Regulation of Hematopoiesis and Osteogenesis by Blood Vessel–Derived Signals

Saravana K. Ramasamy, 1,2,* Anjali P. Kusumbe, 1,2,* Tomer Itkin, 3,* Shiri Gur-Cohen, 3,* Tsvee Lapidot, 3 and Ralf H. Adams 1,2

1Department of Tissue Morphogenesis, Max Planck Institute for Molecular Biomedicine, D-48169 Münster, Germany; email: ralf.adams@mpi-muenster.mpg.de
2Faculty of Medicine, University of Münster, D-48149 Münster, Germany
3Department of Immunology, The Weizmann Institute of Science, Rehovot, 76100, Israel; email: Tsvee.Lapidot@weizmann.ac.il

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Abstract
In addition to their conventional role as a versatile transport system, blood vessels provide signals controlling organ development, regeneration, and stem cell behavior. In the skeletal system, certain capillaries support perivascular osteoprogenitor cells and thereby control bone formation. Blood vessels are also a critical component of niche microenvironments for hematopoietic stem cells. Here we discuss key pathways and factors controlling endothelial cell behavior in bone, the role of vessels in osteogenesis, and the nature of vascular stem cell niches in bone marrow.
INTRODUCTION

Endothelial cells (ECs) form an extensive vascular network that extends into every organ and almost all tissues in the body. In addition to the conventional role of blood vessels as a versatile conduit system for the transport of cargo such as gases, nutrients, waste products, and cells, ECs have been functionally linked to a broad range of physiological and pathological processes, including barrier formation, selective transport, scavenging, thrombosis, wound healing, and inflammation. More recently, it has been discovered that ECs are crucial regulators of tissue morphogenesis, which involves the secretion of growth factors and presentation of molecular signals that act on nearby cell populations in a so-called angiocrine fashion (Butler et al. 2010, Rafii et al. 2016, Ramasamy et al. 2015). Moreover, blood vessels are critical components of niche microenvironments that maintain the special properties of stem and progenitor cells in their proximity. Organs have very distinct functional roles, and such distinctions are reflected by differences in the organization of their vascular beds and by the expression of specific molecular signals by ECs (Nolan et al. 2013). Thus, blood vessels adapt and respond dynamically to changes in their local environment and can thereby regulate diverse physiological, regenerative, and pathobiological processes.

Despite its structural strength, the skeletal system harbors very dynamic microenvironments, which is reflected by the lifelong homeostatic remodeling of bone and the constant production of blood cells. The presence of mesenchymal stem cells, osteoprogenitors, and hematopoietic stem and progenitor cells (HSPCs), along with very heterogeneous stromal cell populations, indicates the possible existence of multiple spatially and functionally distinct microenvironments. The recent identification of distinct capillary subtypes in bone further enhances this complexity by adding specialized vascular environments. Advanced imaging approaches and cell type–specific inducible mouse genetics have greatly advanced our understanding of the organization and function of the vasculature in the skeletal system.
REGULATION OF ORGANS BY ENDOTHELIAL CELL–DERIVED SIGNALS

ECs control tissue morphogenesis, patterning, and regeneration by releasing growth factors, cytokines, and extracellular matrix proteins or by presenting signaling molecules on the cell surface. Importantly, such angiocrine functions of the endothelium are not confined to a few specialized settings but appear to represent a fundamental principle controlling many aspects of developmental and regenerative tissue growth. For example, EC-derived instructive signals in the early mouse embryo control the behavior of endodermal cells during liver (Matsumoto et al. 2001) and pancreas (Lammert et al. 2001) development. Similarly, EC-derived paracrine factors and signals play indispensable roles in the morphogenesis of organs such as the heart, kidney, and lung (Ramasamy et al. 2015). In addition to having crucial roles in directing development of the liver and kidney, EC-derived signals also control regeneration after tissue injury (Hu et al. 2014, Rafii et al. 2016, Ramasamy et al. 2015). Interestingly, liver ECs provide divergent factors that can promote regeneration and inhibit fibrosis in response to injury (Ding et al. 2014). EC-derived signals also modulate body metabolism by regulating pancreatic insulin production (Nikolova et al. 2006), adipogenesis, and thereby thermogenesis (Cao 2013) and by fine-tuning the function of cardiac cells (Noireaud & Andriantsitohaina 2014, Rafii et al. 2016). Moreover, ECs provide protective and nurturing niches for multiple adult stem cell populations such as neural stem cells (Shen et al. 2008, Tavazoei et al. 2008), spermatogonial stem cells (Yoshida et al. 2007), muscle stem cells (Christov et al. 2007), hematopoietic stem cells (HSCs) (Acar et al. 2015, Kunisaki et al. 2013, Morrison & Scadden 2014), hepatic progenitors (Wang et al. 2015), and osteoprogenitors (Kusumbe et al. 2014, Ramasamy et al. 2014). Taken together, various studies indicate that EC-derived signals can orchestrate complex multicellular network interactions in health and disease.

THE ROLE OF BLOOD VESSELS IN SKELETAL DEVELOPMENT

The condensation of mesenchyme generates rudimentary structures that serve as templates in bone morphogenesis. Two distinct developmental modes mediate the conversion of these condensations into skeletal elements. Intramembranous ossification involves the direct formation of bone cells from mesenchymal cells, whereas endochondral ossification involves the formation of chondrocyte structures as an intermediate that is subsequently converted into calcified tissue (Clarkin & Olsen 2010, Zelzer & Olsen 2003). Intramembranous ossification generates flat bones such as the cranium and ilium, whereas endochondral ossification generates a large variety of skeletal structures such as the appendicular skeleton and vertebrae. During endochondral ossification, chondrocytes in the center of the cartilage template become hypertrophic and produce signals acting on osteoprogenitors and blood vessels in the perichondrium (Figure 1), which separates the periphery of the condensed mesenchyme from the surrounding tissue (Long & Ornitz 2013). The invasion of cartilage by blood vessels along with osteoblasts from the perichondrium triggers the formation of the primary ossification center. Growth of this structure is accompanied by extension of the developing bone and by the establishment of growth plates (Colnot et al. 2004, Kronenberg 2003). Hypertrophic chondrocytes express high levels of vascular endothelial growth factor A (VEGF-A), a potent proangiogenic factor that promotes vascular invasion of cartilage (Gerber et al. 1999, Zelzer et al. 2001). Osteoblasts and perivascular cells in the perichondrium also express stimulatory signals such as VEGF-A and Angiopoietin-1 to promote vessel growth (Maes et al. 2010). The interplay between blood vessels, osteoblasts, and chondrocytes at the perichondrium is critical for the initiation of bone formation. Defective chondrocyte differentiation in knockout mice lacking the osteogenic transcription factor Runx2/Cbfα1 is associated
Figure 1
Coupling of angiogenesis and osteogenesis in development, growth, repair, and aging. Displayed are several mechanisms and molecular regulators that mediate communication between blood vessels and bone cells. (a) In primary ossification of condensed mesenchyme during embryonic development, blood vessels (red) invade together with osteoprogenitors (green). (b) Growth of long bones mediated by endochondral ossification involves specialized type H (CD31<sup>high</sup> Endomucin<sup>high</sup>) capillaries and the angiocrine release of factors acting on chondrocytes (blue) and osteoprogenitors (green). (c) Blood vessels and different factors play critical roles in bone repair and remodeling during different stages of fracture healing. (d) Progressive loss of type H capillaries during aging is associated with a decrease in angiocrine, pro-osteogenic factors, which contributes to age-related bone loss. Other abbreviations: BMP, bone morphogenetic protein; dp, diaphysis; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; mp, metaphysis; PTH, parathyroid hormone; type L, CD31<sup>low</sup> Endomucin<sup>low</sup>; VEGF, vascular endothelial growth factor.

with inhibition of both vascular invasion and bone formation (Zelzer et al. 2001). In cultured mesenchymal cells, Runx2/Cbfa1 interacts with hypoxia-inducible factor 1α (HIF-1α) to induce VEGF expression (Kwon et al. 2011). During extension of long bone, the proliferation, maturation, and hypertrophy of growth plate chondrocytes are associated with angiogenic vessel invasion and endochondral ossification. The sum of these processes gradually displace chondrocytes, establish a growth plate, and lead to the generation of distinct metaphysis and diaphysis regions.

Capillaries display distinct morphologies in the metaphysis and diaphysis; such differences also correlate with divergent expression of the markers CD31/Pecam1 and Endomucin (Emcn). Capillaries in the metaphysis have a predominantly columnar organization and show high expression of CD31 and Emcn. Likewise, capillaries in the endosteum, a thin layer of connective tissue lining the inner surface of compact bone, show high levels of the two markers. Accordingly, these
capillaries and their ECs have been classified as type H (CD31\textsuperscript{high} Emcn\textsuperscript{high}) (Kusumbe et al. 2014) (Figure 1). An important common feature of type H capillaries irrespective of their location is the tight association with perivascular osteoprogenitors expressing the transcription factor Osterix. In contrast, the sinusoidal capillaries of the bone marrow (BM) cavity, which show lower expression of Emcn and very low levels of CD31 (CD31\textsuperscript{low} Emcn\textsuperscript{low}) and have therefore been classified as type L, lack association with Osterix-expressing cells and are instead surrounded predominantly by cells of the hematopoietic system (Kusumbe et al. 2014) (Figure 1). In contrast to type L endothelium, ECs of type H capillaries express relatively high levels of pro-osteogenic factors such as fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and bone morphogenetic proteins (BMPs), which may explain the coupling of angiogenesis and osteogenesis at the molecular level (Kusumbe et al. 2014).

Interestingly, both the properties of the capillary endothelium and those of bone formation are controlled by signaling pathways in ECs. Stabilization of HIF-1\(\alpha\) in the endothelium by genetic inactivation of the VHL (von Hippel–Lindau) E3 ubiquitin ligase (Jaakkola et al. 2001), which tags HIF protein for degradation under normoxic conditions, promoted the formation of type H capillaries and enhanced osteogenesis (Kusumbe et al. 2014). Similarly, endothelial Notch signaling promoted the amplification of type H capillaries, leading to enhanced bone angiogenesis and osteogenesis. Surprisingly, disruption of endothelial Notch signaling led to disrupted chondrocyte maturation, hypertrophy, and resorption in the adjacent growth plate, which explains the defective bone extension observed in these mutants. As mature and hypertrophic chondrocytes are a major source of VEGF-A, these defects contribute to reduced blood vessel growth, lower EC proliferation, and loss of type H capillaries (Ramasamy et al. 2014). Notch receptor activation in response to ligand binding requires several proteolytic cleavage processes, one of which is mediated by the ADAM family metalloprotease ADAM10. Similar to defects seen in EC-specific Notch loss-of-function mutants, inactivation of the gene encoding ADAM10 in ECs led to impaired bone growth and to growth plate defects (Glomski et al. 2011). Expression of Noggin, a BMP antagonist that restricts growth plate size (Brunet et al. 1998), is positively controlled by Notch in ECs. Administration of recombinant Noggin was sufficient to restore growth plate defects, VEGF-A expression, and osteogenic defects in EC-specific Notch loss-of-function mutant mice (Ramasamy et al. 2014). Together, these findings indicate that the growth and differentiation of chondrocytes, osteoprogenitors, and ECs are tightly interconnected (Figure 2). Similar links may couple the behavior of ECs and bone-degrading osteoclasts. Deletion of the gene for PDGF-B in the osteoclast lineage led to decreased formation of trabecular and cortical bone without affecting osteoclast cell number in mice (Xie et al. 2014). The abundance of type H capillaries was strongly decreased in these mutants, highlighting the complex interplay between different cell types in the skeletal system.

MECHANISMS COUPLING ANGIOGENESIS AND OSTEOGENESIS

The vasculature forms an integral part of the skeletal system and plays critical roles in bone homeostasis and regeneration. Some of the factors and pathways controlling the interplay between osteogenesis and angiogenesis are discussed below.

Hypoxia-Inducible Factor

During endochondral ossification, vascular invasion of chondrocytes coincides with mineralization of the matrix. The avascular nature of cartilage generates a hypoxic environment in which chondrocytes are adapted to low oxygen tension and stabilize HIF (Rajpurohit et al. 1996). Chondrocytes in the growth plate are spatially arranged as columns, which reflects their life cycle of...
proliferation, differentiation, hypertrophy, and apoptosis (Erlebacher et al. 1995). Apoptosis of hypertrophic chondrocytes is followed by blood vessel invasion, bone formation, and bone mineralization (Harper & Klagsbrun 1999). VEGF-A expression in growth plate chondrocytes is in part controlled by HIF-1α. Chondrocyte-specific inactivation of the gene for HIF-1α in Col2-Cre transgenic mice led to disorganized growth plates because of defects in both chondrocyte proliferation and hypertrophy (Schipani et al. 2001). More recently, fate-mapping studies suggested that Col2-Cre-expressing cells contribute to osteoblasts in addition to chondrocytes (Ono et al. 2014). Osteoblast-specific deletion of HIF-1α in mice resulted in thinner bones with reduced vascularization, whereas deletion of VHL promoted bone vascularization (Wang et al. 2007).
Interestingly, this phenotype was observable in long bones undergoing endochondral ossification, but not during intramembranous ossification (Wang et al. 2007). Oxygen depletion inhibited growth and differentiation of cultured rat osteoblasts in vitro (Utting et al. 2006). Hypoxia also reduced Runx2 expression in human osteoblast lineage cells in vitro and thereby inhibited the formation of mineralizing osteoblasts (Park et al. 2002).

Oxygen is not the only factor controlling HIF expression and activity. Mechanical stress in osteoblasts activates Ras/ERK-mediated mitogen-activated protein kinase (MAPK) signaling, which promotes HIF-1α expression (Kanno et al. 2007, Wang et al. 2004). In addition, growth factors activate mTOR through AKT, leading to stabilization of HIF-1α in many different cell types (Semenza 2003). Insulin-like growth factor 1 (IGF-1) treatment of human osteoblast-like cells can activate HIF in a PI3K/AKT-dependent fashion (Akeno et al. 2002). Similar to HIF stabilization in osteoblasts, which promoted blood vessel growth, EC-specific inactivation of the gene for VHL led to increased angiogenesis and an increase in type II capillaries and associated Osterix-positive osteoprogenitors (Kusumbe et al. 2014). Conversely, EC-specific loss of HIF-1α strongly reduced the abundance of type II vessels and osteoprogenitors (Kusumbe et al. 2014).

**Vascular Endothelial Growth Factor**

VEGF and its main receptor, the tyrosine kinase VEGFR2, are critical regulators of developmental, regenerative, and pathological angiogenesis (Adams & Alitalo 2007). Hypertrophic chondrocytes express VEGF and thereby promote blood vessel growth toward the avascular growth plate (Gerber et al. 1999). VEGF expression is also detected in the osteoblast lineage (Deckers et al. 2000) and induces osteoblast differentiation (Midy & Plouet 1994). Autocrine and paracrine roles of VEGF in osteoblasts have been widely investigated, as hypoxia induces expression of the growth factor in osteoblasts and thereby promotes their differentiation (Mayer et al. 2005; Steinbrech et al. 1999, 2000). Osteoprogenitor-specific loss of VEGF-A reduced bone density, inhibiting the differentiation of these cells into mature, bone-forming osteoblasts (Liu et al. 2012). In addition to hypoxia, numerous factors such as prostaglandins, BMPs, FGF, IGF, and vitamin D3 can induce VEGF expression in osteoblasts (Akeno et al. 2002, Deckers et al. 2000, Harada et al. 1994, Kozawa et al. 2001, Tokuda et al. 2003, Wang et al. 1996). VEGF-A is produced in several isoforms, among which VEGF120, VEGF164, and VEGF188 are the most relevant in mice. Selective expression of the most diffusible isoform, VEGF120, which lacks matrix-binding motifs, in mutant mice was incompatible with endochondral ossification and led to delayed differentiation and impaired vascularization of hypertrophic chondrocytes during embryonic development. Mutants expressing only the intermediate isoform, VEGF164, showed normal skeletal growth, whereas the insoluble VEGF188 led to dwarfism due to disrupted development of growth plates and secondary ossification centers (Maes et al. 2002, 2004). Thus, the evidence indicates that the VEGF is a key player in the skeletal system by regulating the behavior of multiple cell types, including ECs and osteoblasts.

**Notch**

Notch is a highly conserved, cell contact–dependent signaling pathway that regulates a large variety of processes during development and in the adult (Artavanis-Tsakonas et al. 1999). In the vascular system, Notch controls blood vessel growth and function (Adams & Alitalo 2007, Potente et al. 2011) by modulating cellular responses to growth factors such as VEGF. In sprouting angiogenesis, leading endothelial tip cells, which are exposed to high VEGF concentrations, express high levels of the Notch ligand Delta-like 4 (Dll4). Such expression of Dll4 is thought to activate Notch...
signaling in the neighboring stalk ECs and to suppress VEGF signaling and tip cell behavior in these cells (Benedito et al. 2009, Hellstrom et al. 2007). Accordingly, Notch signaling inhibits blood vessel growth in the retina (Hellstrom et al. 2007), zebrafish embryos (Sickmann & Lawson 2007), and tumors (Ridgway et al. 2006), but the pathway surprisingly promotes angiogenesis in the skeletal vasculature (Ramasamy et al. 2014). Angiogenesis in the bone endothelium is mediated through the Dll4-Notch signaling axis, although other Notch ligands such as Jagged1 and Dll1 are dispensable. EC-specific Notch activation in bone promoted the formation of type I1 capillaries and angiocrine release of osteogenic factors that enhance osteogenesis (Kusumbe et al. 2014, Ramasamy et al. 2014). Defective Notch signaling in ECs impaired the maturation and resorption of chondrocytes, compromising VEGF-A expression in the growth plate as well as osteogenesis (Ramasamy et al. 2014).

Notch also controls the differentiation of osteoblasts during skeletal development in a cell-autonomous fashion. Loss of Notch activity in mesenchymal progenitors led to increased bone mass in adults and to subsequent loss of bone during aging. Moreover, mutant mesenchymal progenitors were depleted due to enhanced osteoblast differentiation. Thus, Notch signaling promotes the proliferation of mesenchymal progenitors and inhibits their differentiation (Hilton et al. 2008). Accordingly, overexpression of the constitutively active Notch1 intracellular domain (NICD) promoted proliferation and differentiation of osteoblasts. In addition, complete inhibition of Notch signaling in osteoblasts due to the loss of Presenilin 1 and 2, which are involved in ligand-induced cleavage and activation of Notch receptors, led to osteoporosis in aged mice (Engin et al. 2008). Together, these data indicate that excessive Notch signaling in the osteoblast lineage can cause osteosclerosis due to enhanced osteoblastic proliferation, whereas insufficient Notch activity can result in bone loss, which involves increased osteoblast-dependent osteoclastic activity (Engin et al. 2008). Notch signaling in cultured human BM stromal cells promotes osteoblast differentiation by inhibiting differentiation into the adipogenic lineage (Ugarte et al. 2009). At the mechanistic level, the Notch target gene Hey1 suppresses mineralization and inhibits the activity of Runx2 (Zamurovic et al. 2004). In addition, coimmunoprecipitation analysis showed that the NICD (Engin et al. 2008), Hes1, and Hey1 (Hilton et al. 2008) are binding partners of Runx2 during osteoblast differentiation. Runx2 is also controlled by other pro-osteogenic signals, such as BMP, Wnt, and transforming growth factor β (TGFβ), indicating the integration of multiple signaling pathways during osteoblast differentiation (Lin & Hankenson 2011). It remains to be investigated whether and how these signals converge to couple angiogenesis and osteogenesis.

VASCULAR ALTERATIONS IN SKELETAL AGING AND PATHOLOGY
Skeletal remodeling, which is a lifelong cycle of mineral deposition and resorption on the bone surface, is carefully regulated to maintain a balance between growth and loss of bone mass. Mineral deposition predominates during developmental growth of the skeletal system, whereas aging is associated with elevated bone resorption, loss of bone mass, and increased risk of fracture formation. Osteoporosis is a pathological condition resulting from a progressive decrease in bone mineral density, which is common in postmenopausal women due to decreased estrogen levels. Treatment of osteoclasts with estrogen promoted their apoptosis (Hughes et al. 1996). Increased levels of various cytokines, namely interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF), have also been linked to the high osteoclast activity seen in postmenopausal osteoporosis (Kwan Tat et al. 2004). However, preosteoclasts, immature osteoclast progenitors, release PDGF-B, a growth factor acting on many mesenchymal cell types, and enhance the abundance of type I1 capillaries. These activities are linked with the positive effects of cathepsin K inhibition, a treatment
that inhibits osteoclasts and increases the number of preosteoclasts, in the ovariectomy-induced mouse model of osteoporosis (Xie et al. 2014).

Several other factors promote bone loss in elderly men and women. Growth hormone (GH) regulates growth and stimulates IGF-1 expression. Accordingly, the progressive decrease in GH secretion with age is associated with lower levels of circulating IGF-1 (Sonntag et al. 1999, Yakar et al. 2002). IGF-1 promotes migration and tube formation by cultured human ECs (Shigematsu et al. 1999). Low serum levels of IGF-1 in aged individuals have been associated with an increased risk of vascular dysfunction in the brain and with neurodegenerative disease (Lopez-Lopez et al. 2004). Similarly, intermittent administration of parathyroid hormone (PTH) increased circulating levels of IGF-1 (Canalis et al. 2007). Whereas intermittent PTH treatment is osteoanabolic, chronic administration promotes bone resorption by enhancing osteoclast activity (Jilka et al. 1992). Furthermore, PTH promotes VEGF expression, and administration of VEGF-blocking antibodies blunted PTH-induced increases in bone mass and remodeling (Prisby et al. 2011).

Our own recent work has established that age-related bone loss in mice is associated with profound changes in the organization of the skeletal vasculature. In old mice, sinusoidal type L vessels appeared unchanged, whereas type H capillaries and associated Osterix-positive cells were significantly reduced (Figure 1). Pharmacological reactivation of type H vessels in aged mice led to the reappearance of osteoprogenitors and to increases in trabecular bone (Kusumbe et al. 2014).

Bone damage and fractures are typical features of pathological conditions such as osteoporosis, osteogenesis imperfecta, and osteonecrosis due to the decline of local or total bone mass. Several studies have highlighted the importance of angiogenesis during fracture repair, as the lack of appropriate blood vessel growth is one of the main reasons for delayed or impaired fracture healing (Carano & Filvaroff 2003, Glowacki 1998). Pharmacological inhibition of angiogenesis in fracture animal models suppressed callus formation and resulted in fibrous tissue formation (Hausman et al. 2001). Whereas inhibition of VEGF impaired healing of bone defects, exogenous VEGF promoted angiogenesis, osteogenesis, and fracture repair (Street et al. 2002).

Bone fracture healing is a multistep process involving the formation of hematoma and callus on the fractured site (Carano & Filvaroff 2003, Glowacki 1998) (Figure 2). Bone formation after fracture can occur through either intramembranous ossification or endochondral ossification. Injury-induced hematoma formation attracts inflammatory cells, which secrete cytokines and factors to form a fibrinous clot (Gerstenfeld et al. 2003). Depending on bone and fracture type, the following stages of fracture repair can involve the formation of soft callus, its conversion into a hard callus containing fibrous bone, and the remodeling of the lesioned bone into its original morphology (Schindeler et al. 2008). Soft callus formation involves the hematoma-induced ingrowth of new blood vessels, which is promoted by VEGF (Street et al. 2000). Other growth factors and cytokines that are important during bone development, such as FGF, BMP, IGF, and PDGF, are expressed by multiple cell types present at the injury site to promote the healing process (Barnes et al. 1999, Gerstenfeld et al. 2003, Lieberman et al. 2002, Tatsuyama et al. 2000). VEGF enhances the bone formation potential of BMP4 during skeletal development and fracture healing (Peng et al. 2002). FGF2 displays dose- and time-dependent effects in the various fracture models (Inui et al. 1998, Kato et al. 1998), it promotes angiogenesis (Collin-Osdoby et al. 2002), and its activity is enhanced by TGFβ (Globus et al. 1988). The role of BMPs in fracture healing and bone repair has been extensively studied, and such study has led to the development of therapeutic strategies and grafts to promote bone repair (Garrison et al. 2010, Laurenenc et al. 2006). Interestingly, BMPs promote angiogenesis by direct stimulation of ECs (Beets et al. 2013) and by inducing VEGF expression in osteoblasts (Deckers et al. 2002).
Further research is needed to fully elucidate the role of the vasculature in aging, bone repair, and disease processes. However, the findings presented above raise the interesting possibility that blood vessels may represent an important therapeutic target in these settings.

**ARCHITECTURE OF BONE MARROW VASCULATURE AND SPATIAL LOCALIZATION OF BONE MARROW NICHES**

BM endothelial cells (BMECs) form a mechanical barrier that prevents mature red blood cells and platelets from penetrating into the BM, which is transiently destroyed by total body irradiation preconditioning (Tavassoli 1979). Identification and characterization of BMEC architecture, diverse vascular and perivascular cells, different vascular domains and their spatial localization, and cell surface markers are major challenges. Pioneering studies have defined the unique ultrastructure of BM blood vessels (Brookes 1958, Lichtman 1981), which differs from that of other organs. Oxygenated red blood cells enter the BM circulation via arterial vessels and, after oxygen release, exit via venular BM sinusoidal vessels. This physiological phenomenon creates a distinct phenotype of BM oxygen tension, in which arterial and endosteal regions display higher oxygenation states than in sinusoids and central BM regions (Spencer et al. 2014).

The connection between arterial blood vessels and sinusoidal blood vessels occurs preferentially adjacent to endosteal and trabecular bone, mostly at the bone metaphysis but also in the diaphysis regions, and is mediated by a unique transitional type of capillaries (Itkin et al. 2016, Kusumbe et al. 2014). These capillaries express hybrid markers of both arterial and sinusoidal vessels (Itkin et al. 2016), such as Sca-1 (Hooper et al. 2009) and Nestin (Itkin et al. 2016) for arteries and Emcn (Kusumbe et al. 2014) and VEGFR3 (Hooper et al. 2009, Kusumbe et al. 2014) for sinusoids. However, like arteries, these transitional capillaries (also termed type H) display high-integrity and low-permeability properties and are associated with a second layer of stromal cells (Itkin et al. 2016). These small-diameter capillaries differ from arteries in size and in the type of associated stromal cells (summarized in Figure 3). Whereas the bigger arteries and arterioles are surrounded mostly by α-SMA-expressing cells, the smaller endosteal arterioles and capillaries are associated with mesenchymal stem and progenitor cells (MSPCs), providing a niche for bone-forming cells (Itkin et al. 2016, Kusumbe et al. 2014). Arterial blood vessels are also mostly endosteal, as especially smaller arterioles and adjacent capillaries are located mainly at the trabecular regions and near the cortical bone in the metaphyseal and diaphyseal regions. Of note, self-renewing mesenchymal stem cells—which express the BMP antagonist Gremlin-1 and can differentiate into osteoblasts, chondrocytes, and reticular stromal cells, but not into adipocytes—are found in the trabecular region at the metaphysis and are not perisinusoidal (Worthley et al. 2015). Our own data also show that Nestin- and Sca-1-expressing MSPCs in the BM are spatially localized mainly in the metaphysis and, to some extent, near the cortical bone at the diaphysis (Itkin et al. 2016). Of interest, Sca-1+ and Nestin MSPCs share overlapping markers with their associated arteriole and capillary ECs and with HSPCs, which also functionally express c-kit, Sca-1, and Nestin (Itkin et al. 2016, Ludin et al. 2012). Of interest, similar findings were observed in human bone marrow pathological studies that describe Nestin-positive ECs forming, at steady state, arterial and capillary vascular beds, which are altered during marrow malignancy (Ewalt & Gratzinger 2016). Other markers, which were considered to be HSC specific, were recently shown to overlap with progenitor cells, ECs, and bone-forming stromal cells (Chen et al. 2016).

Previous studies localized some HSC populations to endosteal regions (Arai et al. 2004, Calvi et al. 2003, Lo Celso et al. 2009, Sugimura et al. 2012, Xie et al. 2009, Zhang et al. 2003) and indicated that quiescent HSCs reside mostly in periarterial domains close to bone (Kunisaki et al. 2013). Together with our findings (Itkin et al. 2016), these studies suggest that specialized
osteovascular BM niches regulate HSC maintenance, whereby HSCs are exposed to both perivascular and endosteal elements. Because periarterially located HSCs are quiescent, we compared the metabolic activity in distinct perivascular regions and noted reduced production of reactive oxygen species (ROS) in periarterial microenvironments (Itkin et al. 2016). ROS are pivotal HSC regulators serving as a metabolic feedback loop to control HSC repopulation potential, quiescence, self-renewal, proliferation, maintenance, and life span (Itkin et al. 2012, 2013; Ito et al. 2004, 2006; Ludin et al. 2012; Miyamoto et al. 2007; Takubo et al. 2013). HSCs found in periarterial BM niches were low in ROS, in contrast to perisinusoidal HSCs, which were found in diverse ROS states (Itkin et al. 2016). Considering the essential roles of ROS signaling in HSC migration and development (Dar et al. 2011, Golan et al. 2012, Ludin et al. 2014, Tesio et al. 2011), we suggest that presinusoidal niches serve primarily as activation sites for HSCs, promoting their trafficking, proliferation, and differentiation (Figure 3). We observed that ROS-sensitive (Ota et al. 2007) nonmyelinating Schwann cell fibers, which maintain HSC dormancy (Yamazaki et al. 2011) via secretion of active TGFβ (Yamazaki et al. 2009), exclusively wrap around along less permeable BM arterial blood vessels (Itkin et al. 2016), actively contributing to reduced ROS levels in periarterial microenvironments. Megakaryocytes, found in proximity to both BM arterioles and sinusoids, maintain HSCs (Bruns et al. 2014, Nakamura-Ishizu et al. 2014, Zhao et al. 2014) and keep them in a metabolically low-ROS state (Itkin et al. 2016) by secreting a variety of factors promoting HSC quiescence. Megakaryocytes were suggested to be part of the BMEC barrier regulating leukocyte trafficking (Tavassoli 1986). Mutual regulation between HSCs, BMECs, and niche-forming cells following stress was documented in a recent study showing that HSCs can regulate their own vascular niche, also by affecting BM vascular permeability (Zhou et al. 2015). BM α-SMA- and COX-2-expressing monocytes-macrophages, which reside near Nestin-expressing cells, enwrap HSCs and maintain them in a low-ROS state via prostaglandin E2 secretion (Ludin et al. 2012). Thus, metabolically low HSCs reside mostly near less permeable endosteal and trabecular arterioles and capillaries, in complex with MSPCs, nonmyelinating Schwann cells, and some myeloid cells. Long-term repopulating HSCs (LTR-HSCs) are also more abundant in the endosteal and trabecular regions (Grassinger et al. 2010, Guezguez et al. 2013, Haylock et al. 2007, Xie et al. 2009).

This claim is further supported by the fact that adipocytes, which negatively regulate BM HSC maintenance, become more abundant in the central BM in adulthood and in aging (Naveiras et al. 2009). Interestingly, a recent study suggested that α-catulin-expressing HSCs reside mainly in perisinusoidal areas (Acar et al. 2015). However, α-catulin is expressed by heterogeneous populations of HSPCs, including stromal cells and ECs (Chen et al. 2016). Expression of α-catulin indicates EC activation and migratory state (Bear et al. 2016). Similarly, such expression may also allow for the identification of metabolically activated HSPCs in BM perisinusoidal niches. We propose that perisinusoidal niches prime and activate HSCs metabolically for their migration and development.

PERMEABLE BONE MARROW SINUSOIDS ARE THE EXCLUSIVE SITE FOR CELLULAR TRAFFICKING

Because elevation of ROS levels is crucial for HSPC mobilization and egress to the peripheral blood (Dar et al. 2011, Golan et al. 2012, Tesio et al. 2011), we assumed that sinusoids provide the preferential sites for HSPC trafficking. The concept of endothelial barrier blood vessels that regulate BM cellular trafficking was suggested and extensively studied by the Tavassoli lab almost 40 years ago (Tavassoli 1979), but no in vivo follow-up proof was provided. To further test our hypothesis that distinct vascular niches serve different purposes and that cellular trafficking...
is restricted to sinusoids, we continued defining the different properties of distinct types of BM vessels. Arteries differ from sinusoids in their integrity properties, as arteries express higher levels of VE-cadherin adherens junction and preferentially express tight junction molecules such as ZO-1 and JAM-A (Itkin et al. 2016). JAM-A also labels human LTR-HSCs, which adhere and interact with JAM-A-expressing human BMECs; such interaction involves modulation and activation of the CXCR4 signaling axis (Chang et al. 2016). Moreover, compared with sinusoids, arteries display reduced permeability, as indicated by live dye penetration assays, and exhibit much higher blood flow and shear rates (Itkin et al. 2016). High arterial blood flow suggests that, as in embryonic development, during which blood flow initiates and regulates HSC development (North et al. 2009), mechanical signaling in arterial blood vessels can also regulate HSC...
 maintenanc e in perivascular zones during adulthood. Due to the high flow speed and shear rates in arteries, all cellular trafficking events performed by mature and immature leukocytes, including deceleration, adhesion, rolling, and transendothelial migration, occur exclusively in sinusoidal blood vessels (Itkin et al. 2016). This observation indicates that, during the homing process, HSCs do not directly home to their BM-maintaining niches. We suggest that, after initial homing via the sinusoids, HSCs migrate through the marrow at a later stage and lodge to their periar terial niches, where they undergo metabolic ROS inhibition for prolonged maintenance in a quiescent mode. Melanoma cancer cells elevate ROS levels to dis seminate from the primary tumor and to migrate in the bloodstream but also undergo a metabolic shift and suppress ROS via the production of antioxidant agents so that these cells can successfully metastasize following their exit from blood vessels (Peiris-Pages et al. 2015, Piskounova et al. 2015). Similarly, HSCs may be required to adopt dynamic ROS behavior, elevating ROS levels at perisinusoidal sites to promote egress, trafficking, and homing and to repress ROS upon lodgment to periar terial sites for successful engraftment and durable maintenance.

The CXCL12-CXCR4 signaling axis is a key regulator of HSPC quiescence and BM retention, mobilization, and homing processes (Vagima et al. 2011). The CXCR4 small inhibitor AMD3100 rapidly mobilizes HSCs (Bromeyer et al. 2005) in a ROS-dependent manner (Dar et al. 2011), yet its effect on BM blood vessels and the mechanisms activating HSPCs were poorly understood. We have discovered that AMD3100 preferentially antagonizes endothelial CXCR4 signaling by reducing CXCL12 expression on sinusoidal BMECs and by enhancing endothelial barrier permeability via a preferential decrease in sinusoidal VE-cadherin levels (Itkin et al. 2016). This finding suggests that the CXCL12-CXCR4 axis regulates in vivo blood vessel integrity just like it regulates in vitro endothelial adhesion (Kobayashi et al. 2014). Indeed, when CXCR4 was in vivo conditionally deleted exclusively from ECs, BM blood vessel permeability increased, promoting higher egress of HSPCs (Itkin et al. 2016). Other pathways important for HSPC mobilization may also involve endothelial barrier regulation as part of the complex mobilization process. For example, the Slit2-Robo4 axis, cooperatively with CXCR4, regulates HSPC mobilization and niche localization (Smith-Berdan et al. 2011) and controls BM endothelial integrity (Smith-Berdan et al. 2015). Our results indicate that successful HSPC mobilization, involving enhanced trafficking via sinusoidal sites, requires reduced endothelial integrity and enhanced sinusoidal vascular permeability together with transient ROS elevation in activated HSPCs.

**Figure 3**

Bone marrow (BM) blood vessel (BV) permeability regulates hematopoiesis. The BM vasculature is composed of two major types of BVs: arterial (CD31$^{\text{high}}$/CD45$^{-}$/Sca-1$^{\text{high}}$/Nestin$^{+}$) and sinusoidal (CD31$^{+}$/CD45$^{-}$/Sca-1$^{\text{low}}$/Nestin$^{-}$). The connection between arterial BVs and sinusoidal BVs occurs preferentially in the endosteal region near the cortical bone by a transitional type of endosteal capillaries (type H BVs). As do arteries, these capillaries display high endothelial integrity and low-permeability characteristics, mediated by high expression of tight junction molecules such as VE-cadherin and ZO-1. Whereas arteries present big-diameter BVs and are surrounded mostly by $\alpha$-SMA-expressing cells, smaller arterioles and capillaries are surrounded mostly by Sca-1$^{+}$ and Nestin$^{+}$ mesenchymal stem and progenitor cells (MSCPs). Due to tight barrier integrity state and high shear rates, arterial BVs generally promote a low-ROS metabolic state of cells in their periar terial BM microenvironment. Further support of hematopoietic stem cell (HSC) dormancy and maintenance in a low metabolic state comes from innervating Schwann cell nerve fibers, which secrete transforming growth factor $\beta$ (TGF$\beta$), reducing ROS levels in BM-retained HSCs. These fibers are tightly associated with less permeable, ROS-shielding BM arteries and endosteal arterioles and capillaries. Sinusoids exhibit more permeable and fenestrated BM endothelium characteristics, enabling blood plasma penetration into the BM milieu and thus promoting a higher-ROS state in surrounding cells. These processes hamper HSC long-term repopulation capacity and survival. Displaying higher permeability and low shear rate, BM sinusoids are the exclusive site for cellular trafficking. Megakaryocytes are a major component in the perivascular microenvironment, which supports and maintains hematopoietic stem and progenitor cells (HSPCs) in a low-ROS state. RBC denotes red blood cell.
ENDOTHELIAL BARRIER FENESTRATION DICTATES STEM CELL FATE BY ALLOWING FOR BONE MARROW PLASMATIC FLOW

The application of intravenously injected dyes for permeability and tissue penetration assays, such as albumin binding, Evans blue dye, and fluorescently labeled dextran, indicates concentration gradients of diffusible, plasma-carried molecules. Thus, we hypothesized that BM-penetrating blood plasma, via leaky endothelial barrier cells, plays a part in the regulation of stem cell fate. Initial observations comparing BM-residing HSPCs with those found in the bloodstream indicated that circulating HSPCs are highly metabolically active and exhibit much higher ROS levels. Moreover, exposure of BM-residing HSPCs to peripheral blood plasma elevates ROS levels and primes HSPC migratory capacity in response to CXCL12 gradients. However, this exposure also enhances HSPC differentiation rates and induces higher apoptosis frequencies, resulting in reduced LTR-HSC repopulation capacity (Itkin et al. 2016). Our findings are supported by and provide mechanistic insights into previous reports, which show that transplanted HSPCs minimize their loitering time in the peripheral blood to only a few minutes (Wright et al. 2001). The same is true for steady-state and cytokine-induced egress of HSCs, which suggests that HSC exit to the blood may be a “pathway to death” and that the BM endothelial barrier provides an HSC-protective microenvironment (Abkowitz et al. 2003).

The fact that plasma-derived factors penetrating into the BM prime HSPC migration and development via increasing ROS levels provides an additional mechanism for how leaky perisinusoidal regions generate a niche for HSC activation and serve as a site for HSC migration and development. The less permeable arteries allow for lower levels of plasmatic penetration, reduce the concentration of plasma-derived factors in periarterial BM microenvironments, maintain HSCs in a low-ROS inactive metabolic state, and preserve HSCs stemness during prolonged residence in these regions. To further test our hypothesis, we examined changes in the endothelial barrier during conditions that promote stem cell expansion. We and others showed that FGF signaling promotes HSC expansion and enhances HSC self-renewal during physiological recovery or under enforced therapeutic treatment (Itkin et al. 2012, 2013; Zhao et al. 2012). Indeed, following FGF-2 in vivo treatment inducing BM stem cell expansion, endothelial barrier permeability was reduced, with enhanced expression of adherens junction molecules by BMECs. As a result, HSPC trafficking was reduced, and the egress of LTR-HSCs into the peripheral blood and their differentiation were inhibited (Itkin et al. 2016). Because FGF receptors are widely expressed by all types of BM-residing cells, we examined whether the effects of BM stem cell regulation are EC dependent. FGF signaling regulates endothelial integrity (De Smet et al. 2014, Murakami et al. 2008) and may thus be a perfect candidate to manipulate the endothelial barrier and examine the effects on BM-residing stem cells. Endothelium-specific induction of FGF receptor 1 and 2 (FGFR1 and -2) deletion led to enhanced HSPC bidirectional trafficking, allowing for enhanced HSPC homing into the BM while also resulting in increased HSPC egress from the BM into peripheral blood. Blockade of FGF signaling in ECs hampered endothelial barrier integrity, resulting in enhanced BM permeability and allowing for higher penetration of plasma from the peripheral blood. HSPC numbers and MSPC numbers were thus decreased, and both cell populations exhibited higher intracellular ROS levels in both perisinusoidal and periarterial microenvironments. As a consequence, LTR-HSC capacity in the BM was reduced; apoptosis in HSPCs increased; and there was a developmental skewing in the differentiation ratio, favoring myeloid development (Itkin et al. 2016). The mesenchymal stromal population in the BM was also affected following barrier targeting, displaying an enhanced differentiation rate at the expense of the MSPC pool. Moreover, endothelial development was altered, leading to skewing of the sinusoidal/arterial ratio and resulting in the dramatic decline of arterial blood vessels in the BM (Itkin et al. 2016), very similar to the phenomenon observed during aging (Kusumbe et al. 2016). Reduced arterial blood
vessels may decrease the availability of periarterial niches for both MSPCs and HSPCs and may provide another explanation for the decrease in these two populations.

Because ROS levels were elevated under breached-barrier conditions, administration of ROS scavengers resulted in a complete rescue of the observed phenomenon, restoring the HSC pool and preventing increased HSPC egress (Itkin et al. 2016). Of interest, ROS scavenging–mediated rescue was independent of the endothelium, as such rescue had no effect on endothelial permeability and integrity (Itkin et al. 2016). Because ECs are exposed to the highest oxygen tension, these cells adapted to rely mainly upon glycolysis (De Bock et al. 2013) for energy production and development, thus avoiding excessive ROS levels. ECs evolved to be less sensitive to changes in ROS, and ROS do not play a major part in endothelial signaling events (Vandekeere et al. 2015). Taken together, these results indicate that manipulation and targeting of endothelial integrity–affecting barrier properties can be used to dictate tissue resident stem cell fate decisions that impact developmental programs and cellular trafficking.

In terms of clinical benefit, targeting of BM endothelial barrier can be used to enhance stem cell mobilization, especially in cases of poor mobilizers. Such targeting can also be used to enlarge the number of successfully homed HSPCs following transplantation via opening of the endothelial gates. Finally, in the opposite strategy, the endothelial barrier can be targeted to expand a pool of successfully lodged HSCs by closing the barrier and preventing HSC egress and differentiation. Also, successful stem cell engraftment can be achieved by enhancing the abundance of periarterial BM niches and the numbers of supportive MSPCs.

UNIQUE BONE MARROW BLOOD VESSELS FORM AN ANTICOAGULANT NICHE FOR LONG-TERM REPOPULATING HEMATOPOIETIC STEM CELLS

New emerging paradigms reveal heterogeneity within HSC mature lineage cell development, in which each progenitor cell gives rise to specific lineage-restricted cell types (Mercier & Scadden 2015, Paul et al. 2015). These observations suggest that different BM niches occupy different lineage-restricted progenitors that provide different cues for their development.

Despite technological breakthroughs in high-resolution imaging techniques, the exact location of BM LTR-HSCs and the anatomy of their niches remain controversial topics (Morrison & Scadden 2014). However, recent observations demonstrated that a subtype of LTR-HSCs, which functionally express endothelial protein C receptor (EPCR), occupy unique vascular niches in the murine BM (Gur-Cohen et al. 2015) (Figure 4). EPCR was originally identified as an endothelium-specific receptor (Fukudome & Esmon 1994) that plays critical roles in supporting activated protein C (aPC)-mediated anticoagulant and cytoprotective signaling. Binding of the ligand aPC to EPCR facilitates proteolytic cleavage of cell surface protease-activated receptor 1 (PAR1), initiating antiinflammatory and antipapoptotic responses, and protection of endothelial barrier integrity (Gleeson et al. 2012). A small subpopulation of HSCs in the murine fetal liver and adult BM, endowed with the highest long-term BM repopulation potential, also express surface EPCR (Balazs et al. 2006, Iwasaki et al. 2010, Kent et al. 2009). Murine EPCR+ LTR-HSCs exhibit durable self-renewal potential at the level of a single transplanted stem cell (Wilson et al. 2015) and superior BM retention, which is essential for protection from chemotherapy-induced hematological failure and death (Gur-Cohen et al. 2015). Providing the fuel to retain EPCR+ LTR-HSCs in the BM, unique endothelial structures are enriched with aPC and with thrombomodulin (TM) (Gur-Cohen et al. 2015), which initiates aPC generation (Figure 4). The BM is an organ that lacks active platelets (Junt et al. 2007) or mature red blood cells (Gahmberg et al. 1978). Therefore, coagulation processes are unlikely to take place in the BM. However, the expression of these traditionally
Thrombomodulin (TM)-expressing blood vessels (BVs) maintain endothelial protein C receptor (EPCR)+ long-term repopulating hematopoietic stem cell (LTR-HSC) retention. In the bone marrow (BM), a unique type of BM BVs expressing TM and the anticoagulant protease aPC (activated protein C) form a niche for EPCR+ LT-HSCs. These TM+ BVs have arteriole characteristics, expressing CD31+/CD45-/Sca-1+, and are located mainly in the trabecular bone region. TM facilitates the production of aPC from its circulating precursor, PC. Binding of aPC to its major receptor, EPCR, also expressed by BM LTR-HSCs, induces specific protease-activated receptor 1 (PAR1) signaling, leading to the restriction of nitric oxide (NO) production. This development leads to the initiation of Cdc42-GDP polarity, to downregulation of Cdc42-GTP activity, and to the promotion of VLA4-dependent adhesion and BM-specific stem cell retention. Megakaryocytes are also located in the vicinity of BM BVs and are involved in the regulation and protection of BM stem cell niches by the production of cytokines and growth factors. Megakaryocytes secrete coagulation factors such as factor V, factor X, prothrombin, and platelet factor 4 (PF4). All the above factors may link megakaryocytes to the TM/aPC pathway, as PF4 stabilizes the thrombin-TM complex, thereby promoting aPC generation and EPCR+ LTR-HSC BM retention. RBC denotes red blood cell.

Mechanistically, aPC binding to EPCR initiates PAR1 signaling that leads to inhibition of nitric oxide production and the promotion of VLA4-dependent adhesion and BM-specific stem cell retention. Megakaryocytes are also involved in the regulation and protection of BM stem cell niches by the production of cytokines and growth factors. Megakaryocytes secrete coagulation factors such as factor V, factor X, prothrombin, and platelet factor 4 (PF4). All the above factors may link megakaryocytes to the TM/aPC pathway, as PF4 stabilizes the thrombin-TM complex, thereby promoting aPC generation and EPCR+ LTR-HSC BM retention. RBC denotes red blood cell.
Thrombomodulin (TM)+ endosteal arterioles express Endomucin (Emcn), and eNOS inhibition by L-NAME expands bone marrow (BM) stromal precursors. (a) Representative fluorescence images of a small-diameter blood vessel from the metaphyseal area expressing TM (green), Emcn (red), and nuclei (DAPI) (blue). (b) Frequency of murine mesenchymal progenitors [colony-forming unit fibroblasts (CFU-F)] in vitro following intraperitoneal PBS (phosphate-buffered saline) control or L-NAME treatment for 5 days in vivo.

These two PAR1 signaling cascades regulate the NO generation/inhibition switch, leading to perturbations in NO levels that are orchestrated by endothelial NO synthase (eNOS) activity (Gur-Cohen et al. 2015). The eNOS phosphorylation switch balances NO levels in stem cells, inducing BM retention of NO<sub>low</sub> EPCR-expressing LTR-HSCs. Upon higher levels of thrombin, NO is increasingly generated within PAR1<sup>+</sup> stem cells, leading to loss of retention of these cells in the BM and to their egress to the bloodstream (Gur-Cohen et al. 2015). Directly activating EPCR in vivo by treatment with aPC or mimicking EPCR signaling by injecting the eNOS inhibitor L-NAME expanded EPCR<sup>+</sup> LTR-HSCs in the murine BM (Gur-Cohen et al. 2015). Expansion of the early HSPC population in the BM by systemic inhibition of eNOS was followed by a transient increase in blood neutrophil content (Michurina et al. 2004). Importantly, eNOS inhibition by L-NAME in vivo also expanded the number of BM mesenchymal progenitor cells, as indicated by an increase in the number of colony-forming unit fibroblasts (Figure 5b).

Production of NO by the surrounding niche microenvironment can also regulate, in a paracrine manner, LTR-HSC expansion (Krasnov et al. 2008). NO also plays a key role in the regulation of vascular tone and endothelial permeability (Yang et al. 2015). BM endothelial barrier integrity is an essential element that dictates HSC migration capacity, survival, and maintenance. Another interesting observation links NO production locally in ECs to the response to blood flow, which triggers the onset of definitive hematopoiesis during development (North et al. 2009). Our recent study demonstrates that in BM vasculature the highest blood flow and shear rates occur in arterial blood vessels, suggesting that arteries may be the main endothelial producers of NO and that, as a consequence, NO may promote HSC developmental processes also in adult BM.
Megakaryocytes release a number of factors that can directly regulate aPC generation. Mature megakaryocytes are the major BM source that produces coagulation factors required for the formation of active thrombin (Storan et al. 2015). These secreted factors, which include factor V, factor X, and prothrombin, support thrombin, which generates the cleaved form of osteopontin (OPN) (Storan et al. 2015). HSCs bind to thrombin-cleaved OPN provided by endosteal osteoblasts that induce retention cues, resulting in a decrease in stem cell proliferation and differentiation (Nilsson et al. 2005). Low levels of thrombin can also result in thrombin binding to TM on the surfaces of ECs, leading to loss of the procoagulant function of thrombin and enhancing aPC generation (Esmon 1995). It is therefore tempting to speculate that low-level production of thrombin by mature BM megakaryocytes may contribute to aPC generation in vivo, providing additional insight into EPCR* regulation of HSCs by their mature megakaryocyte progeny. Megakaryocytes also maintain quiescent BM HSCs via the secretion of platelet factor 4 (PF4), providing direct feedback and perhaps differential cues to maintain unique HSC developmental potential (Bruns et al. 2014, Zhao et al. 2014). PF4 (also termed CXCL4) is the most frequently released platelet-derived chemokine and has been recognized as an important marker of the megakaryocytic lineage. The role of megakaryocytes in determining HSC fate may also be linked to the TM/aPC pathway. Secreted PF4 from megakaryocytes stabilizes TM and promotes aPC generation (Slungaard et al. 2003), suggesting that megakaryocytes may participate in the establishment of the stem cell niches for BM EPCR* stem cells, potentially enhancing aPC generation and thereby ensuring EPCR* LTR-HSC retention and protection (Gur-Cohen et al. 2015) (Figure 4). Although this notion needs to be further validated, supportive evidence shows that phenotypically defined HSCs are frequently located adjacent to marrow megakaryocytes (Bruns et al. 2014) and that, under chemotherapeutic stress conditions, PF4 secretion by BM megakaryocytes promotes HSC expansion (Zhao et al. 2014). LTR-HSC BM retention or egress is also regulated by temporal cues such as daily light and darkness rhythms (Mendez-Ferrer et al. 2008). Deletion of Bmal1, which encodes a transcription factor in the core clock gene machinery, abrogates these temporal differences in circulating stem cells (Mendez-Ferrer et al. 2008). Expression of core clock genes also oscillates in vascular ECs, and perturbation of this system regulates TM gene expression (Takeda et al. 2007). Thus, aPC generation may also oscillate due to peaks in surface TM expression, promoting LTR-HSC retention by limiting NO generation and initiating the adhesion machinery (Gur-Cohen et al. 2015). Mature hematopoietic cells, including BM macrophages and B cell precursors, also express TM (Geiger et al. 2012). Importantly, most EPCR-expressing BM LTR-HSCs also express TM (Gur-Cohen et al. 2015), raising the question of whether aPC generation takes place only by specific endothelial niche cells or also by the HSCs themselves. Apart from being the gear for aPC production, TM is a complex molecule that has N-terminal domain sequence similarity with lectins, which are carbohydrate-binding proteins, and is not involved in protein C activation (Conway et al. 2002, Esmon 2003). These lectin-like motifs downregulate nuclear factor-κB and MAPK pathways (Conway et al. 2002), suggesting a possible novel, additional role for TM in LTR-HSC BM retention independent of PAR1 activity.

CONCLUDING REMARKS

It is becoming increasingly clear that the bone vasculature is highly complex, heterogeneously composed of distinct vessel types, and endowed with specialized functions that control bone formation and hematopoiesis. Vascular heterogeneity may be particularly relevant for different niche structures, which are limited in number and are presumably induced or maintained by local interactions between different cell types. Not only would the identification of the cellular and molecular basis of such local microenvironment features enhance our understanding of stem
cell niches in the skeletal system, but the resulting concepts might also apply to other organ systems with vascular niche structures. Deeper insight into the exact organization and molecular regulation of such niches would also be of great relevance for aging and pathobiological processes. In humans, aging is strongly associated with bone loss (Dequeker 1975, Rossi et al. 2005), reduced HSC functionality (Morrison et al. 1996, Rossi et al. 2005), and reduced host immunity (Shaw et al. 2010, Weiskopf et al. 2009). Blood vessels have also been implicated in neural stem cell niches in the adult brain (Shen et al. 2004, Tavazoie et al. 2008) and are thought to contribute to a range of neurodegenerative diseases (Carmeliet 2003, Zlokovic 2008). Similarly, blood vessels and vascular-derived signals play important roles in the homeostasis and regeneration of other organs such as the lung and liver. Thus, understanding the precise localization and organization of these niches would enable the characterization of the changes occurring during disease. Such characterization, in turn, might enable the development of novel therapeutic strategies to restore crucial properties of certain blood vessels or EC subpopulations. Conversely, it might also be desirable to disrupt specific functions of ECs and vascular niches for the treatment of leukemia or other diseases. Despite the many open questions and challenges, we now have the exciting opportunity to unravel critical roles of bone blood vessels and ECs, with important implications for basic research, medicine, and bioengineering.

**DISCLOSURE STATEMENT**

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata
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