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Endothelial and perivascular cells maintain haematopoietic stem cells

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Several 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; also known as KITL) is a key niche component that maintains HSCs. Here, using *Scf*^{gfp} knock-in mice, we found that *Scf* was primarily expressed by perivascular cells throughout the 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. Thus, 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 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 *in vivo*.

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 enhanced green fluorescent protein (*gfp*) into the endogenous *Scf* locus (Supplementary Fig. 1a–c). *Scf*$^{gfp/gfp}$ mice died perinatally (Fig. 1a and Supplementary Fig. 1f, g) with severe anaemia (Fig. 1b and Supplementary Fig. 2c), as observed in mice with a strong loss of SCF/c-Kit function[17]. By quantitative reverse transcription–polymerase chain reaction (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 twofold in *Scf*$^{gfp/+}$ and about fivefold 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 eightfold 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 and Supplementary Fig. 2d). *Scf*$^{gfp/gfp}$ mice therefore have a severe loss of SCF function.

Using flow cytometry we determined that only rare (0.027 ± 0.0099%, mean ± standard deviation (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 have recovered 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^{-} stromal cells (Supplementary Fig. 2f, g), suggesting that 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 and Supplementary Fig. 2h, i).

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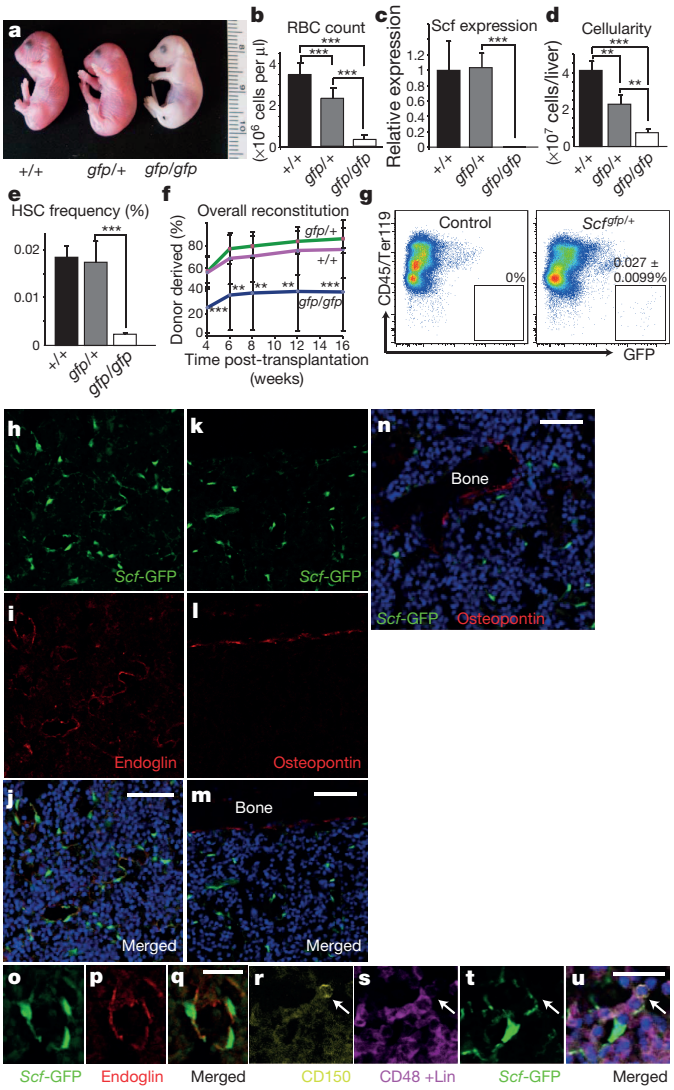

Figure 1 | *Scf*^{gfp} is a strong loss-of-function allele and *Scf* is primarily expressed by perivascular cells in the bone marrow. **a**, **b**, *Scf*$^{gfp/gfp}$ homozygous mice died perinatally and were severely anaemic ($n = 4$–20). RBC, red blood cell. **c**, *Scf* transcripts in livers from newborn mice by qRT–PCR ($n = 3$). **d**, **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 per 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 4′,6-diamidino-2-phenylindole (DAPI; in blue). All data represent mean ± s.d. Two-tailed Student's *t*-tests were used to assess statistical significance. $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars in **j**, **m** and **n** are 50 μm. Scale bars in **q** and **u** are 20 μm.

GFP partially overlapped with endothelial marker staining (Fig. 1h–j, o–q and 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*, *Pdgfra* and *Pdgfrb* were highly elevated in *Scf*-GFP$^+$ cells relative to whole bone marrow cells (Supplementary Table 1). This indicates 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. Sixty-five per cent (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 five cell diameters of GFP-expressing cells. This suggests that *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 perinatally lethal and anaemic (Fig. 2a), similar to other *Scf*-deficient mice (Fig. 1a)[17]. Recombination of the *Scf*^{fl} allele therefore led to 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, and then killed them for analysis. Some of the mice became anaemic 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 towards lower

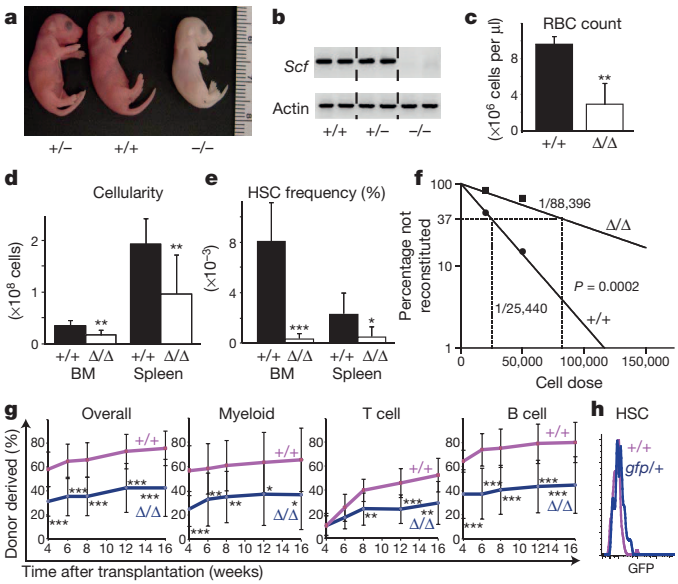

Figure 2 | *Scf* is required for adult HSC maintenance. **a**, Homozygous *Scf*$^{-/-}$ mutant mice generated from germline recombination of the *Scf*^{fl} allele were perinatally lethal and anaemic. **b**, *Scf* transcripts amplified by RT–PCR from the livers of newborn mice. **c**, Global deletion of *Scf* in *Ubc-creER; Scf*$^{fl/fl}$ mice led to anaemia ($n = 5$–6). **d**, **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). BM, bone marrow. **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 assess 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 (three experiments with a total of 12–14 recipients per genotype). **h**, HSCs did not express *Scf*-GFP by flow cytometry. Δ, recombined *Scf*^{fl} allele; +, wild-type allele of *Scf*. All data represent mean ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

white blood cell and platelet counts (Supplementary Fig. 3d). *Ubc-creER; Scf$^{fl/fl}$* mice exhibited approximately twofold 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 *loxP-EYFP* reporter[29] in virtually all HSCs, CD45$^+$ and Ter119$^+$ haematopoietic cells (Fig. 3a and 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 twofold 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 and 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 enhanced yellow fluorescent protein (EYFP) expression among bone-lining cells in *Col2.3-cre; loxP-EYFP* mice (Fig. 3e). To test whether osteoblasts produce SCF for HSC maintenance, we analysed 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 twofold 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 and 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; loxP-EYFP* 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 and 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).

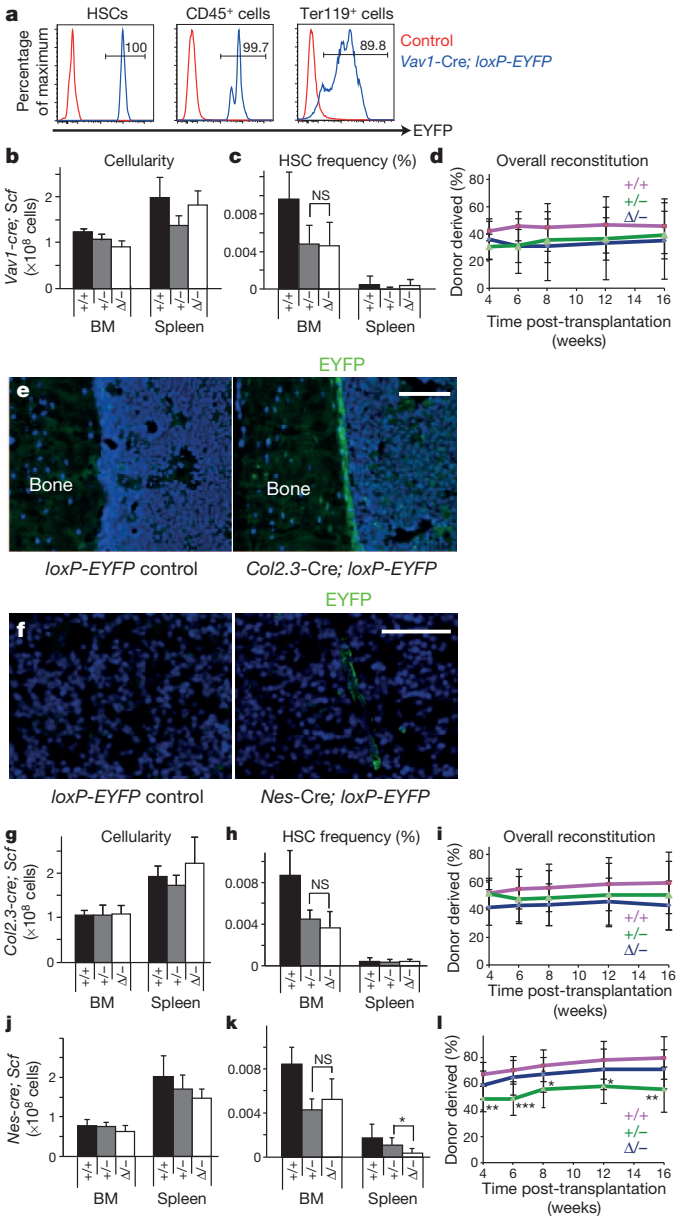

Figure 3 | SCF from haematopoietic cells, osteoblasts and nestin-Cre-expressing stromal cells is not required for HSC maintenance. **a**, *Vav1*-Cre recombined the *loxP-EYFP* reporter in virtually all HSCs, CD45$^+$ and Ter119$^+$ haematopoietic cells. **b**, **c**, Deletion of *Scf* from haematopoietic cells did not significantly affect bone marrow or spleen cellularity or HSC frequency ($n = 4$). **d**, A competitive reconstitution assay with *Vav1-cre; Scf$^{fl/-}$*, *Scf$^{+/-}$* and *Scf$^{+/+}$* bone marrow cells (2 experiments with a total of 10 recipients per genotype). **e**, *Col2.3*-Cre recombined the *loxP-EYFP* reporter in bone-lining osteoblast lineage cells. **f**, Nestin-Cre recombined the *loxP-EYFP* reporter in rare stromal cells around larger blood vessels. **g**, **h**, Bone marrow and spleen cellularity (**g**) and HSC frequency (**h**) 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 per genotype). **j**, **k**, Bone marrow and spleen cellularity (**j**) and HSC frequency (**k**) 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 per genotype). Δ, recombined *Scf^{fl}* allele; +, wild-type allele; −, germline deleted allele. Scale bar is 100 μm in **e** and 50 μm in **f**. All data represent mean ± s.d. NS, not significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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 and 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 haematopoiesis, 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.

Because 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 figure 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, whereas nestin–GFP was detected around both (Supplementary Fig. 8). Thus, different nestin transgenes seem 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 previously 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

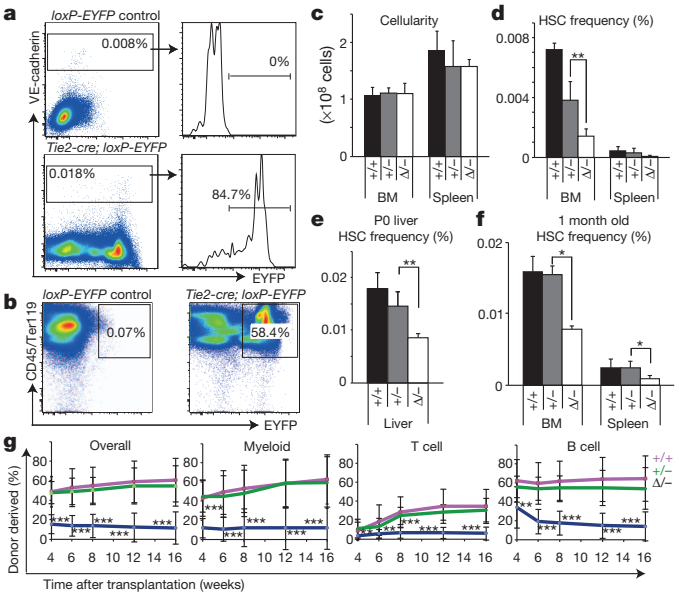

Figure 4 | Deletion of *Scf* from endothelial cells depletes HSCs. **a**, **b**, *Tie2*-Cre recombined the *loxP-EYFP* conditional reporter in VE-cadherin$^+$ endothelial cells and in haematopoietic cells in the bone marrow. **c**, Bone marrow and spleen cellularity in *Tie2-cre*; $Scf^{fl/-}$ mice and 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). P0, postnatal day 0. **f**, HSC frequency in 1-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 per genotype). All data represent mean ± s.d. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

(Fig. 4b) but not in mesenchymal stem/stromal cells from the bone marrow (Supplementary Fig. 9d, e). Because haematopoietic cells do not express *Scf* (Fig. 1g and 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-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 5 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 probably 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 signalling, HSC frequencies return to normal levels upon restoration of normal SCF/c-Kit signalling[19,21]. Nonetheless, we also examined whether SCF expression by endothelial cells during development is required by HSCs. We found a 1.7–2.1-fold reduction in HSC frequency in the liver of newborn *Tie2-cre*; $Scf^{fl/-}$ mice (Fig. 4e) and a twofold reduction in HSC frequency in the bone marrow of 1-month-old *Tie2-cre*; $Scf^{fl/-}$ mice (Fig. 4f) relative to $Scf^{+/-}$ and $Scf^{+/+}$ controls. The magnitude of HSC depletion in adult bone marrow seemed 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 reflect a loss of SCF expression by endothelial cells during development.

HSCs require SCF from perivascular cells

We found that *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*; *loxP-EYFP* 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*; *loxP-EYFP* 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 indicate a mesenchymal identity for the *Lepr*-expressing stromal cells; however, the lack of EYFP expression in bone-lining cells from *Lepr-cre*; *loxP-EYFP* 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

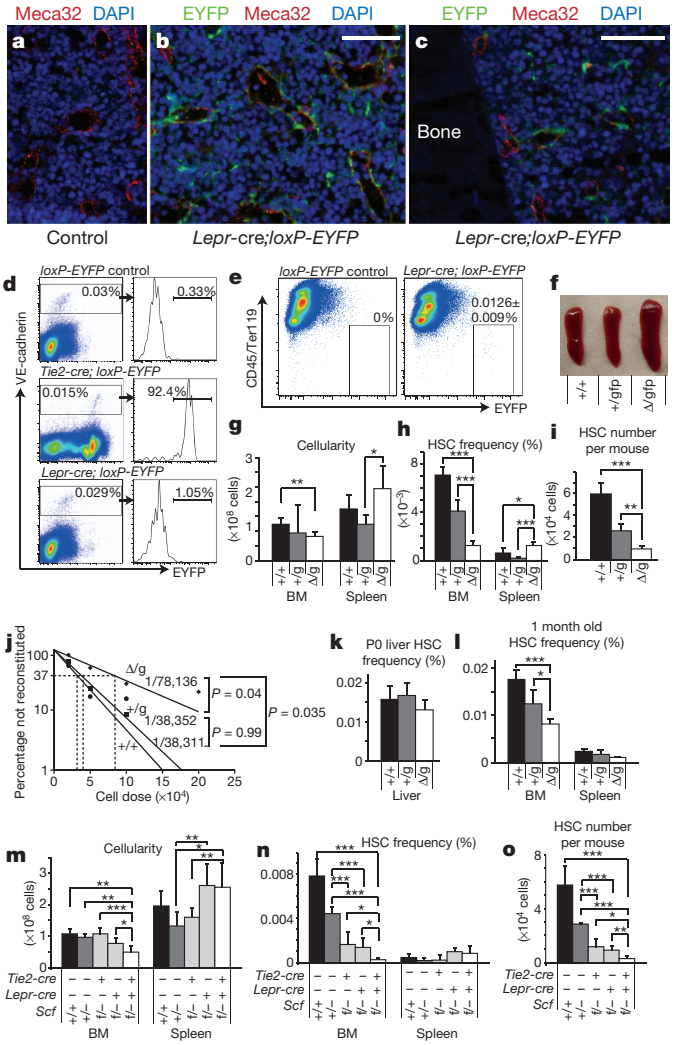

Figure 5 | Deletion of *Scf* from *Lepr-cre*-expressing perivascular stromal cells depletes HSCs in the bone marrow. **a**–**c**, *Lepr*-Cre recombined the *loxP-EYFP* reporter in perisinusoidal stromal cells in the bone marrow but not in bone-lining or haematopoietic cells. **d**, *Lepr*-Cre did not recombine in VE-cadherin$^+$ endothelial cells. **e**, 0.013 ± 0.009% (mean ± s.d.; $n = 3$) of bone marrow cells from *Lepr-cre; loxP-EYFP* mice were EYFP$^+$. **f**, **g**, Spleen size (**f**) and bone marrow and spleen cellularity (**g**) ($n = 4$–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 1-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). g or gfp, *Scf^{gfp}* allele; f, *Scf^{fl}* allele. Scale bars are 50 μm. All data represent mean ± s.d. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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 analysed 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 that 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, although *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 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 probably contribute through mechanisms other than SCF production (for example, 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 and Supplementary Figs 6 and 7). However, *Lepr-cre*-expressing perisinusoidal cells do partially overlap with nestin–GFP-expressing perivascular cells (Supplementary Fig. 8 and Supplementary Fig. 9a–c). The *Lepr-cre*-expressing stromal cells therefore include stromal cells that express certain nestin transgenes, consistent with previous work[12], and may also include *Cxcl12*-abundant reticular cells[10]. Perivascular stromal cells are probably heterogeneous and may include multiple cell types that contribute to HSC maintenance through different mechanisms.

Although 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/Ka 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 *loxP-EYFP*[29] (all from the Jackson Laboratory), *Col2.3-cre*[30], nestin-*creER*[40] and nestin-*GFP*[41]. All were maintained on a C57BL/Ka background. Unless otherwise indicated, data always reflect mean ± s.d. and two-tailed Student's *t*-tests were used to assess statistical significance ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions L.D. performed all of the experiments. T.L.S. helped to design and generate the Scf^{fl} and Scf^{gfp} mice. G.E. generated the nestin-*Cherry* transgenic mice. L.D. and S.J.M. designed the experiments, interpreted the results and wrote the manuscript.

Author Information The microarray data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE33158. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.J.M. (Sean.Morrison@UTSouthwestern.edu).

METHODS

Mice. Targeting vectors for making *Scf*^{gfp} and *Scf*^{fl} mice were constructed by recombineering[34]. Linearized targeting vector was electroporated into W4 (*Scf*^{gfp}) or Bruce4 (*Scf*^{fl}) embryonic stem (ES) cells. Positive clones were identified by Southern blotting and injected into B6 or C57BL/6-Tyr$^{c\text{-}2J}$ blastocysts. Chimaeric mice were bred with B6 or C57BL/6-Tyr$^{c\text{-}2J}$ mice to obtain germline transmission. The *Frt*-flanked *Neo* cassette was subsequently removed by mating with *Flpe* mice[35]. *Scf*^{gfp} mice were backcrossed at least five times and *Scf*^{fl} mice were backcrossed at least three times onto a C57BL/Ka background before analysis. Note that *Scf*^{fl} mice were backcrossed fewer times before analysis because Bruce4 ES cells were from a substantially C57BL/6 background. *Ubc-creER*[36], *CMV-cre*[37], *Vav1-cre*[28], nestin-*cre*[38], *Tie2-cre*[31], *Lepr-cre*[39] and *loxP-EYFP*[29] mice were obtained from the Jackson Laboratory and maintained by crossing with C57BL/Ka mice. *Col2.3-cre* mice on a C57BL/6 background were provided by B. Cream and F. Liu[30]. Nestin-*creER* mice were obtained from G. Fishell and backcrossed onto a C57BL/Ka background[40]. Nestin-*GFP* mice were as previously described[41]. C57BL/6-SJL (CD45.1) mice were used as recipients in transplantation experiments. All mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. All protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Unless otherwise indicated, data are mean ± s.d. and two-tailed Student's *t*-tests were used to assess statistical significance ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Genotyping PCR. Primers for genotyping *Scf*^{gfp} were: OLD291, 5′-CCCGC AGCTCTGGTATATTTGC-3′; OLD292, 5′-CGGACACGCTGAACTTGTGG-3′; and OLD360, 5′-AAGCACTTCAGATTCTAGGG-3′. Primers for genotyping *Scf*^{fl} were: OLD301, 5′-GGAAAAGAACCAAGTGAAGTC-3′; and OLD302, 5′-GTCCGCAGCAAGCTCACCAGC-3′. Primers for genotyping *Scf* were: OLD301, 5′-GGAAAAGAACCAAGTGAAGTC-3′; and OLD304, 5′-ACGG GGAAAGACCTCCGGTCC-3′. Primers for genotyping *Tie2-cre* were: OLD438, 5′-CCTGTGCTCAGACAGAAATG-3′; and OLD435, 5′-GGCAAA TTTTGGTGTACGGTC-3′. Primers for genotyping *Lepr-cre* were: OLD434, 5′-CATTGTATGGGATCTGATCTGG-3′; and OLD435, 5′-GGCAAATTTTG GTGTACGGTC-3′.

Tamoxifen administration. Tamoxifen citrate (Sigma or Spectrum Chemical) was administrated in chow at 400 mg kg^{-1} with 5% sucrose (Harlan). Mice were fed tamoxifen chow for 1–5 months before being analysed.

Long-term competitive reconstitution assay and limit dilution assay. Adult recipient mice were lethally irradiated by a Cesium 137 GammaCell40 Irradiator (MDS Nordia) at 300 rad per minute with two doses of 540 rad (total 1,080 rad) delivered at least 2 h apart. Cells were transplanted by retro-orbital venus sinus injection of anaesthetized mice. 3×10^5 fetal liver or bone marrow cells were transplanted along with 3×10^5 recipient bone marrow cells into each irradiated mouse unless otherwise indicated. Mice were maintained on antibiotic water (neomycin sulphate 1.11 g l^{-1} and polymixin B 0.121 g l^{-1}) for 14 days then switched to regular water. Recipient mice were periodically bled to assess the level of donor-derived blood lineages, including myeloid, B and T cells for at least 16 weeks. Blood was subjected to ammonium chloride potassium red cell lysis before antibody staining. Antibodies including anti-CD45.2 (104), anti-CD45.1 (A20), anti-Gr1 (8C5), anti-Mac-1 (M1/70), anti-B220 (6B2) and anti-CD3 (KT31.1) were added to stain cells. Stained samples were analysed by flow cytometry. For limit dilution assay on *Ubc-creER; Scf*$^{fl/fl}$ mice, three doses of donor bone marrow cells (2×10^4, 5×10^4 and 1.5×10^5) along with 3×10^5 recipient bone marrow cells were transplanted into irradiated mice. For limit dilution assay on *Lepr-cre; Scf*$^{fl/gfp}$ mice, 2×10^4, 5×10^4 or 1×10^5 bone marrow cells along with 3×10^5 recipient bone marrow cells were competitively transplanted into irradiated mice. After 16 weeks, the percentage of mice that were not multilineage reconstituted by donor haematopoietic cells was plotted against cell dose. ELDA software was used to calculate HSC frequency and statistical significance (http://bioinf.wehi.edu.au/software/elda/).

Flow cytometry. Bone marrow cells were isolated by flushing the long bones or by crushing the long bones with mortal and pestle in Ca^{2+} and Mg^{2+} free HBSS with 2% heat-inactivated bovine serum. Spleen cells were obtained by crushing the spleen between two glass slides. The cells were drawn by passing through a 25G needle several times and filtered with a 70 μm nylon mesh. The following antibodies were used to stain HSCs: anti-CD150 (TC15-12F12.2), anti-CD48 (HM48-1), anti-CD41 (MWReg30), anti-Sca-1 (E13-161.7), anti-cKit (2B8), 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). DAPI was used to exclude dead cells. For flow cytometric analysis of *Scf*-GFP$^+$ or *Lepr-cre; loxP*-EYFP$^+$ stromal cells, bone marrow was flushed using HBSS with 2% bovine serum. Then whole bone marrow was digested with Collagenase IV (200 U ml^{-1}) and DNase I (200 U ml^{-1}) at 37 °C for 15 min. Samples were then stained with antibodies and analysed by flow cytometry. Anti-CD140a (APA5), anti-CD140b (APB5), anti-CD45 (30F-11) and anti-Ter119 antibodies were used to stain perivascular stromal cells. For analysis of bone marrow endothelial cells, mice were intravenously injected with 10 μg per mouse Alexa Fluor 647 conjugated anti-VE-cadherin antibody (BV13, eBiosciences)[14]. Ten minutes later, the long bones were removed and bone marrow was flushed, digested and stained as above. Samples were run on FACSAria II or FACSCanto II flow cytometers. Data were analysed by FACSDiva (BD) or FlowJo (Tree Star) software.

Methylcellulose culture. Cells were sorted into methylcellulose culture medium (3434, Stemcell Technology) and incubated at 37 °C as described[8].

Bone sectioning and immunostaining. Freshly dissected long bones were fixed in a formalin-based fixative at 4 °C for 3 h. Then the bones were embedded in 8% gelatin in PBS. Samples were snap frozen with liquid nitrogen and stored at −80 °C. Bones were sectioned using CryoJane (Instrumedics). Sections were dried overnight at room temperature (~22 °C) and stored at −80 °C. Sections were re-hydrated in PBS for 5 min before immunostaining. 5% goat serum in PBS was used for blocking. Primary antibodies were applied to the slides for 1 h at room temperature followed by secondary antibody incubation for 30 min at room temperature with repetitive washes in between. Slides were mounted with antifade prolong gold (Invitrogen) and images were acquired on an Olympus IX81 microscope or a Leica SP5 confocal microscope. Primary antibodies were: chicken-anti-GFP (Aves), rat-anti-endoglin (eBioscience), goat-anti-osteopontin (R&D), rat-anti-mouse pan-endothelial cell antigen (Meca32, Biolegend) and rabbit-anti-Cherry (Clontech).

qRT–PCR. Cells were sorted directly into Trizol. Total RNA was extracted according to manufacturer's instructions. Total RNA was subjected to reverse transcription or amplification using the WT-Ovation Pico RNA Amplification System (Nugen). Quantitative real-time PCR was run using SYBR green on a LightCycler 480 (Roche). β-Actin was used to normalize the RNA content of samples. Primers used in this study were: *Scf*: OLD405, 5′-TTGTTACCT TCGCACAGTGG-3′; and OLD406, 5′-AATTCAGTGCAGGGTTCACA-3′; *Cxcl12*: OLD35, 5′-TGCATCAGTGACGGTAAACCA-3′; and OLD36, 5′-GTTGTTCTTCAGCCGTGCAA-3′; *Alpl*: OLD164, 5′-TTGTGCCAGAGAAA GAGAGAGA-3′; and OLD165, 5′- GTTTCAGGGCATTTTTCAAGGT-3′; *Tie2*: OLD158, 5′-ATGTGGAAGTCGAGAGGCGAT-3′; and OLD159, 5′-CGAATAGCCATCCACTATTGTCC-3′; *Lepr*: OLD418, 5′-TGATGTGTCA GAAATTCTATGTGG-3′; and OLD419, 5′- TGCCAGGTTAAGTGCA GCTAT-3′; *Scf* long form: 5′- GCCAGAAACTAGATCCTTTACTCCTGA-3′; and 5′-ACATAAATGGTTTTGTGACACTGACTCTG-3′; *Scf* short form: 5′-CCCGAGAAAGGGAAAGCCG-3′; and 5′-ATTCTCTCTCTTTCTGTTGCAA CATACTT-3′; β-actin: OLD27, GCTCTTTTCCAGCCTTCCTT-3′; and OLD28, 5′-CTTCTGCATCCTGTCAGCAA-3′.

Gene expression profiling. Three independent, fresh isolated aliquots of approximately 5,000 *Scf*-GFP$^+$ cells or whole bone marrow cells were flow cytometrically sorted into Trizol. Total RNA was extracted and amplified using the WT-Ovation Pico RNA Amplification system (Nugen) following manufacturer's instructions. Sense strand cDNA was generated using the WT-Ovation Exon Module (Nugen). Then, cDNA was fragmented and labelled using FL-Ovation DNA Biotin Module V2 (Nugen). The labelled 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 (RMA) method[43].

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