In the bone marrow, hematopoietic stem cells (HSCs) lodge in specialized microenvironments that tightly control the proliferative state of HSCs to adapt to the varying needs for replenishment of blood cells while also preventing HSC exhaustion\(^1\). All putative niche cells suggested thus far have a nonhematopoietic origin\(^2\)–\(^8\). Thus, it remains unclear how feedback from mature cells is conveyed to HSCs to adjust their proliferation. Here we show that megakaryocytes (MKs) can directly regulate HSC pool size in mice. Three-dimensional whole-mount imaging revealed that endogenous HSCs are frequently located adjacent to MKs in a nonrandom fashion. Selective in vivo depletion of MKs resulted in specific loss of HSC quiescence and led to a marked expansion of functional HSCs. Gene expression analyses revealed that MKs are the source of chemoattractant C-X-C motif ligand 4 (CXCL4, also named platelet factor 4 or PF4) in the bone marrow, and we found that CXCL4 regulates HSC cell cycle activity. CXCL4 injection into mice resulted in a reduced number of HSCs because of their increased quiescence. By contrast, Knockout mice exhibited an increased number of HSCs and increased HSC proliferation. Combined use of whole-mount imaging and computational modeling was highly suggestive of a megakaryocytic niche capable of independently influencing HSC maintenance by regulating quiescence. These results indicate that a terminally differentiated cell type derived from HSCs contributes to the HSC niche, directly regulating HSC behavior.

Recent evidence suggests that progenitors derived from somatic stem cells can provide niches for their own predecessors\(^8\),\(^9\). In the Drosophila system, hematopoietic progenitors are negatively regulated by differentiating cells\(^11\). Using a whole-mount three-dimensional immunofluorescence imaging technique, which allows the visualization of bone marrow tissues to a depth of ~75 µm (Fig. 1a–c), we observed that lineage (Lin)\(^–\)CD48\(^–\)CD41\(^–\)CD150\(^+\) HSCs are generally located close to MKs, with a considerable fraction (20.3 ± 2.6% (mean ± s.e.m.)) lying directly adjacent to MKs (Fig. 1b–d and Supplementary Fig. 1). To test the significance of this association, we ran simulations of random HSC placement on images of whole mount–prepared sternal segments stained for CD41\(^+\) MKs to generate a null distribution of the mean distances of HSCs with nonpreferential localization to MKs (Fig. 1e,f and Supplementary Fig. 2). The observed mean distance of HSCs to MKs was statistically different from the mean distance of randomly placed HSCs to MKs (Fig. 1f). Furthermore, only 7.0 ± 0.6% of randomly distributed HSCs were adjacent to MKs (Fig. 1d). These data indicate that the observed association of HSCs with MKs is statistically different from a random distribution (P = 1.6 × 10\(^{−10}\); Fig. 1e).

Previous studies have suggested a role of MKs in regulating HSC function. After transplantation, HSCs home to MK-rich endosteal areas\(^12\)–\(^14\), and host MKs facilitate donor HSC engraftment after lethal irradiation\(^13\). In addition, coculture with MKs slightly increases HSC numbers ex vivo\(^14\). To assess the role of MKs in HSC function in vivo, we generated transgenic mice in which MKs could be depleted using inducible diphtheria toxin receptor (iDTR) expression by intercrossing Cxcl4\(^−/−\) mice\(^15\) with iDTR animals, in which the diphtheria toxin receptor is expressed by Cre recombination. Time-course studies revealed a reduction in MK numbers after one dose of DT treatment and a >90% reduction after seven daily doses (P < 0.001; Fig. 2a–c and Supplementary Fig. 3), with a concomitant decline in the number of platelets (P < 0.001; Supplementary Fig. 4). Notably, MK depletion led to a marked expansion (up to 11.5-fold) in the number of phenotypic Lin\(^–\)c-KIT\(^+\)SCA1\(^+\)CD105\(^+\)CD150\(^+\) HSCs at day 7 (P < 0.001; Fig. 2d and Supplementary Fig. 3c). We also observed Lin\(^–\)CD48\(^−\)CD41\(^−\)CD150\(^+\) HSC expansion in whole-mount bone marrow images (Fig. 2a,b). Hematopoietic cell proliferation was largely restricted to HSCs and was not evident in other progenitor cell populations, except for a slight increase in multipotent and MK progenitors (Supplementary Fig. 4c–g).
To test the impact of MK depletion on HSC function, we carried out competitive repopulation analyses (Supplementary Fig. 5a) and observed significantly higher reconstitution (CD45.2+ cells) throughout the 16-week observation period in mice transplanted with total bone marrow from DT-treated Cxcl4-cre; iDTR mice than in those transplanted with bone marrow from DT-treated control mice (Fig. 2f and Supplementary Fig. 5b). Extreme limiting dilution analyses revealed 625 HSCs with repopulating capacity per femur in DT-treated control animals (Supplementary Fig. 5a); those from control animals (Supplementary Fig. 5a). The increased HSC numbers after MK depletion were probably due to increased cell proliferation, as BrdU incorporation was increased (5.5-fold) in HSCs from MK-depleted mice compared to controls (P < 0.001; Fig. 2h,i). Enhanced HSC proliferation was also reflected in increased expression of cyclin-dependent kinase 2 (encoded by Cdk2) and cyclin E1 (encoded by Ccne1) (Fig. 2j), which after forming a complex promote entry into S phase46, and a corresponding ~4.3-fold increase in the number of HSCs in non-G0 phases, as seen using Ki67- and Hoechst 33342-based cell cycle analyses (P < 0.05; Supplementary Fig. 5c). Although ~50% of HSCs exited G0 after MK depletion (Supplementary Fig. 5c), the absolute number of G0 HSCs was increased by approximately fourfold, consistent with the increase in HSCs with repopulating capacity observed in the limiting dilution analysis, which is in accordance with the paradigm that long-term engraftment potential resides predominantly in the G0 fraction of HSCs47.

The observation that cell proliferation occurred selectively in the HSC compartment after MK depletion (Fig. 2h,i and Supplementary Fig. 4g) argues that this effect is not caused by an inflammatory milieu emanating from MK death in the marrow. To confirm this idea, we depleted MKs for 6 consecutive weeks using daily DT treatment for the first week and treatment every other day for the next 5 weeks and found that the number of HSCs was still increased (by 2.4-fold) when compared to control mice (Supplementary Fig. 6a,b). However, the number of HSCs after 6 weeks of MK depletion was lower when compared to that after 1 week of MK depletion (Fig. 2d–g), perhaps indicative of compensatory mechanisms or HSC exhaustion, which has been reported previously after loss of quiescence18–20. Consistent with the possibility of exhaustion, we found only a slight but not statistically significant increase in repopulating activity in Cxcl4-cre; iDTR mice after 6 consecutive weeks of DT treatment as compared to control mice (Supplementary Fig. 6c). These results suggest that MKs prevent exhaustion by restraining HSC proliferation.

A recent report indicates that platelets may regulate HSC quiescence through effects on circulating thrombopoietin concentrations21. Hence, to ascertain whether the marked increase of HSCs is mediated by circulating platelets rather than bone marrow MKs, we injected mice with neuraminidase (a glycoside hydrolase enzyme that cleaves the glycosidic linkages of sialic acids on the platelet surface), which depletes platelets without affecting MK numbers22 (Supplementary Fig. 7a–c). Neuraminidase treatment did not alter HSC numbers in bone marrow (Supplementary Fig. 7e,f), suggesting that MKs, but not platelets, regulate bone marrow HSC quiescence.
Figure 2 MKs maintain the quiescence of HSCs in vivo. (a,b) Representative whole-mount images of sternal bone marrow from control (ctrl) and Cxcl4-cre; iDTR (iDTR) mice at 7 d of DT treatment. Arrowheads denote Lin−CD48−CD41−CD150+ phenotypic HSCs. MKs are distinguished by their size, morphology and CD41 expression. Vascular endothelial cells are stained with antibodies to CD31 and CD144. Scale bars, 50 μm (a,b). (c) Quantification of MKs per section of whole-mount images of transverse-shaved femoral bone marrow from control and Cxcl4-cre; iDTR mice after 7 d of DT treatment (representative images are shown in Supplementary Fig. 3a). n = 8 cross-sections from four male mice. (d,e) Number of HSCs per femur in control and Cxcl4-cre; iDTR mice (d) and representative FACS plots (e). n = 5 male mice per group. MPP, multipotent progenitors. (f) Percentage of CD45.2+ cells in the blood of CD45.1+ mice competitively transplanted with femoral bone marrow from control and Cxcl4-cre; iDTR mice after 7 d of DT treatment (representative images are shown in Supplementary Fig. 3a). n = 8 cross-sections from four male mice. (g) Extreme limiting dilution analysis showing the estimated HSC frequency (solid bar) and confidence intervals (dotted lines) in the bone marrow (BM) of control or Cxcl4-cre; iDTR mice after 7 d of DT treatment. n = 4 (control group) and n = 5 (Cxcl4-cre; iDTR group) female recipient mice per dilution, except for the 0.2% BM dose (control group n = 5 and Cxcl4-cre; iDTR group n = 4). CRU, competitive repopulation units. (h,i) Percentage of proliferating HSCs in the BM of control and Cxcl4-cre; iDTR mice (as determined by BrdU incorporation) (h) and representative FACS plots (i). n = 5 male mice per group. (j) Quantitative PCR (qPCR) analysis of cell cycle–related genes within sorted HSCs. n = 4 independent experiments per group. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test). Error bars (c,d,f,h,j), s.e.m. As MKs have been reported to promote the expansion of osteolineage cells and thereby indirectly enhance stem cell engraftment after irradiation11,12, we investigated whether HSC quiescence was maintained indirectly by MKs through stromal components of the HSC niche. After DT treatment of Cxcl4-cre; iDTR, Nes−GFP mice (which express GFP-tagged Nes and were generated by breeding Cxcl4-cre; iDTR mice with Nes−GFP mice), we found no changes in the number of osteolineage cells in the compact bone (Supplementary Fig. 8a) or the number of endothelial cells (Supplementary Fig. 8b). Nestinlin cells (which overlap highly with leptin receptor (LEPR)+ and CD51+ platelet-derived growth factor receptor-α (PDGFR-α)+ perivascular cells5,7,23; Supplementary Fig. 8c) or Nestinbright cells (which enshath arterioles; Supplementary Fig. 8d)7 in the bone marrow. These results suggest that MK depletion does not enhance HSC numbers indirectly by altering an endothelial or mesenchymal niche.

To determine the mechanism by which MKs maintain HSC quiescence, we purified MKs using FACS on the basis of CD41 expression and cell size (Fig. 3a). As expected, immunofluorescence analyses of sorted cells revealed CD41+ multinucleated cells (Fig. 3a) enriched for MK markers, including FLJ1, C-type lectin domain family 2 (CLEC2), GATA-binding protein 2 (GATA2) and CD41 (data not shown). We evaluated the mRNA expression of various factors that have been shown to have roles in cell quiescence or proliferation by real-time PCR. Cxcl4 was by far the most abundant of these factors (by 9.7-fold to 5.8 × 106-fold, P < 0.001; Fig. 3a). Previous studies have shown that CXCL4 can negatively regulate the proliferation of human and mouse hematopoietic progenitors24-27. In addition, CXCL4 has been shown to reduce the chemosensitivity of human hematopoietic progenitors, and mice injected with CXCL4 before treatment with 5-fluorouracil or cytarabine (antimetabolites that selectively kill proliferating cells) show increased hematopoietic recovery27,28. CXCL4 expression is almost exclusively restricted to MKs and platelets (ref. 15 and Supplementary Fig. 8d)7 in the bone marrow. These results significantly reduced the proliferation of cultured mouse HSCs (Fig. 3b), which we found that the addition of recombinant mouse CXCL4 significantly reduced the proliferation of cultured mouse HSCs (Fig. 3d).

To evaluate this concept, we performed in vitro experiments in which we found that the addition of recombinant mouse CXCL4 significantly reduced the proliferation of cultured mouse HSCs (Fig. 3d). This effect was abolished in the presence of heparin, a known CXCL4 inhibitor29 (Fig. 3d). In addition, injection of CXCL4 into C57BL/6 WT mice led to a dose-dependent decrease in the number of phenotypic HSCs in the bone marrow (Fig. 3e). Consistent with this effect, we observed a dose-dependent reduction in the engraftment of bone marrow mononuclear cells (BMNCs) obtained from CXCL4-treated mice, as assessed at 16 weeks after transplantation (Fig. 3f). In keeping with a key role for CXCL4 in the regulation of HSC quiescence, we found that the number of native phenotypic HSCs and the fraction of proliferating BrdU+ HSCs were significantly higher in the bone marrow of Cxcl4−/− animals30 as compared to WT controls (Fig. 3g). We confirmed these differences by competitive transplantation assays, which revealed increased (twofold) repopulating activity in Cxcl4−/− compared to WT bone marrow (Fig. 3h). To assess...
whether the HSC phenotype of Cxcl4−/− mice could be rescued, we administered CXCL4 or vehicle for 7 days and found a substantial reduction of HSC numbers and BrdU incorporation by HSCs in the CXCL4-treated animals (Fig. 3i), indicating that MK-derived CXCL4 has a relevant role in regulating HSC quiescence. However, administration of CXCL4 to MK-depleted Cxcl4-cre; iDTR mice could only partially rescue HSC numbers and proliferation (Fig. 3j). Given the relatively large number of proteins produced or stored by MKs that could potentially affect HSC quiescence, other factors probably contribute to MK-mediated HSC quiescence (as shown for transforming growth factor-β1 (TGFβ1) by Zhao et al. in this issue31). Moreover, indirect effects of CXCL4 on HSCs cannot be formally excluded, as the identity of a CXCL4 receptor on HSCs remains unknown.

Because recent data suggest that a microenvironment surrounding arterioles confers quiescence of both mesenchymal stem cells and HSCs7, we explored the spatial arrangement of the arteriolar niche and the putative megakaryocytic niche. We first sought to determine whether the association of HSCs with MKs was secondary to an association with arterioles or whether HSCs associate with MKs independently of arterioles. We measured the distances of actual and randomly placed HSCs to arterioles (dtart) or to MKs (dMK) (Fig. 4a-d and Supplementary Tables 1–4) on images of whole-mount sternal preparations. In one null model (random 1), we randomized only HSC localization, whereas in a second null model (random 2), we applied random placement of both HSCs and MKs to remove any potential inherent bias of MK localization near or far from arterioles (Fig. 4d). The distance distributions of randomly placed HSCs were not biased to either the arterioles or the MKs, as expected, in either null model (Fig. 4d). The null model distributions were not statistically different from each other (two-dimensional Kolmogorov-Smirnov (2D-KS) test22,33; P = 0.079). We observed that actual HSCs found in sternal preparations were enriched in two configurations: in the first, 15% of HSCs had dMK < 25 μm but were distant (>100 μm) from arterioles (Fig. 4d, suggesting an MK niche that is spatially distinct from the arteriolar niche. We did not see this configuration in random models 1 and 2, where only 5.1% and 4.1% of HSCs, respectively, fell into this configuration (Fig. 4d). We did not observe this enrichment in random models 1 and 2, where only 5.1% and 4.1% of HSCs, respectively, fell into this configuration (Fig. 4d). In the second configuration, 21% of HSCs were localized in close proximity to MKs (<25 μm) but were distant (>100 μm) from arterioles (Fig. 4d), suggesting an MK niche that is spatially distinct from the arteriolar niche.

The role of CXCL4 in maintaining the MK niche, we evaluated the dMK of HSCs in Cxcl4−/− mice (Fig. 4d). We found that...
Figure 4 MKs regulate HSC quiescence independently of the arteriolar niche.

(a–c) Representative whole-mount image of a sternum compartment (a) and magnified high-power views (b,c). Arterioles are identified by CD31-CD144-SCA1- expression. Yellow arrowheads denote Lin-CD48-CD41-CD150+ phenotypic HSCs, and MKs are distinguished by their size, morphology and CD41 expression. The dotted outline denotes the bone–bone marrow border. (b,c) Illustrative images of the measured distances (yellow dashed lines) between HSCs and MKs and between HSCs and arterioles in the bone marrow (BM). Scale bars, 100 µm (a–c). The images in b correspond to the left box in a, and the images in c correspond to the right box in a. (d) 2D probability distribution of the distances between HSCs and MKs or arterioles in the sternum compartment (µg). Ctrl (n = 128 HSCs) and Cxcl4−/− (n = 150 HSCs) mice. The association of HSCs to arterioles is independent from the association of HSCs with MKs distant from arterioles was significantly disturbed (P = 0.0001; Fig. 4g). Consistent with the notion that the association of HSCs to arterioles is independent from the association of HSCs to MKs, neither the distribution of HSCs in relation to arterioles (P = 0.2837; Fig. 4g) nor the absolute number of HSCs adjacent to (darteriole = 0 µm; Fig. 4h and Supplementary Fig. 9a,b) or in close proximity to (darteriole < 20 µm; Fig. 4i and Supplementary Fig. 9a,b) arterioles was significantly altered after MK depletion. Indeed, after MK depletion, clusters of HSCs, which are rarely found in control mice, occurred distant from arterioles (darteriole > 20 µm, P < 0.05; Fig. 4j and Supplementary Fig. 9a,c), suggesting that HSC quiescence away from arterioles depends on the presence of MKs. These results thus suggest the possibility of at least two spatially and functionally distinct niches maintaining HSC quiescence, one near MKs and one near arterioles.

Whereas differentiated progenitors have been shown previously to provide a specialized niche in the intestine6, influence bulge stem cell quiescence in the skin35 or indirectly affect HSC retention in the bone marrow by targeting the stromal niche cells36–38, only cells of nonhematopoietic origin have been implicated so far as candidate HSC niche cells in the mammalian bone marrow2–8. Our current data indicate that a terminally differentiated cell type derived from HSCs, the MK, may control its own replenishment through a direct feedback loop (Supplementary Fig. 10), adding further complexity to the HSC niche. As a subset of HSCs is platelet biased39, it will be of interest to determine whether the MK supports its own lineage by forming a niche selectively for this type of HSC. Because MKs are a source of multiple cytokines, it is probable that other factors in addition to CXCL4 contribute to MK-mediated HSC quiescence, as is suggested for TGFβ1 in the accompanying paper (Zhao et al.40). The role of CXCL4 in HSC quiescence may have implications in certain malignancies (for example, myelodysplastic syndromes) where hyperproliferative HSCs are found alongside dysplastic MKs, which secrete reduced amounts of CXCL4 (ref. 40). Further studies will determine the individual functions of, and interplay between, distinct HSC niches, as well as their implications in disease.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.
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AUTHOR CONTRIBUTIONS

I.B., D.L. and S.P. designed the study; performed the majority of the experiments and analyzed data; J.A. and A.B. performed computational modeling and statistical analysis of the data. M.P.L. performed imaging of CXCL4 in MKs and provided mice. C.S. and Y.K. contributed to the processing and imaging of whole-mount bone marrow tissues. L.S. genotyped mice. M.P. provided mice and interpreted data. P.S.F. and A.B. supervised the study. I.B., D.L., S.P., A.B. and P.S.F. wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Mice.** C57BL/6-Tg(Pf4-cre)Q3Rsko/J (Cxl4-cre) and C57BL/6-Gt(Rosa)26Sortm1 (H2BEGF)Awai (iDTR) mice were purchased from the Jackson Laboratory. C57BL/6-CD45.1 (B6.SJL-Ptprc Pepc<sup>b</sup>Boj) and C57BL/6-CD45.2 (B6.SJL-Ptprc<sup>a</sup> Pepc<sup>b</sup>Boj) congenic strains were purchased from the US National Cancer Institute. Nes-GFP mice<sup>41</sup>, Cx4-cre; iDTR; Nes-GFP mice and Cx4<sup>flx/flx</sup> mice<sup>39</sup> were bred in our facilities. All experimental procedures were approved by the Animal Care and Use Committees of Albert Einstein College of Medicine.

**In vivo treatments.** For DT-mediated MK depletion, 250 ng of DT (Sigma) was injected intraperitoneally (i.p.) every 24 h over 7 d. 0.02 or 0.1 µg CXCL4 (PeproTech) and neuraminidase type IV (0.1 U) were injected i.p. every 24 h over 7 d and then every 48 h for the remaining 5 weeks. Mice were subjected to HSC analyses on the day of the last injection. No blinding or randomization procedures were used for the allocation of mice to experimental and control groups, and no samples size or power calculations were performed.

**Preparations, immunofluorescence staining and imaging of whole-mount sternum tissues and frozen sections of the long bones.** Whole-mount tissue preparation, immunofluorescence staining and imaging of the sternum and femoral bones were performed as described previously<sup>32</sup>.

**Computational modeling of random HSC localization.** Simulations were performed with Matlab software (MathWorks). Images of whole mount–prepared sternal segments were used to generate spatial maps of arterioles and MKs onto which 20 HSCs were randomly placed, consistent with an average density of 20 HSCs per sternal segment sampling field. Thresholds were applied to generate binary maps, followed by median filtering to remove small scatter background (nonspecific background staining) to accurately represent arteriole and MK localization within the marrow space.

Boundaries of the marrow, defined by the bone-to-marrow interface, were demarcated on the binary maps of MKs and arterioles so that random HSCs were only placed in the bone marrow space. Random HSC coordinates were selected from the region within the boundary, and the shortest Euclidean distance was calculated for each HSC to either the MK or to the arteriole. In random model 1 (random 1), each simulated run of 20 randomly placed HSCs was repeated 1,000 times on images of arterioles and MKs in each run. In random model 2 (random 2), each simulated run of 20 randomly placed HSCs was repeated, as before, and 135 randomly placed MKs was repeated 1,000 times. Distances of randomly placed HSCs to randomly placed MKs were adjusted by subtraction of the mean radius of MKs (15.4 µm). 2D distance distributions were compared using a modified non-parametric 2D-KS test<sup>33,34</sup> and a 2D-2S-KS test<sup>44</sup>.

**Cell sorting and flow cytometry.** For isolation of CD41<sup>+</sup> MKs, bone marrow cells were cultured in DEMEM supplemented with 10% FBS, 0.5% penicillin-streptomycin and 50 ng ml<sup>−1</sup> recombinant thrombopoietin (THPO; Prospect) over 3 d, enriched in a BSA gradient as described<sup>42</sup> and isolated by FACS sorting. All cell sorting experiments were performed using an Aria Cell Sorter (BD Biosciences). Flow cytometric analyses were carried out using an LSRII flow cytometer (BD Biosciences). For analyses of Nestin<sup>dim</sup> and Nestin<sup>bright</sup> cells as well as endothelial cells, BM cells were processed, stained and gated as described<sup>2</sup>. For osteoblastic-lineage cell quantification, flushed bones were chopped into small fragments and incubated in 1 mg ml<sup>−1</sup> collagenase type I at 37 °C for 90 min and then filtered. Osteoblastic-lineage cells were defined as CD41<sup>+</sup>CD10<sup>+</sup>CD51<sup>+</sup>SCA1<sup>−</sup> as described<sup>31</sup>. Dead cells and debris were excluded by FSC, SSC and DAPI staining profiles. Data were analyzed with FlowJo (Tree Star) or FACSDiva 6.1 software (BD Biosciences).

**Antibodies and staining reagents.** The following antibodies were used in this study: biotin-anti-Lin (TER-119, RB6-8C5, RA3-6B2, M1/70, 145-2C11, 1:50 dilution), FITC/phycoerythrin (PE)-anti-CD45.2 (104), FITC/PE-PE-Cy7-anti-Ly6A/E (D7), BV421/PE/PE-Cy7-anti-CD117 (2B8), biotin-anti-CD48 (HM48-1), biotin-anti-CD41 (MWReg30, 1:2,500 dilution), allopurinol (1 mg ml<sup>−1</sup>), and DAPI (1:50 dilution). 0.1–0.25 × 10<sup>6</sup> competitor CD45.1 + cells was added to assess CXCL4-related effects on HSCs. Proliferation of HSCs was determined by adding 10 µl of a 1 mM BrdU solution to the cell cultures, and staining and analysis were performed as described above.

**Competitive reconstitution.** Competitive repopulation assays were performed using the CD45.1/CD45.2 congenic system. Equal volumes of BMNCs harvested from Cx4<sup>cre</sup>-iDTR mice, Cx4<sup>flx/flx</sup> mice or C57BL/6WT mice were transplanted together with 0.25 × 10<sup>6</sup> competitor CD45.1<sup>+</sup> cells. Mice were injected with DT (Sigma) was added to assess CXCL4-related effects on HSCs. Proliferation of HSCs was determined by adding 10 µl of a 1 mM BrdU solution to the cell cultures, and staining and analysis were performed as described above.

**Cell culture.** For in vitro analyses of HSCs, lineage-depleted BMNCs were cultured for 7 d in StemSpan medium (StemCell Technologies) supplemented with KIT ligand (KITL) (10 ng ml<sup>−1</sup>), fibroblast growth factor 1 (FGF1) (10 ng ml<sup>−1</sup>) and THPO (20 ng ml<sup>−1</sup>; all R&amp;D Systems). Recombinant mouse CXCL4 (100 ng ml<sup>−1</sup>; PeproTech) or vehicle with or without heparin (30 µg ml<sup>−1</sup>; Sigma) was added to assess CXCL4-related effects on HSCs. Proliferation of HSCs was defined by adding 10 µl of a 1 mM BrdU solution to the cell cultures, and staining and analysis were performed as described above.

**Statistics.** All data are shown as the mean ± s.e.m. Comparisons between two samples were done using paired and unpaired Student’s t tests. One-way analysis of variance (ANOVA) analyses followed by Tukey’s multiple comparison tests were used for multiple group comparisons. Statistical analyses were performed with GraphPad Prism. *P < 0.05, **P < 0.01, ***P < 0.001.

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