

REVIEW

Blood vessel formation and function in bone

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ABSTRACT

In addition to their conventional role as a conduit system for gases, nutrients, waste products or cells, blood vessels in the skeletal system play active roles in controlling multiple aspects of bone formation and provide niches for hematopoietic stem cells that reside within the bone marrow. In addition, recent studies have highlighted roles for blood vessels during bone healing. Here, we provide an overview of the architecture of the bone vasculature and discuss how blood vessels form within bone, how their formation is modulated, and how they function during development and fracture repair.

KEY WORDS: Bone, Angiogenesis, Blood vessels, Endothelial cell, Chondrocyte, Osteoblast, Osteoclast

Introduction

The skeletal system – a framework of bones and connective tissues – provides essential structural support to the body and facilitates movement. However, it is also surprisingly dynamic and is subjected to constant absorption and rebuilding processes throughout the lifetime of an organism (Karsenty and Wagner, 2002). Mammals have two main types of skeletal elements – long bones (e.g. the femur and tibia) and flat bones (e.g. bones of the skull) – that form in distinct ways (Kronenberg, 2003). Long bones form via the process of endochondral ossification, during which mesenchymal stem cells (MSCs) differentiate into different types of bone-forming cells, namely chondrocytes (cartilage cells), osteoprogenitors and osteoblasts. This avascular cartilage serves as a template and is subsequently replaced by new bone and marrow. By contrast, flat bones form via the process of intramembranous ossification, which involves the clustering of MSCs that directly differentiate into cells of the osteoblast lineage. These cells accumulate locally, form an ossification center and secrete an extracellular matrix (ECM) that promotes bone formation (Erlebacher et al., 1995).

A number of studies have shown that both of these processes of bone formation are coupled to the process of angiogenesis – the growth of blood vessels from existing vessels (Brandi and Collin-Osdoby, 2006). For example, bone cells secrete pro-angiogenic factors, most importantly vascular endothelial growth factor (VEGF), that can trigger signaling responses in different cell populations expressing VEGF receptors, including the endothelial cells that make up blood vessels as well as chondrocytes, osteoblasts and osteoclasts (Dai and Rabie, 2007; Eshkar-Oren et al., 2009; Gerber and Ferrara, 2000). Conversely, bone endothelial cells release factors that can act

upon chondrocytes and cells of the osteoblast lineage (Kusumbe et al., 2014; Ramasamy et al., 2014). Vascular cells have also been shown to be part of the niche that controls the maintenance and differentiation of hematopoietic stem cells (HSCs) that reside in bone marrow (Ugarte and Forsberg, 2013). Notably, angiogenesis also plays a major role in bone fracture healing and repair (Beamer et al., 2010), and changes in the local vasculature are also associated with the progression of numerous diseases affecting bone, such as osteoporosis, osteonecrosis, rheumatoid arthritis, bone cancer and metastasis (Carulli et al., 2013).

Here, we discuss this intimate link between bone formation and angiogenesis. We first provide an overview of the process of angiogenesis and then focus on the architecture of the bone vasculature, its growth, and its roles in bone formation and repair.

An introduction to blood vessels and angiogenesis

Blood vessels are made up of several different cell types. The inner layer of blood vessels is composed of endothelial cells (ECs), which are covered on their outer, abluminal surface by perivascular (or mural) cells. Based on marker expression profiles and morphological criteria, these mural cells can be classified as pericytes, which are embedded in the subendothelial basement membrane and make direct cell-cell contact with capillary ECs, and vascular smooth muscle cells, which generally cover larger caliber vessels, namely arteries and veins, and lack physical contact with ECs (Armulik et al., 2011). Blood vessels can form via two processes. In early embryogenesis, mesodermal cells differentiate into hemangioblasts (the progenitors of ECs and blood cells), which migrate to specific locations and aggregate to form the first primitive vessels in a process termed vasculogenesis (Risau and Flamme, 1995). Subsequently, most if not all new blood vessels arise by the process of angiogenesis – the expansion of existing vascular networks through a series of processes such as EC sprouting, migration, proliferation, vessel anastomosis and pruning (Geudens and Gerhardt, 2011; Herbert and Stainier, 2011; Potente et al., 2011). Angiogenesis requires extensive coordination between the different vascular cell types to ensure that new vessels are fully functional and stable. The expansion of capillary beds, for example, typically involves arteriovenous specification of a subset of ECs, allowing the formation of either arteries or veins. Pericytes and smooth muscle cells are also required, notably for vascular remodeling, stabilization and maturation (Adams and Alitalo, 2007; Carmeliet and Jain, 2011; Potente et al., 2011). There is also increasing evidence indicating that blood vessels form and become specialized in an organ-specific fashion, controlled by local microenvironmental signals and leading to specific molecular signatures in ECs (Kuhnert et al., 2010; Nolan et al., 2013; Raffi et al., 2016; Ramasamy et al., 2015). As we discuss below, this specialization also applies to the bone vasculature.

Architecture of the bone vasculature

Historical studies using dye injections and radiomicrographs to visualize blood vessels have provided important insights into the

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organization of the bone vasculature, in particular that of the long bone with a special focus on the arterial vessels entering the femoral head (Crock, 1965; Trueta and Harrison, 1953; Trueta and Morgan, 1960). Such early studies also suggested that the organization of the bone vasculature is similar in different mammalian species, including rats, rabbits, guinea pigs and humans (Trueta and Morgan, 1960). In recent years, various technological advancements, such as the identification of cell type-specific markers, transgenic fluorescent reporters, imaging by confocal and two-photon microscopy, microcomputed tomography (micro-CT), three-dimensional (3D) reconstruction of imaging data, and improved protocols for tissue processing and immunostaining, have advanced our understanding of the organization of the bone vasculature and its functional specialization (Acar et al., 2015; Kunisaki et al., 2013; Kusumbe et al., 2014, 2015; Roche et al., 2012).

Bone is a highly calcified, matrix-rich tissue that typically contains a core (marrow) of hematopoietic cells or, depending on age and the specific bone in question, adipocytes. Bone is also highly vascularized (Fig. 1A) and, with a few exceptions (such as the growth plate and articular cartilage), blood vessels are found in all regions of the skeletal system. As in other organs, the vasculature in bone shows a typical hierarchical organization into an arterial

(afferent) branch that feeds into an extensive capillary network, which is drained into a large vein in the center of the diaphysis – the main shaft of the bone that contains the marrow (Fig. 1A,D) (Kusumbe et al., 2014; Trueta and Morgan, 1960). Based on marker expression and functional characteristics, two subtypes of bone capillaries can be distinguished: H and L (Kusumbe et al., 2014). However, these are interconnected and are therefore part of a single network. Type H capillaries are found in the metaphysis, which is the region containing the avascular growth plate. Type H capillaries in the metaphysis are organized as vessel columns that are interconnected at their distal end in proximity to the growth plate (Fig. 1B). These vessels, as well as the endosteal type H capillaries that are proximal to compact bone, are associated with perivascular osterix (or SP7)-expressing osteoprogenitor cells (Fig. 2) and express high levels of the junctional protein CD31 (or PECAM1) and the sialoglycoprotein endomucin (EMCN) (CD31^{hi} EMCN^{hi}) (Fig. 1B,C). In transverse sections through distal long bone, type H vessels appear densely packed (Fig. 1D) and are interconnected preferentially in proximity to the growth plate (Fig. 1B). By contrast, type L vessels form the dense, highly branched capillary network in the bone marrow cavity of the diaphysis, which corresponds to the sinusoidal vasculature described previously (Aird, 2007; Kopp et al., 2005). These vessels express lower levels

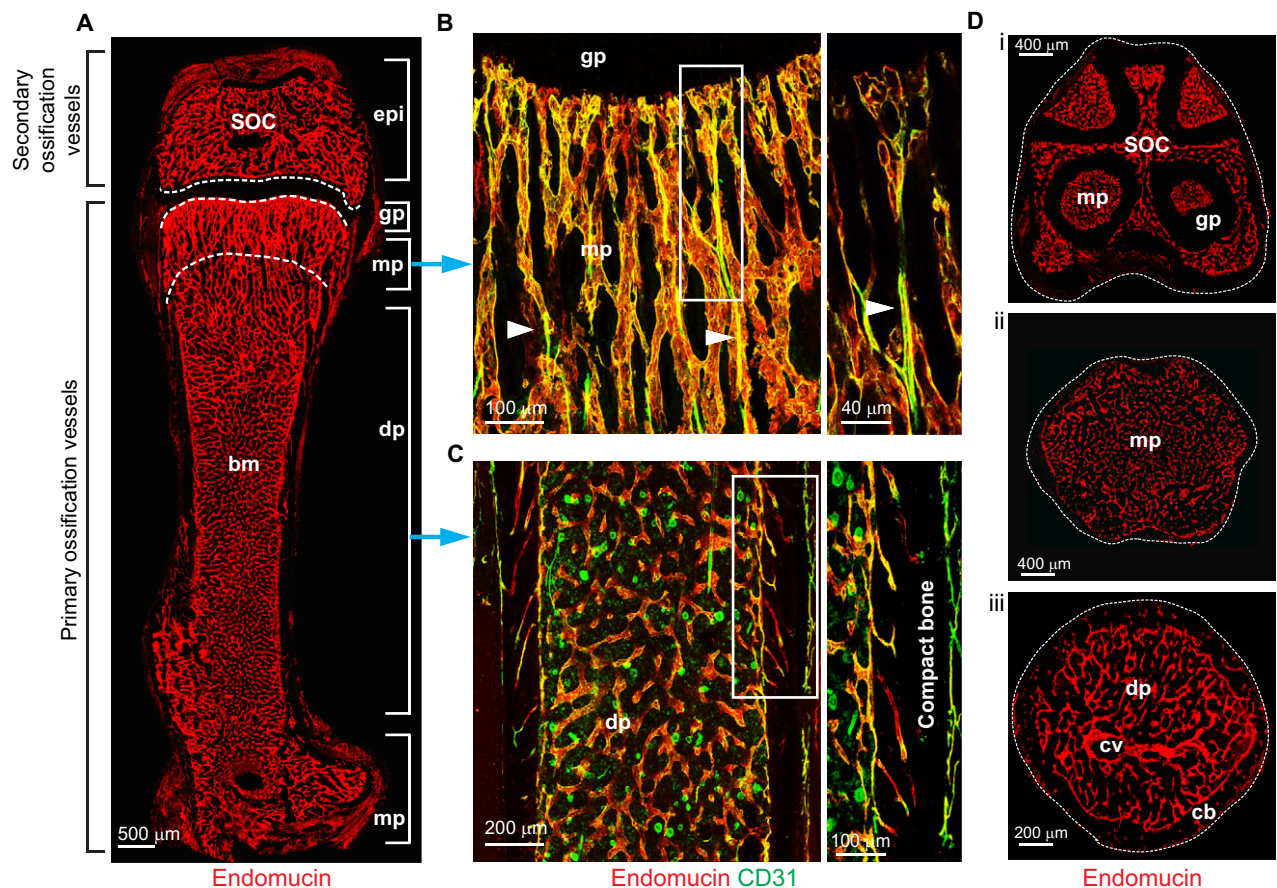


Fig. 1. Architecture of the long bone vasculature. (A) Confocal image of endomucin (EMCN)-immunostained (red) endothelium in a 100 µm thick section of P21 murine femur. Regional differences in the organization of the vasculature are evident, as highlighted in the higher magnification images (B,C) of the regions marked by blue arrows. (B) In the metaphysis, type H vessels (CD31^{hi} EMCN^{hi}) exhibit a columnar organization and arterial connections (arrowheads); the panel on the right shows a higher magnification of the boxed region. (C) In the diaphysis, highly branched sinusoidal type L capillaries (CD31^{lo} EMCN^{lo}) are found; these connect to endosteal type H vessels in the proximity of compact bone. (D) Confocal images of transverse sections through a P21 femur in the region of the growth plate (i), metaphysis (ii) and diaphysis (iii). SOC, secondary ossification center; epi, epiphysis; bm, bone marrow cavity; gp, growth plate; mp, metaphysis; dp, diaphysis; cv, central vein; cb, cortical bone.

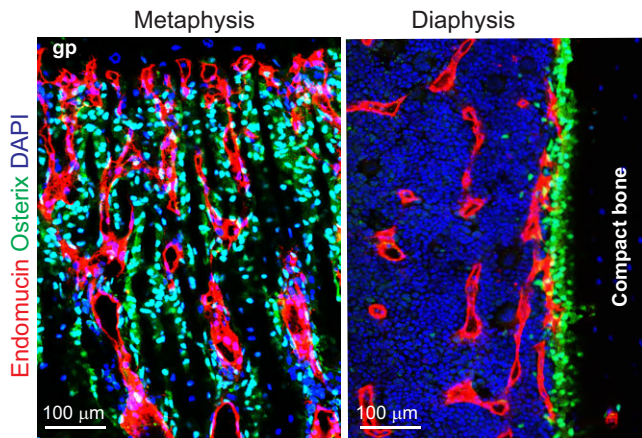


Fig. 2. Associations between blood vessels in the bone and osteoprogenitors. Vessels associate with osteoprogenitors in a subtype-specific manner. Osterix-positive osteoprogenitors (marked by green nuclear staining) associate with EMCN-immunostained (red) type H capillaries found in the metaphysis (left panel) and with endosteal type H vessels found in the proximity of compact bone in the diaphysis (right panel) but not with diaphyseal type L vessels found in the bone marrow cavity (right panel). gp, growth plate.

of the markers CD31 and endomucin ($CD31^{lo} EMCN^{lo}$) (Fig. 1C). Sinusoidal type L capillaries, which are surrounded by densely packed hematopoietic cells, connect to the large ($\sim 100 \mu\text{m}$ diameter) central vein (Fig. 1D). Interestingly, arteries and distal arterioles do not deliver blood directly into type L sinusoidal capillaries, but instead arteries exclusively connect to type H vessels in the metaphysis and endosteum (Fig. 3) (Kusumbe et al., 2014; Ramasamy et al., 2014). Owing to this peculiar organization of the bone vasculature, blood flows through arteries into type H capillaries, enters the type L sinusoidal network at the interface between the metaphysis and diaphysis, and is finally drained into the large central vein. As a consequence, distinct metabolic environments can be detected in postnatal long bone: the diaphysis is highly hypoxic due to the lack of direct arterial supply and the large number of hematopoietic cells, whereas the metaphysis of postnatal and adolescent mice is comparably well oxygenated (Kusumbe et al., 2014; Ramasamy et al., 2014).

The bone vasculature also contains several types of mural cells. In the bone marrow, type L sinusoidal vessels are surrounded by two types of perivascular cells, namely leptin receptor ($LEPR$)⁺ cells (Ding et al., 2012) and CXCL12-abundant reticular (CAR) cells (Sugiyama et al., 2006) (Fig. 3). Substantial evidence indicates that these perivascular cells play important roles in the regulation of hematopoiesis by secreting molecular signals such as stem cell factor (SCF, or KITL), CXCL12 and angiopoietin (Mendelson and Frenette, 2014; Ugarte and Forsberg, 2013). $LEPR$ ⁺ cells also express platelet-derived growth factor receptor α ($PDGFR\alpha$) but are negative for the pericyte markers $PDGFR\beta$ and neural/glial antigen 2 (NG2, or CSPG4). These cells, which show low expression of the nestin-GFP reporter, have the capability to differentiate to different mesenchymal lineage cells, such as bone, cartilage and adipocytes (Zhou et al., 2014). As in soft tissues, arteries in bone are covered with alpha smooth muscle actin (αSMA , or ACTA2)-positive smooth muscle cells, which also express NG2 (Kusumbe et al., 2016). Arteriolar perivascular cells express NG2, are nestin-GFP^{high} and have the potential to differentiate into different mesenchymal lineages (Kunisaki et al., 2013; Mendez-Ferrer et al., 2010). In type H capillaries in the metaphysis, columns are also surrounded by $PDGFR\beta$ ⁺ and $NG2$ ⁺ perivascular cells, which are regulated by

$PDGFB$ secreted from ECs in type H vessels (Kusumbe et al., 2016) (Fig. 3).

Taken together, it is well established that bone contains several distinct perivascular mesenchymal cell populations that play important functional roles in the regulation of hematopoiesis, osteogenesis and vascular homeostasis. What is less clear, however, is the extent to which these mesenchymal cells represent overlapping, completely distinct or interconvertible cell subtypes.

Bone angiogenesis: an introduction

The vasculature in bone appears to be formed mainly or perhaps even exclusively by angiogenesis. In murine long bones, blood vessels start to invade the cartilage template at embryonic day (E) 13.5 to 14.5, and vascular growth is largely completed in adolescent and young adult animals (Maes et al., 2010b) (Fig. 4). This process of endochondral angiogenesis involves a series of events. First, chondrocytes in the location of the future primary ossification center (POC) cease proliferation, become hypertrophic and secrete pro-angiogenic factors to stimulate angiogenesis; osteoprogenitors in the POC are also a source of pro-angiogenic factors. Next, blood vessels invade the hypertrophic chondrocytes and form an initial vascular network, which is accompanied by ossification processes (Kronenberg, 2003). The release of signals by maturing and hypertrophic growth plate chondrocytes at the two ends of the developing long bone further promotes vessel growth and ossification along the longitudinal axis, which leads to the extension of the growing skeletal element. This also involves the formation of distinguishable metaphyseal and diaphyseal capillary networks (Kusumbe et al., 2014). Later in development, vessels invade the epiphyseal chondrocytes at the two distal ends of the long bone and thereby initiate secondary ossification center (SOC) formation (Fig. 4).

As mentioned above, flat bones form via the process of intramembranous ossification and therefore without an intermediate chondrocyte template (Abzhanov et al., 2007). However, just like long bones, flat bones are highly vascularized.

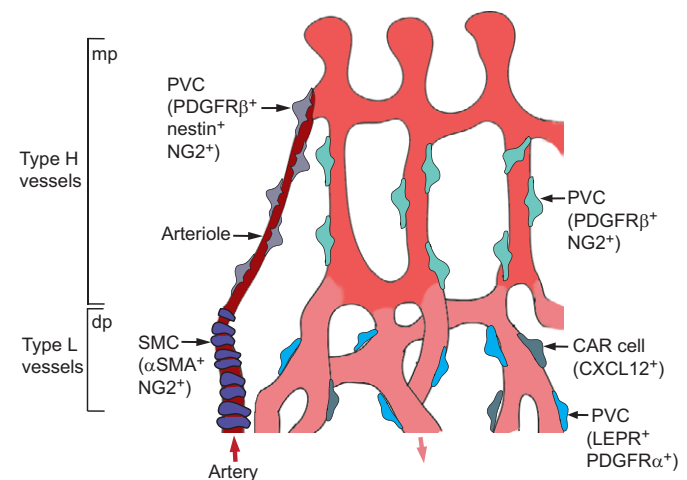


Fig. 3. Perivascular cells associated with blood vessels in the bone.

Larger arteries within bone are covered by smooth muscle cells (SMCs) that are $\alpha\text{SMA}^+ NG2^+$. Distal arterioles, which connect to type H capillaries, are surrounded by perivascular cells (PVCs) expressing nestin, $PDGFR\beta$ and NG2. $PDGFR\beta^+ NG2^+$ PVCs are also found around type H vessel columns. By contrast, two types of PVCs – $LEPR^+ PDGFR\alpha^+$ mesenchymal cells and CXCL12-abundant reticular (CAR) cells – are found on sinusoidal type L vessels. Red arrows indicate the direction of blood flow. Abbreviations as Fig. 1.

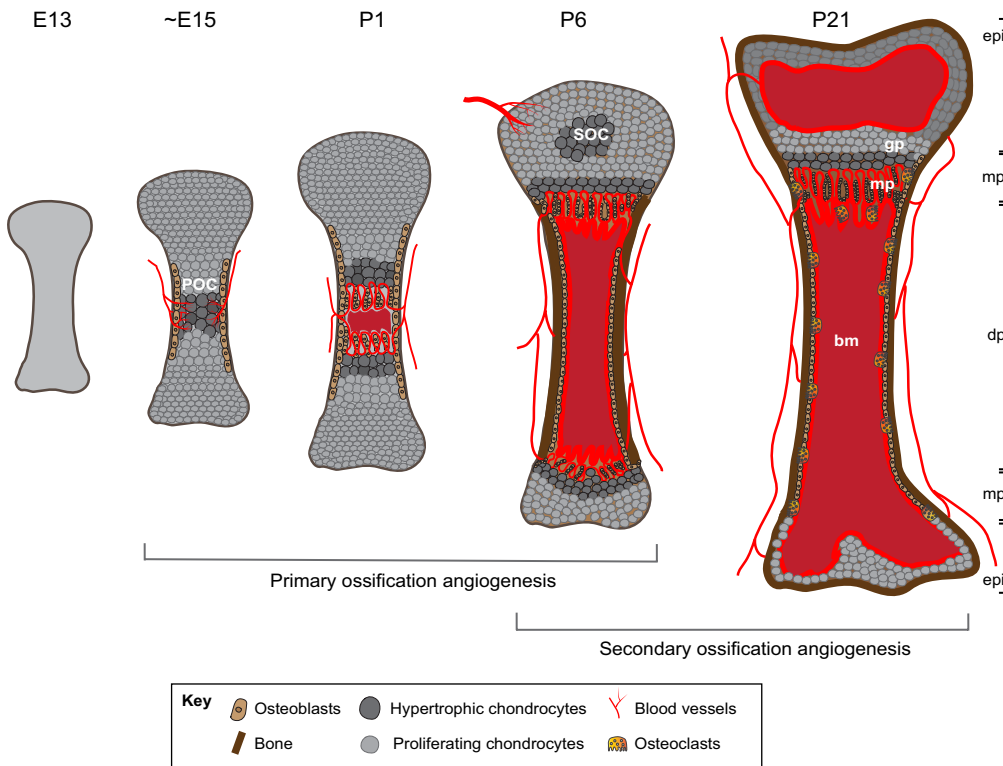


Fig. 4. Endochondral angiogenesis. At ~E13, mesenchymal cells condense, differentiate into chondrocytes and form cartilage. At ~E15, non-proliferative chondrocytes in the center of the primary ossification center (POC) become hypertrophic and secrete pro-angiogenic factors that stimulate blood vessel invasion. Blood vessels then extend within the growing bone and grow towards the epiphysis in both directions at P1. By P6, morphologically distinct capillary beds can be readily distinguished in the metaphysis and diaphysis. Blood vessels start to invade the epiphysis and contribute to the formation of the SOC. At P21, bone growth and vascularization are far advanced and all major structures have been established. Abbreviations as Fig. 1.

This vascularization event (Fig. 5) occurs in a similar fashion to that seen during endochondral angiogenesis, which suggests that similar molecular mechanisms are involved (Percival and Richtsmeier, 2013). However, as most studies concern endochondral angiogenesis, this Review will focus on the latter process.

Factors that regulate endochondral angiogenesis

In recent years, some of the molecular factors that control bone vascularization have been identified (Fig. 6). Several studies, for example, have established that oxygen tension (hypoxia) and the production of VEGF family growth factors are major signals driving endochondral angiogenesis (Maes et al., 2012; Schipani et al., 2009). Notably, many of these signals affect both cells within blood vessels and those of the bone lineage, highlighting that the processes of angiogenesis and osteogenesis are coupled.

VEGF signaling during endochondral angiogenesis

VEGFA, which is a master regulator of angiogenesis, is highly expressed and secreted by hypertrophic chondrocytes. VEGFR2 (or KDR) is the main receptor for VEGFA, and signaling via this receptor triggers processes inducing cell migration, proliferation and survival in ECs (Olsson et al., 2006). VEGFR2 is also expressed by VEGF-producing cells of the osteoblast lineage and controls their migration, differentiation and survival in an autocrine fashion (Duan et al., 2015; Hu and Olsen, 2016) (Fig. 6). Inactivation of the gene encoding VEGFA in chondrocytes affects vessel invasion but also impairs chondrocyte survival and growth (Schipani et al., 2001; Zelzer et al., 2004).

VEGFA exists in three major isoforms: VEGFA¹²⁰ is soluble and does not bind to the ECM; VEGFA¹⁶⁴ can be soluble or matrix bound; and VEGFA¹⁸⁸ is poorly soluble and binds ECM strongly (Harper and Bates, 2008; Robinson and Stringer, 2001). These isoforms elicit different biological responses. For example, mice expressing only VEGFA¹²⁰ show decreased endochondral

angiogenesis, mineralization and reduced expression of osteoblast markers in the POC (Zelzer et al., 2002). By contrast, expression of VEGFA¹⁶⁴ or VEGFA¹⁸⁸ is sufficient for angiogenesis in the developing POC (Maes et al., 2004). It was further shown that overexpression of VEGFA¹⁶⁴ in the osteoblast lineage results in elevated bone angiogenesis and osteogenesis through activation of β -catenin signaling (Maes et al., 2010a). However, expression of a matrix-bound VEGFA¹⁸⁸ isoform only leads to impaired SOC vascularization in knock-in mice, suggesting that the diffusion of soluble VEGFA is required for blood vessel recruitment and angiogenesis in this context (Maes et al., 2004).

Systemic administration of soluble, recombinant VEGF receptor, which captures VEGFs and thereby inhibits signaling, also suppresses bone angiogenesis, chondrogenesis and osteogenesis in mice (Gerber et al., 1999). In addition, EC-specific inactivation of the gene encoding VEGFR2 leads to strong reduction of metaphyseal vessels in proximity to the growth plate (Wang et al., 2013). One of the major signaling pathways triggered by activated VEGF receptors is the PI3K-Akt pathway. The kinase AKT1 is highly expressed in ECs relative to AKT2 and AKT3, and *Akt1* knockout in mice leads to reduced long bone development and vessel formation (Ulici et al., 2009).

The role of hypoxia in endochondral angiogenesis

Hypoxia activates various intracellular signaling pathways and regulates target gene expression through the transcriptional regulator hypoxia inducible factor (HIF). HIF heterodimers are formed by one of three α -subunits [HIF1 α , HIF2 α (or EPAS1) and HIF3 α] and a β -subunit (HIF1 β , or ARNT). HIF1 α and HIF2 α are ubiquitously expressed and stabilized in the hypoxic state (<5% oxygen). In normoxia (>5% oxygen), HIFs are marked for proteasomal degradation by prolyl hydroxylation and the E3 ligase von Hippel-Lindau (VHL) protein, which binds to HIFs and mediates their polyubiquitylation. HIF target genes are involved

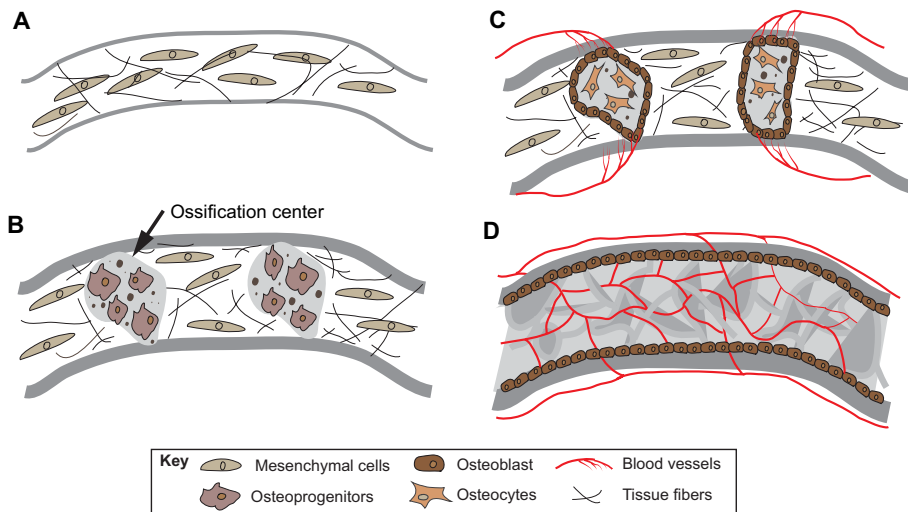


Fig. 5. Intramembranous angiogenesis.

Mesenchymal cells condense to form sponge-like structures (A) and differentiate into osteoprogenitors and osteoblasts, which secrete ECM and form ossification centers and, ultimately, fully differentiated osteocytes (B). Matrix proteins and pro-angiogenic factors generated by the ossification centers then attract blood vessels (C). The subsequent vascularization of the developing flat bone promotes osteogenesis (D).

in a variety of biological processes, such as anaerobic metabolism and angiogenesis. Remarkably, chondrocytes survive in hypoxic conditions by utilizing an anaerobic metabolism, and HIF1 α regulates a number of metabolic genes that play a vital role in this process (Bentovim et al., 2012; Dunwoodie, 2009). Accordingly, the conditional deletion of *Hif1a* in mice causes massive cell death in the inner hypoxic zone of the growth plate (Schipani et al., 2001).

Hypoxia is also a major regulator of VEGF expression (Aragones et al., 2009) and hence angiogenesis. Indeed, HIFs are expressed in osteogenic cells where they regulate VEGF expression (Fig. 6). Accordingly, the deletion of *Hif1a* specifically in osteoblasts leads to reduced bone angiogenesis and osteogenesis. Conversely, the osteoblast-specific overexpression of HIF1 α or loss of VHL results in increased bone angiogenesis and osteogenesis (Wang et al., 2007). HIF1 α and HIF2 α also transcriptionally regulate the expression of ECM genes, and their absence affects chondrogenesis and osteogenesis owing to impaired ECM secretion (Bentovim et al., 2012; Saito et al., 2010; Yang et al., 2010). The activation of hypoxia signaling in ECs also causes an increase in type H capillaries, as well as enhanced endochondral angiogenesis and osteogenesis (Kusumbe et al., 2014). Taken together, there is strong evidence to indicate that hypoxia and VEGF signaling contribute to the coupling of angiogenesis and osteogenesis (Maes and Clemens, 2014; Riddle et al., 2009; Schipani et al., 2009) (Fig. 6).

The role of matrix metalloproteases

Even though the skeletal system is highly mineralized and rich in ECM, bone is subjected to remodeling during growth and homeostasis, and it retains the ability to undergo repair (Bonnans et al., 2014). Matrix molecules secreted by chondrocytes (e.g. collagen type II, COL2) and osteoblasts (e.g. COL1) (Lu et al., 2011) are essential for mineralization (Murshed et al., 2004). During vascular invasion into the POC, matrix metalloproteases (MMPs) induce proteolytic breakdown of ECM leading to matrix remodeling (Ortega et al., 2004; Vu et al., 1998), which, in turn, supports EC migration and proliferation (Vu and Werb, 2000). MMPs are predominantly secreted by osteoclasts and vascular cells. In particular, MMP9 (a member of the gelatinase family) and MMP13 (a collagenase) play major roles in bone angiogenesis and bone remodeling (Stickens et al., 2004; Vu et al., 1998; Yu and Han, 2006). The proteolytic activity of MMPs is inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) (Bonnans et al., 2014).

The ECM is known to induce intracellular signaling through integrin family heterodimeric receptors, and such matrix-integrin signaling is crucial for angiogenesis and vascular integrity (Avraamides et al., 2008). For example, matrix-bound VEGF induces prolonged activation of VEGFR2 and downstream activation of mitogen-activated protein (MAP) kinase p38 relative to soluble VEGF. Matrix-bound VEGF can also induce the association of the integrin $\beta 1$ subunit with VEGFR2 and focal adhesion kinase (FAK, or PTK2) (Chen et al., 2010). Furthermore, and as discussed above, matrix binding of VEGF is crucial for bone angiogenesis (Allerstorfer et al., 2010; Maes et al., 2004). In line with these findings, it is not surprising that MMPs and their matrix-modifying properties can influence angiogenesis and bone formation. MMP9-deficient mice, for example, show decreased vascularization during bone formation (Vu et al., 1998). The administration of exogenous VEGF rescues endochondral ossification defects in *Mmp9* knockout mice (Ortega et al., 2010), arguing that this defect involves insufficient bioavailability of VEGF. This is consistent with earlier studies showing that MMP9 is involved in the release of VEGF from the ECM (Gerber et al., 1999) (Fig. 6). *Mmp13* mutant mice show increased growth plates and tabular bone but no overt changes in the vasculature. By contrast, double-mutant mice lacking both MMP13 and MMP9 show reduced endochondral angiogenesis, diminished ECM remodeling and various bone formation defects (Stickens et al., 2004). These findings highlight the key roles of MMPs, suggesting that they control many aspects of angiogenesis and osteogenesis through cell-matrix and growth factor signaling.

The regulation of angiogenesis and osteogenesis by fibroblast growth factor signaling

Fibroblast growth factors (FGFs), which form a large family of 18 different ligands, can signal through four different tyrosine kinase receptors (FGFR1–4) to induce responses such as cell survival, proliferation and differentiation (Turner and Grose, 2010). During osteogenesis, FGF2, FGF9 and FGF18 and the receptors FGFR1–3 are expressed in a stage- and cell type-dependent fashion (Ornitz and Marie, 2015). The receptors FGFR1 and FGFR2 are expressed in bone vasculature (Coutu et al., 2011), whereas FGFs are secreted from chondrocytes and osteogenic cells (Kozhemyakina et al., 2015; Ornitz and Marie, 2015). Among other responses, FGF can induce VEGFA (Seghezzi et al., 1998) and VEGFR2 (Murakami et al., 2011) expression. Systemic injection of FGF2 (or basic FGF) increases expression of the vascular endothelial adhesion molecule

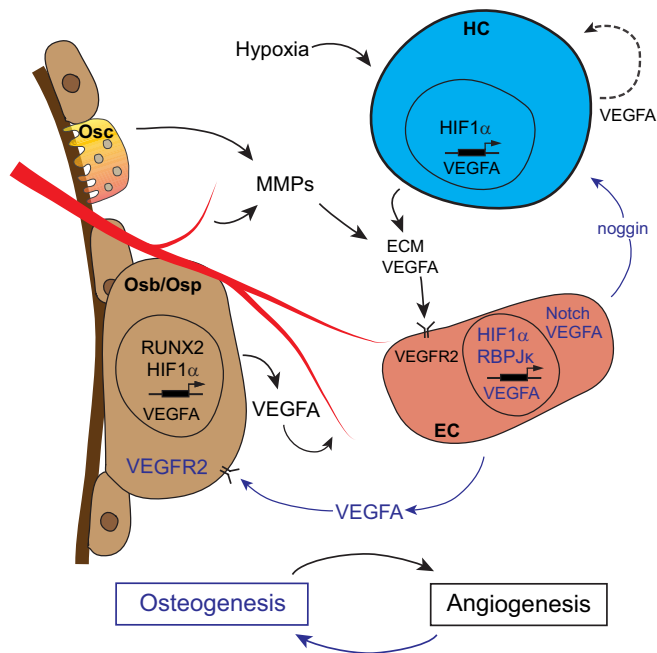


Fig. 6. Molecular coupling of angiogenesis and osteogenesis. During bone formation, hypertrophic chondrocytes (HCs) and osteoblasts/osteoprogenitors (Osbs/Osps, brown) are in a hypoxic environment. Hypoxia inducible factor (HIF) signaling triggers the expression of genes controlling angiogenesis (such as *Vegfa*), metabolism and ECM production. These molecules act as autocrine signals that promote the survival of bone cells but also stimulate angiogenesis in a paracrine fashion. MMPs are secreted from osteoclasts (Osc) and vascular endothelial cells (ECs), induce proteolytic breakdown of ECM and enhance VEGF signaling. RUNX2, a transcription factor expressed in osteoprogenitor cells, is a major regulator of osteoblast differentiation and also regulates VEGF expression in this cell type. VEGFR2 is expressed by ECs and osteoblast lineage cells. VEGFA- and VEGFR2-mediated signaling induces cell migration, proliferation and survival. Conversely, EC-derived signals (such as growth factors and noggin) control chondrocyte maturation and stimulate osteogenesis.

VE-cadherin (or cadherin 5) and the tight junction protein ZO-1 (or TJP1), which stimulates expansion of the arterial vasculature in bone (Itkin et al., 2016). Trabecular bone volume, mineral apposition and bone formation rates are markedly reduced in mutant mice lacking FGF2 (Montero et al., 2000). FGF9 and FGF18 induce chondrocyte proliferation, whereas the absence of these growth factors leads to reduced chondrocyte proliferation and a reduction in hypertrophic chondrocytes. VEGFA expression is reduced in *Fgf9* and *Fgf18* mutant mice resulting in delayed POC vascularization (Hung et al., 2007; Liu et al., 2007). The EC-specific inactivation of genes encoding FGFR1/2 results in increased vessel permeability and loss of perivascular cells associated with bone vessels. These data indicate that FGFR1/2 signaling maintains arterial function and vascular integrity in bone (Itkin et al., 2016). The sum of these studies indicates that the FGF signaling pathway is an important regulator of vessel growth and bone formation.

Notch signaling during bone angiogenesis

The pro-angiogenic effect of VEGF signaling in ECs is modulated by the Notch pathway. Inactivation of Notch signaling in ECs results in hypervascularization in soft tissues due to increased EC proliferation and sprouting (Roca and Adams, 2007; Jakobsson et al., 2009). Surprisingly, activation of Notch signaling in bone ECs was found to promote local angiogenesis and osteogenesis. The latter involves the Notch-controlled secretion of noggin, an

antagonist of the bone morphogenetic protein (BMP) pathway, by ECs, which was found to promote the formation of hypertrophic, VEGF-producing chondrocytes in the adjacent growth plate (Ramasamy et al., 2014) (Fig. 6). EC-specific Notch loss-of-function mutants, namely mice lacking the Notch ligand DLL4 or the mediator of Notch-controlled gene regulation RBPJk, exhibit reduced angiogenesis, loss of type H vessels and bone formation defects (Ramasamy et al., 2014). Remarkably, chondrocyte maturation and hypertrophy in the avascular growth plate are also defective in these EC-specific mutants, resulting in shortening of bone, the formation of irregular and enlarged growth plates, and defective VEGF expression (Ramasamy et al., 2014). Notch receptor activation after ligand binding requires a series of proteolytic cleavage processes, one of which is mediated by the ADAM family metalloprotease ADAM10. Accordingly, the loss of ADAM10 in ECs also leads to impaired bone growth and growth plate defects (Glomski et al., 2011), consistent with the phenotypes seen in other EC-specific Notch loss-of-function mutants (Ramasamy et al., 2014).

Other growth factors and transcription factors that modulate bone angiogenesis

A number of other growth factors, such as connective tissue growth factor (CTGF, or CCN2) and the kinase c-RAF (RAF1), are involved in the regulation of VEGFA expression and, thereby, bone angiogenesis. CTGF is a member of the CCN family of secreted matricellular proteins (Jun and Lau, 2011) and it interacts with growth factors and integrins (Jun and Lau, 2011). CTGF is strongly expressed in the perichondrium and in hypertrophic chondrocytes during endochondral angiogenesis. CTGF is important for osteoclast recruitment but is also necessary for VEGFA expression by hypertrophic chondrocytes, normal ECM production and bone development (Ivkovic et al., 2003). c-RAF is an upstream activator of MEK1/2 (or MAP2K1/2) kinases, which control the signaling activity of MAP kinases (Pearson et al., 2000). c-RAF is strongly expressed in hypertrophic chondrocytes and its absence in these cells leads to reduced apoptosis and expansion of hypertrophic chondrocytes. Vascular invasion into the POC at E15.5 and vascularization of the metaphysis at postnatal day (P) 35 are reduced in chondrocyte-specific c-RAF loss-of-function mutants. Furthermore, it was suggested that these changes are linked to enhanced VEGFA degradation but not to alterations in the transcripts encoding VEGFA¹⁶⁴ and VEGFA¹⁸⁸ (Liu et al., 2016).

As mentioned above hypertrophic chondrocytes secrete pro-angiogenic factors and promote angiogenesis but, perplexingly, proliferative chondrocytes are hypoxic and actually inhibit blood vessel growth. *In vivo* and *in vitro* studies have shown that proliferating chondrocytes inhibit vascularization of the growth plate and cartilaginous bone via secretion of factors such as chondromodulin 1 (or LECT1) and tenomodulin (Hiraki and Shukunami, 2005). It was also shown that the genetic inactivation of SOX9, a transcription factor that regulates chondrocyte differentiation, hypertrophy and survival (Bi et al., 1999; Ikegami et al., 2011), in limb mesenchymal cells leads to vascularization defects and reduced expression of VEGFA. The same study also showed that widespread misexpression of SOX9 in limb mesenchyme is not sufficient to induce ectopic VEGFA expression (Eshkar-Oren et al., 2009). Based on overexpression experiments in hypertrophic chondrocytes, it was also suggested that SOX9 can suppress VEGFA expression leading to reduced angiogenesis and osteogenesis (Hattori et al., 2010).

The regulation of VEGF signaling thus appears to involve a complex network of different molecular players, including CTGF, c-RAF and SOX9. It is, however, not always clear whether the effects of these players on VEGFA levels are direct or whether they simply reflect defective chondrocyte maturation and function. In addition, the interplay of different upstream regulators remains insufficiently understood and requires further investigation.

Angiogenesis in bone repair

The reports summarized above indicate the close interplay between different cell types in the skeletal system. Chondrocytes and cells of the osteoblast lineage produce factors promoting vascular growth, such as VEGFA. In turn, blood vessels are a source of signals acting on bone cells. This reciprocal signaling influences both bone development and bone repair. Blood vessels promote trabecular bone formation by supporting the translocation of osterix-positive osteoprogenitor cells from the perichondrium into the metaphysis (Maes et al., 2010b). Osterix-positive cells are preferentially associated with type H capillaries in the metaphysis and endosteum due to the local release of growth factors and the BMP antagonist noggin by ECs (Ramasamy et al., 2014) (Fig. 2). As distal arterioles directly connect to metaphyseal and endosteal type H vessels but not to the diaphyseal type L vasculature, local differences in oxygenation and presumably the availability of nutrients might also help to generate an appropriate niche environment for osteoprogenitors (Kusumbe et al., 2014; Ramasamy et al., 2014; Spencer et al., 2014) (Fig. 2). These findings might be also relevant in the context of bone repair, which involves blood vessel growth and pro-angiogenic signaling interactions.

The healing of fractured bones is a complex process that differs from wound healing in soft tissues (Einhorn and Gerstenfeld, 2015). Bone damage and the disruption of local blood vessels lead to the exudation of inflammatory cells and hematoma formation. Vessels from three different sites, namely from the bone marrow (medulla), from compact bone and from the periosteum that covers the outer surface of all bones, have been implicated in the re-establishment of local blood supply (Rhineland, 1974). The remodeling and reorganization of blood vessels during bone repair is incompletely understood and it is also unknown whether this process leads to the intermingling of ECs from different sources. The ingrowth of blood vessels is crucial for the formation of a soft callus containing fibroblasts and chondroblasts. Intramembranous ossification leads to the generation of a bone cuff; endochondral bone formation converts the callus into rigid calcified tissue (i.e. hard callus). Further mineralization and vascular remodeling processes in the callus result in repair of the damaged bone (Bahney et al., 2015; Stegen et al., 2015).

A number of studies have shown that pro-angiogenic factors such as VEGF influence fracture healing. For example, in rabbits the treatment of fracture areas with VEGF results in improved healing and vascularization processes (Kleinheinz et al., 2005). Conversely, blocking endogenous VEGF with soluble, recombinant VEGFR1 (or FLT1) fusion protein decreases bone angiogenesis and callus mineralization, and inhibits fracture healing in mice (Street et al., 2002). VEGFR1 is closely related to VEGFR2, but can be produced in a secreted form and acts a negative regulator of blood vessel growth (Cao, 2009). The full-length, membrane-anchored form of VEGFR1 is expressed by inflammatory cells and controls their recruitment to sites of tissue damage (Murakami et al., 2008). Placental growth factor (PIGF, or PGF), a member of the VEGF family and a ligand for VEGFR1, also regulates bone healing. Although PIGF is not required for developmental angiogenesis, it does control a range of regenerative and pathological processes

(Fischer et al., 2007). Indeed, *Pigf* knockout mice show reduced inflammation, osteogenic response and callus remodeling, resulting in impaired fracture healing (Maes et al., 2006).

In addition to its role in developmental bone formation and angiogenesis, FGF signaling is important for bone repair (Du et al., 2012). FGF and FGF receptor expression are increased during fracture healing (Schmid et al., 2009). It was also shown that FGF2 stimulates angiogenesis and osteogenesis during bone repair (Dirckx et al., 2013; Kigami et al., 2014). DJ-1 (PARK7), which was identified as an angiogenic factor secreted from human MSCs, can activate FGFR1-mediated signaling and stimulates both angiogenesis and osteoblast differentiation *in vitro*. Consistent with these findings, DJ-1 enhances the healing of experimental lesions in a rat model (Kim et al., 2012). *Fgf9* heterozygous mutant mice show impaired bone healing, reduced expression of VEGF and VEGFR2 in the lesion, reduced osteoclast recruitment to the callus, and lower MMP9 expression. These defects can be partially rescued by administration of recombinant VEGFA, whereas full rescue results from treatment with FGF9 (Behr et al., 2010). The combined administration of recombinant VEGFA and FGF9 also leads to increased angiogenesis, osteogenesis and bone regeneration in a mouse model of type 2 diabetes (Wallner et al., 2015).

Transforming growth factor β (TGF β), BMPs and growth differentiation factor (GDF) also control bone formation during development and stimulate bone repair (Filvaroff et al., 1999; Salazar et al., 2016; Tang and Alliston, 2013). TGF β 1 and TGF β 2 are the most studied TGFs in the repair context (Chen et al., 2012; Tang et al., 2009). Systemic injection of TGF β increases callus volume and bone strength (Bostrom and Asnis, 1998; Nielsen et al., 1994; Schmidmaier et al., 2003). The effects of TGF β are mainly mediated by the differentiation of chondroblast and osteoprogenitor cells as well as the stimulation of matrix production during the healing process (Edwards et al., 2010; Tang et al., 2009); their potential roles in angiogenesis are not yet sufficiently understood. BMP signaling stimulates mesenchymal and osteoprogenitor cell proliferation and differentiation (Salazar et al., 2016). BMP2 and BMP7 can induce bone formation and stimulate bone repair, which also involves the induction of VEGF expression and stimulation of angiogenesis (Carano and Filvaroff, 2003; Chen et al., 2012).

The examples above show that a number of pathways control not only angiogenesis but also the behavior of bone-forming cells during fracture healing. This is also the case for Notch signaling. Notch in mesenchymal progenitor cells promotes their proliferation and inhibits their differentiation (Hilton et al., 2008; Salazar et al., 2016; Schmid et al., 2009). Overexpression of the constitutively active intracellular domain of NOTCH1 promotes proliferation and differentiation of cells in the osteoblast lineage, whereas inhibition of Notch signaling in osteoblasts causes osteoporosis in aged mice (Engin et al., 2008). Further work is required to investigate the interplay between different signaling pathways and to explore potential therapeutic applications that aim to prevent bone loss or to stimulate fracture healing.

Future perspectives

It is becoming increasingly clear that the formation and homeostasis of the skeletal system rely on the integrated activity of several different cell types. Strikingly, however, ECs are emerging as a crucial source of signals that control chondrocytes and osteoblastic cells, thereby allowing the coupling of angiogenesis and

osteogenesis during both development and regeneration. This is an unexpected addition to the conventional and well-established role of the endothelium as an essential part of the circulatory system.

This link between osteogenesis and angiogenesis also has important implications for understanding bone diseases and aging. Bones undergo constant remodeling throughout the lifetime of the organism, and this involves the continuous activity of osteogenic cells and osteoclasts. The balance between bone formation and resorption is key in maintaining strong and fully functional bones throughout life and, accordingly, an imbalance between these processes can result in age-related reductions in bone mineral density (osteopenia), osteoporotic bone loss and increased risk of fracturing. During aging, bone resorption increases due to hormonal changes and other factors, while osteogenesis declines gradually (Hendrickx et al., 2015). Interestingly, type H vessels and their associated osteoprogenitors are also reduced in aged mice, whereas the total number of ECs in murine bone does not change significantly, owing to an equivalent increase in type L vessels (Kusumbe et al., 2014). CD31^{hi} vessels are also reduced in the ovariectomy mouse model of osteoporosis (Xie et al., 2014), which suggests that changes in the local vasculature might contribute to pathological processes. These findings raise the interesting possibility that the reactivation of developmental processes controlling bone angiogenesis could be utilized to prevent age-related or disease-induced bone loss. Likewise, the reactivation of pathways promoting artery formation during development (Herbert and Stainier, 2011; Simons and Eichmann, 2015) might potentially prove useful for the treatment of avascular necrosis, which involves the death of bone tissue due to a lack of local blood supply (Eltzschig and Eckle, 2011; Silvestre et al., 2013), although whether findings in the skeletal system of mice are applicable to humans needs to be investigated. Despite many open questions and caveats, we are presented with the exciting opportunity to investigate the precise role of bone blood vessels and EC subpopulations in a variety of physiological and pathobiological settings, which will undoubtedly enhance our mechanistic understanding of the cellular and molecular processes in the developing, adult, ageing and diseased skeletal system.

Competing interests

The authors declare no competing or financial interests.

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