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Endothelial and perivascular cells maintain haematopoietic stem cells

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Abstract

Multiple cell types have been proposed to create niches for haematopoietic stem cells (HSCs). However, the expression patterns of HSC maintenance factors have not been systematically studied and no such factor has been conditionally deleted from any candidate niche cell. Thus, the cellular sources of these factors are undetermined. Stem Cell Factor (SCF) is a key niche component that maintains HSCs. Using Scf^{gfp} knock-in mice we found *Scf* was primarily expressed by perivascular cells throughout bone marrow. HSC frequency and function were not affected when *Scf* was conditionally deleted from haematopoietic cells, osteoblasts, *Nestin-Cre*, or *Nestin-CreER*-expressing cells. However, HSCs were depleted from bone marrow when *Scf* was deleted from endothelial cells or *Leptin receptor* (*Lepr*)-expressing perivascular stromal cells. Most HSCs were lost when *Scf* was deleted from both endothelial and *Lepr*-expressing perivascular cells. HSCs reside in a perivascular niche in which multiple cell types express factors that promote HSC maintenance.

Stem cells are maintained in specialized microenvironments in tissues, termed niches, in which supporting cells secrete factors that promote stem cell maintenance[1]. In most mammalian tissues, including the haematopoietic system, the identities of the cells that promote stem cell maintenance remain uncertain[1,2]. One popular model of the HSC niche proposed that osteoblasts express many factors that promote HSC maintenance[3], including SCF, CXCL12, Angiopoietin-1, and Thrombopoietin[4-7]. However, none of these factors

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have been conditionally deleted from osteoblasts, so there is no direct evidence that osteoblasts are a functionally important source of these factors.

We found that most HSCs localize adjacent to sinusoidal blood vessels throughout the bone marrow[8,9]. This led us to hypothesize that the HSC niche is perivascular[2,9]. Others found that perivascular stromal cells secrete high levels of CXCL12 and other factors proposed to regulate HSC maintenance[10,11]. *Nestin*-expressing mesenchymal stem cells also localize adjacent to blood vessels in the bone marrow and express factors thought to promote HSC maintenance[12]. Ablation of the *Cxcl12*-expressing cells or the *Nestin*-expressing cells reduced HSC frequency[12,13]. Administration of antibodies against endothelial cells in vivo impairs HSC engraftment and transformed endothelial cells promote HSC expansion in culture[14,15]. Nonetheless, no factor that promotes HSC maintenance has been conditionally deleted from any perivascular cell so there is no direct evidence that endothelial or perivascular cells are functionally important sources of factors for HSC maintenance.

SCF is non-cell-autonomously required for HSC maintenance in vivo [16-19]. Differential splicing and proteolytic cleavage lead to the expression of a membrane-bound form and a soluble form of SCF. HSCs are depleted in Sl/Sl^{d} mutant mice[20], which express soluble SCF but lack the membrane-bound form, indicating that membrane-bound SCF is particularly important for HSC maintenance[21]. Mice with a mixture of wild-type and Sl/Sl^{d} stromal cells only exhibit normal haematopoiesis in the immediate vicinity of the wild-type cells, demonstrating that SCF acts locally in creating the niche[22].

Scf has been suggested to be expressed by endothelial cells, bone marrow fibroblasts, osteoblasts, *Cxcl12*-expressing perivascular stromal cells, and *Nestin*-expressing mesenchymal stem cells[5,12,13,23-25] but *Scf* has not been conditionally deleted to test which source(s) are functionally important for HSC maintenance. We generated Scf^{gfp} and Scf^{fl} mice to systematically examine *Scf* expression and to conditionally delete *Scf* from subpopulations of bone marrow cells.

Scf is expressed by perivascular cells

We generated Scf^{gfp} knock-in mice by inserting *Egfp* into the endogenous *Scf* locus (Supplementary Fig. 1a-c). $Scf^{gfp/gfp}$ mice died perinatally (Fig. 1a; Supplementary Fig. 1f, g) with severe anemia (Fig.1b; Supplementary Fig. 2c) as observed in mice with a strong loss of SCF/c-Kit function[17]. By quantitative reverse transcription PCR (qRT-PCR) *Scf* transcripts were nearly undetectable in $Scf^{gfp/gfp}$ newborn liver (Fig. 1c).

The overall cellularity of the newborn liver was reduced about 2-fold in $Scf^{gfp/+}$ and about 5-fold in $Scf^{gfp/gfp}$ mutant mice compared to $Scf^{+/+}$ controls (Fig. 1d). The frequency of HSCs ($CD150^{+}CD48^{-}CD41^{-}Sca1^{+}cKit^{+}$ cells[9,26]) in the newborn liver was reduced about 8-fold in $Scf^{gfp/gfp}$ mutant mice compared to littermate $Scf^{gfp/+}$ or $Scf^{+/+}$ controls (Fig. 1e). Consistent with this, newborn $Scf^{gfp/gfp}$ liver cells gave significantly lower levels of donor cell reconstitution in irradiated mice compared to $Scf^{gfp/+}$ or $Scf^{+/+}$ controls (Fig. 1f; Supplementary Fig. 2d). $Scf^{gfp/gfp}$ mice therefore have a severe loss of SCF function.

By flow cytometry, only rare (0.027±0.0099%, mean±s.d.) enzymatically dissociated bone marrow cells were positive for GFP. The actual frequency of GFP^+ cells in the bone marrow may be somewhat higher as our dissociation conditions may not recover all of the GFP^+ stromal cells. These GFP^+ cells were negative for CD45 and Ter119, indicating a non-haematopoietic source of SCF (Fig. 1g). Endogenous *Scf* transcripts were highly enriched in GFP^+ stromal cells and highly depleted in GFP negative stromal cells (Suppl. Fig. 2f, g), suggesting GFP expression faithfully reflected endogenous *Scf* expression.

GFP was mainly expressed by cells surrounding sinusoids throughout the $Scf^{gfp/+}$ bone marrow, with some expression by cells surrounding venuoles and arterioles (Fig. 1h-m; Supplementary Fig. 2h, i). GFP partially overlapped with endothelial marker staining (Fig. 1h-j; o-q; Supplementary Fig. 2i), suggesting that both endothelial and perivascular stromal cells express *Scf*. In contrast, GFP was not concentrated near the endosteum (Supplementary Fig. 2h) and we did not detect any GFP expression by bone-lining cells that expressed osteoblast markers in either the diaphysis (Fig. 1k-m) or trabecular bone (Fig. 1n). Perivascular stromal and endothelial cells therefore appeared to represent the major sources of SCF in bone marrow.

We isolated *Scf*-GFP+ cells by flow cytometry and performed gene expression profiling. Several mesenchymal stem/stromal cell markers, including *Cxcl12, alkaline phosphatase, Vcam1, Pdgfr*α and *Pdgfr*β were highly elevated in *Scf*-GFP+ cells relative to whole bone marrow cells (Supplementary Table 1). This suggests that *Scf*-GFP+ cells included mesenchymal stem/stromal cells[27] and *Cxcl12*-expressing perivascular stromal cells[10]. *Nestin* was not detected in *Scf*-GFP+ perivascular cells (Supplementary Table 1).

As we observed previously[8,9], $CD150^+CD48^-Lineage^-$ candidate HSCs were mainly found adjacent to sinusoidal blood vessels throughout the bone marrow. 65% (47/73) of all $CD150^+CD48^-Lineage^-$ cells were immediately adjacent to GFP-expressing stromal cells (Fig. 1r-u). Almost all of the remaining cells (30%; 22/73) were within 5 cell diameters of GFP-expressing cells. This suggests *Scf*-GFP-expressing cells form a perivascular niche for HSCs.

Scf is required by adult HSCs

We generated a floxed allele of *Scf* (Scf^{fl}) to conditionally delete *Scf* from candidate niche cells (Supplementary Fig. 3a-c). Mice homozygous for the germline recombined allele of *Scf*, $Scf^{-/-}$, were perinatal lethal and anemic (Fig. 2a) similar to other *Scf*-deficient mice (Fig. 1a and ref[17]). Recombination of the Scf^{fl} allele therefore gave a strong loss of SCF function. We were unable to amplify *Scf* transcripts by PCR from the liver of $Scf^{-/-}$ newborns (Fig. 2b).

We generated *Ubc-CreER;* $Scf^{fl/fl}$ mice to ubiquitously delete *Scf* upon tamoxifen administration. We administered tamoxifen-containing chow to *Ubc-CreER;* $Scf^{fl/fl}$ mice and littermate controls for 1-2 months beginning at 8 weeks of age then sacrificed them for analysis. Some of the mice became anemic and ill during tamoxifen administration. The *Ubc-CreER;* $Scf^{fl/fl}$ mice had significantly lower red blood cell counts than controls (Fig. 2c) and a trend toward lower white blood cell and platelet counts (Supplementary Fig. 3d). *Ubc-*

CreER; Scf$^{fl/fl}$ mice exhibited approximately 2-fold reductions in the overall cellularity of bone marrow and spleen compared to controls (Fig. 2d).

CD150^{+}CD48^{-}Lin^{-}Sca1^{+}c-Kit^{+} HSCs were also depleted in the bone marrow and spleen of *Ubc-CreER; Scf*$^{fl/fl}$ mice compared to controls treated concurrently with tamoxifen (Fig. 2e). Limit dilution analysis demonstrated that long-term multilineage reconstituting cells were 3.5-fold less frequent in the bone marrow of *Ubc-CreER; Scf*$^{fl/fl}$ mice compared to controls upon transplantation into irradiated mice (Fig. 2f). Bone marrow cells from *Ubc-CreER; Scf*$^{fl/fl}$ mice gave significantly lower levels of donor cell reconstitution in irradiated mice (Fig. 2g). These data confirmed that SCF is required for HSC maintenance in adult mice.

CD150^{+}CD48^{-}Lin^{-}Sca1^{+}cKit^{+} HSCs from *Scf*$^{gfp/+}$ mice did not express GFP by flow-cytometry (Fig. 2h). This is consistent with prior studies[17,21,22] in suggesting that *Scf* non-cell-autonomously regulates HSC maintenance. To test the role of other haematopoietic cells we conditionally deleted *Scf* using *Vav1-Cre*. As expected[28], *Vav1-Cre* recombined a conditional *loxpEYFP* reporter[29] in virtually all HSCs, CD45^{+} and Ter119^{+} haematopoietic cells (Fig. 3a; Supplementary Fig. 4a). Eight week-old *Vav1-Cre; Scf*$^{fl/-}$ mice exhibited normal blood cell counts, bone marrow composition (Supplementary Fig. 4b, c), and bone marrow and spleen cellularity (Fig. 3b). *Scf*$^{+/-}$ heterozygous mice exhibited a 2-fold decline in the frequency of CD150^{+}CD48^{-}Lin^{-}Sca1^{+}c-Kit^{+} HSCs relative to wild-type littermates. However, deletion of the second allele of *Scf* from haematopoietic cells in *Vav1-Cre; Scf*$^{fl/-}$ mice did not further reduce HSC frequency in the bone marrow or spleen (Fig. 3c). Bone marrow cells from adult *Vav1-Cre; Scf*$^{fl/-}$ mice had a normal capacity to reconstitute irradiated mice (Fig. 3d; Supplementary Fig. 4d) and to form colonies in methylcellulose (Supplementary Fig. 4e, f). Therefore, *Scf* expression by haematopoietic cells is not required for HSC maintenance in adult bone marrow.

HSCs do not require SCF from osteoblasts

Col2.3-Cre recombines genes in fetal and postnatal osteoblasts[30]. Consistent with this we found strong EYFP expression among bone-lining cells in *Col2.3-Cre; loxpEYFP* mice (Fig. 3e). To test whether osteoblasts produce SCF for HSC maintenance we analyzed 8 week-old *Col2.3-Cre; Scf*$^{fl/-}$ mice. *Col2.3-Cre; Scf*$^{fl/-}$ mice had normal blood counts (Supplementary Fig. 5a), normal lineage composition in the bone marrow and spleen (Supplementary Fig. 5b) and normal bone marrow and spleen cellularity (Fig. 3g). Although *Scf*$^{+/-}$ germline heterozygous mice exhibited a 2-fold decline in the frequency of CD150^{+}CD48^{-}Lin^{-}Sca1^{+}c-Kit^{+} HSCs relative to wild-type littermates, conditional deletion of the second allele of *Scf* from osteoblasts in *Col2.3-Cre; Scf*$^{fl/-}$ mice did not further reduce HSC frequency in the bone marrow or spleen (Fig. 3h). Bone marrow cells from *Col2.3-Cre; Scf*$^{fl/-}$ mice had a normal capacity to reconstitute irradiated mice (Fig. 3i; Supplementary Fig. 5c) and to form colonies in methylcellulose (Supplementary Fig. 5d, e). Therefore, *Scf* expression by osteoblasts is not required for HSC maintenance in adult bone marrow.

HSCs do not require SCF from *Nestin*$^{+}$ cells

In *Nestin-Cre; loxpEYFP* mice we found rare EYFP-expressing perivascular stromal cells around larger blood vessels, not sinusoids, in the bone marrow (Fig. 3f). These cells

exhibited a very different distribution than *Scf*-expressing cells (compare Fig. 3f to Fig. 1h-m and Supplementary Fig. 2h, i). Eight week-old *Nestin-Cre; Scf*$^{fl/-}$ mice had normal blood cell counts (Supplementary Fig. 6b), normal lineage composition and cellularity in the bone marrow and spleen (Supplementary Fig. 6c; Fig. 3j). Comparing *Nestin-Cre; Scf*$^{fl/-}$ mutants with *Scf*$^{+/-}$controls, deletion of *Scf* from *Nestin-Cre*-expressing cells did not reduce HSC frequency in the bone marrow (Fig. 3k). *Nestin-Cre; Scf*$^{fl/-}$ mice did exhibit a significant decline in HSC frequency in the spleen (Fig. 3k), raising the possibility that *Nestin-Cre*-expressing cells are a component of the HSC niche in the spleen. Bone marrow cells from adult *Nestin-Cre; Scf*$^{fl/-}$ mice had a normal capacity to reconstitute irradiated mice (Fig. 3l; Supplementary Fig. 6d). Conditional deletion of *Scf* by administering tamoxifen for 2-5 months to adult *Nestin-CreER; Scf*$^{fl/fl}$ mice also did not affect hematopoiesis, HSC frequency, or reconstituting capacity in irradiated mice (Supplementary Fig. 7). Therefore, *Scf* expression by *Nestin-Cre*-expressing or *Nestin-CreER*-expressing perivascular cells is not required for the maintenance of HSCs in adult bone marrow.

Since *Nestin*-GFP-expressing bone marrow cells express *Scf*[12], we independently characterized *Nestin*-GFP expression. Consistent with the prior report[12], we observed strong *Nestin*-GFP staining along larger vessels in the bone marrow (Supplementary Fig. 8; see Supplementary Fig. 1 from ref[12]). *Nestin*-GFP was also observed in perisinusoidal stromal cells in a pattern that resembled *Scf*-GFP+ expression (Supplementary Fig. 8a). This appeared to be different from the *Nestin-Cre* expression pattern, which we detected only around larger blood vessels in the bone marrow (Fig. 3f). In *Nestin-Cherry* and *Nestin-GFP* double transgenic mice we detected *Nestin*-Cherry expression around larger vessels but not around sinusoids while *Nestin*-GFP was detected around both (Supplementary Fig. 8). Thus, different *Nestin* transgenes appear to be expressed by different subpopulations of perivascular stromal cells. *Nestin*-GFP appears to exhibit more expression in perisinusoidal stromal cells than other *Nestin* transgenes. Our data are therefore consistent with the possibility that *Nestin*-GFP-expressing stromal cells contribute to the HSC niche as suggested[12], even though conditional deletion of *Scf* with *Nestin-Cre* and *Nestin-CreER* did not affect HSC frequency.

HSCs require SCF from endothelial cells

We conditionally deleted *Scf* from endothelial cells using *Tie2-Cre*[31]. *Tie2-Cre* recombined in endothelial (Fig. 4a) and haematopoietic cells (Fig. 4b) but not in mesenchymal stem/stromal cells from the bone marrow (Supplementary Fig. 9d, e). Since haematopoietic cells do not express *Scf* (Fig. 1g; Fig. 2i) and conditional deletion of *Scf* from haematopoietic cells did not affect HSC frequency (Fig. 3a-d), the use of *Tie2-Cre* allowed us to test whether SCF expression by endothelial cells regulates HSC frequency.

Eight week-old *Tie2-Cre; Scf*$^{fl/-}$ mice exhibit normal blood cell counts (data not shown), bone marrow and spleen cellularity (Fig. 4c). However, the frequency of $CD150^{+}CD48^{-}Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ HSCs in the bone marrow was significantly reduced in *Tie2-Cre; Scf*$^{fl/-}$ mice relative to controls (Fig. 4d). Consistent with this, 300,000 bone marrow cells from *Tie2-Cre; Scf*$^{fl/-}$ mice gave significantly lower levels of donor reconstitution upon transplantation into irradiated mice (Fig. 4g). In five independent experiments, 24 of 25

recipients of *Scf*$^{+/+}$ cells, 15 of 15 recipients of *Scf*$^{+/-}$ cells, and only 7 of 21 recipients of *Tie2-Cre; Scf*$^{fl/-}$ cells were long-term multilineage reconstituted. By poisson statistics this corresponds to an HSC frequency in control bone marrow of at least 1/93,200 cells but only 1/739,900 in *Tie2-Cre; Scf*$^{fl/-}$ mice. Endothelial cells are therefore an important source of SCF for HSC maintenance.

The HSC depletion in *Tie2-Cre; Scf*$^{fl/-}$ mice likely reflects an ongoing need for SCF expression by endothelial cells in adult bone marrow because when HSCs are depleted as a consequence of reduced SCF/c-Kit signaling, HSC frequencies return to normal levels upon restoration of normal SCF/c-Kit signaling[19,21]. Nonetheless, we also examined whether SCF expression by endothelial cells during development is required by HSCs. We found a 1.7 to 2.1-fold reduction in HSC frequency in the liver of newborn *Tie2-Cre; Scf*$^{fl/-}$ mice (Fig. 4e) and a 2-fold reduction in HSC frequency in the bone marrow of one month-old *Tie2-Cre; Scf*$^{fl/-}$ mice (Fig. 4f) relative to *Scf*$^{+/-}$ and *Scf*$^{+/+}$ controls. The magnitude of HSC depletion in adult bone marrow appeared to increase as we found a 2.7-fold and 5.2-fold reduction in HSC frequency in the bone marrow of 8 week-old *Tie2-Cre; Scf*$^{fl/-}$ mice relative to *Scf*$^{+/-}$ and *Scf*$^{+/+}$ controls, respectively (Fig. 4d). These data suggest that ongoing SCF expression by endothelial cells in adult bone marrow contributes to HSC maintenance; however, HSC depletion in adult bone marrow may reflects a loss of SCF expression by endothelial cells during development.

HSCs require SCF from perivascular cells

We found that *Leptin receptor* (*Lepr*) is highly restricted in its expression within the bone marrow to *Scf*-GFP-expressing perivascular stromal cells (Supplementary Table 1). Consistent with this, *Lepr-Cre; loxpEYFP* mice exhibited EYFP expression in perivascular stromal cells (Fig. 5b, e) but not in haematopoietic cells (Fig. 5b, c, e), bone-lining cells (Fig. 5c), or endothelial cells (Fig. 5d).

Consistent with the gene expression profile of *Scf*-GFP+ cells (Supplementary Table 1), EYFP+ cells from *Lepr-Cre; loxpEYFP* mice did not detectably express *Nestin* but did express mesenchymal stem/stromal cell markers including *Cxcl12*, *alkaline phosphatase*, PDGFRα, and PDGFRβ (Supplementary Fig. 9a-c). These data suggest a mesenchymal identity for the *Lepr*-expressing stromal cells; however, the lack of EYFP expression in bone-lining cells from *Lepr-Cre; loxpEYFP* mice suggests that the *Lepr-Cre*-expressing perivascular cells did not give rise to osteoblasts during normal development. Future studies will be required to assess the relationship between *Lepr-Cre*-expressing perivascular cells, mesenchymal stem cells, and other perivascular stromal cells.

Bone marrow cellularity was significantly reduced in *Lepr-Cre; Scf*$^{fl/gfp}$ mice compared to *Scf*$^{+/+}$ controls, but not compared to *Scf*$^{+/gfp}$ controls (Fig. 5g). Spleen size (Fig. 5f) and cellularity were significantly increased in *Lepr-Cre; Scf*$^{fl/gfp}$ mice (Fig. 5g). Sections through the spleen revealed increased extramedullary haematopoiesis in *Lepr-Cre; Scf*$^{fl/gfp}$ mice (data not shown). The frequency of CD150^{+}CD48^{-}Lin^{-}Sca1^{+}c-Kit^{+} HSCs was significantly reduced in the bone marrow of *Lepr-Cre; Scf*$^{fl/gfp}$ mice, but significantly increased in the spleen (Fig. 5h). The total number of bone marrow and spleen HSCs per

mouse was significantly reduced in *Lepr-Cre; Scf*$^{fl/gfp}$ mice (Fig. 5i). *Lepr-Cre*-expressing cells thus promote HSC maintenance in the bone marrow, but not in the spleen, by producing SCF.

In limit dilution transplantation studies the frequency of long-term multilineage reconstituting cells in *Scf*$^{+/+}$ and *Scf*$^{+/gfp}$ control cells was 1/38,311 and 1/38,352, respectively (Fig. 5j). In *Lepr-Cre; Scf*$^{fl/gfp}$ bone marrow cells the frequency of long-term multilineage reconstituting cells was 1/78,136, significantly lower than in *Scf*$^{+/+}$ and *Scf*$^{+/gfp}$ controls (Fig. 5j). Thus conditional deletion of *Scf* from *Lepr-Cre*-expressing perivascular stromal cells depletes HSCs from adult bone marrow. The frequency of GFP+ cells in the bone marrow of *Lepr-Cre; Scf*$^{fl/gfp}$ mice did not significantly differ from *Scf*$^{gfp/+}$ controls (Supplementary Fig. 10), suggesting that *Scf* deletion did not lead to the death of *Lepr*-expressing cells.

The HSC depletion observed in *Lepr-Cre; Scf*$^{fl/gfp}$ mice did not reflect a developmental effect of SCF expression by *Lepr-Cre*-expressing cells as no HSC depletion was detected in the liver of newborn *Lepr-Cre; Scf*$^{fl/gfp}$ mice (Fig. 5k). Furthermore, the magnitude of the HSC depletion increased with time in the adult bone marrow (Fig. 5h, l).

To test whether deletion of *Scf* from endothelial and *Lepr*-expressing perivascular cells has additive effects on HSC depletion we analyzed 8 week-old *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$ mice. Bone marrow cellularity was significantly reduced in *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$ mice compared to *Tie2-Cre; Scf*$^{fl/-}$ and *Lepr-Cre; Scf*$^{fl/-}$ mice (Fig. 5m). Spleen cellularity was significantly increased in *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$ mice compared to *Scf*$^{+/-}$ or *Tie2-Cre; Scf*$^{fl/-}$ mice (Fig. 5m). HSC frequency was significantly reduced in the bone marrow of *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$ mice compared to *Tie2-Cre; Scf*$^{fl/-}$ or *Lepr-Cre; Scf*$^{fl/-}$ mice (Fig. 5n). The frequency and absolute number of HSCs in the bone marrow of *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$ mice was less than 5% of wild-type levels (Fig. 5m-o). This suggests endothelial and *Lepr*-expressing perivascular stromal cells are the major sources of SCF for HSC maintenance in normal adult bone marrow and that deletion of *Scf* from each cell population has additive effects on HSC depletion.

qRT-PCR revealed that endothelial and *Lepr*-Cre-expressing perivascular cells expressed both long and short splice isoforms of *Scf* rendering both cell types capable of expressing membrane-bound and soluble SCF (Supplementary Fig. 11). The levels of both isoforms of *Scf* in the two cell populations were significantly higher than in whole bone marrow cells, though *Lepr*-Cre-expressing cells expressed much higher levels of both isoforms compared to endothelial cells (Supplementary Fig. 11).

DISCUSSION

Our data demonstrate that HSCs reside in a perivascular niche in which endothelial and *Lepr*-expressing perivascular stromal cells are two functionally important components of the niche (Supplementary Fig. 12). The simplest interpretation of our data is that both cell types produce SCF for the maintenance of HSCs in adult bone marrow; however, endothelial cells also produce SCF for HSC maintenance/expansion during development so it is formally

possible that the depletion of bone marrow HSCs in adult *Tie2-Cre; Scf*$^{fl/-}$ mice reflects a developmental effect of endothelial SCF. Endothelial cells and perivascular stromal cells are probably not the only components of the HSC niche as other cell types likely contribute through mechanisms other than SCF production (e.g. refs[32,33]).

Lepr-Cre-expressing stromal cells did not express endogenous *Nestin* (Supplementary Fig. 9c). *Nestin-Cre, or Nestin-CreER* mediated deletion of *Scf* did not deplete HSCs (Fig. 3j-l; Supplementary Fig. 6-7). However, *Lepr-Cre*-expressing perisinusoidal cells do partially overlap with *Nestin-GFP* expressing perivascular cells (Supplementary Fig. 8; Supplementary Fig. 9a-c). The *Lepr-Cre*-expressing stromal cells therefore include stromal cells that express certain *Nestin* transgenes, consistent with Mendez-Ferrer et al[12] and may also include *Cxcl12*-abundant reticular (CAR) cells[10]. Perivascular stromal cells are likely heterogeneous and may include multiple cell types that contribute to HSC maintenance through different mechanisms.

While we have partially characterized the bone marrow niche for HSCs in adult mice under homeostatic conditions, other studies will be required to functionally characterize HSC niches in other haematopoietic tissues and after haematopoietic stress.

METHODS SUMMARY

Targeting vectors for making *Scf*^{gfp} and *Scf*^{fl} mice were constructed by recombineering[34]. The *Frt* flanked *Neo* cassette was removed by mating with *Flpe* mice[35]. *Scf*^{gfp} and *Scf*^{fl} mice were backcrossed onto a C57BL background before analysis. Mice used in this study included *Ubc-CreER* [36], *CMV-Cre*[37], *Vav1-Cre*[28], *Nestin-Cre*[38], *Tie2-Cre*[31], *Lepr-Cre*[39], and *LoxpEYFP*[29] (all from the Jackson Laboratory), *Col2.3-Cre*[30], *Nestin-CreER*[40] and *Nestin-GFP*[41]. All were maintained on a C57BL background. Unless otherwise indicated, data always reflect mean±s.d. and two-tailed student's t-tests were used to assess statistical significance (*p<0.05, **p<0.01, ***p<0.001). Detailed methods are available online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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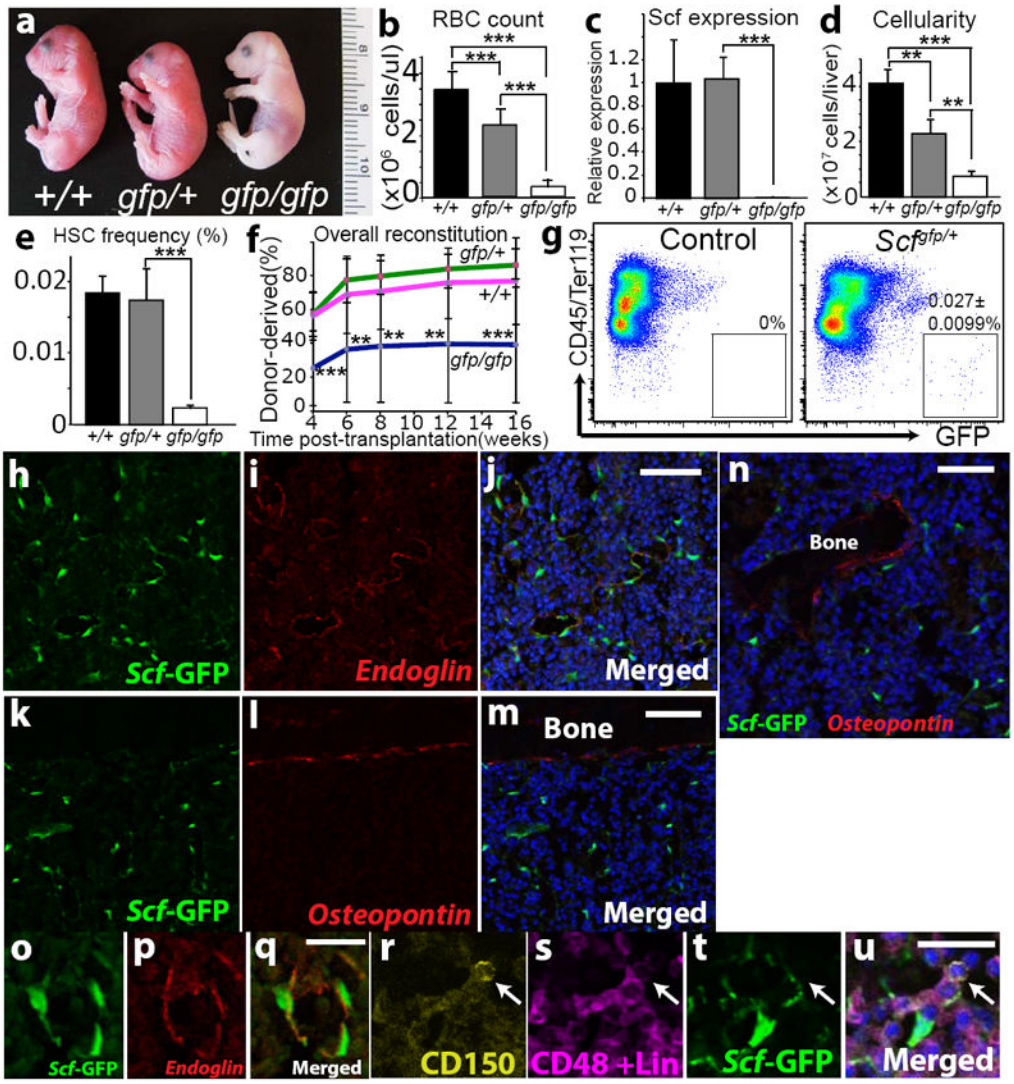

Figure 1. *Scf*^{gfp} is a strong loss-of-function allele and *Scf* is primarily expressed by perivascular cells in the bone marrow

a and **b**, *Scf*$^{gfp/gfp}$ homozygous mice died perinatally and were severely anemic (n=4-20). **c**, *Scf* transcripts in livers from newborn mice by qRT-PCR (n=3). **d** and **e**, Newborn liver cellularity and HSC frequency (n=4). **f**, Irradiated mice (CD45.1+) were transplanted with 3×10^5 newborn liver cells from *Scf*$^{gfp/gfp}$, *Scf*$^{gfp/+}$ or *Scf*$^{+/+}$ donor (CD45.2+) mice along with 3×10^5 recipient (CD45.1+) bone marrow cells (3-4 experiments with 13-18 mice/genotype). **g**, *Scf-GFP* was expressed by rare non-haematopoietic stromal cells (n=8). **h-j**, GFP was primarily expressed by perivascular cells in the bone marrow of *Scf*$^{gfp/+}$ mice. Endothelial cells were stained with an anti-*Endoglin* antibody. **k-n**, GFP was not detected in bone-lining osteoblast lineage cells (Osteopontin) in the diaphysis (**k-m**) or in trabecular bone (**n**). **o-q**, Higher magnification images of a sinusoid. **r-u**, A CD150$^+$CD48$^-$Lineage$^-$ candidate HSC (arrow) localized adjacent to a GFP-expressing perivascular cell. Nuclei were stained with DAPI (in blue). All data represent mean±s.d. Two-tail student's t-tests

were used to assess statistical significance: ** $p<0.01$, *** $p<0.001$. Scale bars in (**j**), (**m**) and (**n**) are 50um. Scale bars in (**q**) and (**u**) are 20um.

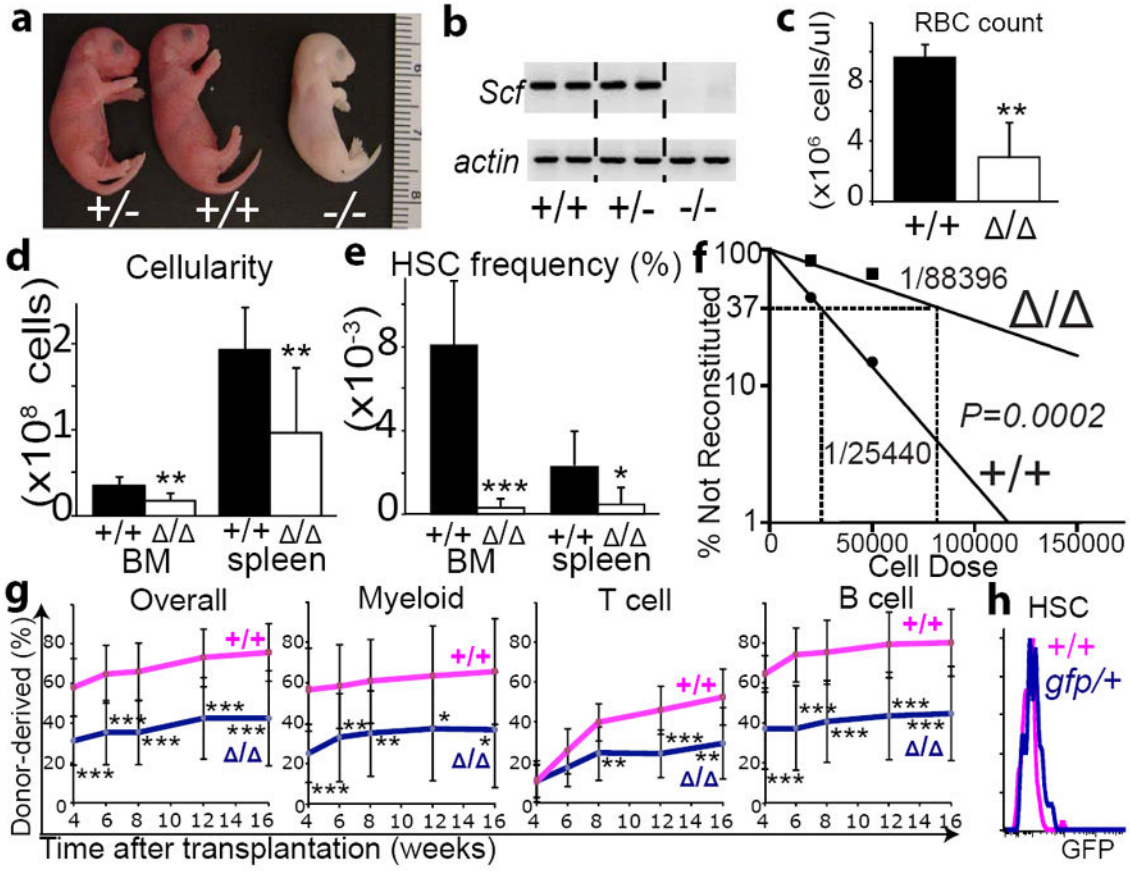

Figure 2. *Scf* is required for adult HSC maintenance

a, Homozygous $Scf^{-/-}$ mutant mice generated from germline recombination of the Scf^{fl} allele were perinatal lethal and anemic. **b**, *Scf* transcripts amplified by RT-PCR from the livers of newborn mice. **c**, Global deletion of *Scf* in *Ubc-CreER;* $Scf^{fl/fl}$ mice led to anemia (n=5-6). **d** and **e**, Global deletion of *Scf* in adult mice significantly reduced cellularity and HSC frequency in bone marrow (two femurs and two tibias) and spleen (n=8-10). **f**, To perform a limit dilution analysis[42], three doses of donor bone marrow cells were competitively transplanted into irradiated mice. ELDA software (http://bioinf.wehi.edu.au/software/elda/) was used to calculate HSC frequency and statistical significance (two experiments). **g**, 3×10^5 donor bone marrow cells were transplanted with 3×10^5 recipient bone marrow cells into irradiated recipient mice (3 experiments with a total of 12-14 recipients/genotype). **h**, HSCs did not express *Scf*-GFP by flow cytometry. Δ, recombined Scf^{fl} allele; +, wild-type allele of *Scf*.

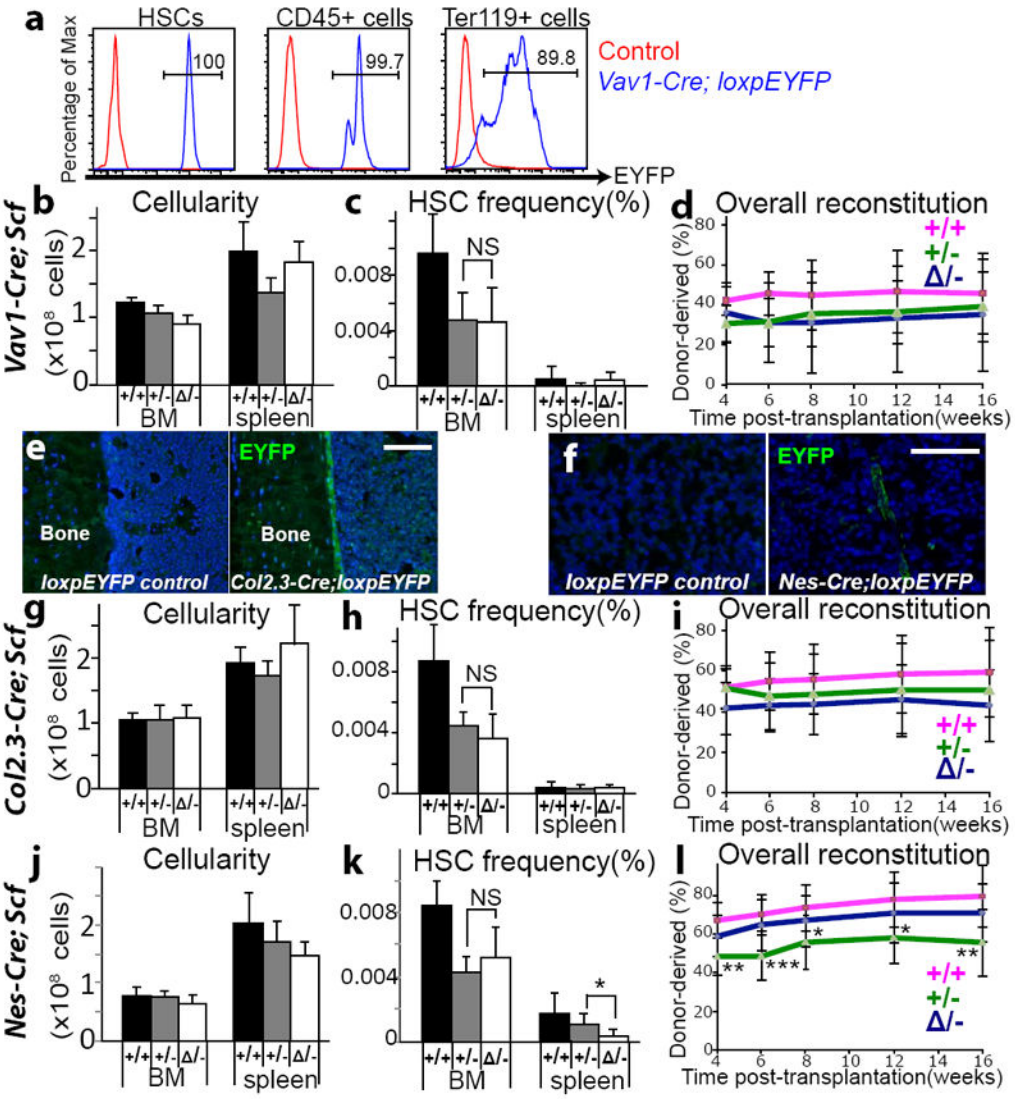

Figure 3. SCF from haematopoietic cells, osteoblasts, and *Nestin-Cre*-expressing stromal cells is not required for HSC maintenance

a, *Vav1-Cre* recombined the *loxpEYFP* reporter in virtually all HSCs, CD45+, and Ter119+ haematopoietic cells. **b** and **c**, Deletion of *Scf* from haematopoietic cells did not significantly affect bone marrow or spleen cellularity or HSC frequency (n=4). **d**, A competitive reconstitution assay with *Vav-1-Cre; Scf*$^{fl/-}$, *Scf*$^{+/-}$ and *Scf*$^{+/+}$ bone marrow cells (two experiments with a total of 10 recipients/genotype). **e**, *Col2.3-Cre* recombined the *loxpEYFP* reporter in bone-lining osteoblast lineage cells. **f**, *Nestin-Cre* recombined the *loxpEYFP* reporter in rare stromal cells around larger blood vessels. **g**, Bone marrow and spleen cellularity and **h**, HSC frequency in *Col2.3-Cre; Scf*$^{fl/-}$ mice relative to controls (n=5-6). **i**, A competitive reconstitution assay with *Col2.3-Cre; Scf*$^{fl/-}$, *Scf*$^{+/-}$ and *Scf*$^{+/+}$ bone marrow cells (3-5 experiments with a total of 14-22 recipients/genotype). **j**, Bone marrow and spleen cellularity and **k**, HSC frequency in *Nestin-Cre; Scf*$^{fl/-}$ mice relative to controls (n=5-7). **l**, 3×10^5 donor bone marrow cells from *Nestin-Cre; Scf*$^{fl/-}$ and *Scf*$^{+/+}$ mice gave similar levels of donor cell reconstitution in irradiated mice. Reconstitution levels from *Scf*$^{+/-}$ cells were

modestly but significantly lower (3-5 experiments with a total of 14-24 recipient mice/genotype). Δ, recombined Scf^{fl} allele; +, wild-type allele; −, germline deleted allele. NS, not significant. Scale bar is 100um in (**e**) and 50um in (**f**).

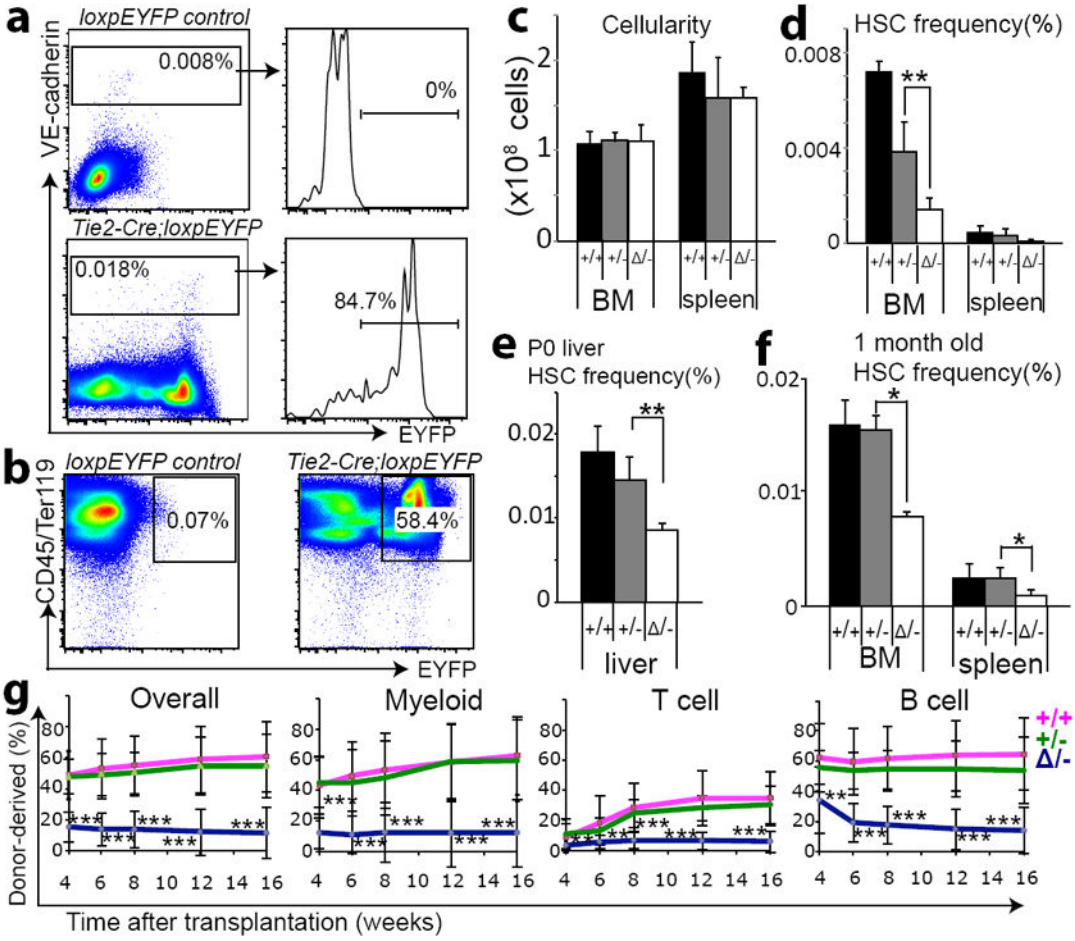

Figure 4. Deletion of *Scf* from endothelial cells depletes HSCs

a and **b**, *Tie2-Cre* recombined the *loxpEYFP* conditional reporter in VE-cadherin+ endothelial cells and in haematopoietic cells in the bone marrow. **c**, Bone marrow and spleen cellularity in *Tie2-Cre; Scf*$^{fl/-}$ mice and littermate controls (n=4-7). **d**, HSC frequency in *Tie2-Cre; Scf*$^{fl/-}$ mice and controls (n=4-7). **e**, HSC frequency in the liver of newborn *Tie2-Cre; Scf*$^{fl/-}$ mice and controls (n=3-6). **f**, HSC frequency in one month-old *Tie2-Cre; Scf*$^{fl/-}$ mice and controls (n=3-4). **g**, Bone marrow cells from *Tie2-Cre; Scf*$^{fl/-}$ mice gave significantly lower levels of reconstitution relative to cells from *Scf*$^{+/-}$ and *Scf*$^{+/+}$ mice (3-5 experiments with a total of 15-25 recipients/genotype).

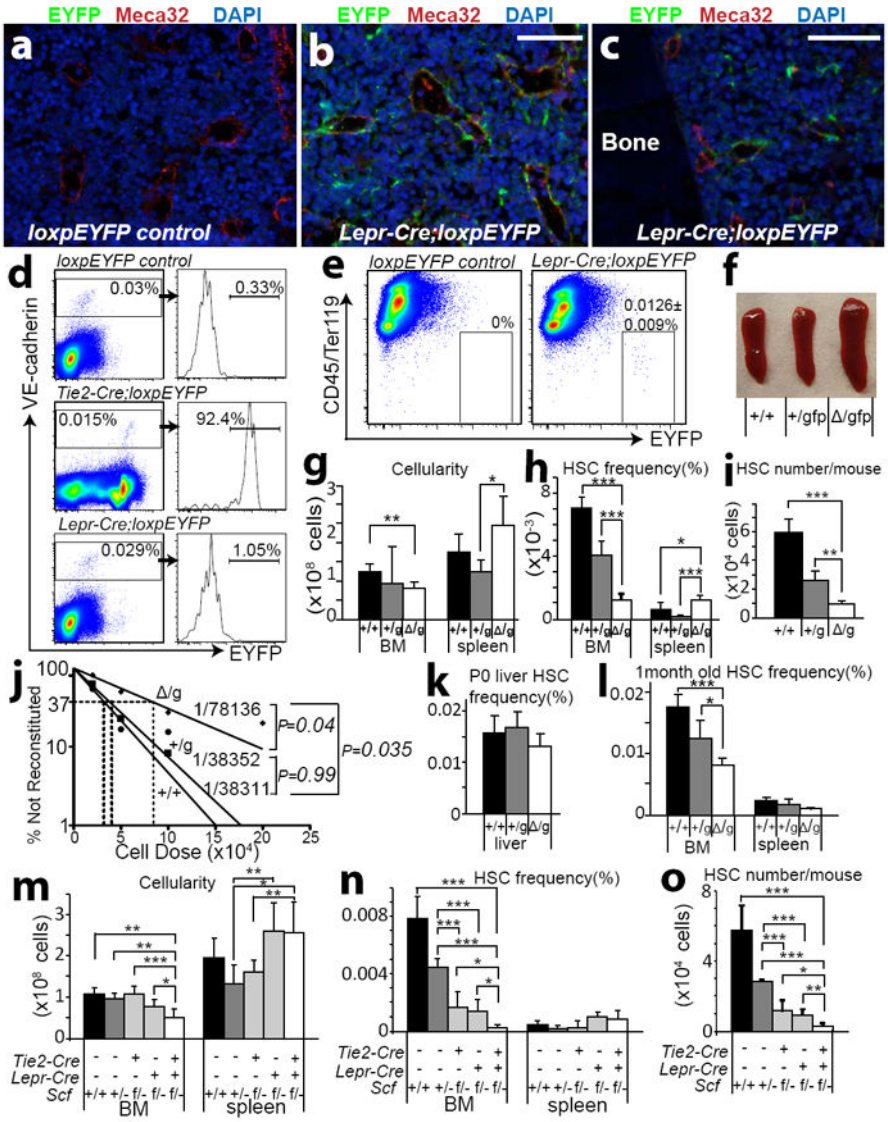

Figure 5. Deletion of *Scf* from *Lepr-Cre*-expressing perivascular stromal cells depletes HSCs in the bone marrow

a-c, *Lepr-Cre* recombined the *loxpEYFP* reporter in perisinusoidal stromal cells in the bone marrow but not in bone-lining or haematopoietic cells. **d**, *Lepr-Cre* did not recombine in VE-cadherin+ endothelial cells. **e**, 0.013±0.009% (mean±s.d.; n=3) of bone marrow cells from *Lepr-Cre; loxpEYFP* mice were EYFP+. **f**, Spleen size and **g**, bone marrow and spleen cellularity (n=4-7). **h**, HSC frequency (n=4-7). **i**, Total HSC numbers (including bone marrow and spleen) in *Lepr-Cre; Scf*$^{fl/gfp}$ mice (n=4-7). **j**, Limit dilution analysis[42] of the frequency of long-term multilineage reconstituting cells in the bone marrow of *Lepr-Cre; Scf*$^{fl/gfp}$ mice relative to controls (two experiments). **k**, HSC frequency in the newborn liver (n=4-11). l, HSC frequency in one month-old *Lepr-Cre; Scf*$^{fl/gfp}$ mice and controls (n=3-6). **m**, *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$ mice had significantly reduced bone marrow cellularity and increased spleen cellularity compared to *Scf*$^{+/-}$ or *Tie2-Cre; Scf*$^{fl/-}$ controls (n=4-11). **n**, Deletion of *Scf* from endothelial and perivascular stromal cells in *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$

mice greatly depleted HSCs from adult bone marrow (n=4-11). **o**, Total HSC number was significantly reduced in *Tie2-Cre; Lepr-Cre; Scf*$^{fl/-}$ mice compared to *Tie2-Cre; Scf*$^{fl/-}$ or *Lepr-Cre; Scf*$^{fl/-}$ mice (n=4-11). Scale bars are 50um.