Fetal hematopoietic stem cell homing is controlled by VEGF regulating the integrity and oxidative status of the stromal-vascular bone marrow niches

Graphical abstract

Highlights

- Establishment of BM hematopoiesis is coupled to development of the skeletal niches
- Primary HSPC seeding of bone depends on balanced molecular crosstalk in the niche
- Stromal VEGF triggers EC activation and controls stromal-vascular niche integrity
- Excessive skeletal VEGF deranges cell metabolism and induces oxidative stress in BM

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In brief

Mesnieres et al. show that increased VEGF in fetal bone disrupts stromal-vascular integrity, alters cell metabolism, and induces oxidative stress in the developing bone marrow niche, blocking the homing of endogenous hematopoietic stem/progenitor cells. Post-natal irradiation, a common stem cell transplantation pretreatment, induces similar niche alterations, thus opening therapeutic strategies.
Hematopoietic stem and progenitor cell (HSPC) engraftment after transplantation during anticancer treatment depends on support from the recipient bone marrow (BM) microenvironment. Here, by studying physiological homing of fetal HSPCs, we show the critical requirement of balanced local crosstalk within the skeletal niche for successful HSPC settlement in BM. Transgene-induced overproduction of vascular endothelial growth factor (VEGF) by osteoprogenitor cells elicits stromal and endothelial hyperactivation, profoundly impacting the stromal-vessel interface and vascular architecture. Concomitantly, HSPC homing and survival are drastically impaired. Transcriptome profiling, flow cytometry, and high-resolution imaging indicate alterations in perivascular and endothelial cell characteristics, vascular function and cellular metabolism, associated with increased oxidative stress within the VEGF-enriched BM environment. Thus, developmental HSPC homing to bone is controlled by local stromal-vascular integrity and the oxidative-metabolic status of the recipient milieu. Interestingly, irradiation of adult mice also induces stromal VEGF expression and similar osteo-angiogenic niche changes, underscoring that our findings may contribute targets for improving stem cell therapies.
govern HSC behavior. Increased understanding of this interplay in health, disease, and therapy can help design strategies to enhance BM engraftment of transplanted HSCs and improve the success of stem cell therapies for life-threatening diseases. Because endogenous HSPC homing to BM takes place during embryogenesis under physiological, optimal conditions, this process may provide vital insights in the ideal niche characteristics and requirements for successful HSPC settlement.

In fetal life, HSCs migrate through different anatomical locations before finding their final destination in the BM (Dzierzak and Bigas, 2018; Mikkola and Orkin, 2006). First, blood cells emerge in the yolk sac, around embryonic day (E) 7.0 in mice. Next, HSC generation and hematopoiesis take place in the placenta and aorta-gonad-mesonephros region (E9.0–E10.5). Around E11.0, HSCs migrate to the liver, where they vigorously expand. Finally, around E16.5, the liver HSCs home toward the skeleton, where they continue to reside throughout life (Ciriza et al., 2013; Coskun and Hirschi, 2010).

There is only very limited information on the identity, function, and molecular signature of the fetal BM niche constituents interacting with incoming HSPCs. Initial seeding of the BM cavity by HSCs requires CXCL12 acting on its receptor CXCR4 in HSCs; mice deficient for these molecules display normal fetal liver hematopoiesis but fail to establish BM hematopoiesis (Ara et al., 2003; Nagasawa et al., 1996; Zou et al., 1998). A few other chemo-attractive cytokines and adhesion molecules have been implicated, including stem cell factor (SCF) (Christensen et al., 2004) and β1 integrin on HSCs (Potocnik et al., 2000). However, it is not known how the local, tightly orchestrated process of bone development is coupled with the generation of a supportive environment fostering incoming HSPCs. Endochondral ossification starts around E12.5 with mesenchymal condensations forming cartilaginous templates of the long bones, which initially remain completely avascular. Between E14.5 and E16.5, an osteo-angiogenic transformation takes place, excavating the cartilage and creating the primary ossification center, i.e., the central vascularized area within the bone shaft corresponding to the nascent BM cavity (Maes, 2013). This process is driven by blood vessels co-invading the cartilage together with perivascular SSPCs and migratory osteoprogenitors expressing osterix (Osx) (Maes et al., 2010b). The establishment of BM hematopoiesis is dependent on this stromal-vascular invasion, accommodating local extravasation of HSPCs infiltrating the developing bones (Chan et al., 2009; Coşkun et al., 2014; Deguchi et al., 1999).

Yet, the regulatory control and impact of the interplay between perivascular/stromal SSPCs and skeletal blood vessels in mediating fetal HSPC homing has not been investigated.

Here, using mouse models to modulate stromal cell-derived vascular endothelial growth factor (VEGF) signaling, combined with transcriptional profiling and high-resolution imaging, we found that developmental HSPC homing to bone critically depends on controlled local crosstalk among the stromal-vascular cell populations, determining the integrity and functionality of the niche. Excessive skeletal VEGF caused endothelial and perivascular cell hyper-activation, altered cellular metabolism, and increased oxidative stress within the fetal BM environment. Interestingly, irradiation of adult mice also induced VEGF in bone and phenocopied several of the stromal and vascular responses of the fetal genetic model. The mechanistic findings made in this study could thus help improve stem cell homing for treatment of hematologic diseases and malignancies.

RESULTS

Osteolineage VEGF overexpression disrupts osteo-angiogenic bone development and impairs BM cavity formation

To alter the stromal-vascular communication in the fetal skeleton and assess the repercussions on hematopoietic development, we conditionally overexpressed the angiogenic factor VEGF in osteoprogenitors by crossing Osx-Cre:GFp mice (Rodda and McMahon, 2006) to mice carrying a conditional transgene (cTg) encoding VEGF164 (Maes et al., 2010a). Herein, Cre-mediated recombination excises a stop cassette, enabling expression of transgenic VEGF164, the prime VEGF-A isoform, in surplus of the normal VEGF expression from the endogenous gene. VEGF cTg embryos (Osx-Cre:GFp+/−;VEGF(cTg/+)) exhibited 2-fold increased VEGF levels in their bones compared with control littermates (Osx-Cre:GFp+/−;VEGF(+/-)) (Figure 1A). Serum VEGF levels were unaltered (Figure 1B), supporting restricted local actions in the skeleton.

Osteolineage VEGF overexpression led to drastic skeletal malformations and stunted bone growth. At E18.5, VEGF cTg limb bones showed bending and thickening of the central shaft (diaphysis) (Figure 1C) and excessive mineralization (Figures 1D and S1A). Ossification was also increased in the sternum and ribs (Figure S1B), as observed previously using a Col2-Cre driver too (Maes et al., 2010a). Despite the normal serum VEGF levels, the mutant mice did not survive past birth, presumably because the

Figure 1. Osteo-angiogenic development of long bones is disrupted in VEGF cTg mice

(A) Total VEGF mRNA (qRT-PCR) and VEGF164 protein levels (ELISA) in bones of control and VEGF cTg embryos (n = 4–8).
(B) VEGF serum levels (ELISA) (n = 6).
(C) Skeletal preparations at E18.5. Middle panels, dissected hindlimbs of control (top) and VEGF cTg (bottom).
(D) Longitudinal (top) and transversal (bottom) 3D micro-computed tomography (micro-CT) reconstructions of E18.5 tibias.
(E) Histology of E18.5 tibias; (left) Safranin O and (right) H&E. Brackets, BM cavity.
(F) BM cavity volume at E18.5 (n = 7).
(G) CD31 IHC on tibia sections. Arrows, aberrant perichondrial vascularization.
(H) Maximum intensity projection (MIP) of 20-μm depth confocal imaging of Osx-Cre:GFp (nuclear green, OPCs), Emcn (red, blood vessels), and Hoechst (blue, nuclei) in E18.5 tibias. Left, overview; middle, metaphysis; right, diaphysis.
(I) Flow cytometry of CD45 Ter119 Osx/GFP+ cells in E18.5 bones (n = 4–5).

All graphs represent mean ± SEM and depict individual data points derived from different mice (also in the next figures); *p < 0.05, **p < 0.01, ***p < 0.001, by Student’s t test between the genotypes for each time point; no symbol implies p > 0.05. See also Figure S1.
thorax deformities impaired breathing (Figures S1C and S1D). Histology of the limb bones showed a grossly normal growth plate morphology throughout development, whereas the distal and proximal epiphyses (both ends of the bone) became misaligned and separated by an underdeveloped and disrupted primary ossification center, representing the nascent BM cavity (Figure 1E; Figures S1E and S1F). Accordingly, the volume of the BM cavity was vastly reduced (Figure 1F). Thus, VEGF cTg mice failed to develop a normally sized and structured bone shaft and BM cavity.

Excavation of developing bones is initiated by angiogenic invasion of the cartilage template (Maes, 2013). Immunohistochemistry (IHC) for the pan-EC marker CD31 revealed that VEGF overexpression led to early and sustained abnormalities in the skeletal vascular density and pattern at all stages from E14.5 on (Figure 1G). By E18.5, VEGF cTg bones showed excessive numbers of randomly oriented and undersized vessels throughout the widened perichondrium and diaphysis (Figure 1G). Vascularization of developing bones is coupled to their invasion by vessel-associated Osx+ osteoprogenitor cells (OPCs), a subset of SSPCs (Maes et al., 2010b). Control bones at E18.5 showed ample Osx-Cre:GFP+ cells in the vascularized metaphysis (region below the growth plate), as visualized by co-IHC for the EC marker endomucin (Emcn) (Figure 1H). In VEGF cTg mice, along with the altered vessel distribution, Osx-Cre:GFP+ cells were homogeneously abundant throughout the bone (Figure 1H), and their overall number was increased (Figure 1I; Osx/GFP+ gating in Figure S1G).

Thus, constitutive overexpression of VEGF164 by Osx+ progenitor cells drastically altered the osteo-angiogenic development of the skeleton, resulting in a disrupted bone architecture and impaired BM cavity formation.

Osteolineage VEGF overexpression impairs the establishment of BM hematopoiesis
To assess how impaired formation of a vascularized bone center would impact the dynamics and characteristics of hematopoietic development, we next quantitatively mapped HSPCs (Lineage [Lin]- stem cell antigen (Sca)-1- c-Kit+ [LSK] cells) and HSCs (LSK-SLAM cells) (illustration of gating strategy in Figure S2A), as well as the global CD45+ hematopoietic fraction and blood lineages, in control versus VEGF cTg embryos across the time frame of BM genesis. At E15.5, prior to active BM hematopoiesis (Coşkun et al., 2014), we detected only very few LSK and no LSK-SLAM cells in the BM of either genotype (Figure 2A). CD45+ cells were detected (Figure 2B), likely representing circulating blood cells contained within the bone vasculature. Consistently, the number of CD45+ cells was slightly elevated in VEGF cTg embryos and remained relatively stable at E15.5–E16.5, prior to the initiation of bona fide BM hematopoiesis (observed from E18.5 in control mice) (Figure 2B). By E16.5, very few LSK and LSK-SLAM cells had migrated to the BM in control mice, whereas mutant bones showed transiently higher numbers of HSCs and HSPCs (Figure 2A), suggesting accelerated homing or, alternatively, increased entrapment in the excessive and smaller blood vessels of VEGF cTg bones (also see Figure 1G). Strikingly, however, further expansion or colonization of the mutant BM appeared blocked. The number of LSK and CD45+ cells stagnated in VEGF cTg bones by E18.5, in sharp contrast with the steep rise in control bones between E16.5 and E18.5 (Figures 2A and 2B). Although LSK-SLAM cells were preserved, the LSK and CD45+ cell fractions were significantly reduced in VEGF cTg bones (Figures 2C and 2D).

Despite the severely impaired HSPC settlement in the BM of E18.5 VEGF cTg embryos, their blood profile was rather normal (Figure S2B), suggesting compensatory extramedullary hematopoiesis. Therefore, we extended the HSC trafficking analysis to non-skeletal hematopoietic organs. There were no differences in circulating LSK cells (Figure 2E), and the VEGF cTg fetal spleen actually showed fewer LSK-SLAM cells (Figure 2F), yet without major impact on the LSK or CD45+ cells in the spleen (Figure 2F). In the mutant liver, though, the LSK, LSK-SLAM, and CD45+ populations were markedly increased at E18.5 (Figure 2G), paralleling the timing of impaired BM hematopoiesis (see Figures 2A and 2B). In line with a compensational function, lineage-committed progenitors were also more abundant in the E18.5 mutant liver (Figure 2H), as was the expression of niche factors known to regulate HSCC maintenance in the fetal liver (Figure 1). Of note, livers of VEGF cTg mice showed no off-target or secondary VEGF upregulation and no vascular alterations (Figure S2C).

Thus, increased skeletal VEGF affects bone vascularization and BM cavity formation, leading to impaired establishment of BM hematopoiesis and triggering compensational hematopoiesis in the fetal liver.

Slightly delaying the onset of osteolineage VEGF overexpression rescues BM cavity formation while still inhibiting HSPC homing to the skeleton
The constitutive Osx-Cre:GFP-targeted VEGF overexpression model suggested functional links between developmental bone vascularization and hematopoietic settlement in the BM. Yet, we could not exclude the possibility that impaired excavation and growth of the bone shaft merely spatially precluded HSPC infiltration. To uncouple these aspects, we established a hypomorph model by slightly delaying the VEGF overexpression. This was achieved by silencing the Tet-off-engineered Osx-Cre:GFP driver with doxycycline (dox) from conception until E13.5. Perinatal (E18.5 and post-natal day [P] 2) VEGF cTgDOX mice showed skeletal VEGF upregulation (Figure 3A) locally, but again not systemically, as serum VEGF levels (Figure 3B) and non-skeletal tissue vascularity (liver, Figure 3A) were normal. In sharp contrast with the constitutive model, temporarily holding back the VEGF overexpression did allow the development of a normally shaped and sized skeleton (Figure 3C), including a bone shaft of normal length (Figure 3D). Although E18.5 VEGF cTgDOX tibias showed evident abnormalities in the distribution of the bone matrix (Figure 3E), these did not impact the overall bone density (Figure 3F), and importantly, the BM cavity volume was normal (Figure 3G).

Despite the relatively normal bone architecture, the osteo-angiogenic BM compartments were still substantially altered in VEGF cTgDOX fetuses. CD31 IHC revealed higher numbers of blood vessels of smaller average size, eventually leaving the total vascularized area of the BM unaltered (Figures 3H and 3I). The Osx-Cre:GFP+ OPC population was again vastly expanded throughout the mutant bone, including the diaphyseal BM cavity, contrasting the more confined metaphyseal and endosteal localization of Osx-Cre:GFP+ cells in controls (Figures 3J and 3K).

Intriguingly, as in the constitutive model, VEGF cTgDOX mice also showed drastic defects in HSPC homing and establishment...
of BM hematopoiesis. At E18.5 and even more pronounced at P2, the number of LSK cells in the BM was dramatically decreased, associated with a striking lack of local CD45+ hematopoietic cell production (Figure 3L; gating strategy in Figure S3B). Again, despite the blocked HSPC settlement in VEGF cTgDOX BM, the circulating blood cell types (Figure S3C), including LSK cells (Figure S3D), were normal. LSK and CD45+ cell numbers in the liver were normal (Figure S3E), suggesting hematopoietic compensation by another organ. Accordingly, VEGF cTgDOX mice showed elevated LSK, LSK-SLAM, and CD45+ cell numbers in their spleens (Figure 3M).

These data show that slightly postponing the excess VEGF production created a hypomorph model, displaying more moderate bone architectural changes and a normal-size BM cavity, while still exhibiting evident stromal-vascular alterations. Despite the rescued BM cavity formation, VEGF cTgDOX mice still showed impaired endogenous HSPC homing to bone and compromised establishment of BM hematopoiesis.

Local over-production of VEGF by the osteogenic stroma impairs HSPC settlement in fetal bones through extrinsic, micro-environmental control of HSPC homing

The data above indicated that increased osteolineage VEGF production altered the dynamics of developmental HSPC trafficking and settlement in bone, even when cellular infiltration into the BM cavity was not hampered by a lack of physical space. We next aimed to define whether the actual HSPC entry process (homing) was impaired or whether, alternatively, reduced local proliferation or increased apoptosis of HSPCs underlay the reduced HSPC pool in the mutant BM environment.
Analysis of cell proliferation (Figure 4A) revealed no deviations in the proportions of BM-resident LSK cells in the G0, G1, and G2/S/M phases of the cell cycle in the VEGF cTg and VEGF cTgDOX models (Figures 4B and 4C), nor in Kit+/S LSK cells (Figure S4A). These data thus exclude a deficit in local HSPC proliferation. Testing apoptosis, we found an increased fraction of annexin V+ LSK cells in VEGF cTgDOX limbs at P2 (Figure 4D). Although a 3% raise in apoptotic rate can be substantial, the drastic 80% reduction in BM HSPCs (see Figure 3L) suggested another or additional cause.

Primary HSPC homing encompasses the traveling of HSPCs from the fetal liver to the developing bone and their seeding for subsequent long-term settlement in the BM cavity. To experimentally test the capacity of the control versus mutant BM environments in supporting HSPC homing, we injected membrane-labeled, wild-type Lin− cells isolated from the liver of newborn donor mice into the circulation of P2 control and VEGF cTgDOX recipient pups (Figure 4E). Sixteen hours after the transplantation, dramatically reduced numbers of labeled LSK cells were retrieved in the bones of VEGF cTgDOX mice compared with control recipients (Figure 4F). Instead, homing to the spleen, not the liver, was increased in VEGF cTgDOX mice (Figures 4G and 4H), similar to the endogenous situation (see Figures 3M and S3E). Of note, virtually all of the ∼5,000-6,000 LSK cells present in the injected Lin− cell suspensions (Figure S4B) were effectively retrieved in these three hematopoietic organs in both genotypes (Figure 4I), with only very few remaining circulating cells (Figure S4C). Yet the relative distribution strongly differed; in control mice, the vast majority (almost 60%) of retrieved LSK cells had homed to the skeleton, foremost to the limbs and vertebrae (see Figure 4F) with hardly any infiltration into the calvaria (Figure S4D), followed by the spleen (25%) and liver (17%). In contrast, in VEGF cTgDOX mice, only 21% of the cells seeded into the skeleton versus 68% in the spleen (Figure 4I). This short-term homing experiment confirmed the intrinsic inhospitality of the mutant bone microenvironment to incoming HSPCs.

Together, these data indicate that the defective developmental BM hematopoiesis in mice with stromal VEGF overproduction resulted from a niche-determined incapacity to support fetal HSPC homing and survival in the challenging environment.

Defective HSPC homing in VEGF transgenic mice is not explained by a deficit in classical niche signals

To identify the basic features leading to the unsupportive microenvironment for HSPC homing in VEGF cTg mice, we performed RNA sequencing (RNA-seq) on the prime osteo-angiogenic niche constituents at an early stage following VEGF over-exposure, preceding the initial HSPC seeding, to favor primary changes. Therefore, we sorted Osx-Cre:GFP+ OPCs and skeletal ECs (CD31+) from E14.5 control and VEGF cTg limbs (Figures 5A, 5B, and S5A). The specificity of the cell populations was validated by the distinct expression of osteolineage markers in sorted OPCs and the restricted expression of endothelial markers in the EC samples (Figure S5B). Specific VEGF-A overexpression in Osx+ OPCs from VEGF cTg mice was also confirmed (Figure S5C).

There were relatively few significantly differentially expressed (DE) genes between the genotypes (Figure 5C). These included several genes related to osteogenic differentiation, which were downregulated in VEGF cTg versus control OPCs (Figure S5D). In line therewith, E18.5 VEGF cTg mice showed increased early (Runx2, Alp) yet reduced late osteoblast markers (osteocalcin [Ocn], Dmp1) by qRT-PCR on full bones (Figure S5E), suggesting a shift to immature cells. In the VEGF cTgDOX model, the overall spectrum of osteolineage markers, early and late, was increased at P2 (Figure S5F), indicating an expanded osteoblast pool.

Osteolineage cells, stromal cells, and ECs all constitute sources of homing factors and secreted or cell-bound signals regulating HSPCs. Surprisingly, though, the RNA-seq databases did not uncover differences in known hematopoietic niche factors produced by Osx+ OPCs (osteocrine factors) and/or CD31+ ECs (angiocrine factors) (Figure 5D). Even at later stages, key secreted HSPC niche factors were expressed at normal levels in VEGF cTg and VEGF cTgDOX bones, with the exception of a mild rise in CXCL12 in VEGF cTg bones only (qRT-PCR, Figure S5G).

Thus, no consistent or pronounced changes, or deficits, were recorded in the expression of classical hematopoietic niche factors and key homing signals, suggesting that other mechanisms had to be responsible for the impaired HSPC homing in VEGF transgenic mice.

Increased VEGF triggers stromal-vascular hyperactivity and alters the metabolic and oxidative status of the fetal BM environment

We next conducted Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) on the RNA-seq data. Intriguingly, these analyses revealed prominent parallels in the impact of the mutation on Osx+ OPCs and CD31+ ECs. First, in line with VEGF strongly stimulating angiogenesis, ECs from VEGF cTg limbs showed activated DNA replication, ribosome synthesis and transcription pathways, and upregulated genes related...
Figure 4. Increased skeletal VEGF expression impairs HSPC homing to fetal bones
(A–C) Gating strategy (A) for LSK cell-cycle analysis in VEGF cTg (B) and VEGF cTgDOX (C) BM (n = 3–9).
(D) AnnexinV+ LSK cells in BM at P2 (n = 4–6).

Short-term homing experiment

- Harvest
- Magnetic separation
- Cell labeling
- Injection
- Flow cytometry

Wild Type
P2 Liver

Lineage negative
Cell isolation

Membrane dye:
Vybrant™ DIL

500,000 Lin neg
Cells in P2 mice

Labeled LSK
16h post-injection

F  
- skeleton
- limbs
- vertebra

G  
- spleen

H  
- liver

I

ControlDOX

VEGF cTgDOX

57.7% ± 9.3  
Skeletal

17.4% ± 1.6  
Liver

24.8% ± 5.0  
Spleen

21.1% ± 2.1  
Skeletal

11.9% ± 2.5  
Liver

67.9% ± 11.6  
Spleen

Average total number of cells retrieved = 6924

Average total number of cells retrieved = 6727

(legend continued on next page)
to cell proliferation (Figures 5E and S5H). Likewise, VEGF cTg OPCs showed enriched DNA replication and cell-cycle pathways/genes (Figures 5F and S5H), implying an overall hyperactivation in the osteo-angiogenic compartments. Pathways related to matrix remodeling and cell growth and migration were also stimulated in both cell types exposed to high VEGF in vivo (Figures S5I and S5J).

Consistently, various pathway/gene set databases (Kyoto Encyclopedia of Genes and Genomes [KEGG], Gene Ontology [GO], Reactome, Hallmark, IPA) implied significant changes between the genotypes in cellular metabolism and redox homeostasis in both OPCs and ECs (Figures 5G–5I). Transcriptomes of ECs from VEGF cTg mice pointed at increased oxidative phosphorylation and mitochondrial respiration (Figures 5G and 5H), while mutant OPCs displayed marked downregulation of pathways associated with glycolysis, glucose metabolism, and hypoxia (Figure 5H), along with reduced expression of hypoxia-regulated genes, including many glycolytic enzymes (Figure 5I).

In both cell types, these changes were associated with disturbed redox regulatory pathways (Figure 5H) and unbalanced lipid metabolism (Figure 5J), characterized by altered transport, accumulation, and metabolism of fatty acids and impaired cholesterol homeostasis. Moreover, ECs and OPCs from VEGF cTg bones showed transcriptional marks of increased production of reactive oxygen species (ROS), along with activation of DNA repair mechanisms in ECs (Figures 5K and 5L).

Thus, based on transcriptome analyses, forced stromal over-expression of VEGF appeared to quickly lead to cellular hyperactivation in the osteo-angiogenic unit and a relative hypoxic state, associated with altered cell metabolism and oxygen use, disturbed redox homeostasis, and increased ROS levels, overall creating excessive oxidative stress in the mutant BM milieu.

**Altered blood vessel architecture, stromal-vascular interplay, and EC phenotype in bones with elevated VEGF levels, associated with increased ROS production**

We next sought in vivo validation for the RNA-seq outcome of stromal VEGF over-production inducing excessive oxidative stress in the BM environment. Increased ROS levels were indeed detected in both the skeletal endothelium (CD31^+CD45^-Ter119^-) and the hematopoietic marrow (CD45^+ and/or Ter119^) of E16.5 VEGF cTg fetuses (Figure 6A). VEGF cTg^{DOX} mice displayed elevated EC ROS also at P2, corresponding to the time of blocked HSPC settlement, both in arteriolar- (CD45^-Ter119^-CD31^+Sca-1^+) and sinusoidal-like (CD45^-Ter119^-CD31^+Sca-1^-) subtypes (Figure 6B).

Global alterations in oxygenation and cellular metabolism within an environment can stem from a dysfunctional local vascular system. We therefore assessed the consequences of the altered stromal VEGF production on the blood vessel architecture and EC phenotype and on the association with perivascular SSPCs in the developing BM niche. Deep-tissue 3D confocal microscopy of bone sections stained for CD31 and Emcn revealed pronounced alterations in the overall architecture and distribution of blood vessels within the mutant BM cavity (Figure 6C; 3D models in Video S1). Longitudinal oriented CD31^{high}Emcn^{low} arterial/arteriolar vessels were largely lost, as was the overall diversification of the blood vessels’ structure, size, and distinct organization along the vascular tree (Figures 6C–6F; quantitative image analysis detailed in Figure S5). Metaphyseal vessels in VEGF cTg bones failed to show normal bulb structures at the interface with the growth plate (Figure 6C, upper magnified panels); diaphyseal vessels displayed a uniform appearance, random distribution, loss of directionality, narrow lumens, and complex connections (Figure 6C, lower magnified panels). Quantitative image analysis (see Figures S6A–S6D) confirmed the loss of longitudinal arrangement of blood vessels along the bone axis (Figure 6D), increased vascular density (Figure 6E), excessive vessel branching (Figure S6E), and loss of smaller vessels and increased prevalence of smaller-diameter vessels (Figures 6F, S6F, and S6G). Overall, the changes led to blunted structural diversity in the vascular pattern of E18.5 VEGF cTg bones, albeit maintaining the global vascularized versus nonvascular volume fractions of the BM cavity (Figure S6E). Newborn VEGF cTg^{DOX} mice similarly showed a dense network of narrower and virtually exclusively CD31^{high}Emcn^{high} vessels of homogenous morphology, with abundant protrusions supporting excessive EC activation (Figures 6G and S6D and see Figure 3I).

In line with the early cellular hyperactivity implied by the RNA-seq analysis, the absolute number of ECs (CD31^+) populating the BM was increased in the mutants (Figure 6H), with abundant EC proliferation even in the later delayed-onset model (Figure 6I). Although both arteriolar-type (CD31^-Sca-1^-) and more permeable sinusoidal-type (CD31^-Sca-1^+) ECs had expanded massively (Figures S7A and S7B), a shift was observed in the relative composition of the vessels. In normal developing bones, the predominant EC subtype classifies as CD31^-Sca-1^- (≥70%-80% of ECs), with the CD31^-Sca-1^- fraction constituting only ≥20%-30% (Figures 6J and S7B, lower graphs). The mutant bones consistently contained increased proportions of sinusoidal-type ECs at the expense of the CD31^-Sca-1^- fraction (Figures 6J and S7B). Despite the overall EC expansion being only manifest by P2 in the delayed-onset VEGF cTg^{DOX} model (see Figure 6H), this EC phenotypic switch was observed already by E18.5 (Figure 6J), suggesting it to be an early effect of increased VEGF exposure. The frequency of ECs with evident ROS and the mean ROS levels were significantly elevated in each EC subtype in both models (Figures S7C and S7D).

Along with the changes in the vasculature, Osx^+ cells in fetal VEGF cTg bones were not only expanded (see Figure 1I) and under-differentiated (see Figures S5D and S5E) but also generally located closer to blood vessels than normal (Figures 6K and 6L; Video S1). In line with the primitive nature of perivascular SSPCs, VEGF cTg bones contained increased fractions of Sca-1^-CD31^+ immature osteogenic progenitor cells, along with decreased fractions of the more mature osteogenic precursors

(E) Design of short-term (ST) homing analysis of transplanted HSPCs. (F–H) Number of Dil^+ LSK cells retrieved from (F) skeleton, limbs, and vertebrae; (G) spleen; and (H) liver (n = 4–8). (I) Distribution of retrieved Dil^+ LSK cells. Bar charts, mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001; t test. See also Figure S4.
that had lost Sca-1 expression (Sca-1−CD51+ cells) (Figure 6M). Earlier mesenchymal stromal populations known to associate with ECs, and to provide niches controlling HSC self-renewal and differentiation in adult murine BM, were expanded as well in the fetal VEGF-enriched BM environment. These included cells marked by expression of PDGFRα and Sca-1 (P+ cells) (Monkawa et al., 2009) and, most prominently, cells expressing PDGFRβ (Böhm et al., 2019; Kusumbe et al., 2016) (Figure 6N). The VEGF cTgPOB model recapitulated particularly the expansion of Ox+ (see Figure 3K) and PDGFRβ+ SSPC populations (Figure 6N). Although co-IHC for PDGFRβ and Emcn showed relatively restricted metaphyseal localization of PDGFRβ+ cells in control tibias, extended areas of PDGFRβ+ stromal-vascular association were observed throughout the mutant bones (Figure 6O), further evidencing coupled disturbances in the stromal-vascular niches.

At the (sub-)cellular levels, independent in vivo endorsement of the RNA-seq findings was sought by transmission electron microscopy (TEM) of the developing BM in control bones. In control bones, blood vessels with pronounced lumens were lined by thin and elongated ECs, containing limited cytoplasm and showing a smooth cell surface (Figure 6P, left panels). In sharp contrast, VEGF cTg bones showed abundant but severely narrowed capillaries (in line with Figures 6E and 6F), formed by numerous and strongly thickened ECs, whose unusually plumb cell bodies partially obstructed the capillary lumens (Figure 6P). These ECs contained more cytoplasm, an irregular and plumb nucleus, and excessive mitochondria, many appearing irregular and swollen (Figure 6P), consistent with the RNA-seq indications of mitochondrial dysfunction and oxidative stress (see Figures 5H and 5K). The ECs also displayed very irregular cell borders with numerous projections and pinocytotic vesicles (Figure 6P, middle), and altered intercellular contacts (Figure 6P, right). In contrast with the normal tight junctions (visible as dark spots of high electron density) in control limbs, ECs in mutants appeared only loosely adherent (small regions of cell-cell contact) with irregular junctions displaying multiple gaps or pores (Figure 6P, right), suggesting more fenestrated capillaries and altered permeability. VEGF cTgPOB bones also showed selective alterations in expression of genes implicated in endothelial junctions and phenotype (Figures 5E and 5F). Thus, TEM analysis provided ultrastructural evidence for distorted vascular integrity, mitochondrial dysfunction, and increased vessel permeability in the VEGF-enriched BM.

Altogether, these data indicate that excessive stromal cell-derived VEGF in the developmental BM environment induced profound changes in endothelial phenotype, organization, and interplay with perivascular stromal cells, presumably associated with vascular dysfunction. Changes were observed in the oxidative-metabolic status of the stromal-vascular niches, and increased oxidative stress occurred broadly and non-cell-autonomously within the BM environment, concomitant with blocked initial HSPC homing and settlement in the skeleton.

Irradiation induces increased stromal VEGF production, coupled stromal-vascular hyper-responses, and oxidative stress in the adult murine BM niche

Irradiation therapy, a conventional anticancer treatment and procedure preceding HSC transplantation, increases ROS levels in the tumor niche and employs the cytotoxicity of ROS for killing tumor cells through a sudden, localized, and intense oxidative burst. Irradiation also severely damages the BM environment (Batsivari et al., 2020). We here analyzed the stromal-vascular compartments during post-irradiation BM recovery. Interestingly, even 2 weeks after sub-lethal irradiation (7 Gy), recovering bones contained increased ROS levels within the non-hematopoietic BM niche (Figure 7A). Elevated ROS production was seen in ECs (Figure 7B) and OPCs, with the Sca-1−CD51+ cells being the most sensitive subtype (Figure 7C). BM vascularization was massively increased and characterized by tortuous, relatively random oriented and dilated blood vessels throughout the metaphysis and diaphysis (Figures 7D and 7E). The EC frequency was increased (Figure 7F) and shifted toward the arteriolar subtype (Figure 7G) by vastly increased CD31+Sca-1− cells (Figure 7H). This hyper-vascularization was associated with expansion of primitive stromal cells, particularly PDGFRβ+ (Figure 7I) and Sca-1−CD51+ (Figure 7J) cells. Given the parallels with the fetal VEGF transgenic models, we analyzed the production of VEGF by the niche following non-lethal radiotherapy. Interestingly, irradiation induced VEGF expression in the recovering bones, with Vegf mRNA levels increasing with dose and time (Figure 7K), as well as in cultured primary murine BMSCs (Figure 7L).

DISCUSSION

In recent years our understanding of the BM niches has advanced immensely. Besides the specifics of individual
HSC-niche interactions, an emerging complexity is to decipher the interplay between the constituents of the multi-component niches. Elucidating how integrated and coordinated inter/ intra-niche signaling is achieved, lending perfectly balanced support to promote HSC stemness and direct HSPC differentiation, will be coupled to uncovering how it is disturbed in disease and therapy settings. VEGF is upregulated in multiple hematologic diseases and malignancies, including myelofibrosis and acute myeloid leukemia (Chand et al., 2016; Duarte et al., 2018; Kampen et al., 2013; Medinger et al., 2010; Panteli et al., 2007; Poulos et al., 2014; Song et al., 2020; Steurer et al., 2007) and has been implicated in vascular remodeling following irradiation and chemotherapy (Chen et al., 2019; Hooper et al., 2009; Passaro et al., 2017). Here, studying the natural homing process of fetal HSPCs toward their BM destination, we found that tightly controlled skeletal VEGF levels are critical for ensuring local vascular integrity and functionality, regulating the oxidative environment and redox homeostasis, and defining the success of endogenous HSPC homing. Exposure to irradiation, often used in cancer treatment preceding stem cell therapy, amplified VEGF signaling and affected the BM stroma and vasculature in ways resembling the developmental genetic models. Understanding the impact of VEGF signaling in the crosstalk within the stromal-vascular niches may thus possibly be applicable toward promoting engraftment of transplanted HSCs and/or improving anticancer treatment.

Fetal hematopoietic development is coupled to skeletogenesis and is dependent on the establishment of a receptive stromal-vascular niche

Severe obstruction of BM cavity formation in VEGF cTg embryos as a result of an altered osteo-angiogenic interplay underscores the tight coupling of hematopoietic development and skeletogenesis. Timely vascular invasion of the cartilaginous bone template and establishment of a perfused BM cavity is a prerequisite for HSC infiltration via the circulation (Ciriza et al., 2013) and is controlled by VEGF (Maes, 2013). Blocking either VEGF or Osx inhibited the hematopoietic conversion of fetal endochondral constructs grafted under the kidney capsule of transplant recipient mice (Chan et al., 2009; Colnot et al., 2004). In vivo, fetal Osx+ cells contribute to bone tissue generation and to the primitive BM stroma (Maes et al., 2010b; Mizoguchi et al., 2014), and they are indispensable contributors to the fetal HSC niche (Chan et al., 2009; Coşkun et al., 2014). In Osx−/− mice lacking differentiating osteolineage cells (Nakashima et al., 2002), initial HSPC seeding took place, but the defective niche was unable to maintain HSC quiescence and long-term engraftment potential (Coşkun et al., 2014). Embryos lacking the earlier osteogenic transcription factor Runx2 also completely lacked osteolineage cells, but additionally failed to upregulate VEGF (Komori et al., 1997; Zelzer et al., 2001) and to undergo stromal-vascular invasion and BM cavity formation, consequently failing to develop BM hematopoiesis (Deguchi et al., 1999; Komori et al., 1997). Thus, the vasculature, invading fetal bones along with its associated primitive stromal cells (Maes et al., 2010b), seems the most critical requirement for allowing HSPC homing, while the osteogenic stroma further supports HSPC maintenance subsequently. Adding to the essential role of VEGF in steering vascular invasion initiating bone development (Duan et al., 2015; Maes et al., 2002, 2010a), the current data establish that local VEGF levels determine stromal-vascular niche creation and HSPC settlement in the fetal BM cavity.

Our findings also underscore the concept that HSPC colonization of newly developing hematopoietic sites relies on the receptiveness of the local microenvironment by having created the required niches. At the stages studied here (E14.5 to P2), several organs actively participate in the ontogeny of the hematopoietic system, including the liver (Morrison et al., 1995) and spleen (Walber et al., 2002). HSPCs constitutively circulate at low levels in this time frame but colonize specific organs stage-restrictively, probably only when local niches capable...
Figure 7. Irradiation increases stromal VEGF production, stromal-vascular BM niche hyper-activation, and ROS levels in adult bone
(A–C) ROS analysis in femurs of irradiated mice (n = 3), in (A) non-hematopoietic cells (CD45−Ter119−), (B) ECs (CD45−Ter119−CD31+), and (C) OPCs (TN CD51+).
MFI, mean fluorescence intensity, relative to non-irradiated mice (n = 4).
(D and E) Emcn IHC on tibias of irradiated (n = 3) versus non-irradiated mice (n = 4).
(F–H) Flow cytometry for the (F) CD31+ EC fraction among non-hematopoietic BM cells, (G) Sca-1+ and Sca-1− EC fractions, and (H) absolute CD31+Sca-1+ cell numbers.

(legend continued on next page)
of supporting HSPC maintenance and self-renewal have been established (Christensen et al., 2004). Seeding of HSPCs into the BM normally initiates around E16.5 (Coskun et al., 2014); if hindered, as in VEGF transgenic mice, yet unknown signals trigger compensational hematopoiesis, but surprisingly, this takes place in different organs depending on the stage. When the block occurred earlier during embryogenesis (as in the constitutive model), the liver provided compensation, whereas defective BM engrafment in perinatal animals (in the delayed-onset model) led HSPCs to enter the spleen. The shift between these extramedullary hematopoietic organs seems to position around E18.5, coinciding with spleen hematopoiesis normally starting. Runx2−/− mice, lacking BM hematopoiesis, develop excessive extramedullary hematopoiesis in both liver and spleen at E18.5 (Deguchi et al., 1999), and livers from Osx−/− mice show higher engraftment potential at E17.5 (Coskun et al., 2014). Later in post-natal life, BM obstruction or niche dysfunction commonly leads to BM egress of HSPCs and migration to the spleen for extramedullary hematopoiesis, causing splenomegaly, which was also seen upon short-term induction of skeletal VEGF in adult mice (Maes et al., 2010a).

Increased skeletal VEGF leads to redox and metabolic changes in the niche cells and oxidative stress in the BM milieu

Our results suggest that, besides the need for adequate initial vascular invasion, successful HSPC homing, survival, and functioning in the developing BM depend on optimal vascular integrity and redox homeostasis. Excess VEGF triggered osteoangiogenic hyper-activation and profoundly changed the EC phenotype, vessel architecture, and stromal-vascular interface. Early-stage changes in osteogenic cells and ECs were dominated by altered cellular metabolism, redox homeostasis, and lipid handling, including cholesterol synthesis and fatty acid oxidation, and associated with increased ROS production. The molecular, metabolic, and morphological alterations in the endothelium may have been detrimental for the vascular barrier function and/or the rolling, attachment, and extravasation of HSPCs, precluding their infiltration of the BM. Alternatively, or additionally, increased vascular permeability and excess ROS in the stromal-vascular niche may have hampered HSPC retention and survival, leading to an overall defective settlement in the BM. The lack of HSPC infiltration could further have negatively impacted on the integrity of the vascular network too, given the mutual dependency of ECs and hematopoietic cells (Chen et al., 2019).

Although knowledge on the metabolic pathways employed by ECs residing in BM is sparse, generally ECs mostly rely on glycolytic metabolism, for up to 85% of their ATP production (Eelen et al., 2018; Potente and Carmeliet, 2017). By maintaining a low oxidative metabolism, ECs minimize the production of ROS, thereby providing protection against their high-oxygen milieu; mitochondria in ECs are correspondingly thought to function as signaling hubs rather than metabolic powerhouses (De Bock et al., 2013; Eelen et al., 2018). The switch to increased endothelial oxidative phosphorylation and mitochondrial activity implied by our data would expectedly alter the metabolic communication in the niche, which might impact its functioning in supporting HSPCs. We consistently documented increased ROS levels in ECs in both VEGF transgenic models; high ROS can induce lipid peroxidation and impact the integrity of the membrane lipid bilayer and tissue permeability. Itkin et al. (2016) showed that HSC maintenance and leukocyte trafficking are regulated by distinct vessel types with different permeability: less permeable arterial vessels maintain quiescent HSCs, which are in a low ROS state, whereas more active, high ROS-containing HSCs are found around permeable sinusoidal vessels promoting HSC differentiation and exchange of blood cells with the circulation. In our model, high stromal-derived VEGF induced a fenestrated sinusoidal EC phenotype and molecular and morphological changes consistent with increased ROS and impaired vascular barrier function, suggesting that similar concepts might apply to the control of HSC homing by the fetal BM microenvironment.

Whether these metabolic alterations may be direct VEGF effects is not clear. VEGF signaling in primary ECs can promote mitochondrial biogenesis (Wright et al., 2008), fitting the abundant mitochondria seen in VEGF cTg ECs. Yet, in our hands, administration of recombinant VEGF to cultured endothelial MS1 cells did not notably affect the rate or balance of glycolysis and oxidative phosphorylation (extracellular flux analysis, data not shown). Others found that VEGF stimulation actually led ECs to elevate their glycolytic flux (De Bock et al., 2013). Environment-dominated causes thus seem more likely for explaining the metabolic shift, especially given the complementary changes in the osteolineage cells. Skeletal progenitor cells normally use glycolysis as their main source of energy (Lee et al., 2017), but reduced glycolysis associated with reduced hypoxia-regulated gene expression prevailed in the transcriptome of VEGF cTg Osx+ cells. Hence combined, the altered metabolic functioning of both cell types in the VEGF-enriched milieu suggests a relative hyperoxic status of the mutant BM. Normally, BM is quite hypoxic (Nombela-Arrieta et al., 2013), and precisely regulated signaling by hypoxia-inducible factors (HIFs) is essential for bone development, homeostasis, and vascularization (Dirckx et al., 2018; Kusumbe et al., 2014; Maes et al., 2012; Schipani et al., 2009; Wang et al., 2007), as well as for hematopoiesis and HSC homing and engrafment (Speth et al., 2014; Takubo et al., 2010). Whether and how the altered oxidative environment may impact metabolism in the HSCs proper remains to be investigated (Chandel et al., 2016; Nakamura-Ishizu et al., 2020). We conclude that fetal HSPC homing to bone is controlled by the local stromal-vascular integrity and the metabolic and oxidative status of the niche, with the establishment of BM hematopoiesis being highly sensitive to the developing tissue’s oxygenation.

† and J Flow cytometry for osteogenic stromal populations after irradiation.
(K) VEGF qRT-PCR in femurs from irradiated mice (n = 2–5; one-way ANOVA with multiple comparison against the non-irradiated group).
(L) VEGF qRT-PCR in BMSCs exposed to 7 Gy (n = 4 cell pools, each split into a non-irradiated and irradiated batch; paired t test).
Graphs, mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, by (except in K and L) unpaired t test.
Parallels between fetal VEGF overexpression and adult post-irradiation niche recovery underscore therapeutic prospects

Our finding that increased stromal VEGF production elicited oxidative stress in the fetal BM milieu inspired us to assess the potential relevance to the post-irradiation BM, as induction of ROS is a hallmark of ionizing radiation therapy (Azzam et al., 2012; Batsivari et al., 2020). Myelosuppression also severely damages the BM vascular system (Batsivari et al., 2020; Li et al., 2008), and engraftment of donor HSPCs and hematopoietic reconstitution after radiotherapy depends on vascular regeneration, a process requiring VEGFR2 signaling in sinusoidal ECs (Hooper et al., 2009) and VEGF provided by HSPCs (Chen et al., 2019). We found increasing VEGF expression following non-lethal irradiation recovery and hyper-responses in the stromal-vascular compartment resembling the fetal VEGF cTg models, including expansion of specific SSPC populations and bias toward CD31⁺Sca-1⁺ ECs. The latter largely typify the fetal bone vasculature and may correspond to the VEGF-responsive Apelin⁺ ECs shown to control hematopoiesis and post-irradiation vascular regeneration (Chen et al., 2019). The fact that such niche changes associated with poor homing of endogenous fetal HSPCs raises the possibility that excess VEGF could similarly be disadvantageous to transplanted HSCs. Recently, though, administration of VEGF shortly after irradiation was shown to be disadvantageous to transplanted HSCs. Furthermore, it was shown that VEGF in the niche may become unwarranted.

Further insights into the dynamic control of HSPC homing in development and disease will be instrumental toward therapeutically exploiting the niche for steering stem cell behavior. Possibly, the links uncovered here may help establish strategies to render the irradiated BM environment more favorable for incoming donor HSCs. Antioxidant treatment reducing ROS levels, for instance, improved hematopoietic engraftment in immunodeficient mice (Hu et al., 2014). Further research can clarify the value of anti-VEGF or antioxidant therapy, or uncover better targets, e.g., mediating vascular normalization, metabolic shift correction, or redox balance restoration, to enhance homing of infused stem cells and disease treatment outcomes. Although compound delivery in fetal systems can in some cases be challenging, the models described here may represent useful tools to explore candidate strategies in an in vivo context.

**STAR+METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109618.

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**AUTHOR CONTRIBUTIONS**

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**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393, 595–599.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CD105, rat monoclonal anti-mouse, Alexa Fluor 488 (used for flow cytometry (FC)) | BioLegend | Cat# 120406; RRID: AB_961053 |
| CD140a (PDGFRα), rat monoclonal anti-mouse, APC (FC) | eBioscience | Cat# 17-1401-81; RRID: AB_529482 |
| CD140b (PDGFRβ), goat polyclonal anti-mouse (used for immunohistochemistry (IHC)) | Santa Cruz Biotechnology | Cat# SC-1627; RRID: AB_631067 |
| CD140b (PDGFRβ), rat monoclonal anti-mouse, PE (FC) | eBioscience | Cat# 12-1402-81; RRID: AB_529484 |
| CD150, rat monoclonal anti-mouse, PE (FC) | eBioscience | Cat# 12-1502-80; RRID: AB_1548767 |
| CD31, goat polyclonal anti-mouse/rat (IHC) | R&D Systems | Cat# AF3628; RRID: AB_2161028 |
| CD31, rat monoclonal anti-mouse (IHC) | BD PharMingen | Cat# 550274; RRID: AB_393571 |
| CD31, rat monoclonal anti-mouse, Alexa Fluor 647 (FC) | BD Biosciences | Cat# 563608; RRID: AB_2738313 |
| CD31, rat monoclonal anti-mouse, PE-Cy7 (FC) | BioLegend | Cat# 102417; RRID: AB_830756 |
| CD31, rat monoclonal anti-mouse, PerCP-Cy5.5 (FC) | BioLegend | Cat# 102419; RRID: AB_10612742 |
| CD41, rat monoclonal anti-mouse, eFluor450 (FC) | BioLegend | Cat# 133911; RRID: AB_10960744 |
| CD45, rat monoclonal anti-mouse, PE (FC) | eBioscience | Cat# 12-0451-82; RRID: AB_465668 |
| CD45, rat monoclonal anti-mouse, PerCP-Cy5.5 (FC) | BioLegend | Cat# 103132; RRID: AB_893340 |
| CD48, armenian hamster monoclonal anti-mouse, APC-eFluor780 (FC) | eBioscience | Cat# 47-0481-80; RRID: AB_2573961 |
| CD51, rat monoclonal anti-mouse, biotin (FC) | BioLegend | Cat# 104103; RRID: AB_313072 |
| ckit (CD117), rat monoclonal anti-mouse/pig, APC (FC) | eBioscience | Cat# 17-1171-82; RRID: AB_469430 |
| Donkey polyclonal anti-rat IgG (H+L), DyLight 550 (IHC) | Invitrogen | Cat# SA5-10027; RRID: AB_2556607 |
| Donkey polyclonal anti-goat IgG (H+L), Alexa Fluor 647 (IHC) | Abcam | Cat# ab150131; RRID: AB_2732857 |
| Endomucin (Emcn), rat monoclonal anti-mouse (IHC) | Santa Cruz Biotechnology | Cat# SC-65495; RRID: AB_2100037 |
| Goat polyclonal anti-rat, biotin conjugated (IHC) | BD Biosciences | Cat# 554014; RRID: AB_395209 |
| Ki67, rat monoclonal anti-mouse, Pacific Blue (FC) | BioLegend | Cat# 652421; RRID: AB_2564489 |
| Ly-6A/E (sca-1), rat monoclonal anti-mouse, Super Bright 436 (FC) | eBioscience | Cat# 62-5981-80; RRID: AB_2637287 |
| Ly-6A/E (sca-1), rat monoclonal anti-mouse, PE-Cy7 (FC) | eBioscience | Cat# 25-5981-82; RRID: AB_469669 |
| Mouse Hematopoietic Lineage Biotin Panel (FC) | eBioscience | Cat# 88-7774-75; RRID: AB_476399 |
| PHH3 (phospho-histone H3) (IHC) | Abcam | Cat# ab47297; RRID: AB_880448 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Ter-119, rat monoclonal anti-mouse, PerCP-Cy5.5 (FC) | BioLegend | Cat# 116227; RRID: AB_893638 |
| VE-cadherin (IHC) | R&D systems | Cat# AF1002; RRID: AB_2077789 |
| **Chemicals, peptides, and recombinant proteins** | | |
| 2,2'-Thiodiethanol | Sigma-Aldrich | Cat# 88561 |
| 7-AAD Viability Staining Solution | Thermo Fisher Scientific | Cat# 00699350 |
| Albunin Bovine Fraction V | Serva | Cat# 11926.02 |
| Annexin V - PE (FC) | BioLegend | Cat# 640907 |
| Antibiotic-Antimycotic (100x) | Thermo Fisher Scientific | Cat# 15240-062 |
| 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) | Thermo Fisher Scientific | Cat# C6827 |
| CEB (cell extraction buffer), Invitrogen | Thermo Fisher Scientific | Cat# FN0011 |
| Collagenase Type II | Thermo Fisher Scientific | Cat# 17101015 |
| Dispase | Thermo Fisher Scientific | Cat# 17105041 |
| dNTP Set 100mM Solutions | Thermo Fisher Scientific | Cat# R0181 |
| Donkey serum | Sigma-Aldrich | Cat# S30 |
| Doxycycline hydrate | Sigma-Aldrich | Cat# D9891-25G |
| Dulbecco’s Modified Eagle Medium (DMEM) | Thermo Fisher Scientific | Cat# 31966-021 |
| EDTA disodium salt dihydrate | Fisher Scientific | Cat# BP120-1 |
| Fetal Bovine Serum (FBS) South Africa | BioWest | Cat# 51300-500 |
| Freeze Gel Q Path | VWR International | Cat# 0711245 |
| Gelatin from porcine skin | Sigma-Aldrich | Cat# G6144 |
| Goat serum | Sigma-Aldrich | Cat# G9023 |
| Hard epoxy resin Agar 100 (TEM) | Agar Scientific | Cat# AGR1043 |
| Hoechst 33342 | Life Technologies | Cat# HI3570 |
| HyClone Fetal Bovine Serum | GE Healthcare Life Sciences | Cat# SV30160.03 |
| Minimum Essential Media alpha | Thermo Fisher Scientific | Cat# 12561056 |
| Osmium tetroxide (OsO4) (TEM) | Electron Microscopy Sciences | Cat# 19152 |
| Polyvinylpyrrolidone | Sigma-Aldrich | Cat# 856568 |
| Potassium ferrocyanide (K4Fe(CN)6) (TEM) | Sigma-Aldrich | Cat# 455989 |
| Protease Inhibitor Cocktail | Sigma | Cat# P2714 |
| Protein Assay Dye Reagent Concentrate | Bio-Rad | Cat# 5000006 |
| Pyronin Y | Sigma-Aldrich | Cat# P9172-1G |
| Random Primers | Thermo Fisher Scientific | Cat# 48190011 |
| Streptavidin - APC/Cy7 (FC) | BioLegend | Cat# 405208 |
| Streptavidin - PerCP/Cy5.5 (FC) | eBioscience | Cat# 45-4317-80 |
| Streptavidin - Alexa Fluor 488 (FC) | BioLegend | Cat# 405235 |
| TRITC™ Reagent | Thermo Fisher Scientific | Cat# 15596026 |
| Uranyl acetate (TEM) | Electron Microscopy Sciences | Cat# 22400 |
| Verapamil hydrochloride | Sigma-Aldrich | Cat# V4629-1G |
| Vybrant DiI-Labeling Solution | Thermo Fisher Scientific | Cat# V22885 |
| **Critical commercial assays** | | |
| DNA 1000 kit | Agilent Technologies | Cat# 5067-1504 |
| Fast SYBR Green Master Mix | Thermo Fisher Scientific | Cat# 4385612 |
| RNeasy Micro Kit | QIAGEN | Cat# 74004 |

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Continued

| REAGENT or RESOURCE                      | SOURCE                   | IDENTIFIER     |
|------------------------------------------|--------------------------|----------------|
| RNasey Mini Kit                          | QIAGEN                   | Cat# 74104     |
| Lineage Cell Depletion Kit, mouse        | Milteny Biotec           | Cat# 130-090-858 |
| Mouse VEGF Quantikine ELISA Kit          | R&D Systems              | Cat# MMV00     |
| NucleoSpin RNA XS, Micro kit for RNA purification | Macherey-Nagel                | Cat# 740902.50 |
| SuperScript II Reverse Transcriptase     | Thermo Fisher Scientific  | Cat# 18064014  |
| TruSeq Stranded Total RNA Library Prep   | Illumina                 | Cat# 2002097   |
| TSA Indirect System                      | Perkin Elmer             | Cat# NEL700001KT |

Deposited data

RNA Seq databases: E14.5 perichondrial Osx-GFP+ OPCs and CD31+ ECs; from control and VEGF cTg embryos

This paper

Study accession numbers: ECs: European Nucleotide Archive (ENA): PRJEB46121. OPCs: European Nucleotide Archive (ENA): PRJEB47015.

Experimental models: Organisms/strains

Mouse: 'Crl:CD1(ICR)'

Charles River Laboratories

Strain Code 022

Rodda & McMahon, 2006, Development

Mouse 'Osx-Cre:GFP': B6.Cg-Tg(Sp7-tTA, tetO-EGFP/cre)1Amc/J

Andrew McMahon, University of Southern California, Los Angeles, USA

Maes et al., 2010a, EMBO J

Mouse 'VEGF cTg': Gt(ROSA)26Sor < tm1(Vegfa*)Jhai > ; ROSA26-VEGF164

Andras Nagy and Jody Haigh, Mount Sinai Hospital, Samual Lunenfeld Research Institute, Toronto, Canada

Oligonucleotides

Table for Oligonucleotide Sequences: Table S1

This paper

N/A

Software and algorithms

CellSense

Olympus

https://www.olympus-lifescience.com/en/software/cellsens/powerful-analysis-tools/

CTAn, CTVox

Bruker

https://www.bruker.com/en/services/software-downloads.html

Cuffquant (Cufflinks 2.2.1)

Cole Trapnell Lab

http://cole-trapnell-lab.github.io/cufflinks/getting_started/

DESeq and Cufflinks 2.2.1

Bioconductor

https://bioconductor.org

Fiji

Schindelin et al., 2012

https://imagej.net/software/fiji/downloads

FlowJo

Becton, Dickinson & Company

https://www.flowjo.com

GSEA (MSigDBv6,)

Broad Institute

http://www.gsea-msigdb.org/gsea/index.jsp

HT-Seq count v0.5.3p3

SoureForge

https://sourceforge.net/projects/htseq/

ImageJ 1.52a

NIH

https://imagej.nih.gov/ij/download.html

Ingenuity Pathway Analysis (IPA)

QIAGEN

https://www.qiagen.com/us/products/discovery-and-translational-research/next-generation-sequencing/informatics-and-data/interpretation-content-databases/ingenuity-pathway-analysis/

NIS-elements

Nikon

https://www.microscope.healthcare.nikon.com/products/software/nis-elements

Prism 8

Graphpad Software

https://www.graphpad.com

R v3.5.1 (CRAN)

https://cran.r-project.org/

Samtools v0.1.19.24

Wellcome Trust Sanger Institute

http://samtools.sourceforge.net

TopHat v2.0.13

John Hopkins University

https://ccb.jhu.edu/software/tophat/index.shtml
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christa Maes (christa.maes@kuleuven.be).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- The RNA-seq datasets generated in this study have been deposited at the European Nucleotide Archive (ENA) repository and are publicly available as of the date of publication. Accession numbers are listed in the Key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse models
All animals were housed in a temperature- and humidity-controlled environment, with a 12h light/dark cycle, and access to food and water ad libitum. Animal experiments and care were performed in accordance with the guidelines of institutional authorities and approved by the Animal Ethics Committee of the KU Leuven.

Embryos were harvested by cesarean section at the indicated stage (embryonic day (E)14.5-E18.5), as mentioned in the respective figure legends. Embryos at all stages and newborn mice at postnatal day (P)2 were euthanized by decapitation; adult mice were euthanized by CO2 inhalation. Young adult wild-type females with CD1 genetic background were used for experimental purposes at 8-12 weeks of age. Mice were irradiated with a single dose of 5Gy or 7Gy using a RS 2000 X-ray irradiator (Rad Source).

Constitutive and delayed-onset conditional VEGF164 transgenic (cTg) mice were used in this study, based on the use of a Rosa26-VEGF164 transgene located in the genomic Rosa26 locus and consisting of the full-length cDNA sequence of mouse VEGF164 with an upstream floxed cassette containing a transcriptional stop sequence (Maes et al., 2010a). To direct constitutive overexpression of the VEGF164 isoform of VEGF-A to Osx+ skeletal stem/progenitor cells (SSPCs) and their osteolineage descendants, female VEGF(cTg/+), mice carrying one copy of the conditional VEGF164 transgene in the Rosa26 locus (Maes et al., 2010a) were crossed with male mice carrying one copy of the Osx-Cre:GFP transgene (Rodda and McMahon, 2006). Mutant mice (Osx-Cre:GFP+;VEGF(cTg/+)) were termed VEGF cTg mice, and the control littermates were Osx-Cre:GFP+;VEGF(+/+). The primers used for genotyping are listed in Table S1.

The Osx-Cre:GFP construct contains a Tet-Off system, such that the expression and activity of the Cre recombinase is prevented when a tetracycline analog, such as doxycycline, is given to the mice (Rodda and McMahon, 2006). To establish a delayed-onset VEGF overexpression transgenic mouse model, doxycycline (dox) (Sigma-Aldrich) was administered via the drinking water of the pregnant dams at 0.2mg/ml from conception (E0.5, the morning a vaginal plug was observed) until E13.5. The dox solution was protected from light and changed three times per week. The resulting mice with delayed activation of VEGF164 cTg expression were termed VEGF cTgDOX (Osx-Cre:GFP+;VEGF(cTg/+); given dox) and the control mice were Osx-Cre:GFP+;VEGF(+/+), littermates that similarly received dox via the mother during embryogenesis.

Primary murine bone cells
Murine bone marrow-derived stromal cells (mBMSCs) were isolated from 8-week-old females of CD1 genetic background, housed in standard conditions as described above (‘Mice’). Primary cell isolation and culture conditions are described in detail below. Cells were cultured at 37°C with 5% CO2.

METHOD DETAILS

Mouse bone tissue processing and image analysis
Micro-computed tomography (micro-CT)
Micro-CT analysis was performed using the NanoCT phoenix nanotom® system (GE Healthcare). Embryonic limbs were scanned at 50kV/260μA, 3μm voxel size. Images were analyzed using CTAn software (Bruker) and 3D reconstructed models were made using CTVox software (Bruker). Scans of tibias were used for 3D representation and the quantification of bone volume/tissue volume (BV/TV, %) and BM cavity volume (μm3) was performed using a region of interest (ROI) containing the entire BM cavity.

Skeletal staining
Full embryos were dissected and prepared for skeletal staining with Alizarin red and Alcian blue to visualize mineralized tissue and cartilage respectively. Briefly, dissected embryos were fixed in 95% ethanol for at least 3 days and stained with Alcian blue for 24h.
Samples were further cleared using a 1% KOH solution and stained with Alizarin red for 3h. Finally, samples were placed in 2% KOH for the last removal of soft tissues and progressively in a gradient of 2% KOH to glycerol, until storage and imaging in a 20:80 ratio KOH:glycerol solution.

**Paraffin histology and histomorphometry**

The histological methods using paraffin sections of fetal and adult mouse bones have been described in detail previously (Böhm et al., 2019; Dejaeger et al., 2017; Dirckx et al., 2018). Here, the bones were fixed overnight using freshly prepared 2% paraformaldehyde (PFA) at 4°C, decalified in 0.5M EDTA for 2 days (E18.5 and P2 samples) or 2 weeks (adult samples), embedded in paraffin and sectioned at 5 μm. Paraffin sections were stained with standard hematoxylin and eosin (H&E) or with safranin O to visualize cartilage proteoglycans. To visualize the vasculature, sections were subjected to immunohistochemistry (IHC) for CD31 using TSA Indirect Biotin System (PerkinElmer) with 3,3'-diaminobenzidine (DAB)-based visualization and brightfield detection, as before. Stained sections were imaged using an Olympus IX83 inverted microscope equipped with a DP73 camera. For blood vessel (BLV) quantification (shown in Figure 3I), the average BLV size (lumen surface area of individual vessels (μm²)), density (number of vessels per BM area unit (/mm²)), and vascularized area denoting the fraction of the BM occupied by vessels (BLV area/BM area, %), were determined using the entire BM cavity as a region of interest. Each parameter was quantified on 2 to 3 sections per bone, to generate a representative average value for the animal, and a total of 4 animals were analyzed per group.

**Immunohistochemistry (IHC) of frozen tissue sections and confocal microscopy.**

For histological analysis of embryonic and newborn bones, dissected samples were fixed for 4-6 h in 4% PFA. Frozen samples were either embedded in Freeze Gel Q Path (VWR International) and cut into thin sections (5-10 μm) or, embedded in 8% gelatin diluted in cryoprotection medium as described in Caire et al. (2019). For the staining of blood vessels with CD31 and Emcn together, half trimmed cryo-embedded bones were first blocked and permeabilized using 10% serum, 0.1% tween and 0.2% v/v Triton-X in PBS overnight. Samples were then stained with goat polyclonal anti-CD31 (Santa Cruz) and goat polyclonal anti-mouse PDGFRβ (Santa Cruz) overnight, followed by secondary staining for 75 min using donkey-anti-rat IgG DyLight 550 (Invitrogen) and donkey-anti-goat IgG Alexa Fluor 647 (Abcam). The detection of endogenous GFP+ cells together with Emcn staining, and/or with additional phospho-histone H3 (PHH3) proliferation marker or junctional VE-cadherin (Cdh5) staining, was also prepared on the same type of samples (gelatin sections of 10 or 50 μm) and following this protocol.

Staining for PDGFRβ and endomucin (Emcn) together was performed on 50 μm thick gelatin sections. Samples were block with 5% donkey serum for 45 min and stained with rat monoclonal anti-mouse Eemcn (Santa Cruz) and goat polyclonal anti-mouse PDGFRβ (Santa Cruz) overnight, followed by secondary staining for 75 min using donkey-anti-rat IgG DyLight 550 (Invitrogen) and donkey-anti-goat IgG Alexa Fluor 647 (Abcam). Nuclear staining was performed using Hoechst 33342 (Invitrogen). The stained sections were imaged by laser scanning confocal microscopy on a Nikon C2 Eclipse Ni-E, inverted microscope (Nikon) equipped with 20x (Plan Apo VC NA 0.75), 40x (Oil Plan Fluor NA 1.30) and 60x (Oil Apo NA 1.40) objectives.

For the staining of blood vessels with CD31 and Emcn together, half trimmed cryo-embedded bones were first blocked and permeabilized using 10% serum, 0.1% tween and 0.2% v/v Triton-X in PBS overnight. Samples were further stained with goat polyclonal anti-mouse CD31 (R&D Systems) and rat monoclonal anti-mouse Emcn (Santa Cruz) for 48 h in blocking solution, followed by secondary staining for 48 h using donkey-anti-goat IgG Alexa Fluor 647 (Abcam) and donkey-anti-rat IgG DyLight 550 (Invitrogen). Nuclear staining was performed with Hoechst 33342 (Invitrogen) and 4’6-diamidino-2-phenylindole (DAPI) for imaging. The stained sections were imaged with a 20x (Multi-immersion Plan Fluor NA 0.75) objective using an Andor HSU spinning-disk module mounted on a Nikon upright Ni-E microscope, and acquired with a microscope and Andor Ixon3 camera (Ultra EMCCD 885). The setup was steered by NIS-Elements AR 5.21.01 software (Nikon).

**Image processing and segmentation**

**2D blood vessel analysis and PHH3 analysis.** Blood vessels marked with CD31 or Emcn within single confocal optical sections were segmented by training a machine learning pixel classification software (Ilastik 1.3.2) to distinguish between blood vessels and background. Binary versions of these images were then imported into FIJI (Schindelin et al., 2012) for further processing. As blood vessels are hollowed structures, they were closed and filled within their lumen to make quantifications possible. To exclude CD31+ hematopoietic cells and debris from the final analysis, we used a 50 μm² size filter.

Vascularized area fraction and perimeter were determined using the “analyze particles tool” from FIJI for area and perimeter. Final values were obtained by summing either the area or the perimeter of detected blood vessels and then normalizing them to the analyzed tissue area.

Blood vessel width was determined using the local thickness tool in FIJI. To allow for frequency width distribution comparison between multiple samples, we normalized the maximum blood vessel width to be 200 μm and stacked the data into 10 μm bins.

For directionality analysis, previously filled blood vessel binary images were iteratively eroded to obtain the center-line using the “skeletonization” tool in FIJI. Skeletonized blood vessel images were rotated such that the line traversing the proximal growth plate was parallel to the right y axis of the image (no interpolation was used in this step). We defined a range from −90° to 90° with respect to the growth plate, meaning that 0° represents blood vessels oriented orthogonal to the growth plate, hence running along the longitudinal axis of the bone shaft. The fraction of blood vessels oriented in each direction was calculated using the “directionality” tool.
The blood vessel, junction, triple, and quadruple point numbers were obtained by running the “analyze skeleton tool” on the skeletonized blood vessel images.

To quantify proliferating ECs, Emcn+ cells also showing nuclear staining for the proliferation marker PHH3 were counted in tissue areas of 250 μm x 500 μm of the metaphyseal and diaphyseal areas of P2 bones. The density of double positive (Emcn+ PHH3+) cells per surface area was calculated.

**3D Osx-GFP cells nearest distance to blood vessel surface analysis.** Confocal image stacks were stitched together, denoised, and deconvolved using proprietary software (NIS-Elements AR 5.21.01). Images were then imported into Imaris v9.5.1 (Bitplane AG) and rendered as 3D volumes. CD31 and Emcn signals were modeled using the surface tool of Imaris using the absolute intensity algorithm. Osterix-GFP cells were modeled as 3D spots with an estimated XY diameter of 8 μm. To quantify the distance of Osx-GFP spots to the nearest blood vessel surface, we used a distance transformation algorithm (MATLAB R2017b) on the modeled surfaces generated from either CD31 or Emcn signals and calculated the intensity value of the distance transformed image at the center of each modeled Osx-GFP spot. Growth plate Osx-GFP spots were filtered out of the analysis. All Osx+ cells were analyzed in a representative bone of each genotype.

**Transmission electron microscopy (TEM)**

E16.5 tibia samples were harvested and cleaned. The epiphyses were cut to improve fixative infiltration, and the samples were immediately fixed with a 4% PFA and 2.5% glutaraldehyde solution (both from Electron Microscopy Sciences) in a 0.1 M phosphate buffer. Samples were then stained with 2% osmium (OsO4) (Electron Microscopy Sciences) and 1.5% potassium ferrocyanide (K4Fe(CN)6) (Sigma) for 4 h, followed by incubation with 0.5% uranyl acetate (Electron Microscopy Sciences) in 25% methanol overnight and en bloc staining with Walton’s lead aspartate (Walton, 1979) for 30 min at 60°C. Samples were then dehydrated with a graded series of ethanol and incubated with propylene oxide (Agar Scientific) twice for 10 min. Finally, samples were gradually infiltrated with hard epoxy resin (Agar Scientific)/propylene oxide mixtures followed by embedding in pure epoxy resin and polymerization at 60°C for 48 h. Blocks were sectioned at 70 nm thickness, post stained with 4% uranyl acetate (SPI) for 8 min and Reynold’s lead citrate (Reynolds, 1963) for 3 min and imaged at an acceleration voltage of 80 kV using a JEOL JEM14400-LaB6 Transmission Electron Microscope equipped with a EMSIS Quemesa 11 Mpxl camera.

**Primary Cell Culture and in vitro Irradiation**

For primary murine (m) BMSC cultures, femurs and tibias were isolated from 8-week-old wild-type CD1 mice and thoroughly cleaned to remove muscles and connective tissues. Epiphyses were removed with a sterile scalpel, and each bone shaft was placed in a heparin-coated Microvette CB tube (Sarstedt) for a 2-min centrifugation step at 4500 rpm. The BM plug in the collection tube was resuspended and plated in DMEM containing 20% non-heat inactivated FBS and 1% AA. During the first 3 days, medium was changed every day to remove hematopoietic cells. Next, cells were grown until 50% confluency and irradiated with 7Gy; parallel control cultures were kept non-irradiated. The medium was changed every 2 days subsequently, and the cells were harvested 7 days after irradiation by lysing them in RLT buffer containing 1% β-mercaptoethanol. RNA was extracted for qRT-PCR, as described below.

**Analysis of gene expression**

**Real-time quantitative RT-PCR (qRT-PCR)**

For RNA extraction, bones were crushed while frozen in liquid nitrogen and homogenized in Trizol (Sigma-Aldrich), as described before (Dejaeger et al., 2017; Dirckx et al., 2018). For embryos, we used both tibias and femurs in a pooled hind limb sample of each individual mouse; for newborn mice at P2 we pooled the two femurs; adult bone RNA samples were extracted from a single femur per mouse. For RNA isolation of livers, the samples were homogenized in Trizol (Sigma-Aldrich). Total RNA from these samples was isolated using the RNeasy Mini kit (Qiagen), all according to the manufacturer’s protocol. cDNA was synthesized from 1 or 2 μg RNA by a standard protocol as described before (Böhm et al., 2019). Gene expression was quantified using the Fast SYBR Green Master Mix (Thermo Fisher Scientific) on a Step One Plus Real-Time PCR system (Applied Biosystems) according to standard protocols. Primers are listed in Table S1. Relative mRNA levels were calculated by the ΔΔCt method, using hypoxanthine-guanine phosphoribosyltransferase (HPRT) as housekeeping/reference gene.

**Bulk RNA-sequencing**

Mice were dissected at E14.5 to isolate osteoprogenitor cell (OPC) and endothelial cell (EC) populations from the perichondrium of fetal long bones (forelimbs and hind limbs), according to the procedure described in detail previously (Böhm et al., 2019). Briefly, bones were subjected to a digest using 2 mg/mL collagenase type II and 3 mg/mL dispase in αMEM for 30 min, in a shaking water bath at 37°C. Medium containing 10% FBS was added and the isolated cells were filtered (70 μm), centrifuged and resuspended at 1x 106 cells/mL in PBS containing 2% FBS. Cells were stained for 30 min with anti-CD31-AlexaFluor 647 (BD Biosciences) and anti-CD45-PE (eBioscience). Populations of interest were isolated using fluorescence-activated cell sorting (FACS) on a BD FACSAria II. Three independent samples for each group (two cell types, from control and VEGF cTg fetuses) were collected, except for OPCs from control mice where 4 samples were collected. Each individual sample contained the pooled cell yield from 12 to 31 embryos as detailed in Figure S5A.
The extraction of total RNA (RNeasy Micro kit, QIAGEN), library preparation using 100 ng RNA (TruSeq Stranded mRNA Sample Preparation Kit, Illumina), indexing, quantification, validation of library quality and size range (DNA 1000 kit, Agilent Technologies), sequencing Illumina HiSeq2500, generating 50 bp single-end reads with a depth of 30–40 Mb per sample, and processing of results and bioinformatics was all done according to the procedures described before (Böhm et al., 2019). Count-based differential expression analysis was done with R-based Bioconductor package DESeq and Cufflinks 2.2.1. Reported p values were adjusted for multiple testing with the Benjamini-Hochberg procedure, which controls the false discovery rate (FDR). For differential expression analysis of a single gene, DESeq datasets were used. To directly compare the differential expression of multiple genes (in Figure S5C), cufflinks software datasets were used because these include a correction for gene length; all other analyses were based on DESeq datasets. Differentially expressed (DE) genes were identified based on the FDR, with FDR < 0.1 considered statistically significant. Gene Set Enrichment Analysis (GSEA) was performed with GSEA software (MSigDBv6, Broad Institute) (Mootha et al., 2003; Subramanian et al., 2005) using the full gene lists with normalized reads and Ingenuity Pathway Analysis (IPA) (QIAGEN) was performed using the gene list of DE genes.

Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Hind limbs and forelimbs (from constitutive and delayed-onset (DOX) mice, respectively) were crushed on liquid nitrogen and extracted in cell extraction buffer (CEB buffer, Invitrogen #FNN0011) including 350 mM of sodium fluoride (NaF), 2.3 mM of sodium orthovanadate (Na3VO4) and a protease inhibitor cocktail (Sigma #P2714). Samples were cleared by centrifugation at 14,000 g at 4°C and the protein concentration was quantified by the Protein Assay Dye Reagent (BioRad). For ELISA, 18 μg protein lysate was assayed using the Mouse VEGF Immunossay Quantikine ELISA Kit (R&D Systems MMVO). Serum VEGF protein levels were determined using the same. Briefly, blood was collected and centrifuged for 10 min at 4000 rpm to obtain the serum and samples were stored at −80°C until use. The ELISA was performed using 10 μL of serum, according to the manufacturer’s protocol, and analyzed on a Synergy HT (BioTek) plate reader.

Peripheral blood analysis

Blood samples were collected via the jugular vein and kept in EDTA-coated tubes (Sarstedt) until analysis. Total nucleated blood cell counts were performed using an automated scil Vet abc Plus+ hemocytometer (SCIL Animal Care Company).

Flow cytometry

Single-cell samples preparation

For flow cytometry, all skeletal tissue digestions were performed at 37°C with an enzymatic digest solution consisting of α-MEM medium containing 3 mg/mL collagenase II and 4 mg/mL dispase (both from Thermo Fisher Scientific). Full embryonic femurs and tibias were digested for 2×30 min. Newborn mouse-derived femurs and tibias were longitudinally cut and digested for 40 min. Spleens and livers were mechanically dissociated. Blood was taken via the jugular vein and kept in EDTA-coated tubes. Samples from bones, livers and spleens were filtered through 70 μm nylon cell strainers to obtain single-cell suspensions. Cell suspensions derived from livers were additionally incubated for 10 min with red blood cell (RBC) lysis solution. For bones from adult mice, the BM was flushed and incubated in the enzymatic digest solution for 15 min; the remaining bone shaft was further cut into pieces and incubated with the same digestion medium formula for 30 min, all at 37°C. Bone pieces and BM flush were then combined and filtered through 70 μm nylon cell strainers to obtain single cell suspensions.

Staining procedure

For staining of cell surface markers, cells were kept in FACS buffer (0.5% BSA, 2 mM EDTA in PBS) and incubated for 30 min at 4°C unless indicated otherwise. All antibodies used for flow cytometry analyses are listed in the Key resources table and the populations analyzed are described in the following section (‘Sample recording and analysis”).

Cell cycle analysis was performed either with Hoechst and Pyronin Y (Shen et al., 2008) or with Ki67 (Kim and Sederstrom, 2015). For cell cycle analysis by Hoechst and Pyronin Y, cells were kept in FACS buffer with 50 μg/mL verapamil (Sigma-Aldrich) through the entire procedure and stained with 20 μg/mL Hoechst 33342 (Invitrogen) for 30 min and with 2 μg/mL Pyronin Y (Sigma) for an additional 30 min, all at 37°C. After centrifugation, cells were subsequently incubated with antibodies detecting specific cell surface markers. For cell cycle analysis using Ki67, the cells were first incubated with antibodies against specific cell surface markers, and after centrifugation, fixed with 4% PFA for 20 min and permeabilized with 1% (w/v) saponin solution in 10 mM HEPES buffer for another 20 min. After this step, cells were maintained in a 0.5% saponin buffer and incubated with Ki67-Pacific Blue antibody (BioLegend) for 30 min. Detection of apoptotic cells was performed by incubating the cells with Annexin V-PE (BioLegend), along with antibodies for selected cell surface markers, for 30 min at 4°C in binding buffer (10 mM HEPES pH 7.5, 140 mM NaCl, 2.5 mM CaCl2 in PBS).

Detection of reactive oxygen species (ROS) in cells was done using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Thermo Fisher Scientific). CM-H2DCFDA passively diffuses into cells, where it reacts with intracellular components and is finally oxidized, leading to fluorescent 2',7'-di-chlorofluorescein (DCF). After staining for cell surface markers, cells were additionally incubated with 3 μg/mL CM-H2DCFDA for 30 min at 37°C in FACS buffer. The results are displayed as the ratio between control and VEGF cTg samples of the mean fluorescent intensity (MFI) for 2',7'-di-chlorofluorescein (DCF) (relative DCF MFI (%)).

All flow cytometry experiments included corresponding unstained, single color and fluorescence minus one (FMO) controls for determining compensation and gating.
Sample recording and analysis

All samples were run on a BD FACSCanto II High Throughput Sampler (BD Biosciences) flow cytometer followed by analysis with FlowJo® software. Hematopoietic stem and progenitor cells (HSPCs) and HSCs were identified in all organs as CD45+ Lineage- (Lin-; with the lineage cocktail including antibodies against CD3, CD45R (B220), Ter119, and Gr1 (Ly-6G), but not CD11b for fetal/newborn analyses of endogenous HSC/HSPC numbers, because CD11b is expressed by a subfraction of developmental HSCs and we did not want to risk excluding a specific subset of the cells of interest from the analyses) Sca-1+ c-kit+ (LSK) and LSK CD48- CD150+ (LSK-SLAM) (Kiel et al., 2005; Kim et al., 2006), respectively. Committed progenitors were identified in liver samples as pre-colony forming unit – erythroid (pre-CFU-E: Lin- Sca-1- c-kit+ CD41+ CD105+ CD150+), megakaryocyte progenitors (MkP: Lin- Sca-1- c-kit+ CD41+ CD150+), pre-granulocytes and monocytes (preGM: Lin- Sca-1+ c-kit+ CD41 CD105 CD150) (Pronk et al., 2007). Osx-Cre:GFP+ cells were recorded as CD45- Ter119- CD31- Osx-Cre:GFP+. BM ECs were identified as CD45- Ter119- CD31+ and further divided into arterial (CD45- Ter119- CD31+ Sca-1+) and sinusoidal (CD45- Ter119- CD31+ Sca-1-) fractions (Hooper et al., 2009). SSPC populations including stromal cells in the BM were identified as either PDGFRα+ cells (CD45- Ter119- CD31- PDGFRα+) (Bohm et al., 2019; Kusumbe et al., 2016), P2S cells (CD45- Ter119- CD31- PDGFRα+ Sca-1-) (Morikawa et al., 2009), immature osteolineage progenitors cells (CD45- Ter119- CD31- CD51+ Sca-1-) or more mature osteolineage progenitors (CD45- Ter119- CD31- CD51+ Sca-1+) (Semerad et al., 2005).

Short-term (ST) homing assay.

To experimentally test the homing capacity of transplanted wild-type HSPCs toward the control versus mutant BM environment, we opted for using the VEGF cTgDOX mouse model at P2 as recipient to facilitate transplantation and survival for 16 hours subsequently until tissue harvesting. To model the natural migration process, we used HSPCs derived from the liver as donor cells; furthermore, no irradiation was included to not introduce new changes to the BM environment. Liver cells were isolated from wild-type donor mice at P2 by mechanical dissociation as described above, and Lin- cells were subsequently selected by magnetic separation using the Lineage Cell Depletion Kit (cocktail containing antibodies against CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7/4-antigen, and Ter-119), with MS columns and MiniMACS Separator (all from Milteny Biotec). Lineage-depleted cells were then labeled with the membrane dye Vybrant DIIL Cell-Labeling Solution (Invitrogen) and injected into the jugular vein of P2 control and VEGF cTgDOX newborn pups. Sixteen hours after the transplantation, the bones, liver, spleen and blood were harvested and prepared for flow cytometry as described above. For the analysis, total LSK cells were first identified and then further gated for the detection of LSK cells labeled with the VibrantTM DIIL dye (Excitation 549nm / Emission 565nm). The number of labeled LSK cells retrieved from the limbs (fore- and hind limb bones), vertebra, calvaria, skeleton (sum of cells homed to the limbs, vertebra and calvaria), spleen, liver, and circulation were documented.

Quantification and statistical analysis

Analysis of the RNA-seq data is detailed in the respective section above. Briefly, padj < 0.1 by the Benjamini-Hochberg procedure was used as threshold for statistical significance. All other results are expressed as mean ± standard error of the mean (SEM). With the exception of a few selective cases, indicated in the figure legends, statistical testing entailed the comparison of samples of two conditions or genotypes, using a two-sided two-tailed unpaired Student’s t test (GraphPad prism), whenever applicable applied to distinct time points separately. P values (p) < 0.05 were considered as significant, and indicated as * p < 0.05, ** p < 0.01, *** p < 0.001. Absence of a symbol implies that the difference between the groups was not statistically significant (p > 0.05). n numbers are biological repeats, representing the number of individual mice or distinct sets of pooled mice for each genotype group or condition.
Supplemental information

Fetal hematopoietic stem cell homing is controlled by VEGF regulating the integrity and oxidative status of the stromal-vascular bone marrow niches

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Figure S1. Increased ossification and bone deformations in VEGF cTg embryos as likely cause of fatal breathing impairment, Related to Figure 1.

(A) Quantification of mineralized bone volume relative to tissue volume (BV/TV, in %) in tibias derived from control and VEGF cTg mice at E18.5, based on the 3D micro-CT scan reconstructions represented in Figure 1D (n=7). Graphs represent the average values ± SEM and show data points derived from individual embryos. ***p<0.001, unpaired Student’s t-test.

(B) Skeletal preparations by Alizarin red and Alcian blue staining for bone and cartilage, respectively, showing lateral views of the rib cage of a control and a VEGF cTg embryo harvested at E18.5. Note the malformed ribs and sternum in the mutant embryos, reducing the overall rib cage volume.

(C) Macroscopic overviews (upper panels) and magnified whole-mount views (lower panels) of the lungs of control and VEGF cTg mice delivered by cesarean section at E18.5 and stimulated to start breathing autonomously. Consistent with the breathing impairment of the VEGF cTg mice as observed by the investigator performing the study, the images illustrate the smaller organ size and reduced air infiltration in the mutant animals; white arrows point at air-filled alveoli detected abundantly in control animals, but not in the lungs of VEGF cTg littermates.

(D) Histology of the E18.5 lungs, showing representative images of H&E stained paraffin sections. These observations confirm the scarcity in air-filled (pre-)alveoli in the mutant lungs and support the conclusion that VEGF cTg mice die shortly after birth due to hampered breathing, likely caused by the malformed thoracic cage.

(E) H&E staining on E14.5 tibia paraffin sections, demonstrating that no major morphological differences are discernable in the pre-invasion cartilaginous bone template of control and VEGF cTg fetuses at this stage.

(F) Histology of the tibia at E16.5, showing safranin O (left panels, red color marks cartilage proteoglycans) and H&E (right panels) staining on adjacent paraffin sections.

(G) Representative flow cytometry gating examples for the quantification of Osx-Cre:GFP-expressing (Osx/GFP+) cells in bones of control and VEGF cTg embryos harvested and digested at E18.5.
Figure S2. Compensatory hematopoiesis in the fetal liver results in normal levels of circulating blood cells in VEGF cTg embryos, Related to Figure 2.

(A) Representative plots illustrating the flow cytometry gating strategy as used to quantify the CD45+ cells (from single cells), LSK cells (Lin− Sca1+ c-Kit+ HSPCs), and LSK-SLAM cells (CD150+ CD48− LSK cells, representing HSCs) throughout this study. Example plots obtained for the different fetal hematopoietic organs are shown, for both control and VEGF cTg mice at E18.5. The gating strategy applied to an adult BM control sample is shown for reference.

(B) Blood cell counts in the peripheral blood of Control and VEGF cTg embryos at E16.5, E17.5 and E18.5. Graphs represent the average values ±SEM; n=7-14; ***p<0.001, unpaired Student’s t-tests between the genotypes.

(C) (Left panel) qRT-PCR for VEGF mRNA in liver samples from control and VEGF cTg embryos at E16.5 and E18.5. (Right panels) Histomorphometric analysis of tissue vascularization on liver sections from E16.5 control and VEGF cTg embryos, stained for Emcn. No significant differences between the genotypes were detected. Graphs represent the average values ± SEM and show the individual data points.
### A
**liver**

|          | Control | VEGF cTg^{Dox} |
|----------|---------|----------------|
| Blood vessel # | ![Image] | ![Image] |
| Mean blood vessel diameter (µm) | ![Image] | ![Image] |
| Mean blood vessel thickness (µm) | ![Image] | ![Image] |
| Vascularized surface % | ![Image] | ![Image] |

### B
**bone**

|          | Control | VEGF cTg^{Dox} |
|----------|---------|----------------|
| PECAM1 | ![Image] | ![Image] |
| CD45 | ![Image] | ![Image] |
| Sca-1 | ![Image] | ![Image] |

### C
**blood**

|          | granulo-/monocytes | lymphocytes | erythrocytes | platelets |
|----------|--------------------|-------------|--------------|-----------|
| Concentration (10^3/mm³) | ![Image] | ![Image] | ![Image] | ![Image] |

### D
**blood**

|          | E18.5 | P2 |
|----------|-------|----|
| % LSK cells from CD45+ cells | ![Image] | ![Image] |

### E
**liver**

|          | E18.5 | P2 |
|----------|-------|----|
| Number of LSK cells | ![Image] | ![Image] |
| Number of CD45+ cells | ![Image] | ![Image] |
Figure S3. Compensatory hematopoiesis in the spleen results in normal levels of circulating blood cells in the delayed-onset VEGF 164 cTg mice, Related to Figure 3.

(A) Representative liver sections immunostained for Emcn and quantified vasculature parameters in P2 control and VEGF cTg<sup>DOX</sup> mice (n=4 per genotype).

(B) Flow cytometry gating strategy for CD45+ cells (from single cells) and LSK cells in bones of P2 control and VEGF cTg<sup>DOX</sup> mice.

(C) Peripheral blood analysis showing counts of the circulating granulocytes/monocytes, lymphocytes, erythrocytes and platelets, in E18.5 and P7 control and VEGF cTg<sup>DOX</sup> animals (n=7-11 per group). Graphs show all the data points for each individual animal, and average ± SEM.

(D) Percentage of circulating LSK from CD45+ cells at E18.5 and P2 (n=4-8).

(E) Number of LSK and CD45+cells in E18.5 and P2 liver samples (n=4-6).

All graphs represent the average values ± SEM and show individual data points; no significance was observed between the genotypes by two-sided unpaired Student’s t-test for each time point.
Figure S4. The decreased abundance of HSPCs in VEGF cTg fetal bones is not due to impaired HSPC proliferation but is caused by impaired BM homing of the cells, Related to Figure 4.

(A) Cell cycle analysis of LSK cells derived from hind limbs of E18.5 control and VEGF cTgDOX mice, based on Ki67 staining detection by flow cytometry (n=4-11). The Ki67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis) but absent in quiescent or resting cells (G0). No significant differences were detected between the Ki67+ and Ki67− LSK fractions of the respective genotypes, indicating that HSPC proliferation was unaffected in VEGF cTgDOX embryos at E18.5.

(B) Number of LSK cells contained in n=2 representative samples of 500,000 lineage-negative (lin−) cells isolated from the liver of newborn wild-type mice at P2, as determined by flow cytometry. Similar membrane-labeled cell pools were used for the homing experiment shown in Figures 4, E-I.

(C) Quantification of LSK cells carrying the Vybrant™ DII membrane label, expressed as fraction of CD45+ circulating cells, in the blood of control and VEGF cTgDOX mice 16h following transplantation of 500,000 labeled liver-derived lin− cells (short-term homing experiment, see Figure 4E) (n=4-5).

(D) Number of retrieved labeled LSK cells in the calvaria of control and VEGF cTgDOX mice 16h after the transplantation (n=4-6).

All graphs represent average values ± SEM. No significant differences were detected between the genotypes, by two-sided unpaired Student’s t-test.
Figure S5. Design and validation of RNA-seq analysis of fetal osteogenic and endothelial populations after short-term exposure to increased skeletal VEGF, Related to Figure 5.

(A) Details on the control (CON) and VEGF cTg samples used for bulk RNA-sequencing, indicating the number of E14.5 embryos of which the cell isolates were pooled and the total number of cells contained within each sample of osteoprogenitor cells (OPCs) and endothelial cells (ECs), isolated by flow cytometry as described in Figure 5A.

(B) Heat map showing the expression of osteolineage markers and endothelial markers across all samples, validating the cell type-specific nature of the isolated populations.

(C) Expression levels of the VEGF family members VEGF-A, VEGF-B, VEGF-C and placental growth factor (PGF) in OPCs and ECs, expressed as fragments per kilobase million (FPKM) deducted from Cufflinks-based datasets, confirming selective differential expression of VEGF-A in OPCs, in line with the Osx-Cre:GFP-driven VEGF overexpression in this model. Asterisks (**) represent the false discovery rate (FDR)-adjusted p value (padj)<0.01, by the Benjamini-Hochberg procedure.

(D) Expression levels of osteolineage markers, displayed as log2 fold change of normalized read counts deducted from DESeq-based databases, in VEGF cTg OPCs relative to control OPC values.

(E and F) qRT-PCR analysis determining the mRNA expression levels of a panel of osteolineage cell differentiation markers in hind limbs from (E) constitutive VEGF cTg mice at E18.5 and (F) delayed-onset VEGF cTg<sup>DOX</sup> mice at P2 (n=5-8; *p<0.05, **p<0.01, ***p<0.001, by two-sided unpaired Student’s t-test between genotypes). Abbreviations of (early to late) differentiation marker genes: Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; Osx, osterix; Col1, collagen type I alpha 1; Opn, osteopontin; Ocn, osteocalcin; Dmp1, dentin matrix acidic phosphoprotein 1.

(G) qRT-PCR analysis showing the mRNA expression levels of the secreted HSC niche factors CXCL12, SCF, and angiopoietin 1 (ANGPT1) in hind limb bones from E18.5 VEGF cTg (left) and P2 VEGF cTg<sup>DOX</sup> (right) mice (n=5-8; *p<0.05, by unpaired Student’s t-test between genotypes).

(H) Heat maps representing the expression of genes implicated in DNA replication, selected from KEGG pathways, in sorted ECs and OPCs from control and VEGF cTg mice.

(I) Enrichment plots derived from Reactome pathway analysis, showing that pathways related to ECM remodeling are intensified in the transcriptomes of both OPCs and ECs derived from the mutant mice (degradation of the ECM in OPCs, normalized enrichment score (NES)=1.678 and q-value= 0.080; ECM organization in ECs, NES=1.891 and q-val=0.017).

(J) Significantly enriched pathways in sorted OPCs from VEGF cTg fetuses, related to cell growth and migration, as found by Ingenuity Pathway Analysis (IPA) (p-values all <0.001; number of significantly contributing molecules per pathway: 10 – 76).
Figure S6. Image analysis and quantitative morphology of VEGF-induced alterations in skeletal vascularization architecture, Related to Figure 6.

(A-B) Confocal microscopy of combined immunohistochemistry (co-IHC) for CD31 (green) and endomucin (Emcn, red) on halved bones of tibias from (A) E18.5 VEGF cTg and (B) P2 VEGF cTg^{DOX} mice, showing maximum intensity projections (MIPs) spanning an imaging depth (z-axis depth) of 100µm in (A) and 60µm in (B). Inset in bottom left corners of (A) represent segmented arterioles (CD31^+ Emcn^{low or negative}). Dotted lines delineate the BM cavity. Also shown in Figure 6; repeated here to link to the image quantification in the following panels.

(C-D) Illustrations showing segmentation of the Emcn signal from stained and confocal imaged specimens as shown in panel A and B. The 3D rendered segmented signal is visualizing the metaphyseal (top panels) and diaphyseal (bottom panels) blood vessels from (C) E18.5 VEGF cTg tibias and (D) P2 VEGF cTg^{DOX} tibias. Note randomized orientation of blood vessels in the constitutive mutant and abundant protrusions emanating from the vessels in the delayed-onset model (red arrowheads in (D)).

(E) Morphometric blood vessel analysis performed in 2D on confocal images segmented for the CD31 signal, showing the vascularized area (as percentage of BM area), the number of vascular junctions/bifurcations relative to the BM area, and the density of triple and quadruple points (where 3 or 4 vascular branches coalesce) in the tissue. Bar graphs represent mean ± SEM, with individual data points superimposed (n=4); *p<0.05, by two-sided unpaired Student’s t-test.

(F) Example of CD31-signal image analysis of blood vessel width, illustrating increasing fitted widths by a pink-to-yellow color gradient, superimposed on the CD31 IHC view in green.

(G) Quantitative analysis of the blood vessel width processed as illustrated in (F), showing the distribution of bone marrow blood vessels along discrete width ranges (left graph) or grouped into small (<50µm) and large (>50µm) vessels (right graph). Note the increase in the proportion of small vessels at the expense of the larger blood vessels in the VEGF cTg fetuses compared with control littermates. Bar graphs, mean ± SEM (n=4); *p<0.05 for unpaired Student’s t-test applied in (G).
Supplemental Information

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A

B

Constitutive VEGF cTg

Control

VEGF cTg

Arteriolar fraction

Sinusoidal fraction

Delayed-onset VEGF cTg

Control

VEGF cTg

Arteriolar fraction

Sinusoidal fraction

C

EC subtype fractions

D

ROS+ cell fractions

Mean ROS levels

D

In CD31+ cells

In CD31+; sca1+

In CD31+; sca1-

In CD31+; sca1+

In CD31+; sca1-

In CD31+ cells

E

Rel. mRNA

Oxidadd, Zn-2+, JAK2, Twist-1, Notch-1, HIF-1α, VE-cadherin, CD31, Emcn, E-selection, Enk

Control

VEGF cTg

Control

VEGF cTg

F

Control

VEGF cTg
Figure S7. Increased VEGF in the skeletal environment alters the abundance and phenotype of local EC subtypes and elicits a rise in endothelial ROS production, Related to Figure 6.

(A) Flow cytometry gating strategy for CD45−Ter119−CD31+ ECs and the subpopulations thereof that are marked by presence or absence of Sca-1, generally considered arteriolar-type ECs (CD45−Ter119−CD31+Sca1+) and sinusoidal-type ECs (CD45−Ter119−CD31−Sca1−), respectively.

(B) Flow cytometry analysis of arteriolar and sinusoidal ECs in limbs derived from constitutive (left panels) and delayed-onset (right panels) VEGF over-expressing mice at the indicated ages, showing (upper graphs) the number and (lower graphs) the relative proportions (within the total CD31+ population) of the respective EC subsets.

(C, D) Flow cytometry analysis of ROS by quantifying the presence and levels of DCF in bones from VEGF cTg fetuses at E16.5 (left panels) and VEGF cTgDOX newborns at P2 (right panels), showing (C, upper panels) the fraction of DCF+ cells within each endothelial subpopulation, (C, lower panels) the DCF mean fluorescence intensity (MFI), indicative of the level of ROS production, expressed relative to the control mice, and (D) representative flow cytometry plots for the respective cell subsets.

(E) mRNA levels determined by qRT-PCR of genes representing EC markers, implicated in endothelial junctions (left graph) and EC subtype (right graph), in limb bones derived from VEGF cTgDOX embryos at E18.5.

(F) Co-IHC detecting VE-cadherin (green) and Emcn (red) in bone sections from control and VEGF cTgDOX mice at P2. Note increased VE-cadherin staining intensity in the metaphysis of the mutant (yellow signal denotes VE-cadherin in ECs). Scale bar, 200 µm; gp, growth plate. Representative image of n=2-3 mice per group.

All bar graphs show the average ± SEM, and dots represent individual data points (n=4-9); *p<0.05, **p<0.01, ***p<0.001, by unpaired Student’s t-test comparing values for control and mutant mice; absence of an asterisk implies that the results for the groups were not statistically significantly different (p>0.05).
Table S1. Oligonucleotide sequences used in this study, Related to STAR Methods.

### Primers used for genotyping

| Target gene | Forward primer (5'→3')          | Reverse primer (5'→3')          |
|-------------|---------------------------------|---------------------------------|
| Cre         | CGCGGTCTCGGAGTAAAAACTATC        | CCCACCGTCAGTACGTGAGATATC        |
| ROSA (WT)   | AAAGTCGCTCTGAGTTGTAT            | GGAGCGGGGAGAAATGGATATG          |
| ROSA (cTg)  | GCGAAGAGTTTGTCTCAACC            | GGACGCCGGAGAAATGGATATG          |

### Primers used with SYBR Green for real-time quantitative (q)RT-PCR

| Target gene | Forward primer (5'→3')          | Reverse primer (5'→3')          |
|-------------|---------------------------------|---------------------------------|
| Aplp        | CGCACGGCAAGGTACAAAAACTATC       | CCGACCTCCACAGATCTCCT            |
| Angpt1      | CTCGTCAGACATTCACTCAG            | AGCAAGGAAACCTTCTTTATGGA         |
| Angptl3     | TCTACTGTGTACCAATCGACGGGC        | CATGTTTGGTAGAAGTCTGTA           |
| Apln        | ATTCGACGTGATGGAGGTGG            | ACTGCGGCTTTAAGCATAGC            |
| Bglap (Osteocalcin) | GGCCCTGAGTCTGAGACAAAGC | GCTCGTCACAGACAGGTTTA |
| Bmx         | GATCTGACATCGCAATCAGT            | TGGAAATAGCGTGATTCTGTG           |
| Cbfa1 / Runx2 | TACCCAGCGACAGACCAGAA           | AGAGGCTGTTTGGACGCCATAG          |
| Cdh5 (VE-cadherin) | CACTGCTTGGGGAGCTCTTC  | GGGGCAGCGATTCATTCTTCT          |
| Col1a1      | GCTCCCTTGAAGGGCCACT            | CCACGTCTCCAGATTGGGG             |
| CXCL12      | CCGCGGGGTTTCTGAA               | CACACTGGTCTGTGTTTGTCTTCA        |
| Dmp1        | AGTGGAGGGAGACAGCTGAA           | GACGGGCTGCTTGGAGCTCAC           |
| Emcn        | CTTTGTCCAAACAGTCTGTCG          | GACACGATGCCCTGATTTGTG           |
| Hprt        | TGCTGACCTGCCTGGATTACA          | TATGCTCCCCGTGACTGAT             |
| Pecam1 (CD31) | ACGTGGTGTCCTATGCAAG            | TCGATGGCTGCCCATTCATCA            |
| Kitl (SCF)  | GGTAGCTGATTTCTCATCCATGCCTG    | CCTGTAAGGACTTTTCTGAGAGCTCT      |
| Sele (E-selectin) | ATGCCTGCGGTCTTCTCTC   | GTAGTCCGGTGACAGTATGC            |
| Sp7 (Osterix) | ATGGCGTCCTCTCTGGCTTGGA        | AGTCCCGCAGAGGGCTAGAG            |
| Spp1 (Osteopontin) | AGCAAGAATACTTCTCAAAGCAA    | GTGAGATTCGTCAGATTCATCCG         |
| Tjp1 (Zo-1) | GCCGCTAAGAGACAGCAAGCA         | TCCCCACTCTGAAATGGAGGA           |
| Tpo         | GGCCATGCTTCTTGCAGTG            | AGTCGGCTGTAAGGGAGGT             |
| VEGF (total) (*) | AGTCCCATGAAGTGATCAAGTTCA    | CACTCCAGGGCTTCATCGGT            |

(*) Total VEGF mRNA is detected, including endogenous VEGF, all isoforms, and transgenic VEGF164.