**Hepatic stellate and endothelial cells maintain hematopoietic stem cells in the developing liver**

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The liver maintains hematopoietic stem cells (HSCs) during development. However, it is not clear what cells are the components of the developing liver niche in vivo. Here, we genetically dissected the developing liver niche by systematically determining the cellular source of a key HSC niche factor, stem cell factor (SCF). Most HSCs were closely associated with sinusoidal vasculature. Using ScfGFP knockin mice, we found that Scf was primarily expressed by endothelial and perisinusoidal hepatic stellate cells. Conditional deletion of Scf from hepatocytes, hematopoietic cells, Ng2+ cells, or endothelial cells did not affect HSC number or function. Deletion of Scf from hepatic stellate cells depleted HSCs. Nearly all HSCs were lost when Scf was deleted from both endothelial and hepatic stellate cells. The expression of several niche factors was down-regulated in stellate cells around birth, when HSCs egress the developing liver. Thus, hepatic stellate and endothelial cells create perisinusoidal vascular HSC niche in the developing liver by producing SCF.

**Introduction**

Hematopoietic stem cells (HSCs) rely on niche for their maintenance. In adults, HSCs are sustained by bone marrow perivascular niche composed of multiple cellular components, including endothelial cells (Ding et al., 2012), and mesenchymal stromal cells (Ding and Morrison, 2013; Ding et al., 2012; Greenbaum et al., 2013; Sugiyama et al., 2006), along with other cell types (Morrison and Scadden, 2014). But the bone marrow is not the only organ that supports HSCs. During development, the liver is the major hematopoietic organ in which HSCs expand and mature (Mikkola and Orkin, 2006). It is only after birth that HSCs complete their migration to the bone marrow and reside there throughout adult life (Lee et al., 2017).

In contrast to the adult bone marrow niche, the cellular components of the developing liver niche remain largely undefined in vivo. Stromal cell lines derived from murine fetal liver and fibroblasts derived from human fetal liver can support hematopoiesis in vitro (Hackney et al., 2002; Moore et al., 1997; Tsai et al., 1986). Cells undergoing epithelial-to-mesenchymal transition can also maintain hematopoietic cells in vitro (Chagraoui et al., 2003; Zhang et al., 2005). But the in vivo identities of these cells are not clear. DLK+ hepatic progenitors have been shown to express key HSC niche factors and to possess the ability to support HSCs in vitro (Chou and Lodish, 2010). In addition, fetal liver Lyve+ endothelial cells can maintain HSCs short-term in vitro (Iwasaki et al., 2010). However, it is not clear whether any of these cells maintain HSCs in vivo. Recently, Nestin+NG2+ perportal stromal cells in the fetal liver have been suggested to be an important component of the niche (Khan et al., 2016), as genetic ablation of NG2+ cells in a Ng2-cre/iDTA mouse model leads to an estimated 30% reduction of phenotypic HSCs. However, fetal liver cells from Ng2-cre/iDTA mice have higher reconstituting activity in T and B cell lineages, but not myeloid lineage, compared with littermate controls (Khan et al., 2016), suggesting that functional HSCs are not compromised in these mice. Therefore, there must be other cellular components with critical roles in maintaining HSCs in the developing liver. Overall, it is not clear what cells create a niche for HSCs in the developing liver in vivo.

A niche is defined as the cellular microenvironment that generates necessary cytokines and growth factors for HSC maintenance in vivo. To uncover the HSC niche components in the developing liver, we focused on identification of the cellular sources of key factors required for HSC maintenance in vivo. Stem cell factor (SCF) is one of the very few cytokines known to be genetically required by HSCs. Signaling through the cKIT receptor on HSCs, SCF is essential for HSC maintenance in vivo (Barker, 1994; Ding et al., 2012). Scf transcript is alternatively spliced into a short and a long form, with the exclusion or inclusion of an exon containing a proteolytic cleavage site. This leads to two forms of SCF protein, a membrane-bound form and a...
soluble form with protease-mediated cleavage (Broudy, 1997). The membrane-bound form of SCF is particularly important for HSC maintenance and implies that its engagement with HSCs is within a local niche (Barker, 1997; Wolf, 1978). Scf−/− knockout mice have a profound loss of fetal liver HSCs and are perinatal lethal due to severe hematopoietic defects (Ding et al., 2012; Ikuta and Weissman, 1992). Furthermore, administration of a cKIT functionally blocking antibody during development at times after but not before the liver hematopoietic stages abolishes hematopoietic progenitors, suggesting that the SCF–cKIT pathway is required for HSC maintenance starting from the fetal liver stage (Ogawa et al., 1993). Based on antibody staining, PCR, and a bacterial artificial chromosome transgenic Scf reporter allele with likely incomplete promoter, Scf has been suggested to be expressed by hepatic progenitors (Azzoni et al., 2018; Chou and Lodish, 2010), Nestin ‘NG2’ periportal cells (Khan et al., 2016), and endothelial cells (Azzoni et al., 2018). Yet no study has systematically examined which sources of SCF are functionally important for HSCs in the developing liver in vivo. Here, we used Scffl/fl conditional knockout mice to systemically characterize the expression of Scf and to uncover functionally important cellular sources of SCF for HSC maintenance in the developing liver in vivo. We found that most HSCs localized close to the sinusoidal vasculature, and endothelial and stellate cells are the major functional source of SCF in the perinatal liver. Interestingly, stellate cells down-regulated SCF and several other putative HSC maintenance factors around birth, suggesting that the developing liver is uniquely equipped with HSC-supporting function.

Results

HSCs reside in a perisinusoidal vascular niche in the developing liver

HSCs mature and expand in the fetal liver (Ema and Nakauchi, 2000; Morrison et al., 1995). Around birth, HSCs migrate to and take up residence in the bone marrow (Lee et al., 2017). However, the perinatal time window during which the liver maintains HSCs has not been assessed. We found that liver CD150+CD48−Lin− candidate HSCs were closely associated with Laminin+ sinusoidal endothelial cells throughout the liver (Fig. 1, D–H; and Fig. S1, B–E). Similarly, E15.5 fetal liver HSCs were also closely associated with sinusoidal endothelial cells (Fig. S1, F–M). Compared with random mononuclear cells or Lin−CD48+ mature bone marrow cells, HSCs localized significantly closer to sinusoidal endothelial cells (Fig. 1 I). Similar results were obtained when vascular endothelial (VE)-cadherin was used as an endothelial cell marker (Fig. S1, N–S). These data suggest that HSCs localize within a perisinusoidal vascular niche in the developing liver.

Scf is primarily expressed by endothelial cells and hepatic stellate cells

The close association of HSCs to sinusoidal vasculature prompted us to investigate what cells are the source of a key cytokine, SCF, within the perivascular niche. We systematically analyzed Scf expression pattern in the developing liver using Scffl/fl conditional knockout mice (Ding et al., 2012). Flow-cytometric analyses of enzymatically dissociated P0 Scf+/−/− livers showed that GFP+ cells were rare (~1%) and negative for CD45 and Ter119, markers of hematopoietic cells, indicating that Scf is expressed by non-hematopoietic cells (Fig. 2 A and Fig. S2 A). About 83% of CD45+Ter119+CD31+ endothelial cells were positive for GFP, demonstrating that endothelial cells are a source of SCF (Fig. 2 B).

At P0, ~20% of GFP+ stromal cells were CD31+, suggesting that Scf is also expressed by stromal cells other than endothelial cells in the developing liver (Fig. 2 C). To elucidate the identity of these cells, we sorted CD45+Ter119+CD31−SCf-GFP− cells by flow cytometry and analyzed their gene expression by quantitative RT-PCR (qRT-PCR). Compared with whole liver, hematopoietic, or CD31− endothelial cells, CD45+Ter119+CD31− Scf-GFP− cells expressed significantly higher levels of Desmin, Lrat, Lhx2, and Pdgfra, markers of liver-specific perisinusoidal stellate cells (Mederacke et al., 2013; Tsutsumi et al., 1987; Wandzioch et al., 2004; Wright et al., 2014; Yin et al., 2013; Fig. 2 D), suggesting that these Scf-GFP− cells are hepatic stellate cells. Cxcl2, a chemokine required for HSC maintenance and homing (Ara et al., 2003; Ding and Morrison, 2013; Greenbaum et al., 2013; Sugiyama et al., 2006), was also highly expressed by the non-endothelial CD45+Ter19+CD31− Scf-GFP− cells (Fig. 2 D). It has been reported that fetal liver stellate cells express Cxcl2 (Kubota et al., 2007). Consistently, using Cxcl2DsRed+/− knockin reporter mice (Ding and Morrison, 2013), we found that virtually all (95%) Cxcl2-DsRed−/− cells were Desmin+ in P0 livers (Fig. 2 E–J). CD45+Ter19 Cxcl2-DsRed−/− stromal cells from P0 livers of Cxcl2DsRed+/− mice consistently expressed significantly higher levels of Desmin, Lrat, Lhx2, and Pdgfra compared with whole liver cells (Fig. S2 B). We systematically examined the expression of Cxcl2 in P0 livers and found that Cxcl2 was not expressed by hematopoietic, hepatic, or CD31− endothelial cells (Fig. S2, C and D). Mature hepatic stellate cells have the unique characteristic of storing vitamin A in their cytoplasm.
Indeed, the majority of CD45−Ter119−Cxcl12−DsRed+ stromal cells were vitamin A+ by flow cytometry analysis (Fig. 2 K). Taken together, our data established Cxcl12−DsRed as a specific marker for hepatic stellate cells in the developing liver.

We generated Scfgfp/+;Cxcl12DsRed/+ mice to directly test whether hepatic stellate cells are indeed a cellular source of SCF in the developing liver. By flow cytometry, ∼78% of CD45−Ter119−Cxcl12−DsRed+ stellate cells were Scf-GFP+ (Fig. 2 L). Cxcl12+ stellate cells accounted for the majority (81%) of nonendothelial CD45−Ter119−CD31−Scf-GFP+ cells (Fig. 2 M). Thus, besides endothelial cells, stellate cells are a major source of SCF in the developing liver.

qRT-PCR analyses revealed that endothelial cells and hepatic stellate cells expressed Scf at ~155-fold and ~50-fold the levels found in whole P0 liver cells, respectively (Fig. 2 N). Consistent with our flow cytometry data (Fig. 2 A and Fig. S2 A), hematopoietic cells and hepatocytes expressed very little if any Scf (Fig. 2 N). Ng2+ cells expressed an ∼12-fold higher level of Scf compared with whole liver cells (Fig. 2 N). The short (membrane-bound) and long (with the potential to become soluble) forms of Scf showed similar expression patterns in endothelial cells.
Figure 2. Endothelial cells and hepatic stellate cells are the major sources of SCF in the developing liver. (A) Scf-GFP was expressed by rare CD45-Ter119- nonhematopoietic cells in the P0 liver (n = 7 mice for control, and n = 13 mice for Scfgfp/+). (B) The majority of CD45-Ter119-CD31+ endothelial cells in the P0 liver expressed Scf-GFP (n = 5 mice for control, and n = 12 mice for Scfgfp/+). (C) Scf-GFP was also expressed by CD31- nonendothelial stromal cells (n = 13 mice for Scfgfp/+). CD45-Ter119-Scf-GFP+ cells are shown. (D) qRT-PCR revealed that CD45-Ter119-CD31-Scf-GFP+ cells had significantly higher expression of Desmin, Lrat, Lhx2, Pdgfra, and Cxcl12 compared with P0 whole liver cells [WL], hematopoietic cells, or endothelial cells (n = 5 for whole liver cells, n = 4 for CD45-Ter119-CD31-Scf-GFP+ cells, n = 3 for CD45+ or Ter119+ hematopoietic cells [Hema], and n = 3 for CD45-Ter119-CD31+ endothelial cells [Endo]). (E-I) Cxcl12-DsRed was expressed by Desmin+ cells in the P0 liver. Nuclei were stained with DAPI, in blue. I is an inset of the selected area in H. Roman numerals i–iv indicate individual channels and merged images. (J) Quantification of DsRed+Desmin+ cells and DsRed+Desmin− cells from Cxcl12DsRed/+ P0 liver tissue sections. About 98% of DsRed+ cells were Desmin+. Three representative confocal images from two independent experiments were used for quantification. (K) The majority of P0 liver CD45-Ter119-Cxcl12-DsRed+ stromal cells were vitamin A+ (VitA; n = 5 mice for Cxcl12DsRed/+). (L) The majority of P0 liver CD45-Ter119-Cxcl12-DsRed+ stellate cells expressed Scf-GFP in Scfgfp/+. Cxcl12DsRed+ mice (n = 4 mice for control, and n = 7 mice for Scfgfp/+; Cxcl12DsRed/+).
and stellate cells (Fig. S2 E). Endothelial and stellate cells were also the major source of Scf in E15.5 fetal livers, although at this stage, stellate cells expressed Scf at a higher level than endothelial cells (Fig. S2, F-O). Throughout development, stellate cells gradually mature with up-regulated expression of Desmin and Lrat, and accumulation of vitamin A (Fig. S2, P and Q). Taken together, these data suggest that endothelial cells and hepatic stellate cells are the major sources of SCF in the developing liver.

CD150^CD48^Lin^-^HSCs were closely associated with Desmin^-^perisinusoidal stellate cells (Fig. S3, A-F). This raised the possibility that HSCs reside in a perisinusoidal vascular niche composed of endothelial cells and hepatic stellate cells in the developing liver.

**HSCs do not require SCF from hematopoietic cells in the developing liver**

To test which cells are the functionally important sources of SCF for HSC maintenance in the developing liver in vivo, we systematically deleted Scf from candidate niche cells. Hepatic lineage cells have been proposed to be a candidate niche cell type for fetal liver HSCs (Chou and Lodish, 2010). But it is not clear whether hepatocytes are a source of SCF for developing liver HSC maintenance in vivo. Albumin-cre effectively recombines genes in fetal and postnatal hepatocytes (Weiend et al., 2009). Consistent with this, we found that Albumin-cre recombined a loxp-tdTomato$^+$ reporter in HNF4a$^+$ hepatic cells of P0 livers efficiently (Fig. 3, A-E). To test whether hepatocytes are an important source of SCF for HSC maintenance in the developing liver, we generated and analyzed newborn Albumin-cre; Scf$^+/-$ mice. P0 livers from Scf$^+/-$ germine heterozygous mice exhibited a normal HSC frequency but an approximately twofold decline in cellularity compared with WT controls (Fig. 3, F and G). No discernable morphological abnormalities were observed in the P0 livers from Scf$^+/-$ mice, suggesting that the decrease in cellularity is largely due to loss of hematopoietic cells (Fig. S3, G-V). Deletion of Scf from hepatic cells in Albumin-cre; Scf$^+/-$ mice did not significantly reduce HSC frequency in the developing liver compared with either WT or germine Scf$^+/-$ heterozygous mice (Fig. 3 F). Compared with Scf$^+/-$ heterozygous mice, deletion of the second allele of Scf from hepatic cells in Albumin-cre; Scf$^+/-$ mice did not lead to a further loss of liver cellularity or HSC number (Fig. 3, G and H). To determine whether functional HSCs were impaired in the developing livers of Albumin-cre; Scf$^+/-$ mice, we transplanted 500,000 P0 liver cells from Albumin-cre; Scf$^+/-$, littermate Scf$^+/+$, or Scf$^+/-$ mice along with 500,000 recipient-type bone marrow cells into lethally irradiated recipient mice. Consistent with the normal HSC frequency in Scf$^+/-$ livers (Fig. 3 F), 500,000 P0 liver cells from Scf$^+/-$ and Scf$^+/-$ mice gave similar reconstitution (Fig. S3 W). Thus, hereafter throughout this study, reconstitution data from liver cells of Scf$^+/-$ and/or Scf$^+/-$ mice were combined as controls unless otherwise noted. Importantly, P0 liver cells from Albumin-cre; Scf$^+/-$ mice had normal reconstitution activity compared with controls (Fig. 3 I). These data demonstrate that Scf from hepatocytes is not required for HSC maintenance in the developing liver in vivo.

**HSCs do not require SCF from hematopoietic cells in the developing liver**

Although hematopoietic cells express very little if any Scf (Fig. 2, A and N; and Fig. S2 A), it was still possible that hematopoietic cells are an important functional source of SCF for HSC maintenance in the developing liver. We thus conditionally deleted Scf from hematopoietic cells using Vav1-cre (de Boer et al., 2003). Consistent with prior reports (de Boer et al., 2003; Ding et al., 2012), recombination was highly efficient in HSCs and other hematopoietic cells from Vav1-cre; Scf$^+/-$ P0 livers (Fig. 4, A and B). Vav1-cre; Scf$^+/-$ mice had normal HSC frequency in the P0 livers relative to WT or germine Scf$^+/-$ heterozygous littermate control mice (Fig. 4 C). Compared with Scf$^+/-$ mice, Vav1-cre; Scf$^+/-$ mice had similar cellularity and HSC numbers in the P0 livers (Fig. 4, D and E). P0 liver cells from Vav1-cre; Scf$^+/-$ mice had normal reconstitution capacity in all major blood lineages (Fig. 4 F). Therefore, Scf expressed by hematopoietic cells is not required for HSC maintenance in the developing liver.

**HSCs do not require SCF from Ng2$^+$ cells in the developing liver**

Ng2$^+$ periporal stromal cells have been proposed to be a component of the fetal liver niche (Khan et al., 2016). Consistent with the prior report, we found that Ng2$^+$-recombined in α-SMA$^+$ periporal stromal cells in the developing liver (Fig. 5, A-E). Deletion of Scf from periporal stromal cells in Ng2$^+$-cre; Scf$^+/-$ mice did not significantly reduce HSC frequency in the developing liver (Fig. 5 F). P0 liver cellularity and HSC number did not significantly differ in Ng2$^+$-cre; Scf$^+/-$ mutants compared with littermate Scf$^+/-$ controls (Fig. 5, G and H). Moreover, P0 liver cells from Ng2$^+$-cre; Scf$^+/-$ mice had normal long-term myeloid and T cell reconstituting activity and a slight increase in B cell lineage reconstitution compared with controls (Fig. 5 I), consistent with a lack of hematopoietic reconstitution defects when Ng2$^+$ cells are genetically ablated (Khan et al., 2016). These data demonstrate that Scf from Ng2$^+$-cre$^+$ cells do not contribute to HSC maintenance in the developing liver.

**Deletion of Scf from endothelial cells does not cause significant HSC depletion**

Tie2-cre recombines in endothelial and hematopoietic cells (Ding et al., 2012; Kisanuki et al., 2001). We generated Tie2-cre; Scf$^+/-$ mice to conditionally delete Scf from these cells. Since hematopoietic cells do not express Scf (Fig. 2, A and N; and Fig. S2 A) and Scf from hematopoietic cells is not required for HSC maintenance in the developing liver (Fig. 4), this model allowed us to...
investigate whether endothelial cells are a critical source of SCF for HSC maintenance. As expected, Tie2-cre efficiently recombined in Scf-expressing CD31+ endothelial cells in P0 livers (Fig. 6, A and B). Tie2-cre; Scffl/− mutants had normal HSC frequency compared with littermate controls in the developing liver (Fig. 6 C). Liver cellularity and the number of HSCs in the developing livers of Tie2-cre; Scffl/− mutants did not differ significantly from littermate Scf+/− controls (Fig. 6, D and E). Competitive reconstitution assays revealed that P0 liver cells from Tie2-cre; Scffl/− mice had normal reconstitution capacity compared with controls (Fig. 6 F). Thus, deletion of Scf from endothelial cells alone does not significantly affect either the number or the function of HSCs in the developing liver.

Deletion of Scf from hepatic stellate cells leads to loss of HSCs in the developing liver

We found that Pdgfra, a mesenchymal cell marker (Andrae et al., 2008; Li et al., 2018), was highly expressed by CD45−Ter119−Cxcl12−DsRed+ hepatic stellate cells in the developing liver (Fig. 2 D and Fig. S2 B), raising the possibility that we could conditionally delete Scf from stellate cells using a Pdgfrα-cre transgenic line (Roesch et al., 2008). We generated Pdgfrα-cre; loxp-tdTomato mice to examine its recombination pattern in the developing liver. About 90% of tdTomato+ cells were Desmin+ stellate cells (Fig. 7, A–E), with minimal if any recombination in endothelial cells (0%), hematopoietic cells (0.3%), or hepatocytes (0.6%; Fig. S4, A–J). A minimal number of α-SMA+ cells (5%) were targeted by Pdgfrα-cre in P0 livers (Fig. S4, K–O). Thus, the Pdgfrα-cre transgenic line recombines specifically and efficiently in perisinusoidal stellate cells in the developing liver.

To test whether Pdgfrα-cre-expressing stellate cells are an important source of SCF for HSC maintenance, we generated Pdgfra-cre; Scffl/fl and Pdgfra-cre; Scffl/− mice. These mice had normal HSC frequency in the developing liver compared with controls (Fig. 7 F). However, liver cellularity as well as HSC number in the P0 livers from Pdgfra-cre; Scffl/fl or Pdgfra-cre; Scffl/− mice were significantly reduced compared with either Scf+/+ or Scf+/− littermate controls (Fig. 7, G and H). Importantly, 500,000 P0 liver cells from...
Pdgfra-cre; Scf−/− or Pdgfra-cre; Scffl/fl mice gave modest but significantly lower levels of reconstitution compared with controls when transplanted into lethally irradiated recipient mice (Fig. 7I). We also analyzed these mice at E15.5 and did not observe significant decreases in liver cellularity or HSC number compared with Scf+/+ littermate controls (Fig. S5, A–C). E15.5 liver cells from these mutant mice had normal long-term multilineage reconstitution activity (Fig. S5 D), suggesting that the depletion of HSCs in P0 livers (Fig. 7, K and L) was not due to developmental defects before E15.5. These data suggest that HSC niche in the mutant mice was functionally compromised in the developing liver. We also analyzed these mice at P5, when the liver still contains a substantial number of HSCs (Fig. 1, A–C). Compared with controls, Pdgfra-cre; Scf−/− or Pdgfra-cre; Scffl/fl mice had normal levels of reconstitution in irradiated mice (data represent three independent experiments with a total of n = 14 recipients per genotype). Scf+/+ and Scf−/− mice were used as controls. +, WT allele; Δ, recombined Scf allele; −, germline deleted allele. Scf+/+ mice include Cre; Scf+/+, Cre; Scf−/+, Cre−/−; Scf+/±, and Cre−/−; Scf−/− mice. All data represent mean ± SD. Two-tailed Student’s t tests were used to evaluate statistical significance. *, P < 0.05.

**Figure 4.** Scf from hematopoietic cells is not required for HSC maintenance in the developing liver. (A) Genotyping of individual colonies formed by single HSCs isolated from Vav1-cre; Scf−/− mice. Vav1-Cre recombined the Scf−/− allele in all 22 colonies. (B) Quantification of recombined Scf−/− allele in CD45+ and/or Ter119+ hematopoietic cells purified from control (Scffl/fl) or Vav1-cre; Scf−/− mice by qPCR (n = 3 mice for control, n = 3 mice for Vav1-cre; Scf−/−). gDNA, genomic DNA. (C–E) HSC frequency (C), cellularity (D), and HSC number (E) in P0 livers from Vav1-cre; Scf−/− mice compared with littermate controls (n = 6 mice for Scf+/+, n = 4 mice for Scf−/−, and n = 7 mice for Vav1-cre; Scf−/−). (F) 5 × 10⁵ liver cells from Vav1-cre; Scf−/− mice gave normal levels of reconstitution in irradiated mice (data represent three independent experiments with a total of n = 11 independent litters). Scf−/− and Scf+/− mice were used as controls. +, WT allele; Δ, recombined Scf+ allele; −, germline deleted allele. Scf+/+ mice include Cre; Scf+/+, Cre; Scf−/+, Cre−/−; Scf+/±, and Cre−/−; Scf−/− mice. All data represent mean ± SD. Two-tailed Student’s t tests were used to evaluate statistical significance. *, P < 0.05.

HSCs require SCF from both endothelial and hepatic stellate cells

Both endothelial and hepatic stellate cells are the major source of SCF in the developing liver (Fig. 2 and Fig. S2). However, deletion of Scf from endothelial cells did not result in significant HSC depletion (Fig. 6, C–F), while deletion of Scf from stellate cells...
resulted in only modest reconstitution defects (Fig. 7 I). This raised the question of whether SCF from endothelial and hepatic stellate cells synergistically maintains HSCs in the developing liver. To directly test this, we generated Pdgfrα-cre; Tie2-cre; Scfl−/− mice. Pdgfrα-cre; Tie2-cre; Scfl−/− mice were born smaller with pale livers, suggesting a hematopoietic defect in the liver (Fig. 8, A and B). P0 livers from Pdgfrα-cre; Tie2-cre; Scfl−/− mice had significant decreases in HSC frequency, cellularity, and HSC number compared with controls (Fig. 8, C–E). P0 liver cells from Pdgfrα-cre; Tie2-cre; Scfl−/− mice had a significantly reduced capacity to reconstitute lethally irradiated recipient mice in competitive reconstitution assays (Fig. 8 F), with significantly lower contribution to bone marrow HSCs and Lin−Sca1+cKit+ (LSK) progenitors in the recipient mice compared with controls (Fig. 8 G). The hematopoietic defects of the mutant mice became more prominent at P5 (Fig. 8, H and I). Strikingly, ∼90% of HSCs were depleted from the P5 livers of Pdgfrα-cre; Tie2-cre; Scfl−/− mice (Fig. 8, J–L). Importantly, multilineage reconstitution activity was largely depleted from P5 liver cells of Pdgfrα-cre; Tie2-cre; Scfl−/− mice upon transplantation, with mutant liver cells contributing significantly lower levels of HSCs and LSK progenitors in the recipients (Fig. 8, M and N). These data suggest that endothelial and stellate cells are the major source of SCF in the developing liver.

At E14.5, livers from Pdgfrα-cre; Tie2-cre; Scfl−/− mice had normal HSC frequency, cellularity, and HSC number compared with controls (Fig. 8, C–E). P0 liver cells from Pdgfrα-cre; Tie2-cre; Scfl−/− mice had a significantly reduced capacity to reconstitute lethally irradiated recipient mice in competitive reconstitution assays (Fig. 8 F), with significantly lower contribution to bone marrow HSCs and Lin−Sca1+cKit+ (LSK) progenitors in the recipient mice compared with controls (Fig. 8 G). The hematopoietic defects of the mutant mice became more prominent at P5 (Fig. 8, H and I). Strikingly, ∼90% of HSCs were depleted from the P5 livers of Pdgfrα-cre; Tie2-cre; Scfl+/− mice (Fig. 8, J–L). Importantly, multilineage reconstitution activity was largely depleted from P5 liver cells of Pdgfrα-cre; Tie2-cre; Scfl+/− mice upon transplantation, with mutant liver cells contributing significantly lower levels of HSCs and LSK progenitors in the recipients (Fig. 8, M and N). These data suggest that endothelial and stellate cells are the major source of SCF in the developing liver.

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At E14.5, livers from Pdgfrα-cre; Tie2-cre; Scfl−/− mice had normal HSC frequency, cellularity, and HSC number compared with Scfl+/− heterozygous mice (Fig. S5, M–O). Notably, E14.5 liver cells from Pdgfrα-cre; Tie2-cre; Scfl+/− mice had normal reconstitution capacity (Fig. S5 F). These data suggest that the HSC depletion observed in the P0 developing livers from Pdgfrα-cre; Tie2-cre; Scfl−/− mice (Fig. 8) does not reflect developmental defects before E14.5. Taken together, these data strongly suggest that endothelial cells cooperate
with hepatic stellate cells to promote HSC maintenance in the developing liver by producing SCF.

**Hepatic stellate cells progressively lose HSC niche gene expression signature in the neonatal liver**

From E15.5 to P5, stellate cells significantly down-regulated Scf-GFP expression (10.8-fold), while endothelial cells did so to a much lesser extent (2.7-fold; Fig. 9 A). These data suggest that gene expression changes in stellate cells may deplete HSC-supporting activity and drive HSC egress from the liver around birth. To study these gene expression changes in detail, we conducted a genome-wide expression profiling of hepatic stellate cells from E15.5, P5, and P10, when HSCs gradually migrate out of the liver (Fig. 1 C and Fig. S1 A). 2,441 genes were significantly up-regulated and 2,253 genes were significantly down-regulated (P ≤ 0.05, fold-change two or greater) in hepatic stellate cells comparing E15.5 to either P5 or P10 (Fig. 9 B). Gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery comparing stellate cells at E15.5 and P10 revealed several significantly enriched biological processes, including oxidation reduction, metabolic process, and liver development (Fig. 9 C).

Consistent with the loss of HSC-supporting activity from the liver by P10, gene set enrichment analysis (GSEA) showed that P10 hepatic stellate cells have expression profiles significantly depleted of hematopoietic stem and progenitor cell–supporting genes (Charbord et al., 2014) compared with those from E15.5 livers (Fig. 9 D). The expression levels of HSC-supporting factors, such as Scf (Barker, 1994), Vcam1 (Dutta et al., 2015), Pleiotrophin (Himburg et al., 2012), and Jagged1 (Poulos et al., 2013), in hepatic stellate cells decreased significantly over time from E15.5 to P10 (Fig. 9 E), correlating with the gradual depletion of HSC-supporting activity in the livers from E15.5 to P10. Interestingly, principal component analysis (PCA) showed that E15.5 hepatic stellate cells are more similar to bone marrow Scf-GFP+ mesenchymal stromal cells, the key niche cells in the adult bone
Figure 7. Scf from hepatic stellate cells is required for HSC maintenance in the developing liver. (A–D) Confocal images showing that Pdgfra-cre recombinant in hepatic stellate cells in the developing liver of Pdgfra-cre; loxp-tdTomato mice. Hepatic stellate cells were stained with an anti-Desmin antibody. Nuclei were stained with DAPI, in blue. (E) Quantification of hepatic stellate cell specificity of Pdgfra-cre in Pdgfra-cre; loxp-tdTomato P0 livers. Cells were quantified from three independent confocal images. (F–H) HSC frequency (F), liver cellularity (G), and HSC number (H) in P0 livers of Pdgfra-cre; Scffl/fl or Pdgfra-cre; Scffl−/− mice versus littermate controls (n = 8 mice for Scf+/+, n = 6 mice for Scf−/−, and n = 12 mice for Pdgfra-cre; Scffl/fl or Pdgfra-cre; Scffl−/−). (I) 5 × 10^5 liver cells from P0 Pdgfra-cre; Scffl/fl or Pdgfra-cre; Scffl−/− mice gave significantly lower levels of reconstitution in irradiated mice relative to controls (data represent five independent experiments with a total of n = 23 recipients for controls and n = 22 recipients for mutants). Scf+/+ and Scf−/− mice were used as controls. (J–L) HSC frequency (J), liver cellularity (K), and HSC number (L) in P5 livers of Pdgfra-cre; Scffl/fl or Pdgfra-cre; Scffl−/− mice compared with littermate controls (n = 10 mice for Scf+/+, n = 6 mice for Scf−/−, and n = 6 mice for Pdgfra-cre; Scffl/fl or Pdgfra-cre; Scffl−/−). (M) Representative images of P5 Pdgfra-cre; Scffl/fl mice and littermate controls. N and O) Representative images of spleens (N) and livers (O) of P5 Pdgfra-cre; Scffl/fl mice and littermate controls. +, WT allele; Δ, recombinant Scfl allele; −, germline deleted allele. Scf+/+ mice include Cre; Scf−/−, Cre; Scfl+/−, and Cre(−); Scf−/− mice. Scf+/+ and
marrow (Ding and Morrison, 2013; Ding et al., 2012; Lee et al., 2017), than adult hepatic stellate cells at the transcriptome level (Fig. 9 F). Collectively, these data suggest that fetal stellate cells are endowed with HSC-supporting activity, similar to adult bone marrow mesenchymal cells, and this activity is lost around birth.

Discussion

Here, we undertook a systematic genetic approach to functionally dissect the HSC niche in the developing liver in vivo. We found that HSCs localized in a perisinusoidal vascular niche where endothelial cells and hepatic stellate cells are two major cellular sources of SCF. Both cell types are functionally important as combined deletion of Scf from endothelial cells and hepatic stellate cells led to profound HSC depletion (Fig. 8). Hepatic stellate cells play important roles in storage of vitamin A in adult livers (Yin et al., 2013). But their role during hematopoietic development was previously unknown. Our results suggest that fetal hepatic stellate cells are a critical component of the HSC niche. Whole body deletion of stellate cell-specific transcriptional factors, Lhx2 and Hlx, resulted in fetal liver hematopoietic defects in a non-cell-autonomous manner (Hentsch et al., 1996; Porter et al., 1997), supporting the notion that stellate cells are a critical component of the liver hematopoietic niche, although HSCs were not analyzed in these studies. In line with these data, fetal hepatic stellate cells are associated with hematopoietic foci and have a greater capacity in maintaining hematopoiesis in vitro than other stromal cells in the fetal liver (Kordes et al., 2013).

Our genetic approach does not distinguish the function of membrane-bound or soluble SCF, although it likely acts locally (Wolf, 1978). Consistent with this, we found that most HSCs are close to endothelial and stellate cells, the major source of SCF, in the developing liver. Through systematic conditional deletion in candidate cell types, we found that endothelial and stellate cells are the major, if not exclusive, source of SCF, as ~90% HSCs are lost when Scf is deleted from both endothelial and hepatic stellate cells (Fig. 8). It is not clear why endothelial and stellate cells synergistically promote HSC maintenance. The redundancy between these cells as sources of SCF may offer extra robustness to the system, and thus an evolutionary advantage. Khan et al. (2016) identified Nestin-NG2+ periportal stromal cells as an important cellular component of the fetal liver niche. However, ablation of these cells resulted in enhanced reconstitution activity, mainly in the T and B cell lineages (Khan et al., 2016), suggesting that NG2+ periportal stromal cells are not required for the maintenance of functional HSCs in the fetal liver. Consistent with this, we did not observe depletion of HSC number or function when Scf was deleted from Ng2-cre-expressing cells (Fig. 5, F–I). Hepatocytes are a critical source of thrombopoietin for bone marrow HSC maintenance (Decker et al., 2018). But it appears that thrombopoietin is not required for E14.5 fetal liver HSC maintenance, suggesting distinct fetal and adult HSC maintenance mechanisms (Qian et al., 2007). Additional cell types may also contribute to the HSC niche in the developing liver by generating other HSC-supporting factors.

Murine fetal liver hematopoiesis starts from around E12.5 (Mikkola and Orkin, 2006). Blockade of the SCF-cKIT pathway by administration of cKIT functional blocking antibody after E12.5 quickly eliminates hematopoietic progenitors (within 2 d), while applying the same blocking antibody before E12.5 does not affect those progenitors, suggesting that hematopoiesis before the fetal liver stage is SCF independent (Ogawa et al., 1993). Alternatively, using a whole-body knockout approach, a recent report suggests that SCF is required for preHSCs before the fetal liver stage (Azzoni et al., 2018). Although further investigation is clearly needed to address the role of SCF in early hematopoiesis, it is well established that SCF is required for fetal liver HSCs (Broudy, 1997; Ding et al., 2012; Ikuta and Weissman, 1992). We found that conditional deletion of Scf from Pdgfra-cre and/or Tie2-cre-expressing cells does not lead to HSC depletion in early (E14.5 or E15.5) livers (Fig. S5). In contrast, we observed significant HSC depletion in later (P0 and P5) developing livers (Fig. 7 and Fig. 8). Although antibody blockade has faster kinetics (Ogawa et al., 1993) than our conditional genetic models, our data suggest that the observed HSC depletion in later developmental stages in our study reflects disruption of niche function and a critical role of SCF for HSCs in the developing liver.

Materials and methods

Mice

Scfl−/−, Scffl−/−, Scffl−/−, and Cxcl2−/− mice were described previously (Ding and Morrison, 2013; Ding et al., 2012). Vav1-cre (de Boer et al., 2003), Albumin-cre (Weisend et al., 2009), Tie2-cre (Kisanuki et al., 2001), Pdgfra-cre (Roesch et al., 2008), Ng2-cre (Zhu et al., 2008), and loxp-tdTomato (Madisen et al., 2010) mice were obtained from The Jackson Laboratory and maintained on C57BL/6 background. All mice were housed in a specific pathogen–free, Association for the Assessment and Accreditation of Laboratory Animal Care–approved unit at Columbia University Medical Center. All protocols were approved by Columbia University Committee on the Institute Animal Care and Use. Unless otherwise noted, data are mean ± SD, and two-tailed Student’s t tests were used to evaluate statistical significance (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
Figure 8. Scf from endothelial cells and hepatic stellate cells is synergistically required for HSC maintenance in the developing liver. (A) Representative images of P0 Pdgfra-cre; Tie2-cre; Scffl/− mice and littermate controls. (B) Representative images of P0 Pdgfra-cre; Tie2-cre; Scffl/− mice and littermate controls. (C) HSC frequency (C), cellularity (D), and absolute HSC number (E) in P0 livers of Pdgfra-cre; Tie2-cre; Scffl/− mice compared with littermate controls (n = 11 mice for Scf+/+, n = 4 mice for Scf+/−, and n = 7 mice for Pdgfra-cre; Tie2-cre; Scffl/−). (F) 5×10⁵ liver cells from P0 Pdgfra-cre; Tie2-cre; Scffl/− mice gave significantly lower levels of donor reconstitution in all major hematopoietic lineages in irradiated recipient mice relative to controls (data represent three independent experiments with a total of n = 9 recipients for Scf+/+ mice and n = 13 recipients for mutants). Scf+/+ and Scf+/− mice were used as controls. (G) Donor-derived HSC and LSK chimerism in recipient mice 16 wk after transplantation (n = 3 mice for control, and n = 4 mice for Pdgfra-cre; Tie2-cre; Scffl/−). (H) Representative images of P5 Pdgfra-cre; Tie2-cre; Scffl/− mice and littermate controls. (I) Representative images of livers and spleens of P5 Pdgfra-cre; Tie2-cre; Scffl/− mice and littermate controls. (J–L) HSC frequency (J), cellularity (K), and absolute HSC number (L) in P5 livers of Pdgfra-cre; Tie2-cre; Scffl/− mice compared with littermate controls (n = 16 mice for Scf+/+, n = 6 mice for Scf+/−, and n = 12 mice for Pdgfra-cre; Tie2-cre; Scffl/−). (M) 5×10⁵ liver cells from P5 Pdgfra-cre; Tie2-cre; Scffl/− mice gave significantly lower levels of donor reconstitution in all major hematopoietic lineages in irradiated recipient mice relative to controls (data represent two independent experiments with a total of n = 9 recipients and n = 7 recipients from three independent pairs of mutant and control mice). Scf+/+ includes Pdgfra-cre; Tie2-cre; Scffl/+, Pdgfra-cre; Scffl/−, Tie2-cre; Scffl/−, Pdgfra-cre; Scffl/−, Tie2-cre; Scffl/−, and Cre(−); Scffl/− mice. Scf+/− mice include Pdgfra-cre; Tie2-cre; Scffl/−, Pdgfra-cre; Scffl/−, Tie2-cre; Scffl/−, and Cre(−); Scffl/− mice. Littermate controls include Scf+/+ and Scf+/− mice. All data represent mean ± SD. Two-tailed Student’s t tests were used to evaluate statistical significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. +, WT allele; Δ, recombinant Scffl allele; −, germline deleted allele; Con, control; Mut, mutant.

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wk up to at least 16 wk after transplantation to evaluate the level of donor-derived hematopoietic lineages including myeloid, B, and T cells. Blood was incubated with ammonium chloride potassium red cell lysis before proceeding to antibody staining. Cells were stained with anti-CD45.2 (104), anti-CD45.1 (A20), anti-Gr1 (8C5), anti-Mac-1 (M1/70), anti-B220 (6B2), and anti-CD3 (KT31.1; Biolegend) and analyzed by flow cytometry. For transplantation of neonatal mice, 1 million freshly prepared bone marrow cells from CD45.1 mice were injected into the liver or temporal vein of neonatal mice (P0–P3). The mice were then followed for up to 4 mo.

**Flow cytometry**

Liver cells were isolated by crushing the tissue between two glass slides. The cells were passed through 25-G syringes with Ca2+ and Mg2+ free HBSS with 2% heat-inactivated bovine serum for single cell suspension. Then cells were filtered through 70-μm nylon mesh. For staining HSCs, the following antibodies were used: lineage markers (anti-Ter119, anti-B220 [6B2], anti-Gr1 [8C5], anti-CD2 [RM2-5], anti-CD3 [17A2], anti-CD5 [53–7.3], and anti-CD8 [53–6.7]), anti-CD150 (TC15-12F12.2), anti-CD48 (HM48-1), anti-Sca-1 (E13-161.7), and anti-cKit (2B8; Biolegend). DAPI was used to preclude dead cells. For flow-cytometric analysis of Scf-GFP+, Cxcl12-DsRed+, and endothelial cells, the tissue was digested with Collagenase IV (200 U ml−1) and DNase I (200 U ml−1) at 37°C for 20 min. Digested tissue was further dissociated by repetitive pipetting and passed through 70-μm nylon mesh. Samples were then stained with antibodies and assessed by flow cytometry. Anti-CD45 (30F-11) and anti-Ter119 antibodies (Biolegend) were used to label hematopoietic cells. For staining endothelial cells, samples were incubated with anti-CD31 (MEC13.3; Biolegend) antibody. To detect autofluorescence...
of vitamin A, cells were excited with a 405-nm laser, and the emission signal was detected with a 450/50-nm bandpass filter. To exclude dead cells, 7-AAD (BD Pharmingen) was used. Samples were run on FACSaria II, BD LSR II, FACSCelesta, or FACScanto flow cytometers. FACSDiva (BD) or FlowJo (Tree Star) software was used for data analysis.

**Immunostaining of liver sections**

Freshly dissected livers were fixed in 4% paraformaldehyde for 1–3 h followed by overnight incubation in 30% sucrose in PBS. The tissues were embedded in optimal cutting temperature compound and snap-frozen on dry ice. Liver sections were cut at 10 μm using a CryoJane system (Intrumedics) and air-dried overnight at room temperature. Sections were rehydrated in PBS for 5 min and blocked using either 5% goat serum or 5% donkey serum in PBS for 30 min. Primary antibodies were applied overnight at 4°C. Then slides were incubated in secondary antibodies at room temperature for 2 h with washes in between. Primary antibodies were rabbit-anti-Laminin antibody (L9393; Sigma-Aldrich), rabbit-anti-Desmin antibody (GTX103557; GeneTex), goat-anti-Desmin antibody (AF3844; R&D Systems) rabbit-anti-HNF4α antibody (ab201460; Abcam), goat-anti-VE-cadherin antibody (BAF1002; R&D Systems), goat-anti-Albumin-FITC antibody (GTX103557; GeneTex), goat-anti-Desmin antibody (AF3844; R&D Systems) rabbit-anti-HNF4α antibody (A90-234F; Bethyl Laboratories), and mouse-anti-α-SMA-FITC antibody (F3777; Sigma-Aldrich). Secondary antibodies were anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific), anti-goat Alexa Fluor 555 (Thermo Fisher Scientific), anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific), anti-goat Alexa Fluor 555 (Thermo Fisher Scientific), Alexa Fluor 647-conjugated streptavidin (Jackson ImmunoResearch), and anti-CD48 antibodies (Biolegend) at 4°C overnight. Then slides were incubated in secondary antibodies at room temperature for 2 h with washes in between. Primary antibodies were anti-rabbit Laminin (L9393; Sigma-Aldrich), rabbit-anti-Desmin antibody (GTX103557; GeneTex), goat-anti-Desmin antibody (AF3844; R&D Systems) rabbit-anti-HNF4α antibody (ab201460; Abcam), goat-anti-VE-cadherin antibody (BAF1002; R&D Systems), goat-anti-Albumin-FITC antibody (GTX103557; GeneTex), goat-anti-Desmin antibody (AF3844; R&D Systems) rabbit-anti-HNF4α antibody (A90-234F; Bethyl Laboratories), and mouse-anti-α-SMA-FITC antibody (F3777; Sigma-Aldrich). Secondary antibodies were anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific), anti-goat Alexa Fluor 555 (Thermo Fisher Scientific), anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific), anti-goat Alexa Fluor 555 (Thermo Fisher Scientific), Alexa Fluor 647-conjugated streptavidin (Jackson ImmunoResearch), and anti-CD48 antibodies (Biolegend) at 4°C overnight. Alexa Fluor 647-conjugated streptavidin (Jackson ImmunoResearch) was applied for 2 h at room temperature.

**Quantitative PCR (qPCR)**

Enzymatically digested cells were sorted directly into Trizol. Total RNA was purified according to the manufacturer’s instructions and subjected to reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen). The levels of gene transcripts were quantified by quantitative real-time PCR using GoTaq qPCR Master Mix (Promega) on a CFX Connect Real-Time PCR Detection System (Bio-Rad). β-Actin was used to normalize the expression of genes. Primers used in this study were as follows: Scf: 5’-GTCACAGATTCCCGCAG-3’ and 5’-AGCGCT GCTTTTCTTATG-3’; shortScf: 5’-GAGGGCCGAAACTAGAC GCTTT-3’ and 5’-TAAGGCTCAAAGGCAAGC-3’; longScf: 5’- GCCGAAACTAGATCCTTTACTCTGA-3’ and 5’-ACATAAATT GTTTGTGACACTGACTCTG-3’; Cxcl2: 5’-GTCCGAATGTC GCCAAATC-3’ and 5’-GTGTTTCTTCCGGCCTGCA-3’; Desmin: 5’-CCTGGAGGCCGAAGATCAGAT-3’ and 5’-TGAAGTCACTAGT CAAAACTTTGA-3’; Lrat: 5’-GCAGTGGGACTGACTCATCAT-3’ and 5’-CAGATTGGCAGAAAGGCTCAT-3’; Lhx2: 5’-AGTGACCG CGAGGTTGTGT-3’ and 5’-AGGCCGGCACCATCATTGCA-3’; Pdxf1: 5’-TTGACCTCTGTCAGAGAGG-3’ and 5’-CAGACGGTC GAGGAATCTA-3’; β-actin: 5’-GCTCTTTTCCAGCTCTTCTT-3’ and 5’-CTCTGCTATGCTAGACGCAA-3’; VE-cadherin: 5’-TCTCT GCCACCTCACATCA-3’ and 5’-GTAAGTCACTAGTGCTGTA-3’; Tie2: 5’-ATGTGGAAGTCCAGAGGCGT-3’ and 5’-GAATACCCATCTATGTC-3’. For quantifying deletion efficiency of Vav1 cre, CD45/Ter119+ hematopoietic cells were sorted into tail DNA digestion buffer (Viagen Biotech) and digested. Genomic DNA was used as template for qPCR. The Cxcl2 genomic region was used to normalize the input concentrations. The following primers were used: Cxcl2 genomic region: 5’-GAG CCCGAGACTCTGCCACC-3’ and 5’-TTTGCAAGAAGCATTCCC CTC-3’; and Scf genomic region: 5’-GGAGAGGACCAAGTTGAA GT-3’ and 5’-GTCGAGCAGAAGCTTACACCAGC-3’. Scf β/β genomic DNA was used as control.

**Gene expression profiling and analysis**

50,000 CD45−Ter119−Cxcl2−DsRed− stromal cells from freshly prepared livers of E15.5, P5, and P10 Cxcl2LoxP/LoxP mice were sorted into Trizol by flow cytometry. Total RNA was isolated and amplified with the WT-Ovation Pico RNA Amplification system (Nugen) according to the manufacturer’s instructions. Sense strand cDNA was generated using the WT-Ovation Exon Module (Nugen). Then cDNA was fragmented and labeled using FL-Ovation DNA Biotin Module V2 (Nugen). The labeled cDNA was hybridized to Affymetrix Mouse Gene ST 1.0 chips following the manufacturer’s instructions. Expression values for all probes were normalized and determined using the robust multi-array average method with an Affymetrix Expression Console (Irizarry et al., 2003). Normalized data were analyzed using Transcriptome Analysis Console 2.0. Significantly up- or down-regulated genes (P ≤ 0.05 and fold-change two or greater) were used to carry out gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery online tools (Huang et al., 2009). GSEA analysis was conducted as previously described (Subramanian et al., 2005).
A gene set of 297 genes up-regulated in the HSC supportive stromal cell lines compared with nonsupportive stromal lines (Charbord et al., 2014) was downloaded from Broad Institute website. Heatmap analysis and PCA were performed using MATLAB2016. The microarray data were deposited to the Gene Expression Omnibus under accession no. GSE159308.

**Online supplemental material**

Fig. S1 shows multilineage reconstitution of perinatal liver cells and perivascular localization of HSCs in the developing liver. Fig. S2 shows hepatic stellate cells are a major source of SCF in the developing liver, in addition to endothelial cells. Fig. S3 shows that HSCs are close to hepatic stellate cells in the developing liver, and Scf−/- developing liver structure and reconstitution activity are normal. Fig. S4 shows that the recombination pattern of Pagfra-cre is specific to stellate cells in the developing liver. Fig. S5 shows that deletion of Scf from stellate cells does not lead to HSC depletion before E15.5 but leads to severe hematopoietic defects at PS. It also shows that deletion of Scf from both stellate and endothelial cells does not lead to HSC depletion before E14.5.

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Author contributions: Y. Lee, J. Leslie, and L. Ding performed all of the experiments. Y. Yang provided technical help and all of the experiments. Y. Lee and L. Ding designed the experiments, interpreted the results, and wrote the manuscript.

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Figure S1. HSCs are closely associated with perisinusoidal endothelial cells in the P0 liver. (A) Long-term multilineage reconstitution assays as described in Fig. 1C (n = 4 embryos for E15.5, n = 62 mice for P0, n = 31 mice for P5, and n = 20 mice for P10). (B–E) Representative images of CD150+CD48−Lin− candidate HSCs (arrows) localized adjacent to sinusoidal endothelial cells in the P0 liver. Endothelial cells were stained with an anti-Laminin antibody. Nuclei were stained with DAPI, in blue.

(F–M) Representative images of CD150+CD48−Lin− candidate HSCs (arrows) localized adjacent to sinusoidal endothelial cells in the E15.5 liver. Endothelial cells were stained with an anti-Laminin antibody. Nuclei were stained with DAPI, in blue.

(N) An expanded inset of the image shown in R. (O–R) A CD150+CD48−Lin− candidate HSC (arrow) is adjacent to VE-cadherin+ endothelial cells. (S) Quantification of HSC localization relative to VE-cadherin+ endothelial cells. A total of 27 HSCs were quantified from two biological replicates. Scale bars are 20 µm in E, I, M, N, and R.

**P < 0.01; ***P < 0.001; ****P < 0.0001.

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Figure S2. Endothelial cells and hepatic stellate cells are the major sources of SCF in the developing liver. (A) Representative flow-cytometric plots showing that CD45+ cells from livers of P0 Scfgfp/+ mice did not express SCF-GFP (n = 5 mice for control, and n = 5 mice for Scfgfp/+). (B) CD45+ cells from P0 livers had significantly higher expression of Desmin, Lrat, Lhx2, and Pdgfra compared with whole P0 liver cells as revealed by qRT-PCR analysis (n = 5 for whole P0 liver cells, and n = 3 for CD45+ cells). (C) Cxcl12qRT-PCR levels in different cell populations of P0 liver as quantified by qRT-PCR (n = 3 for whole liver cells [WL], n = 3 for CD45+ or Ter119+ hematopoietic cells [Hema], n = 3 for CD45−Ter119−CD31+ endothelial cells [Endo], n = 4 for CD45−Ter119−Cxcl12−DsRed+ stellate cells [DsRed], and n = 4 for CD45−Ter119−tdTomato+ hepatocyte lineage cells from Albumin-cre, loxp-tdTomato mice [Hepato]). Cxcl12 was highly expressed by Cxcl12−DsRed+ hepatic stellate cells with little if any expressed by other cells in the P0 liver. (D) Representative flow-cytometric plots of enzymatically digested P0 Cxcl12−DsRed−livers show that CD45+ Cxcl12−DsRed−stromal cells did not express Cxcl12, an endothelial cell marker (n = 5 mice from two independent experiments). (E) qRT-PCR analysis of short and long forms of Scf transcript levels in endothelial and stellate cells of P0 livers (n = 5 for WL, n = 3 for Endo, and n = 5 for DsRed+ stellate cells). (F) Representative flow-cytometric plots showing that Scf−GFP was expressed by rare nonhematopoietic cells in E15.5 livers. Among Scf−GFP+ stromal cells, there was a subset that expressed higher levels of Scf−GFP−, denoted as GFPhigh (n = 3 embryos for control, and n = 6 embryos for Scfgfp/+). (G) Representative flow-cytometric plots showing the majority of CD45−Ter119−CD31+ endothelial cells in the E15.5 livers expressed Scf−GFP+ (n = 3 embryos for control, and n = 6 embryos for Scfgfp/+). (H–J) Representative confocal images showing that virtually all Cxcl12−DsRed+ cells expressed Desmin, a stellate cell marker, in the E15.5 liver. (K) Quantification of Cxcl12−DsRed+ cells that expressed Desmin in E15.5 Cxcl12−DsRed+/livers. The data are from three independent confocal images. (L) Representative flow-cytometric plots showing that the majority of CD45−Ter119−CD31−Scf−GFP− nonendothelial stromal cells were Cxcl12−DsRed− stellate cells in E15.5 livers (n = 3 embryos for control, and n = 3 embryos for Scfgfp/+). Note that at this stage, stellate cells expressed higher levels of Scf compared with endothelial cells. (M) The majority of CD45−Ter119−Cxcl12−DsRed− stellate cells express Scf−GFP+ in Scfgfp/+; Cxcl12−DsRed− mice (n = 3 embryos for control, and n = 3 embryos for Scfgfp/+; Cxcl12−DsRed−). (N) The vast majority of CD45−Ter119−GFPhigh cells, denoted as GFPhigh cells in F, were Cxcl12−DsRed− stellate cells in E15.5 livers (n = 3 embryos for control, and n = 3 embryos for Scfgfp/+; Cxcl12−DsRed−). (O) CD45−Ter119−Cxcl12−DsRed− cells expressed higher levels of Scf−GFP compared with CD45−Ter119−CD31+ endothelial cells in the Scfgfp/+; E15.5 livers. The fluorescence intensity of GFP in CD45−Ter119− hematopoietic cells in each samples was used for normalization of the expression of Scf-GFP (n = 6 for Endo, and n = 3 for DsRed+ stellate cells). (P) qRT-PCR analyses show that CD45−Ter119−Cxcl12−DsRed− stellate cells gradually up-regulate the expression of Desmin and Lrat during development (n = 4–7 embryos or mice per time point). All data represent mean ± SD. Two-tailed Student’s t tests were used to evaluate statistical significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Scale bar is 20 μm in J. a.u., arbitrary units; MFI, mean fluorescence intensity.

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HSCs localize adjacent to Desmin+ hepatic stellate cells in the P0 liver, and Scf+/− P0 livers have normal number, distribution of major stromal cells, and reconstitution activity. (A) An expanded inset of the image shown in E. (B–E) Representative confocal images showing that HSCs were closely associated with perisinusoidal hepatic stellate cells. HSCs were marked as CD150+CD48 Lin− cells. Hepatic stellate cells were stained with an anti-Desmin antibody. Nuclei were stained with DAPI, in blue. (F) Quantification of distance distribution between CD150+CD48 Lin− candidate HSCs and Desmin+ stellate cells. Measurements for all images were performed using ImageJ software. A total of 41 HSCs were quantified from two independent experiments. (G–R) Scf+/− P0 livers had normal numbers and distribution of VE-cadherin+ endothelial (G–J), Albumin+ hepatic (K–N), and Desmin+ stellate (O–R) cells compared with Scf+/+ littermate controls. Nuclei were stained with DAPI, in blue. (S–U) Quantification of the numbers of VE-cadherin+ (S), Albumin+ (T), and Desmin+ (U) cells per square millimeter shown in G–R. Three representative independent regions from H, J, L, N, P, and R were used for quantification. (V) A representative flow-cytometric plot showing that CD45+ or Ter119+ hematopoietic cells constitute ∼92% of the total cells of enzymatically digested P0 WT livers (n = 5 from three independent experiments). (W) 5 × 10⁶ P0 liver cells from Scf+/− mice gave similar reconstitution activity compared with littermate Scf+/+ mice (n = 5 mice for Scf+/+, and n = 9 mice for Scf+/−). All data represent mean ± SD. Scale bars are 20 µm in A and E. Scale bars are 100 µm in H, J, L, N, P, and R.
Figure S4. Characterization of Pdgfrα-deletion pattern in the developing liver. (A) Representative flow-cytometric plots showing that minimal if any CD45−Ter119−CD31+ endothelial cells were labeled in the P0 livers from Pdgfrα-cre; loxp-tdTomato mice (n = 3 mice for control, and n = 3 mice for Pdgfrα-cre; loxp-tdTomato). (B) A representative flow-cytometric plot showing that minimal if any CD45+ and/or Ter119+ hematopoietic cells were labeled in the P0 livers from Pdgfrα-cre; loxp-tdTomato mice (n = 3 mice for control, and n = 3 mice for Pdgfrα-cre; loxp-tdTomato). (C) A representative flow-cytometric plot of gating strategy for CD45−Ter119−tdTomato+ stromal cells purified from Pdgfrα-cre; loxp-tdTomato P0 livers used in D and E. (D and E) VE-cadherin (D) and Tie2 (E) transcript levels in different cell populations of P0 livers as measured by qRT-PCR showing that Pdgfrα-cre recombined in minimal if any endothelial cells (n = 4 for whole liver cells [WL], n = 4 for CD45−Ter119−CD31+ endothelial cells [Endo], and n = 3 for CD45−Ter119−tdTomato+ stellate cells from Pdgfrα-cre; loxp-tdTomato P0 livers [tdT]). (F–I) Representative confocal images showing that Pdgfrα-cre does not recombine in HNF4α+ hepatic lineage cells in the P0 liver. Hepatic cells were labeled with an anti-HNF4α antibody. Nuclei were stained with DAPI, in blue. (J) Quantification of HNF4α+ hepatic lineage cells from confocal images of Pdgfrα-cre; loxp-tdTomato P0 liver tissue sections. Only 0.6% of hepatic lineage cells were tdTomato+. The data were from three independent confocal images. (K–N) Representative confocal images showing that Pdgfrα-cre recombines in a minority of α-SMA+ periportal stromal cells in the P0 liver. Periportal stromal cells were stained with an anti-α-SMA antibody. Nuclei were stained with DAPI, in blue. (O) Quantification of α-SMA+ cells from confocal images of Pdgfrα-cre; loxp-tdTomato P0 liver tissue sections. Only 5% α-SMA+ periportal stromal cells are tdTomato+. The data were from three independent confocal images. All data represent mean ± SD. Two-tailed Student’s t tests were used to evaluate statistical significance. ****, P < 0.0001. Scale bars are 20 µm in I and N. SSC-A, side scatter area.
Progressive loss of HSCs in the developing liver when Scf is deleted from stellate cells and endothelial cells. (A–C) HSC frequency (A), cellularity (B), and HSC number (C) in livers from E15.5 *Pdgfra*-cre; Scffl/fl mice and littermate controls (n = 9 embryos for Scf+/+, n = 6 embryos for Scf+/−, and n = 8 embryos for *Pdgfra*-cre; Scffl/fl or *Pdgfra*-cre; Scffl−/−). (D) 5 × 10^5 liver cells from E15.5 *Pdgfra*-cre; Scffl/fl mice gave normal levels of reconstitution in irradiated mice (data represent a total of n = 7 recipients for controls and n = 10 recipients for mutants from two independent experiments). *Pdgfra*-cre; Scf+/+ and *Pdgfra*-cre; Scffl/+ mice were used as controls. (E–G) P5 *Pdgfra*-cre; Scffl/fl mice had significant reduction in white blood cell (WBC) count (E), RBC count (F), and platelet count (G; n = 6 mice for controls [Con], and n = 4 mice for mutants [Mut]). (H) A representative image of P10 *Pdgfra*-cre; Scffl/fl mice and littermate controls. (I and J) Representative images of spleens (I) and livers (J) from P10 *Pdgfra*-cre; Scffl/fl mice and littermate controls. (K) 14 out of 15 *Pdgfra*-cre; Scffl/fl pups died early postnatally, mostly before weaning age, while none of the controls (n = 63) did so. The one surviving *Pdgfra*-cre; Scffl/fl mouse was smaller and paler compared with littermate controls. (L) A representative image of *Pdgfra*-cre; Scffl/fl and control mice at 4 mo after being intravenously transplanted with 1 million bone marrow cells at P0–P3. (M–O) HSC frequency (M), cellularity (N), and absolute HSC number (O) in E14.5 livers from *Pdgfra*-cre; Tie2-cre; Scffl−/− mice compared with littermate controls (n = 3 embryos for Scf+/+, n = 4 embryos for Scf+/−, and n = 3 embryos for *Pdgfra*-cre; Tie2-cre; Scffl/fl). (P) 5 × 10^5 liver cells from E14.5 *Pdgfra*-cre; Tie2-cre; Scffl−/− mice gave normal donor reconstitution in all major hematopoietic lineages in irradiated recipient mice (data represent two independent experiments with a total of n = 8 recipients for controls and n = 6 recipients for mutants). SCF mice were used as controls. +, WT allele; Δ, recombined Scf/fl allele; −, germline deleted allele. Scf/fl includes Scf+/+, Cre(−); Scf/fl, *Pdgfra*-cre; Scf+/+, *Pdgfra*-cre; Scffl−/−, and Cre(−); *Pdgfra*-cre; Scffl/fl mice. Scf+/+ mice include *Pdgfra*-cre; Scf+/+ and Cre(−); Scf+/− mice. All data represent mean ± SD. Two-tailed Student’s t tests were used to evaluate statistical significance. *, P < 0.05.

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