Liver sinusoidal endothelial cell progenitor cells promote liver regeneration in rats

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The ability of the liver to regenerate is crucial to protect liver function after injury and during chronic disease. Increases in hepatocyte growth factor (HGF) in liver sinusoidal endothelial cells (LSECs) are thought to drive liver regeneration. However, in contrast to endothelial progenitor cells, mature LSECs express little HGF. Therefore, we sought to establish in rats whether liver injury causes BM LSEC progenitor cells to engraft in the liver and provide increased levels of HGF and to examine the relative contribution of resident and BM LSEC progenitors. LSEC label-retaining cells and progenitors were identified in liver and LSEC progenitors in BM. BM LSEC progenitors did not contribute to normal LSEC turnover in the liver. However, after partial hepatectomy, BM LSEC progenitor proliferation and mobilization to the circulation doubled. In the liver, one-quarter of the LSECs were BM derived, and BM LSEC progenitors differentiated into fenestrated LSECs. When irradiated rats underwent partial hepatectomy, liver regeneration was compromised, but infusion of LSEC progenitors rescued the defect. Further analysis revealed that BM LSEC progenitors expressed substantially more HGF and were more proliferative than resident LSEC progenitors after partial hepatectomy. Resident LSEC progenitors within their niche may play a smaller role in recovery from partial hepatectomy than BM LSEC progenitors, but, when infused after injury, these progenitors engrafted and expanded markedly over a 2-month period. In conclusion, LSEC progenitor cells are present in liver and BM, and recruitment of BM LSEC progenitors is necessary for normal liver regeneration.

Introduction
The liver is the only human internal solid organ capable of regeneration after resection. The current literature suggests that normal liver regeneration requires increased expression of hepatocyte growth factor (HGF) by liver sinusoidal endothelial cells (LSECs) (1–3) and LSEC proliferation (4–6); however, mature LSECs, like other endothelial cells, are low in HGF (2, 7), whereas endothelial progenitor cells (EPCs) are both rich in HGF (7) and highly proliferative. This raises the question as to whether the increase in HGF indeed occurs in mature LSECs or whether after injury there is an influx of LSEC progenitor cells (SPCs) that proliferate and provide the increase in HGF. The LSEC is unique in its morphology and function (8). One difference from other endothelial cells is that more than 80% of LSECs, i.e., LSECs in the periportal and midlobular regions of the liver, express CD45 on the cell surface (8, 9) and by gene expression (8). CD45 expression by LSECs indicates that they are not derived from the CD45+ EPC that has been described as the progenitor to other endothelial cells.

In addition to BM-derived EPCs, recent studies have suggested the existence of resident EPCs in the vasculature. This evidence, however, is predominantly based on in vitro cell culture assays and phenotypic marker studies (10, 11). Important questions have been raised about what role the resident EPC would play in turnover of endothelial cells, whether it is transiently present, whether it derives from a BM stem cell, and whether a resident stem cell can be identified (10). Given the availability of methods to isolate LSECs with very high purity and in large numbers, the liver is an ideal organ to examine the existence of resident label-retaining cells (LRCs) and resident SPCs.

This study has identified 2 distinct populations of LSEC progenitors: LSEC LRCs and SPCs that reside within the liver and SPCs present in the BM. The study examines the BM progenitor cell response to partial hepatectomy (PHx), persistence of SPC engraftment, and magnitude of SPC expansion in the liver as well as the requirement for SPC recruitment in liver regeneration and the relative contribution of resident and BM SPCs to repair of liver injury.

Results
Identification of resident LSEC LRCs and progenitors. The LSEC expresses both CD31 and CD45 (8, 9). CD133 is a progenitor cell marker, and infusion of CD133+ cells from the BM repopulates the liver endothelium (8). We therefore used CD133+CD45+CD31+ triple positivity as the phenotypic signature for SPCs. To identify resident SPCs, a nonparenchymal cell population was subjected to iodixanol density gradient centrifugation (12, 13), and elutriation fractions were examined for CD133+ cells. CD133+ cells were only present in the elutriation fraction that contains LSECs, indicating that CD133+ LSECs are the same size as mature LSECs. 90.9% ± 2.3% of CD133+ LSECs were CD45+ and 93.1% ± 6.0% of CD133+ LSECs were CD31+ (Figure 1, A–C); the percentages were similar in the CD133-depleted fraction of LSECs, 91.8% ± 7.7% and 90.7% ± 1.7%, respectively (n = 3). The resident CD133+CD45+CD31+ SPCs isolated by this method showed the hallmark ultrastructural features of LSECs, i.e., fenestrae organized in sieve plates, as identified by scanning EM (Figure 1D). Thus, these CD133+ cells were resident SPCs, with ultrastructural features that identify them as LSECs. One hundred percent of resident SPCs stained positive for the endothelial markers VEGF-receptor 1 and VEGF-receptor 1 (Figure 2). The pattern of VEGF receptor staining was identical to the staining pattern of mature LSECs, periportal LSECs (CD45bright LSECs;
ref. 14), and centrilobular LSECs (CD45− LSECs; ref. 14 and Figure 2). The frequency of resident SPCs varied across strains of rats: 1.1% ± 0.1% of LSECs in Sprague-Dawley rats were CD133+, 1.5% ± 0.1% of LSECs in Lewis rats were CD133+, and 7.0% ± 0.3% of LSECs in Fischer rats were CD133+ (n = 3). As described below, infusion of resident SPCs during liver injury leads to persistent engraftment and expansion as LSECs.

To determine whether there was a population of label-retaining LSECs within the liver, BrdU was injected at birth, and cells were isolated at 2 months after birth (n = 3). As expected, none of the BrdU+ cells were present in the CD133− fraction. The BrdU+ cells obtained by immunomagnetic selection for CD133 from the LSEC elutriation fraction were CD133+CD45+CD31+ (Figure 3), indicating that the BrdU+ cells obtained by this method were label-
retaining LSEC progenitors. 1.1% of LSECs in this rat strain were CD133+, and 9.1% ± 2.3% of the CD133+ cells were BrdU+, so that 0.1% of the LSEC population was resident LSEC LRCs.

**BM SPCs and normal LSEC turnover.** To investigate the role of BM SPCs in normal LSEC turnover, gender-mismatched BM transplantation (male into female) was performed, and LSECs were isolated 9 months later. LSECs derived from the BM were identified in recipient rats by FISH detection of the Y chromosome (8). Only 0.8% ± 0.1% of LSECs and 2.7% ± 0.2% of resident SPCs were of BM origin at 9 months, demonstrating that BM SPCs play an insignificant role in normal LSEC turnover and therefore do not contribute significantly in unjured liver to the population of resident SPCs in adult rats.

**Peak of LSEC proliferation.** To investigate the time course of LSEC proliferation during liver regeneration, LSECs were isolated after PHx and immunostained for proliferating cell nuclear antigen (PCNA) (Figure 4A). Fewer than 2% of LSECs were PCNA+ on day 3 after PHx in Lewis rats transplanted with BM from male Lew-Tg(CAG-EGFP)ys Lewis rats; in contrast, 2.7% ± 0.2% of resident SPCs were PCNA+ (Figure 5C) cells along the sinusoid demonstrates engraftment of BM SPCs as endothelial cells.

**Differentiation of BM SPCs to LSECs.** Wild-type Lewis rats transplanted with Lew-Tg(CAG-EGFP)ys BM were used to determine whether BM SPCs that engraft in the liver differentiated into LSECs. LSECs were isolated 2 weeks after PHx, and the GFP+ fraction (i.e., BM-derived cells) was cultured overnight. Scanning EM showed that GFP+ LSECs had developed normal fenestrae grouped into sieve plates (Figure 4H), the hallmark feature of differentiated LSECs. In contrast, SPCs in the BM were not fenestrated (data not shown).

**Persistence of engraftment and expansion.** To determine whether engrafted SPCs persist in the liver, resident SPCs were isolated from GFP+ transgenic rats, and 1 × 10⁶ resident SPCs were infused into the tail veins of wild-type Lewis rats 3 days after PHx (n = 3). Two months later, LSECs were isolated and examined by FACS for GFP expression. 54.2% ± 5.1% of LSECs and 44.4% ± 3.0% of resident SPCs were GFP+, demonstrating persistent engraftment of infused resident SPCs (Figure 6). 1 × 10⁶ GFP+ resident SPCs were infused, and the total number of GFP+ LSECs isolated after 2 months averaged 34.3 × 10⁶ ± 5.1 × 10⁶, so that there was at least a 34-fold expansion of infused SPCs to LSECs.

**Impairment of liver regeneration by BM suppression and effect of SPC infusion.** To examine whether recruitment of BM SPCs is necessary for normal liver regeneration, PHx was performed in BM-suppressed rats. Rats underwent selective hind limb irradiation, which suppressed the peripheral leukocyte count by nearly 40% after 1 week. One week after irradiation, rats underwent PHx. Rats received tail vein infusions of either saline (“no infusion”), 1 × 10⁶ resident SPCs, or 50 × 10⁶ BM cells on day 1 after PHx and were sacrificed on day 5 (Figure 7A). One group of rats underwent PHx without prior irradiation (“PHx only”). Five days after PHx, liver weight of the PHx-only group reached 86% of that of control littermates (Figure 7B). The amount of liver weight regained in the BM-suppressed rats infused with saline (no infusion) was 40% less than that in PHx-only rats (Figure 7B).

Infusion of either resident SPCs or BM cells 1 day after PHx completely reversed the impairment of liver regeneration caused by BM suppression (Figure 7B), whereas infusion on 3 days...
after PHx did not significantly improve liver regeneration (data not shown). Hepatocyte proliferation was examined by PCNA immunostaining in LSECs isolated from sham-operated rats on days 1–4 after PHx. n = 3; ***P < 0.001 compared with sham control. (B) Proliferation of BM SPCs. FACs analysis of PCNA+ BM SPCs on PHx day 3 and sham controls. **P < 0.005 compared with sham surgery control. (C) Mobilization. BM SPCs in peripheral blood (PB) were counted on PHx day 3. n = 4; *P < 0.05 compared with sham surgery control. (D–F) Engraftment of BM SPCs. LSECs were isolated on PHx day 3 from rats that had undergone gender-mismatched (male into female) BM transplantation and subsequent PHx. BM-derived LSECs were identified by FISH staining for Y chromosome (arrows) in (D) sham surgery control and (E) after PHx. Original magnification, ×100. (F) Engraftment data from 3 individual experiments. ***P < 0.001 compared with sham surgery control. (G) Engraftment of BM SPCs. Wild-type rats were transplanted with BM from Lew-Tg(CAG-EGFP)ys rats. Isolation of LSECs on PHx day 3, followed by FACs for GFP, demonstrated that 24.6% ± 3.1% of LSECs were GFP+ and therefore BM derived (n = 3). (H) Differentiation of BM SPCs to LSECs. Wild-type rats received BM transplantation from Lew-Tg(CAG-EGFP)ys rats. BM-derived LSECs (GFP+) were isolated 2 weeks after PHx and examined by scanning EM for fenestration. The black circle demonstrates characteristic fenestrae. Original magnification, ×5,000; scale bar: 5 μm. Note that SPCs isolated from BM were not fenestrated (data not shown).

Discussion

This study examines the contribution of 2 populations of SPCs to repopulation of LSECs after liver injury and their respective roles in liver regeneration. PHx led to a robust BM SPC progenitor cell response, defined here as proliferation of SPCs in the BM, mobilization of BM SPCs to the circulation, engraftment of SPCs in the liver, and differentiation to fenestrated LSECs. Three days after PHx, 25% of LSECs were BM derived. After 2 weeks, the originally nonfenestrated, BM-derived SPCs had developed fenestrated grouped into sieve plates, the hallmark of differentiated LSECs.

Previous studies suggested that LSEC HGF expression increases after liver injury and that this increased HGF expression in LSECs promotes liver regeneration (1, 2). This study refines the concept by demonstrating that most of the increase in HGF does not come from the LSECs but is derived from an influx of HGF-rich BM SPCs that engraft after liver injury and that are present in the isolated LSEC fraction. This influx of BM SPCs promotes hepatocyte proliferation. Of note, hepatic stellate cells are a rich source of HGF, and contamination by these
cells could confound the results. The method used to isolate LSECs, however, provides a population that is 99% pure LSEC, as determined by uptake of formaldehyde-treated serum albumin. Thus, SPCs isolated from the LSEC fraction were not contaminated by stellate cells, and contamination cannot account for the high HGF expression.

BM SPCs were necessary for normal liver regeneration: BM suppression impaired liver regeneration, whereas infusion of SPCs promoted hepatocyte proliferation and restored normal liver regeneration. There was no difference in the effect of SPC infusion compared with BM infusion on hepatocyte proliferation and liver regeneration, indicating that SPCs are sufficient to account for the whole benefit of BM infusion. Although the infusion of SPCs on day 1 after PHx restored normal liver regeneration, infusion of SPCs on day 3 did not. This temporal divergence in the effect of SPC infusion is pertinent, because, in the rat PHx model, the paracrine effect of HGF released by nonfenestral cells is thought to stimulate the second round of proliferation of hepatocytes, which occurs on day 2 in the rat (15). Furthermore, over 40% of intrahepatic SPCs are still proliferating on day 3 after PHx, and this likely reflects the need to produce additional LSECs to line the microcirculation of the regenerating liver.

The current study has identified and examined resident LSEC LRCs and progenitor cells. The label-retaining assay identifies putative stem cells (16–20), but demonstration that an LRC is a functional stem cell requires evidence of self-renewal, lineage-specific differentiation, and serial repopulation. LSEC LRCs in the liver are relatively numerous: 1 in 1,000 LSECs are resident LRCs. By comparison, slightly fewer than 1 in 10,000 BM mononuclear cells are stem cells (21). The resident SPC is identified by the presence of CD133+, a classic progenitor cell marker, but it also has fenestrae organized in sieve plates, which shows that it is differentiating into an LSEC. On day 3 after PHx, 57% of all SPCs within the liver were resident progenitor cells, but the BM-derived SPCs are the major source of HGF and have much higher proliferation rates. Thus, although our studies show that resident SPCs that have been isolated and infused into the liver after PHx can promote hepatocyte proliferation and liver regeneration, resident SPCs in their niche play a less important role in liver regeneration than BM SPCs.

The current study has 2 clinical implications. First, the observation that BM suppression impairs liver regeneration is an unexpected finding that may shed light on worsening liver disease and acute liver failure in BM-suppressed patients. Lack of compensatory liver regeneration may contribute to the 12% incidence of acute hepatic failure and death in patients with hepatitis B after hematopoietic cell transplantation (22) and to the high case-fatality rate of sinusoidal obstruction syndrome after myeloablative conditioning for hematopoietic cell transplantation (23). The finding that infusion of SPCs can overcome the effects of BM suppression provides new avenues for potential therapies for liver disease in BM-suppressed patients. Second, liver regeneration is impaired in chronic liver disease. The finding that recruitment of SPCs is necessary for liver regeneration raises questions about SPC recruitment in chronic liver disease.

In summary, this study has identified 2 sources of LSEC progenitors: within the liver, there are resident LSEC LRCs and fenestrated SPCs and, within the BM, there are nonfenestrated SPCs that substantially repopulate the sinusoids after liver injury and differentiate into fenestrated LSECs. The BM-derived SPC does not contribute significantly to normal turnover of LSECs or resident SPCs in adult rats, but BM SPCs are the major contributor to recovery from liver injury. Perhaps more importantly, the influx of BM SPCs provides most of the increase in LSEC HGF after injury and promotes hepatocyte proliferation and normal liver regeneration. When present in their niche, resident LSEC LRCs and SPCs play a lesser role in LSEC repopulation after liver injury than BM SPCs; however, when resident SPCs are isolated from the liver and infused after PHx, there is marked expansion of the SPCs with persistent engraftment as LSECs and SPCs.

Figure 5
Engraftment of BM SPCs in the liver. Liver from wild-type rats, transplanted with BM from Lew-Tg(CAG-EGFP)ys rats, on day 5 after PHx. (A) GFP+ BM cells engraft along the sinusoid. (B) CD31 staining indicates LSECs. (C) The merged image of A and B demonstrates that GFP+ BM cells engraft as LSECs. Original magnification, ×100.

Figure 6
Persistence of engraftment and expansion. Resident SPCs were isolated from GFP+ transgenic rats, and 1 × 10⁶ resident SPCs were infused into the tail veins of wild-type Lewis rats 3 days after PHx (n = 3). Two months later, LSECs and resident SPCs were isolated and examined by FACS for GFP expression (shown on the x axis). (A) The gated area shows 53.2% of LSECs are GFP+, i.e., BM derived. (B) The gated area shows 47.7% of resident SPCs are GFP+, i.e., BM derived. SSC-H, side scatter height; FL1-H, fluorescence intensity.
Methods

**Materials.** Chemicals were obtained from Sigma-Aldrich unless stated otherwise. The following antibodies were used: mouse anti-CD31 (Abcam); TRITC-conjugated anti-PCNA, rabbit anti-CD31, goat anti-Flt1, rabbit anti-FKlb, mouse anti-rat HGF, TRITC-conjugated donkey anti-rabbit IgG, and FITC-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology Inc.); FITC-conjugated mouse anti-CD45, mouse anti-CD45, Alexa Fluor 488-conjugated mouse anti-rat BrdU, and PE-conjugated goat anti-mouse IgG (BD Biosciences); and Alexa Fluor 405–conjugated goat anti-mouse IgG and Alexa Fluor 488–conjugated goat anti-rabbit IgG (Invitrogen). Microbeads from the following kits were used: CD133 Cell Isolation Kit and Anti-FITC MultiSort Kit (Miltenyi Biotec).

**Animals.** Sprague-Dawley, Lewis, and Fischer rats were obtained from Harlan Corp. Breeding pairs of Lew-Tg(CAG-EGFP)ys Lewis rats were obtained from the NIH Rat Resource and Research Center, University of Missouri.

**PHx.** PHx was performed under general anesthesia. 70% of liver was resected. Sham surgery consisted of laparotomy and gentle mobilization of the liver. This study followed the guidelines outlined in the “Guide for the Care and Use of Laboratory Animals,” prepared by the National Academy of Sciences and published by the NIH (NIH publication no. 86-23, revised 1985).

**LSEC isolation.** LSECs were isolated by collagenase perfusion, iodixanol density gradient centrifugation, and centrifugal elutriation as previously described (12, 13). Yields averaged 84 million cells per normal rat liver, with more than 95% viability. Purity of these cells is 99%, as determined from the NIH Rat Resource and Research Center, University of Missouri.

**SPC isolation.** BM and peripheral blood SPCs were isolated by immunomagnetic selection for CD133 and CD45, followed by FACS sorting for CD31 cells for studies that required PCNA staining or CD133 immunomagnetic selection, followed by FACS sorting for CD45 and CD31 for other experiments. For double-label immunomagnetic selection, BM and peripheral blood mononuclear cells were incubated with anti-CD45 FITC antibody (1:10 dilution, 30 minutes at 4°C) and incubated with anti-FITC microbeads (20 μl beads for up to 107 cells) for 30 minutes at 4°C. After magnetic selection using the autoMACS Pro (Miltenyi Biotec), release reagent was used to clip off the magnetic bead. CD45+ cells were incubated with anti-CD133 microbeads (100 μl beads for up to 106 cells) for 30 minutes at 4°C.

To obtain resident SPCs, a nonparenchymal fraction was obtained by collagenase perfusion and iodixanol density gradient centrifugation, as per LSEC isolation protocol (12). Elutriation fractions from the first elutriation step were examined for CD133+ cells. Virtually all CD133+ cells were recovered from the same elutriation fraction as LSEC during the first elutriation step, and CD133+ cells isolated by this method were confirmed to be LSEC progenitors (see Results). Thus, resident SPCs were obtained by isolation of LSECs and immunomagnetic selection of the CD133 fraction.

**Immunostaining.** Frozen sections of liver tissue were fixed with acetone, and coverslips with LSECs were fixed with 4% paraformaldehyde. Slides were incubated with anti-PCNA, anti–VEGF receptor 1, anti-VEGF receptor 2, anti-CD31, anti-CD45, or anti-BrdU antibody (1:100 dilution) at 37°C for 1 hour and examined by confocal microscopy (Zeiss LSM 510, Carl Zeiss Microimaging Inc.).

**Label-retaining assay.** Day 3 newborn pups (Sprague-Dawley rats) were injected with 7 doses of 50 μg/g BrdU s.c. in saline, given twice daily over 3.5 days. After 60 days, LSECs were isolated; immunomagnetically selected for CD133; immunostained for CD31, CD45, and BrdU; and examined by confocal microscopy.

**Flow cytometry.** 0.5 × 106 LSECs were incubated with PE-conjugated anti-CD31 antibody (1:100 dilution) or TRITC-conjugated anti-PCNA antibody (1:100 dilution) at 4°C for 30 minutes and examined using a FACS Calibur (BD Biosciences). Data were analyzed using Cell Quest Pro software.

**Figure 7**

Infusion of SPCs restored normal liver regeneration in BM-suppressed rats. (A) Cartoon of the experimental design. (B) Liver weight was examined in control rats and in rats 5 days after PHx (day 12) in the following groups: nonirradiated rats (PHx only), irradiated rats without cell infusion (no infusion), irradiated rats rescued with infusion of SPCs (“SPC infusion”), and irradiated rats rescued with BM cell infusion (“BM infusion”) (n = 4). The horizontal line at 2.8 grams indicates the estimated liver weight immediately after two-thirds PHx. (C) Hepatocyte proliferation on day 5 after PHx was determined by PCNA immunostaining (n = 3). ***P < 0.001 compared with no infusion group.

**Figure 8**

Role of BM-derived SPCs in liver regeneration. LSECs were isolated on day 3 after PHx from wild-type rats that had been transplanted with BM from Lew-Tg(CAG-EGFP)ys rats. (A) Hgf mRNA expression was measured by real-time PCR in GFP+ (BM-derived) and GFP− LSECs. n = 3; ***P < 0.001. (B) HGF protein expression was determined by immunoblot and quantified by densitometry in control LSECs (isolated from nonoperated rats) and in GFP+ and GFP− LSECs isolated on day 3 after PHx (n = 3). ***P < 0.05. **P < 0.01 compared with GFP+ LSECs after PHx. (D) CD133+ cells from the LSEC fraction were immunostained for PCNA and sorted by FACS for GFP. n = 3; ***P < 0.0001 comparison of the percentage PCNA+ cells in the GFP+ and GFP− fractions.
**FISH.** Freshly isolated LSECs were applied to slides by cytospin preparation at 30 g for 5 minutes. FISH was performed as previously published (8). Y chromosome was detected with FITC-labeled anti-digoxigenin (Boehringer Mannheim, Roche) and anti-fade mounting medium (Dako Corporation) containing 100 ng/ml propidium iodide (Molecular Probes). Cells were considered positive if a red nucleus with a single green dot was seen using a Zeiss LSM 510 confocal microscope. The presence of Y chromosome was determined in 20 cells per field in 15 random fields. The Y chromosome is only visualized when it is in the plane of visualization. To correct for non-visualization of Y chromosome, LSECs were isolated from a control male rat, and the percentage of Y chromosome-positive cells was determined.

**Irradiation.** The lateral pelvis and hind limbs, with exclusion of the paws, were irradiated with 800 cGy to 2-cm depth using the 6 MeV X-ray beam of a Varian 2300 c/d linear accelerator, located at the University of Southern California Norris Cancer Research Institute.

**BM transplantation.** Cells were obtained from the BM of one tibia and femur from the donor. Recipients underwent 1,000 cGy total body irradiation using a Zeiss LSM 510 confocal microscope. The presence of Y chromosome was determined in 20 cells per field in 15 random fields. The Y chromosome is only visualized when it is in the plane of visualization. To correct for non-visualization of Y chromosome, LSECs were isolated from a control male rat, and the percentage of Y chromosome-positive cells was determined.

**Statistics.** Numerical data represent mean ± SEM from at least 3 separate experiments. Values were compared by 2-factor ANOVA with replication or t test, using Microsoft Excel (Microsoft) Analysis Toolpak. If ANOVA was significant, a posteriori comparison of individual group means was performed by the Bonferroni (Tukey) multiple comparison test. For comparison of proportions, a chi-square test was used.

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