Differential cytokine contributions of perivascular haematopoietic stem cell niches

Noboru Asada¹,², Yuya Kunisaki¹,²,⁵, Halley Pierce¹,², Zichen Wang³, Nicolas F. Fernandez³, Alexander Birbrair¹,²,³, Avi Ma’ayan³ and Paul S. Frenette¹,²,⁴,⁶

Arterioles and sinusoids of the bone marrow (BM) are accompanied by stromal cells that express nerve/glial antigen 2 (NG2) and leptin receptor (LepR), and constitute specialized niches that regulate quiescence and proliferation of haematopoietic stem cells (HSCs). However, how niche cells differentially regulate HSC functions remains unknown. Here, we show that the effects of cytokines regulating HSC functions are dependent on the producing cell sources. Deletion of chemokine C-X-C motif ligand 12 (Cxcl12) or stem cell factor (Scf) from all perivascular cells marked by nestin-GFP dramatically depleted BM HSCs. Selective Cxcl12 deletion from arteriolar NG2+ cells, but not from sinusoidal LepR+ cells, caused HSC reductions and altered HSC localization in BM. By contrast, deletion of Scf in LepR+ cells, but not NG2+ cells, led to reductions in BM HSC numbers. These results uncover distinct contributions of cytokines derived from perivascular cells in separate vascular niches to HSC maintenance.

Haematopoietic stem cells (HSCs) self-renew and differentiate into all blood types in response to various demands during life. HSC functions are finely regulated by specialized ‘niche’ cells in the BM. The location of the HSC niches in the BM remains controversial. Recent analyses with improved surface marker identification and BM imaging have suggested that HSCs are largely perivascular. Knock-in mice of GFP in the chemokine C-X-C motif ligand 12 (Cxcl12) locus revealed that the brightest GFP-expressing stromal cells (commonly referred to as Cxcl12-abundant reticular—CAR—cells) are distributed around sinusoids. Cxcl12 and other niche factors are expressed by perivascular cells marked by Nes-GFP, which contain all mesenchymal stem cell activity in the BM, and are physically associated with HSCs. Nes-GFP+ cells thus overlap with CAR cells as both stromal cell types differentiate into adipocytic and osteoblastic mesenchymal lineages. Perivascular cells marked by constitutive expression of Cre driven by the LepR, osterix-, or Pnx-1 cre-derived cells have also been shown to contribute to HSC maintenance via synthesis of Cxcl12 and Scf, whereas the deletion of the same factors in committed osteoblasts (using osteocalcin-cre) did not reveal a significant HSC phenotype. Knock-in reporter mice for Cxcl12 and Scf revealed a major (/>95%) overlap in the perivascular stromal cells expressing these niche factors. Additionally, no significant alterations in HSC numbers were observed on genetic deletion of Cxcl12 or Scf using nestin-cre transgenic mice, but the significance of these results remains unclear since Cre expression, even if driven by the same promoter, is low among Nes-GFP+ cells. Thus, the exact functional contribution of Nes-GFP+ cells in niche activity remains unclear.

Recent whole-mount three-dimensional imaging of the BM revealed two major subsets of Nes-GFP cells where stromal cells with bright GFP signals are exclusively associated with arterioles of the BM whereas Nes-GFP+ cells with lower GFP levels are distributed ubiquitously around sinusoids. The latter subset largely corresponds to LepR-cre-marked cells, whereas the former is labelled by NG2 pericyte marker. The role of arteriole-associated stromal cells in regulation of HSC quiescence is suggested by significant changes in HSC associations with arterioles, compared with randomly assigned virtual HSCs, following recovery after chemotherapy, after the administration of polyinosinic:polycytidylic acid, or in animals genetically deficient of Pml, all of which lead to HSC proliferation. Schwann cells (GFAP+) in the BM, which are exclusively associated with arterioles, activate TGF-β that promotes HSC quiescence. However, it has recently been argued that HSCs are randomly distributed in the BM and that arteriole-associated NG2+ cells do not contribute to...
**RESULTS**

**NG2-cre targets virtually all perivascular nestin-GFP+ cells in the BM**

To evaluate the contributions of NG2+ cells in the HSC niche, we crossed NG2-cre transgenic mice with ROSA26-loxP-stop-loxP-tdTomato reporter (iTDTomato) and Nes-GFP transgenic mice. Whole-mount imaging analyses of the BM revealed that constitutive NG2-driven Cre expression efficiently labelled Nes-GFP+ stromal cells including both peri-arteriolar Nes-GFP<sup>+</sup> and homogeneously distributed peri-sinusoidal Nes-GFP<sup>-</sup> cells (Fig. 1a,b). Fluorescence-activated cell sorting (FACS) analyses of digested BM nucleated cells isolated from NG2-cre/iTDTomato/Nes-GFP transgenic mice. n = 4 mice, confirmed that 96.9 ± 1.3% of CD45<sup>-</sup>TER119<sup>-</sup>CD31<sup>-</sup>Nes-GFP<sup>+</sup> stromal cells were marked by NG2-cre/iTDTomato (Fig. 1c), suggesting that NG2-cre recombines in the entire Nes-GFP+ stromal cell population of the adult BM. Consistent with the trilineage mesenchymal features of NG2-cre-targeted cells, we found labelling in osteocytes, chondrocytes and adipocytes (Supplementary Fig. 1a–c). However, we found that a small fraction (∼10%) of endothelial cells was labelled (Supplementary Fig. 1d,e). As LepR<sup>+</sup> stromal cells represent a large subset (∼80%) of Nes-GFP<sup>+</sup> cells located around sinusoids<sup>13,19</sup>, we examined the relationships among NG2-cre-targeted cells, arteriolar NG2<sup>+</sup> and sinusoidal LepR<sup>+</sup> cells. Staining of BM from NG2-cre/iTDTomato/Nes-GFP mice with anti-NG2 and anti-LepR antibodies revealed that a high proportion of TdTomato<sup>+</sup> cells (88.5 ± 1.6%) expressed LepR (Fig. 1d,e). While LepR-cre marked a small portion of Nest-GFP<sup>+</sup> cells, NG2-cre labelled all the Nes-GFP<sup>+</sup> cells (Fig. 1f,g). Immunoreactive NG2<sup>+</sup> cells around arterioles were also targeted by NG2-cre (Supplementary

---

**Figure 1** NG2-cre labels perivascular niche cells. (a,b) Whole-mount images of sternums from NG2-cre/iTDTomato/Nes-GFP transgenic mice stained with anti-VE-cadherin antibody. The dashed lines delineate the borders between bone and BM. Representative image from 3 mice. Scale bars, 100 µm in a, 20 µm in b. NG2-cre-targeted cells overlap with both peri-arteriolar Nes-GFP<sup>+</sup> cells (arrows) and peri-sinusoidal Nes-GFP<sup>-</sup> cells (arrowheads). (c) Representative FACS plots showing the percentage of NG2-cre/iTDTomato-positive cells within CD45<sup>-</sup>TER119<sup>-</sup>CD31<sup>-</sup>Nes-GFP<sup>+</sup> BM stromal cells.
NG2+/-cre marked cells are the main source of Cxcl12 in the BM

Since Cxcl12 production in mature osteolineage cells has been shown to be dispensable for HSC maintenance\(^6,11,21\), NG2+/-cre transgenic mice give the opportunity to define functionally the contribution of the entire population of Nes-GFP+ cells in HSC maintenance. We crossed NG2+/-cre/TdtTomato or LepRe+/-cre/TdtTomato with Cxcl12-GFP knock-in mice\(^6\) to interrogate the relationships with CAR cells. As previously reported\(^9\), the vast majority of stromal cells marked by LepRe+ (-~90%) were CAR cells (Supplementary Fig. 2a,b). Likewise, a large proportion of NG2+/-cre-marked cells (-~80%) were CAR cells (Fig. 2a,b). We sorted GFP-negative, low- and bright cells from Cxcl12-GFP knock-in mice and found, as expected, that GFP expression positively correlated with endogenous Cxcl12 messenger RNA (Fig. 2c,d). However, staining for intracellular Cxcl12 protein in the same subpopulations revealed, unexpectedly, that GFP-low stromal cells expressed as much Cxcl12 as GFP-bright canonical CAR cells (Fig. 2e,f). To confirm the staining specificity, we crossed NG2+/-cre/Cxcl12-GFP mice with Cxcl12+/- mice, and evaluated intracellular Cxcl12 level in each population. We found that the positive signal was completely abrogated in stromal cells in NG2+/-cre Cxcl12+/- mice, indicating that the intracellular staining was specific (Supplementary Fig. 2c). Interestingly, all NG2+/-cre-targeted cells, whether from the GFP-bright classical CAR cell gate (Fig. 2g, circled four), the GFP-low (Fig. 2g, circled three), or GFP-negative gate (Fig. 2g, circled two), expressed similar levels of Cxcl12 protein (Fig. 2h,i). These results indicate that all NG2-marked cells in flushed BM produce high levels of Cxcl12 protein.

Cxcl12 from distinct perivascular niche cells contributes differentially to HSC functions

We then crossed Cxcl12+/- mice with CMV+/-cre transgenics to generate animals with one germline knockout allele (Cxcl12+/-) and then with NG2+/-cre animals (or LepRe+/-cre mice for comparison) to determine the contribution of all Nes-GFP+ cells in the synthesis of this niche factor (Supplementary Fig. 3a). There was an ~80% reduction of Cxcl12 expression in LepRe+ expressing cells identified by anti-LepR antibody (which stains 82.2 ± 2.1% of LepR-marked cells), suggesting a high deletion efficiency in this population (Supplementary Fig. 3b). Consistent with a prior study\(^10\), deletion of Cxcl12 in LepRe+ targeted cells led to mobilization of HSCs in blood and spleen, but no significant reduction of HSCs in the BM (Supplementary Fig. 3c and Fig. 3a). We investigated the functional relevance of the Cxcl12 deletion in LepR+ cells and observed no significant alteration in HSC cycling profile and distribution in the BM relative to arterioles by three-dimensional imaging (Fig. 3b,c). Deletion of Cxcl12 in NG2+/-cre-marked cells drastically reduced Cxcl12 expression in Nes-GFP+ stromal cells without affecting the expression levels of other niche factors including Sca1, vascular cell adhesion molecule-1 (Vcam1) and angiopoietin-1 (Angpt1) (Fig. 3d and Supplementary Fig. 3d). Whereas the absolute number of Nes-GFP+ stromal cells was not altered by the deletion of Cxcl12 (Supplementary Fig. 3e), the BM cellularity was significantly reduced (Fig. 3e). Importantly, the number of phenotypic HSCs in the BM was markedly decreased in NG2+/-cre/Cxcl12+/- mice, a finding confirmed by competitive repopulation assays (Fig. 3f,g and Supplementary Fig. 3f). Committed progenitors were also decreased in Cxcl12-depleted BM (Supplementary Fig. 3g). Interestingly, Cxcl12 deletion in NG2+/-cre-marked stromal cells forced HSCs to exit from quiescence (Fig. 3h and Supplementary Fig. 3h) and led to redistribution of remaining HSCs away from arterioles. (Fig. 3i,j). In addition, a robust HSC mobilization was observed in NG2+/-cre/Cxcl12+/- mice (Fig. 3k). Since NG2+/- Nes-GFP+ cells associated with portal vessels form an HSC niche in the fetal liver\(^22\), we also evaluated the impact of constitutive NG2-driven Cre deletion in the neonatal liver to rule out a contribution from defective embryonic HSC specification or fetal HSC proliferation. We found that the cellularity and the number of phenotypic HSCs in the liver of newborn NG2+/-cre/Cxcl12+/- mice were comparable to those of control animals (Supplementary Fig. 3i), which is consistent with prior studies showing that Cxcl12 is dispensable for HSCs in fetal liver\(^22\). These data strongly suggest that Cxcl12 derived from Nes-GFP+ cells play essential roles for HSC maintenance and retention in the BM.

Cxcl12 from peri-arteriolar cells contributes to HSC maintenance

The difference in the phenotype of NG2+/-cre- or LepRe+/-cre-induced deletion, combined with the significant Cxcl12 expression in NG2+/-cre-marked cells outside of the canonical CAR cell population, raised the possibility that adult stromal cells expressing NG2 significantly contributed to Cxcl12 production in the BM. In triple-transgenic NG2+/-creERTM/Nes-GFP+TdtTomato mice, TdtTomato expression was observed in peri-arteriolar Nes-GFP+ stromal cells, as well as in cells of the mature osteolineage (Fig. 4a,b). No labelling was detected in peri-sinusoidal Nes-GFP+ cells or endothelial cells (Fig. 4a,b). We next examined the expression level of the niche factors in TdtTomato+ BM stromal cells that exclusively expressed Nes-GFP using quantitative PCR analyses of Nes-GFP+ TdtTomato+ stromal cells isolated from digested flushed BM cells after 8 weeks of tamoxifen administration (Supplementary Fig. 4a and Fig. 4c) and found that the expression level of Cxcl12 and Scf in Nes-GFP+ TdtTomato+ stromal cells was equal to or above CD31+ endothelial cells, which have been reported to contribute to niche activity\(^4\) (Fig. 4d). Using platelet-derived growth factor receptor β (PDGFRβ), expressed in pericytes but not in osteolineage cells, we were able to enrich for a cell population exhibiting gene expression profiles similar to NG2+/-creERTM targeted cells from NG2-+DsRed mice (Supplementary Fig. 4b,c). Using Cxcl12-GFP mice as a reporter in NG2+/-creERTM/TdtTomato or NG2-+DsRed transgenics, GFP was not detectable in NG2+ cells compared with the bright NG2-negative canonical CAR cells (Supplementary Fig. 4d,e). Since significant endogenous Cxcl12 mRNA is expressed in NG2+ cells, we assessed intracellular Cxcl12 protein level in NG2+/-creERTM/TdtTomato cells, which revealed that NG2+ cells expressed higher Cxcl12 protein than CD31+ BM endothelial cells (Fig. 4e). These results thus suggest that all stromal cells marked by NG2 produce Cxcl12. It remains unclear whether, and if so how, intracellular content of Cxcl12 or GFP content in Cxcl12 knock-in mice reflects Cxcl12 secretion that is highly regulated, notably via cell–cell contact, glycan presentation and gap junctions\(^23\).
To evaluate Cxcl12 functions in NG2-expressing cells of postnatal BM, we generated NG2-creERTM/Cre+ERTM (Supplementary Fig. 4f). Since recombination efficiency in NG2-creERTM mice is ∼30% after tamoxifen administration in adult mice, we injected tamoxifen at 2–3 week age and then analysed 6–8 weeks after treatment to maximize the recombination in this compartment. To evaluate the NG2 promoter activity in TdTomato+ cells after tamoxifen treatment, we confirmed Csgg4 (NG2) expression in TdTomato+ cells 7–8 weeks after tamoxifen injection (Supplementary Fig. 4g). As both Cxcl12 and Grf(Rosa26SorCreERTM/LacZ/tdTomato)Hze alleles are closely linked (208kb apart) on Chromosome 6, we were not able to generate NG2-creERTM/Cxcl12−/+;TdTomato mice to verify the deletion efficiency of Cxcl12 in NG2-creERTM-targeted cells. Targeted deletion of Cxcl12 in NG2+ cells led to a significant reduction...
of HSC numbers in BM, which was confirmed by competitive repopulation assays, without affecting BM cellularity, LSK cells in the BM, HSCs in the spleen, LSK in the blood or composition of mature cells in the blood (Fig. 4f–j and Supplementary Fig. 4h,i). In addition, we found that HSCs were located further away from arterioles in NG2-creERT2/Cxcl12f/−/− mice (Fig. 4k). To confirm these results, we intercrossed Myh11-creERT2 transgenic mice, reported to target vascular smooth muscle cells, with TdTomato and Nes-GFP transgenics. Whole-mount immunofluorescence analysis of the BM of triple-transgenic mice revealed strong and selective recombination with arteriole-associated stromal cells (Fig. 4l), which was also confirmed by the staining with anti-Ng2 antibody (Supplementary Fig. 4j). Myh11-creERT2-labelled cells were uniformly Nes-GFP+ (Supplementary Fig. 4k) and also expressed Cxcl12 as determined by intracellular staining (Supplementary Fig. 4l). We then bred Myh11-creERT2 mice with Cxcl12f/−/− animals to evaluate the impact of Cxcl12 deletion in this subpopulation of arteriole-associated Nes-GFP+ cells using the same tamoxifen regimen. We also confirmed that TdTomato+ cells expressed Myh11 at the time of analyses (Supplementary Fig. 4m). We found that the number of HSCs in the

Figure 3 Cxcl12 from distinct perivascular niche cells contributes differentially to HSC functions. (a–c) Analyses of LepR-cre/Cxcl12f/−/− mice. (a) Absolute numbers of HSCs in BM. n = 6 mice for each group. (b) FACS analyses of cell cycle of HSCs with Ki-67 and Hoechst 33342 staining. n = 5 mice per group. (c) HSC localization relative to arterioles. Error bars, n = 3 mice. The P value has been calculated using n = 129 HSCs for cre (−), 160 HSCs for cre (+), pooled from 3 mice per group. P = 0.9981. (d–k) Analyses of NG2-cre/Cxcl12f/−/− mice. (d) Cxcl12 mRNA expression relative to β-actin in CD45−/Cd31−/Cd48−/Lineage−/Sca-1−/Cd150−/Cd41− (LSK) HSCs (f) per femur. n = 10 mice. (g) Percentages of donor-derived cells after competitive reconstitution. n = 5 mice per group. (h) Quantification of cell cycle of HSCs with Ki-67 and Hoechst 33342 staining. n = 5 mice for cre (−), n = 7 mice for cre (+). (i) Representative images of whole-mount immunofluorescent staining of the sternal BM from 3 mice. Arrows indicate CD150+/CD48+/CD41− Lineage− HSCs. Dashed lines depict the border between bone and BM. Scale bars, 100 μm. (j) HSC localization relative to arterioles. Error bars, n = 3 mice for cre (−), n = 4 mice for cre (+). The P value has been calculated using n = 139 HSCs pooled from 3 mice for cre (−), 105 HSCs pooled from 4 mice for cre (+). P = 0.0001. (k) Absolute numbers of HSCs in the spleen (left) and blood (right). n = 6 mice (cre(−)), 8 mice (cre(+)) for spleen, n = 5 mice (cre(−)), n = 7 mice (cre(+)) for blood. Data are represented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data were analysed with two-tailed t-test (a,b,d-f,h,k) and two-sample Kolmogorov–Smirnov test (c,j).
Figure 4 Cxcl12 deletion in NG2-creERTM-targeted cells alters HSC numbers and location in the BM. (a, b) Whole-mount images of sternum from NG2-creERTM/TdTomato/Nes-GFP mice. Representative images from 3 mice. Scale bars, 100 μm in a and 20 μm in b. (c) Representative FACS plot of BM stromal cells isolated from NG2-creERTM/TdTomato/Nes-GFP mice. (d) Quantitative real-time PCR of Cxcl12 and Scf in CD45−TER119−CD31−, CD45−TER119−CD31−Nestin−GFP−, and CD45−TER119−CD31−Nestin−GFP−NG2-creERTMTDTomato− mice. (e) Representative image of intracellular Cxcl12 levels in NG2-creERTMTDTomato− cells (left). MFI of intracellular Cxcl12 protein (right). n=7 mice. (f–k) Analyses of NG2-creERTMTDTomato− mice. (f) Cellularity in the BM and spleen. BM; n=10 mice for cre (−), n=13 mice for cre (+). Spleen; n=5 mice for cre (−), n=8 mice for cre (+). (g) Numbers of CD150−CD48−LSK HSCs in BM, n=10 mice for cre (−), n=13 mice for cre (+). (h) Percentages of donor-derived cells after competitive reconstitution. n=8 mice for cre (−), n=13 mice for cre (+). (i) LSK cells in BM. n=10 mice for cre (−), n=13 mice for cre (+). (j) HSC numbers in the spleen (left) and LSK cells in blood (right). n=5 mice for cre (−), n=8 mice for cre (+). (k) HSC localization relative to arterioles. Error bars, n=3 mice. The P value has been calculated using n=254 HSCs for cre (−), 238 HSCs for cre (+) pooled from 3 mice per group. P < 0.0001. (l) Whole-mount sternal images from Myh11-creERT2/Nes-GFP/TdTomato mice stained with anti-NG2 antibody. Representative images from 3 mice. Scale bars, 20 μm. (m, n) Analyses of Myh11-creERT2/Cxcl12−/− mice. (m) Numbers of CD150−CD48−LSK HSCs in BM. n=5 mice for cre (−), n=8 mice for cre (+). (n) HSC localization relative to arterioles. Error bars, n=3 mice. The P value has been calculated using n=220 HSCs pooled from 3 mice for cre (−), 239 HSCs pooled from 4 mice for cre (+). P = 0.0007. Data are represented as ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance was assessed using two-tailed t-test (e–j, m), one-way ANOVA (d), and two-sample Kolmogorov–Smirnov test (k, n).
**Figure 5** NG2-cre-, but not NG2-creERT2-, targeted cells are the source of Scf in the BM. (a) Whole-mount sternum from NG2-cre/iTdTomato/Scf-GFP mice, anti-VE-cadherin. Representative images from 3 mice. Scale bars, 20 μm. (b) Representative FACS plot showing percentage of NG2-cre/iTdTomato+ cells within CD45-TER119-CD31-Scf-GFP+ cells. n = 3 mice. (c-e) Analyses of LepR-cre/Scffl/fl mice. (c) Numbers of HSCs (left) in BM and LSK cells in spleen (right), n = 4 mice for cre (-), n = 3 mice for cre (+). (d) FACS analyses of HSC (CD150+CD48-LSK) cell cycle with Ki-67 and Hoechst 33342 staining. n = 5 mice for cre (-), n = 6 mice for cre (+). (e) HSC localization relative to arterioles. Error bars, n = 3 mice. P value has been calculated using n = 272 HSCs for cre (-), 293 HSCs for cre (+) pooled from 3 mice per group. P = 0.3402. (f-i) Analyses of NG2-cre/Scffl/fl mice. (f) Numbers of total BM cells (left) and CD150+CD48-LSK HSCs (right) in BM. n = 5 mice for cre (-), n = 7 mice for cre (+). (g) Percentages of donor-derived cells after competitive reconstitution. n = 5 mice for cre (-), n = 7 mice for cre (+). (h) FACS analyses of HSC cell cycle with Ki-67 and Hoechst 33342 staining. n = 6 mice for cre (-), n = 7 mice for cre (+). (i) HSC localization relative to arterioles. Error bars, n = 3 mice. P value has been calculated using n = 224 HSCs for cre (-), 274 HSCs for cre (+) pooled from 3 mice per group. P = 0.2872. (j-l) Analyses of NG2-creERT2-Scffl/fl mice. (j) Absolute numbers of total cells (left) and HSCs (right) per femur BM. n = 8 mice for cre (-), n = 6 mice for cre (+). (k) Percentages of donor-derived cells after competitive reconstitution. n = 9 mice for cre (-), n = 7 mice for cre (+). (l) HSC localization relative to arterioles. Error bars, n = 3 mice. P value has been calculated using n = 161 HSCs for cre (-), 152 HSCs for cre (+) pooled from 3 mice per group. P = 0.0868. Data are represented as ± s.e.m. (b-l). *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance was assessed using two-tailed t-test (c,d,f,h,j,k) and two-sample Kolmogorov–Smirnov test (e,i,l). Statistics source data are available in Supplementary Table 1.

BM was significantly reduced (by 40%) (Fig. 4m) and the distribution of HSCs was altered in Myh11-creERT2-deleted BM (Fig. 4n), confirming an important contribution of arteriole-associated stromal cells in the synthesis of Cxcl12 for HSC maintenance.

Scf from ubiquitously distributed LepR+ cells, but not peri-arteriolar niche cells, promotes HSC maintenance. The distinct phenotypes of Cxcl12 deletion from NG2+ arteriolar and LepR+ sinusoidal niches suggest differential functions of these

NATURE CELL BIOLOGY VOLUME 19 | NUMBER 3 | MARCH 2017

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Figure 6 Distinct contributions of vascular-associated cells in niche activity. (a) RNA-seq analysis of perivascular niche cells. The heat map of unsupervised hierarchical clustering of significant enriched genes for sorted CD45−TER119−CD31−NG2-cre/TdTomato+, CD45−TER119−CD31−LepR-cre−TER119+CD31−Myh11-creERT2−TER119+CD31−cells created with clustergrammer. 2 mice for each group. Enrichment analysis for each cluster as determined by hierarchical clustering of the rows was performed with Enrichr. (b) Heat map expression levels of selected genes defined by previous studies for HSC niche cells, pericytes, and endothelial cells. The values of log-transformed reads per kilobase per million mapped reads (RPKM) obtained from RNA-seq were visualized using GraphPad Prism7. Genes with absent expression were assigned the lowest value of the gene pool for visualization. 2 mice for each group. (c) Summary of phenotypes of niche-factor-deleted mice in perivascular stromal cells. PB HSPC, peripheral blood hematopoietic stem and progenitor cells.

stromal cells in HSC maintenance. To investigate roles of Scf, the other cytokine shown to be critical for HSC maintenance, we assessed the expression of Scf in these distinct niches. We analysed NG2-cre/iTdTomato/Scf-GFP mice and confirmed Scf-GFP+ cells ∼100% overlapped with NG2-cre-targeted cells (Fig. 5a,b). Quantitative PCR analyses showed that NG2+ peri-arteriolar niche cells marked with NG2-creERTM or NG2DsRed+PDGFRβ+ expressed a modest level of Scf (Fig. 4d and Supplementary Fig. 4c), which was confirmed by immunofluorescence imaging of the BM from NG2-creERTM/TdTomato/Scf-GFP mice (Supplementary Fig. 5a).

We, therefore, assessed the functions of Scf in these distinct niches by analysing LepR-cre, NG2-cre or NG2-creERTM,Scffl/fl lines. Deletion of Scf in LepR-cre-targeted cells, which marked broadly distributed Nes-GFP+, LepR-expressing stromal cells (Supplementary Fig. 5b), led to reductions in HSC numbers in the BM and increased LSK cells in the spleen (Fig. 5c), which is consistent with previous studies. LepR-cre/Scffl/fl mice showed no effects on cell cycle or localization of HSCs in the BM (Fig. 5d,e). Deletion of Scf in NG2-cre-targeted cells led to a reduction of cellularity and HSC numbers in the BM (Fig. 5f). Competitive transplantation assays have confirmed the reduction of long-term reconstitution activity (Fig. 5g and Supplementary Fig. 5c). The numbers of HSCs in spleen and LSK cells in blood were comparable to those of littermate control mice (Supplementary Fig. 5d). As was the case with LepR-cre/Scf−− mice, neither cell cycle of HSCs nor distribution was altered in the NG2-cre/Scf−− BM (Fig. 5h,i). In addition, there was a reduction observed in the numbers of myeloid cell lineage cells in peripheral blood while NG2-cre/Scf−− mice had normal haematopoietic lineage composition in the spleen (Supplementary Fig. 5e). Scf has been shown to be required for fetal liver haematopoiesis, and Scf deletion in LepR-cre-targeted cells showed no effect on HSC number in the liver of newborn mice. We found that HSC number in NG2-cre/Scf−−
newborn liver was decreased without a significant change in cellularity (Supplementary Fig. 5f), suggesting that NG2-cre-targeted cells are an important source of Scf essential for HSCs not only in the BM but also fetal liver.

We next examined whether Scf from peri-arteriolar niche cells played roles in HSC maintenance using NG2-creERTM/Scf−/− mice. After 6–8 weeks of tamoxifen administration, NG2-creERTM/Scf−/− mice showed no significant changes in the numbers of total cells and HSCs in the BM, spleen or blood, or in the location of HSCs in the BM compared to Scf−/− littermates treated with tamoxifen (Fig. 5j–l and Supplementary Fig. 5g,h). Although it is possible that Scf supplied from the neighbouring peri-sinusoidal cells or peri-arteriolar stromal cells that escaped Cre recombination may have compensated the deletion of Scf in NG2-creERTM/Scf−/− mice, these results confirm the importance of LepR+ peri-sinusoidal cells rather than NG2+ peri-arteriolar niche cells as a major source of Scf essential for maintenance of HSCs in the BM.

Distinct contributions of vascular-associated cells in niche activity

To further explore the differential genetic makeup of various perivascular niche cell populations, we analysed the transcriptomes of differentially expressed gene sets across the endothelial, LepR+ stromal cells and NG2-cre- and LepR-cre-marked stromal cells. Consistent with the high degree of overlapping function, NG2-cre- and LepR-cre-marked stromal cells’ expression profiles closely clustered by various unsupervised clustering analyses, whereas Myh11-marked and CD31+ endothelial cells showed distinct expression vectors, while repeats were highly reproducible (Fig. 6a).

Enrichment analyses for differentially expressed gene sets across the cell types revealed enrichment for muscle-related terms in Myh11-creERTM-marked cells, including muscle system process (GO:0003012, adjusted P-value < 9.13 × 10−4, proportion test) and abnormal muscle contraction phenotype based on MGI’s Mammalian Phenotype ontology (MP0005620, adjusted P < 4.16 × 10−10). NG2-cre- and LepR-cre-labelled cells were enriched for extracellular matrix components (GO:0030198, adjusted P < 2.12 × 10−25), cell adhesion pathway members (hsa04510, KEGG, adjusted P < 0.000004), and genes highly expressed in osteoblasts (osteoblast day 21, Mouse Gene Atlas, adjusted P < 8.819 × 10−28) and human skin (GTEX, skin, female, 50–59 yr; P < 1.67 × 10−31). Enriched terms in the genes upregulated in the endothelial cells are angiogenesis (GO:0001525, adjusted P < 4.14 × 10−14) and targets of GATA2 as determined by ChIP-seq experiments from the ENCODE project conducted in umbilical vein endothelial cells (P < 2.52 × 10−21). Generally, all non-endothelial stromal cell populations expressed more genes associated with the HSC niche, except for Vcan1, E-selectin (Sele) or Delta-like ligand-1 (Dll1), which were more selectively expressed in endothelial cells (Fig. 6b). These results suggest distinct contributions of vascular-associated cells in niche activity.

DISCUSSION

Recent evidence indicates that HSCs are uniformly perivascular in the BM3–7. However, whether specific regions or structures of the BM provide defined microenvironments for distinct HSCs remains controversial. Results herein argue that such selected microenvironments exist and highlight the possibility of heterogeneity among niche factor-producing perivascular cells (Fig. 6c and Supplementary Fig. 6). While knock-in reporter mice have suggested stromal cells synthesizing Scf and Cxcl12 nearly completely overlap with each other19, the differential contributions of perivascular cell subsets argue that GFP reporters may not faithfully reflect the complexity of regulated secretion and presentation of niche factors in the local environment. That HSCs are not randomly distributed in the BM is also supported by the association and regulation of an HSC subset by the megakaryocyte27–29, and a recent study that has found that quiescent HSCs with low reactive oxygen species are localized near arterioles and megakaryocytes30. Notch signalling expands a CD31+ arteriole-ending endothelial cell population reduced in the ageing BM34. As lineage-biased HSCs are identified31–33, further studies will determine the extent by which HSC heterogeneity is matched by niche heterogeneity.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS

We thank C. Prophete, P. Ciero and C. Cruz for technical assistance and L. Tesfa, Y. Wang and D. Sun for help with cell sorting. We thank T. Nagasawa and S. Morrison for providing reagents. This work was supported by R01 grants from the National Institutes of Health (NIH) (DK056638, HL116340, HL097819 to P.S.F.), New York Stem Cell Foundation and NIH’s Common Fund (U54HL127624, U54CA189201 to A.M.). We are also grateful to the New York State Department of Health (NYSTEM Program) for shared facility (G29154) and research support (N13G-262) and the Leukaemia and Lymphoma Society’s Translational Research Program. Y.K. is supported by JSPS Grant-in-Aid for Scientific Research (B) (15H04859) and the Takeda Science Foundation. N.A. is supported by JSPS Postdoctoral Fellowships for Research Abroad.

AUTHOR CONTRIBUTIONS

N.A. performed most of the experiments and analysed data; H.P. performed CFU-C experiments; Z.W., N.E.F. and A.M. analysed RNA-seq data; A.B. bred Myh11-creERT2 mice; P.S.F. initiated and directed the study, N.A., Y.K. and P.S.F. interpreted data and wrote the manuscript. All of the authors contributed to the design of experiments, discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/nbt3475

Reprints and permissions information is available online at www.nature.com/reprints

1. Boulais, P. E. & Frenette, P. S. Making sense of hematopoietic stem cell niches. Blood 125, 2621–2629 (2015).
2. Yu, V. W. & Scadden, D. T. Heterogeneity of the bone marrow niche. Curr. Opin. Hematol. 23, 331–338 (2016).
3. Calvi, L. M. & Link, D. C. The hematopoietic stem cell niche in homeostasis and disease. Blood 126, 2443–2451 (2015).
4. Mendelson, A. & Frenette, P. S. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nat. Med. 20, 833–846 (2014).
5. Mendez-Ferrer, S. et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466, 829-834 (2010).
6. Sukujama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 25, 977–988 (2006).
7. Kiel, M. J. et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121, 1109–1121 (2005).
8. Omatsu, Y. et al. The essential functions of adipose-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* **33**, 387–399 (2010).
9. Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain hematopoietic stem cells. *Nature* **481**, 457–462 (2012).
10. Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**, 231–235 (2013).
11. Greenbaum, A. et al. CXCL12 in early mesenchymal progenitors is required for hematopoietic stem-cell maintenance. *Nature* **495**, 227–230 (2013).
12. Frenette, P. S., Pinho, S., Lucas, D. & Scheiermann, C. Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for regenerative medicine. *Annu. Rev. Immunol.* **31**, 285–316 (2013).
13. Kunisaki, Y. et al. Arteriolar niches maintain hematopoietic stem cell quiescence. *Nature* **502**, 637–643 (2013).
14. Yamazaki, S. et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* **147**, 1146–1158 (2011).
15. Acat, M. et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature* **526**, 126–130 (2015).
16. Chen, J. Y. et al. Hoxb5 marks long-term haematopoietic stem cells and reveals a homogenous perivascular niche. *Nature* **530**, 223–227 (2016).
17. Itkin, T. et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature* **532**, 323–328 (2016).
18. Kusumbe, A. P. et al. Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature* **532**, 380–384 (2016).
19. Mizoguchi, T. et al. Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev. Cell* **29**, 340–349 (2014).
20. Zhou, B. O., Yue, R., Murphy, M. M., Peyer, J. G. & Morrison, S. J. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* **15**, 154–168 (2014).
21. Khan, J. A. et al. Fetal liver hematopoietic stem cell niches associate with portal vessels. *Science* **351**, 176–180 (2016).
In vivo treatment. For induction of CreERT2, or Creβ2;β2-mediated recombination, two-week-old mice were injected intraperitoneally with 0.5 μg tamoxifen (Sigma) dissolved in corn oil (Sigma) twice a day for two rounds of 5 consecutive days with a 7-day interval.

Flow cytometry and cell sorting. For the analyses of haematopoietic cells, BM cells were flushed and dissociated by gently passing through a 21G needle. Spleen cells were obtained by grinding and passing through a 70 μm nylon filter. Ammonium chloride was used for red blood cell (RBC) lysis. For analysis of stromal cells, BM cells were flushed and digested with 1 mg/ml collagenase IV (Gibco) and 2 mg/ml dispase (Gibco) in Hank’s balanced salt solution (Gibco) for 30–45 min at 37 °C. After lysing RBCs with ammonium chloride, cells were filtered through 100 μm nylon mesh. For analysis of newborn liver cells, livers were minced into small pieces with scissors and digested with 3 mg/ml type 1 collagenase (Sigma) in HBSS with shaking for 5 min. Digested cells were dissociated by passing through an 18G needle and a 21G needle. After lysing RBCs with ammonium chloride, cells were filtered through 70 μm nylon mesh. For Flow cytometry analysis, cells were stained with antibodies in PBS containing 0.5% BSA and 2 mM EDTA buffer for 20–60 min on ice. For cell sorting, we used L-15 FACS buffer (Invitrogen) as a sheath fluid. The following antibodies were used: allophyocyanin (APC)-anti-Gr-1 (eBioscience, cat. no. 17-5931, clone R6-8C5), phycoerythrin (PE)-anti-CD11b (eBioscience, cat. no. clone M1/70), APC-eFlour780-anti-CD45R (eBioscience, cat. no. 47-0452, clone RA3-682), PerCP-Cy5.5-anti-CD3e (eBioscience, cat. no. 45-0031, clone 145-2C11), biotin-anti-Lineage (TER119, RB6-8C5, RA3-682, MA170, 14–2C11, BD Biosciences, cat. no. 559971), fluorescein isothiocyanate (FITC)-anti-CD41 (eBioscience, cat. no. 555195), PE-anti-CD150 (eBioscience, cat. no. 13-0411, MW6-327), APC-eFlour670-anti-CD144 (Biolegend, cat. no. 130806, clone BV13, 1:100), PE-anti-CD150 (Biolegend, cat. no. 115904, clone TC15-12F12, 1:100). The secondary antibody used was streptavidin eFlour450 (eBioscience, cat. no. 48-4317, 1:100).

For preparation of frozen sections of long bones, femoral bones were perfusion-fixed and then post-fixed with 4% PFA for 30 min. Bones were decalcified in 10% EDTA (pH 7.4) for 3 days at room temperature, and were incubated in 30% sucrose/PBS for cryoprotection and embedded in SCAE embedding medium (SECTION-LAB). Twenty-micrometre-thick frozen sections were cut with a Cryostat (CM3050 S, Leica) using Kawamoto’s tape transfer method. For immunofluorescence staining of femoral bone sections, after rinsing with PBS, slides were blocked with PBS containing species-matched serum. Slides were incubated with a primary antibody overnight at 4 °C, visualized by fluorescent-conjugated secondary antibodies, and mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories). The primary antibodies used were anti-aggreкан (Abcam, AB1031, 1:400), anti-ostecalcin (TAKARA, M188, clone R21C-01A, 1:400), and anti-peri-tpin (Cell Signaling Technology, cat. no. 9349, clone D1D8, 1:1,000). The secondary antibodies used were Alexa Fluor 647-anti-rat IgG (Molecular Probes, A-11011), Alexa Fluor 488-anti-rabbit IgG (Molecular Probes, A-21202), and Alexa Fluor 647-anti-rabbit IgG (Molecular Probes, A32733). All images were acquired using a ZEISS AXIOMI EXAMER 1 microscope (Zeiss) with a confocal scanner unit, CSUX19 (Yokohama), and reconstructed in three dimensions with Slide Book software (Intelligent Imaging Innovations).

Blood cell count. Blood cells were analysed on an ADVIA 120 haematology system (SIEMENS).
cells, and CD31+ endothelial cells was extracted using the RNAeasy Plus Micro kit (Qiagen). The integrity and purity of total RNA were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Complementary DNA was generated using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech Laboratories) from 1 ng of total RNA. The Nextera XT DNA Sample Preparation Kit (Illumina) was used for preparation of DNA libraries. The libraries were then submitted for Illumina HiSeq2500 sequencing (Illumina) according to the standard operation procedure.

**RNA-Seq analysis.** RNA-Seq data generated from Illumina HiSeq 2500 were processed following a recently developed reproducible pipeline\(^\text{37}\). Briefly, paired-end sequencing reads were aligned to the mouse genome using Spliced Transcripts Alignment to a Reference (STAR)\(^\text{38}\). We next used featureCount\(^\text{39}\) to assign aligned reads to genes. Count per million (CPM) was used as the expression quantification method. The CPM matrix was log\(_2\)-transformed and Z-score-scaled before performing principal component analysis (PCA) and hierarchical clustering (HC).

To identify signature genes for each cell population, we normalized the read count matrix using Voom\(^\text{40}\) and performed gene-wise moderated F-tests to test whether a gene is differentially expressed across the four cell populations. Nominal P values were corrected using the Benjamini–Hochberg procedure to adjust for multiple hypothesis testing. Signature genes for each cell population were identified using the following criteria: adjusted P value < 0.001 and log\(_2\)-fold change over at least 2 other cell types. Enrichment analyses for signature genes were performed using Enrichr\(^\text{29}\).

**Competitive transplantation.** Competitive transplantation assays were performed using the CD45.1/CD45.2 congenic system. Equivalent volumes of BM cells collected from gene-deleted mice or control mice (CD45.2) were transplanted into lethally irradiated (12Gy) CD45.1 recipients with 0.3 × 10\(^6\) competitor CD45.1 cells. CD45.1/CD45.2 chimaerism of recipients’ blood was analysed up to 4 months after transplantation.

**Cell cycle analysis.** Cell cycle analysis was performed as described previously\(^\text{41}\). Briefly, BM cells were stained with surface markers, fixed in 2% PFA in PBS for 20 min, washed, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and stained with anti-Ki67 antibody and Hoechst 33342 (Sigma) at 20 μg ml\(^{-1}\) for 30 min.

**CFU-C assay.** CFU-C assay was performed by bleeding animals retro-orbitally into EDTA-containing tubes and subsequently centrifuging the whole blood over lympholyte-M (CEDARLANE) to separate red blood cells from mononuclear cells. The mononuclear cell fraction was collected and plated in CFU-C media (Stem cell technologies, 3534). After one week, colonies were counted and CFU-Cs per millilitre of blood were calculated per animal.

**Statistics and reproducibility.** All data are represented as mean ± s.e.m. of at least three independent experiments, unless otherwise noted in the figure legends. We used two-tailed Student’s t-tests for evaluating the significance of difference unless otherwise indicated. Two-sample Kolmogorov–Smirnov tests were used for comparisons of distribution patterns. Statistical analyses were performed using GraphPad Prism 6 or 7 software. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

No statistical method was used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome analyses.

**Data availability.** RNA sequencing data have been deposited in the Gene Expression Omnibus under accession number GSE89811. Statistical source data supporting Figs 1c,e, 2b and 5b and Supplementary Figs 1d–g and 4a have been provided in Supplementary Table 1. All data supporting the conclusion of this paper are available from the authors on request.

36. Kawamoto, T. & Shimizu, M. A method for preparing 2- to 50-micron-thick fresh-frozen sections of large samples and undecalcified hard tissues. Histochem. Cell Biol. 133, 331–339 (2000).
37. Wang, Z. & Ma’ayan, A. An open RNA-Seq data analysis pipeline tutorial with an example of reprocessing data from a recent Zika virus study. F1000Res. 5, 1574 (2016).
38. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2013).
39. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 15, 1–17 (2014).
40. Law, C. W. et al. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts.
Supplementary Figure 1 Characterization of NG2-cre derived BM stromal cells. (a-c) Fate mapping study of NG2-cre targeted cells. Immunofluorescence staining of osteocalcin (a), perilipin (b), and aggrecan (c) in femur bone marrow sections from NG2-cre/ iTdTomato mice. Representative images from 3 mice. Scale bars, 200 μm in low power field, 20 μm in high power field. (d) Representative FACS plots showing the percentage of TdTomato positive cells within CD45−TER119−VE-cadherin positive endothelial cells. n=3 mice. (e) Representative FACS plots showing the percentage of NG2-cre/ iTdTomato positive cells within CD45−TER119−CD31+ endothelial cells. n=4 mice. (f) Representative FACS plots showing the percentage of Nes-GFPdim and Nes-GFPbright within CD45−TER119−CD31−LepR-cre/ TdTomato+ stromal cells (upper right), and of LepR-cre/ TdTomato+ cells within CD45−TER119−CD31−Nes-GFPbright cells (lower right). n=4 mice. (g) Representative FACS plots showing the percentage of Nes-GFPdim and Nes-GFPbright within CD45−TER119−CD31−NG2-cre/ TdTomato+ stromal cells (upper right), and of NG2-cre/ TdTomato+ cells within CD45−TER119−CD31−Nes-GFPbright cells (lower right). n=3 mice. (h) Whole-mount images of the sternum from NG2-cre/ iTdTomato mice stained with anti-NG2 antibody. Representative images from 3 mice. Scale bars, 20 μm. All panels show the same area for different channels (NG2-cre, NG2 and merged fluorescence images with DAPI). Data are represented as mean ± SEM. Statistics Source Data are available in Supplementary Table1.
Supplementary Figure 2  Cxcl12-GFP expression of LepR-cre marked stromal cells. (a) Whole-mount sternum images from LepR-cre/iTdTomato/Cxcl12-GFP mice stained with anti-VE-cadherin antibody. All panels show the same area for different channels (LepR-cre, Cxcl12-GFP, VE-cadherin and merged fluorescence images). Representative images from 3 mice. Scale bars, 20μm. (b) Representative FACS plots showing the percentage of LepR-cre/iTdTomato positive cells within CD45−TER119−CD31−Cxcl12-GFP+ cells. (c) Representative histograms showing intracellular Cxcl12 protein level of each fraction in CD45−TER119−CD31− cells from NG2-cre/Cxcl12fl/gfp mice. Representative histograms from 3 mice.
Supplementary Figure 3 Deletion of Cxcl12 from peri-sinusoidal niche cells.

(a) NG2-cre/Cxcl12lox/− mice enable Cxcl12 deletion from both arteriole associated Nes-GFP⁺, NG2⁺ cells and more broadly distributed Nes-GFP⁺, LepR⁺ stromal cells. (b) Cxcl12 mRNA expression relative to β-actin in CD45⁻TER119⁻CD31⁻LepR⁺ cells from LepR-cre(-) Cxcl12fl/⁻ and LepR-cre(+) Cxcl12fl/⁻ mice. n=4 mice for cre (-), n=5 mice for cre (+). (c) Analyses of LepR-cre/ Cxcl12fl/⁻ mice. Absolute numbers of lineage⁻ Sca-1⁺ c-kit⁺ (LSK) cells in the blood (left) n=6 mice, CFU-C in the blood (middle) n=5 mice from 2 independent experiments, and HSCs in spleen (right) n=6 mice. (d) Quantitative real-time PCR of Scf, Vcam-1, and Angiopoietin-1 (Angpt-1) relative to β-actin in sorted CD45⁻TER119⁻CD31⁻Nes-GFP⁺ stromal cells from NG2-cre(-) Cxcl12fl/+ or NG2-cre(+) Cxcl12fl/+ mice. n=4 mice for cre (-), n=3 mice for cre (+), from two independent experiments. (e) Absolute numbers of CD45⁻TER119⁻CD31⁻Nes-GFP⁺ cells from NG2-cre(-) Cxcl12fl/⁻ and NG2-cre(+) Cxcl12fl/⁻ mice. n=4 mice for cre (-), n=3 mice for cre (+), from two independent experiments. (f-i) Analyses of NG2-cre/ Cxcl12fl/⁻ mice. (f) The percentages of CD45.2 donor-derived cells in competitive reconstitution of bone marrow cells. The number of X-axis indicates the time (week) after transplantation. n=5 mice per group. (g) Absolute numbers of common myeloid progenitor (CMP), granulocyte monocyte progenitor (GMP), and megakaryocyte erythroid progenitor (MEP) in the BM (left). Absolute numbers of common lymphoid progenitor (CLP) in the BM (right). n=6 mice. (h) Representative FACS plots (CD150⁺CD48⁻LSK gated) of cell cycle of HSCs with Ki-67 and Hoechst 33342 staining. (i) Cellularity (left) and absolute number of phenotypic HSCs (right) in P0 newborn liver. n=7 mice for cre (-), n=5 mice for cre (+). Data are represented as mean ± SEM.
Supplementary Figure 4 Niche factor deletion from peri-arteriolar niche cells. (a) Representative histogram showing the percentage of Nes-GFP<sup>Δ</sup> and Nes-GFP<sup>+</sup> cells within CD45<sup>-</sup> TER119<sup>-</sup> CD31<sup>-</sup> NG2-cre<sup>ERTM</sup>/iTdTomo<sup>+</sup> cells. n=3 mice. (b) Representative FACS plots showing the gating strategy for sorting of Lineage<sup>-</sup> CD31<sup>-</sup> Nes-GFP<sup>-</sup> NG2-DsR<sup>-</sup> PDGFRβ<sup>+</sup> cells. Blue and red lines represent isotype control and anti-PDGFRβ antibody, respectively. (c) Gene expression analysis of Cxcl12 and Scf in sorted Lineage<sup>-</sup> CD31<sup>-</sup> Nes-GFP<sup>-</sup> NG2-DsR<sup>-</sup> cells. Lineage<sup>-</sup> CD31<sup>-</sup> Nes-GFP<sup>-</sup> NG2-DsR<sup>-</sup>, and Lineage<sup>-</sup> CD31<sup>-</sup> Nes-GFP<sup>-</sup> NG2-DsR<sup>-</sup> PDGFRβ<sup>+</sup> cells. n=4 mice from two independent experiments. (d) Whole-mount images of stromum from NG2-cre<sup>ERTM</sup>/Cxcl12<sup>GFP</sup>/ iTdTomo<sup>+</sup> mice stained with anti-VE-cadherin and merged fluorescence images with DAPI. Representative images from 3 mice. Scale bars, 20 µm. (e) Whole-mount images of stromum from NG2-DsRed/ Cxcl12-GFP/iTdTomo<sup>+</sup> mice stained with anti-VE-cadherin antibody and DAPI. Representative images from 3 mice. Scale bars, 20 µm. (f) Arterioles stained with anti-VE-cadherin antibody, respectively. (g) NG2-cre<sup>ERTM</sup>/Cxcl12<sup>GFP</sup> mice were analysed at 7-8 weeks after tamoxifen treatment. n=3 mice from two independent experiments. (h) Analyses of NG2-cre<sup>ERTM</sup>/ Cxcl12<sup>GFP</sup> mice. (h) The percentages of CD45.2 donor-derived cells in competitive reconstitution of bone marrow cells, n=8 mice for cre (-), n=13 mice for cre (+). (i) Absolute numbers of lineage cells in the blood. n=5 mice for cre (-), n=8 mice for cre (+). (j) Whole-mount images of stromum from Myh11-cre<sup>ERT2</sup>/ iTdTomo<sup>+</sup> mice stained with anti-NES antibody and DAPI. Representative images from 3 mice. Scale bars, 100 µm. (k) Representative FACS plots showing the percentage of NES-positive cells within CD45<sup>-</sup> TER119<sup>-</sup> CD31<sup>-</sup> Myh11-cre<sup>ERT2</sup>/ iTdTomo<sup>+</sup> positive cells. Representative data from 3 mice from 2 independent experiments. (l) Histogram showing intracellular Cxcl12 protein level of CD45<sup>-</sup> TER119<sup>-</sup> CD31<sup>-</sup> endothelial cells and CD45<sup>-</sup> TER119<sup>-</sup> TdTomo<sup>+</sup> cells from Myh11-cre<sup>ERT2</sup>/iTdTomo<sup>+</sup> mice (left). Quantification of MFI of intracellular Cxcl12 protein (right). n=3 mice from two independent experiments. (m) Gene expression analysis of Myh11 mRNA in sorted CD45<sup>-</sup> TER119<sup>-</sup> CD31<sup>-</sup>, CD45<sup>-</sup> TER119<sup>-</sup> Cxcl12<sup>-</sup> TdTomo<sup>+</sup>, CD45<sup>-</sup> TER119<sup>-</sup> Cxcl12<sup>-</sup> TdTomo<sup>-</sup> stromal cells. Myh11-cre<sup>ERT2</sup>/iTdTomo<sup>+</sup> mice were analysed at 7-8 weeks after tamoxifen treatment. n=4 mice from two independent experiments. Data are represented as mean ± SEM. Statistical significance was assessed using two-tailed t-test (h, i, l) and one-way ANOVA (c, g, m). Statistics Source Data are available in Supplementary Table1.
**Supplementary Figure 5** Sca deletion from peri-vascular niche cells.

(a) Whole-mount images of the sternum from NG2-cre^{ERTM}/Scf-GFP/ iTdTomato mice stained with anti-VE-cadherin antibodies. All images show the same area for different channels (Scf-GFP, NG2-cre^{ERTM}, VE-cadherin and merged fluorescence images). Representative images from 3 mice. Scale bars, 20 μm. (b) In LepR-cre/Scf^{flox/-} mice, Scf is deleted from broadly distributed Nes-GFP^{+}, LepR^{+} stromal cells, but not from the arteriole-associated Nes-GFP^{+}, NG2^{+} stromal cells. (c-f) Analysis of NG2-cre/Scf^{flox/-} mice. (c) The percentages of CD45.2 donor-derived cells in competitive reconstitution of bone marrow cells. n=5 mice for cre (-), n=7 mice for cre (+). (d) Absolute number of LSK cells in the blood (left) and HSCs in the spleen (right). n=5 mice for cre (-), n=7 mice for cre (+). (e) Differential leukocyte counts in the blood (left) and spleen (right). n=5 mice for cre (-), n=7 mice for cre (+). (f) Cellularity (left) and absolute number of phenotypic HSCs (right) in P0 newborn liver of NG2-cre/Scf^{flox/-} mice. n=8 mice for cre (-), n=10 mice for cre (+). (g,h) Analysis of NG2-cre^{ERTM}/Scf^{flox/-} mice. (g) The percentages of CD45.2 donor-derived cells in competitive reconstitution of bone marrow cells. n=9 mice for cre (-), n=7 mice for cre (+). (h) Absolute numbers of HSCs in the spleen (left) and LSK cells in the blood (right). n=8 mice for cre (-), n=6 mice for cre (+). Data are represented as mean ± SEM. Statistical significance was assessed using two-tailed t-test (c-h).
Supplementary Figure 6 Proposed model of HSC regulation by distinct perivascular niche cells via different cytokines. Cxcl12, derived from NG2-expressing Nes-GFP$^+$ stromal cells, closely associates with arterioles, regulating HSC maintenance. HSC distributions relative to arterioles are altered after Cxcl12 deletion in arteriole-associated stromal cells, suggesting that Cxcl12 derived from these niche cells may promote HSC tethering to the proper microenvironment for their maintenance. Non-myelinating Schwann cells regulating quiescence of HSCs are also tightly associated with arterioles, which suggest the possibility of coordination for HSC regulation in the peri-arteriolar niche. On the other hand, Cxcl12 derived from broadly distributed LepR-expressing, Nes-GFP$^+$ stromal cells controls mobilization of HSCs to the circulation. Uniformly distributed LepR-expressing Nes-GFP$^+$ stromal cells are the main source of Scf for HSC maintenance.
Supplementary Table Legends

Supplementary Table 1 Statistic source data of main and supplementary figures.