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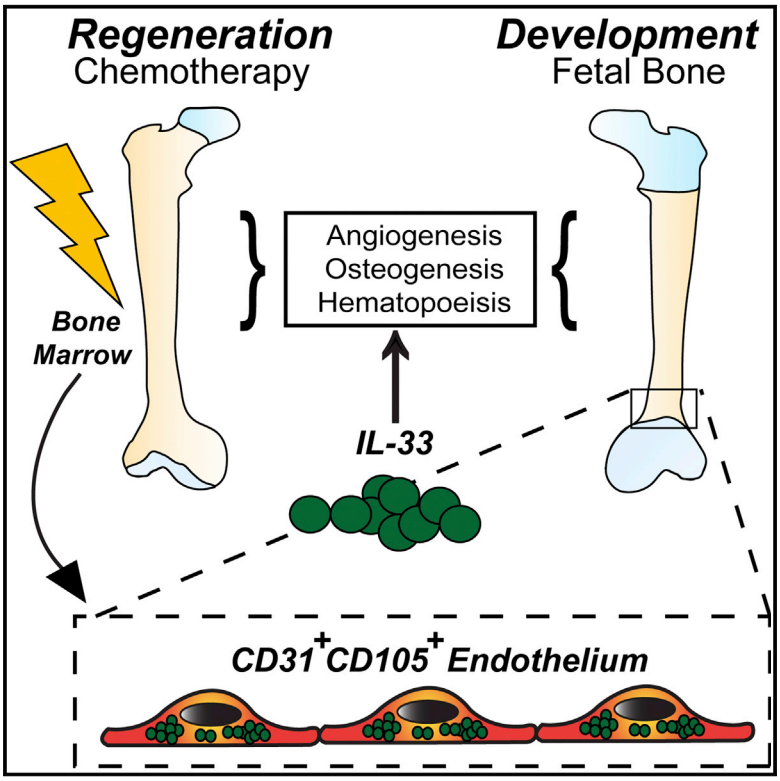
Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 As an Anabolic Factor

Graphical Abstract



Highlights

- Human bone marrow CD105^{+} ECs (hRECs) are associated with regeneration and development
- hRECs display remarkable phenotypic and molecular similarity to murine type H endothelium
- This includes expression of genes implicated in blood, bone, and vessel formation
- IL-33 is identified as a promotor of HPCs, endothelial cells, and osteogenesis

Authors

Keane Jared Guillaume Kenswil, Adrian Christopher Jaramillo, Zhen Ping, ..., Bram van Eerden, Tom Cupedo, Marc Hermanus Gerardus Petrus Raaijmakers

Correspondence

m.h.g.raaijmakers@erasmusmc.nl

In Brief

In this paper, Kenswil et al. report on the identification and molecular characterization of human bone marrow endothelial cells enriched during chemotherapy-induced regeneration and development. These findings give insight into the angiocrine factors and regulatory programs driving bone marrow recovery after injury.

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Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 As an Anabolic Factor

Keane Jared Guillaume Kenswil,[1] Adrian Christopher Jaramillo,[1] Zhen Ping,[1] Si Chen,[1] Remco Michiel Hoogenboezem,[1] Maria Athina Mylona,[1] Maria Niken Adisty,[1] Eric Moniqué Johannes Bindels,[1] Pieter Koen Bos,[2] Hans Stoop,[3] King Hong Lam,[3] Bram van Eerden,[4] Tom Cupedo,[1] and Marc Hermanus Gerardus Petrus Raaijmakers[1,5,*]

[1]Department of Hematology, Erasmus MC Cancer Institute, Rotterdam 3015CN, the Netherlands
[2]Department of Orthopaedics, Erasmus MC, 3015 CE Rotterdam, the Netherlands
[3]Department of Pathology, Erasmus MC Cancer Institute, Rotterdam 3015CN, the Netherlands
[4]Department of Internal Medicine, Erasmus MC Cancer Institute, Rotterdam 3015CN, the Netherlands
[5]Lead Contact
*Correspondence: m.h.g.raaijmakers@erasmusmc.nl
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SUMMARY

Bone marrow formation requires an orchestrated interplay between osteogenesis, angiogenesis, and hematopoiesis that is thought to be mediated by endothelial cells. The nature of the endothelial cells and the molecular mechanisms underlying these events remain unclear in humans. Here, we identify a subset of endoglin-expressing endothelial cells enriched in human bone marrow during fetal ontogeny and upon regeneration after chemotherapeutic injury. Comprehensive transcriptional characterization by massive parallel RNA sequencing of these cells reveals a phenotypic and molecular similarity to murine type H endothelium and activation of angiocrine factors implicated in hematopoiesis, osteogenesis, and angiogenesis. Interleukin-33 (IL-33) was significantly overexpressed in these endothelial cells and promoted the expansion of distinct subsets of hematopoietic precursor cells, endothelial cells, as well as osteogenic differentiation. The identification and molecular characterization of these human regeneration-associated endothelial cells is thus anticipated to instruct the discovery of angiocrine factors driving bone marrow formation and recovery after injury.

INTRODUCTION

Endothelial cells (ECs) govern tissue development and regeneration by signaling molecules on their cell surface and the release of factors, such as cytokines and extracellular matrix proteins. This angiocrine function of the endothelium drives tissue development and regeneration in multiple organs.

In the hematopoietic system, studies in mice have revealed pivotal contributions of ECs to the formation and regeneration of bone and bone marrow (BM) (Hooper et al., 2009; Ramasamy et al., 2016). Coordinated activation of osteogenesis, angiogenesis, and hematopoiesis is required for BM regeneration after tissue injury (Rafii et al., 2016; Ramasamy et al., 2015) induced by irradiation or chemotherapy. ECs support the proper regeneration of the hematopoietic system after myeloablation (Butler et al., 2010; Hooper et al., 2009; Kobayashi et al., 2010). Engraftment and repopulation of hematopoietic stem and progenitor cells (HSPCs) in mice is dependent on the regeneration of sinusoid ECs, which are vulnerable to toxic injury (Hooper et al., 2009). Sinusoid regeneration is mediated through vascular endothelial growth factor receptor 2 (VEGFR2) signaling, the blockage of which resulted in delayed reconstitution of peripheral blood values in irradiated mice (Hooper et al., 2009).

ECs do not seem to be created equally in their ability to drive or contribute to bone and hematopoietic development and regeneration. Recent studies in mice have identified a specialized endothelial subset that controls the number of HSPCs in addition to coupling angiogenesis and osteogenesis (Kusumbe et al., 2014, 2016; Ramasamy et al., 2014). This endothelial subtype, dubbed type H endothelium for its high expression of endomucin and CD31, is enriched in the bone metaphysis at the endosteal surface, where it is adjacent to osteoprogenitor cells and gives rise to sinusoidal endothelial vessels. Importantly, insight into the molecular programs underlying the capacity of this endothelial subtype to drive angiogenesis and osteogenesis enabled its pharmacologic manipulation in mice, resulting in increased bone formation (Kusumbe et al., 2014; Ramasamy et al., 2015).

Taken together, various studies indicate that specified EC-derived signals can orchestrate complex multicellular network interactions in the mammalian marrow, driving bone formation and regeneration under stress conditions. These findings in murine models open the perspective of EC-instructed strategies to regenerate bone and marrow in humans, both in degenerative conditions as well as after injury, such as chemotherapy and irradiation. Translation of these important findings to human regenerative medicine, however, will be critically dependent on our ability to identify and interrogate molecularly equivalent human ECs driving ontogeny and regeneration.

Here, we describe the identification of a human EC subtype that is strongly associated with human fetal BM development and regeneration after chemotherapeutic injury. This EC displays striking immunophenotypic and molecular commonalities with

type H endothelium in mice, including transcriptional activation of programs and angiocrine factors previously related to BM recovery in mice. Interleukin 33 (IL-33) is identified as a putative regenerative factor facilitating hematopoietic expansion and bone mineralization *ex vivo*, thus supporting the notion that the transcriptome of this human EC may serve as an important resource instructing discovery as well as validating the relevance of findings in murine models to human regenerative medicine.

RESULTS

Identification of Endoglin (CD105)-Expressing ECs Associated with BM Regeneration after Chemotherapeutic Injury

To identify niche cells potentially implicated in the regeneration of the hematopoietic system in humans, we interrogated the composition of the hematopoietic niche during recovery after chemotherapeutic injury. Chemotherapeutic exposure causes damage to endothelial, hematopoietic, and osteolineage cells within the BM (Hooper et al., 2009; Kopp et al., 2009; Lerner and Harrison, 1990; Xian et al., 2006), resulting in a prolonged neutropenia associated with considerable morbidity and mortality in humans. Flow cytometric assessment of the niche ($7AAD^-CD45^-CD235a^-$; Figure 1A) composition in the regenerating BM of acute myeloid leukemia (AML) patients (see Experimental Procedures) revealed an unaltered frequency of ECs ($CD31^+CD9^+$) (Barreiro et al., 2005) in comparison to marrow under homeostatic conditions (healthy donors) (22.1% ± 2.24% versus 31.57% ± 9.66%; p = 0.15 by unpaired Student's t test) (Figures 1A and 1B). To identify putative immuno-markers of EC subtypes emerging during regeneration, we performed massive parallel RNA sequencing (RNA-seq) of the fluorescence-activated cell sorting (FACS)-purified endothelial compartment ($CD31^+CD9^+$) (Figure 1A). A total of 903 transcripts were found to be significantly differentially expressed in ECs in the regenerative marrow in comparison to ECs from normal, steady-state marrow (glmLRT function, EdgeR; false discovery rate [FDR] < 0.05). The top 200 overexpressed transcripts contained 16 genes encoding for cluster of differentiation (CD) molecules, among which CD105 (*endoglin*) (Table S1), a co-receptor for transforming growth factor β (TGF-β) promoting angiogenesis (Cheifetz et al., 1992; Duff et al., 2003) (Miller et al., 1999) and previously associated with tissue injury (Wang et al., 1995). CD105 protein levels, assessed by FACS analysis (Figure 1C), identified a distinct subset of CD105-expressing ECs strongly enriched in the regenerative marrow compared with normal BM (10.81% ± 2.14% versus 0.48% ± 0.24% of $CD31^+CD9^+$; p = 0.043) (Figure 1D). The presence of this endoglin-expressing subset was temporally restricted, as it was (virtually) absent in AML patients at diagnosis (0% ± 0% of $CD31^+CD9^+$ cells) or after full recovery of peripheral blood values (in complete remission) (1.27% ± 0.66% of $CD31^+CD9^+$ cells) (Figure 1E).

Endoglin-Expressing ECs Are Enriched in the Mouse BM after Chemotherapeutic Myeloablation

To confirm our observation that the CD105-expressing subset of ECs is enriched during BM regeneration and establish a broader relevance for mammalian biology, we next translated our observations to an experimental setting in which we exposed C57BL/6 wild-type mice to a myeloablative dose of the chemotherapeutic agent 5-fluorouracil (5FU) or PBS (vehicle control). The endoglin-expressing subset constituted a rare subpopulation of endothelial ($CD31^+$) cells in the steady-state adult BM niche (0.05% ± 0.02% of $CD45^-Ter119^-$ cells) and in the collagenased bone niche (0.42% ± 0.12% of $CD45^-Ter119^-$ cells) (Figures 2A, 2B, S1A, and S1B). This fraction increased significantly after administration of 5FU in the BM (0.65% ± 0.15% of $CD45^-Ter119^-$ cells, fold-change (FC) increase of 13.6 ± 3.2, p = 0.026) and collagenased bone (8.18% ± 1.43% of $CD45^-Ter119^-$ cells, FC increase of 19.6 ± 3.4, p = 0.007) (Figures 2A, 2B, S1A, and SB), confirming a relative increase of this specific subset in the regenerative phase after myeloablation. Of note, the absolute number of $CD31^+CD105^+$ ECs in the BM did not increase after exposure to 5FU (Figure 2C), suggesting that selection of these cells under chemotherapeutic pressure (rather than absolute expansion) may be implicated.

CD105 (Endoglin)-Expressing ECs Are Enriched in the Human BM during Fetal Development

The temporally restricted enrichment of CD105-expressing ECs during recovery after chemotherapeutic injury suggests that this cell type could potentially be implicated in regeneration and hematopoietic niche formation. To corroborate this notion, we sought to define other conditions in human biology where angiogenesis, osteogenesis, and hematopoiesis are synergistically activated. In human fetal bone development, hematopoiesis shifts from the fetal liver to the bones starting at week 10 after gestation. During this process, angiogenesis, osteogenesis, and hematopoiesis are tightly coupled to allow coordinated bone and hematopoietic development (Coşkun et al., 2014; Jagannathan-Bogdan and Zon, 2013; Medvinsky et al., 2011). Invasion of blood vessels into the mesenchymal condensate is crucial for the coordinated activity of chondrocytes and osteoblasts, and each of these cell types stands in spatial and molecular interaction with ECs (Maes, 2013; Salazar et al., 2016).

Flow cytometric dissection of the endothelial composition of fetal BM at gestational week 15–20 revealed a striking predominance of CD105-expressing cells within the endothelial compartment (62.8% ± 5.9% of $CD31^+CD9^+$ cells) (Figures 3A and 3B). Massive parallel sequencing of these cells confirmed overexpression of genes encoding CD markers identified in the ECs in regenerating BM (Figure S2A), supporting the notion that the enrichment of these transcripts in ECs in the regenerative marrow is caused by overexpression in the subset of $CD105^+$ ECs.

The frequency of CD105-expressing ECs within the endothelial compartment was even higher when examining collagenased fetal bone fractions (average 82.65% ± 2.5% of $CD31^+CD9^+$ cells) (Figures 3A and 3B), suggesting that this endothelial subpopulation might preferentially localize to the endosteal surface of fetal long bones. In line with this, CD105-expressing ECs were identified, albeit at a considerably lower frequency, in collagenased bone fractions of human adult postnatal bone (average 25.9% ± 5.0% of $CD31^+CD9^+$ cells) (Figures 3A and 3B).

To provide anatomical context for this $CD31^+CD105^+$ EC subset, we performed *in situ* immunohistochemistry and

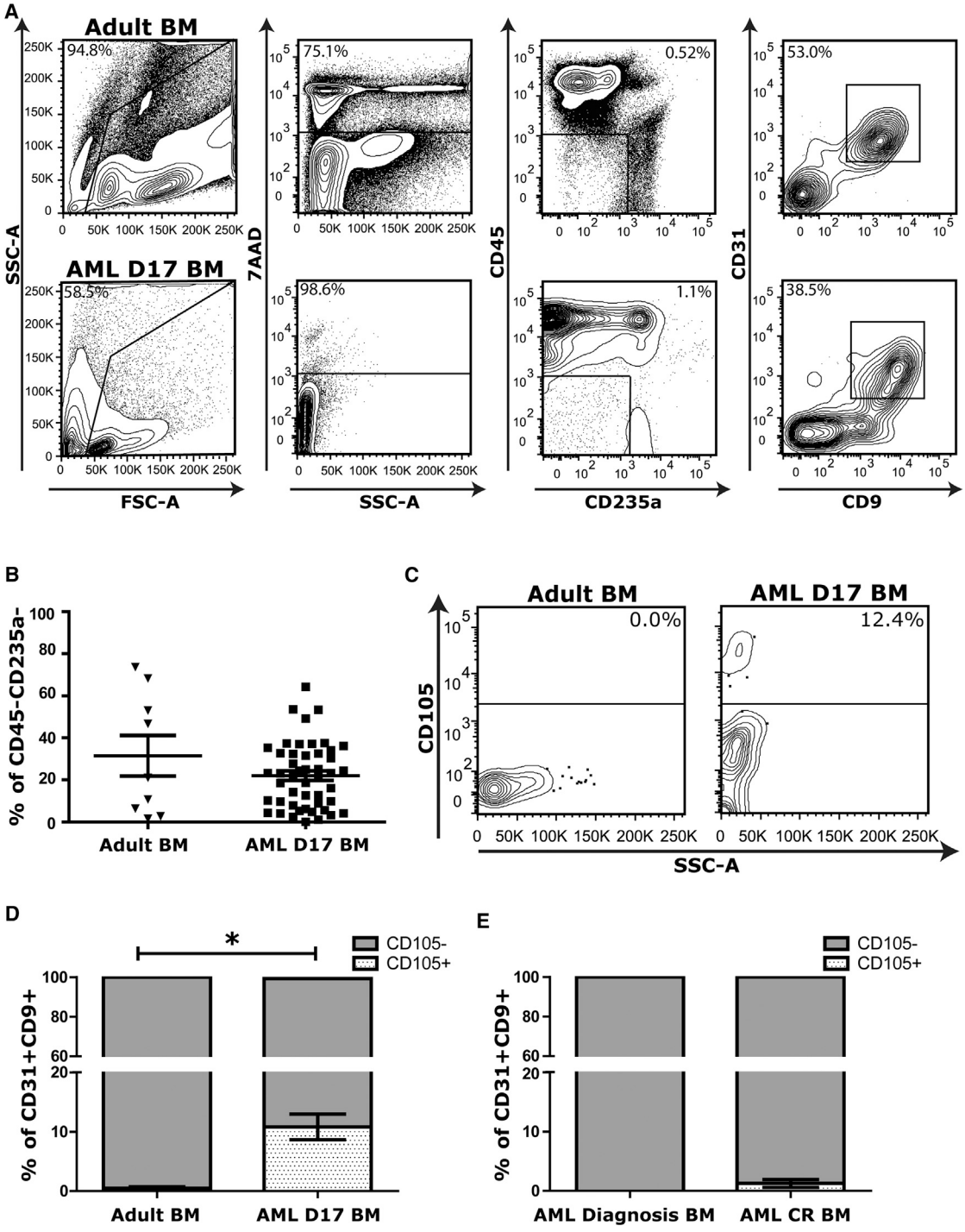

Figure 1. Identification of CD105 (Endoglin)-Expressing ECs Associated with BM Regeneration after Chemotherapeutic Injury

BM obtained by aspirates on recovery after chemotherapeutic injury (AML D17) was compared with normal adult BM.

(A) Gating strategy for identification and isolation of ECs. After doublet exclusion, 7AAD$^-$ mononuclear cells (MNCs) were gated based on low/negative CD45 and CD235a expression to identify niche cells. ECs were identified as CD31$^+$CD9$^+$ cells within the niche.

(B) Frequency of ECs within the niche in adult steady-state (n = 9) and regenerative (n = 48) BM.

(C) Representative FACS plots revealing the existence of a CD105-expressing endothelial subset in regeneration.

(D) The frequency of CD105$^+$ cells in the endothelial niche in normal BM (n = 9) and during regeneration after chemotherapy (n = 48).

(E) The frequency of CD105$^+$ cells in the endothelial niche in BM aspirates of AML patients at diagnosis (n = 4) and after recovery in complete remission (n = 3).

Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test. AML, acute myeloid leukemia; CR, complete remission; D17, day 17.

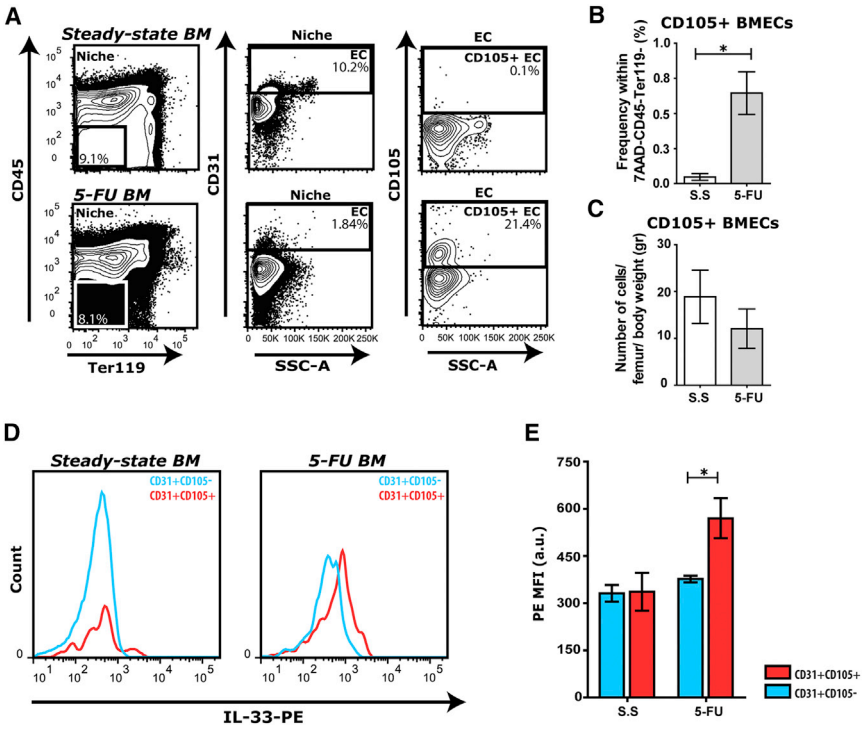

Figure 2. CD105-Expressing ECs Are Enriched in Mice during Regeneration after Chemotherapeutic Injury

(A) Representative FACS plots for identification of murine BM ECs. After doublet exclusion, 7AAD$^-$ mononuclear cells (MNCs) were gated based on low/negative CD45/ Ter119 expression to select for BM niche cells. BM ECs were identified by CD31 and CD105 expression.

(B) Frequency of CD105$^+$ ECs within the murine BM niche during S.S (n = 3 mice) and on recovery after 5-FU (n = 5 mice).

(C) Numbers of CD105$^+$ BM ECs during S.S (n = 3) and after 5-FU treatment (n = 5).

(D and E) CD105$^+$ BM ECs differentially express IL-33 on injury. (D) Representative FACS plots. (E) IL-33 expression in CD105$^+$ BM ECs and CD105$^-$ BM ECs in S.S (n = 3) and after 5-FU treatment (n = 5).

Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test. 5-FU = 5-fluorouracil; S.S = steady-state.

immunofluorescence on fetal femurs and core hip bone biopsies obtained from adults. CD31$^+$CD105$^+$ ECs were observed in the trabecular bone area of the metaphysis of fetal femurs (Figures 3C, S3A, and S3B) at a significantly higher frequency than in the trabecular bone of adult BM. The majority of vascular structures in both fetal and adult human bone were lined with CD31$^-$CD105$^+$ ECs, previously shown to be sinusoid ECs. CD31$^+$CD105$^-$ cells were present sporadically in adult bone.

Collectively, these findings identify a human endothelial subtype that is enriched in collagenased bone fractions, prevalent during human fetal bone development, declines in frequency on aging, and emerges, in a temporally restricted fashion, in the BM during regeneration after chemotherapeutic injury. Henceforward, we will refer to this EC type as "human regeneration-associated ECs" (hRECs).

hRECs Share Immunophenotypic and Molecular Similarities with Murine Type H Endothelium and Express Key Regulators of Angiogenesis and Osteogenesis

Interestingly, similarities exist between hRECs and the recently described specialized endothelium coordinating osteo- and angiogenesis in mice, termed type H endothelium (Kusumbe et al., 2014, 2016; Ramasamy et al., 2014). These include enrichment at the bone surface, reduced frequencies on aging, and an increase in frequency on genotoxic stress, suggesting that hRECs may reflect human equivalents of mouse type H endothelium. To further investigate this, differentially expressed transcripts in CD31$^+$CD9$^+$CD105$^+$ hRECs isolated from human fetal bone (in comparison to steady-state postnatal CD31$^+$CD9$^+$CD105$^-$ cells) were related to genes reported to be overexpressed in type H endothelium. This confirmed elevated expression of many transcripts previously reported to be enriched in type H endothelium, including genes encoding the signature markers CD31 (*PECAM1*) and mucin-like sialoglycoprotein endomucin (*EMCN*) (Figure 4A) and vessel guidance molecules (Ramasamy et al., 2014) (Figure 4B). Most of these genes were similarly enriched in the CD31$^+$CD9$^+$ fraction of regenerative BM (Figures 4A and 4B) (the limited number of CD105$^+$ cells precluded RNA-seq of this specific subset). In total, 3,718 genes were differentially expressed (glmLRT function, EdgeR; FDR < 0.05) in fetal bone hRECs in comparison to steady-state postnatal BM ECs. Among the overexpressed genes were HSPC niche factors (Figure 4C) and known angiocrine anabolic regulators of osteogenesis and angiogenesis (Figure 4D), further suggesting that hRECs might be involved in hematopoietic niche formation.

Next, transcriptional programs and signatures were interrogated in hRECs using gene set enrichment analysis (GSEA) (Subramanian et al., 2005). Activated Notch signaling and stabilization of HIF1a have been identified in type H endothelium as key promotors of the formation of type H capillaries and the release of osteogenic factors that enhance osteogenesis (Kusumbe et al., 2014; Ramasamy et al., 2014). In line with this, key NOTCH regulators, such as Jagged-1, DLL4, NOTCH1, and NOTCH4 (receptors of DLL4), were upregulated in fetal hRECs (Figure 4E), reflected in the activation of downstream transcriptional NOTCH signaling, as demonstrated by GSEA (Figure 4F). Similarly, gene sets related to the HIF1a pathway were enriched in fetal hRECs according to GSEA (Figure S2B). Other relevant molecular signatures that were identified to be significantly enriched in hRECs include "angiogenesis" and "stemness" signatures, among which VEGF and WNT signaling (Figure S2C). Together, the data indicate that hRECs share immunophenotypic and molecular commonalities with murine

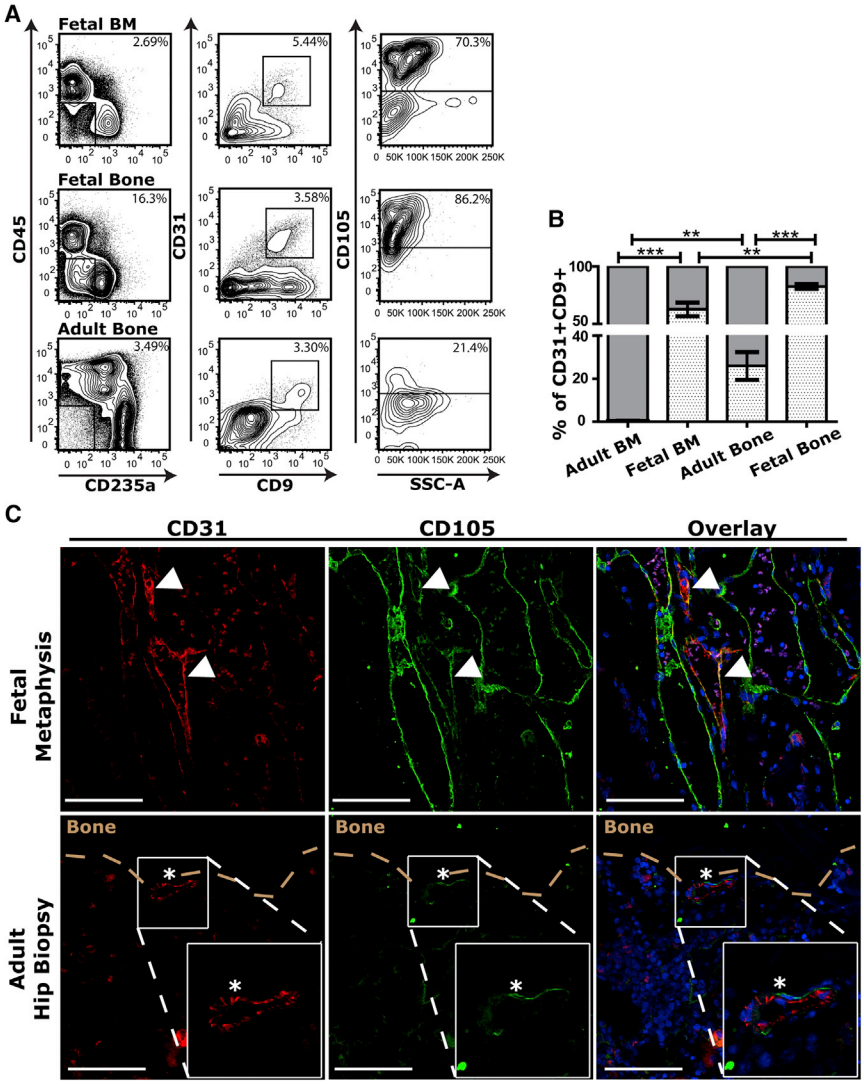

Figure 3. CD105-Expressing ECs Are Enriched in Fetal Development and Collagenized Bone Fractions

(A) Representative FACS plots identifying CD105-expressing ECs in fetal BM, fetal bone, and adult bone.

(B) Frequency of $CD105^+$ cells in the endothelial compartment in healthy adult BM aspirates (n = 9), fetal BM (n = 15), fetal bone (n = 21), and adult bone (n = 9). Data represent mean ± SEM. **p < 0.01, ***p < 0.001, 1-way ANOVA (p < 0.0001) followed by Bonferroni's multiple comparison test.

(C) *In situ* immunofluorescence demonstrating the existence of capillary structures in the trabecular area of fetal long bone comprised of $CD31^+CD105^+$ ECs (arrowheads). Sinusoid structures are $CD31^{lo}CD105^+$. In human adult BM, the majority of $CD31^+$ ECs lacks CD105 expression (asterisks). The area within the dotted line in the adult BM represent bone tissue. Magnification, 20×. White scale bar represents 100 μm.

type H ECs and have a transcriptional wiring that may be congruent with the view that these cells are implicated in the coupling of hematopoiesis, osteogenesis, and angiogenesis in regeneration.

IL-33 Is Expressed by hRECs and Promotes Angiogenesis, Osteogenesis, and the Expansion of Hematopoietic Precursor Cells

We thus hypothesized that elucidation of the transcriptome of ECs related to hematopoietic niche formation might instruct the discovery of pathways or proteins facilitating niche formation. In particular, angiocrine factors may be identified that facilitate angiogenesis, osteogenesis, and hematopoiesis. To provide proof of principle for this assumption, we focused our attention on genes encoding secreted factors that were significantly enriched in both fetal hRECs and regenerative BM ECs. In total, 237 genes were significantly enriched, of which 34 are genes encoding known secreted factors with a strong correlation in levels of expression (Figure 5A). Of interest, the canonical receptors of many of these secreted proteins were overexpressed in fetal hRECs, (MMRN2-CLEC14A, BMP4-BMPR2, EFNA1-EPHA2/EPHA4/EPHA7, EDN1-EDNRB, SEMA3A-NRP1/NRP2/PLXNA2, and ADM-RAMP2/CALCRL) (Figure S2D), suggesting the possibility of autocrine signaling.

In addition to secreted factors with a known role in regulating HSPC behavior, such as plasminogen activator (*PLAT*) (Ibrahim et al., 2014) and tissue factor pathway inhibitor (*TFPI*) (Khurana et al., 2013), we found significant overexpression of *IL33* in hRECs (Figure 5A).

IL-33 is a pro-inflammatory cytokine and a chromatin-associated nuclear factor (Carriere et al., 2007). IL-33 protein expression in fetal hRECs was confirmed using fluorescence microscopy (Figure 5B), and IL-33 was differentially overexpressed in murine $CD31^+CD105^+$ ECs in comparison to their $CD105^-$ counterparts during regeneration (Figures 2D and 2E). The inability to propagate sorted hRECs *ex vivo* (data not shown) precluded the possibility to perform co-culture blocking experiments to assess the contribution of hREC-derived IL-33 to angiogenesis, hematopoiesis, and osteogenesis. As an alternative strategy, we exposed relevant cell types to recombinant human IL-33 (rhIL-33).

Hematopoiesis

To test if IL-33 facilitates human hematopoiesis *in vitro*, we exposed cord blood (CB) $CD34^+$ HSPCs to rhIL-33 or vehicle control for one week in serum-free medium containing stem cell factor (SCF). rhIL-33 expanded the total number of hematopoietic cells (total mononuclear cells [MNCs]) (3.4-fold increase ± 0.29; p < 0.01) with a concomitant expansion of hematopoietic progenitor cells (HPCs), specifically immunophenotypic multipotent progenitors (MPPs) (2.5-fold increase ± 0.43;

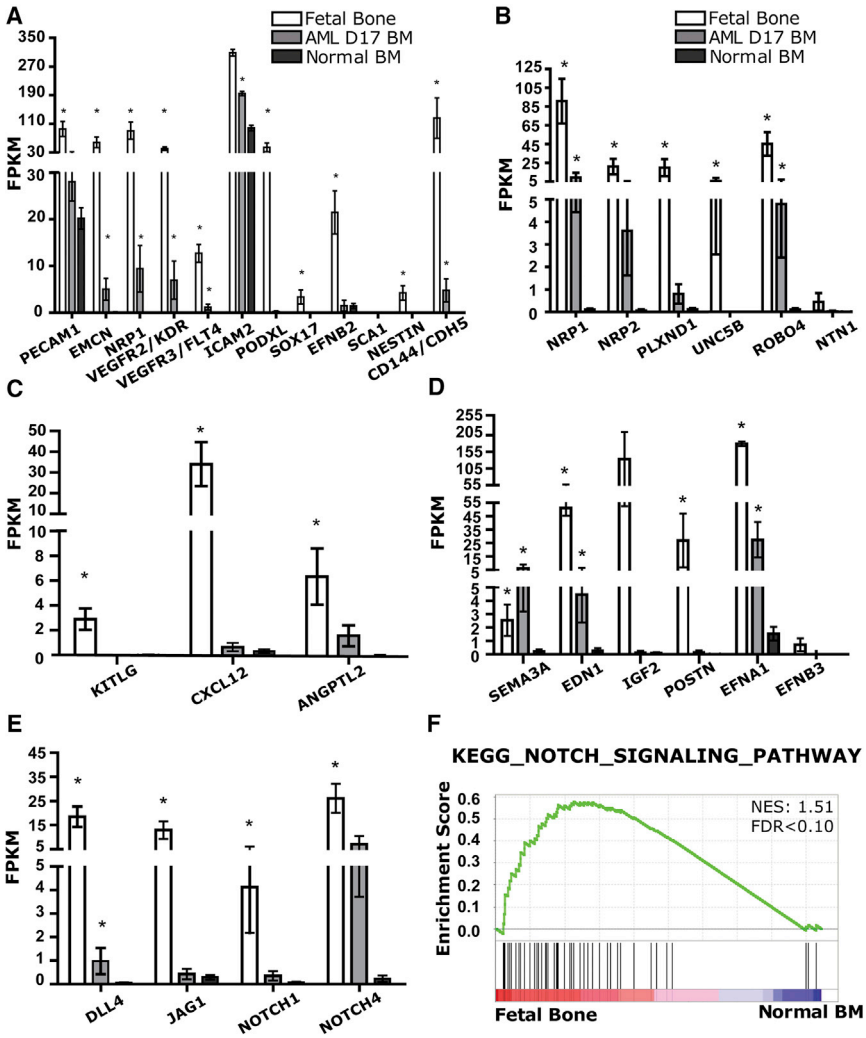

Figure 4. hRECs Express Transcriptional Programs Defining Niche-Forming ECs in Mice

Transcriptomes of hRECS isolated from human fetal bone ($CD45^-CD235^-CD31^+CD9^+CD105^+$) (n = 3) and regenerative BM ($CD45^-CD235^-CD31^+CD9^+$) (n = 3) were compared with postnatal steady-state BM ECs ($CD45^-CD235^-CD31^+CD9^+$) (n = 7).

(A and B) Expression of genes encoding arterial and venous markers (A), and vessel guidance molecules previously reported to be overexpressed in murine type H ECs (B) (Itkin et al., 2016; Kusumbe et al., 2014, 2016; Ramasamy et al., 2014). Note, Sca-1 has no known human homolog (Holmes and Stanford, 2007).

(C) Expression of established hematopoiesis-supporting cytokines (Broudy, 1997; Sugiyama et al., 2006; Zhang et al., 2006).

(D) Expression of genes encoding anabolic regulators of angiogenesis and osteogenesis (Blumenfeld et al., 2002; Clines et al., 2007; Edwards and Mundy, 2008; Oshima et al., 2002; Salani et al., 2000; Salvucci and Tosato, 2012; Serini et al., 2003; Shao et al., 2004; Shigematsu et al., 1999; Tamagnone and Giordano, 2006).

(E and F) Expression of genes encoding critical NOTCH pathway components (E) and activation of Notch- signaling (F) as demonstrated by gene set enrichment analysis (GSEA).

*FDR < 0.05. FDR, false discovery rate; FPKM, fragments per kilobase million; NES, normalized enrichment score.

$p < 0.05$) and multilymphoid progenitors (MLPs) (2.7-fold increase ± 0.15; $p < 0.01$) (Figures 5C and S4). Immunophenotypic HSC numbers were not affected by exposure to IL-33. Expansion of myeloid progenitor cells was confirmed in colony-forming unit cell assays (CFU-C assays, demonstrating an increase in granulocyte-macrophage colony-forming units (CFU-GMs) (2.27-fold increase ± 0.21; $p < 0.05$) (Figure 5D).

Osteogenesis

Next, we interrogated a potential role of IL-33 in bone formation. In human fetal bone development (endochondral ossification), vascular invasion of chondrocytes coincides with expansion of osteoblasts and mineralization of the matrix (Charbord et al., 1996; Ramasamy et al., 2016). To test the role of IL-33 in this process, the effect of rhIL-33 on the osteogenic differentiation of human BM-derived stromal cells (BMDSCs) was assessed. The addition of rhIL-33 to osteogenic induction medium accelerated terminal differentiation of BMDSCs toward matrix-depositing osteoblasts, as suggested by Alizarin red-staining (Figure 6A), indicating increased calcific matrix deposition. To confirm this finding, we performed colorimetric assessment of calcium deposition, demonstrating a striking 3.61-fold ± 0.63 ($p < 0.02$) increase in calcium deposition of IL-33-exposed BMDSCs in comparison to BMDSCs cultured in osteogenic induction medium alone (Figure 6B). The proliferation of BMDSCs was not affected by rhIL-33 (Figures S5A and S5B), suggesting that IL-33 exerted its osteogenic effect by promoting osteoblastic differentiation or the secretion of matrix proteins by osteoblasts rather than by expanding primitive mesenchymal cells.

Angiogenesis

In line with earlier reports (Choi et al., 2009), we confirmed that IL-33 is an angiogenic factor. shRNA-mediated knockdown of IL-33 (Figures S5C and S5D) from human umbilical vein ECs (HUVECs) dramatically impaired their expansion in culture (Figure 6C), indicating that IL-33 promotes angiogenesis in an autocrine fashion.

IL-33 Promotes Expansion of Hematopoietic Precursor Cells and Alters the Architecture of the BM Niche in Mice

We next studied the *in vivo* relevance of these effects of IL-33 on distinct cellular components of human BM. The administration of recombinant murine IL-33 (rmIL-33) compared with PBS control resulted in expansion of immature (Lin^-) (Figure S6A) and primitive progenitor (Lin^-, c-KIT^+, Sca-1^+ [LKS]) hematopoietic cells, in particular the HPC-1 population (Figures 7A and 7B), earlier

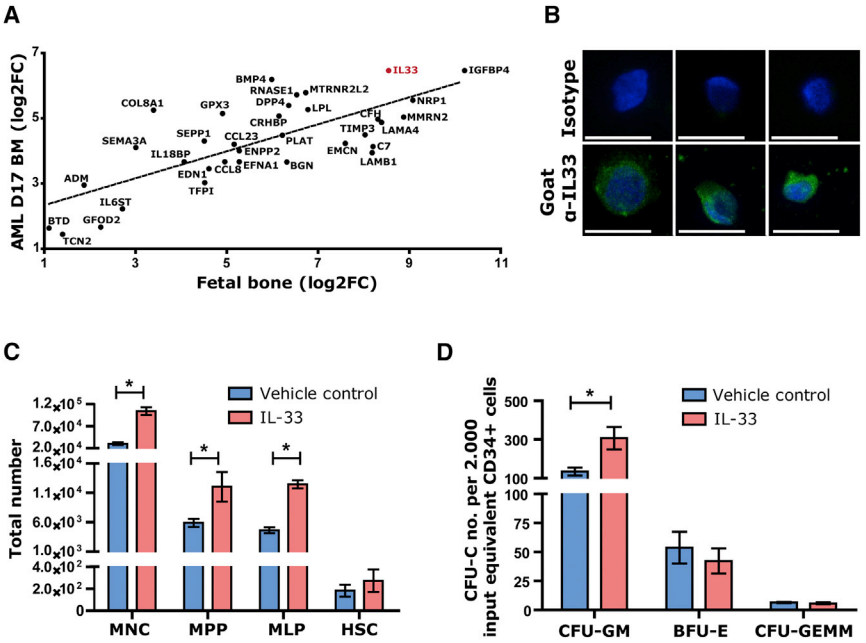

Figure 5. Identification of IL-33 As a Hematopoietic Niche Factor

(A) Transcript expression of 34 genes encoding secreted factors significantly enriched (FDR < 0.05) in fetal bone and regenerating BM ECs (Log2 fold change in comparison to steady-state BM). FDR: false discovery rate. FC: Fold Change.

(B) Cropped pictures (magnification, 63×) of immunostained fetal hRECs demonstrating protein expression of IL-33. White scale bar represents 20 μm.

(C and D) IL-33-mediated expansion of cord-blood-derived myeloid and lymphoid progenitor cells as demonstrated by (C) flow cytometric cell counting (n = 4 independent experiments) and (D) CFU-C confirming an increase in CFU-GMs (n = 3 independent experiments). Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test. HSC, hematopoietic stem cell, $CD90^{+}CD45RA^{-}$; MLP, multilymphoid progenitor, $CD90^{-}CD45RA^{+}$; MPP, multipotent progenitor, $CD90^{-}CD45RA^{-}$.

shown to contain restricted hematopoietic precursor cells with myeloid and lymphoid lineage potential (Oguro et al., 2013). Expansion of a myeloid progenitor population was confirmed with CFU-C assays (Figure S6B). Total BM cellularity and $Lin^{-}Kit^{+}Sca1^{-}$ cell counts remained unchanged (Figure S6C). In addition, the number of granulocyte-macrophage progenitors (GMPs) and myeloid cells increased after IL-33 administration (Figures 7A, 7B, S6D, and S6E), recapitulating the expansion of human hematopoietic myeloid precursors *in vitro*. Congruent with our findings in human hematopoietic cells, immunophenotypic HSCs were not numerically affected by IL-33 (Figures 7A and 7B). Interestingly, IL-33 also significantly expanded the population of $Lin^{-}Kit^{-}Sca1^{+}$ BM cells, previously shown to contain early lymphoid-committed precursors with T cell, B cell, and natural killer (NK) cell potential (Kumar et al., 2008) and innate lymphoid cells (Brickshawana et al., 2011). Hematopoietic changes were accompanied by a relative increase, albeit not reaching statistical significance, in $CD31^{+}CD105^{+}$ ECs as well as $Lin^{-}Ter119^{-}CD51^{+}Sca^{-}$ cells (earlier shown to contain lineage-committed/osteoblastic cells) (Schepers et al., 2012) within the niche compartment (Figures S7A and S7B).

Collectively, the *in vitro* and *in vivo* data indicate that IL-33 modulates distinct cellular components of hematopoietic tissue and has the potential to facilitate angiogenesis, hematopoiesis, and osteogenesis, supporting the view that elucidation of the transcriptome of hRECs may instruct the identification of modulators of these processes.

DISCUSSION

Injury to the hematopoietic system, caused by chemotherapy or irradiation, is a significant cause of morbidity and mortality in the treatment of malignant hematopoietic disease. Studies in mice have demonstrated a pivotal role of specific BM niche cells and secreted molecules in hematopoietic recovery. Translation of these findings to the clinic, however, is hampered, principally by insufficient understanding of the niche cells and molecular programs governing niche formation and hematopoietic recovery in humans. Here, by cellular dissection of the BM niche in humans during fetal development and regeneration after chemotherapeutic injury, we reveal the existence of a specific EC type (hRECs) associated with these conditions. hRECs share phenotypic and molecular similarities with specialized ECs driving hematopoietic niche formation in mice, expressing critical regulators of hematopoiesis, osteogenesis, and angiogenesis. The data comprise, to our knowledge, the first comprehensive molecular characterization of human ECs on tissue regeneration after injury.

We identified the TGF-β1 receptor endoglin (CD105) as a marker of endothelium associated with BM (re)generation in fetal development and after chemotherapeutic injury. Endoglin-expressing ECs have earlier been associated with angiogenesis in tumors and inflammation (Kumar et al., 1996), and loss of endoglin results in defective angiogenesis in mice (Li et al., 1999), supporting the view that it mediates signals governing blood vessel formation. The data indicate that endoglin with concomitant CD31 expression marks a specific subset of angiogenic ECs, which is further supported by observations that endoglin expression is strongly elevated in ECs of small, capillary-like vessels at tumor edges (Miller et al., 1999; Yoshitomi et al., 2008). Endoglin thus likely identifies a subset of endothelium during ontogeny and regeneration that marks an angiogenic subset, in line with observations in different settings.

This subset revealed remarkable molecular congruence with EC subsets identified in murine studies driving bone and BM regeneration after injury. ECs in mice support the regeneration of the hematopoietic system after injury, such as myeloablation

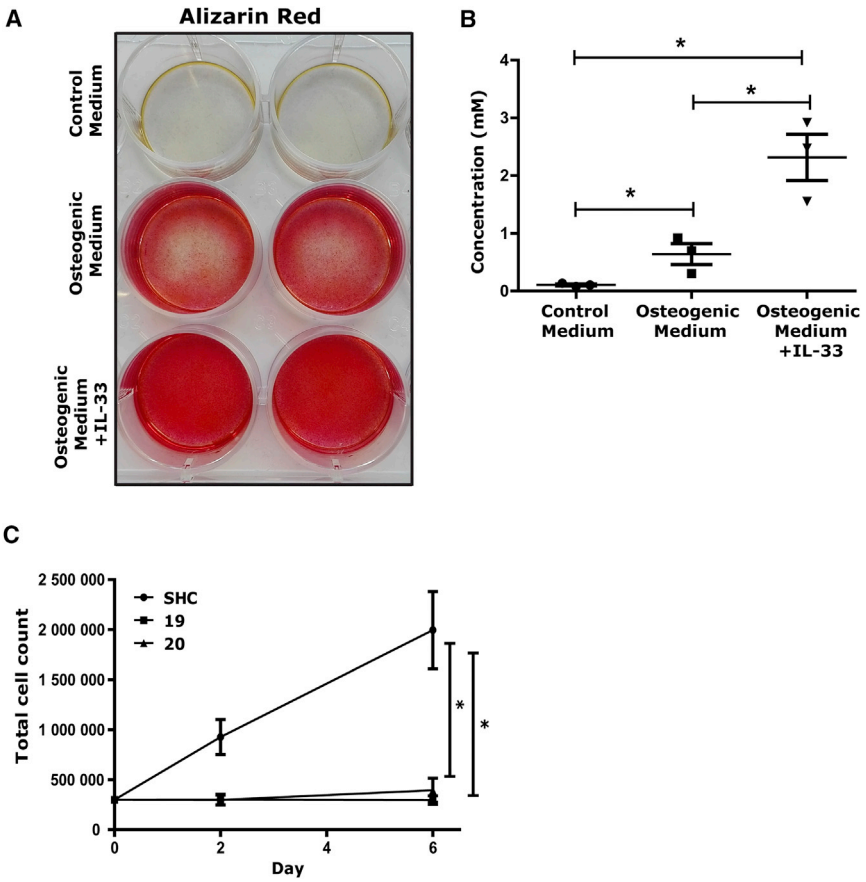

Figure 6. IL-33 Promotes Osteogenesis and Angiogenesis *In Vitro*

(A and B) IL-33 accelerates terminal differentiation of mesenchymal cells toward matrix-depositing osteoblasts as demonstrated by (A) Alizarin red-staining and (B) colorimetric assessment of calcium deposition (n = 3 independent experiments). (C) Knockdown of IL-33 inhibits expansion of HUVECs. Quantification of the total number of HUVECs over time (combined data of n = 3 independent experiments).

Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test.

(Butler et al., 2010; Hooper et al., 2009; Kobayashi et al., 2010), and in recent years, several markers of specified endothelial subsets exerting this function as well as the underlying mechanisms have been revealed. Interestingly, hRECs share many characteristics with the ECs described in these studies, including expression of the cell surface proteins Tie2/TEK (Figure S2E) (Doan et al., 2013; Kopp et al., 2005), EMCN (Kusumbe et al., 2014; Ramasamy et al., 2014), and Jagged1 and activation of specific signaling pathways, including NOTCH (Butler et al., 2010; Poulos et al., 2013). Also, hRECs express many molecules previously shown to regulate HSPC behavior in mice, such as *PLAT* (Ibrahim et al., 2014), *TFPI* (Khurana et al., 2013), E-selectin *(ESELE)* (Figure S2E) (Winkler et al., 2012), thrombomodulin *(TMBD)* (Figure S2E) (Gur-Cohen et al., 2015), and tenascin C (*TNC*) (Figure S2E) (Nakamura-Ishizu et al., 2012).

In particular, hRECs displayed striking commonalities with type H endothelium, a murine EC subtype that has recently been functionally implicated in EC-driven formation of the niche through activation of NOTCH and HIF1 signaling (Kusumbe et al., 2014; Ramasamy et al., 2014). Commonalities with the now identified hRECs include enrichment at the bone surface, reduction in frequency on aging, resistance to stress conditions, expression of markers typical for both arterial (Ephn2b, Nestin, Nrp1, Sox17, and VEGFR2), and sinusoidal vessels (VEGFR3 and EMCN), and activation of the NOTCH and HIF1 pathways driving regeneration.

Expression of CD105 was not addressed in the studies on type H endothelium, but our data in mice show that a rare population of endoglin-expressing ECs increases in frequency in the regenerative phase after chemotherapy, likely reflecting increased resistance to myeloablative stress. It is noteworthy that $CD105^+$ ECs associated with elevated HIF-1a expression have been described in the BM of mice on regeneration after 5-flourouracil (5-FU) treatment (Nombela-Arrieta et al., 2013), making it tempting to hypothesize that these represent similar or overlapping cell types.

Taken together, these immunophenotypic and molecular similarities between hRECs and murine endothelial subtypes implicated in hematopoietic niche formation point toward evolutionary conservation of these cells between mammalian species. They thus provide human relevance to findings in murine studies, supporting the notion that ECs are implicated in niche regeneration in humans.

Providing experimental support for this view is challenging due to limitations inherent to the study of human cells as well as the inability to propagate highly purified hRECs *ex vivo*, precluding co-culture studies. As an alternative approach, we exploited elucidation of their transcriptome to identify candidate factors driving EC-driven formation of the hematopoietic niche and regeneration of HSPCs. We identified IL-33, a cytokine typically associated with innate immunity and inflammation (Cayrol and Girard, 2014), as a candidate factor. IL-33 was overexpressed in human hRECs, and expression was increased in murine $CD31^+CD105^+$ ECs on exposure to 5FU. Hematopoietic niche regenerating properties of IL-33 were demonstrated by its *ex vivo* capacity to facilitate hematopoiesis (increased numbers of HPCs), osteogenesis (accelerating terminal differentiation of BMDSCs toward matrix-depositing osteoblasts), and angiogenesis (expansion of HUVECs). The data follow recent reports demonstrating IL-33 to predominantly act as an "alarmin" released by cells undergoing necrosis after tissue damage or active secretion (Kakkar et al., 2012; Lee et al., 2015) and playing anabolic roles in angiogenesis (Choi et al., 2009; Shan et al., 2016) and osteogenesis (Saleh et al., 2011). Our finding that

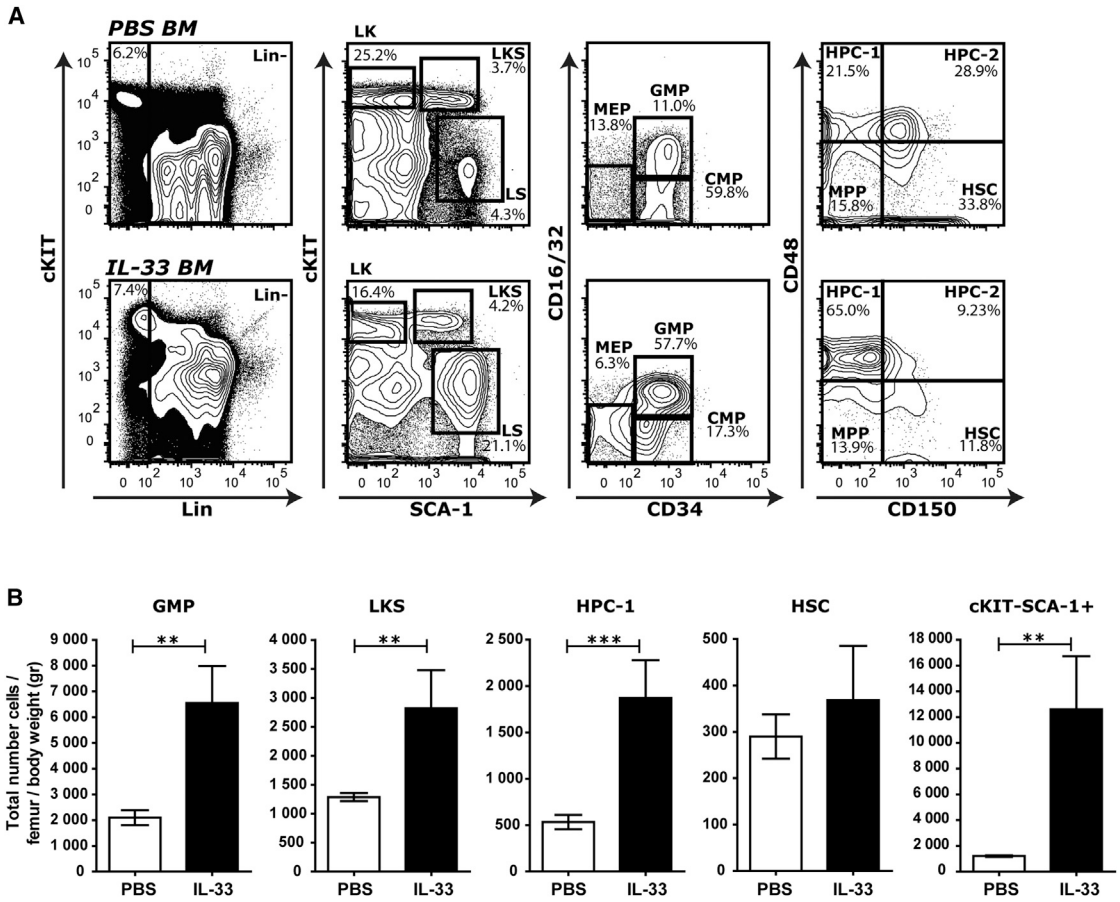

Figure 7. Recombinant IL-33 Expands Hematopoietic Progenitors *In Vivo*

(A) Representative FACS plots of PBS versus IL-33-treated mice depicting the distribution of hematopoietic (progenitor) populations. CMP, LK, CD16/32$^-$, CD34$^+$ cells, common myeloid progenitor; GMP, LK, CD16/32$^+$, CD34$^+$ cells, granulocyte-macrophage progenitor; HSC, LKS, CD48$^-$CD150$^+$ cells, hematopoietic stem cell; HPC-1, LKS, CD48$^+$, CD150$^-$ cells, hematopoietic progenitor-1; HPC-2, LKS, CD48$^+$, CD150$^+$ cells, hematopoietic progenitor-2; Lin$^-$, Lineage-negative cells; LK, Lin$^-$, c-KIT$^+$, Sca-1$^-$ cells; LKS, Lin$^-$, c-KIT$^+$, Sca-1$^+$ cells; LS, Lin$^-$, c-KIT$^-$, Sca-1$^+$ cells. MEP, LK, CD16/32$^-$, CD34$^-$ cells, megakaryocyte-erythroid progenitor; MPP, LKS, CD48$^-$CD150$^-$ cells, multipotent progenitor.

(B) Total BM counts of GMP, LKS, HPC-1, HSC, and LS populations in PBS-treated (n = 10) and IL-33-treated (n = 5) mice. Data represent mean ± SEM. *p < 0.05, **p < 0.01, 2-tailed unpaired Student's t test.

rhIL-33 increased the numbers of HPCs *ex vivo* seems congruent with recent observations in mice, where administration of IL-33 promoted myelopoiesis (Kim et al., 2014). Of considerable interest, rhIL-33 in our experiments expanded both immunophenotypic MLPs and MPPs, in line with observations in experiments in mice where expansion of splenic lymphoid progenitors after IL-33 administration resulted in enhanced defense against opportunistic infection (Kim et al., 2014). Formal demonstration that secretion by a defined subset of ECs is required for the regenerative actions of IL-33 will have to await *in vivo* targeted deletion experiments, because it is currently challenging to maintain this particular subset of ECs *ex vivo* to enable co-culture experiments. The profound effect of IL-33 knockdown on HUVEC proliferation and maintenance precluded the use of this *ex vivo* system to address this question.

The combined findings point to a unique role of IL-33 in mammalian species in facilitating the reconstitution of both hematopoietic lineages, which may be of considerable importance to prepare the hematopoietic system for the extra-uterine environment in ontogeny, but also for immune reconstitution after injury (e.g., hematopoietic stem cell transplantation characterized by long-term lymphocyte depletion and ensuing opportunistic infections).

The exact molecular mechanisms by which IL-33 exerts these effects (either direct or indirect) remain to be fully elucidated. IL-33 expression in HUVECs has been associated with a quiescent cellular state (Küchler et al., 2008), and although we did not examine the cell cycle status of CD31$^+$CD105$^+$, IL-33^{high} hRECs, this might help explain the notion that they may be relatively resistant to chemotherapeutic myeloablation. We can speculate that quiescent CD31$^+$CD105$^+$, IL-33^{high} hRECs survive chemotherapy and are "activated" to release IL-33 as an anabolic hematopoietic factor. In this context, it is noteworthy that hRECs display transcriptional activation of DLL4 and the Notch pathway, earlier shown to be an important driver of IL-33 expression (Sundlisaeter et al., 2012).

Regardless of the underlying molecular mechanisms of IL-33 expression, the data support the notion that elucidation of the transcriptome of hRECs may instruct the identification of the proteins and pathways driving niche formation after injury. It is conceivable that receptor-ligand interactions allow targeting of these cells to drive regeneration (as previously shown by pharmacologic modulation of NOTCH signaling in mice [Ramasamy et al., 2014]). In this context, it is noteworthy that transcriptional profiling of fetal hRECs revealed overexpression of genes encoding secreted factors as well as their receptors, suggesting the potential relevance of autocrine signaling in the biology of hRECs. Alternatively, it would be worthwhile to test the ability of identified secreted factors to expand human HSPCs *ex vivo*, either directly or in co-culture settings with mesenchymal elements.

Collectively, the identification of human ECs associated with hematopoietic niche formation and the elucidation of their transcriptome are anticipated to provide a valuable resource for the regenerative community to relate findings in animal models to human biology and to instruct *in vivo* and *ex vivo* approaches to foster EC-driven regeneration of the hematopoietic system after injury.

EXPERIMENTAL PROCEDURES

Human BM Samples

BM aspirates of AML patients were collected at diagnosis, 17 days after the start of chemotherapy (3+7 schedule of chemotherapy with anthracycline and cytarabin), and on achievement of complete remission (median age: 65 years, range: 28–76 years). The time point of 17 days after the start of chemotherapy represents the neutropenic phase, 10 days after administration of chemotherapy and, on average, 4 days before recovery of neutropenia. Control marrow was obtained by aspiration from donors for allogeneic transplantation (median age: 40 years, range: 39–48 years) after obtaining written informed consent. In addition, trabecular hip bone samples were collected from patients undergoing hip replacement surgery (median age: 55 years, range: 22–71 years). Human fetal long bones (median age: 18 gestational weeks, range: 15–20 gestational weeks) were obtained from elective abortions. Gestational age was confirmed by ultrasonic measurement of skull diameter and femoral length. The use of human samples with informed consent was approved by the Institutional Review Board of the Erasmus Medical Center (the Netherlands) in accordance with the Declaration of Helsinki.

RNA-seq and GSEA Analysis

RNA of sorted cells was extracted according to the manufacturer's instructions for RNA isolation with GenElute LPA (Sigma). cDNA was prepared using the SMARTer procedure (SMARTer Ultra Low RNA Kit, Clonetech). Library preparation and RNA-seq was performed as previously described and validated for low input (Chen et al., 2016). Finally, GSEA was performed on the fragments per kilobase million (FPKM) values using the curated C2 collection of gene sets within the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005).

Mice and *In Vivo* Procedures

C57BL/6JOlaHsd wild-type mice were purchased from Envigo. Animals were maintained in specific pathogen-free conditions in the Experimental Animal Center of Erasmus MC (EDC). To study the murine niche in regenerative conditions, adult mice (7–12 weeks old) were intraperitoneally administered 250 mg/kg 5-FU and then sacrificed 7 days after 5-FU treatment. To study the effect of rmIL-33 on steady-state hematopoiesis and the BM niche, adult mice (7–14 weeks old) were intraperitoneally injected with 2 μg of recombinant IL-33 (catalog no. 580504, BioLegend) or PBS vehicle control daily for 6 consecutive days and then sacrificed.

All mice were sacrificed by cervical dislocation. Mouse BM and bone fraction cells were isolated as previously described (Zambetti et al., 2016). Peripheral blood was collected from the submandibular vein in K2EDTA-coated microtainers (BD) and analyzed using a Vet ABC counter (Scil Animal Care). Animal studies were approved by the Animal Welfare/Ethics Committee of the EDC in accordance with legislation in the Netherlands (approval no. EMC 4015).

Liquid Culture of $CD34^+$ CB Cells

A total of 20,000 $CD34^+$ CB cells in 200 μL per well were cultured in StemSpan SFEM (Stem Cell Technologies, catalog no. 9600) with SCF (50 ng/mL, Cellgenix, Freiburg, Germany), in a flat-bottom, 96-well plate at 37°C and 5% CO_2. Two μL mQ solution containing only rhIL-33 protein (ProSpec, catalog number CYT-425) or just mQ (vehicle control) was added to the medium for a final concentration of 25 ng/mL rhIL-33 or 1% mQ, respectively. The medium was refreshed every 3 or 4 days, and cells were collected at day 7 for FACS analysis or for hematopoietic colony forming-unit assay.

Culture of Human BMDSCs

Human BMDSCs (catalog no. PT-2501, Lonza) were cultured as described previously (Brum et al., 2015). For osteogenic differentiation, BMDSCs were cultured in osteogenic induction medium (αMEM medium containing 10% heat-inactivated fetal calf serum [FCS] supplemented with 100 nM dexamethasone and 10 mM β-glycerophosphate) with rhIL-33 (250 ng/mL) or mQ vehicle control for 3 weeks. Medium was refreshed every 3–4 days.

Culture and shRNA-Mediated Knockdown of IL-33 in HUVECs

HUVECs were expanded in EGM-2 Bulletkit medium (CC-3156 and CC-4176, Lonza). RNAi was achieved by lentiviral transduction. Briefly, shRNAs against *IL-33* (sh19: TRCN0000135845 and sh20: TRCN0000135846) and a non-target control (shControl: SHC002 [SHC]) cloned in the pLKO.1 backbones were obtained from the Mission TRC shRNA library (Sigma-Aldrich). Lentiviral shRNAs were produced in HEK293T cells after co-transfection of shControl, sh19, or sh20 together with the packaging plasmids pSPAX2 and pMDG.2. HUVECs were infected with lentivirus for 24 hr and selected for 5 days with 2μg/mL of puromycin.

Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software). Unless otherwise specified, unpaired, 2-tailed Student's t test (single test for comparison of 2 means) or 1-way ANOVA followed by Bonferroni correction for multiple comparisons were used to evaluate statistical significance, defined as $p < 0.05$. All results in bar graphs are means ± SEMs.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data derived from human healthy adults, AML day 17 patients, and fetal bone specimens is European Genome-phenome Archive: EGAS00001002736.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.070.

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AUTHOR CONTRIBUTIONS

K.J.G.K. designed and performed the experiments, analyzed the data, made the figures, and wrote the manuscript. A.C.J. designed and performed the experiments and analyzed the data. Z.P., S.C., E.M.J.B., and H.S. provided input on and performed the experiments. R.M.H performed the bioinformatics analysis for the RNA-seq data. M.A.M. and M.N.A. performed FACS and RNA-seq. K.H.L. provided core hip bone biopsies and input on the experiments. P.K.B. provided the human hip bone samples and input on the experiments. B.v.E. provided the BMDSCs, necessary reagents, and input on the experiments. T.C. provided the human fetal samples and input on the experiments. M.H.G.P.R. designed the experiments, analyzed the data, wrote the manuscript, supervised the study, and acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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