). Interestingly, clonal growth of myeloid colonies was also noticed in the hematopoietic fraction of cells, at much higher numbers than the hepatic colonies (Figure 4E). It may be noted that few fibroblastic colonies was observed in the non-hematopoietic fraction of cells (Figure 4, left bottom). Thus, *in vitro* bulk culture experiments and clonal analysis suggest that the E10.5 hematopoietic fraction of cells has potential to provide both myeloid and hepatic colonies, depending on the conditions of the culture.

In Utero Transplantation Confirms that Early Hematopoietic Cells Differentiate into Hepatocytes

Finally, we confirmed the results of *in vitro* experiments by *in utero* transplantation of E10.5 GFP$^+$Ter119$^-$CD45$^+$ and GFP$^+$Ter119$^-$CD45$^-$ cells in congenic pregnant mice of the same gestation period. The details of the experimental protocols have been explained in Figure 5A. Mice were allowed to give birth and the pups were maintained for 8 weeks before analysis of the donor cells. Chimerism of BM (13.7 ± 4.5%, $n = 5$) with donor cells was observed when CD45$^+$ cells were transplanted (Figure 5B, right). Liver lobules of these mice were engrafted with GFP$^+$ cells, which expressed albumin and CK-18 (Figure 5C). To determine whether GFP$^+$Ter119$^-$CD45$^+$-derived hepatocytes participate actively in the normal liver regeneration process, three chimeric mice underwent partial hepatectomy followed by a BrdU incorporation assay (Figure 5A). The quantitative analysis by flow cytometry showed that 13.0 ± 1.2% liver cells were donor-derived, and almost 85% of them incorporated BrdU (Figure 5D, right). These results were supported by immunohistochemical analysis, which indicated that GFP$^+$ hepatocytes proliferate in response to partial hepatectomy. Like other host cells (non-GFP), the donor-derived cells incorporated BrdU in the nuclei (Figure 5E). Mice transplanted with E10.5 GFP$^+$Ter119$^-$CD45$^+$ did not show donor-derived CK-19$^+$ cholangiocytes (data not shown), suggesting that these cells had the potential to differentiate into hepatocytes but not biliary epithelial cells.

After confirming that E10.5 GFP$^+$Ter119$^-$CD45$^+$ cells were able to differentiate into hepatocytes during liver development, we analyzed mice that received GFP$^+$Ter119$^-$CD45$^-$ cells at the same stage of developmental. The results of these experiments were quite surprising. Donor cells were engrafted (3.25 ± 2.05%) in the BM (Figure 6A, lower) at significantly ($P < 0.01$) lower levels than GFP$^+$Ter119$^-$CD45$^+$ cell engraftment (Figure 5B). In this case GFP$^+$ cells also engrafted in the liver lobules, but did not express albumin (Figure 6B). GFP$^+$ cells were located on the lining of intrahepatic bile ducts and expressed CK-19, hence making bile ductular cells (Figure 6B, lower panel). The donor-derived cells did not respond to partial hepatectomy, as BrdU was not incor-

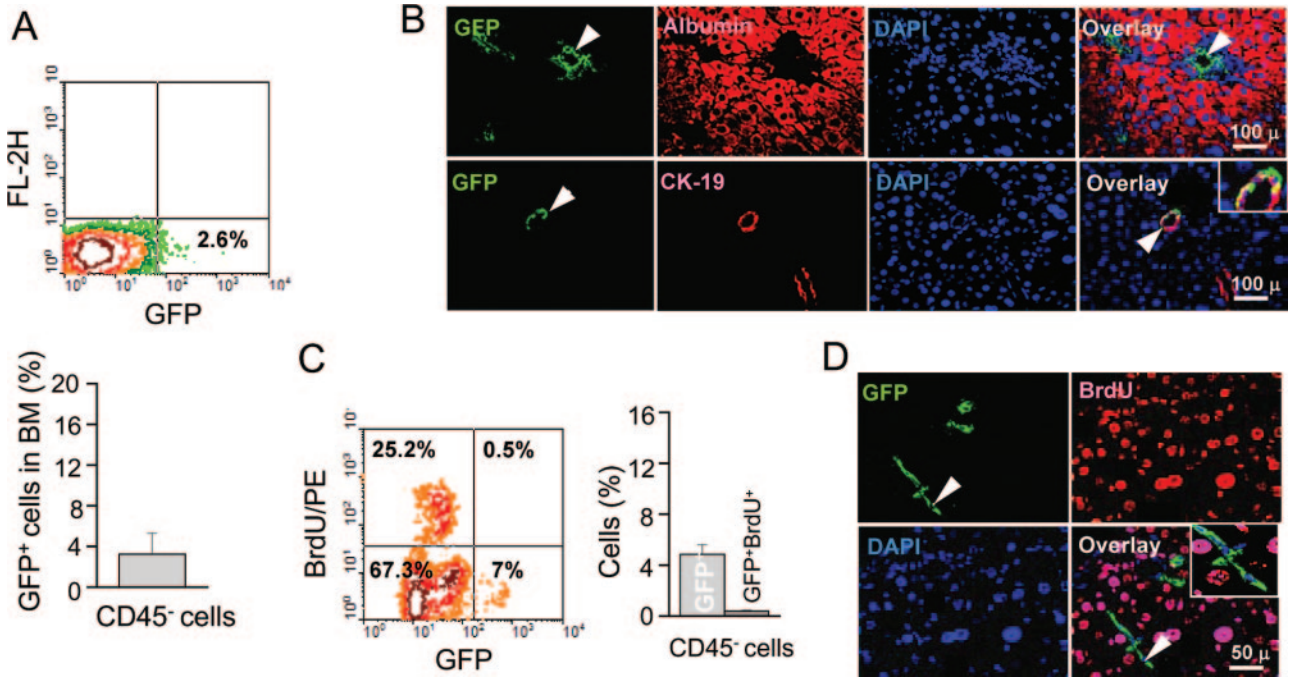

Figure 6. *In utero* transplantation of E10.5 Ter119$^-$CD45$^-$ FLCs. **A:** Flow cytometric analysis of GFP$^+$ cells in BM. Bar diagram (bottom) shows that the presence of CD45$^-$ cells in BM was significantly lower than that of CD45$^+$ cells. A representative dot plot to determine the presence of donor cells is shown (top) (Number of mice = 3). **B:** The engrafted GFP$^+$ cells in the liver lobules did not express albumin (**arrows, upper panel**), but expressed CK-19 (**arrows, lower panel**; **inset** ×600) in the bile ductular region (Number of mice = 3; magnification = original ×200; scale bar = 100 µm). **C:** Flow cytometric analysis of BrdU incorporated cells. Bar diagram (**right**) shows very few GFP$^+$ cells incorporated BrdU in the nuclei. A representative dot plot shows BrdU incorporation in the test (GFP$^+$ cells transplanted) mice (**left**) (Number of mice = 3). **D:** Immunohistochemical analysis of BrdU incorporated cells (**arrows, upper panel**). GFP-expressing bile ductular cells did not incorporate BrdU in the nuclei (**inset** ×400) and remain blue due to 4,6-diamidino-2-phenylindole (DAPI) (Number of mice = 3; magnification = original ×400; scale bar = 50 µm).

porated by them (Figure 6, C and D). The results of these *in utero* transplantation experiments suggest that E10.5 hematopoietic cells have the potential for hepatocytic differentiation (other than usual hematopoietic reconstitution), whereas non-hematopoietic fetal liver cells of same stage give rise to cholangiocytes.

Discussion

In mammals, stem cells possess highly diverse potential for their development. It was felt that repression and depression of gene expression were primarily responsible for developmental plasticity in stem cells.[28] Blau et al[29] suggested that differentiation is an actively maintained state of a cell, which implies that no restriction is imposed to the cells undergoing differentiation. In the recent past, new hypotheses on cellular plasticity have emerged on the basis of theories of genomic completeness, cellular uncertainly, and stochasticity of cell fate.[30] The molecular basis of stochasticity model suggests that each cell has the inherent capacity of gene expression, which is governed by exposure of its euchromatic genes to the transcription factor in nucleoplasm. Irrespective of the prevailing mechanism of stem cells plasticity, our study showed that hematopoietic cells from early stage liver development can engraft into congenic liver during development and differentiate into functional hepatocytes.

The origin of hepatocytes remains elusive. The classical explants culture suggested that they were derived from endodermal tissue.[31] Later, it was shown that bipotential cells of non-hematopoietic origin, the hepatoblasts, give rise to both hepatocytes and biliary epithelial cells in mammals.[9,11,32] Most of the reports related to the origin of hepatocytes have been on the basis of experiments in which the tissues were not examined at the beginning of the liver development.[31,33] Again, hepatoblastic cells at E14.5 were shown to express Dlk-1,[25] but no comparative analysis was available for its expression during early stages of liver development. The migration of hematopoietic cells from aorta-gonad-mesonephros to liver bud and the formation of hepatoblasts are believed to occur in parallel during early stages of liver development.[5,6] Thus, it is crucial to investigate different cell lineages at the beginning of the development of liver.

Our study suggests that though Dlk-1$^+$ hepatoblasts at E14.5 were CD45$^-$, they originate from CD45$^+$ cells of E10.5. An earlier report showed the expression of Dlk-1 in liver bud by whole-mount *in situ* hybridization technique.[25] Dlk-1 protein may be expressed in early fetal liver cells, but the above study did not ensure that its expression was limited to non-hematopoietic cells. We observed that Dlk-1 was first expressed in CD45$^+$ cells, later restricted to CD45$^-$ cells, as shown by others.[25] Two possible explanations can be proposed to clarify our results: (i) Dlk-1 is originated in a fraction of CD45$^+$ cells, later hematopoietic genes are completely down-regulated in these cells for commitment to hepatocytes, and (ii) in addition to (i), Dlk-1 is also expressed in CD45$^-$ cells, in the later stage of the development.

Earlier investigations demonstrated that the OSM/OSMRβ signaling pathway is crucial in development of fetal liver.[19,34] It was shown that OSMRβ is expressed on the adhered fetal liver cells (presumably on fetal hepatocytes) at E14 and its ligand OSM is secreted by the hematopoietic cells.[19,34] The present report indicates the possibility of expression of this receptor and ligand in two different subsets on E10.5 CD45$^+$ FLCs. At later timepoints during development, the expression of OSMRβ shifted to non-hematopoietic lineage of FLCs. Since Dlk-1 was also expressed in E10.5 Ter119$^-$CD45$^+$ cells, we presumed that they were related to OSMRβ expressing cells.

Our results strongly suggest that a subpopulation of hematopoietic cells at E10.5 fetal liver possessed hepatic differentiation potential. We observed that during culture the hematopoietic activity was declined in the Dlk-1$^+$ fraction of cells when they committed to hepatocytes. Similarly, the hepatic activity was reduced in the CD45 fraction, as they committed to hematopoietic cells. The contribution of hematopoietic cells in the formation of hepatocytes was further supported by coculture experiments. The clonal analysis suggested functional heterogeneity in E10.5 CD45$^+$ cells, as all of them did not produce either hepatic or hematopoietic colonies.

Finally, the conclusive evidence on the hepatic differentiation potential of hematopoietic cells came from the results of *in utero* experiments. It is known from the earlier studies that bipotential hepatoblasts, appearing in the liver bud at early stage of development, are endodermally (CD45$^-$) originated. Hence, we presumed that a fraction of GFP$^+$Ter119$^-$CD45$^-$ cells isolated from E10.5 liver were hepatoblastic in nature, which could differentiate into hepatocytes and biliary epithelial cells in developing liver. Paradoxically, the engrafted GFP$^+$ cells were identified in the lining of intrahepatic bile ducts, which expressed CK-19. They did not express any hepatic marker or respond to partial hepatectomy. A few GFP$^+$Ter119$^-$CD45$^-$ cells were also detected in the BM, though it was not known whether they participated in the formation of blood cells. The reason that GFP$^+$Ter119$^-$CD45$^-$ cells did not differentiate into hepa-

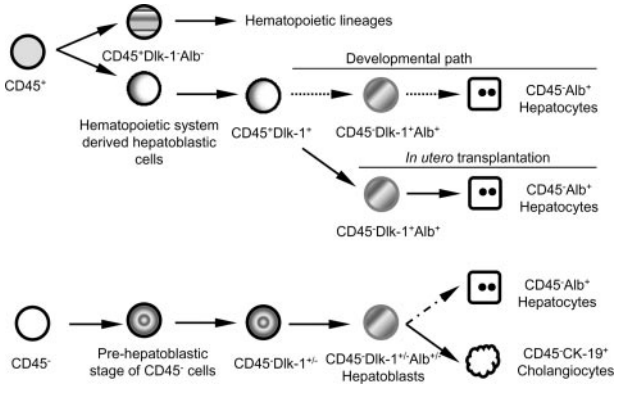

Figure 7. Summarizing cellular contribution of E10.5 FLCs. Dlk-1$^+$ cells originate from E10.5 Ter119$^-$CD45$^+$ cells, which later mature as hepatocytes when transplanted *in utero*. These cells might be involved in the developmental path (hidden **arrow**) for matured hepatocytes. The CK-19 expressing cholangiocytes are derived from CD45$^-$ cells. According to the present belief, hepatocytes originate from CD45$^-$Dlk-1$^+$Alb$^-$ cells (hidden **arrow**), though our results do not explain that.

tocytes was not clear. We concluded that either they are not bipotential or their hepatic differentiation potential was lost. Most interestingly, E10.5 $GFP^+Ter119^-CD45^+$ cells not only differentiated into albumin and CK-18 expressing cells in the liver, they responded to partial hepatectomy like normal hepatocytes. However, they did not differentiate into biliary epithelial cells.

The origin of ancestor cells of any lineage could be better analyzed by lineage tracking approach using Cre/loxP technology, as described in the hematopoietic system.[35] Using the same system, it was shown that hematopoietic cells contribute to hepatocytes in unperturbed mice.[31] In bulk as well as clonal culture, we showed that a fraction of E10.5 hematopoietic cells are capable of differentiating into hepatocytes. Earlier report[31] has proposed a modest role of hematopoietic plasticity in maintenance and regeneration of adult liver. Whether the above analysis is legitimate in systems other than hematopoietic (eg, liver) has been questioned.[35,36] This is due to inactivation of *vav* promoter-based expression system in non-hematopoietic cells, like hepatocytes.[35] Overall, we propose that a subpopulation of E10.5 fetal liver hematopoietic cells serves as progenitor of hepatocytes. These cells might be involved in the development of hepatocytes, confirmation of which warrants further investigation. An emerging model for the development of hepatocytes in mouse is shown in Figure 7.

Acknowledgments

Authors are indebted to The Jackson Laboratories for providing the GFP-expressing mice for this investigation.

References

1. Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS: Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev 1996, 10:1670–1682
2. Zaret KS: Liver specification and early morphogenesis. Mech Dev 2000, 92:83–88
3. Shiojiri N: The origin of intrahepatic bile duct cells in the mouse. J Embryol Exp Morphol 1984, 79:25–39
4. Johnsosn GR, Jones RO: Differentiation of the mammalian hepatic primordium in vitro. I. Morphogenesis and the onset of haematopoiesis. J Embryol Exp Morphol 1973, 30:83–96
5. Bonifer C, Fausto N, Geiger H, Muller AM: Developmental changes in the differentiation capacity of haematopoietic stem cells. Immunol Today 1998, 19:236–238
6. Johnson GR, Moore MAS: Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. Nature 1975, 275:726–728
7. Tanimizu N, Miyajima A: Molecular mechanism of liver development and regeneration. Inter Rev Cytol 2007, 259:1–48
8. Houssaint E: Differentiation of the mouse hepatic primordium. I. An analysis of tissue interactions in hepatocyte differentiation. Cell Diff 1980, 9:269–279
9. Thorgeirsson SS: Hepatic stem cells in liver regeneration. FASEB J 1996, 10:1249–1256
10. Le Douarin NM: An experimental analysis of liver development. Med Biol 1975, 53:427–455
11. Haruna Y, Saito K, Spaulding S, Nalesnik MA, Gerber MA: Identification of bipotential progenitor cells in human liver development. Hepatology 1996, 23:476–481
12. Godin I, Cumano A: The hare and the tortoise: an embryonic haematopoietic race. Nat Rev Immunol 2002, 2:593–604
13. Lipp W: Early development of architecture of the liver parenchyma in guinea pig. Anat-Ges (Jena) 1952, 50:241–249
14. Gilbert SF: The early development of vertebrates- fish, birds and mammals. Developmental Biology. Sunderland, Massachusetts, Sinauer Associates, 1994, pp 234–253
15. Petersen BE, Grossbard B, Hatch H, Pi L, Deng J, Scott EW: Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology 2003, 37:632–640
16. Jang YY, Collector MI, Baylin SB, Diehl AM, Sharkis SJ: Hematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol 2004, 6:532–539
17. Khurana S, Mukhopadhyay A: Characterization of the potential subpopulation of bone marrow cells involved in the repair of injured liver tissue. Stem Cells 2007, 25:1439–1447
18. Almeida-Porada G, Porada CD, Chamberlain J, Torabi A, Zanjani ED: Formation of human hepatocytes by human hematopoietic stem cells in sheep. Blood 2004, 104:2582–2590
19. Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A: Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J 1999, 8:2127–2136
20. Richards MK, Liu F, Iwasaki H, Akashi K, Daniel C: Link Pivotal role of granulocyte colony stimulating factor in the development of progenitors in the common myeloid pathway. Blood 2003, 102:3562–3568
21. Khurana S, Mukhopadhyay A: In vitro transdifferentiation of adult hematopoietic stem cells: an alternative source of engraftable hepatocytes. J Hepatol, DOI: 10.1016/j.jhep. 2008.05.019
22. Carrier E, Lee TH, Busch MP, Cowan MJ: Induction of tolerance in nondefective mice after in utero transplantation of major histocompatibility complex-mismatched fetal hematopoietic stem cells. Blood 1995, 86:4681–4690
23. Higgins GM, Anderson RM: Experimental pathology of the liver. Arch Pathol 1931, 12:186–202
24. Smas CM, Sul HS: Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 1993, 73:725–734
25. Tanimizu N, Nishikawa M, Saito H, Tsujimura T, Miyajima A: Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J Cell Sci 2003, 116:1775–1786
26. Darlington GJ: Molecular mechanisms of liver development and differentiation. Curr Opin Cell Biol 1999, 11:678–682
27. Kanwar YS, Pan X, Lin S, Kumar A, Wada J, Haas CS, Liau G, Lomasney JW: Imprinted mesodermal specific transcript (MEST) and H19 genes in renal development and diabetes. Kidney Int 2003, 63:1658–1670
28. Dennis JE, Charbord P: Origin and differentiation of human and murine stroma. Stem Cells 2002, 20:205–214
29. Blau HM, Chiu CP, Webster C: Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell 1983, 32:1171–1180
30. Theise ND: Implications of 'postmodern biology' for pathology: the cell doctrine. Lab Invest 2006, 86:335–344
31. Stadtfeld M, Graf T: Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. Development 2005, 132:203–213
32. Shiojiri N, Lemire JM, Fausto N: Cell lineages and oval cell progenitors in rat development. Cancer Res 1990, 51:2611–2620
33. Blouin MJ, Lamy I, Noel M, Corlu A, Gugen-guillouzo C, Marceau N: Specialization switch in differentiating embryonic rat liver progenitor cells in response to sodium butyrate. Exp Cell Res 1995, 217:22–30
34. Kinoshita T, Sekiguchi T, Xu M-J, Ito Y, Kamiya A, Tsuji K-I, Nakahata T, Miyajima A: Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. Proc Natl Acad Sci USA 1999, 96:7265–7270
35. Ogilvy S, Metcalf D, Gibson L, Bath ML, Harris AW, Adams JM: Promoter elements of vav drive transgene expression in vivo throughout the hematopoietic compartment. Blood 1999, 94:1855–1863
36. Thowfeequ S, Myatt E-J, Tosh D: Transdifferentiation in developmental biology, disease, and in therapy. Dev Dyn 2007, 236:3207–3217