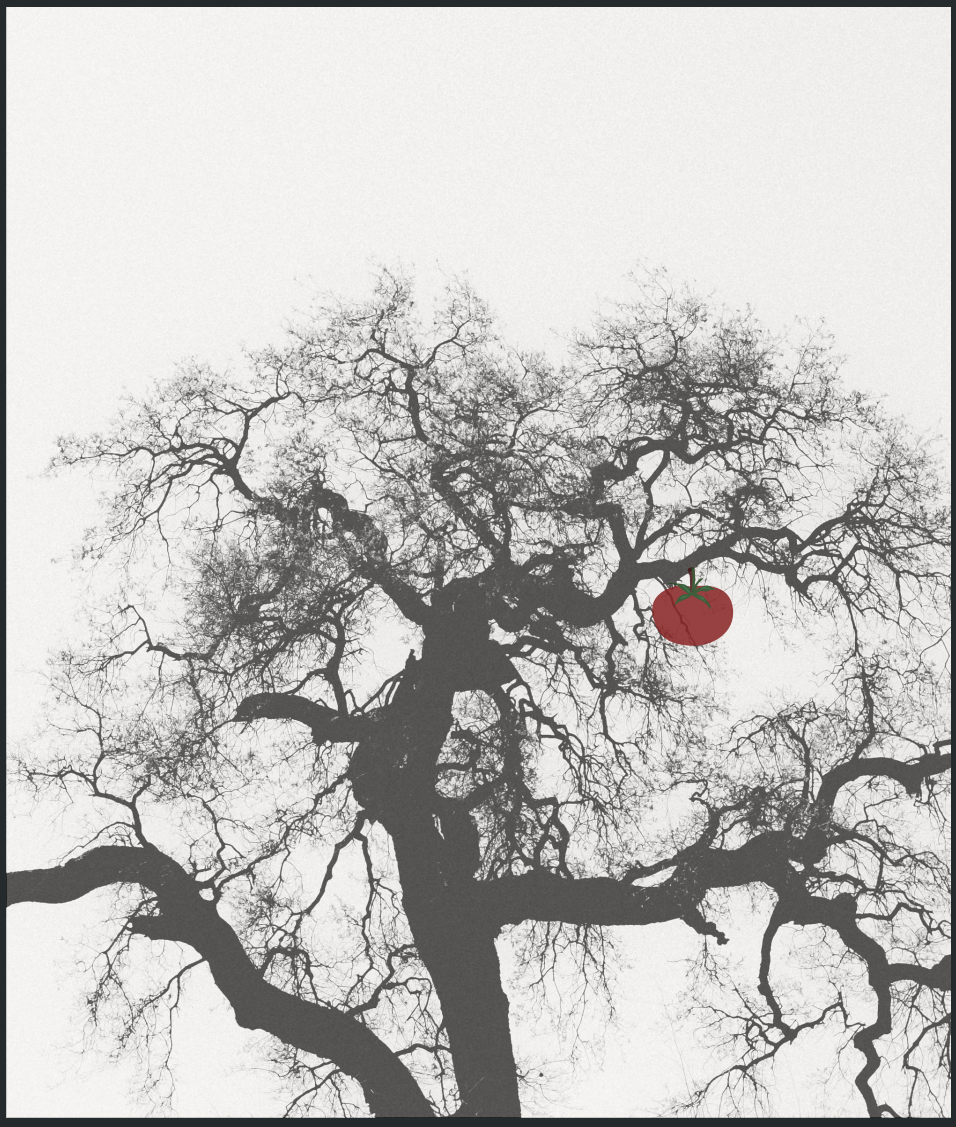


Niche Contributions to Bone Marrow (Re)generation

Keane Jared Guillaume Kenswil

Niche Contributions to Bone Marrow (Re)generation

Keane Jared Guillaume Kenswil

Erasmus MC
University Medical Center Rotterdam

ISBN: 978-94-6361-426-9
Layout: E.C.M.M. Simons
Cover: L. Rebollar, N. Orteu, E.C.M.M. Simons, K.J.G. Kenswil
Printing: Optima Grafische Communicatie (www.ogc.nl)

The work described in this dissertation was performed at the Department of Hematology of the Erasmus Medical Center, Rotterdam, the Netherlands. Printing of this dissertation was financially supported by Erasmus University Rotterdam and the Erasmus MC Vriendenfonds.

Niche Contributions to Bone Marrow (Re)generation

De rol van de niche in beenmerg vorming en herstel

Doctoral dissertation

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. R.C.M.E. Engels

and in accordance with the decision of the Doctorate Board.

The public defence shall be held on

Tuesday, 19 May 2020 at 15:30 hrs

by

Keane Jared Guillaume Kenswil

born in Paramaribo, Suriname

Erasmus University Rotterdam

DOCTORAL COMMITTEE

Promotors: Prof.dr. H.G.P. Raaijmakers
Prof.dr. I.P. Touw

Other members: Prof.dr. P. ten Dijke
Prof.dr. W. E. Fibbe
Dr. E. Farrell

CONTENTS

1

INTRODUCTION

INTRODUCTION

Blood cells have a limited life-span and must be continuously replenished throughout mammalian life, in a process termed hematopoiesis. Hematopoiesis is maintained by the proliferation and differentiation of a very small population of pluripotent hematopoietic stem cells (HSCs) and their progeny that reside in bone marrow (BM) (Figure 1). Together they provide a steady-supply of red blood cells (that enable O_2/CO_2 exchange), thrombocytes (needed for blood clot formation), and leukocytes (essential for the protection against pathogens).

Chemo- and radiotherapy (chemo/radiotherapy) are among the most commonly used treatments for patients with cancer. Both inhibit cell division by differing modes of action, and once combined, can synergistically arrest growth and induce death of rapidly dividing malignant (cancerous) cells. However, this means that "healthy" non-malignant cells undergoing DNA replication are also affected, which may result in unwanted deleterious effects, such as BM suppression.

BM suppression, also known as myelosuppression, generally refers to the depletion of hematopoietic cells: red blood cells (anemia), thrombocytes (thrombocytopenia), and leukocytes (leukopenia). Anemia is associated with fatigue, dizziness, and heart palpitations, while thrombocytopenia is commonly accompanied by increased risks of bruises and bleedings. Leukopenia in particular is concerning since it leaves patients susceptible to opportunistic infections (including viral infections and fungal infections and fever/febrile episodes. The time needed for patients to achieve full hematopoietic recovery can be considerable and is a significant cause of morbidity, especially for patients with a delayed hematopoietic recovery.

In response to chemo/radiotherapy-induced damage, HSCs become activated to generate progenitors and mature blood cells to replenish those lost during injury. However, how and what drives this activation of HSCs remains incompletely understood. Over the past two decades we have come to realize that HSC function can be greatly influenced by its surrounding microenvironment in the BM. The BM microenvironment for HSCs, often referred to as the HSC niche, can be considered an ancillary meshwork of heterogeneous cells that may facilitate HSC fate by various mechanisms, such as paracrine signaling mediated by secretion of growth factors (cytokines).

Given the key role of niche cells in HSC maintenance and proliferation, it is reasonable to think that a better understanding of the HSC niche, specifically upon regeneration (after chemotherapeutic injury), may lead to the identification of niche cells and niche-derived factors that promote hematopoietic recovery. This may enable us to develop novel treatments that reduce the time needed for patients to recover from chemotherapy-induced myelosuppression.

1.1 BM regeneration

1.1.1 Homeostatic hematopoiesis

Under homeostatic conditions, the hematopoietic system depends on a small pool of pluripotent HSCs. In general, HSCs rarely divide and can generate virtually identical HSCs (a hallmark ability termed self-renewal) or progeny that are more highly proliferative but also more lineage-committed. These progenitors in turn mature into terminally differentiated cells that constitute the entire blood system. A simplified view of hematopoiesis (Figure 1) is to consider the hematopoietic system as a hierarchy of different blood cells that either fall under the myeloid or lymphoid branch with HSCs at the apex.

HSCs are heterogeneous and can be subdivided into long-term and short-term HSCs (LT-HSCs and ST-HSCs) based on their self-renewal capacity and degree of quiescence, with the more dormant LT-HSCs giving rise to multipotent progenitors (MPPs) via the more proliferative ST-HTCs (Passegue et al., 2003). MPPs lack self-renewal ability but retain full-lineage differentiation potential. MPPs in turn differentiate into oligo-potent progenitors, namely the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). Further downstream, the CLP develops into cells of the lymphoid lineage, such as the natural killer (NK) cells, B and T lymphocytes. The CMP gives rise to the granulocytic/macrophage progenitor (GMP) and the megakaryocytic/erythroid progenitor (MEP) that together will generate granulocytes (neutrophils, eosinophils, and basophils), mononuclear cells (monocytes, macrophages), megakaryocytes, and erythrocytes.

Erythrocytes, also known as red blood cells (RBCs), provide O_2/CO_2 exchange and are the most abundant cell-type in peripheral blood (PB). Granulocytes and mononuclear cells are the first line of defense against pathogens and foreign material (innate immunity), and are involved in the removal of damaged cells. Megakaryocytes associate with BM sinusoidal vessels and release thrombocytes (platelets) in the peripheral circulation, which are essential for clot formation in case of damage to blood vessels. The lymphoid cells provide long-term memory for protection against recurring pathogens (adaptive immunity).

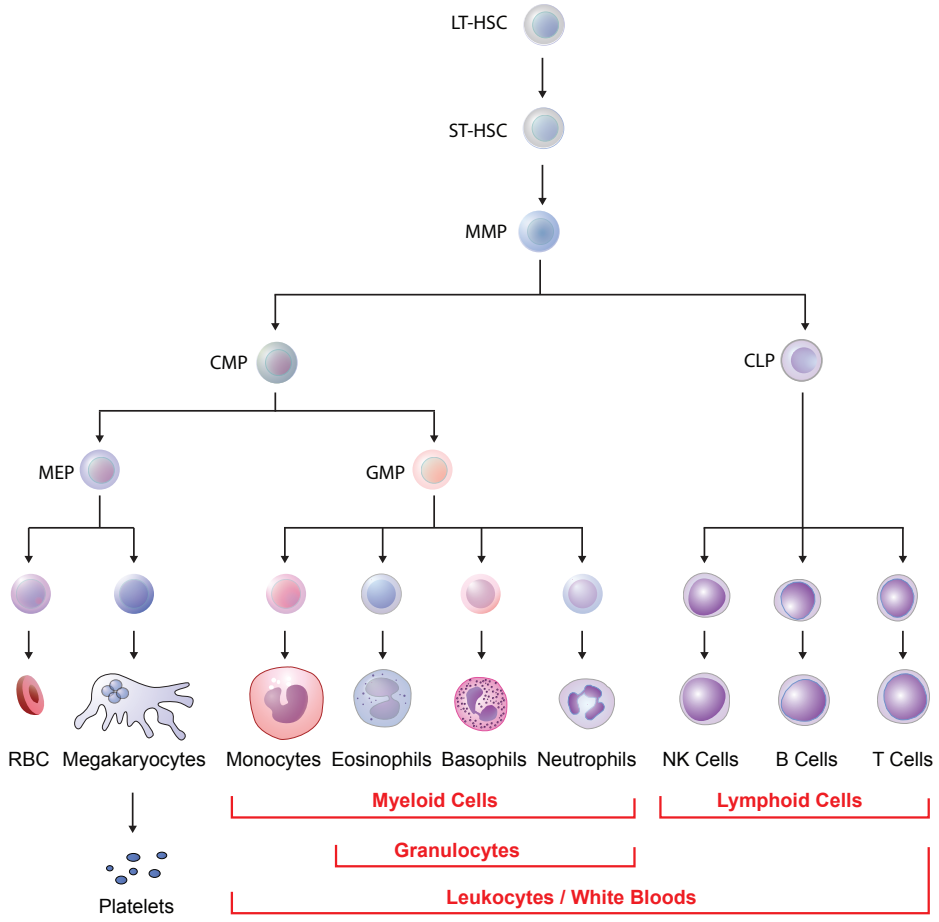


Figure 1. Simplified overview of hematopoiesis. Continuous production of mature blood cells and components depends on the self-renewal and differentiation of HSCs. HSCs give rise to MPPs that develop into more committed progenitors. CMPs give rise to erythrocytes, platelets, and myeloid cells, whereas CLPs differentiate into lymphoid cells. The myeloid cells consist of monocytes/macrophages, and granulocytes, the latter consisting of eosinophils, basophils and neutrophils. The lymphoid cells include the NK cells, and the B and T lymphocytes. Collectively, the granulocytes and lymphoid cells are referred to as leukocytes/white blood cells.

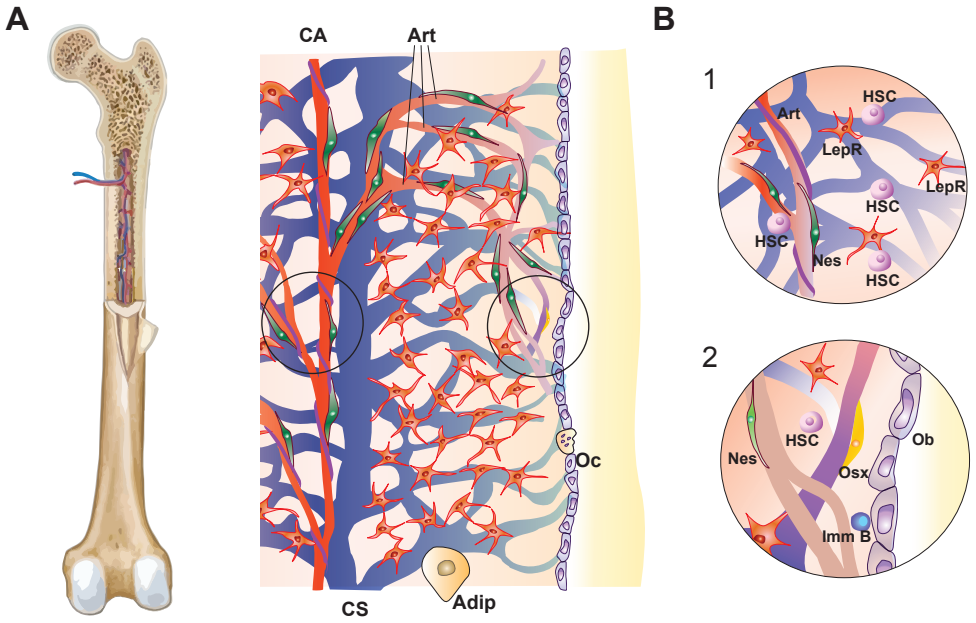


Figure 2. Structural organization of BM in long bones of mice. Adapted from (Nombela-Arrieta and Manz, 2017).

a. Simplified overview of the BM microarchitecture in the mouse femur. The central artery (CA) divides into arterioles (Art) that extend to the endosteal surface which develop into smaller endosteal capillaries (purple) that connect to the complex sinusoidal network (blue) which merges into the central sinus (CS) in the middle of the BM cavity. The distinct blood vessels are aligned by various stromal cells. Bone-degrading cells, called osteoclasts (Oc), line the endosteal surface. Adipocytes (Adip) also reside in the marrow and progressively increase upon aging.

b. 1. While most hematopoietic stem cells (HSCs) are adjacent to the sinusoids (blue), some HSCs preferentially localize to arteries (red). Arteries are enwrapped by sympathetic nervous system (SNS) fibers (purple) and Nestin-GFP^{bright}NG2^{+} cells (green), whereas sinusoids are associated with LEPR^{+}Nestin-GFP^{dim} cells. 2. Endosteal capillaries (purple) are associated with Osterix^{+} cells (Osx, orange). Bone-lining osteoprogenitors/osteoblasts (Ob) regulate lymphoid progenitor (Imm B) number.

1.1.2 HSC niches

Architecture of the BM microenvironment

Whether hematopoietic stem and progenitor cells (HSPCs) become activated (to differentiate and proliferate) or remain in a dormant state is partially determined by extracellular cues derived from their BM microenvironment. These extrinsic cues include secretion of regulatory factors, cell-cell interactions, interaction with the extracellular matrix, and regulation of reactive species oxygen (ROS) levels. In long bones, the architecture of BM is characterized by a complex and dynamic arrangement of hematopoietic niches with distinct compositions (Figure 2A).

BM is a very cell-dense and highly vascularized tissue (Draenert and Draenert, 1980; Nombela-Arrieta and Manz, 2017; Ramasamy, 2017). Blood is supplied to BM by multiple

arteries which divide into smaller arterioles that move towards the endosteal region (the inner-surface of bone), where they further divide into small-diameter endosteal arterioles/capillaries. These endosteal capillaries transit into downstream sinusoidal vessels, which form a complex network that extends inwards to the medullary cavity, and merge with the central sinus/vein. Despite high vascular density, BM is relatively hypoxic (<32 mmHg), with the lowest oxygen tensions found near sinusoids in the central cavity, due to high oxygen demand associated with its high cellular density (Spencer et al., 2014). Many niche cells are adjacent to these diverse BM blood vessels (Crane et al., 2017), which, besides oxygen, also provide access to nutrients and other metabolites derived from circulating blood, and regulate cellular trafficking. Of note, both bone and BM are extensively innervated by the sympathetic nervous system (SNS). Furthermore, while human BM hardly contains any fat at birth, adipocytes (fat-producing cells) gradually increase with age, leading to a shift from red (hematopoietic-rich) marrow to yellow (fatty) marrow.

The discovery of surface markers for HSCs and BM microenvironmental cells, as well as, advances in imaging techniques and transgenic mouse models, have enabled studies to better define the cellular components and function of the HSC niche. Studies in mice suggest that within adult BM numerous cell-types constitute the HSC niche, with the majority being non-hematopoietic (stromal) cells and an increasingly recognized minor subset consisting of (mature) hematopoietic cells, as will be discussed in more detail in the following section. In particular, BM blood vessels seem to take on a critical role, which will be highlighted in their own separate section following the introduction of the HSC niche.

HSC-regulatory factors derived from niche cells essential for HSC maintenance

One of the ways to define niche cells is by their ability to synthesize factors that sustain HSC survival. Stem cell factor (SCF) and C-X-C motif chemokine ligand 12 (CXCL12) are the quintessential example of such HSC-regulatory factors and are probably among the best characterized niche-derived cytokines.

SCF is an agonist for the c-KIT receptor (Williams et al., 1990), which is expressed by HSCs (Kiel et al., 2005). Membrane-bound and soluble forms of SCF exist, and *in vivo* studies revealed that transgenic mice only expressing the soluble form of SCF have decreased HSC number (Barker, 1994, 1997), indicating that the membrane-bound form is critical for HSC maintenance. This finding also implies that SCF-expressing niche cells are in direct cell-cell contact with HSCs.

CXCL12, in addition to regulating HSC number, is also required for HSC retention in BM (Ara et al., 2003; Sugiyama et al., 2006; Tzeng et al., 2011). Mutant mice lacking CXCL12 fail to initiate hematopoiesis in fetal BM due to impaired colonialization by LT-HSCs (Ara et al., 2003). Additional genetic studies showed that abrogating expression of CXCL12 or its receptor CXCR4 in adulthood lead to depletion of BM HSCs (Sugiyama et al., 2006; Tzeng et al., 2011).

Advances in mouse genetics have enabled conditional and inducible abrogation of *CXCL12*, *SCF*, and other genes encoding for HSC regulatory factors in restricted cell populations by means of various Cre recombinases expressed under the control of (in theory) cell type-specific promoters. This has become an essential tool to test if and when expression of a single factor by a candidate cell type is required for HSC regulation.

HSC maintenance during steady-state: Osteoblasts

Osteoblasts reside at the endosteal surface of BM and are indispensable for *de novo* bone formation, secreting numerous extracellular proteins, such as type 1 collagen, osteocalcin (Ocn), and alkaline phosphatase (Figure 2B). Initially, *in vivo* studies using genetically engineered mice suggested that osteoblasts were important for regulating HSC number (Calvi et al., 2003; Zhang et al., 2003). Mice deficient for bone morphogenetic protein receptor type IA (BMPR1A) have an increased number of spindle-shaped N-cadherin-expressing osteoblastic cells which was associated with increased HSC number. Using 5-bromodeoxyuridine (BrdU)-labelling, researchers found that BrdU-retaining (a surrogate marker for LT-HSCs) cells were adjacent to these osteoblasts (Zhang et al., 2003). In line with these findings was the observation that expansion of osteoblasts, mediated by constitutively active PTH/ PTHrP receptor (PPR) signaling that was restricted to maturing osteoblastic cells, also lead to more HSCs (Calvi et al., 2003).

However, follow-up studies using other transgenic mouse lines and more specific markers for HSCs have since disputed a direct role for osteoblasts in regulating HSC number under homeostatic conditions. While conditional ablation of maturing osteoblasts in transgenic mice (that express herpesvirus thymidine kinase under control of a rat type 1 collagen promotor (*Col 2.3*)) upon ganciclovir injection eventually resulted in loss of BM HSCs (Visnjic et al., 2004); subsequent analyses showed that the decrease in HSC number (28 days post-injection) is preceded by early loss of B-lymphoid progenitors (8 days post-injection), suggesting that osteoblasts play a more direct role in B lymphopoiesis rather than in HSC regulation (Zhu et al., 2007).

This view was later supported by two companion papers from the laboratories of Drs. Daniel Link (using *Ocn*-Cre transgenic mice) and Sean Morrison (using *Col2.3*-Cre transgenic mice) both demonstrating that conditional deletion of *CXCL12* in osteoblasts did not deplete HSC number (Ding et al., 2012; Greenbaum et al., 2013). Furthermore, it had been shown that BM osteoblasts lack SCF expression and conditional deletion of *SCF* in mature osteoblasts had no effect on HSCs (Ding et al., 2012). Lastly, the group lead by Sean Morrison also demonstrated that c-kit^{+}Sca-1^{+}Lin^{-} BM cells with distinct expression of signaling lymphocyte activation molecules (SLAM) – CD150^{+}, CD48^{-}, and CD41^{-} – enriched for functional HSCs and the majority of these cells localized close to sinusoidal blood vessels, rather than the endosteum (Kiel et al., 2005) (Figure 2B).

Taken together, these studies still support a regulatory function of osteoblasts in unperturbed hematopoiesis, albeit at more committed stages of hematopoietic differentiation and perhaps not directly at HSC level. Hence, focus had shifted to other cellular components in the BM to uncover which niches directly control the HSC population.

HSCs niches under steady-state conditions: Perivascular stromal cells

Multipotency is not limited to HSCs in the BM. BM also contains stromal cells that form fibroblast colonies in culture (colony-forming unit-fibroblasts (CFU-Fs)) that exhibit multi-lineage differentiation capacity; fibroblasts expand extensively and can differentiate into cells of different mesenchymal fates, including osteoblasts, adipocytes, and chondrocytes (*ex vivo*). These multipotent bone marrow stromal cells (BMSCs) are very heterogeneous *in situ* (ability to give rise to different mesenchymal lineages, ancestry, and marker expression) and can be found on the abluminal surface of BM blood vessels (Crisan et al., 2008; Sacchetti et al., 2007). Intriguingly, *ex vivo* expanded BMSCs have previously been shown to form ectopic BM upon transplantation, forming donor-derived bone housing recipient-derived hematopoietic cells, making them attractive HSC niche candidates (Friedenstein et al., 1974). Considering that the majority of immunophenotypic HSCs were found to be associated with sinusoidal blood vessels (Kiel et al., 2005), and also with CXCL12-abundant reticular (CAR) cells – the main source of BM CXCL12 – which themselves often surrounded sinusoids (Sugiyama et al., 2006), studies started dissecting (1) the precise role of perivascular stromal cells in HSC maintenance and (2) their relation to BMSCs due to similar perivascular localization and reticular morphology.

In 2010, two independent studies by Mendez-Ferrer *et al* and Omatsu *et al* were among the first to demonstrate that perivascular stromal cells in postnatal marrow of mice are essential for regulating BM HSCs number and that some physiologically resembled BMSCs (Méndez-Ferrer et al., 2010; Omatsu et al., 2010). Using *Nes*-GFP transgenic mice, in which GFP expression is driven by the second intronic enhancer of *nestin*, the group of Paul Frenette demonstrated that *Nes*-GFP+ cells marked perivascular stromal cells that expressed *CXCL12* and *SCF*, and ablation of Nestin-expressing cells lead to reduced BM HSC number and HSC mobilization to the spleen. Provocatively, *Nes*-GFP+ cells (1%) were enriched for CFU-Fs and were able to differentiate into osteoblasts and chondrocytes *in vivo*, and proved to be serially transplantable: demonstrating their self-renewal capacity (Méndez-Ferrer et al., 2010). Congruent with these findings were the observations that (1) CAR cells depletion in postnatal marrow lead to a decreased HSC number and (2) CAR cells were able to differentiate into adipocytes and osteogenic cells *in vivo* (Omatsu et al., 2010). These landmark studies were the first to provide proof of principle that HSC and BMSC biology may be coupled under physiological conditions.

Subsequent work demonstrated that the *Nes*-GFP+ population could be subdivided in two populations based on GFP expression and their specific perivascular localization (Kunisaki et al., 2013). *Nes*-GFP^{bright} cells marked rare peri-arteriolar cells and were labelled by the

pericyte marker NG2, while *Nes*-GFP^{dim} cells were much more abundant reticular stromal cells that largely associated with sinusoidal vessels (peri-sinusoidal). Though the authors also confirmed that the majority of the HSCs were closest to sinusoids, a subset of quiescent HSCs localized closer than expected to *Nes*-GFP^{bright}-enwrapped arterioles assuming a random distribution pattern (Figure 2B). Of note, most of the CFU-F content was confined to *Nes*-GFP^{dim} cells, despite exhibiting lower CFU-F frequency (1% vs 4%), due to their higher total cell count (3,500 vs 300 cells per femur).

Meanwhile the studies from the laboratories of Drs. Daniel Link and Sean Morrison, in which researchers systematically abolished CXCL12 and SCF expression in candidate niche cells, demonstrated that *Prx1*-Cre and *LepR*-Cre activity also marked perivascular stromal cells that expressed both CXCL12 and SCF in postnatal marrow (Ding and Morrison, 2013; Ding et al., 2012; Greenbaum et al., 2013). It was initially suggested that *LepR*-Cre cells partially overlapped with *Nes*-GFP+ cells (Ding et al., 2012). In fact, it was later demonstrated that *LepR*-Cre cells highly overlapped (98%) with Nes-GFP+ cells (Zhou et al., 2014) and that 80-90% Nes-GFP^{dim} cells coincided with *LepR*-Cre cells (Kunisaki et al., 2013; Zhou et al., 2014). Nes-GFP^{bright} cells did not strongly overlap with *LepR*-Cre (Kunisaki et al., 2013), only 9% of Nes-GFP^{bright} cells coincided with LepR-Cre (Zhou et al., 2014). In addition, Zhou *et al* showed that nearly all Leptin receptor (LepR)-expressing cells in postnatal BM were positive for *Prx1*-Cre and vice versa.

Conditional deletion of *CXCL12* and *SCF* revealed distinct contributions of perivascular stromal cells to HSC maintenance; abrogation of CXCL12 expression in *Prx1*-Cre and *LepR*-Cre transgenic mice induced HSC mobilization in both cases, but only HSC depletion in *Prx1*-Cre mice (Ding and Morrison, 2013; Greenbaum et al., 2013), whereas abrogation of SCF expression in *LepR*-Cre did deplete HSC number (Ding and Morrison, 2013). This was later confirmed in follow-up work by the group of Dr. Paul Fernette, in which they reproduced the effects of *LepR*-Cre mediated *SCF* and *CXCL12* deletion on HSC maintenance. They also showed that inducible targeted deletion of *CXCL12* induced in 2 week-old *NG2*-CreER mice (supposedly targeting peri-arteriolar *Nes*-GFP^{bright} stromal cells), resulted in HSC number depletion, while observing no effect of *SCF* deletion on HSC number.

Although it was also demonstrated that the majority of LepR-expressing perivascular cells enwrapped sinusoids, a minority was also found around some arterioles. Intriguingly, 10% of the BM cells marked by *LepR*-Cre gave rise to CFU-Fs that were able to differentiate into osteoblasts, adipocytes and chondrocytes *ex vivo* (Zhou et al., 2014). Lineage-tracing studies revealed that *LepR*-Cre cells arose postnatally and were a major source of bone and fat in postnatal BM.

Collectively, these studies further redefined the role of perivascular stromal cells as key components of the HSC niche and their associated BMSC activity, and indicated that perivascular stromal cells are diverse and consist of distinct subsets that exhibit specific spatial characteristics in the BM, and differentially contribute to the HSC niche (Figure 2B).

HSCs niches under steady-state conditions: Other cells

Hematopoietic cells derived from HSCs can convey signals to HSCs and thereby create a feedback loop. Three-dimensional whole-mount imaging demonstrated that a subset of HSCs associate with megakaryocytes (Bruns et al., 2014). Depletion of megakaryocytes leads to HSC proliferation and expansion, and megakaryocytes-derived chemokine C-X-C motif ligand 4 (CXCL4) (Bruns et al., 2014) and TGFβ-1 (Zhao et al., 2014) have been suggested to contribute to the maintenance of HSC quiescence (Figure 2B).

Recent work by Hur *et al* also proposed that HSC quiescence is controlled by niche-mediated activation of TGFβ signaling in HSCs. These researchers suggested that activation of TGFβ–SMAD3 signaling was dependent on stabilization of CD82 (expressed on dormant HSCs) by engaging with its binding partner DARC/CD234, which is expressed by a subset of macrophages (Hur et al., 2016). Previously, macrophages were thought to have more indirect contributions on HSCs by acting via niche cells. In mice, HSC lodgment in the BM was severely disrupted upon targeted depletion of CD169+ macrophages and this coincided with a substantial loss of CXCL12, indicating that macrophages may act via niche cells by regulating their expression of HSC-retention factors (such as CXCL12 by Nestin-GFP+ cells) (Chow et al., 2011). CXCL12 expression can also be regulated by the removal of aged neutrophils from the circulation and their subsequent engulfment by macrophages (Casanova-Acebes et al., 2013), suggesting that interactions between mature hematopoietic cells (macrophages and neutrophils) can affect the HSC niche. A more direct role for (mature) myeloid cells was suggested by a recent study that demonstrated that myeloid cells form a spatial cluster around a subset of HSCs and conferred their quiescence by histamine secretion (Chen et al., 2017).

Differentiated lymphoid cells have also been implicated to play a role in hematopoiesis. IFNγ produced by cytotoxic CD8+ T cells was shown to induce IL-6 production by BMSCs, and thereby enhanced proliferation and myeloid differentiation of early hematopoietic progenitor cells during acute viral infection (Schürch et al., 2014). More recently, a subset of FoxP3+ regulatory T cells marked by CD150 expression was shown to closely associate with HSCs and maintained HSC quiescence by protecting HSCs from oxidative stress under homeostatic conditions (Hirata et al., 2018).

The SNS also seems to play an important role in HSC regulation. Adrenergic nerves from the SNS have been shown to control HSC egress into the circulation by regulating cyclical expression of niche-derived CXCL12 (in a circadian manner) (Méndez-Ferrer et al., 2008) and it was later revealed that these sympathetic nerves innervated Nes-GFP+ stromal cells (Méndez-Ferrer et al., 2010). Furthermore, it has been reported that sympathetic nerve-associated nonmyelinating Schwann cells can promote HSC maintenance by regulating proteolytic activation of latent TGFβ in the BM (Yamazaki et al., 2011).

Lastly, adipocytes have been suggested to play a negative role in the HSC niche; adipocyte-rich BM (such as the vertebrae of the mouse tails) was shown to contain fewer HSPCs

compared to adipocyte-free BM (vertebrae of the thorax) (Naveiras et al., 2009), but direct evidence, particularly during homeostasis, is lacking (Figure 2).

HSC niches in humans

Knowledge on the specific cellular components of the human HSC niche (in adult BM) has until recently been limited compared to that generated by studies performed on mice. Much attention has been placed on identifying human BMSCs considering data from murine models strongly argue that mesenchymal progenitor cells fulfill a crucial role in regulating HSC function.

Ironically, one of the first studies that managed to prospectively isolate human BMSCs with bona-fide self-renewal capacity and the ability to create a hematopoietic-supporting microenvironment, preceded the transgenic mice studies that initially identified BMSCs as important HSC niche components (Méndez-Ferrer et al., 2010; Zhou et al., 2014). By using a single positive marker, CD146, the group of Paolo Bianco demonstrated that CD146 labelled adventitial (abluminal surface of blood vessels) reticular cells in adult BM that were enriched for CFU-Fs and upon xenogenic transplantation in mice, formed heterotopic ossicles harboring (1) murine-derived hematopoietic tissue and (2) phenotypically identical CD146+ BMSCs (Sacchetti et al., 2007). Later it was revealed that CD146 marked a perivascular subset of the CD271-expressing BMSCs, while the CD271+ subset lacking CD146 expression exhibited an endosteal localization, and together contained virtually all the CFU-Fs in adult BM (Tormin et al., 2011). Both CD271+ subsets co-localized with CD34+ HSPCs *in situ* and were equally capable of forming ectopic bone and BM upon transplantation (Tormin et al., 2011). Of note, work of the group of Dr. Simón Méndez-Ferrer suggested that a combination of the cell surface markers CD105 and CD146 could be used to prospectively isolate the human equivalent of Nestin-GFP+ cells. CD105+CD146+ cells obtained from adult BM were able to generate mesenchymal spheres *in vitro* that were able to support the expansion of HPCs capable of engrafting immunocompromised mice (Isern et al., 2014).

Despite the considerable effort spent on identifying human BMSCs, evidence for their hematopoietic-supporting capacity and potential underlying mechanisms is still relatively lacking compared to that generated by studies using genetically engineered mice. It remains to be seen to what extent findings in mice are relevant for human biology.

1.1.3 HSCs and their niche during regeneration

Impact of chemo/radiotherapeutic stress on the hematopoietic system

In the treatment of various malignancies, chemotherapy and radiotherapy are often used to eradicate malignant cells, but can also have unwanted harmful effects on hematopoiesis. In general, chemo/radiotherapy works by causing DNA damage and consequently inducing senescence or death of cells. The severity of the damage to the hematopoietic system depends on the treatment regimen.

Ionizing radiation (IR) can induce DNA damage in cells by the formation of reactive oxygen species (ROS). Cells harbor antioxidant systems to scavenge excess ROS, but can become overwhelmed if ROS reach high enough levels. This overabundance of ROS can damage macromolecules within cells (such as lipids, proteins, and DNA) and if not timely repaired, may lead to cell death (Azzam et al., 2012). In severe cases, this oxidative stress persists because pro-inflammatory cytokines are often up-regulated by ROS, and these re-inforce the production of ROS (cytokine storm) (Kim et al., 2014). IR can also directly damage cells by inducing double-stranded breaks in DNA.

Chemotherapy makes use of cytotoxic drugs that inhibit cell division. Chemotherapeutic agents are diverse, and include alkylating agents, antimetabolites, and anthracyclines (Cheung-Ong et al., 2013). Alkylating agents, such as cyclophosphamide act by forming cross-links in DNA double-helix strands, while antimetabolites, like cytarabine, compete with nucleosides for incorporation into DNA. Anthracyclines can interact with DNA in different ways, including intercalation (insertion between the base pairs) and poisoning of topoisomerase II.

In general, cycling cells are in particular sensitive to the effects of chemo/radiotherapy, which means that committed myeloid progenitors giving rise to mature blood cells are more affected compared to the more dormant/quiescent HSCs (Lerner and Harrison, 1990; Mohrin et al., 2010). Consequently, peripheral blood cell counts often decrease after chemotherapy, and exhibit a progressive decline based on the intrinsic lifespan of the cell, with granulocytes declining prior to platelets followed by erythrocytes (Mauch et al., 1995). This sequence and rate of decline of peripheral blood cells is similar for radiotherapy. The resulting granulocytopenia and thrombocytopenia leave patients especially at risk for opportunistic infections and bleedings, and are major cause of morbidity in the treatment of cancer.

Upon stress conditions dormant HSCs become activated to replenish lost cells (Wilson et al., 2008), but how the niche might mediate dormant HSC activation has remained obscure until recently. Spurred on by the increased knowledge on which BM microenvironmental cells constitute the HSC niche and with advances in the tools to identify specific subsets of HSPC populations, studies have started focusing on dissecting the role of the niche cells in BM regeneration in response to chemoradiotherapeutic stress.

Impact of chemoradiotherapeutic stress on the HSC niche and consequences

Cytotoxic damage resulting from chemo/radiotherapy is not limited to the hematopoietic system, micro-environmental cells in the BM can also suffer damage depending on the treatment regimen.

Cao *et al* reported that femur irradiation at a dose of 20 Gray can robustly alter the BM architecture of mice and saw that BM exhibited increased adipogenesis, reduced CFU-F efficiency, and a disrupted vasculature network (Cao et al., 2011). Similarly, chemotherapeutic agents, such as cyclophosphamide and methotrexate (an antimetabolite), have also been associated with increased marrow adipocyte content, bone loss, and regression of BM blood vessels in murines (Georgiou et al., 2012; Shirota and Tavassoli, 1991).

Osteolineage cells

Although recent work has toned down the importance of osteoblasts in regulating HSC number during homeostasis, numerous studies have suggested that bone-lineage cells play a prominent role after BM injury. For instance, studies have demonstrated that transplanted HSPCs localize near the endosteal surface and osteolineage cells of irradiated mice (Lo Celso et al., 2009; Dominici et al., 2009; Jiang et al., 2009). Irradiation was associated with a reversible expansion of N-cadherin+ osteoblasts and concomitant CXCL12 expression (Dominici et al., 2009). These findings were in line with previous work that demonstrated that CXCL12 levels were increased after conditioning with DNA-damage agents (ionizing irradiation, cyclophosphamide, and 5-fluorouracil) (Ponomaryov et al., 2000). This is especially clinically relevant in the context of HSPC recovery after BM transplantation, which is preceded by conditioning regimens consisting of chemo/radiotherapy.

Recent work has suggested that the subset of osteoblasts expressing N-cadherin can normally maintain HSC quiescence via non-canonical WNT signaling and after 5-FU challenge this signaling becomes attenuated and HSCs subsequently enter cell cycle (Sugimura et al., 2012). Furthermore, single-cell analysis of osteoblasts from irradiated mice in direct contact with transplanted HSCs lead to the discovery of 3 novel HSPC regulatory factors: Embigin, IL-18 and angiogenin (Silberstein et al., 2016). Remarkably, angiogenin was found to regulate hematopoietic recovery after myeoablation by specifically promoting proliferation of committed progenitors, while inducing quiescence in HSCs (Goncalves et al., 2016). More recently, it was reported that dickkopf-1 (DKK1) released by osteoprogenitors could promote HSC reconstitution following irradiation by suppressing mitochondrial ROS levels and senescence (Himburg et al., 2017).

Together these studies indicate that bone-lining/osteoprogenitor cells take on a prominent role in hematopoietic recovery after chemoradiotherapeutic insult.

Perivascular stromal cells

The group of Dr. Paul Frenette was one of the first to describe the role of BMSCs upon regeneration by challenging *Nes*-GFP mice with 5-FU (Kunisaki et al., 2013). Kunisaki et al observed that the *Nes*-GFP^{bright} cells were relatively quiescent compared to sinusoid-associated *Nes*-GFP^{dim} cells, and were subsequently also more chemoresistant upon 5-FU challenge. Intriguingly, the proportion of HSCs near *Nes*-GFP^{bright} cells was initially increased after 5-FU treatment, and as regeneration progressed this proportion steadily decreased. The authors proposed that *Nes*-GFP^{bright} cells might somehow shield HSCs from genotoxic insult by mediating HSC quiescence in a protective milieu, but the direct role of *Nes*-GFP^{bright} cells or protective signals involved remains unclear.

The role of LepR-expressing BMSCs is more clear and has been recently described by multiple groups (Himburg et al., 2018; Zhou et al., 2017). The Morrison laboratory reported that SCF derived from cells marked by *LepR*-cre mediated hematopoietic regeneration after HSC transplantation in irradiated mice, as well as, in mice treated with 5-FU (Zhou et al., 2017). Notably, Zhou et al observed that LepR+ cells actually declined after chemo/radiotherapy and that SCF derived from adipocytes, presumably descendants of LepR+ cells (95% of the BM adipocytes were previously shown to be marked Lepr-Cre after irradiation), promoted hematopoietic regeneration. In addition, pleiotrophin (PTN), a novel HSPC regulatory factor, was identified in BM cells marked by *LepR*-cre and was suggested to mediate acute hematologic recovery and survival following irradiation (Himburg et al., 2018).

Collectively these studies suggest that BMSC niche cells respond differently to cytotoxic challenge. While Nestin-GFP^{bright} cells may be relatively chemoresistant and are associated with a subset of quiescent HSCs, their exact role in BM regeneration is not well described. LepR+ cells on the other hand seem to be more sensitive to chemoradiotherpeutic stress, but together with their descendants mediate hematopoietic recovery by secreting SCF and PTN.

Nerve cells

Impaired adrenergic signaling in 5-FU treated mice resulted in reduced number of Nestin-GFP+, as well as, endothelial cells and delayed hematopoietic recovery (Lucas et al., 2013). These findings imply that the SNS indirectly plays a role in BM regeneration by ensuring survival of other HSC niche components after chemotherapy. Interestingly, while the number of sympathetic fibers was unaltered after IR or 5FU treatment, peripheral nerve damage did occur in mice that received cisplatin, indicating that the response of HSC niche cells depends on the type of cytotoxic stressor.

Megakaryocytes

In response to chemotherapeutic stress megakaryocytes were shown to up-regulate fibroblast growth factor (FGF1) to promote HSC expansion (Zhao et al., 2014). Furthermore,

megakaryocytes accumulate in the BM after cytotoxic stress (Hérault et al., 2017; Kopp et al., 2005) and megakaryocyte-derived cytokines were previously implicated as a possible mediator of the transient osteoblast expansion after irradiation (Dominici et al., 2009).

Conclusion

Taken together, emerging evidence from mice studies indicate that the HSC niche is not limited to a single cell type or anatomical location in BM. It has been proposed that there are multiple HSC niches situated in BM, perhaps reflecting (or contributing to?) the heterogeneity within the HSC pool (Haas et al., 2018). Individual niche components may have a specific contribution to HSC regulation, but most likely there is a complex interdependency and crosstalk between the different niches that enable fine-tuned regulation of the HSC pool under specific circumstances. In the following section, the specialized role of BM blood vessels will be discussed.

1.2 The role of endothelial cells in BM regeneration

1.2.1 General

Endothelial cells (ECs), collectively termed endothelium, form a monolayer of cells that constitute the inner-lining of blood vessels and are most commonly known for facilitating blood flow and enabling exchange of nutrients and waste products. ECs are also widely recognized for their role in regulating vascular tone, blood clotting, and migration of hematopoietic cells. However, these cells perform other critical physiological tasks as well – signals from ECs guide the development, maintenance, and regeneration of their surrounding tissue. This has been shown to be especially true for BM physiology, and by extension, BM hematopoiesis.

ECs have been shown to play a critical role during endochondral ossification, which is required for the formation of long bones and subsequent BM (Chan et al., 2009). Invading blood vessels are attracted to the cartilage template of bone by VEGF from hypertrophic chondrocytes (Gerber et al., 1999), and together with the latter, as well as, with osteoblasts and osteoclasts, will coordinate the development of bone. It is thought that during this process ECs express factors (angiocrine factors) that support osteogenesis. The expression of EC-derived osteogenic factors has been reported for specific endosteal blood vessels recently characterized in (postnatal) mice (Kusumbe et al., 2014).

1.2.2 Endothelial cells

The importance of BM ECs for hematopoiesis first emerged in studies that focused on hematopoietic recovery following chemo/radiotherapy. The group of Dr. Shahin Rafii revealed that chemo/radiotherapy can severely disrupt the sinusoidal network, with the extent of damage depending on the type and magnitude of the cytotoxic agent used (Hooper et al., 2009; Kopp et al., 2005). Importantly, blocking regeneration of sinusoidal vessels after

irradiation by inhibiting VEGFR2 signaling impaired engraftment and reconstitution of HSPCs. In line with these findings was the observation that targeted deletion of pro-apoptotic genes *Bak* and *Bax* in endothelium (using either *Tie2*-Cre or *VEcadherin*-Cre mice) preserved the integrity of BM vasculature following lethal irradiation, which was associated with improved survival of mice (Doan et al., 2013a). These studies indicate that BM ECs might mediate HSPC reconstitution after myelosuppresive injury, but the precise mechanisms involved were unclear.

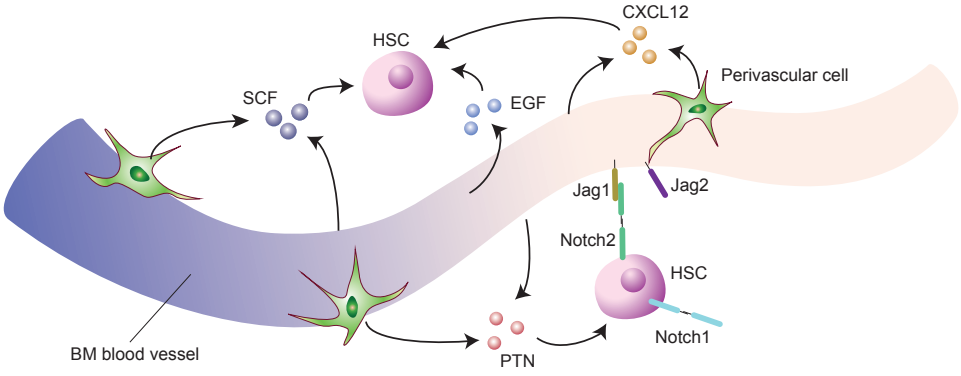


Figure 3. Regeneration-associated paracrine factors in the bone marrow (BM) vascular niche. Adapted from (Sasine et al., 2017). A schematic diagram of a BM blood vessel aligned with perivascular stromal cells in longitudinal view and representation of several paracrine factors that are secreted by BM endothelial cells and perivascular cells. Abbreviations: CXCL12, C-X-C chemokine ligand 12; CXCR4, C-X-C chemokine receptor type 4; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HSC, hematopoietic stem cell; Jag1, Jagged-1; Jag2, Jagged-2; PTN, pleiotrophin; PTP-z, protein receptor tyrosine phosphatase-z; SCF, stem cell factor.

1.2.3 Endothelial cell-derived instructive (angiocrine) factors

Follow-up work revealed that BM ECs can mediate hematopoietic recovery following injury in a perfusion-independent manner (Figure 3). Butler *et al* demonstrated that inhibition of VEGFR2 signaling diminished endothelial expression of Notch ligands, Jagged1 and Jagged2, indicating that BM ECs may mediate the reconstitution of hematopoiesis by expressing HSPC-instructive factors (Butler et al., 2010). Indeed, it was later suggested by specifically disrupting Jagged1 and Jagged2 expression in ECs (using *VEcadherin*-Cre mice) that both Notch ligands can contribute to hematopoietic recovery after myelosuppressive injury (Guo et al., 2017; Poulos et al., 2013). Subsequently, other studies have identified additional angiocrine factors that may regulate HSPC recovery by mediating cell-to-cell or cell-extracellular matrix interactions, such as sinusoidal E-selectin (Winkler et al., 2012) and extracellular matrix protein developmental endothelial locus (Del)-1 (Mitroulis et al., 2017). The team of Dr. John Chute reported that paracrine factors released by BM ECs can also mediate HSPC regeneration following myelosuppression. These include epidermal growth factor (EGF) (Doan et al., 2013b) and PTN (Himburg et al., 2010, 2018), with EGF preventing

radiation-induced apoptosis of HSCs, while PTN was associated with HSC expansion via activation of the RAS pathway (Himburg et al., 2014). Of note, it has been demonstrated that during steady-state conditions BM ECs also release CXCL12 and SCF, and abrogating CXCL12 or SCF expression in BM ECs lead to decreased HSC number (Ding and Morrison, 2013; Ding et al., 2012; Greenbaum et al., 2013). The effects on HSPC regeneration following injury were not explored, but a previous report did describe that 5FU treated mice had a dramatic increase in SCF expression by sinusoidal ECs (Kimura et al., 2011). These studies indicate that ECs express instructive factors that may be involved in both HSC maintenance and regeneration.

1.2.4 Crosstalk with other niche cells

Other niche cells can indirectly regulate hematopoietic recovery via BM ECs. DKK1 released by osteoprogenitors induced upregulation of EGF expression by BM ECs, which contributed to hematopoietic recovery following irradiation (Himburg et al., 2017). In addition, BM granulocytes were recently reported to promote EC survival and vessel growth following irradiation by delivering tumor necrosis factor-alpha (TNFα) to regenerative vasculature, which resulted in improved hematopoietic engraftment (Bowers et al., 2018). Also, as previously mentioned, the SNS has been suggested to mediate EC survival following chemotherapy.

1.2.5 Specialized endothelium

It has recently become clear that BM blood vessels are very heterogeneous. Besides structure and morphology, ECs of BM blood vessels differ in respect to their sensitivity to cytotoxic stress, expression of angiocrine factors, and their associated function.

For example, the endosteal capillaries that connect arterioles with sinusoids have been attributed with a number of specific characteristics (Kusumbe et al., 2014; Ramasamy et al., 2014) (Figure 2B). Endosteal capillaries, also termed Type H capillaries due to their high expression of CD31 and endomucin, were reported to be relatively radioresistant compared to sinusoidal ECs, and intriguingly, were suggested to couple BM angiogenesis and osteogenesis in a Notch-dependent manner by angiocrine release of Noggin. These endosteal capillaries were also tightly associated with perivascular osteoprogenitors marked by Osterix expression. Remarkably, activated Notch signaling in BM ECs also lead to HSC expansion (Kusumbe et al., 2016).

Another study demonstrated that some non-sinusoidal BM ECs expressed thrombomodulin and maintained the retention of a small subset of LT-HSCs (marked by EPCR expression) in the BM by inhibiting NO production (Gur-Cohen et al., 2015). Subsequent work by the same lab indicated that due to leaky nature of sinusoidal ECs, HSCs exposed to plasma components exhibit increased ROS and are more active, while less permeable arterial ECs maintain lower levels of ROS and thereby confer quiescence to HSCs (Itkin et al., 2016).

Further supporting the notion that sinusoidal and arterial ECs exhibit specific roles was a very recent report that indicated that arterial ECs are the main source of endothelial SCF, which regulated HSC number under steady-state conditions, as well as, HSPC reconstitution after 5-FU challenge (Xu et al., 2018).

Taken together these studies indicate that ECs are important and versatile. In addition to their conventional function, ECs are needed for HSC maintenance and regeneration, and can mediate these functions in a perfusion-independent manner by expressing angiocrine factors. Not only do they affect HSPCs, but they can also couple angiogenesis and osteogenesis by secreting osteogenic factors.

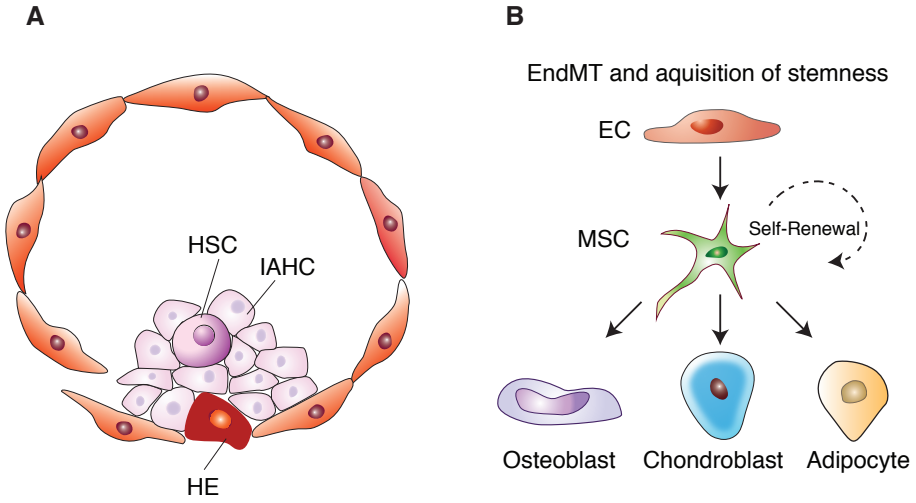


Figure 4. Plasticity of endothelium.

a. Adapted from (Kauts et al., 2016). Hemogenic endothelium gives rise to intra-aortic hematopoietic clusters that contain hematopoietic stem cells (HSCs) at embryonic day 10.5 (E10.5).

b. Hypothetical model of endothelium giving rise to mesenchymal multipotent stromal cells (MSCs) capable of self-renewal and differentiating into multiple skeletal lineages.

1.3 Plasticity of endothelial cells

1.3.1 Endothelial-to-Hematopoietic transition and Endothelial-to-Mesenchymal transition

The intimate relationship between ECs and HSCs is not surprising considering that definitive HSCs arise from ECs during embryogenesis. In mice at embryonic day (E) 10.5, specialized ECs in the aorta-gonad-mesonephros (AGM) region, called hemogenic endothelium, start giving rise to hematopoietic clusters by undergoing endothelial to hematopoietic transition (EHT) (Figure 4A). These hematopoietic clusters contain the HSCs that will give a life-long supply of blood cells and components (de Bruijn et al., 2002). Of note, the actual number of HSCs in these clusters is very low (2/700) (Yokomizo and Dzierzak, 2010), indicating that

in addition to EHT, HSC-precursors need to undergo a molecular program that specifies HSC fate. The AGM itself is thought to originate from embryonic mesoderm, specifically the splanchnopleural mesoderm (Rosselló Castillo et al., 2013). Ultimately, the generated HSCs migrate to fetal liver where they will undergo massive expansion prior to colonizing the BM of fetal long bones.

In addition to giving rise to HSPCs at the AGM, ECs of other tissues can directly contribute to organogenesis by transforming into other cell types. For example, ECs of the embryonic heart (endothelial endocardial cells) can give rise to mesenchymal cells necessary for proper heart development by undergoing endothelial to mesenchymal transition (EndMT). EndMT is required for the generation of the atrioventricular valves, as well as, the formation of endocardial cushion (Liebner et al., 2004) and a substantial portion of the cardiac pericytes and vascular smooth muscle cells (Chen et al., 2016). Intriguingly, endothelial endocardium-derived cells have been reported to give rise to osteogenic and adipogenic cells (Pu et al., 2016; Wylie-Sears et al., 2011). This remarkable plasticity of endothelium was also observed in ECs of human skeletal muscle that were reported to be able to differentiate into myogenic, osteogenic and chondrogenic cells in culture (Zheng et al., 2007) (Figure 4B).

1.4 Scope and outline of this thesis

Recent studies in mice have led to the recognition of BM ECs as critical HSC niche components that can mediate hematologic recovery after injury. Furthermore, these studies also identified specialized subsets of endothelium that not only regulate the hematopoietic system, but also couple osteogenesis and angiogenesis. Importantly, insights into the molecular programs underlying the specialized function of these endothelial subsets have enabled targeted interventions in mice, such as pharmacologic manipulation that enhanced bone formation or administration of recombinant proteins that promoted hematologic recovery after injury. However, the relevance of these findings for human biology remains relatively unknown. In particular, studies in humans that have attempted to unravel the specific contributions of BM ECs to hematologic recovery are scarce. To identify potential targets for therapeutic modulation to accelerate hematologic recovery in humans, a better understanding of the relevant cellular constituents and molecular pathways underlying hematologic recovery is needed.

In this thesis, we aimed to better define cellular and molecular events that occur in the human bone marrow during (re)generation, in order to identify novel cellular programs and mechanisms that might be exploitable for therapeutic modulation.

To this end, we defined two distinct (patho)physiologic conditions associated with BM (re)generation, requiring coordinated activation of osteogenesis, angiogenesis, and hematopoiesis, namely (1) regeneration after chemotherapeutically-induced injury and (2) bone marrow generation in human fetal development upon migration from hematopoiesis from the fetal liver to the bone at week 15–20 post gestation.

In **chapter 2**, we characterized human BM ECs associated with BM (re)generation in these conditions by flow-cytometry. Massive parallel sequencing was employed in EC subsets to identify candidate angiocrine factors that might drive hematologic recovery. This led to the identification of IL-33 which was subsequently functionally interrogated for its regenerative potential in bone and marrow. In **chapter 3**, we build upon the observation in chapter 2 that BM ECs exist that co-express mesenchymal markers, suggesting the possibility that specific BM ECs might contribute to BM regeneration by giving rise to mesenchymal progenitors (via EndMT). This notion is tested by extensive experimentation in both human cells and mice, leading to the postulation of EndMT as novel concept in bone marrow (re)generation and the *de novo* generation of MSCs in the mammalian BM. IL-33 is subsequently identified as a driver of this process.

As immune cells have also been implicated to regulate HSPC behavior, we dissected the BM lymphoid composition of the AML patients recovering (from remission induction chemotherapy) to determine whether specific immune subsets are associated with hematologic recovery in **chapter 4**. Finally, in **chapter 5** the findings in this thesis are summarized and their relevance for the field of BM regeneration, including future perspectives, are discussed.

REFERENCE LIST

Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., and Nagasawa, T. (2003). Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. Immunity *19*, 257–267.

Azzam, E.I., Jay-Gerin, J.-P., and Pain, D. (2012). Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. *327*, 48–60.

Barker, J.E. (1994). Sl/Sld hematopoietic progenitors are deficient in situ. Exp. Hematol. *22*, 174–177.

Barker, J.E. (1997). Early transplantation to a normal microenvironment prevents the development of Steel hematopoietic stem cell defects. Exp. Hematol. *25*, 542–547.

Bianco, P., Cao, X., Frenette, P.S., Mao, J.J., Robey, P.G., Simmons, P.J., and Wang, C.Y. (2013). The meaning, the sense and the significance: Translating the science of mesenchymal stem cells into medicine. Nat. Med. *19*, 35–42.

Bowers, E., Slaughter, A., Frenette, P.S., Kuick, R., Pello, O.M., and Lucas, D. (2018). Granulocyte-derived TNFα promotes vascular and hematopoietic regeneration in the bone marrow. Nat. Med. *24*, 95–102.

de Bruijn, M.F.T.., Ma, X., Robin, C., Ottersbach, K., Sanchez, M., and Dzierzak, E. (2002). Hematopoietic Stem Cells Localize to the Endothelial Cell Layer in the Midgestation Mouse Aorta. Immunity *16*, 673–683.

Bruns, I., Lucas, D., Pinho, S., Ahmed, J., Lambert, M.P., Kunisaki, Y., Scheiermann, C., Schiff, L., Poncz, M., Bergman, A., et al. (2014). Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. Nat. Med. *20*, 1315–1320.

Butler, J.M., Nolan, D.J., Vertes, E.L., Varnum-Finney, B., Kobayashi, H., Hooper, A.T., Seandel, M., Shido, K., White, I.A., Kobayashi, M., et al. (2010). Endothelial Cells Are Essential for the Self-Renewal and Repopulation of Notch-Dependent Hematopoietic Stem Cells. Cell Stem Cell *6*, 251–264.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841–846.

Cao, X., Wu, X., Frassica, D., Yu, B., Pang, L., Xian, L., Wan, M., Lei, W., Armour, M., Tryggestad, E., et al. (2011). Irradiation induces bone injury by damaging bone marrow microenvironment for stem cells. Proc. Natl. Acad. Sci. U. S. A. *108*, 1609–1614.

Casanova-Acebes, M., Pitaval, C., Weiss, L.A., Nombela-Arrieta, C., Chèvre, R., A-González, N., Kunisaki, Y., Zhang, D., van Rooijen, N., Silberstein, L.E., et al. (2013). Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell *153*, 1025–1035.

Lo Celso, C., Fleming, H.E., Wu, J.W., Zhao, C.X., Miake-Lye, S., Fujisaki, J., Côté, D., Rowe, D.W., Lin, C.P., and Scadden, D.T. (2009). Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature *457*, 92–96.

Chan, C.K.F., Chen, C.-C., Luppen, C.A., Kim, J.-B., DeBoer, A.T., Wei, K., Helms, J.A., Kuo, C.J., Kraft, D.L., and Weissman, I.L. (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. Nature *457*, 490–494.

Chen, Q., Zhang, H., Liu, Y., Adams, S., Eilken, H., Stehling, M., Corada, M., Dejana, E., Zhou, B., and Adams, R.H. (2016). Endothelial cells are progenitors of cardiac pericytes and vascular smooth muscle cells. Nat. Commun. *7*, 12422.

Chen, X., Deng, H., Churchill, M.J., Luchsinger, L.L., Du, X., Chu, T.H., Friedman, R.A., Middelhoff, M., Ding, H., Tailor, Y.H., et al. (2017). Bone Marrow Myeloid Cells Regulate Myeloid-Biased Hematopoietic Stem Cells via a Histamine-Dependent Feedback Loop. Cell Stem Cell *21*, 747–760.e7.

Cheung-Ong, K., Giaever, G., and Nislow, C. (2013). DNA-damaging agents in cancer chemotherapy: Serendipity and chemical biology. Chem. Biol. *20*, 648–659.

Chow, A., Lucas, D., Hidalgo, A., Méndez-Ferrer, S., Hashimoto, D., Scheiermann, C., Battista, M., Leboeuf, M., Prophete, C., van Rooijen, N., et al. (2011). Bone marrow CD169 + macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. J. Exp. Med. *208*, 261–271.

Crane, G.M., Jeffery, E., and Morrison, S.J. (2017). Adult haematopoietic stem cell niches. Nat. Rev. Immunol. *17*, 573–590.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. Cell Stem Cell *3*, 301–313.

Ding, L., and Morrison, S.J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature *495*, 231–235.

Ding, L., Saunders, T.L., Enikolopov, G., and Morrison, S.J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. Nature *481*, 457–462.

Doan, P.L., Russell, J.L., Himburg, H.A., Helms, K., Harris, J.R., Lucas, J., Holshausen, K.C., Meadows, S.K., Daher, P., Jeffords, L.B., et al. (2013a). Tie2 + Bone Marrow Endothelial Cells Regulate Hematopoietic Stem Cell Regeneration Following Radiation Injury. Stem Cells *31*, 327–337.

Doan, P.L., Himburg, H.A., Helms, K., Russell, J.L., Fixsen, E., Quarmyne, M., Harris, J.R., Deoliviera, D., Sullivan, J.M., Chao, N.J., et al. (2013b). Epidermal growth factor regulates hematopoietic regeneration after radiation injury. Nat. Med. *19*, 295–304.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Krause, D.S., Deans, R.J., Keating, A., Prockop, D.J., and Horwitz, E.M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315–317.

Dominici, M., Rasini, V., Bussolari, R., Chen, X., Hofmann, T.J., Spano, C., Bernabei, D., Veronesi, E., Bertoni, F., Paolucci, P., et al. (2009). Restoration and reversible expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation. Blood *114*, 2333–2343.

Draenert, K., and Draenert, Y. (1980). The vascular system of bone marrow. Scan. Electron Microsc. 113–122.

Friedenstein, A.J., Chailakhyan, R.K., Latsinik, N. V, Panasyuk, A.F., and Keiliss-Borok, I. V (1974). Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation *17*, 331–340.

Georgiou, K.R., Scherer, M.A., Fan, C.-M., Cool, J.C., King, T.J., Foster, B.K., and Xian, C.J. (2012). Methotrexate chemotherapy reduces osteogenesis but increases adipogenic potential in the bone marrow. J. Cell. Physiol. *227*, 909–918.

Gerber, H.P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., and Ferrara, N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. *5*, 623–628.

Goncalves, K.A., Silberstein, L., Li, S., Severe, N., Hu, M.G., Yang, H., Scadden, D.T., and Hu, G. fu (2016). Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells. Cell *166*, 894–906.

Greenbaum, A., Hsu, Y.M.S., Day, R.B., Schuettpelz, L.G., Christopher, M.J., Borgerding, J.N., Nagasawa, T., and Link, D.C. (2013). CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature *495*, 227–230.

Guo, P., Poulos, M.G., Palikuqi, B., Badwe, C.R., Lis, R., Kunar, B., Ding, B.-S., Rabbany, S.Y., Shido, K., Butler, J.M., et al. (2017). Endothelial jagged-2 sustains hematopoietic stem and progenitor reconstitution after myelosuppression. J. Clin. Invest. *127*, 4242–4256.

Gur-Cohen, S., Itkin, T., Chakrabarty, S., Graf, C., Kollet, O., Ludin, A., Golan, K., Kalinkovich, A., Ledergor, G., Wong, E., et al. (2015). PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. Nat. Med. *21*, 1307–1317.

Haas, S., Trumpp, A., and Milsom, M.D. (2018). Causes and Consequences of Hematopoietic Stem Cell Heterogeneity. Cell Stem Cell *22*, 627–638.

Hérault, A., Binnewies, M., Leong, S., Calero-Nieto, F.J., Zhang, S.Y., Kang, Y.-A., Wang, X., Pietras, E.M., Chu, S.H., Barry-Holson, K., et al. (2017). Myeloid progenitor cluster formation drives emergency and leukaemic myelopoiesis. Nature *544*, 53–58.

Himburg, H.A., Muramoto, G.G., Daher, P., Meadows, S.K., Russell, J.L., Doan, P., Chi, J.T., Salter, A.B., Lento, W.E., Reya, T., et al. (2010). Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nat. Med. *16*, 475–482.

Himburg, H.A., Yan, X., Doan, P.L., Quarmyne, M., Micewicz, E., McBride, W., Chao, N.J., Slamon, D.J., and Chute, J.P. (2014). Pleiotrophin mediates hematopoietic regeneration via activation of RAS. J. Clin. Invest. *124*, 4753–4758.

Himburg, H.A., Doan, P.L., Quarmyne, M., Yan, X., Sasine, J., Zhao, L., Hancock, G. V., Kan, J., Pohl, K.A., Tran, E., et al. (2017). Dickkopf-1 promotes hematopoietic regeneration via direct and niche-mediated mechanisms. Nat. Med. *23*, 91–99.

Himburg, H.A., Termini, C.M., Schlussel, L., Kan, J., Li, M., Zhao, L., Fang, T., Sasine, J.P., Chang, V.Y., and Chute, J.P. (2018). Distinct Bone Marrow Sources of Pleiotrophin Control Hematopoietic Stem Cell Maintenance and Regeneration. Cell Stem Cell *23*, 370–381.e5.

Hirata, Y., Furuhashi, K., Ishii, H., Li, H.W., Pinho, S., Ding, L., Robson, S.C., Frenette, P.S., and Fujisaki, J. (2018). CD150highBone Marrow Tregs Maintain Hematopoietic Stem Cell Quiescence and Immune Privilege via Adenosine. Cell Stem Cell *22*, 445–453.e5.

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.-G., Shido, K., Petit, I., Yanger, K., et al. (2009). Engraftment and Reconstitution of Hematopoiesis Is Dependent on VEGFR2-Mediated Regeneration of Sinusoidal Endothelial Cells. Cell Stem Cell *4*, 263–274.

Hur, J., Choi, J. Il, Lee, H., Nham, P., Kim, T.W., Chae, C.W., Yun, J.Y., Kang, J.A., Kang, J., Lee, S.E., et al. (2016). CD82/KAI1 Maintains the Dormancy of Long-Term Hematopoietic Stem Cells through Interaction with DARC-Expressing Macrophages. Cell Stem Cell *18*, 508–521.

Isern, J., García-García, A., Martín, A.M., Arranz, L., Martín-Pérez, D., Torroja, C., Sánchez-Cabo, F., and Méndez-Ferrer, S. (2014). The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. Elife *3*, 1–28.

Itkin, T., Gur-Cohen, S., Spencer, J.A., Schajnovitz, A., Ramasamy, S.K., Kusumbe, A.P., Ledergor, G., Jung, Y., Milo, I., Poulos, M.G., et al. (2016). Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature *532*, 323–328.

Jiang, Y., Bonig, H., Ulyanova, T., Chang, K., and Papayannopoulou, T. (2009). On the adaptation of endosteal stem cell niche function in response to stress. Blood *114*, 3773–3782.

Kauts, M.L., Vink, C.S., and Dzierzak, E. (2016). Hematopoietic (stem) cell development — how divergent are the roads taken? FEBS Lett. *590*, 3975–3986.

Kiel, M.J., Yilmaz, Ö.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109–1121.

Kim, J., Kim, W., Le, H.T., Moon, U.J., Tran, V.G., Kim, H.J., Jung, S., Nguyen, Q.-T., Kim, B.-S., Jun, J.-B., et al. (2014). IL-33–Induced Hematopoietic Stem and Progenitor Cell Mobilization Depends upon CCR2. J. Immunol. *193*, 3792–3802.

Kimura, Y., Ding, B., Imai, N., Nolan, D.J., Butler, J.M., and Rafii, S. (2011). c-Kit-Mediated Functional Positioning of Stem Cells to Their Niches Is Essential for Maintenance and Regeneration of Adult Hematopoiesis. PLoS One *6*, e26918.

Komada, Y., Yamane, T., Kadota, D., Isono, K., Takakura, N., Hayashi, S.-I., and Yamazaki, H. (2012). Origins and Properties of Dental, Thymic, and Bone Marrow Mesenchymal Cells and Their Stem Cells. PLoS One *7*, e46436.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., Shmelkov, S. V., Ramos, C.A., Zhang, F., and Rafii, S. (2005). Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. Blood *106*, 505–513.

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., et al. (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. Nature *502*, 637–643.

Kusumbe, A.P., Ramasamy, S.K., and Adams, R.H. (2014). Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature *507*, 323–328.

Kusumbe, A.P., Ramasamy, S.K., Itkin, T., Mäe, M.A., Langen, U.H., Betsholtz, C., Lapidot, T., and Adams, R.H. (2016). Age-dependent modulation of vascular niches for haematopoietic stem cells. Nature *532*, 380–384.

Lerner, C., and Harrison, D.E. (1990). 5-Fluorouracil spares hemopoietic stem cells responsible for long-term repopulation. Exp. Hematol. *18*, 114–118.

Liebner, S., Cattelino, A., Gallini, R., Rudini, N., Iurlaro, M., Piccolo, S., and Dejana, E. (2004). B-Catenin Is Required for Endothelial-Mesenchymal Transformation During Heart Cushion Development in the Mouse. J. Cell Biol. *166*, 359–367.

Lucas, D., Scheiermann, C., Chow, A., Kunisaki, Y., Bruns, I., Barrick, C., Tessarollo, L., and Frenette, P.S. (2013). Chemotherapy-induced bone marrow nerve injury impairs hematopoietic regeneration. Nat. Med. *19*, 695–703.

Mauch, P., Constine, L., Greenberger, J., Knospe, W., Sullivan, J., Liesveld, J.L., and Deeg, H.J. (1995). Hematopoietic stem cell compartment: Acute and late effects of radiation therapy and chemotherapy. Int. J. Radiat. Oncol. *31*, 1319–1339.

Méndez-Ferrer, S., Lucas, D., Battista, M., and Frenette, P.S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. Nature *452*, 442–447.

Méndez-Ferrer, S., Michurina, T. V, Ferraro, F., Mazloom, A.R., MacArthur, B.D., Lira, S. a, Scadden, D.T., Ma□ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature *466*, 829–834.

Mitroulis, I., Chen, L.-S., Singh, R.P., Kourtzelis, I., Economopoulou, M., Kajikawa, T., Troullinaki, M., Ziogas, A., Ruppova, K., Hosur, K., et al. (2017). Secreted protein Del-1 regulates myelopoiesis in the hematopoietic stem cell niche. J. Clin. Invest. *127*, 3624–3639.

Mizuhashi, K., Ono, W., Matsushita, Y., Sakagami, N., Takahashi, A., Saunders, T.L., Nagasawa, T., Kronenberg, H.M., and Ono, N. (2018). Resting zone of the growth plate houses a unique class of skeletal stem cells. Nature *563*, 254–258.

Mohrin, M., Bourke, E., Alexander, D., Warr, M.R., Barry-Holson, K., Le Beau, M.M., Morrison, C.G., and Passegué, E. (2010). Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. Cell Stem Cell *7*, 174–185.

Morikawa, S., Mabuchi, Y., Niibe, K., Suzuki, S., Nagoshi, N., Sunabori, T., Shimmura, S., Nagai, Y., Nakagawa, T., Okano, H., et al. (2009). Development of mesenchymal stem cells partially originate from the neural crest. Biochem. Biophys. Res. Commun. *379*, 1114–1119.

Naveiras, O., Nardi, V., Wenzel, P.L., Hauschka, P. V., Fahey, F., and Daley, G.Q. (2009). Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature *460*, 259–263.

Niibe, K., Morikawa, S., Mabuchi, Y., Araki, D., Nakagawa, T., Okano, H., and Matsuzaki, Y. (2011). Mesp1+ early paraxial mesodermal cells supply initial bone marrow mesenchymal stem cells capable of differentiating into neural crest lineage cells. Inflamm. Regen. *31*, 116–124.

Nombela-Arrieta, C., and Manz, M.G. (2017). Quantification and three-dimensional microanatomical organization of the bone marrow. Blood Adv. *1*, 407–416.

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., and Nagasawa, T. (2010). The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche. Immunity *33*, 387–399.

Ono, N., Ono, W., Mizoguchi, T., Nagasawa, T., Frenette, P.S., and Kronenberg, H.M. (2014a). Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. Dev. Cell *29*, 330–339.

Ono, N., Ono, W., Nagasawa, T., and Kronenberg, H.M. (2014b). A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. Nat. Cell Biol. *16*, 1157–1167.

Passegue, E., Jamieson, C.H.M., Ailles, L.E., and Weissman, I.L. (2003). Normal and leukemic hematopoiesis: Are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc. Natl. Acad. Sci. *100*, 11842–11849.

Ponomaryov, T., Peled, A., Petit, I., Taichman, R.S., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Magerus, A., Caruz, A., Fujii, N., et al. (2000). Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. J. Clin. Invest. *106*, 1331–1339.

Poulos, M.G., Guo, P., Kofler, N.M., Pinho, S., Gutkin, M.C., Tikhonova, A., Aifantis, I., Frenette, P.S., Kitajewski, J., Rafii, S., et al. (2013). Endothelial Jagged-1 Is Necessary for Homeostatic and Regenerative Hematopoiesis. Cell Rep. *4*, 1022–1034.

Pu, W., Hu, S., He, L., Zhang, L., Zhang, H., Liu, Q., Huang, X., Zhou, B., Lui, K.O., Nie, Y., et al. (2016). Endocardium Contributes to Cardiac Fat. Circ. Res. *118*, 254–265.

Ramasamy, S.K. (2017). Structure and Functions of Blood Vessels and Vascular Niches in Bone. Stem Cells Int. *2017*.

Ramasamy, S.K., Kusumbe, A.P., Wang, L., and Adams, R.H. (2014). Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. Nature *507*, 376–380.

Robey, P.G. (2018). Skeletal Stem Cells/Bone Marrow Stromal Cells. In Genetics of Bone Biology and Skeletal Disease, (Elsevier), pp. 241–260.

Rosselló Castillo, C., Lempereur, A., Souyri, M., Speck, N.A., Jaffredo, T., Piwarzyk, E., Bollérot, K., Canto, P.-Y., Drevon, C., Richard, C., et al. (2013). Endothelio-Mesenchymal Interaction Controls runx1 Expression and Modulates the notch Pathway to Initiate Aortic Hematopoiesis. Dev. Cell *24*, 600–611.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., et al. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell *131*, 324–336.

Sasine, J.P., Yeo, K.T., and Chute, J.P. (2017). Concise Review: Paracrine Functions of Vascular Niche Cells in Regulating Hematopoietic Stem Cell Fate. Stem Cells Transl. Med. *6*, 482–489.

Schürch, C.M., Riether, C., and Ochsenbein, A.F. (2014). Cytotoxic CD8+ T cells stimulate hematopoietic progenitors by promoting cytokine release from bone marrow mesenchymal stromal cells. Cell Stem Cell *14*, 460–472.

Shirota, T., and Tavassoli, M. (1991). Cyclophosphamide-induced alterations of bone marrow endothelium: implications in homing of marrow cells after transplantation. Exp. Hematol. *19*, 369–373.

Silberstein, L., Goncalves, K.A., Kharchenko, P. V., Turcotte, R., Kfoury, Y., Mercier, F., Baryawno, N., Severe, N., Bachand, J., Spencer, J.A., et al. (2016). Proximity-Based Differential Single-Cell Analysis of the Niche to Identify Stem/Progenitor Cell Regulators. Cell Stem Cell *19*, 530–543.

Spencer, J.A., Ferraro, F., Roussakis, E., Klein, A., Wu, J., Runnels, J.M., Zaher, W., Mortensen, L.J., Alt, C., Turcotte, R., et al. (2014). Direct measurement of local oxygen concentration in the bone marrow of live animals. Nature *508*, 269–273.

Sugimura, R., He, X.C., Venkatraman, A., Arai, F., Box, A., Semerad, C., Haug, J.S., Peng, L., Zhong, X.-B., Suda, T., et al. (2012). Noncanonical Wnt signaling maintains hematopoietic stem cells in the niche. Cell *150*, 351–365.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. Immunity *25*, 977–988.

Takashima, Y., Era, T., Nakao, K., Kondo, S., Kasuga, M., Smith, A.G., and Nishikawa, S.I. (2007). Neuroepithelial Cells Supply an Initial Transient Wave of MSC Differentiation. Cell *129*, 1377–1388.

Tormin, A., Li, O., Brune, J.C., Walsh, S., Schutz, B., Ehinger, M., Ditzel, N., Kassem, M., and Scheding, S. (2011). CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. Blood *117*, 5067–5077.

Tzeng, Y.-S., Li, H., Kang, Y.-L., Chen, W.-C., Cheng, W.-C., and Lai, D.-M. (2011). Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. Blood *117*, 429–439.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood *103*, 3258–3264.

Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K., March, C.J., Park, L.S., Martin, U., Mochizukl, D.Y., Boswell, H.S., et al. (1990). Identification of a ligand for the c-kit proto-oncogene. Cell *63*, 167–174.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., et al. (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. Cell *135*, 1118–1129.

Winkler, I.G., Barbier, V., Nowlan, B., Jacobsen, R.N., Forristal, C.E., Patton, J.T., Magnani, J.L., and Lévesque, J.-P. (2012). Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat. Med. *18*, 1651–1657.

Worthley, D.L., Churchill, M., Compton, J.T., Tailor, Y., Rao, M., Si, Y., Levin, D., Schwartz, M.G., Uygur, A., Hayakawa, Y., et al. (2015). Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. Cell *160*, 269–284.

Wylie-Sears, J., Aikawa, E., Levine, R.A., Yang, J.H., and Bischoff, J. (2011). Mitral valve endothelial cells with osteogenic differentiation potential. Arterioscler. Thromb. Vasc. Biol. *31*, 598–607.

Xu, C., Gao, X., Wei, Q., Nakahara, F., Zimmerman, S.E., Mar, J., and Frenette, P.S. (2018). Stem cell factor is selectively secreted by arterial endothelial cells in bone marrow. Nat. Commun. *9*, 1–13.

Yamazaki, S., Ema, H., Karlsson, G., Yamaguchi, T., Miyoshi, H., Shioda, S., Taketo, M.M., Karlsson, S., Iwama, A., and Nakauchi, H. (2011). Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. Cell *147*, 1146–1158.

Yokomizo, T., and Dzierzak, E. (2010). Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. Development *137*, 3651–3661.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.-G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., et al. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836–841.

Zhao, M., Perry, J.M., Marshall, H., Venkatraman, A., Qian, P., He, X.C., Ahamed, J., and Li, L. (2014). Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat. Med. *20*, 1321–1326.

Zheng, B., Cao, B., Crisan, M., Sun, B., Li, G., Logar, A., Yap, S., Pollett, J.B., Drowley, L., Cassino, T., et al. (2007). Prospective identification of myogenic endothelial cells in human skeletal muscle. Nat. Biotechnol. *25*, 1025–1034.

Zhou, B.O., Yue, R., Murphy, M.M., Peyer, J.G., and Morrison, S.J. (2014). Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. Cell Stem Cell *15*, 154–168.

Zhou, B.O., Yu, H., Yue, R., Zhao, Z., Rios, J.J., Naveiras, O., and Morrison, S.J. (2017). Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. Nat. Cell Biol. *19*, 891–903.

Zhu, J., Garrett, R., Jung, Y., Zhang, Y., Kim, N., Wang, J., Joe, G.J., Hexner, E., Choi, Y., Taichman, R.S., et al. (2007). Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. Blood *109*, 3706–3712.

2

CHARACTERIZATION OF ENDOTHELIAL CELLS ASSOCIATED WITH HEMATOPOIETIC NICHE FORMATION IN HUMANS IDENTIFIES IL-33 AS AN ANABOLIC FACTOR

Keane Jared Guillaume Kenswil[1], Adrian Jaramillo Mantilla[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 der Eerden[4], Tom Cupedo[1], 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, Rotterdam 3015CN, The Netherlands
[3]Department of Pathology, Erasmus MC, Rotterdam 3015CN, The Netherlands
[4]Department of Internal Medicine, Laboratory for Calcium and Bone Metabolism, Rotterdam, The Netherlands.
[5]Lead Contact: m.h.g.raaijmakers@erasmusmc.nl
*Correspondence: m.h.g.raaijmakers@erasmusmc.nl

Cell Reports 2018

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 H endothelium, and activation of angiocrine factors implicated in hematopoiesis, osteogenesis and angiogenesis. 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.

KEYWORDS

Endothelial, bone marrow, niche, regeneration, development, hematopoietic stem/progenitor cell, bone formation, interleukin-33

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 following 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 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, 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 HSPC number in addition to coupling angiogenesis and osteogenesis (Kusumbe et al., 2016, 2014; 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 bone marrow regeneration after chemotherapeutic injury

To identify niche cells potentially implicated in regeneration of the hematopoietic system in humans, we interrogated the composition of the hematopoietic niche upon 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 vs 31.57% ± 9.66; p=0.15 by unpaired student t-test) (Figure 1A, B). To identify putative immuno-markers of EC subtypes emerging during regeneration, we performed massive parallel sequencing (RNAseq) of the fluorescence-activated cell sorting (FACS)-purified endothelial compartment ($CD31^+CD9^+$) (Figure 1A). 903 transcripts were found to be significantly differentially expressed in ECs in the regenerative marrow in comparison to ECs from normal, steady-state, marrow (GLM LRT Edge R; 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 (TGFβ) 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 to normal BM (10.81% ± 2.14 vs 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 $CD31^+CD9^+$) or after full recovery of peripheral blood values (in complete remission) (1.27% ± 0.66 of $CD31^+CD9^+$) (Figure 1E).

Endoglin-expressing ECs are enriched in the mouse bone marrow after chemotherapeutic myeloablation

To confirm our observation that the CD105 expressing subset of endothelial cells is enriched during bone marrow 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 ($CD31^+CD9^+$) 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) (Figure

2A, B and S1A, B). 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) (Figure 2A, B and S1A, B), 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 bone marrow 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.

Endoglin-expressing ECs are enriched in the human bone marrow 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 from 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, 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 bone marrow at gestational week 15-20 revealed a striking predominance of CD105-expressing cells within the endothelial compartment (62.8% ± 5.9 of $CD31^+CD9^+$) (Figure 3A, B). Massive parallel sequencing of these cells confirmed overexpression of genes encoding CD markers identified in the endothelial cells in regenerating bone marrow (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 $CD31^+CD9^+$) (Figure 3A, B), 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 considerably lower frequency, in collagenased bone fractions of human adult postnatal bone (average 25.9% ± 5.0 of $CD31^+CD9^+$) (Figure 3A,B).

To provide anatomical context for this $CD31^+CD105^+$ EC subset, we performed *in situ* immunohistochemistry and immunofluorescence on fetal femurs and core hip bone biopsies obtained from adults. $CD31^+CD105^+$ EC were observed in the trabecular bone area of the

metaphysis of fetal femurs (Figure 3C and S3A, B) at a significant higher frequency than in the trabecular bone of adult bone marrow. The majority of vascular structures in both fetal and adult human bone were lined with $CD31^{-}CD105^{+}$ endothelial cells, previously shown to be sinusoid endothelial cells. $CD31^{+}CD105^{-}$ 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 upon 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 EC' or hREC.

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., 2016, 2014; Ramasamy et al., 2014). These include the enrichment at the bone surface, reduced frequencies upon aging and an increase in frequency upon 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 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 (Figure 4A, B) (the limited number of $CD105^{+}$ precluded RNA-sequencing of this specific subset). In total, 3718 genes were differentially expressed (GLM LRT Edge R; 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 hREC 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 H endothelium as key promotors of the formation of type H capillaries and 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, NOTCH 1 and NOTCH4 (receptors of DLL4) were upregulated in fetal hRECs (Figure 4E), reflected in activation of downstream transcriptional NOTCH signaling, as demonstrated by GSEA (Figure 4F). Similarly, gene sets related to the HIF1a pathway were enriched in fetal hREC 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 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.

Interleukin-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 both significantly enriched in 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, 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 interleukin-33 (*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 hREC was confirmed using fluorescence microscopy (Figure 5B) and IL-33 was differentially overexpressed in murine $CD31^{+}CD105^{+}$ endothelial cells in comparison to their $CD105^{-}$ counterparts during regeneration (Figure 2D, E). 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 a week in serum-free medium containing SCF. rhIL-33 expanded the total number of hematopoietic cells (total MNCs) (3.4-fold increase ± 0.29; $p<0.01$) with a concomitant expansion of hematopoietic progenitors cells (HPCs), specifically immunophenotypic multipotent progenitors (MPPs) (2.5-fold increase ± 0.43; $p<0.05$) and multilymphoid progenitors (MLPs) (2.7-fold increase ± 0.15; $p<0.01$) (Figure 5C,

S4). Immunophenotypic HSC numbers were not affected by exposure to IL-33. Expansion of myeloid progenitor cells was confirmed in colony-forming assays (CFU-C) demonstrating an increase in 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. Addition of rhIL-33 to osteogenic induction medium accelerated terminal differentiation of BMDSCs towards 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 (Figure S5A, B), suggesting that IL-33 exerted its osteogenic effect by promoting osteoblastic differentiation or the secretion of matrix proteins by osteoblasts, rather than 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 (Figure S5D, E) from 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 bone marrow niche in mice

We next studied the *in vivo* relevance of these effects of IL-33 on distinct cellular components of the human bone marrow. Administration of recombinant murine IL-33 (rmIL-33) compared to PBS control resulted in expansion of immature (Lin^-) (Figure S6A) and primitive progenitor (LKS) hematopoietic cells, in particular the HPC-1 population (Figure 7A, B), earlier 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 (Figure 7A, B, and Figure S6D, E), 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 (Figure 7A, B). Interestingly, IL-33 also expanded significantly the population of $Lin^-Kit^-Sca1^+$ bone marrow cells, previously shown to contain

early lymphoid-committed precursors with T cell, B-cell and NK cell potential (Kumar et al., 2008) and innate lymphoid cells (Brickshawana et al., 2011). Hematopoietic changes were accompanied by a relative increase in $CD31^{+}CD105^{+}$ ECs as well as $Lin^{-}Ter119^{-}CD51^{+}Sca^{-}$ cells (earlier shown to contain lineage committed/osteoblastic cells) (Schepers et al., 2012), albeit not reaching statistical significance, within the niche compartment (Figure S7A, B).

Collectively, the *in vitro* and *in vivo* data indicate that IL-33 modulates distinct cellular components of the 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 (hREC) associated with these conditions. hRECs share phenotypic and molecular similarities with specialized EC driving hematopoietic niche formation in mice, expressing critical regulators of hematopoiesis, osteogenesis and angiogenesis. The data comprises, to our knowledge, the first comprehensive molecular characterization of human ECs upon 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 endothelial cells 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 following injury such as myeloablation (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, hREC 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), 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 behaviour 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), tenascin C (*TNC)* (Figure S2E) (Nakamura-Ishizu et al., 2012).

In particular, hRECs displayed striking commonalities with 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 upon ageing, resistance to stress conditions, expression of markers typical for both arterial (Ephn2b, Nestin, Nrp1, Sox17, VEGFR2), and sinusoidal vessels (VEGFR3, EMCN) and activation of the NOTCH and HIF1 pathways driving regeneration.

Expression of CD105 was not addressed in the studies on H endothelium but our data in mice show that a rare population of endoglin expressing endothelial cells 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 upon regeneration following 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 towards 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 by 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. IL33 was overexpressed in human hRECs and expression was increased in murine $CD31^+CD105^+$ ECs upon exposure to 5FU. Hematopoietic niche regenerating properties of IL33 was demonstrated by its *ex vivo* capacity to facilitate hematopoiesis (increased numbers of HPCs), osteogenesis (accelerating terminal differentiation of BMDSCs towards 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 human recombinant IL-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, as it is currently challenging to maintain this particular subset of ECs *ex vivo* to enable co-culture experiments. The profound effect of IL33 knockdown on HUVEC proliferation and maintenance precluded use of this *ex vivo* system to address this question.

The combined findings point to a unique role of IL-33 in mammalian species facilitating the reconstitution of both hematopoietic lineages, which may be of considerable importance to prepare the hematopoietic system for 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 (Sundlisæter et al., 2012).

Regardless 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 proteins and pathways driving niche formation after injury. It is conceivable that receptor-ligand interactions allows 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 potential relevance of autocrine signaling in the biology of hRECs. Alternatively, it will be worthwhile testing the ability of identified secreted factors to expand human HSPC *ex vivo*, either direct or in co-culture settings with mesenchymal elements.

Collectively, the identification of human ECs associated with hematopoietic niche formation and elucidation of their transcriptome is 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.

Figure 1

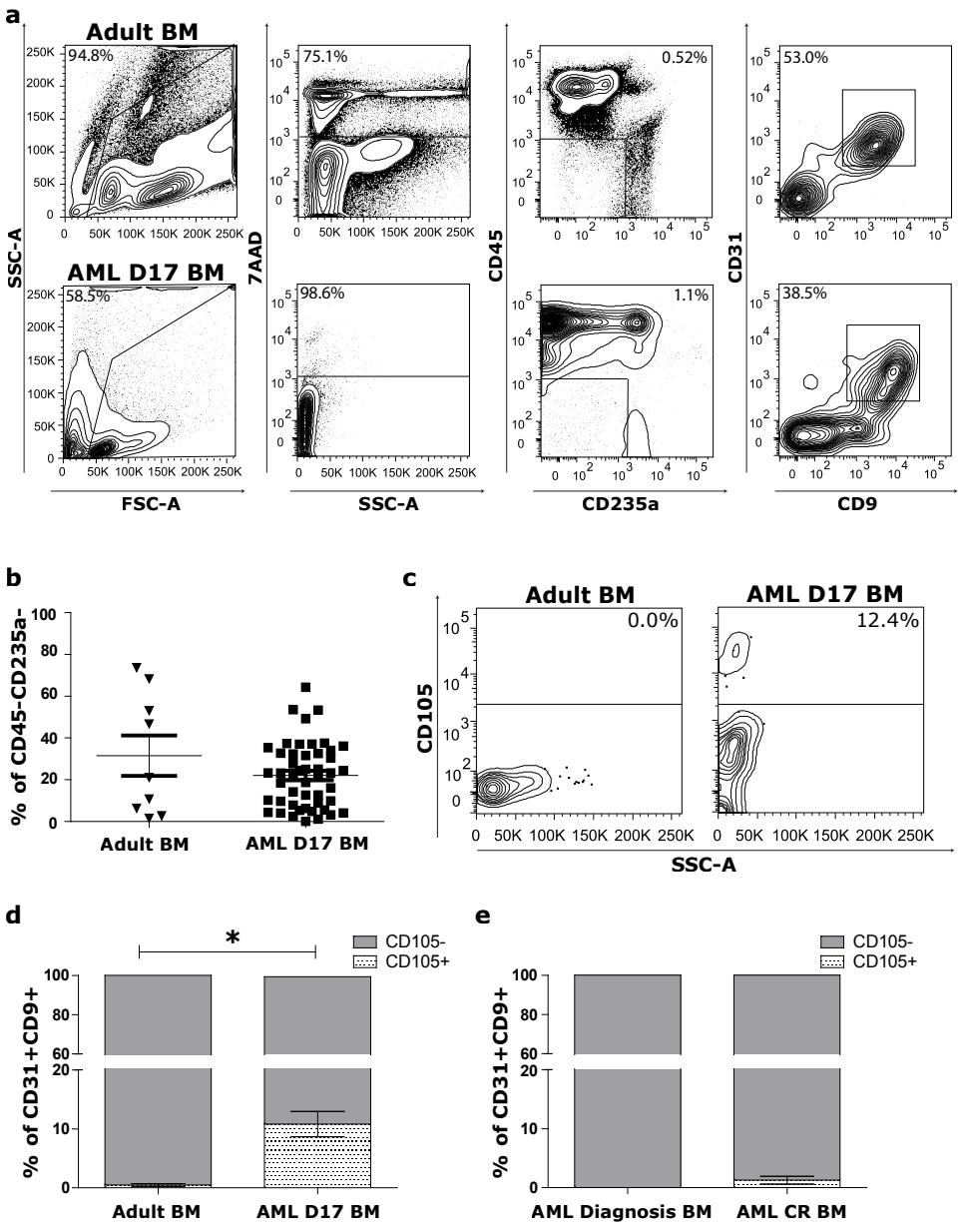

a
Adult BM
AML D17 BM
94.8%
75.1%
0.52%
53.0%
58.5%
98.6%
1.1%
38.5%
SSC-A
7AAD
CD45
CD31
FSC-A
SSC-A
CD235a
CD9
b
% of CD45-CD235a-
Adult BM
AML D17 BM
c
Adult BM
AML D17 BM
0.0%
12.4%
CD105
SSC-A
d
CD105-
CD105+
% of CD31+CD9+
Adult BM
AML D17 BM
e
CD105-
CD105+
% of CD31+CD9+
AML Diagnosis BM
AML CR BM

Figure 1. Identification of CD105 (endoglin)-expressing endothelial cells associated with bone marrow regeneration after chemotherapeutic injury

Bone marrow (BM) obtained by aspirates upon recovery after chemotherapeutic injury (AML D17) was compared to normal adult BM.

a. Gating strategy for identification and isolation of endothelial cells (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 ± s.e.m. *p <0.05, two-tailed unpaired t-test. AML=Acute Myeloid Leukemia. BM=Bone marrow. D17=Day 17. CR=Complete Remission.

Figure 2

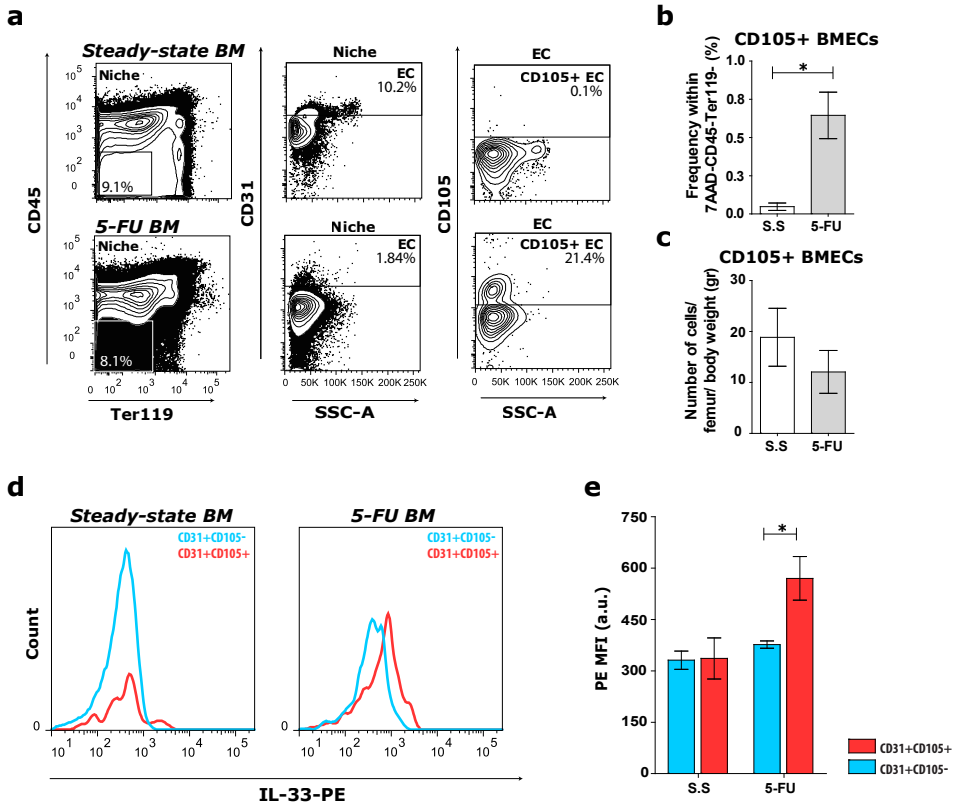


Figure 2. CD105-expressing endothelial cells are enriched in mice during regeneration after chemotherapeutic injury

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 upon 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,e. CD105$^+$ BM ECs differentially express IL-33 upon 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 ± s.e.m. *p <0.05, two-tailed unpaired t-test. S.S=Steady-state. 5-FU=5-fluorouracil.

Figure 3

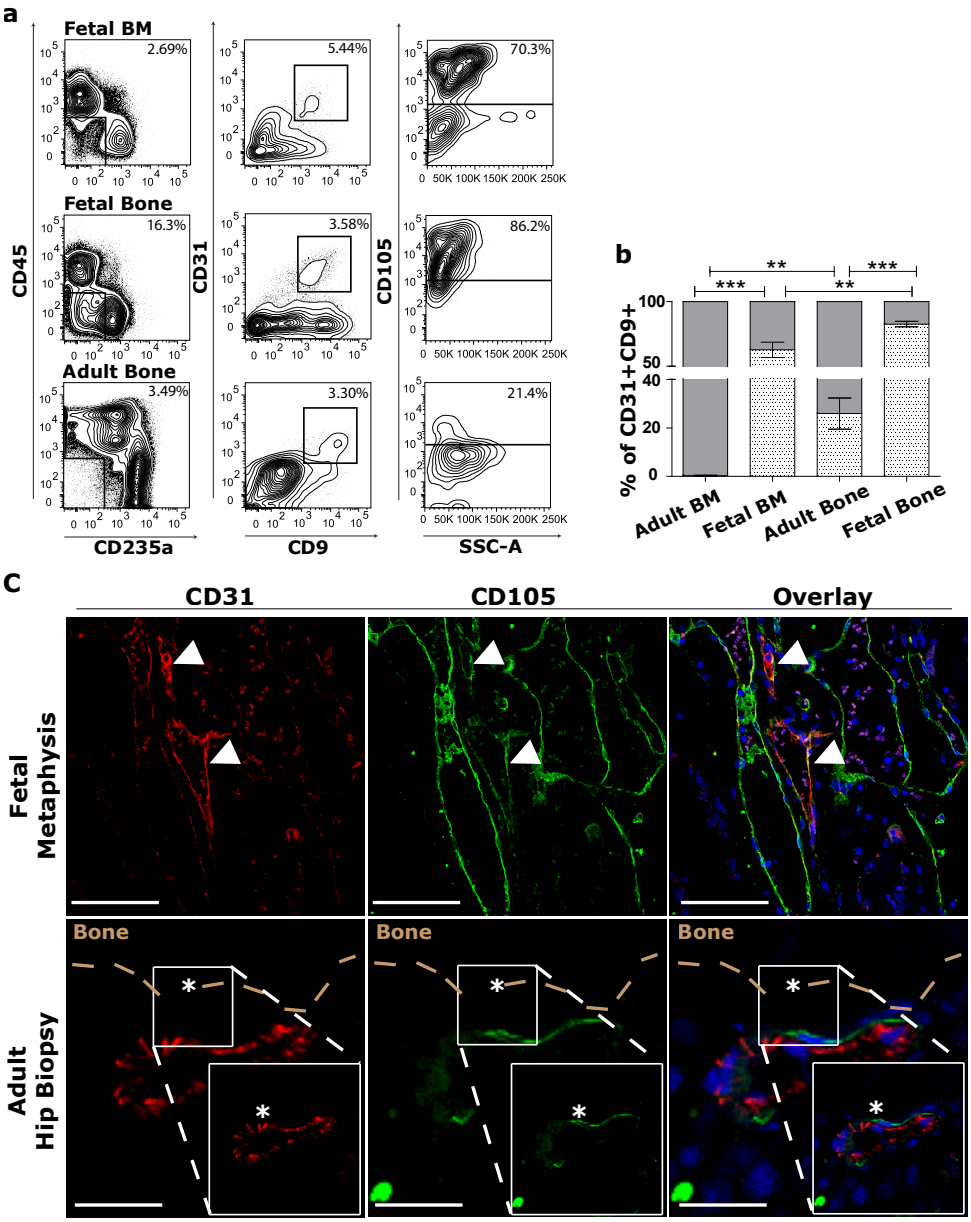


Figure 3. CD105-expressing endothelial cells are enriched in fetal development and collagenized bone fractions

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 ± s.e.m. **p <0.01, ***p <0.001, one-way ANOVA (p< 0.0001) followed by Bonferroni's Multiple Comparison Test. BM= Bone marrow.

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

Figure 4

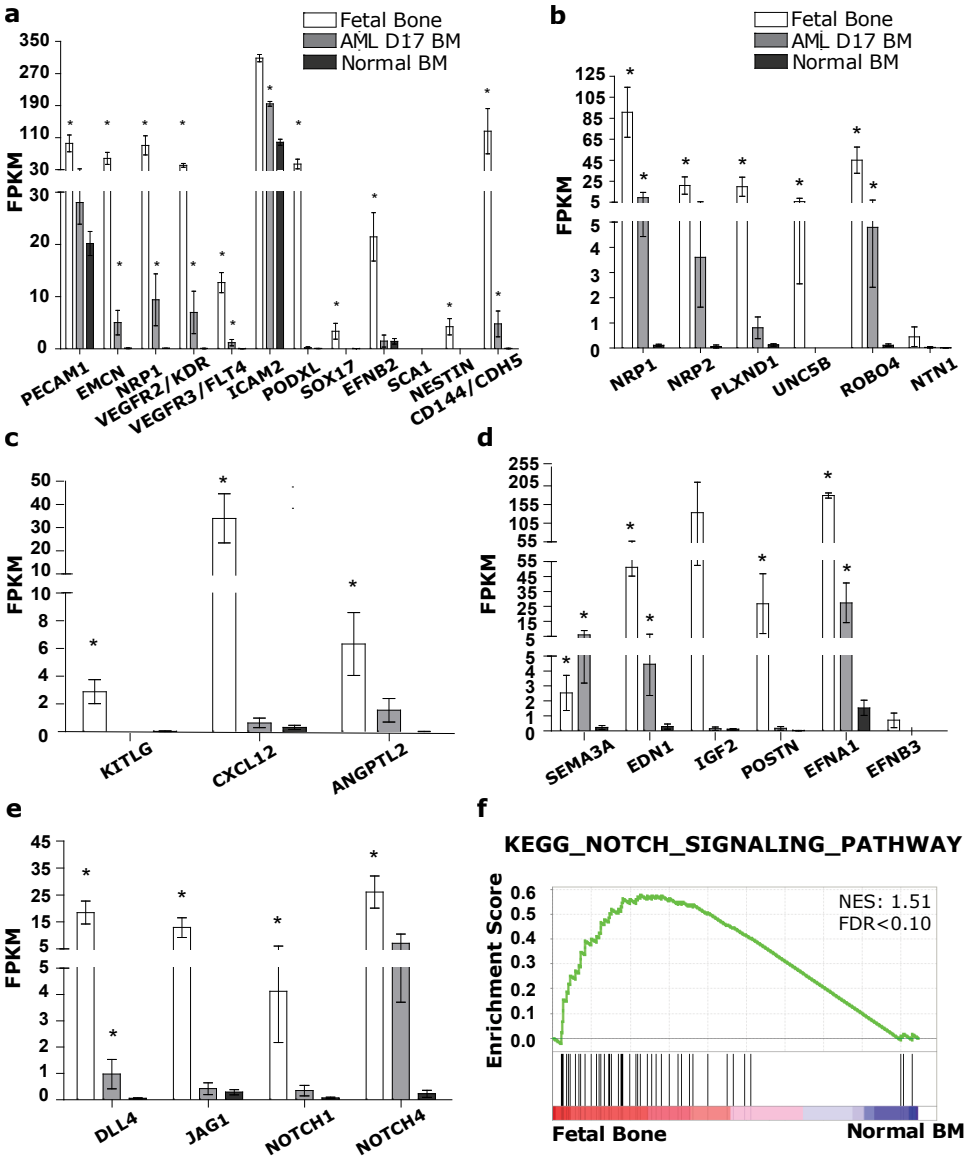

a
Fetal Bone
AML D17 BM
Normal BM
FPKM
PECAM1
EMCN
NRP1
VEGFR2/KDR
VEGFR3/FLT4
ICAM2
PODXL
SOX17
EFNB2
SCA1
NESTIN
CD144/CDH5
b
Fetal Bone
AML D17 BM
Normal BM
FPKM
NRP1
NRP2
PLXND1
UNC5B
ROBO4
NTN1
c
FPKM
KITLG
CXCL12
ANGPTL2
d
FPKM
SEMA3A
EDN1
IGF2
POSTN
EFNA1
EFNB3
e
FPKM
DLL4
JAG1
NOTCH1
NOTCH4
f
KEGG_NOTCH_SIGNALING_PATHWAY
Enrichment Score
NES: 1.51
FDR<0.10
Fetal Bone
Normal BM

Figure 4. hRECs express transcriptional programs defining niche-forming endothelial cells in mice
Transcriptomes of hRECS isolated from human fetal bone ($CD45^-CD235^-CD31^+CD9^+CD105^+$)(n=3) and regenerative bone marrow ($CD45^-CD235^-CD31^+CD9^+$)(n=3) were compared to postnatal steady-state BM ECs ($CD45^-CD235^-CD31^+CD9^+$)(n=7).

a,b. Expression of genes previously reported to be overexpressed in murine type H endothelial cells (Itkin et al., 2016; Kusumbe et al., 2016, 2014; 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,f. Expression of genes encoding critical NOTCH pathway components (e) and activation of Notch- signaling (f) as demonstrated gene sets enrichment analysis (GSEA).

FPKM: fragments per kilobase of exon per million fragments mapped. NES: normalized enrichment score. FDR: false discovery rate. *FDR<0.05.

Figure 5

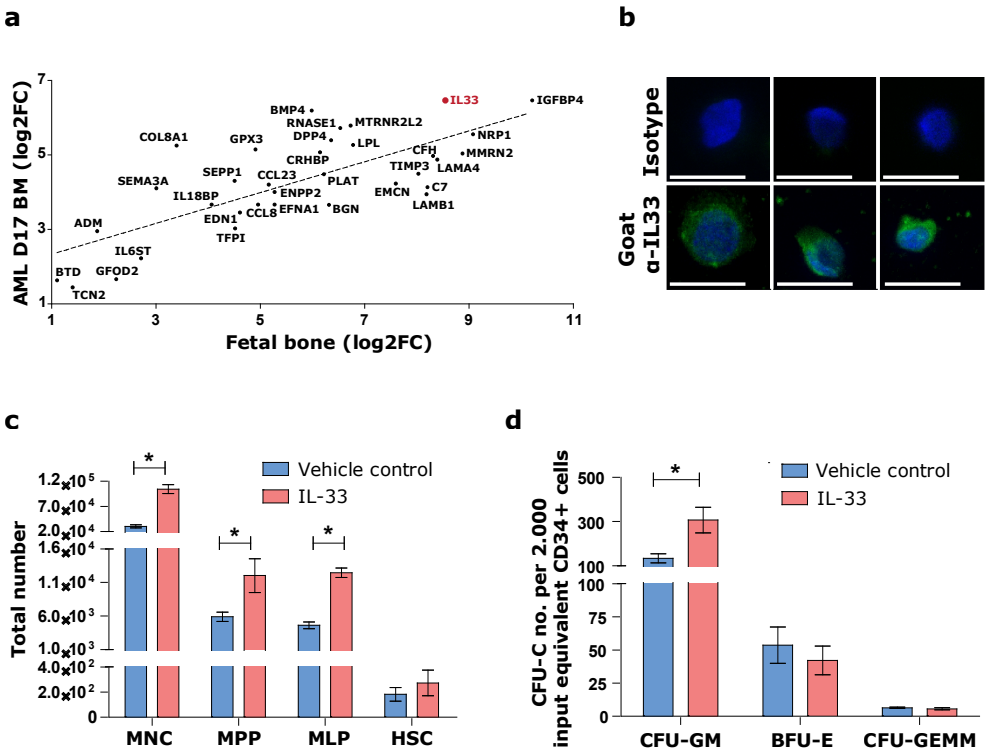


Figure 5. Identification of IL-33 as a hematopoietic niche factor

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 bone marrow). FDR: false discovery rate. FC: Fold Change.

b. 3x magnification cropped pictures of immunostained fetal hRECs demonstrating protein expression of IL-33. White scale bar represents 20 µm.

c,d. IL-33 mediated expansion of cord blood derived myeloid and lymphoid progenitor cells as demonstrated by flow-cytometric cell counting (c) (MPP, multipotent progenitor, $CD90^{-}CD45RA^{-}$; MLP, multilymphoid progenitor, $CD90^{-}CD45RA^{+}$; HSC, hematopoietic stem cell, $CD90^{+}CD45RA^{-}$) (n= 4 independent experiments) and colony-forming assays (CFU-C) (d) confirming an increase in CFU-GMs (n= 3 independent experiments).

Data represent mean ± s.e.m. *p<0.05, two-tailed unpaired t-test.

Figure 6

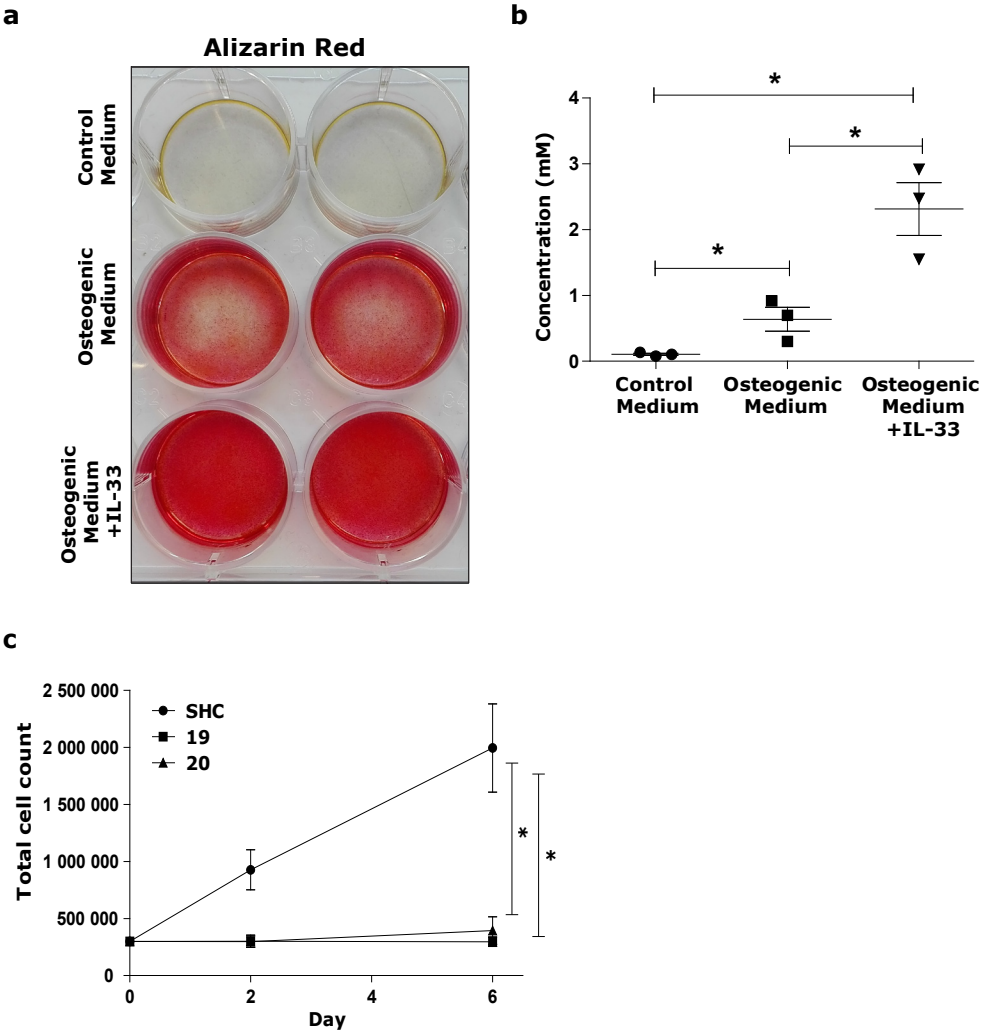


Figure 6. IL-33 promotes osteogenesis and angiogenesis *in vitro*

a,b. IL-33 accelerates terminal differentiation of mesenchymal cells towards matrix-depositing osteoblasts as demonstrated by Alizarin red-staining (a) and colorimetric assessment of calcium deposition (b) (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 ± s.e.m. *p <0.05, two-tailed unpaired t-test.

Figure 7

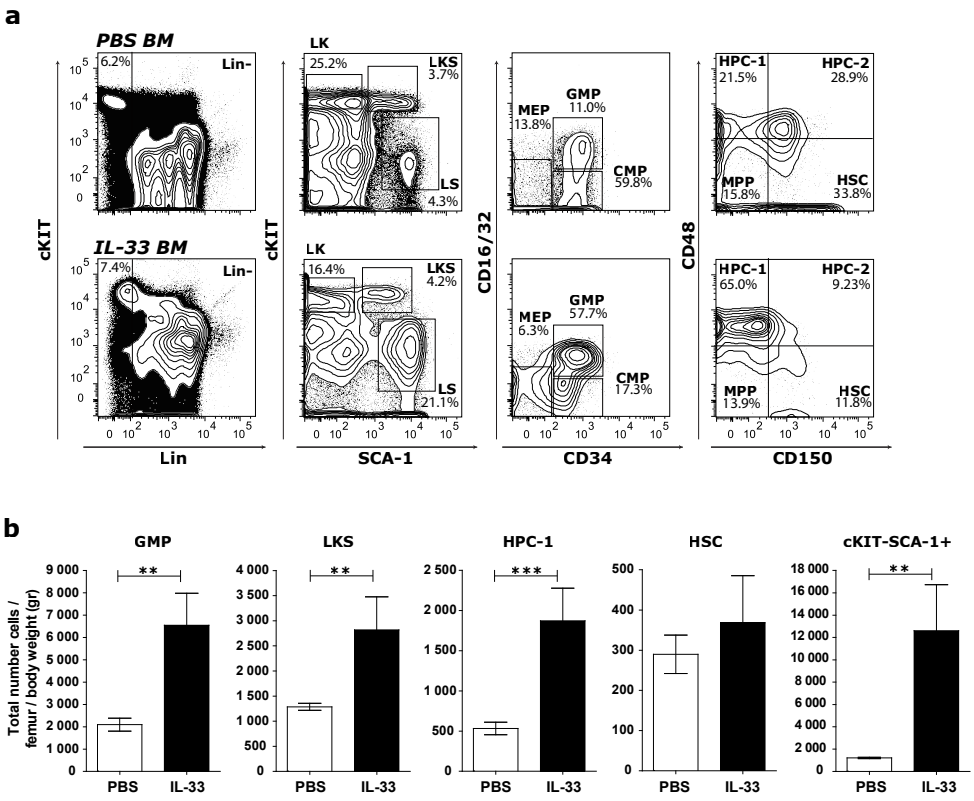


Figure 7. Recombinant IL-33 expands hematopoietic progenitors *in vivo*

a. Representative FACS plots of PBS vs IL-33 treated mice depicting the distribution of hematopoietic (progenitor) populations. **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. **GMP**: LK, CD16/32$^+$, CD34$^+$ cells, granulocyte-macrophage progenitor. **CMP**: LK, CD16/32$^-$, CD34$^+$ cells, common myeloid progenitor. **HPC-1**: LKS, CD48$^+$, CD150$^-$ cells, hematopoietic progenitor -1. **HPC-2**: LKS, CD48$^+$,CD150$^+$ cells, hematopoietic progenitor-2. **MPP**: LKS, CD48$^-$CD150$^-$ cells, multipotent progenitor. **HSC**: LKS, CD48$^-$CD150$^+$ cells, hematopoietic stem cell.

b. Total BM counts of GMP, LKS, HPC-1, HSC and LS populations in PBS (n=10) and IL-33 (n=5) treated mice.

Data represent mean ± s.e.m. *p<0.05, **p<0.01, two-tailed unpaired t-test.

EXPERIMENTAL PROCEDURES

Human bone marrow samples

Bone marrow (BM) aspirates of AML patients were collected at diagnosis, 17 days after start chemotherapy (3+7 schedule anthracycline and cytarabin) and upon achievement of complete remission (median age: 65 years, range 28-76). The time point of 17 days after 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) after written informed consent. In addition, trabecular hip bone was collected from patients undergoing hip replacement surgery (median age: 55 years, range 22-71). Human fetal long bones (median age: 18 gestational weeks, range 15-20) were obtained from elective abortions. Gestational age was confirmed by ultrasonic measurement by measurement of skull diameter and femoral length. The use of human samples was approved by the Institutional Review Board of the Erasmus Medical Center, the Netherlands, in accordance with the declaration of Helsinki with informed consent.

RNA Sequencing 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-sequencing was performed as previously described and validated for low-input (Chen et al., 2016). Finally gene set enrichment analysis (GSEA) was performed on the FPKM values using the curated C2 collection of gene sets within 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 with 250 mg/kg 5-fluorouracil (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 (580504, Biolegend) or PBS vehicle control daily for 6 consecutive days and then sacrificed.

All mice were sacrificed by cervical dislocation. Mouse bone marrow (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$^+$ cord blood cells

20,000 CD34$^+$ cord blood (CB) cells in 200 µl per well were cultured in StemSpan™ SFEM (Stem cell technologies, cat. 9600) with Stem Cell Factor (SCF) (50 ng/ml, Cellgenix, Freiburg, Germany), in a flat-bottom 96-well plate, at 37ºC and 5% CO2. 2 µl mQ solution containing only recombinant human IL-33 protein (ProSpec, cat. CYT-425) or just mQ (vehicle control) was added to the medium for a final concentration of 25 ng/ml IL-33 or 1% mQ, respectively. The medium was refreshed every 3 or 4 days and cells collected at day 7 for FACs analysis or for hematopoietic colony forming-unit assay.

Culture of human BM-derived mesenchymal stromal cells

Human BM-derived mesenchymal stromal cells (BMDSCs) (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 FCS supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate) with recombinant human IL-33 (250 ng/ml) or mQ vehicle control for 3 weeks. Medium was refreshed every 3-4 days.

Culture of and shRNA-mediated knockdown of IL-33 in HUVECs

Human umbilical vein endothelial cells (HUVECs) were expanded in EGM-2 Bulletkit medium (CC-3156 & CC-4176, Lonza). RNA interference was achieved by lentiviral transduction. Briefly, short hairpin RNAs 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 cotransfection of shControl, sh19, or sh20 together with the packaging plasmids pSPAX2 and pMDG.2. HUVECs were infected with lentivirus for 24 hours 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 two means) or 1-way analysis of variance followed by Bonferroni correction for multiple comparisons were used to evaluate statistical significance, defined as $P<0.05$. All results in bar graphs are mean value ± standard error of the mean.

AUTHOR CONTRIBUTIONS

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

ACCESSION NUMBERS

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

ACKNOWLEDGEMENTS

We would like to thank Dr. Eric Braakman, Lucia Duinhouwer, and Mariëtte ter Borg for providing $CD34^+$ cells; Nathalie Papazian for helping to process fetal tissue. Kirsten van Lom for coordinating human sampling. Marijke Koedam for preparing and culture of BMDSCs. Dr. Moniek de Maat for providing HUVECs. Dr. Elwin Rombouts, Peter van Geel, Paulette van Strien and Michael Vermeulen for operating the BD FACSARIA III; Noemi Zambetti, Onno Roovers, Jyotirmaya Behera, Lucia Duinhouwer, Marijke Koedam, Inge Snoeren, Almira Henić, Dr. Rebekka Schneider and Gert-Jan Kremers for providing technical assistance; members of the Erasmus MC Department of Hematology for providing scientific discussion.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Barreiro, O., Yáñez-Mó, M., Sala-Valdés, M., Gutiérrez-López, M.D., Ovalle, S., Higginbottom, A., Monk, P.N., Cabañas, C., Sánchez-Madrid, F., 2005. Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. Blood 105, 2852–2861. https://doi.org/10.1182/blood-2004-09-3606

Blumenfeld, I., Srouji, S., Lanir, Y., Laufer, D., Livne, E., 2002. Enhancement of bone defect healing in old rats by TGF-β and IGF-1. Exp. Gerontol. 37, 553–565. https://doi.org/10.1016/S0531-5565(01)00215-7

Brickshawana, A., Shapiro, V.S., Kita, H., Pease, L.R., 2011. Lineage(-)Sca1+c-Kit(-)CD25+ cells are IL-33-responsive type 2 innate cells in the mouse bone marrow. J. Immunol. 187, 5795–804. https://doi.org/10.4049/jimmunol.1102242

Broudy, V.C., 1997. Stem cell factor and hematopoiesis. Blood 90, 1345–64.

Brum, A.M., van de Peppel, J., van der Leije, C.S., Schreuders-Koedam, M., Eijken, M., van der Eerden, B.C.J., van Leeuwen, J.P.T.M., 2015. Connectivity Map-based discovery of parbendazole reveals targetable human osteogenic pathway. Proc. Natl. Acad. Sci. 112, 12711–12716. https://doi.org/10.1073/pnas.1501597112

Butler, J.M., Nolan, D.J., Vertes, E.L., Varnum-Finney, B., Kobayashi, H., Hooper, A.T., Seandel, M., Shido, K., White, I.A., Kobayashi, M., Witte, L., May, C., Shawber, C., Kimura, Y., Kitajewski, J., Rosenwaks, Z., Bernstein, I.D., Rafii, S., 2010. Endothelial Cells Are Essential for the Self-Renewal and Repopulation of Notch-Dependent Hematopoietic Stem Cells. Cell Stem Cell 6, 251–264. https://doi.org/10.1016/j.stem.2010.02.001

Carriere, V., Roussel, L., Ortega, N., Lacorre, D.-A., Americh, L., Aguilar, L., Bouche, G., Girard, J.-P., 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proc. Natl. Acad. Sci. 104, 282–287. https://doi.org/10.1073/pnas.0606854104

Cayrol, C., Girard, J.P., 2014. IL-33: An alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr. Opin. Immunol. 31, 31–37. https://doi.org/10.1016/j.coi.2014.09.004

Charbord, P., Tavian, M., Humeau, L., Peault, B., 1996. Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. Blood 87, 4109–4119. https://doi.org/10.1182/blood.V87.10.4109.bloodjournal87104109

Cheifetz, S., Bellón, T., Calés, C., Vera, S., Bernabeu, C., Massagué, J., Letarte, M., 1992. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J. Biol. Chem. 267, 19027–30.

Chen, S., Zambetti, N.A., Bindels, E.M.J., Kenswill, K., Mylona, A.M., Adisty, N.M., Hoogenboezem, R.M., Sanders, M.A., Cremers, E.M.P., Westers, T.M., Jansen, J.H., van de Loosdrecht, A.A., Raaijmakers, M.H.G.P., 2016. Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context-dependent activation of inflammatory programs. Leukemia 30, 1938–42. https://doi.org/10.1038/leu.2016.91

Choi, Y., Choi, H., Min, J., Pyun, B., Maeng, Y., Park, H., Kim, J., Kim, Y., Kwon, Y., 2009. ST2 / TRAF6-mediated endothelial nitric oxide production Interleukin-33 induces angiogenesis and vascular permeability through ST2 / TRAF6-mediated endothelial nitric oxide production. Blood 114, 3117–3126. https://doi.org/10.1182/blood-2009-02-203372.The

Clines, G. a, Mohammad, K.S., Bao, Y., Stephens, O.W., Suva, L.J., Shaughnessy, J.D., Fox, J.W., Chirgwin, J.M., Guise, T. a, 2007. Dickkopf Homolog 1 Mediates Endothelin-1-Stimulated New Bone Formation. Mol. Endocrinol. 21, 486–498. https://doi.org/10.1210/me.2006-0346

Coşkun, S., Chao, H., Vasavada, H., Heydari, K., Gonzales, N., Zhou, X., de Crombrugghe, B., Hirschi, K.K., 2014. Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. Cell Rep. 9, 581–90. https://doi.org/10.1016/j.celrep.2014.09.013

Doan, P.L., Russell, J.L., Himburg, H.A., Helms, K., Harris, J.R., Lucas, J., Holshausen, K.C., Meadows, S.K., Daher, P., Jeffords, L.B., Chao, N.J., Kirsch, D.G., Chute, J.P., 2013. Tie2 + Bone Marrow Endothelial Cells Regulate Hematopoietic Stem Cell Regeneration Following Radiation Injury. Stem Cells 31, 327–337. https://doi.org/10.1002/stem.1275

Duff, S.E., Li, C., Garland, J.M., Kumar, S., 2003. CD105 is important for angiogenesis: evidence and potential applications. FASEB J. 17, 984–992. https://doi.org/10.1096/fj.02-0634rev

Edwards, C.M., Mundy, G.R., 2008. Eph receptors and ephrin signaling pathways: A role in bone homeostasis. Int. J. Med. Sci. 5, 263–272. https://doi.org/10.7150/ijms.5.263

Gur-Cohen, S., Itkin, T., Chakrabarty, S., Graf, C., Kollet, O., Ludin, A., Golan, K., Kalinkovich, A., Ledergor, G., Wong, E., Niemeyer, E., Porat, Z., Erez, A., Sagi, I., Esmon, C.T., Ruf, W., Lapidot, T., 2015. PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. Nat. Med. 21, 1307–17. https://doi.org/10.1038/nm.3960

Holmes, C., Stanford, W.L., 2007. Concise Review: Stem Cell Antigen-1: Expression, Function, and Enigma. Stem Cells 25, 1339–1347. https://doi.org/10.1634/stemcells.2006-0644

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.-G., Shido, K., Petit, I., Yanger, K., James, D., Witte, L., Zhu, Z., Wu, Y., Pytowski, B., Rosenwaks, Z., Mittal, V., Sato, T.N., Rafii, S., 2009. Engraftment and Reconstitution of Hematopoiesis Is Dependent on VEGFR2-Mediated Regeneration of Sinusoidal Endothelial Cells. Cell Stem Cell 4, 263–274. https://doi.org/10.1016/j.stem.2009.01.006

Ibrahim, A.A., Yahata, T., Onizuka, M., Dan, T., Van Ypersele De Strihou, C., Miyata, T., Ando, K., 2014. Inhibition of plasminogen activator inhibitor type-1 activity enhances rapid and sustainable hematopoietic regeneration. Stem Cells 32, 946–958. https://doi.org/10.1002/stem.1577

Itkin, T., Gur-Cohen, S., Spencer, J.A., Schajnovitz, A., Ramasamy, S.K., Kusumbe, A.P., Ledergor, G., Jung, Y., Milo, I., Poulos, M.G., Kalinkovich, A., Ludin, A., Kollet, O., Shakhar, G., Butler, J.M., Rafii, S., Adams, R.H., Scadden, D.T., Lin, C.P., Lapidot, T., 2016. Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature 532, 323–328. https://doi.org/10.1038/nature17624

Jagannathan-Bogdan, M., Zon, L.I., 2013. Hematopoiesis. Development 140, 2463–7. https://doi.org/10.1242/dev.083147

Kakkar, R., Hei, H., Dobner, S., Lee, R.T., 2012. Interleukin 33 as a mechanically responsive cytokine secreted by living cells. J. Biol. Chem. 287, 6941–6948. https://doi.org/10.1074/jbc.M111.298703

Khurana, S., Margamuljana, L., Joseph, C., Schouteden, S., Buckley, S.M., Verfaillie, C.M., 2013. Glypican-3-mediated inhibition of CD26 by TFPI: a novel mechanism in hematopoietic stem cell homing and maintenance. Blood 121, 2587–2595. https://doi.org/10.1182/blood-2012-09-456715

Kim, J., Kim, W., Le, H.T., Moon, U.J., Tran, V.G., Kim, H.J., Jung, S., Nguyen, Q.-T., Kim, B.-S., Jun, J.-B., Cho, H.R., Kwon, B., 2014. IL-33–Induced Hematopoietic Stem and Progenitor Cell Mobilization Depends upon CCR2. J. Immunol. 193, 3792–3802. https://doi.org/10.4049/jimmunol.1400176

Kobayashi, H., Butler, J.M., O'Donnell, R., Kobayashi, M., Ding, B.-S., Bonner, B., Chiu, V.K., Nolan, D.J., Shido, K., Benjamin, L., Rafii, S., 2010. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. Nat. Cell Biol. 12, 1046–1056. https://doi.org/10.1038/ncb2108

Kopp, H.G., Avecilla, S.T., Hooper, A.T., Shmelkov, S. V., Ramos, C.A., Zhang, F., Rafii, S., 2005. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. Blood 106, 505–513. https://doi.org/10.1182/blood-2004-11-4269

Kopp, H.G., Hooper, A.T., Avecilla, S.T., Rafii, S., 2009. Functional heterogeneity of the bone marrow vascular niche. Ann. N. Y. Acad. Sci. 1176, 47–54. https://doi.org/10.1111/j.1749-6632.2009.04964.x

Küchler, A.M., Pollheimer, J., Balogh, J., Sponheim, J., Manley, L., Sorensen, D.R., De Angelis, P.M., Scott, H., Haraldsen, G., 2008. Nuclear Interleukin-33 Is Generally Expressed in Resting Endothelium but Rapidly Lost upon Angiogenic or Proinflammatory Activation. Am. J. Pathol. 173, 1229–1242. https://doi.org/10.2353/ajpath.2008.080014

Kumar, P., Wang, J.M., Bernabeu, C., 1996. CD 105 and angiogenesis. J. Pathol. 178, 363–6. https://doi.org/10.1002/(SICI)1096-9896(199604)178:4<363::AID-PATH491>3.0.CO;2-8

Kumar, R., Fossati, V., Israel, M., Snoeck, H.-W., 2008. Lin-Sca1+Kit- Bone Marrow Cells Contain Early Lymphoid-Committed Precursors That Are Distinct from Common Lymphoid Progenitors. J. Immunol. 181, 7507–7513. https://doi.org/10.4049/jimmunol.181.11.7507

Kusumbe, A.P., Ramasamy, S.K., Adams, R.H., 2014. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature 507, 323–328. https://doi.org/10.1038/nature13145

Kusumbe, A.P., Ramasamy, S.K., Itkin, T., Mäe, M.A., Langen, U.H., Betsholtz, C., Lapidot, T., Adams, R.H., 2016. Age-dependent modulation of vascular niches for haematopoietic stem cells. Nature 532, 380–384. https://doi.org/10.1038/nature17638

Lee, E.-J., Kim, J.W., Yoo, H., Kwak, W., Choi, W.H., Cho, S., Choi, Y.J., Lee, Y.-J., Cho, J., 2015. Single high-dose irradiation aggravates eosinophil-mediated fibrosis through IL-33 secreted from impaired vessels in the skin compared to fractionated irradiation. Biochem. Biophys. Res. Commun. 464, 20–26. https://doi.org/10.1016/j.bbrc.2015.05.081

Lerner, C., Harrison, D.E., 1990. 5-Fluorouracil spares hemopoietic stem cells responsible for long-term repopulation. Exp. Hematol. 18, 114–8.

Li, D.Y., Sorensen, L.K., Brooke, B.S., Urness, L.D., Davis, E.C., Taylor, D.G., Boak, B.B., Wendel, D.P., 1999. Defective angiogenesis in mice lacking endoglin. Science. 284, 1534–7. https://doi.org/10.1126/science.284.5419.1534

Maes, C., 2013. Role and regulation of vascularization processes in endochondral bones. Calcif. Tissue Int. 92, 307–323. https://doi.org/10.1007/s00223-012-9689-z

Medvinsky, A., Rybtsov, S., Taoudi, S., 2011. Embryonic origin of the adult hematopoietic system: advances and questions. Development 138, 1017–31. https://doi.org/10.1242/dev.040998

Miller, D.W., Graulich, W., Karges, B., Stahl, S., Ernst, M., Ramaswamy, A., Sedlacek, H.H., Müller, R., Adamkiewicz, J., 1999. Elevated expression of endoglin, a component of the TGF-beta-receptor complex, correlates with proliferation of tumor endothelial cells. Int. J. cancer 81, 568–72.

Nakamura-Ishizu, A., Okuno, Y., Omatsu, Y., Okabe, K., Morimoto, J., Uede, T., Nagasawa, T., Suda, T., Kubota, Y., 2012. Extracellular matrix protein tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. Blood 119, 5429–5437. https://doi.org/10.1182/blood-2011-11-393645

Nombela-Arrieta, C., Pivarnik, G., Winkel, B., Canty, K.J., Harley, B., Mahoney, J.E., Park, S.-Y., Lu, J., Protopopov, A., Silberstein, L.E., 2013. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. Nat. Cell Biol. 15, 533–543. https://doi.org/10.1038/ncb2730

Oguro, H., Ding, L., Morrison, S.J., 2013. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell 13, 102–16. https://doi.org/10.1016/j.stem.2013.05.014

Oshima, A., Tanabe, H., Yan, T., Lowe, G.N., Glackin, C.A., Kudo, A., 2002. A novel mechanism for the regulation of osteoblast differentiation: Transcription of periostin, a member of the fasciclin I family, is regulated by the bHLH transcription factor, Twist. J. Cell. Biochem. 86, 792–804. https://doi.org/10.1002/jcb.10272

Poulos, M.G., Guo, P., Kofler, N.M., Pinho, S., Gutkin, M.C., Tikhonova, A., Aifantis, I., Frenette, P.S., Kitajewski, J., Rafii, S., Butler, J.M., 2013. Endothelial Jagged-1 Is Necessary for Homeostatic and Regenerative Hematopoiesis. Cell Rep. 4, 1022–1034. https://doi.org/10.1016/j.celrep.2013.07.048

Rafii, S., Butler, J.M., Ding, B.-S., 2016. Angiocrine functions of organ-specific endothelial cells. Nature 529, 316–325. https://doi.org/10.1038/nature17040

Ramasamy, S.K., Kusumbe, A.P., Adams, R.H., 2015. Regulation of tissue morphogenesis by endothelial cell-derived signals. Trends Cell Biol. 25, 148–157. https://doi.org/10.1016/j.tcb.2014.11.007

Ramasamy, S.K., Kusumbe, A.P., Itkin, T., Gur-Cohen, S., Lapidot, T., Adams, R.H., 2016. Regulation of Hematopoiesis and Osteogenesis by Blood Vessel–Derived Signals. Annu. Rev. Cell Dev. Biol. 32, 649–675. https://doi.org/10.1146/annurev-cellbio-111315-124936

Ramasamy, S.K., Kusumbe, A.P., Wang, L., Adams, R.H., 2014. Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. Nature 507, 376–380. https://doi.org/10.1038/nature13146

Salani, D., Taraboletti, G., Rosanò, L., Di Castro, V., Borsotti, P., Giavazzi, R., Bagnato, A., 2000. Endothelin-1 Induces an Angiogenic Phenotype in Cultured Endothelial Cells and Stimulates Neovascularization In Vivo. Am. J. Pathol. 157, 1703–1711. https://doi.org/10.1016/S0002-9440(10)64807-9

Salazar, V.S., Gamer, L.W., Rosen, V., 2016. BMP signalling in skeletal development, disease and repair. Nat. Rev. Endocrinol. 12, 203–221. https://doi.org/10.1038/nrendo.2016.12

Saleh, H., Eeles, D., Hodge, J.M., Nicholson, G.C., Gu, R., Pompolo, S., Gillespie, M.T., Quinn, J.M.W., 2011. Interleukin-33, a target of parathyroid hormone and oncostatin m, increases osteoblastic matrix mineral deposition and inhibits osteoclast formation in vitro. Endocrinology 152, 1911–1922. https://doi.org/10.1210/en.2010-1268

Salvucci, O., Tosato, G., 2012. Essential Roles of EphB Receptors and EphrinB Ligands in Endothelial Cell Function and Angiogenesis, in: Advances in Cancer Research. pp. 21–57. https://doi.org/10.1016/B978-0-12-386503-8.00002-8

Schepers, K., Hsiao, E.C., Garg, T., Scott, M.J., Passegue, E., 2012. Activated Gs signaling in osteoblastic cells alters the hematopoietic stem cell niche in mice. Blood 120, 3425–3435. https://doi.org/10.1182/blood-2011-11-395418

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A.W., Bussolino, F., 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391–397. https://doi.org/10.1038/nature01784\rnature01784 [pii]

Shan, S., Li, Y., Wang, J., Lv, Z., Yi, D., Huang, Q., Corrigan, C.J., Wang, W., Quangeng, Z., Ying, S., 2016. Nasal administration of interleukin-33 induces airways angiogenesis and expression of multiple angiogenic factors in a murine asthma surrogate. Immunology 148, 83–91. https://doi.org/10.1111/imm.12589

Shao, R., Bao, S., Bai, X., Blanchette, C., Anderson, R.M., Dang, T., Gishizky, M.L., Marks, J.R., Wang, X., 2004. Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression. Mol. Cell. Biol. 24, 3992–4003. https://doi.org/10.1128/MCB.24.9.3992

Shigematsu, S., Yamauchi, K., Nakajima, K., Iijima, S., Aizawa, T., Hashizume, K., 1999. IGF-1 regulates migration and angiogenesis of human endothelial cells. Endocr. J. 46 Suppl, S59–S62. https://doi.org/10.1507/endocrj.46.Suppl_S59

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M. a, Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. 102, 15545–15550. https://doi.org/10.1073/pnas.0506580102

Sugiyama, T., Kohara, H., Noda, M., Nagasawa, T., 2006. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. Immunity 25, 977–988. https://doi.org/10.1016/j.immuni.2006.10.016

Sundlisæter, E., Edelmann, R.J., Hol, J., Sponheim, J., Kchler, A.M., Weiss, M., Udalova, I.A., Midwood, K.S., Kasprzycka, M., Haraldsen, G., 2012. The alarmin IL-33 is a notch target in quiescent endothelial cells. Am. J. Pathol. 181, 1099–1111. https://doi.org/10.1016/j.ajpath.2012.06.003

Tamagnone, L., Giordano, S., 2006. Semaphorin pathways orchestrate osteogenesis. Nat. Cell Biol. 8, 545–547. https://doi.org/10.1038/ncb0606-545

Wang, J.M., Kumar, S., Van Agthoven, A., Kumar, P., Pye, D., Hunter, R.D., 1995. Irradiation induces up-regulation of E9 protein (CD105) in human vascular endothelial cells. Int. J. Cancer 62, 791–796. https://doi.org/10.1002/ijc.2910620624

Winkler, I.G., Barbier, V., Nowlan, B., Jacobsen, R.N., Forristal, C.E., Patton, J.T., Magnani, J.L., Lévesque, J.-P., 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat. Med. 18, 1651–1657. https://doi.org/10.1038/nm.2969

Xian, C.J., Cool, J.C., Pyragius, T., Foster, B.K., 2006. Damage and recovery of the bone growth mechanism in young rats following 5-fluorouracil acute chemotherapy. J. Cell. Biochem. 99, 1688–1704. https://doi.org/10.1002/jcb.20889

Yoshitomi, H., Kobayashi, S., Ohtsuka, M., Kimura, F., Shimizu, H., Yoshidome, H., Miyazaki, M., 2008. Specific expression of endoglin (CD105) in endothelial cells of intratumoral blood and lymphatic vessels in pancreatic cancer. Pancreas 37, 275–281. https://doi.org/10.1097/mpa.0b013e3181690b97

Zambetti, N.A., Ping, Z., Chen, S., Kenswil, K.J.G., Mylona, M.A., Sanders, M.A., Hoogenboezem, R.M., Bindels, E.M.J., Adisty, M.N., Van Strien, P.M.H., van der Leije, C.S., Westers, T.M., Cremers, E.M.P., Milanese, C., Mastroberardino, P.G., van Leeuwen, J.P.T.M., van der Eerden, B.C.J., Touw, I.P., Kuijpers, T.W., Kanaar, R., van de Loosdrecht, A.A., Vogl, T., Raaijmakers, M.H.G.P., 2016. Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. Cell Stem Cell 19, 613–627. https://doi.org/10.1016/j.stem.2016.08.021

Zhang, C.C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., Lodish, H.F., 2006. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat. Med. 12, 240–245. https://doi.org/10.1038/nm1342

SUPPLEMENTAL INFORMATION

Table S1

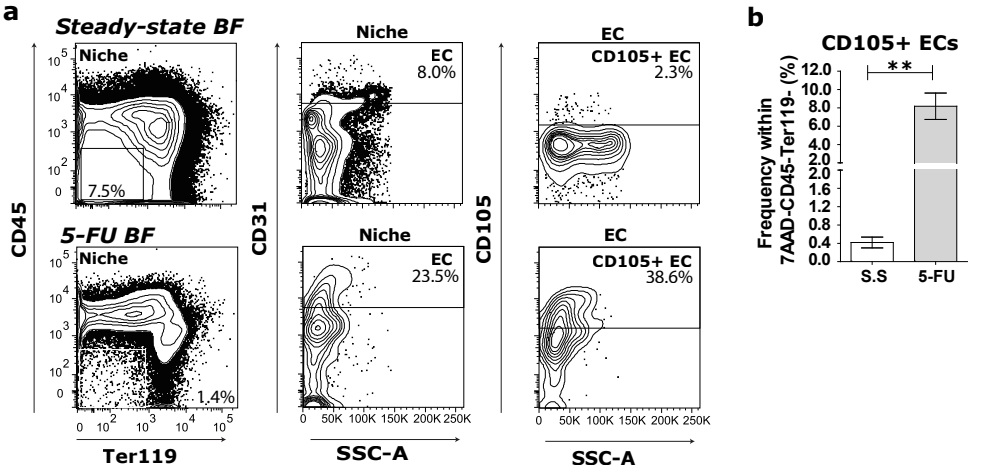
Gene Name	Cluster of differentation	FDR	FC	Avg FPKM AML D17	Avg FPKM Healthy CTRL
DPP4	CD26	4.30E-06	42.06162224	1.447326333	0.018322079
ENG	CD105	1.61E-03	8.55449896	15.62384567	1.547395714
VCAM1	CD106	3.81E-06	50.16296233	88.93116733	0.804735029
LIFR	CD118	2.08E-02	10.1185124	0.1064362	0.016143224
IL1R1	CD121a	1.54E-03	22.29341884	1.835577597	0.040494024
CDH5	CD144	4.26E-03	22.72755944	4.79532	0.072456899
CXCR3	CD183	1.04E-02	6.439581287	4.524633333	1.938153714
CCR2	CD192	1.09E-03	5.554609242	17.7306	4.429592857
PROCR	CD201	1.03E-03	19.40383328	7.131228667	0.224176357
TSPAN7	CD231	2.61E-05	24.32565573	54.17457	1.558463086
TNFSF10	CD253	1.16E-02	5.826945634	10.84400633	1.776891571
CD300LG	CD300g	1.90E-04	38.86795899	14.576502	0.166514471
NRP1	CD304	3.82E-06	47.01754435	9.396109867	0.113237357
KDR	CD309	1.69E-02	18.71674562	6.977216	0.077225637
FZD4	CD344	1.50E-06	45.00592407	2.850081333	0.043208416
S1PR1	CD363	1.33E-03	13.72492829	11.43101533	0.575994457

Supplemental Table 1. Related to Figure 1. Differential expression of candidate membrane markers of endothelial cells associated with regeneration after chemotherapy

Genes encoding cluster of differentiation antigens overexpressed in endothelial cells ($CD45^-CD235^-CD31^+CD9^+$) in aspirates obtained from regenerative marrow (n= 3) compared to postnatal steady-state BM (n= 7). FC: Fold change. FPKM: fragments per kilobase of exon per million fragments mapped. BM: bone marrow. FDR: false discovery rate.

SUPPLEMENTAL FIGURES

Figure S1

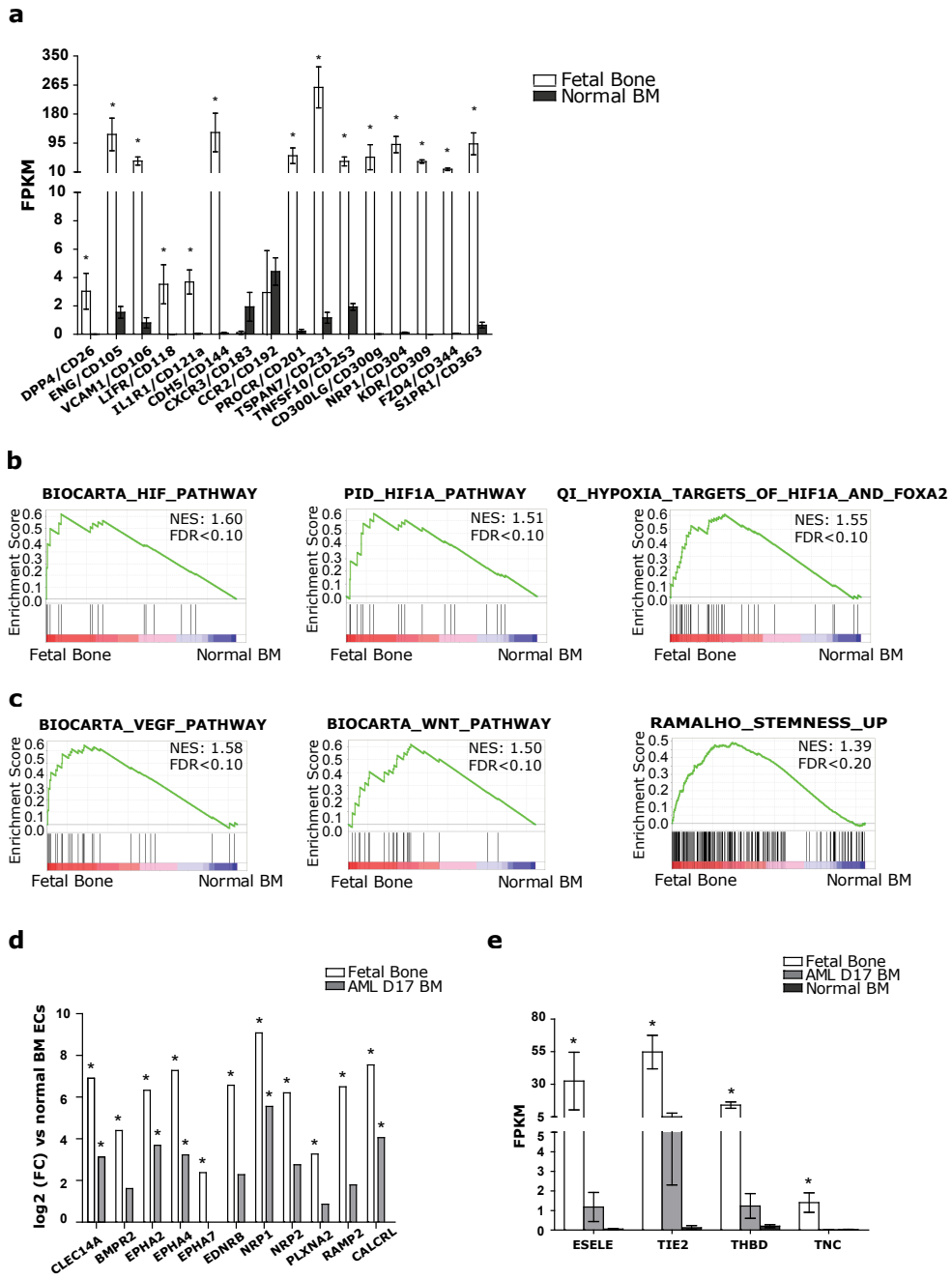


Supplementary Figure 1. Related to Figure 2. CD105-expressing endothelial cells are enriched in collagenased bone of mice after chemotherapeutic injury

a. Representative FACS plots revealing the enrichment of a CD105-expressing endothelial cells (ECs) in collagenased bone after administration of 5-fluoroucil. Gating strategy for identification of murine ECs is also depicted (see also figure 2). BF=Bone fraction.

b. Frequency of $CD105^{+}$ ECs within collagenased bone of mice in steady-state (n=3) and after administration of 5-fluoroucil (n=5). Data represent mean ± s.e.m. **p <0.01, two-tailed unpaired t-test. S.S=Steady-state. 5-FU=5-fluorouracil.

Figure S2


a
FPKM
Fetal Bone
Normal BM
DPP4/CD26
ENG/CD105
VCAM1/CD106
L1FR/CD118
IL1R1/CD121a
CDH5/CD144
CXCR3/CD183
CCR2/CD192
PROCR/CD201
TSPAN7/CD231
TNFSF10/CD253
CD300LG/CD300g
NRP1/CD304
KDR/CD309
FZD4/CD344
S1PR1/CD363
b
BIOCARTA_HIF_PATHWAY
NES: 1.60
FDR<0.10
PID_HIF1A_PATHWAY
NES: 1.51
FDR<0.10
QI_HYPOXIA_TARGETS_OF_HIF1A_AND_FOXA2
NES: 1.55
FDR<0.10
Enrichment Score
Fetal Bone
Normal BM
c
BIOCARTA_VEGF_PATHWAY
NES: 1.58
FDR<0.10
BIOCARTA_WNT_PATHWAY
NES: 1.50
FDR<0.10
RAMALHO_STEMNESS_UP
NES: 1.39
FDR<0.20
d
Fetal Bone
AML D17 BM
log2 (FC) vs normal BM ECs
CLEC14A
BMPR2
EPHA2
EPHA4
EPHA7
EDNRB
NRP1
NRP2
PLXNA2
RAMP2
CALCRL
e
Fetal Bone
AML D17 BM
Normal BM
FPKM
ESELE
TIE2
THBD
TNC

Supplementary Figure 2. Related to Supplemental Table 1, Figure 4 and 5. Transcriptional signatures of fetal CD105+ ECs

a. Transcript expression of membrane proteins (CD markers) in fetal bone $CD31^{+}CD105^{+}$ ECs compared to steady-state BM ECs. The overlap with transcripts identified in ECs isolated from D17 BM (Table S1) supports the notion that the enrichment of these transcripts in ECs in the regenerative marrow is caused by overexpression in the subset of $CD105^{+}$ ECs.

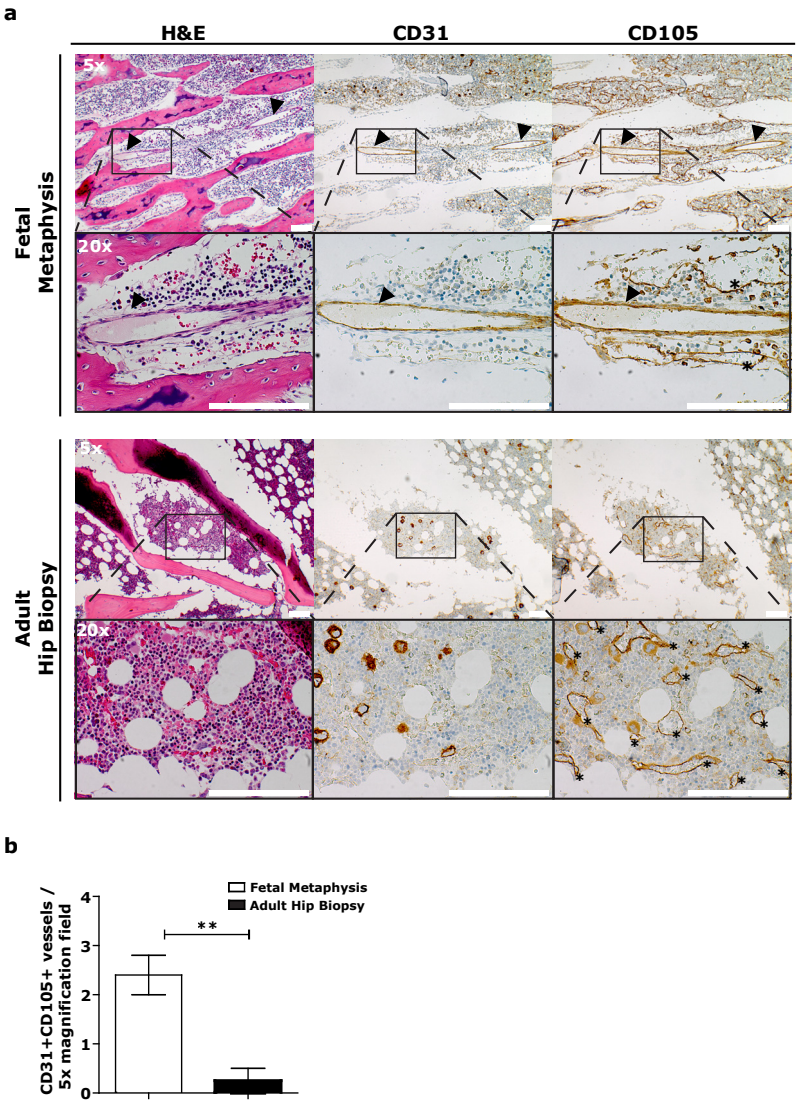
b,c. GSEA plots demonstrating activation of HIF1a signaling (b) and gene sets associated with VEGF, WNT and stem cells (c) in fetal hRECs compared to steady-state BM. GSEA: gene sets enrichment analysis.

d. Transcript expression (Log 2 fold change) of cognate receptors of candidate secreted proteins (Figure 5A) in fetal hRECs and ECs of regenerative marrow compared to steady-state bone marrow.

e. Transcript abundance of molecules associated with regeneration previously described in murine studies (Doan et al., 2013; Gur-Cohen et al., 2015; Kopp et al., 2005; Nakamura-Ishizu et al., 2012; Winkler et al., 2012).

*<FDR 0.05. FDR: false discovery rate. FPKM: fragments per kilobase of exon per million fragments mapped. BM: bone marrow. NES: normalized enrichment score. FC: Fold Change.

Figure S3

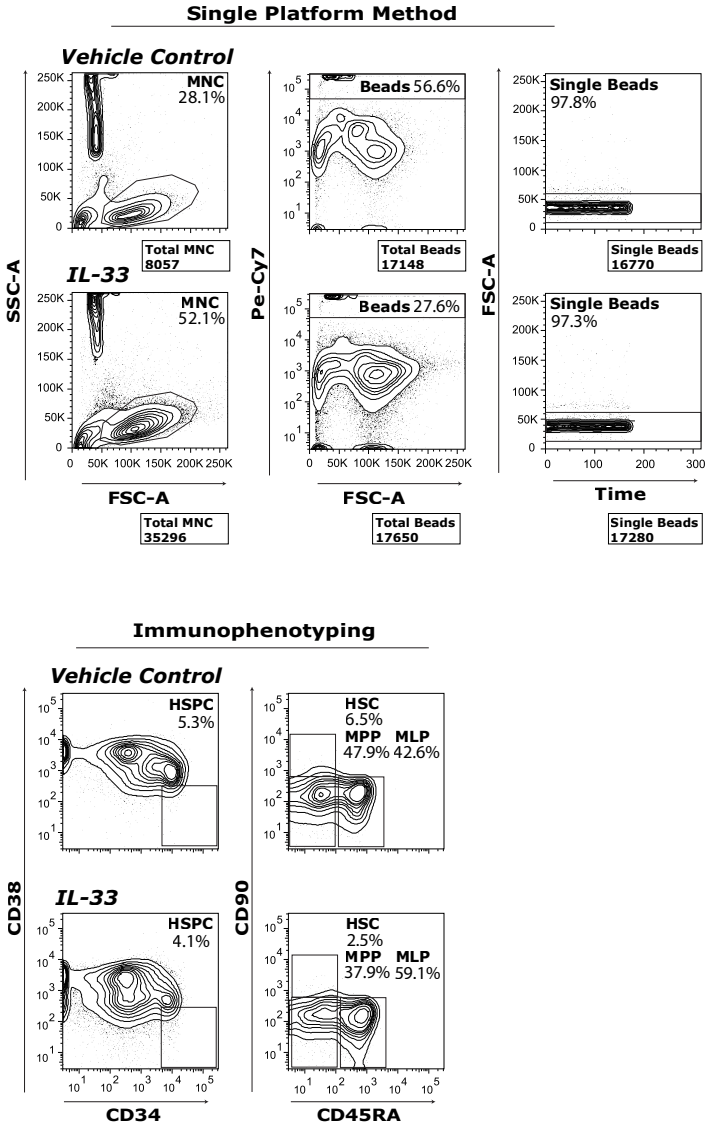


Supplementary Figure 3. Related to Figure 3. *In situ* immunohistochemistry of CD31⁺CD105⁺ endothelial cells in human fetal and postnatal bone marrow

a. Hematoxylin and eosin, CD31, and CD105 staining in consecutive serial sections of fetal and adult trabecular bone. CD31⁺CD105⁺ vessels (indicated by black arrowheads) were readily detected in the fetal bone, but at much lower frequency in postnatal trabecular bone. Asterisks highlight sinusoidal endothelium, marked by CD105 expression and concomitant low or lack of CD31 expression. Note: hematopoietic elements with high CD31 expression (indicated by black arrows) represent megakaryocytes. Representative 5x and 20x magnified images. Scale bars represent 100 µm.

b. Quantified total number of CD31⁺CD105⁺ vessels per 5x magnification field in trabecular areas of human fetal (n=5) and postnatal trabecular bone (n=4). Data represent mean ± s.e.m. **p <0.01, two-tailed unpaired t-test.

Figure S4

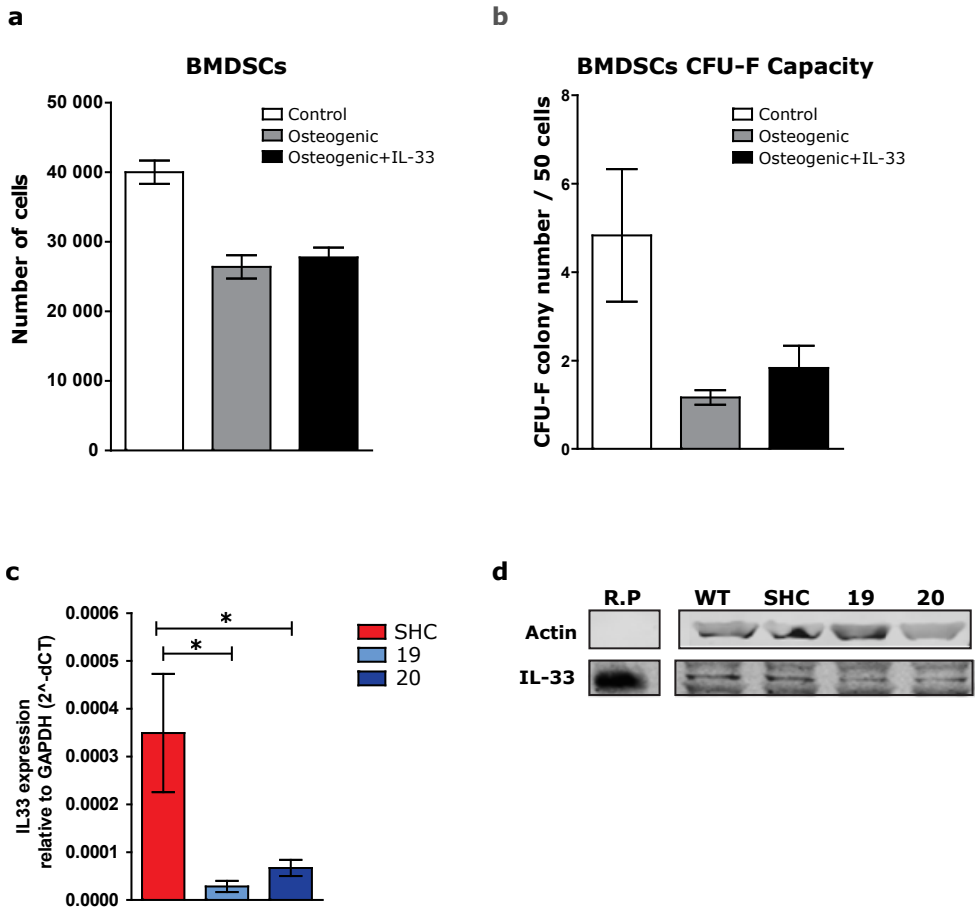


Supplementary Figure 4. Related to Figure 5. Representative FACS plots of cord blood stem/progenitor cells exposed to recombinant IL-33 for one week

Top, representative example of the single platform flow cytometric assay to quantify total cell number. Total number of mononuclear cells were calculated using the total number of MNC and beads events recorded using the quantification formula described in Experimental Procedures. Note that the MNC/Beads ratio in the vehicle control (VC) sample approximates 0.5, whereas the IL-33 treated sample is 2.0, indicating an a 4-fold increase of MNC in comparison with VC.

Bottom, characterization of the MNCs based on CD marker expression. $CD34^{+}CD38^{lo}$ MNCs were identified as hematopoietic stem and progenitor cells (HSPCs). HSPCs were further subdivided based on CD90 and CD45RA expression. $CD90^{+}CD45RA^{-}$ cells were defined as hematopoietic stem cells (HSCs), CD90-$CD45RA^{-}$ cells classified as multipotent progenitor cells (MPP), and $CD90^{-}CD45RA^{+}$ cells as multilymphoid progenitors (MLP).

Figure S5



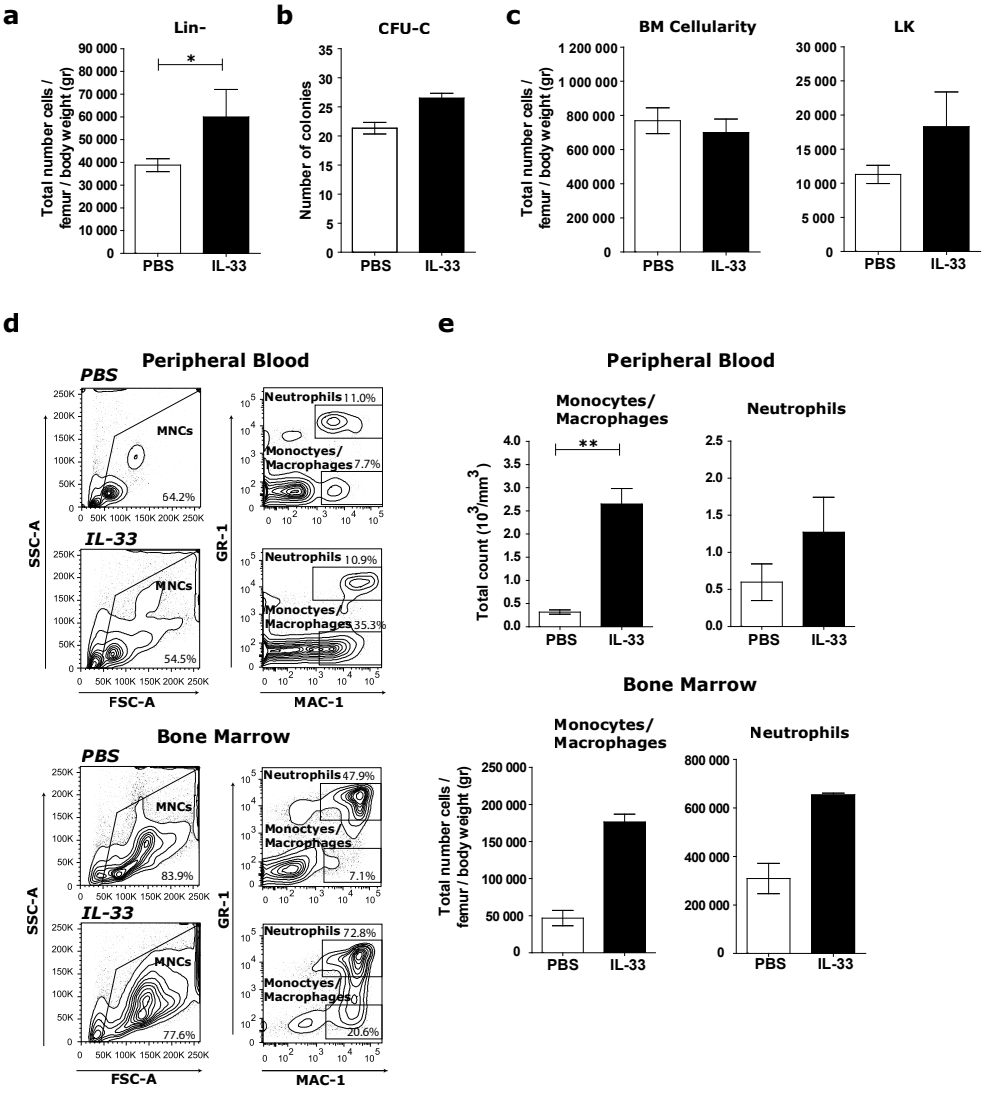
Supplementary Figure 5. Related to Figure 6. Effect of recombinant IL-33 on bone marrow-derived stromal cells and validation of shRNA-mediated IL-33 knockdown in HUVECs

a,b. The total number of bone marrow-derived mesenchymal cells (BMDSCs) and their CFU-F capacity is not affected after treatment with recombinant IL-33 in osteogenic conditions. (a) Quantification of the total number of BMDSCs kept in regular culture medium (n=2), osteogenic induction medium (n=2) and osteogenic induction medium containing recombinant IL-33 (n=2). (b) Quantification of the colony-forming unit-fibroblast (CFU-F) capacity of BMDSCs kept in regular culture medium (n=2), osteogenic induction medium (n=2) and osteogenic induction medium containing recombinant IL-33 (n=2).

c,d. Validation of shRNA-mediated IL-33 knockdown in HUVECs. (c) Transcript analysis of IL-33 by quantitative polymerase chain reaction (qPCR) demonstrates efficient knockdown of IL-33 in HUVECs transduced with two independent shRNA (sh19 and sh20) compared to a scramble hairpin control (SHC). (n= 4 independent experiments). (d) Representative Western Blot analysis confirming knockdown of IL33 at the protein level. R.P=recombinant protein IL-33. Protein ladder has been cropped out from both gel figures.

Data represent mean ± s.e.m. *p <0.05, two-tailed unpaired t-test.

Figure S6



Supplementary Figure 6. Related to Figure 7. IL-33 promotes expansion of myeloid cells

a. Total lineage-negative cell counts in PBS (n=10) and IL-33 treated (n=5) mice. Data represent mean ± s.e.m. *p<0.05, unpaired t-test.

b. Colony-forming assay (CFU-C) on total BM of PBS (n=2) and IL-33 (n=2) treated mice. Data represent mean ± s.e.m. Experiments were performed in triplicate.

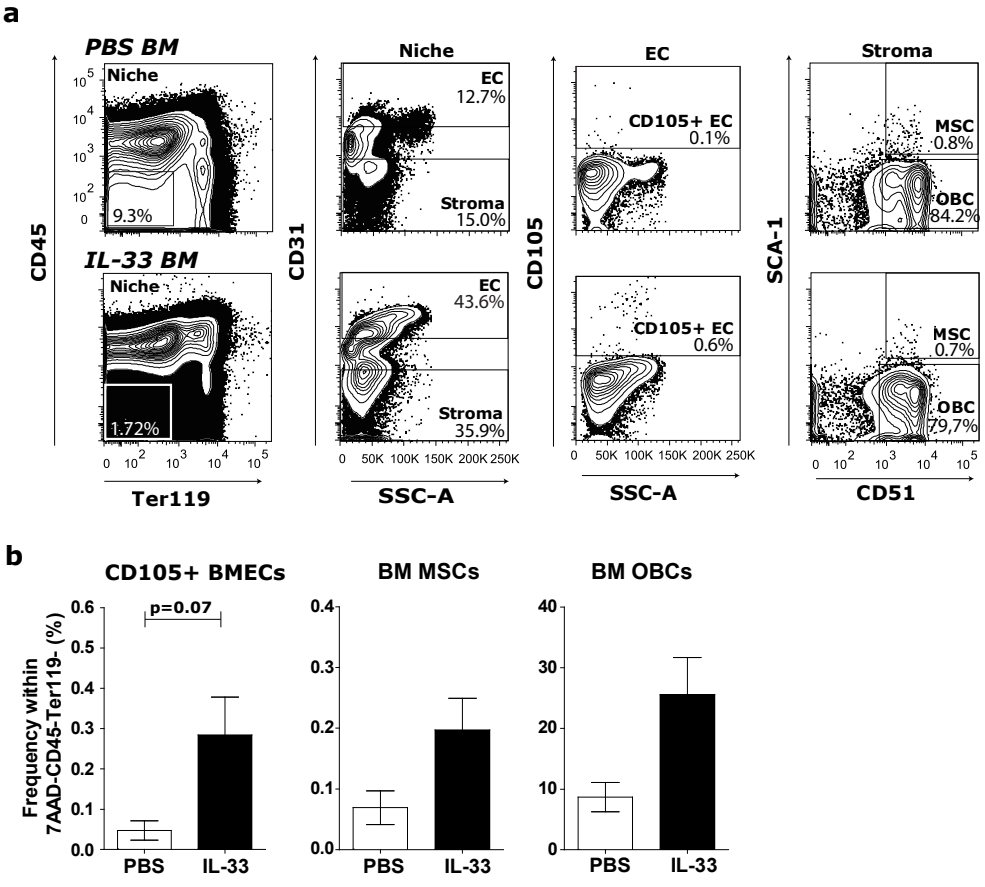
c. Total bone marrow cellularity and Lin-, c-KIT+, Sca-1- cell counts in PBS (n=10) and IL-33 treated (n=5) mice. Data represent mean ± s.e.m.

d. Representative FACS plots demonstrating an increased frequency of monocytes/macrophages in the peripheral blood (PB) and bone marrow (BM) of IL-33 treated mice.

e. Total PB and BM counts of monocytes/macrophages and neutrophils in PBS-treated (n=3 for PB and n=2 for BM) and IL-33 treated (n=3 for PB and n=2 for BM) mice.

Data represent mean ± s.e.m. **p<0.01, two-tailed unpaired t-test.

Figure S7



Supplementary Figure 7. Related to Figure 7. Recombinant IL-33 alters the architecture of the BM niche

a,b. Representative FACS plots of PBS (n=3) vs IL-33 treated (n=3) mice depicting the distribution of niche ($CD45^-$ $Ter119^-$) cells within the BM. ECs were identified by CD31. Stromal ($CD31^-$) cells were further defined as SCA-1+CD51+ cells (MSCs) or SCA-1-CD51+ cells (OBCs). IL33 administration resulted in a relative increase of $CD31^+CD105^+$, MSC and OBC populations within the niche.

Data represent mean ± s.e.m. *p<0.05, two-tailed unpaired t-test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell isolation from human bone (marrow)

Freshly obtained BM aspirates of AML Day 17 patients were diluted 1:25 with red blood cell lysis solution (NH4Cl 0.155 M, KHCO3 0.01 M, EDTA-Na2.2H2O 0.1 M, pH 7.4) and incubated for 10 min at room temperature. Mononuclear cells (MNCs) were collected by centrifugation and washed once with PBS+0.5%FCS.

Fetal BM MNCs were isolated by gently crushing fetal long bones (femora, tibiae, fibulae, humeri, radii and ulna) in PBS+0.5%FCS using a mortar and pestle and passing subsequent cell suspension through a 40-µm filter in a 50 ml collection tube. Simultaneously, bone fragments were digested with 0.25% collagenase type I (Stem Cell. Cat#07902) for 45 minutes at 37°C, vortexing every 15 minutes. Excess PBS was added to the solution and filtered through a 40-µm filter in a collection tube. Next, both BM and bone fragment cell suspensions were washed once with PBS+0.5%FCS and were subsequently cleared from erythrocytes with IOtest3 lysing solution (Beckman Coulter. Cat#A07799), according to manufacturer's instructions.

For adult hip bone cells, trabecular bone was gently crushed using a mortar and pestle and resulting bone fragments were processed as described for fetal bone cells.

Fluorescence active cells-sorting (FACS) of human BM niche cells

Prior to cell sorting, FACS antibody incubations were performed in PBS+0.5%FCS for 20 minutes on ice in the dark with the following antibodies using optimized dilutions: CD45 (clone HI30, 1:100), CD271 (clone ME20.4, 1:100), CD235a (clone HI264, 1:100), CD31 (clone WM59, 1:100), CD9 (clone HI9a, 1:100) from Biolegend, and CD105 (clone SN6, 1:50) from eBioscience. The indicated populations of interest were sorted using a FACS ARIAIII Cell Sorter (BD Biosciences). Dead cells were gated out using 7AAD (Stem-Kit Reagents) after MNC selection and doublets exclusion. For RNASeq, cells were directly sorted in 800µl Trizol (Ambion) for RNA isolation. RNAse free non-stick micro-tubes (Ambion) were used to prevent pre-digestion of RNA.

Flow cytometry on murine peripheral blood and bone (marrow) cells

To identify murine hematopoietic stem and progenitor cells (HSPCs), BM cells were first co-stained with a cocktail of biotin-labelled antibodies against the following lineage (Lin) markers: Gr1 (RB6-8C5), Mac1 (M1/70), Ter119 (TER-119), CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7) and B220 (RA3-6B2) (all from BD Biosciences). After washing, cells were incubated with Pacific Orange-conjugated streptavidin (Life Technologies) and the following antibodies: APC anti-c-Kit (2B8), PE anti-CD34 (HM34), Pacific Blue anti-Sca1 (D7), Alexa Fluor 700 anti-CD48 (HM48-1), PE-Cy7 anti-CD150 (TC15-12F12.2) (all from Biolegend), and APC/Cy7 anti-CD16/32 (56054, from BD Biosciences). To analyze murine differentiated cells,

we used APC anti-Gr1 (RB6-8C5), PE-Cy7 anti-Mac1 (M1/70), Pacific Blue anti-B220 (RA3-6B2) antibodies (all from Biolegend). To define murine niche cells, BM and bone fraction cell suspensions were stained with the following antibodies: APC-Cy7 anti-CD45.2 (104), BV510 anti-Ter119 (TER-119), PE-Cy7 anti-CD105 (MJ7/18), PE anti-CD51 (RMV-7), Pacific Blue anti-Sca1 (D7) (all from Biolegend), and PE-CF594 anti-CD31 (MEC 13.3, BD Biosciences). IL-33 expression was assessed in BM cells fixed and permeabilized with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) by incubating cells with PE anti-IL33 (IC3626P, R&D Systems) diluted in 1X Perm/Wash buffer (BD Biosciences).

CD31 and CD105 in situ immunofluorescence and immunohistochemistry

4 µm thick sections of paraffin-embedded BM biopsies were obtained from benign lymphoma patients without evidence of intramedullary localization. Fetal femurs were fixed in 4% formaldehyde for 24 hours and decalcified in 12.5% EDTA overnight. Fetal femurs were subsequently embedded in paraffin and sliced with a Micron HM355Smicrotome (Thermo Scientific) to create 4 µm thick sections.

For in situ immunofluorescence, deparaffinizing and antigen retrieval of the sections were performed with CC1 (Ventana 900-124) at 97oC for 64 minutes in a Ventana BenchMark ULTRA automatic staining system (Ventana Medical Systems, Tuscon, AZ). Next, murine anti-human CD31 (Cell Marque 760-4378) and rabbit anti-human CD105 (Genetex GTX100508_25ul,1:100) primary antibodies were manually added to the sections and incubated in the Ventana BenchMark ULTRA automatic staining system for 60 minutes at 36oC. After thorough washing with PBS/Tween 0.1%, sections were incubated for 30 minutes with a biotinylated rabbit anti-mouse secondary antibody lacking the FC fragment (DAKO E413), followed by washing in PBS/Tween20 0.1% and a 30 minute incubation with Cy™3-streptavidin (Jackson ImmunoResearch 016-0160-084). Next, avidin/biotin blocking was performed (Vector Laboratories P-2001). After thorough washing with PBS/Tween 0.1%, sections were incubated for 30 minutes with a biotinylated swine anti-rabbit secondary antibody (DAKO E431), followed by washing in PBS/Tween20 0.1% and a 30 minute incubation with FITC- streptavidin (Jackson ImmunoResearch 016-010-084). Finally, sections were mounted with DAPI-containing (1:2.000) vectashield (Vector Laboratories H-1000). 20x magnified Z-series images were acquired with a Leica TCS SP5 confocal microscope using the LAS software (Leica Microsystems). Briefly, after deparaffinization sectioned specimens were processed for 64-minute antigen retrieval with CC1. After 20-minute incubation at 36°C with either previously described primary antibodies for CD31 and CD105 (1:500), detection was performed using UltraView DAB IHC Detection Kit (760-500, Ventana). The sections stained with CD31 and CD105 were counterstained with hematoxylin II (Ventana Ref.: 790-2208). Images were acquired with a ZEISS AxioPhot microscope using cellSens Entry 1.9 software (Olympus Corporation).

IL-33 immunofluorescence

FACS-isolated hRECs, obtained from fetal bones and BM, were collected in PBS, cytospun on a glass slide for 3 minutes at 500 rpm using a Cytospin 4 centrifuge (Thermo Scientific), and fixed in 3% PFA/PBS for 15 minutes on ice. After washing with PBS 3 times, cells were permeabilized for 2 minutes in 0.15% Triton-X100/PBS and then incubated in 1%BSA/PBS for 1 hour at room temperature to block aspecific binding sites. Cells were next stained overnight at 4°C with polyclonal goat anti-human IL-33 antibody (AF3625, R&D, 20 µg/ml in 1%BSA/PBS) or goat IgG isotype control antibody(Catalog # AB-108-C). Slides were washed twice with PBS for 5 minutes and incubated for 1 hour at 37°C with Alexa Fluor 488-conjugated donkey anti-goat antibody (A-11055, Invitrogen, 1:200, in 1%BSA/PBS). After 2 washes in PBS, slides were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). 63x magnified Z-series images were acquired with a Leica TCS SP5 confocal microscope using the LAS software (Leica Microsystems). For *in situ* immunohistochemistry consecutive serial sections of the benign lymphoma patients and fetal femur biopsies were stained for hematoxylin and eosin, CD31 and CD105 separately with the Ventana Benchmark Ultra automated staining system.

Hematopoietic stem progenitor cell isolation from umbilical cord blood

Umbilical cord blood (CB) was collected in a 300 ml bag filled with 20ml of the anticoagulant citrate phosphate dextrose (T2950, Fresenius Kabi), after receiving informed consent from the mother, and retrieved within 24 hours after collection. MNCs were obtained by using a standard Ficoll gradient protocol (1114547, Axis-Shield). CD34+ CB HSPCs were further isolated by magnetic-activated cell sorting (MACS). Briefly, MNCs were incubated with a magnetically coated anti-CD34 antibody (120-000-268, Miltenyi Biotec) and FcR blocking reagent (120-000-265, Miltenyi Biotec) in MACS buffer at 4°C for 30 minutes. After washing, CD34+ CB HSPCs were isolated using a LS column (130-042-401, Miltenyi Biotec) and purity was further enriched by performing an additional MACS selection using two MS columns (130-042-201, Miltenyi Biotec). Purity of the isolated cell population was confirmed with fluorescence-activated cell sorting (FACS).

Quantification and multiparametric immunophenotyping of cultured CB CD34+ cells

Cultured CB cells were thoroughly resuspended and collected for the single platform flowcytometric assay (to determine absolute cell counts) and for immunophenotyping (to specify HSPC subpopulations), respectively. For the single platform flowcytometric assay, cultured cells were incubated with CD34-PE-Cy7 (348811, BD Biosciences, 1:50) and CD45-PE-Cy5 (557075, BD Biosciences, 1:50) antibodies, and a calibrated number of flow-count fluorosphere beads (7547053, Beckman Coulter). In addition, 4',6-diamidino-2-phenylindole (DAPI) (1:5.000) was used to distinguish between living and dead cells. The total number of living MNC per µl in (n) a well was calculated using the following formula: n= ((number of

MNC events recorded * bead concentration)/number of recorded single beads)/ (volume cells/ volume beads). For immunophenotyping, cultured cells were incubated with Lin-FITC (22-7778-72, eBiosciences, 1:25), CD34-PE-Cy7 (348811, BD Biosciences, 1:50), CD38-PerCP-Cy5.5 (561106, BD Pharmingen, 1:60), CD90-PE (12-0909-42, eBiosciences, 1:30), CD45RA-APC-H7 (560674, BD Pharmingen, 1:30) and DAPI. Antibody incubation for FACs took place as previously described. The total number of a specific subpopulations was determined by multiplying the absolute number of living MNCs with the frequencies of the populations, determined with immunophenotyping, of interest. Flowcytometric analysis was performed using a BD LSRII (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Human hematopoietic colony forming-unit assay

To further assess the effect of recombinant human IL-33 on the hematopoietic potential of cultured CB cells, we performed a colony-forming unit assay (CFU). Input equivalent of 2000 7-days cultured CD34+ cells of each condition (rhIL-33 vs vehicle control) was resuspended in 400 µl Iscove's Modified Dulbecco's Medium (IMDM) and transferred to 3.6 ml of methylcellulose (MethoCult H84434, StemCell Technologies). Cells were then plated in triplicate in 1 cm^2 petri-dishes(1 ml/dish) and were kept at 37ºC and 5% CO2. Colonies were counted after 12-14 days.

Osteogenic differentiation and mineralization assays

BMDSC extracts were harvested at the end of culture by scraping the cells in PBS/triton 1% and storing them at −80 °C for biochemical analyses (Bruedigam et al., 2011). After scraping, PBS/triton 1%/0.24 M HCL was added to the wells and kept overnight at 4 °C to release calcium ions from the matrix deposition. Cell extracts were briefly sonicated (Soniprep 150, Sanyo) to produce cell lysates and incubated overnight with 6 M HCL. In short, calcium content was determined colorimetrically by adding o-cresolphthalein complexone to the well contents and cell lysates and measuring the absorbance of the resulting chromophore-complex at 595 nm.

Alternatively, cells were fixed in 70% ethanol (vol/vol) on ice for an hour and after washing with PBS, stained for 10–20 min with alizarin Red S solution (saturated Alizarin Red S (Sigma) in demineralized water adjusted to pH 4.2 using 0.5% ammonium hydroxide).

Human bone marrow fibroblast colony-forming unit assay

BMDSCs were first either cultured in αMEM medium containing 10% heat-inactivated FCS, or osteogenic induction medium containing recombinant human IL-33 (250 ng/ml) or mQ vehicle control for 1 week. After culture, total number of cells per condition were quantified with a Bürker counting chamber. Next, 50 cells per 0.32 cm2 (1 well of a 96-well plate) were seeded in αMEM supplemented with 20% fetal bovine serum and 1% penicillin/

streptomycin. On day 14, dishes were fixed with 70% ethanol (vol/vol) and stained with Giemsa. CFU-F colonies were counted as previously described (Chen et al., 2016).

Validation of IL-33 knockdown in HUVECs

For transcriptional analyses, HUVECs were harvested and collected after puromycin selection in TRIzol Reagent (Life Technologies). RNA isolation, conversion to cDNA and qPCR were performed accordingly to previously described methods (Zambetti et al., 2015) using the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). For qPCR, expression levels were obtained using the ddCt method using GAPDH as an internal control. The following primers were used: GAPDH-Fw: GTCGGAGTCAACGGATT; GAPDH-Rv: AAGCTTCCCGTTCTCAG; IL-33-Fw: GGAAGAACACAGCAAGCAAAGCCT; IL-33-Rv: TAAGGCCAGAGCGGAGCTTCATAA.

For protein analyses, cells were lysed in Carin lysis buffer (20 mM Tris-HCl pH 8.0, 138 mM NaCl, 10mM EDTA, 100 mM NaF, 1% Nonidet P-40, 10% glycerol, 2mM NA-vanadate) supplemented with 0.5 mM DTT and the protease inhibitor SigmaFast (Sigma Aldrich). Equal amounts of proteins were denatured and separated on a Novex NuPage 4-12% Bis-Tris Gradient gel (Life Technologies) and transferred to Protran BA83 blotting paper (GE Healthcare Sciences). After blocking with 5% BSA, membranes were incubated overnight at 4°C with a polyclonal goat anti-IL-33 primary antibody (AF3625, R&D systems). Actin was used as a loading control and detected with a mouse anti-actin antibody (A5441 Sigma Aldrich). Western blots were scanned and processed using an Odyssey Infrared Imager (Li-COR Biosciences).

To monitor the expansion of HUVECs after shRNA mediated knockdown of IL-33 after puromycin selection, HUVECs were re-plated and seeded at 300.000 cells in a T-25 flask and grown in EGM-2 medium containing 2µg/ml of puromycin. HUVECs were passaged every 2 to 4 days and total number of cells were quantified using a Casy counter (Roche Innovatis).

Murine hematopoietic colony forming-unit assay

To assess the myeloid progenitor capacity of whole BM from mice treated with PBS or IL-33, BM cells were cultured in methylcellulose containing murine SCF (10 ng/ml), IL-3 (100 ng/ml, purified from supernatant of CHO cells), IL-6 (10 ng/ml) and GM-CSF (10 ng/ml). Cytokines were purchased from PeproTech, if not otherwise mentioned. Specifically, 20000 BM cells per condition were resuspended in 400 µl IMDM and transferred to 3.6 ml of Methocult 3231 (StemCell Technologies). Cells were then plated in triplicate in 1 cm^2 petri-dishes(1 ml/dish) and were kept at 37ºC and 5% CO2. Colonies were counted after 5-7 days.

SUPPLEMENTAL REFERENCES

Bruedigam, C., van Driel, M., Koedam, M., van de Peppel, J., van der Eerden, B.C.J., Eijken, M., van Leeuwen, J.P.T.M., 2011. Basic techniques in human mesenchymal stem cell cultures: Differentiation into osteogenic and adipogenic lineages, genetic perturbations, and phenotypic analyses. Curr. Protoc. Stem Cell Biol. 1–20. doi:10.1002/9780470151808.sc01h03s17

Doan, P.L., Russell, J.L., Himburg, H.A., Helms, K., Harris, J.R., Lucas, J., Holshausen, K.C., Meadows, S.K., Daher, P., Jeffords, L.B., Chao, N.J., Kirsch, D.G., Chute, J.P., 2013. Tie2(+) bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. Stem Cells 31, 327–37. doi:10.1002/stem.1275

Gur-Cohen, S., Itkin, T., Chakrabarty, S., Graf, C., Kollet, O., Ludin, A., Golan, K., Kalinkovich, A., Ledergor, G., Wong, E., Niemeyer, E., Porat, Z., Erez, A., Sagi, I., Esmon, C.T., Ruf, W., Lapidot, T., 2015. PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. Nat. Med. 21, 1307–17. doi:10.1038/nm.3960

Kopp, H.G., Avecilla, S.T., Hooper, A.T., Shmelkov, S. V., Ramos, C.A., Zhang, F., Rafii, S., 2005. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. Blood 106, 505–513. doi:10.1182/blood-2004-11-4269

Nakamura-Ishizu, A., Okuno, Y., Omatsu, Y., Okabe, K., Morimoto, J., Uede, T., Nagasawa, T., Suda, T., Kubota, Y., 2012. Extracellular matrix protein tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. Blood 119, 5429–5437. doi:10.1182/blood-2011-11-393645

Winkler, I.G., Barbier, V., Nowlan, B., Jacobsen, R.N., Forristal, C.E., Patton, J.T., Magnani, J.L., Lévesque, J.-P., 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat. Med. 18, 1651–7. doi:10.1038/nm.2969

Zambetti, N.A., Bindels, E.M.J., Van Strien, P.M.H., Valkhof, M.G., Adisty, M.N., Hoogenboezem, R.M., Sanders, M.A., Rommens, J.M., Touw, I.P., Raaijmakers, M.H.G.P., 2015. Deficiency of the ribosome biogenesis gene Sbds in hematopoietic stem and progenitor cells causes neutropenia in mice by attenuating lineage progression in myelocytes. Haematologica 100, 1285–1293. doi:10.3324/haematol.2015.131573

3

UNDER EMBARGO UNTIL PUBLISHED

4

UNDER EMBARGO UNTIL PUBLISHED

5

UNDER EMBARGO UNTIL PUBLISHED

A

ADDENDUM

LIST OF ABBREVIATIONS

5FU	5-fluorouracil
α-SMA	Alpha smooth muscle actin
ADM	Adrenomedullin
AGM	Aorta-gonad-mesonephros
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
ANOVA	One-way analysis of variance
APLN	Apelin
BF	Bone fraction
BM	Bone marrow
BMP4	Bone morphogenetic protein 4
BMDSC	BM-derived stromal cells
BMSC	Bone marrow stromal cell
BrdU	5-bromodeoxyuridine
CAR	CXCL12-abundant reticular
CD	Cluster of differentiation
CFU-C	Colony-forming unit cell
CFU-F	Colony-forming unit fibroblast
CFU-GM	Colony-forming unit granulocyte-monocyte
CLP	Common lymphoid progenitor
CM	Central memory
CMP	Common myeloid progenitor
CXCL4	C-X-C motif ligand 4
CXCL12	C-X-C motif chemokine ligand 12
D17	Day 17
DKK1	Dickkopf-1
E	Embryonic day
EC	Endothelial cell
ECFC	Endothelial colony forming cell
EDN1	Endothelin 1
EFNA1	Ephrin A1
EGF	Epidermal growth factor
EHT	Endothelial-to-hematopoietic transition
EM	Effector memory
EMT	Epithelial-to-mesenchymal transition
EndMT	Endothelial-to-mesenchymal transition
ES	Enrichment score

ESELE	Selectin E
FACS	Fluorescence activated cell sorting
FC	Fold change
FDR	False discovery rate
FGF1	Fibroblast growth factor 1
FOP	Fibrodysplasia ossificans progressiva
FPKM	Fragments per kilobase of exon per million fragments mapped
G-CSF	Granulocyte colony-stimulating factor
GAG	Glycosaminoglycan
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocytic/macrophage progenitor
GO	Gene ontology
GSEA	Gene set enrichment analysis
HPC	Hematopoietic progenitor cell
hREC	Human regeneration-associated EC
HUVEC	Human umbilical vein endothelial cell
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IL-1β	Interleukin 1 β
IL-33	Interleukin 33
IR	Ionizing radiation
Jag1	Jagged-1
Jag2	Jagged-2
LEPR	Leptin receptor
LNGFR	Low-affinity nerve growth factor receptor
LT-HSC	Long-term HSC
MACS	Magnetic-activated cell sorting
MEP	Megakaryocytic/erythroid progenitor
MFI	Mean fluorescence intensity
MLP	Multilymphoid progenitor
MMRN2	Multimerin 2
MNC	Mononuclear cell
MPP	Multipotent progenitor
MSC	Mesenchymal stromal cell
NES	Normalized enrichment score
NK	Natural killer cell
N	Naive
OBC	Osteoblastic cell
OLC	Osteo/chondrolineage (progenitor) cell

Ocn	Osteocalcin
PCA	Principal component analysis
PLAT	Tissue-type plasminogen activator
PTN	Pleiotrophin
RBC	Red blood cell
rmIL-33	Recombinant murine IL-33
rhIL-33	Recombinant human IL-33
ROS	Reactive oxygen species
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
S.E.M	Standard error of the mean
SEMA3A	Semaphorin-3A
SLAM	Signaling lymphocyte activation molecule
SNAI1	Snail family transcriptional repressor 1
SNAI2	Snail family transcriptional repressor 2
SNS	Sympathetic nervous system
ST-HSC	Short-term HSC
TEMRA	CD45RA re-expressing cells
TNC	Tenascin C
TFPI	Tissue Factor Pathway Inhibitor
TGF-β	Transforming Growth Factor Beta
TMBD	Thrombomodulin
TNFα	Tumor necrosis factor-alpha
TPO	Thrombopoietin
TWIST1	Twist Family BHLH Transcription Factor 1
UCB	Umbilical cord blood
WBC	White blood cell
VC	Vehicle Control
VEGFR2	Vascular endothelial growth factor receptor 2

ENGLISH SUMMARY

UNDER EMBARGO UNTIL PUBLISHED

DUTCH SUMMARY (NEDERLANDSE SAMENVATTING)

UNDER EMBARGO UNTIL PUBLISHED

CURRICULUM VITAE

Keane Kenswil was born on the 9th of May 1989 in Paramaribo, Suriname. In 2001 he moved to the Netherlands after living in St. Maarten for 6 years. He attended Het Citycollege St. Franciscus high school in Rotterdam (The Netherlands) from 2001 until 2007 where he obtained his VWO diploma. In 2007 he started his study Biomedical Sciences at the LUMC in Leiden, where he did his bachelor and master with a focus on biomedical research. He did his first master internship in 2011 at the department of Surgery at LUMC in the group of Prof. dr. Paul Quax, where he tried to unravel the signaling pathways mediating arteriogenesis in a murine model of hind limb ischemia. His second master internship was performed at the Erasmus MC in Rotterdam in the group of Prof.dr. Raaijmakers at the deparment of Hematology in 2013. During this internship he focused on setting up an *ex vivo* culture system to study the potential effects of candidate (niche-derived) ligands on hematopoietic stem/progenitor cell proliferation and differentiation. At the end of this internship, in 2014, he decided to continue this project as a PhD-candidate, and also expanded the scope of his work. This included characterizing the human niche cells involved in bone marrow formation during fetal development, as well as bone marrow regeneration after chemotherapy-induced injury. Here he found that endothelial cells have an important role and express regulatory factors that can facilitate angiogenesis, osteogenesis, and hematopoiesis. Detailed results are presented in this thesis.

LIST OF PUBLICATIONS

Keane J.G. Kenswil, Adrian C. Jaramillo, Zhen Ping, Si Chen, Remco M. Hoogenboezem, Maria A. Mylona, Maria N. Adisty, Eric M. J. Bindels, Pieter K. Bos, Hans Stoop, King Hong Lam, Bram van der Eerden, Tom Cupedo, Marc H.G.P. Raaijmakers. Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 As an Anabolic Factor. *Cell Rep*. 2018 Jan 16;22(3):666-678

Keane J.G. Kenswil, Gonzalo Sánchez-Duffhues, Andrea Lolli, Claire van Dijk, Callie Knuth, Byambasuren Vanchin, Adrian Christopher Jaramillo, Remco Michiel Hoogenboezem, Tom Cupedo, Eric Moniqué Johannes Bindels, Bianca Blom, Pieter Koen Bos, Marie-Jose Goumans, Peter ten Dijke, Eric Farrell, Guido Krenning, Marc Hermanus Gerardus Petrus Raaijmakers. Endothelial-derived Mesenchymal Cells Contribute to Hematopoietic Niche Formation in Humans. *Manuscript Submitted*

Jasper J. Koning, Tanja Konijn, Kim A. Lakeman, Tom O'Toole, **Keane J. G. Kenswil**, Marc H. G. P. Raaijmakers, Tatyana V. Michurina, Grigori Enikolopov and Reina E. Mebius. Nestin-Expressing Precursors Give Rise to Both Endothelial as well as Nonendothelial Lymph Node Stromal Cells. *The Journal of Immunology*. 2016 Oct 1;197(7):2686-94

Noemi A. Zambetti, Zhen Ping, Si Chen, **Keane J.G. Kenswil**, Maria A. Mylona, Mathijs A. Sanders, Remco M. Hoogenboezem, Eric M.J. Bindels, Maria N. Adisty, Paulina M.H. van Strien, Cindy S. van der Leije, Theresia M. Westers, Eline M.P. Cremers, Chiara Milanese, Pier G. Mastroberardino, Johannes P.T.M. van Leeuwen, Bram C.J. van der Eerden, Ivo P. Touw, Taco W. Kuijpers, Roland Kanaar, Arjan A. van de Loosdrecht, Thomas Vogl, Marc H.G.P. Raaijmakers. Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. *Cell Stem Cell*. 2016 Nov 3;19(5):613-627

Zhen Ping, Si Chen, Sjoerd J. F. Hermans, **Keane J. G. Kenswil**, Jacqueline Feyen, Claire van Dijk, Eric M. J. Bindels, Athina M. Mylona, Niken M. Adisty, Remco M. Hoogenboezem, Mathijs A. Sanders, Eline M. P. Cremers, Dicky J. Lindenbergh-Kortleve, Janneke N. Samsom, Arjan A. van de Loosdrecht, Marc H. G. P. Raaijmakers. Activation of NF-κB driven inflammatory programs in mesenchymal elements attenuates hematopoiesis in low-risk myelodysplastic syndromes. *Leukemia*. 2019 Feb;33(2):536-541

Hongzhe Li, Hooi Ching Lim, Dimitra Zacharaki, Xiaojie Xian, **Keane J.G. Kenswil**, Sandro Bräunig, Marc HGP Raaijmakers, Niels-Bjarne Woods, Jenny Hansson, Stefan Scheding. Early Growth Response 1 Regulates Hematopoietic Support And Proliferation In Human Primary Bone Marrow Stromal Cells. *Haematologica*. 2019 Aug 1. pii: haematol.2019.216648

PHD PORTFOLIO

Name PhD Student: Keane Kenswil
Erasmus MC Department: Hematology
Research School: Molecular Medicine (MolMed)

PhD Period: January 2014 – January 2020
Promoters: Prof.dr. H.G.P. Raaijmakers
Prof.dr. I.P. Touw

1. PhD Training Year

	Year	ECTS
General Courses		
Photoshop and Illustrator workshop	2014	0.3
Research management for PhD-students	2014	1.0
Research integrity course	2016	0.3
Biomedical English writing course	2017	2.0
Annual Course on Molecular Medicine	2018	0.6
In-depth Courses and Workshops		
Molecular aspects of hematological disorders (4x)	2014-2017	2.8
The course on R	2016	1.8
Basic course on SPSS	2018	1.0
Basic course on Python	2019	1.7
Basic course on Microsoft Access	2019	0.3
Two-days Course Bayesians statistics and JASP: Basic & Advanced	2019	0.6
Advanced course on Excel	2019	0.4
Scientific Meetings Department of Hematology		
Work discussions (Weekly)	2013-2018	8
Journal club / literature discussions (bi-montly)	2013-2018	7
PhD lunch with invited speaker (Monthly)	2014-2018	2.5
Erasmus Hematology Lectures (Monthly)	2014-2018	2
National/International conferences		
Molecular Medicine Day (4x) (Rotterdam)	2014-2017	1.5
European School of Haematology International Conference (Lisbon, Portugal)	2015	0.3
Dutch Hematology Congress (2x) (Arnhem)	2016-2017	0.6
Annual Conference of American Society of Hematology (Atlanta, USA)	2018	0.3
Presentations		
Departmental work discussions (Oral, 6x) (Rotterdam)	2014-2017	3.0
Journal clubs (Oral, 3x) (Rotterdam)	2014-2017	1.5
Dutch Hematology Congress (Oral, 1x)	2017	1.0
Academic Center for Stem Cells and Organoids in REgenerative medicine (ACE-SCORE) Day (Oral, 1x)	2017	1.0
Annual Conference of American Society of Hematology (Atlanta, US (Poster, 1x)	2018	1.0
2. Teaching, Supervision & Organization Activities		
Organization and supervision PhD lunch with invited speakers	2015-2016	0.2
Total		**41.7**

WORD OF THANKS

Perhaps I'm exaggerating by stating that I never expected to be writing my acknowledgments (at least any time soon), but there were times I genuinely thought I'd never reach this point in my PhD programme. Achieving this milestone would not have been possible without my own niche; in particular the people – to whom I will forever be indebted to for their continuous support over the past years – who I would like to thank in the following paragraphs.

Allereerst wil ik mijn promotor **Prof.dr. Raaijmakers**, **Marc**, bedanken: U nam mij niet alleen aan als een masterstudent, heel lang geleden al, maar u zag (gelukkig voor mij) genoeg potentie in mij om mijn onderzoek als volwaardige promovendus voort te zetten. De afgelopen jaren heeft u duidelijk aan mij gemaakt wat nodig is om succesvol te zijn in wetenschappelijk onderzoek. Naast imponeren met uw gladde praatjes, bent u ook gewoon het schoolvoorbeeld geweest van hard werken, hoe vaak ben ik u wel niet tegengekomen nog laat in de avond, of in het weekend, op werk. En misschien was de allerbelangrijkste les: gericht werken en bewust zijn van wat "the bigger picture" is. Dit heeft mij uiteindelijk ook immens geholpen met het (leren) schrijven. Verder heeft u mij ook geleerd om door te zetten als het tegenzit en dat tegenslagen in de wetenschap de normaalste zaak van de wereld is; uw enthousiasme en gedrevenheid voor onderzoek wist me vaak ook gewoon weer te motiveren wanner het nodig was. We waren misschien regelmatig niet met elkaar over eens over hoe bepaalde vraagstukken (realistisch) aangepakt moesten worden, maar dat was waarschijnlijk ook beter zo, anders had ik niet veel geleerd tijdens mijn periode in de Raaijmakers' groep. Ik ben heel dankbaar hiervoor en wens u en uw groep veel succes toe in het maken van nieuwe wetenschappelijke ontdekkingen.

Prof.dr. Touw, **Ivo**, mijn tweede promotor, ik heb veel geleerd, en me ook goed vermaakt, van uw moeilijke (en ook gewoon legitieme) vragen die vaak levendige discussies deed oplaaien tijdens de werkbesprekingen. U stelde ook vaak relevante vragen over mijn experimenten, deze hielpen ons het onderzoek naar een hoger niveau te brengen, maar voorkwam ook dat we voorbarige conclusies trokken. Verder kon u ook tegengewicht bieden aan Marc en kon ik gelukkig naar jou toe gaan voor duidelijk advies wanneer ik er soms niet uitkwam met hem. Misschien op de eerste oogopslag niet heel erg wetenschappelijk, maar ik stelde ook altijd op prijs hoe je altijd verschillende gerechten wist klaar te maken voor de eet-evenementen van onze afdeling. Oh en natuurlijk ook bedankt voor het grondig nakijken van mijn proefschrift!

I would also like to thank my assessment committee for critically evaluating and approving my manuscript. **Prof.dr. P. ten Dijke**, ik heb u persoonlijk (nog) niet ontmoet, maar u bent wel nauw betrokken geweest bij mijn onderzoek, vooral met de meest recente studie over EndMT, waar u (en uw groep) ook kritisch aan heeft bijgedragen. Nog bedankt hiervoor. **Prof.dr. W. E. Fibbe**, ook u heb ik (nog) niet ontmoet, maar u heeft ook een belangrijke bijdrage geleverd door mijn proefschrift snel op tijd grondig door te nemen, zelfs na wat

kleine mankementen met het *Hora Finita* systeem. Dank hiervoor. **Dr. E. Farrell**, I'm very grateful for our long-time ongoing collaboration that has turned out to be very fruitful, and which will hopefully lead to a very nice publication! Your willingness to extend a helping hand to our group helped us answer some very important questions brought up by the peer reviewers. I would also like to thank you for being the secretary of the committee, and keeping Marc updated through the whole process, who in turn could keep me informed on the latest developments.

I would also like to extend my gratitude to the other members of my doctoral committee for willing to participate in my defence ceremony. **Prof.dr. J.P.T.M van Leeuwen**, ik voel me vereerd dat u als decaan deel kan nemen in mijn promotieplechtigheid. Ik herinner me dat één van mijn eerste collaboratieve bijeenkomsten met jouw onderzoeksgroep was, namelijk de "Bone-HSC" meetings op de vijfde verdieping. Dit leidde gelijk naar één van de eerste samenwerkingen in mijn onderzoek, wat heel goed verliep, bedankt hiervoor. **Dr. T. Cupedo**, **Tom**, ik ben u ook heel erg dankbaar voor de verschillende manieren hoe jij mij hebt geholpen. Jij hebt je niet alleen (letterlijk) ingespannen om foetaal materiaal voor mijn onderzoek te regelen, maar jij heb ook veel constructieve feedback geleverd in twee van mijn belangrijkste studies. Ik vond de collaboratieve bijeenkomsten met jouw onderzoeksgroep ook altijd heel erg leerzaam. Jouw onderzoeksgroep was ook altijd bereid om mij te helpen, en is in zekere mate een weerspiegeling van jou. **Dr. G. Krenning**, **Guido**, jouw groep heeft ook belangrijke bijdragen geleverd aan mijn werk naar EndMT. Jij hebt ook zelf ervoor gezorgd dat nadat jouw PhD-student, Byamba, terug naar Mongolië was vertrokken dat onze samenwerking nog vlekkeloos verliep. Bedankt hiervoor. **Dr. R. Schneider**, **Rebekka**, while I maybe have not worked as closely as my other (former) colleagues with your group, I really still appreciate your input and the help you and your group have provided me along the way. I hope the common themes shared between our groups will continue to foster close (future) collaborations, in particular when it comes to resolving the potential role of IL-33 in myelofibrosis.

Verder wil ik ook de andere groepsleiders van de afdeling Hematologie bedanken die ik heb meegemaakt. Speciale dank aan **Bob** die ik nog regelmatig tegenkwam en mij altijd heel erg vrolijk groette, ook al kende u mij eigenlijk niet, en die ook nog vaak de Friday floor meetings bijwoonde en daar ook interessante vragen stelde. Dit geldt ook voor **Pieter**, **Frank**, **Jan**, **Ruud**, **Moniek**, **Peter**, **Eric**, **Mojca**, **Stefan** en **Emma**, jullie hebben allemaal op één of andere manier mij geholpen, bijvoorbeeld door het doneren van bepaalde protocollen, reagenten, cellen, weefsel, of zelfs met het opzetten van nieuwe muizen-breedings. Ook was het gezellig om met jullie te babbelen of grappen te maken over jullie eigen PhD-studenten tijdens de borrels.

Of course I would also like to thank my fellow group members, be it former, contemporary, as well as recently arrived colleagues, you all had your part in helping me to complete my PhD studies.

First of all I would like to thank **Noemi**, you were my daily supervisor during my master internship despite you not really benefiting from this task due to our projects not being directly related. Nonetheless you still took your time to teach me basic stuff, such as cell culture and genotyping, and helped me to get on my way to conduct my own research independently. I appreciated your more hands-off (progressively over time) approach in supervising me and liked that you didn't mind me slacking off from time to time to socialize with other fellow (master)students. I think you also set a great example for the rest of the students in the lab, and I think this is partly why Marc has such high standards for his PhD students, so thanks for that ☺.

Athina, while I did not end up being your student, you still helped me out with any questions I had for you, even though you already had left the group by then. Also thank you for helping to set up the group before I arrived!

Niken, apppaaa, how I miss our KFC feasts we shared with our fellow (Asian) group members. Thank you so much for helping with out with genotyping, FACS and RNA-Sequencing, without you the group barely managed to scrape by.

Si, another of the original KFC-crew members! I'm impressed by how you always managed to stick to your goals and never got too distracted, despite whatever stressful situations were occurring in the lab. It's particularly impressive that you managed to graduate with minimal delays, which is not an easy task in this group. You also have a real knack for presenting, and I hope you keep putting it to good use in Singapore! I would like to thank you for giving me a lot of constructive feedback during our group-meetings, at least when you were not falling asleep haha, and also for organizing a lot of fun social (read: eating a lot of food) events with our other lab-mates.

Ping, the honour and privilege is mine. We have been through so much, and even lived together for a while, though I actually did not see you much socially back then since you were usually slaving away in the lab until the early morning hours. This was often, if you were not procrastinating in the lab or getting distracted by me, due to your dedication to getting stuff done perfectly. Because, indeed, what's the point of doing something if it's not done properly? This is a sentiment I agree strongly with you, and I think this is also why I get a long with you not only at professional level, but also a personal level. In fact, we see eye to eye on a lot on a lot stuff, in particular what it means to have honor. That said we also had some disagreements, and inevitable tensions in the lab, like about Tibet/Taiwen, but we always managed to keep it respectful. I have to keep it brief, but I'm sincerely grateful that I shared an office, our own private fridge, many dinners, karaoke-borrels, fun festivals, and many crazy parties over the years. Also special thanks for helping me out with my mice experiments whenever my hands were too shaky!

Adrian, you bloody Mexican! We both know I would never have gotten to this point without you laying the groundwork for me when it comes to the Day 17 and fetal samples. For this I will always be grateful to you. Besides these critical scientific contributions, you

were also one hell of a roommate to have! The many nights out, but also the nights at our place hosting costume parties, including the toga, yoga, Halloween, murder-mystery, and Christmas sweater themed events; the utter debauchery would have made Caligula blushed. And then not to speak of the countless concerts we attended, such as Nils Vroom, The Beach Pixies, Blossoms, Corvalis, Small Blacks, Yucks, Dinosaur Sr., Sonic Seniors, The Serial Killer from No Country for Old Men, and The Robert Smiths, what a time to be alive indeed. I really hope we manage to attend another festival with the gang~~bang~~ before all it is said and done. There are many more stuff I'm grateful for, but I have to keep it brief. I will spare you the platitudes, and simply wish you good luck in whatever you still need to do to finish your PhD. Trust the dice.

Claire, ik wilde net schrijven dat alles gelukkig eindelijk achter de rug is, maar we hebben misschien binnenkort nog revisies voor de EndMT paper :')! Maar nu even serieus, ik ben je zeer dankbaar dat jij mijn projecten voorlopig (en niet eventjes) wist over te nemen. Zonder jou zouden de foetale monsters in Amsterdam niet verwerkt zijn geweest, en dat was ook allemaal net op tijd gebeurd. Jij hebt niet alleen praktisch werk gedaan, zoals FACs en RNASeq, maar jij hebt ook gewoon in zijn geheel kunnen bijdragen door jouw eigen wetenschappelijke inzichten te verschaffen, zoals bijvoorbeeld bij het analyseren van de scRNASeq data, en andere momenten die ik me nu natuurlijk niet meer kan herinneren :p. Ik geloof oprecht dat je geschikt zou zijn om zelf een PhD-traject te voltooien! Jij hebt in ieder geval het IQ en passie ervoor. Verder was het ook heel gezellig met jou; en hoop ik binnenkort met jou en PaOla te kunnen sushi'n.

Jacqueline, you are the bridge from the former generation to the current one. I'm really impressed to see how you've developed over time as researcher, like me also starting off as a master student, and becoming more confident and taking charge of your own experiments. Not only that, but you have also kind of taken up a senior-ish role in the group, making sure everyone is doing well or doing stuff properly in the group. I'm really grateful for the several times you helped me out with my projects, be it with HiX to help me out with the AML day 17 samples, or preparing antibody orders, or even performing flow cytometry experiments for the EndMT project. I'm sure you will continue to do well in your PhD, and wish you good luck in carrying the torch for the newer generation!

To the other former Post-Docs in our group: **Yongyi** and **Tamar**, it's too bad our periods didn't overlap much in the lab, I would have really liked to soak up more of your knowledge. I wish you well in your future scientific pursuits! All active PhD-candidates or Post-Docs in our group, **Eline**, **Isabel**, **LanPeng**, and **Martijn**, I haven't worked or interacted much with you at all, but I still want to thank you for the times I chatted or distracted you in the office or hallways and wish you well with your studies! Also a special mention to all former students in our group, like **Sjoerd**, I enjoyed nerding out about stuff like Twitch, I felt like I was teenager again.

Last but not least of the Raaijmakers' group, I want to thank **PaOla**, I often say time is a flat circle, and working closely with you these past weeks on the submission for the EndMT paper, made me believe this all the more: I started my studies on the niche with a feisty, short, intelligent Italian brunette, only for it to finish with another fire-y, small, smart, Italian brunette. Though my thesis was technically already approved, you still helped me elevate the EndMT paper to another level, not only stylistically with your choice of colours, but also literally at a single cell level. For this I really want to say thank you. I'm happy "my project" is in your hands now, and I'm sure you will do well. Also like I told Claire, I look forward to our sushi dinner to discuss not only how the project is progressing, but also to figure out which political system, instead of democracy, would be the best to meet the needs of society.

I would also like to name a lot of other people from the lab that have helped me out. Special thanks to **Eric-Sensei**, I don't think I'm exaggerating when I call you the Godfather, or maybe even the babysitter of our group, without your guidance we would not gotten much done (efficiently or properly) over the past years. I'm especially grateful for the many times you helped me out with those pesky western blots and with the shRNA knockdowns. In addition, I also appreciated your fine taste in music (most of the times), in particular when it comes to our shared love for (post)punk music or The National. We even attended a Sigur Rós concert together once with Mr.O! **Mr.O**, what up my ginger! It's time to plan Iceland part II, it has to happen! Thanks for organizing the many epic (ambrosia) nights, and being part of all the crazy adventures we've experienced over the years. We also worked a bit in this period, and I also want to thank you for helping me out in the lab, be it with confocal, getting me extra screen for the PC, or providing me shiny antibodies for immunofluorescence. Thanks man! **Paulette!** Hoe vaak moest jij wel niet lang op het lab blijven, omdat jij mij aan het helpen was met FACS-sorteren. Verder hielp jij mij ook zo vaak met het preppen van mijn samples voor RNASeq, en was jij ook altijd oprecht geïnteresseerd in hoe het met mij ging, en ook met de anderen in de groep, door een luisterend oor aan te bieden. Je was soms eigenlijk gewoon een technician binnen onze groep (en ik zou het helemaal niet erg gevonden hebben als jij naar de Raaijmakers' groep was overgelopen), en wil je hiervoor hartelijk bedanken! Ik wil ook **Nathalie** bedanken, jij hebt me ook zo vaak geholpen met het sorteren en het verwerken van de foetale botten. Ook vond jij het nooit een probleem als ik regelmatig antilichamen kwam gebruiken van jouw groep. Verder kon ik wel lachen om je grappen, zoals over "The Great Wall of China".
Remco, ik vond het heel gezellig met jou op de 19de verdieping toen ik aanvankelijk begon als een masterstudent, en dat bleef ook zo nadat we zelf een eigen plek op de 13de hadden gevonden. Ik wil jou ook hartelijk bedanken voor jouw inzet om gedegen RNASeq/GSEA analyses uit te voeren. Dit geldt ook voor **Mathijs**, en ik wil jullie allebei bedanken voor jullie geduld met onze vragen over statistiek, bijvoorbeeld wanneer iets nou echt als significant beschouwd kan worden. Ik wil ook **Elodie** bedanken voor het bouwen van de RNASeq-database website.

Verder wil ik nog meer mensen bedanken, waaronder de flow-cytometrie operators: **Elwin**, **Peter** en **Michael**. Jullie werkten altijd nauwkering en bleven geduldig en flexibel wanneer ik samples (nog laat) moest sorteren. Ik wil in het bijzonder Elwin verder bedanken, want jij hebt mij ook ontzettend veel geholpen met het vierde hoofdstuk van mijn proefschrift. Samen met **Lucia** hebben jullie mij ook geholpen met opzetten van mijn masterstage. Ik wil ook **Mariëtte** bedanken, jij hebt mij veel geholpen met het isoleren van $CD34^+$ cellen uit navelstrengbloed, en je hebt mij vaak cellen gedoneerd. **Jasper** en **Joyce**, ik heb misschien niet veel direct met jullie gewerkt, maar ik kon altijd leuke grappen met jullie maken. To err is human to arr is pirate.

I would also like to give special thanks to my collaborators, who helped me finish my studies. **Bram** en **Marijke**, dank jullie wel voor de MSCs en ook voor het duidelijk maken aan mij hoe ik ALP-assays moest uitvoeren. **Byamba** and **Callie**, thank you for all the cell culture and (differentiation assays) you guys performed for the EndMT paper. Callie, it's about time we find another festival or event to celebrate that we can put our PhD-adventure behind us! **Gonzalo** and **Andrea Lolli**, I also want to thank you for your critical contributions to the EndMT paper, I hope you guys don't mind if I keep pestering you about experimental details if any questions pop-up, especially if we end up doing revisions! **Dr. Pieter Koen Bos** en **Dr. King Lam** bedankt voor de humane (bot) monsters die ik heb kunnen gebruiken in mijn studies. **Dr. Hans Stoop**, jou wil ik ook bedanken voor al de immunohistochemie die jij voor mij hebt gedaan, en ik wens je nog succes toe met je jazz-optredens! Ik wil ook het **BMT** lab bedanken voor al de BM samples die jullie aan mij hebben gegeven. Speciale dank aan **Hennie** die mij heeft geleerd hoe ik kolonies moet tellen.

I'm also much obliged to the colleagues who I shared my office with over the years. **Emanuele**, my paranymph and dear friend, hopefully you have survived the corona-scare with Matteo, and will manage to make my ceremony. If not, you will be very difficult to replace, as I don't see anyone easily replicating your rather care-free, bordering on the lackadaisical, attitude that is very much appreciated by me. In fact, I'm rather fond of this approach to life as it led to many eggtastic eggperiences. At the same time your attitude keeps my rather neurotic and impulsive behaviour in check, and whenever we almost came to blows you always knew when to walk away (cause you'd get your ass kicked). Kidding aside, as Ping would put it, it's been an honour to have gotten to know you. I look forward to visiting your hometown one day so we can play some basketball and eat arrosticini with the locals. Having said that I hope I already partially repaid my debt to you by finding you your first Dutch GF. P.S. Sorry for dragging you to Welcome to the Future, but it's your own fault you got sick from eating bad fish. Anywho, good luck with your own writing. **Cansuuu**, thank you for bestowing upon me my cute "Keannuu" nickname, I think it actually suits me quite well. You and your daddy have made my final years of my PhD so much fun, I'm happy that we not only saw each other so often in the lab, but also outside of it! I'm in particularly impressed by how you can keep up with me, Ping and your daddy when it comes to eating yummy food. With Ping, me, and

also your daddy leaving soon, I hope you won't get too lonely, but I'm sure you'll manage, since you probably will just keep laughing like a maniac, as you always do. Good luck with finishing your projects, you can do it!! **Mira**, you were never officially my officemate, but you practically were with how often you came to visit us. Luckily, you never did become part of our office, otherwise I would have gone crazy with the nonstop chit-chat between you and Cansu. That said I did appreciate your presence most of the times, even though I admittedly was a bit too mean sometimes (sorry, not sorry, for that). I am grateful for the times you helped me out with my experiments. While things didn't work out at the EMC, I'm pretty sure you'll do fine in the LUMC, and I hope I'm also invited for your PhD ceremony when that time arrives!! **Michiyo**, you were one of the most polite officemates I had, and it was so much fun talking to you about Japan, and even going out and eating Ramen with you and the rest of the gang. **Farshid**, thank you for providing me a lot of career advice, and welcoming me into your office that you were already sharing with **Lucilla**, who always had a story or two to tell.

I still have many people to thank, and I'd like to continue with the technicians in the Delwel Group. **Claudia** and **Andrea**, thank you for the pleasant and very considerate conversations, you guys were always nice to me. Andrea one day we will definitely attend a salsa party or bachata event with one of my good friends, ahah. **Marijke**, bedankt voor jouw hulp in het ML-II lab. Ook wil ik de andere technici van onze afdeling bedanken, in het bijzonder **Hans** en **Dennis**; ik vond de bierproeverij en andere sociale evenementen altijd goed geslaagd, alleen was het jammer dat ik geen pittige ketchup meer van jullie kon krijgen. **Inge**, ik ben je ook dank verschuldigd, jij had me geholpen met het verwerken van muizen-botten voor immuno-kleuringen, nog bedankt hiervoor!

I also want to thank former and current Post-Docs in the lab, **Julien** (also magic cola lol), **Ferry**, **Leonie**, **Jess, and Nils**. Thank you for all the times you helped me out with FACS-sorting, providing general career advice, engaging scientific discussions, or just exchanging simple pleasantries.

I also owe a lot to the (PhD-)students that have come before me. Very special thanks to **Kasia** and **Jana**, original founders of the EMC-techno club, it was such a blast to get to know the playful side of my fellow colleagues of the lab, and much respect to you OGs for setting the tone for the younger generation. I'd like to give even more props to Jana for introducing me to her fellow MolMed students. And thank you Kasia for giving me not only career advice, but also advice about life in general. **Monica** you deserve a special mention as well, you also helped me out a couple of times with FACS-sorting, and you were also very fun to party with, especially with Julien, I hope you are doing well in Vancouver! Shout-out to **Patricia D** (you also had a very fine musical taste), and **Marshall**, hope you are doing well in MAGA-land! As for you, **Roberto**, I hope our lengthy conversations, which still have continued and hopefully will keep on going for a long time, made it clear how much I appreciate having you around. You have also been very considerate of others, in addition to being a deep-thinker

without ever being pretentious or arrogant. Having you and Adrian as roommates was so much fun, and I'm happy we managed to arrange this and experience so many, as you put it, "moments" together. Cause in the end, that's all what matters no? I wish you all the best, and hopefully I will see you again soon. Perhaps one day we can all see Solar Fields together? Ostia.

I also want to thank the other PhD students that are close to graduating or are getting there: **Davine**, **Tim**, **Patricia O**, **Adil**, **Sophie**, and **Roger**, zet hem op! I wish you the best with finishing your studies. To the newer PhD students that I have gotten to know a bit: **Bella** totally awesome that you returned to Hematology, hope you keep listening to good indie music, and perhaps I'll bump into you in one of those random ADE parties. **Madelon**, het was leuk om je te leren kennen tijdens de Python cursus, houd me op de hoogte als je ooit een documentaire heb geregisseerd! Verder wens ik je nog succes met Paola in je kantoor, ik ben er heel bewust van dat ze best veel kan praten ☺.

Ik wil ook nog andere collega's van de afdeling Hematologie bedanken: **Ans**, **Annelies**, **Eaudia**, **Tessa**, en **Leenke**, ik wil jullie bedanken voor het regelen van al mijn afspraken met Marc en Ivo, verder waren jullie altijd behulpzaam als ik vragen had, bijvoorbeeld over hoe ik bepaalde reagenten het beste kon verzenden. **Egied**, ik wil jou ook hartelijk bedanken voor al jouw hulp met mijn proefschrift, zonder jou zou ik de tijd niet hebben gehad om mij goed te concentreren op mijn schrijfwerk. Verder ben ik heel tevreden over hoe mijn proefschrift eruit gaat zien.

Over the years I also met a lot of fellow students outside of Hematology that made my EMC-experience all the more fun! Special thanks to **Lena**, I enjoyed our talks about free will/ determinism, you playing my musical requests during your DJ-set at BAR, and not to forget the many karaoke events! You also helped me realize that for some reason most of the cool people from Greece are from Thessaloniki, case in point **Irene** and **Isabella**! I had a great time with you girls at BKS, and many other events, perhaps we can find another festival soon. Maybe the one Ping will be attending this August, the music won't be Radiohead or The Cure, but it I'm pretty sure it will be awesome! I also want to wish you both good luck with your PhDs. Also a special thank you to Irene for introducing me to the girl who took over your old room, haha. Also please give some hugs and kisses to Nefeli and Len for me! I also want to thank another expat from Thessaloniki, **Simy**, especially for giving me plenty of extra discounts at the EMC-cafeteria, good look with your egg studies! I also want to mention **Anika** and **Liz**, you guys were one of the first master students I started to hang out with when I first started out at the EMC. Thanks for having me over in Atlanta Liz, and I wish you good luck at the CDC! Anika good luck finishing up your PhD in Switzerland, and get that €€€. Also shout-out to the people I met from Infection & Immunology, **Jurre, Anusha**, and **Simone** who were always up for parties! I'd also like to give special thanks to the people form MolMed-school, **Rodrigo** and **Pablo** it was fun playing basketball with you guys from

time to time, and having many BBQ sessions (in the park)! **Diana** and **Adna**, you guys also joined for basketball a couple of times and even more special, I really had great time visiting you guys in your home countries and showing me around. I think it made me understand you guys better knowing where you came from, I guess haha. Diana, I wish you good luck in Memphis, just don't get shot by those crazy Americans okay? Adna, hopefully you can manage finding a position in the EU and maybe see you at **Tania**'s and **Alex**' wedding?! Very special thanks to these lovebirds for letting me stay at your place, so many times. It really felt like my third home. And besides that it has been so much fun going to (karaoke-)parties and festivals, such as Expedition and Bangface, with you guys. Take care of each other, and also good luck with writing your thesis Tania!

The fun was not limited to the EMC, and I also have to mention some other awesome people, in particular the my fellow Bisons. Special thanks to **Raz** who has been the driving force behind all the awesome Bisons events. Shout-out to **Ian**, maybe the craziest Bison of them all, and that says a lot considering I know Adrian. I also want to thank **Lorenzo**, **Thomas**, **Livia**, **Matteo**, the Italian (Schopare?) branch of the Bison group, thanks for all the memorable parties and BBQs in the park.

Ik heb gelukkig nog meer vrienden, denk ik, onder andere **Esther** en **Sanders**, die ik ook wil bedanken voor het bijwonen van allerlei concerten, en hopelijk blijft dit ook de komende jaren zo doorgaan. Alleen moet DIIV wel weer goede muziek maken. Ik moet natuurlijk de Lamers-squad ook opnoemen, zonder jullie zou ik mij veel minder hebben vermaakt de afgelopen jaren, vooral bedankt voor jullie geduld met mijn trollings, in het bijzonder **Nilton** en **Kachon** (die het ook vaak verdienen). Jullie hebben mij regelmatig geholpen met verhuizen, en ik ben eigenlijk de tel kwijt, thanks boys. **Nahuel** en **Ali**, cool dat jullie naar IJsland mee waren gekomen, kom maar nog een keer, alleen wel met extra verzekering voor de huurauto. Ik ben nu wel betere driver, of niet Nahuek? Verder GG met alle gaming sessies, en kom snel weer een paar potjes PES doen. Shout-out to fellow OG SXM'r strongman **Rugene**, I appreciated your heavy-lifting, not only during my moving, but also during my mom's. It was also cool playing Pokemon Go with you for a bit, and I wish you the best in becoming a Pokemon master, if not yu-gi-oh/salsa/bachata master. Oh ja, ik wil ook nog succes aan **Shiraaz** toewensen met het afronden van jouw PhD. Ik wil nog één speciaal iemand van het EMC bedanken, en dat is **Frank van Vliet**, dank je voor al die keren dat je naar me luisterde over mijn problemen, advies hebt gegeven, fondsen voor mij hebt geregeld, en ook gewoon de gezelligheid die jij altijd aanbood met ons etentjes en drankjes. Hopelijk doe jij zelf de komende tijd ook rustig aan en hoef jij het niet zo druk te hebben met werk.

Ik wil ook even mijn familie bedanken, waaronder mijn neven en nichten, **Bryan**, **Edsard**, en **Amy**, wat leuk dat **oma** ons allemaal heeft weten samen te brengen, door haar hebben we allerlei familie-uitjes kunnen maken afgelopen tijd. Wel jammer dat we voorlopig niet veel kunnen tot 1 juni, maar ik weet zeker dat we daarna snel iets vinden om mijn promotie goed te vieren. Verder echt tof dat je nog mee was gekomen naar Malta Bryan toen we

Roberto opzochten, alleen volgende keer niet zo goedkoop parkeren in Eindhoven! Sowieso nog bedankt dat je me paranimf wil zijn! Verder wil ik ook mijn zusje **Abygail** bedanken, jij hebt me zo vaak moeten ophalen bij het vliegveld als er weer eens problemen waren, zelfs ook helemaal uit Brussel. Ook heb je vaak andere dingen voor mij gedaan en vervoerd, bijvoorbeeld wanneer **mama** eten wilde langsbrengen. Ik zal mijn best doen om aardiger tegen jou te zijn, en ook vaker bubbeltea meennemen naar huis voor jou. Ik wens je nog succes met het afronden van je studie. **Ma**, bedankt voor al het eten, en alle ontelbare dingen die jij voor mij hebt geregeld. Jij hebt misschien gelijk dat ik vaker naar je moet luisteren, maar ik zal altijd koppig blijven. Ik ben wel dankbaar naar **jou** en **papa**, dat jullie Abygail en mij naar Nederland hebben gebracht, want uiteindelijk hebben we hier een heel goed leven weten op te bouwen. **Pa**, ook bedankt voor al je aanmoedigingen en steun, onder andere door telkens te vragen wanneer mijn proefschrift af zal zijn. Het is eindelijk gelukt.

And to cap it all off, I want to also to give special thanks **Nina**, my cutiepie. I have put you through a lot all these years of me trying to obtain my PhD title. Still the longer you waited the more I felt I had to finish; otherwise that waiting would have been for nothing. Luckily you are very patient (haha) and the time has finally arrived that we can take the next step, not only in our career, but life in general. I want to thank you for hanging in there, and also for the pretty cover you and your friend **Lydia**, made for me. Where-ever our adventures may lead us, I hope I'll be good enough to stand by your side.

Well that's it, I probably forgot to mention a couple of people, and for that I have to apologize, but I need to print this dissertation in time, kthxbye.