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Loss of *EfnB1* in the osteogenic lineage compromises their capacity to support hematopoietic stem/progenitor cell maintenance

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The bone marrow stromal microenvironment contributes to the maintenance and function of hematopoietic stem/progenitor cells (HSPCs). The Eph receptor tyrosine kinase family members have been implicated in bone homeostasis and stromal support of HSPCs. The present study examined the influence of EfnB1-expressing osteogenic lineage on HSPC function. Mice with conditional deletion of *EfnB1* in the osteogenic lineage (*EfnB1*$_{OB}^{-/-}$), driven by the *Osterix* promoter, exhibited a reduced prevalence of osteogenic progenitors and osteoblasts, correlating to lower numbers of HSPCs compared with *Osx:Cre* mice. Long-term culture-initiating cell (LTC-IC) assays confirmed that the loss of EfnB1 within bone cells hindered HSPC function, with a significant reduction in colony formation in *EfnB1*$_{OB}^{-/-}$ mice compared with *Osx:Cre* mice. Human studies confirmed that activation of EPHB2 on CD34^{+} HSPCs via EFNB1-Fc stimulation enhanced myeloid/erythroid colony formation, whereas functional blocking of either EPHB1 or EPHB2 inhibited the maintenance of LTC-ICs. Moreover, EFNB1 reverse signaling in human and mouse stromal cells was found to be required for the activation of the HSPC-promoting factor CXCL12. Collectively, the results of this study confirm that EfnB1 contributes to the stromal support of HSPC function and maintenance and may be an important factor in regulating the HSPC niche.

The hematopoietic stem/progenitor cell (HSPC) niche is composed of diverse cellular components that contribute to HSPC maintenance and niche function within the bone marrow. These include arterioles, sinusoidal endothelial cells, Nestin+ mesenchymal stem cells, stromal populations including bone marrow stromal stem cells/perivascular reticular cells, and cells of the osteogenic lineage [1–9]. These cells are key mediators of hematopoietic stem cell (HSC) maintenance, proliferation, differentiation, and maturation, acting in a non-cell-autonomous manner through membrane-dependent or soluble chemokines and growth factors. A number of molecules, including CXCL12, stem cell factor (SCF), WNT, NOTCH, and SLIT ligands, FGF1/2, BMPs, TGFβ, angiogenin, angiopoietin-1 (Ang-1), osteopontin (OPN), and interleukin-6 (IL-6), have been attributed to mediating HSPC support and regulation in physiological and pathophysiological settings [4,5,9–16]. Recently, the Eph receptors, the largest membrane-bound, contact-dependent receptor tyrosine kinase family, have been implicated in HSPC niche function and maintenance [17].

The Eph receptors and their cognate Efn (ephrin) ligands are clustered into two subclasses referred to as the A and B subclasses. The division into their respective subclasses is based on the structure of the Efn ligands; EfnA molecules are anchored by glycophosphatidylinositol to the membrane, whereas EfnB ligands possess transmembrane

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domains [17,18]. The EphA or EphB receptors are segregated based on their binding affinity for their respective ligands, with minimal cross-communication between subclasses; this is limited to EphA4 and EphB2 [19–21]. However, there is extensive promiscuous binding within subclasses, with multiple EphA or EphB receptors interacting with numerous EfnA or EfnB ligands, respectively. Furthermore, the members of both the Eph receptors and their Efn ligands can mediate signaling unidirectionally through either the Eph- or Efn-expressing cell or bidirectionally through both cell types [18,22,23]. Due to these unidirectional and bidirectional signaling properties, these molecules are essential for a multitude of cellular processes during embryonic and postnatal development and in many pathologies [18,23–29]. More specifically, Eph and ephrin molecules have been associated with bone marrow stromal stem cell (BMSC) adhesion, migration, differentiation, and apoptosis [30–33]. In the context of hematopoiesis, the Eph and Efn molecules have been implicated, not only in the function of mature hematopoietic populations such as B cells, T cells, and platelets [34–44], but also in HSPC migration and differentiation [17,37,39,45,46].

Mutations in the human *EFNB1* gene can lead to craniofrontonasal syndrome and other skeletal deformities [47–49]. Similarly, global and targeted deletion of *EfnB1* in mouse resulted in cranial and skeletal defects [24,25,50,51]. More specifically, the loss of *EfnB1* expression under the control of the *Osterix* (*Sp7*) promoter in osteogenic/stromal populations hindered skeletal development through cell-autonomous and non-cell-autonomous signaling [24]. These mice displayed reduced cortical and trabecular bone to tissue volume and increased fragility due to the loss of EfnB1 reverse signaling within the osteogenic population. Furthermore, this homozygous deletion of *EfnB1* in the osteogenic lineage was also associated with an increase in the number of monocytic lineage-derived osteoclasts.

In the present study, we investigated whether disruption of skeletal development following deletion of *EfnB1* in the osteogenic lineage could affect the integrity of the HSPC niche. We performed experiments to determine whether conditional deletion of *EfnB1* in osteogenic/stromal populations leads to a diminished capacity to support HSPC maintenance and function.

Methods

All methods were performed in accordance with the relevant institutional (The University of Adelaide) and Australian Federal Government guidelines and regulations as indicated below.

Animal breeding and maintenance

129S-Efnb1tm1Sor/J (*EfnB1*$^{fl/fl}$) mice purchased from The Jackson Laboratory (Bar Harbor, ME, USA; catalog no. 007664) were backcrossed 10 generations to a C57BL/6 genetic background. To conditionally delete *EfnB1* in osteoprogenitors/pre-osteoblastic cells and chondrocytes [52], tTA:Osx1-GFP:Cre (hereafter referred to as *Osx:Cre*) male mice [53] were bred with *EfnB1*$^{fl/fl}$ females. The hemizygote *Osx:Cre-EfnB1*$^{fl/O}$ male offspring were then bred with *EfnB1*$^{fl/fl}$ females, generating homozygote *Osx:EfnB1*$^{fl/fl}$ females and *EfnB1*$_{OB}^{-/0}$ males. The *Osx:Cre* strain was maintained by breeding *Osx:Cre* males with C57BL/6 females. Because homozygous female and hemizygous male mice both lack EfnB1 in osteoprogenitors/pre-osteoblastic cells and chondrocyte populations, hereafter, both genders will be referred to as *EfnB1*$_{OB}^{-/-}$. The loss of EfnB1 in osteogenic populations of *EfnB1*$_{OB}^{-/-}$ has been previously reported [24]. The SA Pathology Animal Ethics Committee approved the animal breeding (BC BC01/11) and all animal experiments and analyses were approved by both SA Pathology (102/13) and the University of Adelaide (M-2013-144) Animal Ethics Committees. Note that, for all animal studies, two femurs and two tibias were collected and used in the studies outlined below.

Flow cytometric analysis of stromal and hematopoietic populations

Murine stromal and hematopoietic populations were isolated using previously described methods [46]. Briefly, a single-cell suspension of cells was filtered (70 μm) and the red blood cells lysed with ammonium chloride potassium buffer. The remaining cells were blocked with mouse gamma globulin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). A total of 1×10^6 compact bone cells were stained with the antibodies previously reported to isolate osteoprogenitor cells (OP: $Lin^-CD45^-CD31^-Sca1^+CD51^+$) and osteoblasts (OB: $Lin^-CD45^-CD31^-Sca1^-CD51^+$); 1×10^6 nucleated cells isolated from the bone marrow were also stained with antibodies that define primitive hematopoietic stem cells (SLAM HSCs: $Lin^-Sca1^+ckit^+CD48^-CD150^+$), multipotent progenitors (MPPs: $Lin^-Sca1^+ckit^+CD48^-CD150^-$), and hematopoietic progenitor cells (LSKs: $Lin^-Sca1^+ckit^+$). A total of 1×10^5 cells were stained to identify myeloid cells, T cells, and B cells [46]. Flow cytometric analysis was performed on the BD LSRFortessa X20 Analyzer (BD Biosciences). Data were acquired using BD FACSDiva analysis software and analyzed using FlowJo data analysis software (BD Biosciences).

Isolation of stromal and hematopoietic cell populations from humans

Human mononuclear cells were prepared by Ficoll separation of bone marrow aspirates from normal adult donors following informed consent (Royal Adelaide Hospital Human Ethics #940911a) as described previously [54]. Primary BMSCs were cultured (5×10^4/cm^2 of STRO-1$^+$ mononuclear cells) in alpha modification of Eagle's medium containing 10% (v/v) fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 μmol/L L-ascorbate-2-phosphate, and penicillin (50 i.u./mL)/streptomycin sulfate (50 μg/mL) at 37°C and 5% CO_2 [33,55].

Primitive HSCs ($CD34^+CD38^-$) were isolated by fluorescence-activated cell sorting (FACS) as described previously [46,54,56]. Briefly, human mononuclear cells were stained with phycoerythrin-conjugated anti-CD34 and fluorescein isothiocyanate-conjugated

anti-CD38 (BD Biosciences) and then sorted and analyzed on a FACSAria cell sorter (BD Biosciences). Isolated cells were then processed for gene expression.

Gene expression analysis

RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction were conducted as described previously [46]. Briefly, TRIzol (Invitrogen Life Technologies, Waltham, MA, USA) was used to isolate total RNA. Superscript III reverse transcriptase (Invitrogen) was used to reverse transcribe total RNA. Gene expression of *EPHB* receptors, *EFNB1*, *CXCL12*, and *IL-6* was performed using human-specific primers as described previously [33,46,57].

Mouse and human CFC and LTC-IC assays

Mouse [7,58] and human [59] colony-forming cell (CFC) assays and LTC-IC assays were performed as described previously [46].

CFC assay. Briefly, mouse bone marrow nucleated cells (1×10^5 cells/mL) or human purified $CD34^+$ HSPCs (1.5×10^4 cells/mL) isolated from the bone marrow were cultured in methylcellulose medium (STEMCELL Technologies, Vancouver, Canada) for 10–14 days at 37°C and 5% O_2. In human studies, cells were cultured in the presence of EfnB1-Fc or the human IgG-Fc control (10 μg/mL, R&D Systems, Minneapolis, MN, USA). Plates were enumerated for erythroid (BFU-E), granulocyte and/or macrophage (CFU-GM), and granulocyte-erythrocyte-monocyte/macrophage-megakaryocyte colony (CFU-GEMM) formation.

LTC-IC assay. Stromal cells, used for the feeder layers, were gamma-irradiated (30 Gy) and plated (3×10^4 mouse stromal cells/well of 96-well plate and 1.5×10^4 human BMSCs/well of 24-well plate). Twenty-four hours later, freshly isolated mouse bone marrow nucleated cells or human purified $CD34^+$ HSPCs were resuspended in myeloid LTC medium (STEMCELL Technologies) containing freshly added hydrocortisone sodium hemisuccinate (10^{-6} mol/L, STEMCELL Technologies). The murine bone marrow nucleated cells and human HSPCs were plated onto pre-established stromal feeder layers. Cultures were maintained for 4 weeks at 33°C for mouse studies and for 5 weeks at 37°C for human studies with weekly half medium replacement. Total cells from each well were harvested, plated in methylcellulose medium (STEMCELL Technologies), and cultured for 14 days. Colonies were scored based on their morphology described in the CFC assay. In human studies designed to investigate the contribution of EphB receptor to colony formation, EphB receptor-blocking peptides (100 μmol/L) were added to LTC-IC assays at weekly half-medium changes. Blocking peptides for specific EphB receptors included: EphB1 (EWLSPNLAPSVR), EphB2 (SNEWILPRLPQH), and scramble control peptide (RTVAHHGGLYHTNAEVK) (Mimotopes, VIC, Australia).

siRNA knockdown

Small interfering RNA (siRNA) knockdown of *EFNB1* in human BMSCs was performed as described previously [60]. Briefly, BMSCs were seeded ($1.5 \times 10^4/cm^2$) and, 24 hours later, *EFNB1-A* siRNA (s4512), *EFNB1-B* siRNA (s446539), or scramble siRNA control (12 pmol, Ambion/Thermo Fisher Scientific) was combined with RNAiMAX lipofectamine (Ambion/Thermo Fisher Scientific) as per the manufacturer's instructions and the siRNAs were added to the cells for 72 hours.

Letriviral transduction

Primary stromal cells were isolated and cultured from 4-week-old $EfnB1^{fl/fl}$ mice as described previously [32]. Cells from $EfnB1^{fl/fl}$ mice were infected with a lentivirus containing the tamoxifen-inducible self-deleting Cre recombinase LEGO-CreERT2-iG2) in the presence of Polybrene (4 μg/mL) as described previously [61,62]. Stably transduced cells were selected by FACS based on enhanced green fluorescent protein expression. Cells were expanded and treated with either the vehicle (0.05% ethanol) or 0.5 mmol/L 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich) for 8 days. Treatment was not included in any subsequent cultures.

Statistical analysis

Prism software (GraphPad, La Jolla, CA, USA) was used to determine statistical significance. Treated groups were compared with their corresponding controls using a two-tailed unpaired Student *t* test. $p < 0.05$ was considered statistically significant.

Results

Loss of EfnB1 *reduces the frequency of osteogenic cell populations*

The present study investigated the importance of EfnB1 in maintaining osteogenic populations within the bone marrow microenvironment [63–65]. These populations were isolated with specific cell-surface antigens and analyzed by flow cytometry to assess the incidence of OP ($Lin^-CD45^-CD31^-Sca1^+CD51^+$) and OBs ($Lin^-CD45^-CD31^-Sca1^-CD51^+$) (Figures 1A and 1B). Flow cytometric analysis demonstrated a significant reduction ($p < 0.05$) in the number of OP and OB in $EfnB1_{OB}^{-/-}$ mice compared with *Osx:Cre* control mice (Figures 1C and 1D). No significant difference in the number of endothelial cells was observed between $EfnB1_{OB}^{-/-}$ mice and *Osx:Cre* control mice (Figure 1E). These data suggest that EfnB1 is a critical factor in directing mesenchymal stem/progenitor cell development toward the osteogenic cell lineage.

EfnB1 *deficiency in the osteogenic cell lineage leads to diminished levels of HSPCs within the bone marrow*

Studies were performed to determine whether the loss of EfnB1 in Osterix-expressing cells could influence HSPC populations within the bone marrow using flow cytometric analysis of SLAM HSCs, MPPs, and LSKs (Figures 2A and 2B). As shown in Figure 2, there was a significant reduction ($p < 0.05$) in the prevalence of SLAM HSCs, MPPs, and LSKs in the bone marrow of

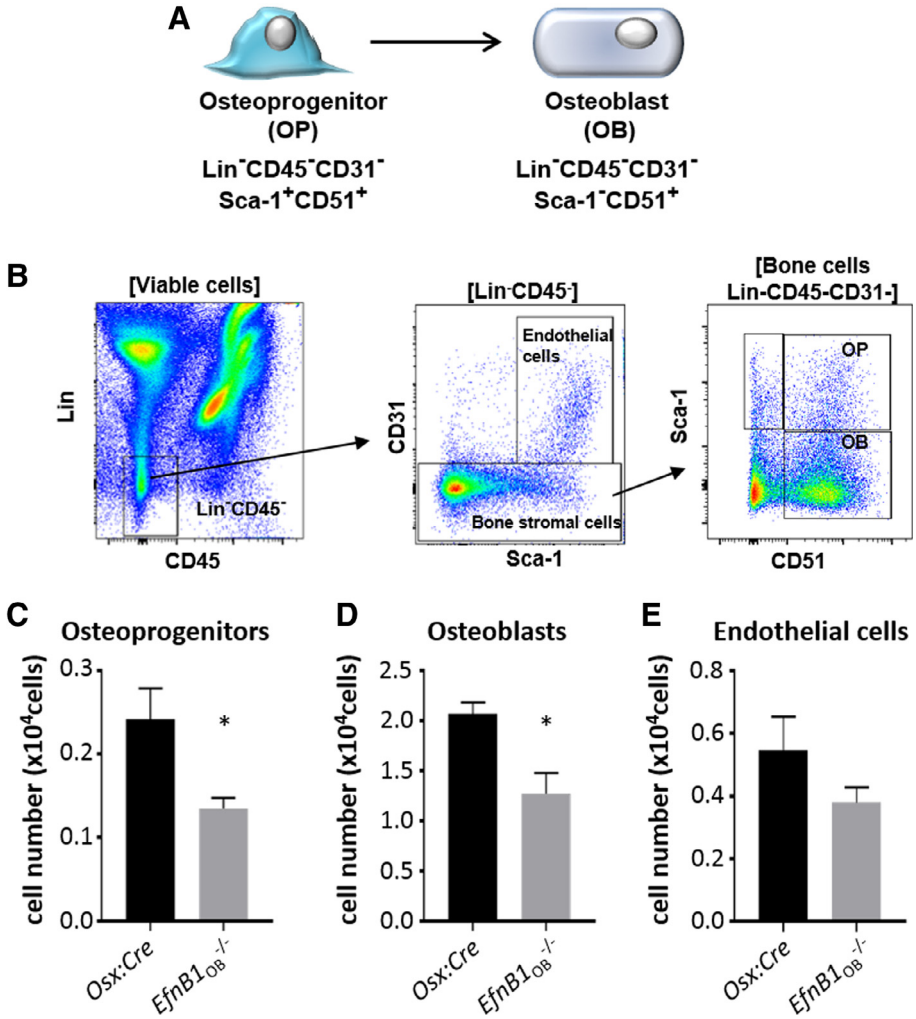

Figure 1. Loss of EfnB1 in osteoprogenitors reduces the amount of osteogenic derivatives. **(A,B)** Representative **(A)** schematic and **(B)** flow cytometry gates of cell surface markers used to identify OPs and OBs. **(C,D)** Flow cytometry analysis of endothelial and mesenchymal populations derived from 4-week-old *Osx:Cre* and *EfnB1*$_{OB}^{-/-}$ female and male mice represented as the number of **(C)** OPs ($Lin^-CD45^-CD31^-Sca1^+CD51^+$), **(D)** OBs ($Lin^-CD45^-CD31^-Sca1^-CD51^+$), and **(E)** endothelial cells isolated from compact bones of two tibias and two femurs ($n = 6–8$ mice/strain) Data represent mean ± SEM. *$p < 0.05$, unpaired Student t test.

EfnB1$_{OB}^{-/-}$ mice compared with age-matched *Osx:Cre* control mice (Figures 2C–2E). These observations correlated with a significant ($p < 0.05$) reduction in total colony formation, including BFU-E, CFU-GM, and CFU-GEMM (Figure 3A) of bone marrow nucleated cells isolated from *EfnB1*$_{OB}^{-/-}$ compared with age-matched *Osx:Cre* control mice (Figure 3B). LTC-IC assays were used to determine whether HSPC maintenance was influenced by EfnB1-deficient osteogenic cells. Bone marrow nucleated cells isolated from *Osx:Cre* or *EfnB1*$_{OB}^{-/-}$ mice were cultured on irradiated BMSC feeder layers derived from *Osx:Cre* mice. HSPCs derived from *EfnB1*$_{OB}^{-/-}$ mice produced significantly ($p < 0.05$) fewer colonies compared with age-matched *Osx:Cre* control mice (Figure 3C). LTC-IC analyses were subsequently performed to determine whether the loss of EfnB1 in the osteogenic lineage could influence the function of primitive HSPCs. Bone marrow nucleated cells isolated from *Osx:Cre* mice were seeded onto an irradiated BMSC feeder layer derived from either *Osx:Cre* or *EfnB1*$_{OB}^{-/-}$ mice. Bone marrow nucleated cells cultured on the BMSC feeder layers from *EfnB1*$_{OB}^{-/-}$ mice produced significantly ($p < 0.05$) fewer colonies compared with those cultured on the *Osx:Cre* feeder layers (Figure 3D). These observations confirm that EfnB1, which is expressed by osteogenic cells, influences the maintenance of HSPCs.

Frequency of mature hematopoietic populations is influenced by the lack of EfnB1 in the osteogenic population within the marrow

We next assessed the cellularity of nucleated cells isolated from the bone marrow, peripheral blood, and spleens of

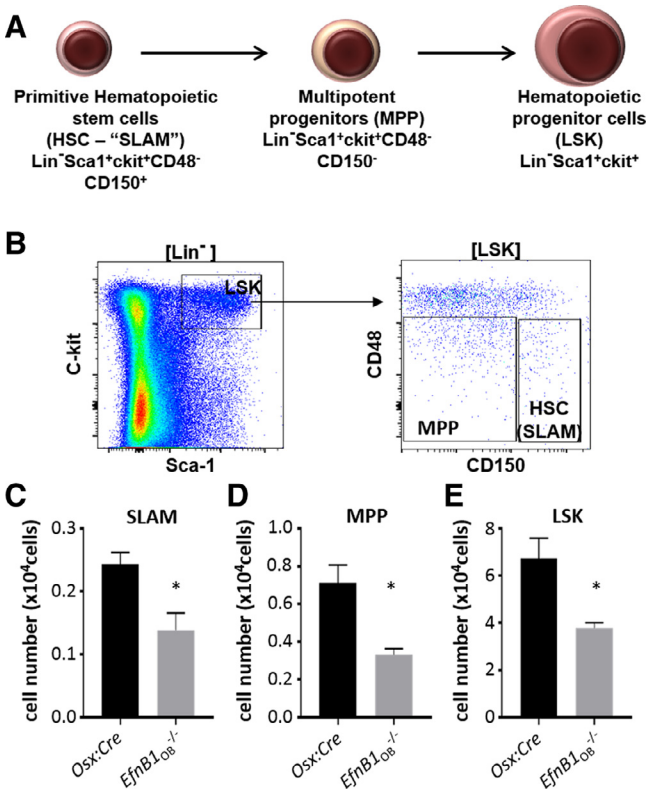

Figure 2. Mice lacking EfnB1 in osteogenic populations have diminished frequency of HSPC populations within the bone marrow. **(A,B)** Representative **(A)** schematic and **(B)** flow cytometry gates of cell surface markers used to characterize SLAM HSCs, MPPs, and LSKs. **(C–E)** Flow cytometry analysis of hematopoietic populations derived from the bone marrow of 4-week-old *Osx:Cre* and *EfnB1*$_{OB}^{-/-}$ female and male mice represented as the number of **(C)** SLAM HSCs, **(D)** MPPs, and **(E)** LSKs isolated from two tibias and two femurs ($n = 5–8$ mice/strain) Data represent mean ± SEM, unpaired Student *t* test, $*p < 0.05$.

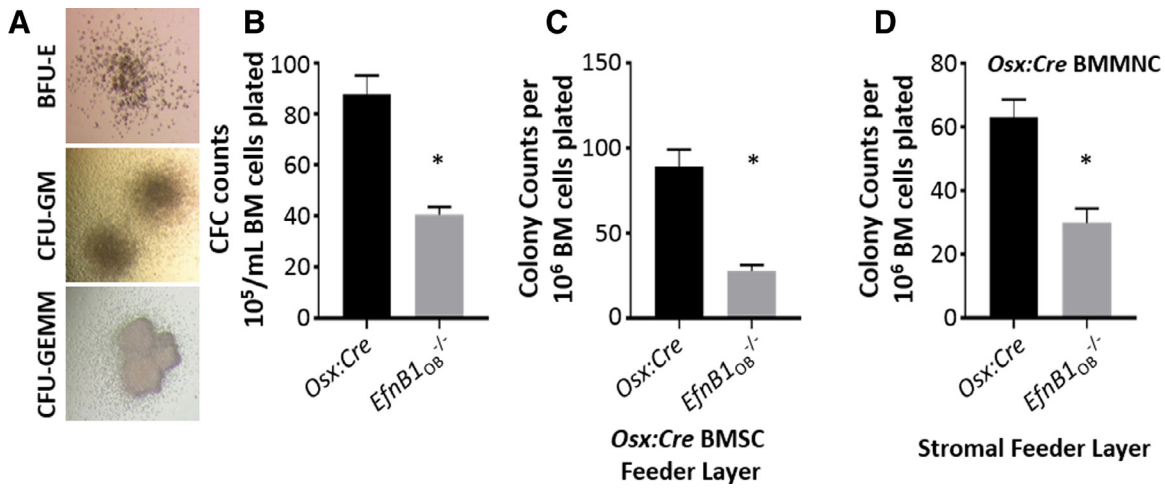

Figure 3. The lack of EfnB1 in osteogenic populations compromises the stromal support of hematopoietic colony formation. **(A–C)** Bone marrow nucleated cells isolated from 4-week-old *Osx:Cre* and *EfnB1*$_{OB}^{-/-}$ female and male mice were placed **(A,B)** directly into colony formation assays **(A)** represented pictorially (BFU-E, CFU-GM and CFU-GEMM), and **(B)** enumerated and represented as total colony counts. Alternatively the bone marrow nucleated cells were cultured in **(C)** LTC-IC assays, grown on a irradiated BMSC feeder layer isolated from *Osx:Cre* mice ($n = 4$) and assessed for the colony formation capacity. **(D)** bone marrow nucleated cells isolated from 4-week-old *Osx:Cre* mice were engaged in LTC-IC assays, cultured on a irradiated BMSC feeder layer isolated from 4-week-old *Osx:Cre* or *EfnB1*$_{OB}^{-/-}$ female and male mice. The cells were subsequently assessed for their colony formation capacity ($n = 4$ mice/strain). Data represent mean ± SEM. $*p < 0.05$, unpaired Student *t* test.

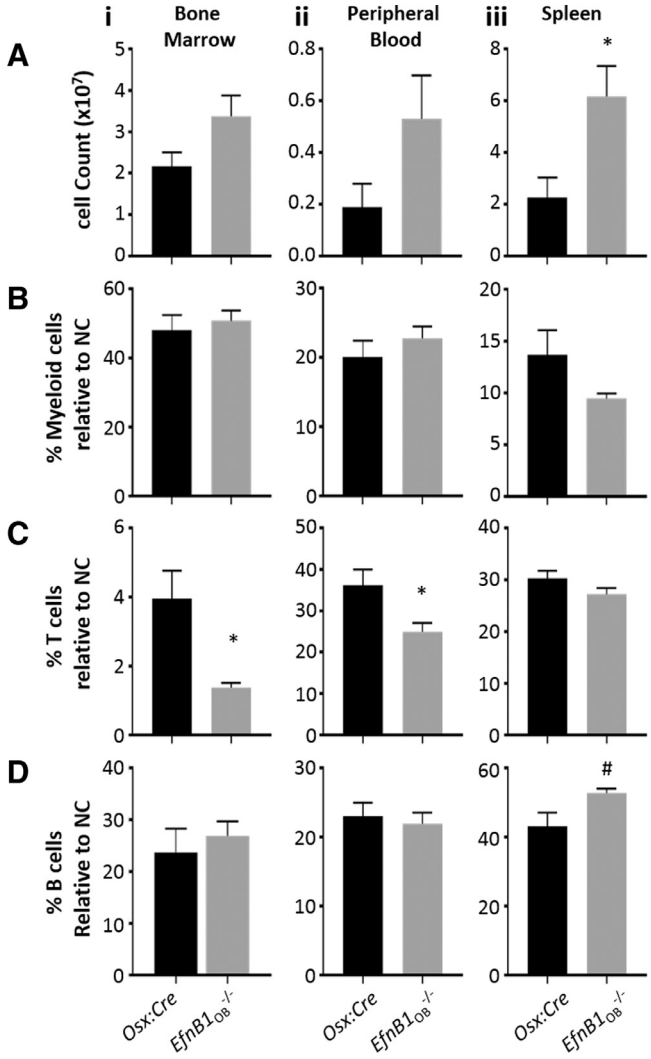

Figure 4. Abundance of hematopoietic populations within the bone marrow, peripheral blood, and spleen are influenced by the loss of EfnB1 in osteogenic cells. **(A)** Nucleated cells isolated from the **(Ai)** bone marrow, **(Aii)** peripheral blood, and **(Aiii)** spleens of 4-week-old *Osx:Cre* and *EfnB1*$_{OB}^{-/-}$ female and male mice were enumerated and represented as the total mononuclear cell count. **(B–D)** Flow cytometry analysis of **(B)** myeloid, **(C)** T cells, and **(D)** B cells expressed as a percentage (%) relative to the total number of nucleated cells (NCs) analyzed from the **(Di)** bone marrow, **(Dii)** peripheral blood, and **(Diii)** spleen. $n = 7$-10 mice/strain. Data represent mean ± SEM. *$p < 0.05$, unpaired Student t test.

Osx:Cre and *EfnB1*$_{OB}^{-/-}$ mice (Figures 4Ai–4Aiii). The loss of *EfnB1* in the osteogenic lineage was associated with significantly ($p < 0.05$) elevated levels of nucleated cells in the spleens of *EfnB1*$_{OB}^{-/-}$ mice, but not in the bone marrow or peripheral blood, compared with *Osx:Cre* control mice (Figures 4Ai–4Aiii). This increase in spleen cellularity (splenomegaly) was consistent with a significant increase in spleen weight of *EfnB1*$_{OB}^{-/-}$ mice (Supplementary Figure E1A, online only, available at www.exphem.org). Assessment of mature hematopoietic lineages within the bone marrow, peripheral blood, and spleen revealed no significant difference in the proportion of myeloid cells in these tissues when comparing *EfnB1*$_{OB}^{-/-}$ and *Osx:Cre* mice (Figures 4Bi–4Biii). A significant ($p < 0.05$) reduction in the incidence of T cells in the bone marrow and the peripheral blood of *EfnB1*$_{OB}^{-/-}$ mice was observed compared with *Osx:Cre* control mice (Figures 4Ci–4Cii); however, this was not associated with difference in thymus weight between *EfnB1*$_{OB}^{-/-}$ and *Osx:Cre* mice (Supplementary Figure 1B, online only, available at www.exphem.org). Although there was no difference in the relative number of B cells within the bone marrow and peripheral blood (Figures 4Di–4Dii), an increase ($p = 0.059$) in the frequency of B cells within the spleens of *EfnB1*$_{OB}^{-/-}$ mice was observed (Figure 4Diii). Collectively, these findings imply that the loss of EfnB1 in osteogenic populations can influence either the production or the maturation of hematopoietic populations within the

bone marrow and associated peripheral lymphoid organs such as the spleen, where Osterix is not expressed [52,61,66,67].

EPHB-EFNB1 communication is critical for human stromal cell support of HSPCs

We have previously reported that human BMSCs express high levels of *EFNB1* [33,55] and *EPHB1* and *EPHB2*, the highest-affinity binding partners of EFNB1, are highly expressed by human primitive HSCs ($CD34^+CD38^-$) (Supplementary Figure 2, online only, available at www.exphem.org). To confirm that human stromal-cell-mediated maintenance of HSPCs is regulated by EFNB1 signaling, $CD34^+$ sorted human HSPCs were cultured in the presence of either soluble EFNB1-Fc or IgG-Fc control in short-term CFC assays (Figures 5A and 5B). Human $CD34^+$ HSPCs cultured in the presence of EFNB1-Fc significantly ($p < 0.05$) enhanced the total number of colonies formed (Figure 5B) compared with the IgG-Fc control. This elevation in colony formation was attributed to an elevation in both erythroid (Figure 5C) and granulocyte/macrophage lineages (Figure 5D). To further demonstrate the contribution of EPHB1 or EPHB2 receptor forward signaling in human HSPC maintenance, LTC-IC assays were performed with $CD34^+$ cells in the presence or absence of blocking peptides specific to either EPHB1 or EPHB2. Blocking either EPHB1 (EWLS) or EPHB2 (SNEW) forward signaling significantly ($p < 0.05$) reduced LTC-IC incidence (Figure 5E) compared with the scramble (RTVA) control peptide. Further experiments were performed to determine whether EFNB1 signaling in human BMSCs affected their expression of key hematopoietic supportive factors. Studies showed that siRNA-mediated knockdown of *EFNB1* gene expression in human BMSCs (Figure 5F) resulted in a significant ($p < 0.05$) reduction of *CXCL12* (Figure 5G) and *IL-6* (Figure 5H) gene expression levels compared with BMSCs treated with the scramble control siRNA. Supportive mouse studies utilizing $EfnB1^{fl/fl}$ cells that were stably transduced with LEGO-CreERT2-iG2 and then treated with 4-OHT confirmed that the loss of *EfnB1* (Figure 5I) gene expression in stromal cells also resulted in a significant ($p < 0.05$) reduction of *CXCL12* (Figure 5J). No significant difference in *IL-6* gene expression was observed. Together, these data suggest that EFNB1-EPHB1 or -EPHB2 interactions enhance human BMSC-mediated support of HPSC maintenance. EFNB1 action appeared to influence HSPC maintenance directly through its interaction with EPHB receptors expressed by HSPCs. Moreover, EFNB1-mediated stromal support of HSPCs could also occur indirectly by modulating the gene expression levels of essential hematopoietic supportive factors expressed by BMSCs.

Discussion

The present study demonstrated that conditional ablation of *EfnB1* in osteogenic cells under the control of the *Osterix* promoter resulted in a significant reduction in HSPC populations within the bone marrow. As a transcription factor, *Osterix* is important for osteogenesis, skeletal development, and maintenance of bone homeostasis, as demonstrated in numerous mouse models [52,66–68]. Previous reports have shown that *Osterix* expression is present in perivascular cells, adipocytes, and stromal cells restricted specifically within the bone marrow [67]. It is well established that endothelial, stromal/osteogenic cell populations [5–8], in addition to Nestin+ BMSCs [1], support hematopoiesis through the expression of HSC niche factors. In these studies, we found that mice lacking EfnB1 in the osteogenic lineage exhibited impaired function of osteogenic cells and a reduction in HSPC populations, whereas endothelial cells were not significantly altered. More specifically, we found that EfnB1-mediated stimulation in isolation acts as a promoter of HSPC colony formation capacity. Flow cytometric analysis confirmed a significant decrease in the most primitive HSCs, MPPs, and LSKs within the bone marrow of mice lacking EfnB1 in the osteogenic lineage. Functional CFC assays confirmed that loss of EfnB1 in osteoprogenitor cells of $EfnB1_{OB}^{-/-}$ mice impeded the formation of erythroid and myeloid lineages. LTC-IC assays using irradiated stromal cells from $EfnB1_{OB}^{-/-}$ mice revealed dysregulated HSPC maintenance and function. Conversely, in vitro studies assessing cultured human $CD34^+$ HSPCs showed an increase in myeloid and erythroid colony formation in the presence of a functional soluble EFNB1 molecule. Confirmation of the role of EPHB1 or EPHB2 forward signaling in hematopoiesis was further demonstrated in human LTC-IC assays in the presence of blocking peptides targeting the EPHB1 and EPHB2 receptors. Importantly, human $CD34^+CD38^-$ cells were found to express high levels of the EFNB1 high-affinity receptors EPHB1 and EPHB2 relative to other EphB receptors. Collectively, these observations suggest that both mouse and human BMSC-mediated support of HSPC maintenance is directly regulated via interactions between EfnB1-EphB1 and -EphB2, respectively. Consistent with the observations of this study, we have previously demonstrated that stromal/osteogenic cells of both human and murine origin support HSPC maintenance via EphB4-ephrinB2 signaling [46]. Similarly, EFNA-subclass, EFNA5-expressing human stromal cells were found to support EPHA5- or EPHA7-expressing HSPC colony formation and migration through RAC1-WAVE1 downstream signaling [45].

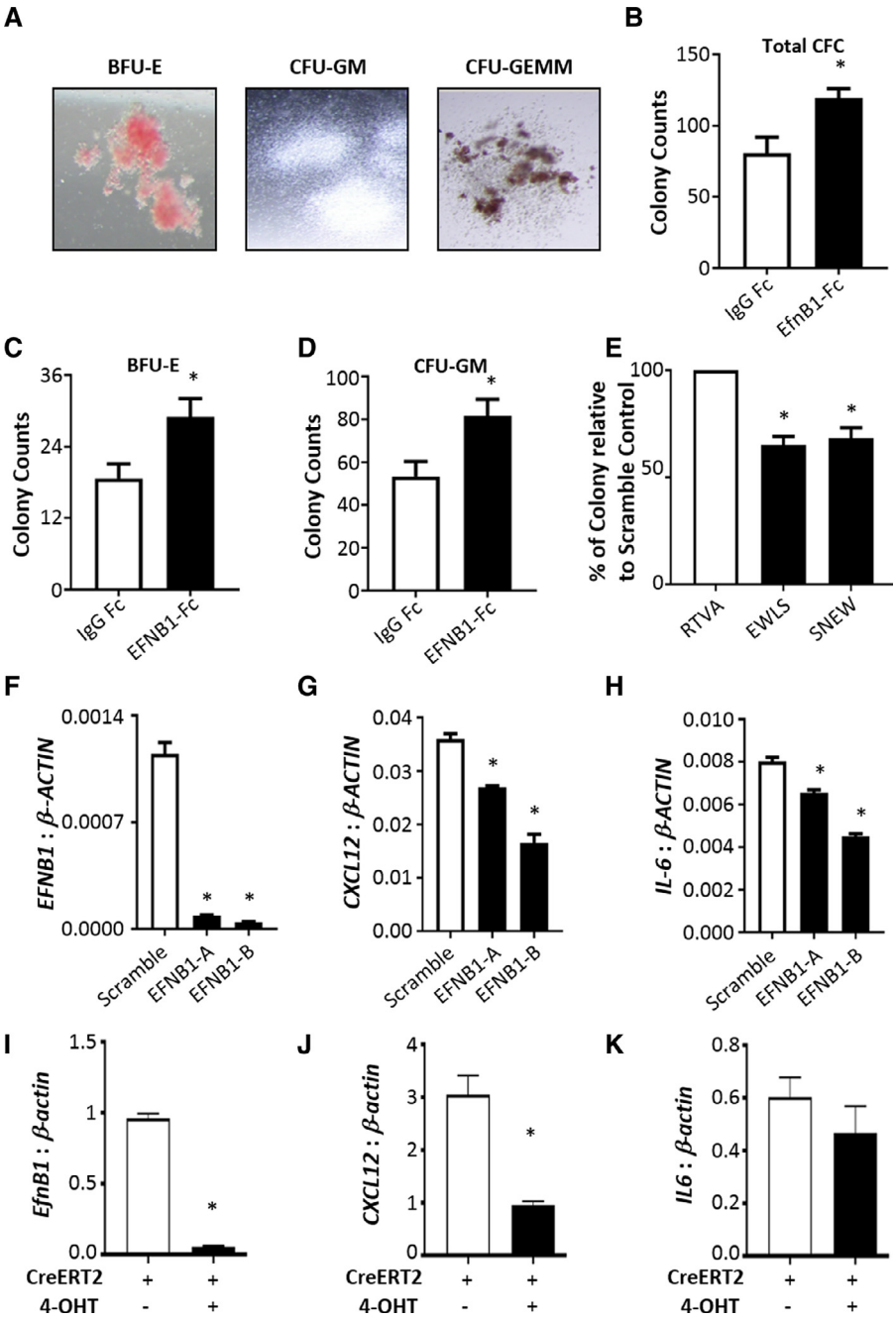

Figure 5. EFNB1/EPHB interactions enhanced hematopoietic stem/progenitor cell support mediated by human BMSCs. **(A–D)** Purified human $CD34^+$ HSPCs were cultured in CFC assays in the presence of clustered human IgG-Fc (IgG-Fc) or EFNB1-Fc fusion protein (10 μg/mL). **(A)** Representative images of BFU-E, CFU-GM, and CFU-GEMM colonies enumerated for **(B)** total colony formation (total CFC, CFU-GM, and BFU-E), **(C)** BFU-E colonies, or **(D)** CFU-GM colonies. **(E)** Human $CD34^+$ HSPCs were seeded onto irradiated human BMSC feeder layers in the presence of a scramble control (RTVA) blocking peptides (100 μmol/L), EPHB1 (EWLS), or EPHB2 (SNEW)-specific blocking peptides in LTC-IC assays ($n = 3$ BMSC donors and three pooled CD34+ HSPC donors). **(F–K)** Gene expression **(F–H)** following siRNA knockdown of *EFNB1* (*EFNB1-A* or *-B*) in human primary BMSCs ($n = 3$) compared with the nonsilencing scramble control and in **(I–K)** EfnB1-floxed cells ($n = 4$) transduced with the tamoxifen-inducible self-deleting Cre recombinase (CreERT2) lentivirus and treated with the vehicle control (−) or tamoxifen (4-OHT). Shown is the relative expression of **(F,I)** *EFNB1* and hematopoietic stimulatory factors **(G,J)** *CXCL12* and **(H,K)** *IL-6*. Data represent mean ± SD of three replicate experiments. *$p < 0.05$, unpaired Student *t* test.

Together, these studies demonstrate that a number of EPH receptors and EFN ligands are important components of the HSPC niche and are involved in direct cell–cell communication and maintenance of HSPCs with different stromal cell populations.

The present study also investigated whether EFNB1 reverse signaling in stromal cells could influence the expression of critical HSPC supportive factors such as SCF, CXCL12, and IL-6 [4,5,11,12]. The expression of SCF within the endothelial cells and *Lepr*-expressing perivascular stromal cells within the bone marrow niche microenvironment is a critical factor in HSPC maintenance [10]. However, SCF was not differentially expressed following the downregulation of EfnB1 in either human or mouse stromal populations (data not shown). Previous studies have shown that CXCL12, which is expressed by stromal cells, including mesenchymal progenitors within the perivascular niche, is a critical HSC supportive factor [11,12]. In the present study, *CXCL12* gene expression levels were dramatically decreased in human and mouse stromal cells following siRNA- or 4-OHT-mediated knockdown of *EFNB1*. Similarly, the expression of the stromal cell-derived HSPC supportive factor IL-6, which is critical for HSPC survival and self-renewal [15,16], was also significantly decreased only in human stromal cells following knockdown of *EFNB1* expression. Therefore, EfnB1 expressed by osteogenic cells not only promotes osteogenesis [24,33,50], but also activates downstream factors within the HSPC cell niche.

Investigation of extramedullary tissues showed that the spleens of $EfnB1_{OB}^{-/-}$ mice were significantly heavier and greater in cellularity compared with *Osx:Cre* control mice. EfnB1-EphB signaling has been implicated in the regulation and function of plasma cells and T-helper cells within the germinal center and is regulated locally [69]. Importantly, the heterogeneity and off-target effects of cells tracked by Osterix drivers has been noted [70]. Lineage tracing studies have demonstrated *Osterix* expression external to the skeletal system, localizing to glomerular cells within the olfactory bulb to a subset of cells within the small and large intestine and the stomach, in addition to vascular smooth muscle cells [66,67,71]. However, despite this heterogeneity, *Osterix* is not expressed in stromal populations within the thymus or the spleen [52,61,66,67]. Therefore, the observed difference in the proportion of B cells within the spleen is likely to be a secondary consequence, potentially due to the dysregulation of the HSPC niche within the bone marrow. However, this observation requires further investigation. A difference in B cells within the bone marrow was not observed, but a significant reduction in the frequency of T cells was identified within the peripheral blood. However, no difference in thymus weight was detected between the different mouse cohorts, suggesting that EfnB1 signaling, which is mediated through the stromal compartment, may contribute to the alteration in T-cell frequency through an unidentified mechanism. The EphB-EfnB subclass has been implicated in T-cell development and maturation, but this was once again local signaling within the thymus rather than through stromal interactions within the bone marrow [72–75]. Studies by Stimamiglio and colleagues suggested that modulation of EphB2 forward signaling, which is mediated by T-cell progenitors and thymocytes following engagement with EfnB1, was important for regulating migration [74].

Collectively, the findings presented herein demonstrate that the loss of EfnB1 within the osteogenic population has a profound influence on bone development, prevalence of stromal/bone cell populations, and bone marrow HSPC maintenance and function. Therefore, EfnB1 may represent novel therapeutic target for manipulating the HSPC niche.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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