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The skeletal vascular system – Breathing life into bone tissue

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ABSTRACT

During bone development, homeostasis and repair, a dense vascular system provides oxygen and nutrients to highly anabolic skeletal cells. Characteristic for the vascular system in bone is the serial organization of two capillary systems, each typified by specific morphological and physiological features. Especially the arterial capillaries mediate the growth of the bone vascular system, serve as a niche for skeletal and hematopoietic progenitors and couple angiogenesis to osteogenesis. Endothelial cells and osteoprogenitor cells interact not only physically, but also communicate to each other by secretion of growth factors. A vital angiogenic growth factor is vascular endothelial growth factor and its expression in skeletal cells is controlled by osteogenic transcription factors and hypoxia signaling, whereas the secretion of angiocrine factors by endothelial cells is regulated by Notch signaling, blood flow and possibly hypoxia. Bone loss and impaired fracture repair are often associated with reduced and disorganized blood vessel network and therapeutic targeting of the angiogenic response may contribute to enhanced bone regeneration.

1. Introduction

A functional vascular network is a prerequisite for normal tissue development, homeostasis and repair. Skeletal blood vessels supply different cell types in the bone environment with oxygen and nutrients, but also serve as a source for hormones, growth factors and calcium and phosphate, the building blocks for matrix mineralization. In addition, skeletal and endothelial cells interact reciprocally by paracrine signaling. Indeed, skeletal cells secrete angiogenic factors whereas endothelial cells produce angiocrine factors that regulate skeletal cell behavior. Finally, the bone marrow vascular system serves as a specialized microenvironment that promote maintenance of stem and progenitor cells. This strong link between blood vessels and skeletal tissue is not only observed during bone development, where there is a close connection between angiogenesis and osteogenesis, but also during aging and in different skeletal pathologies that are associated with altered vasculature. Increasing insight into the molecular and cellular processes orchestrating the angiogenic cascade may help to develop novel treatments for bone healing, especially for clinical situations with a limited angiogenic host response. In this review, we first discuss the current knowledge of developmental skeletal angiogenesis and its regulation by angiogenic growth factors. We here focus on the role of vascular endothelial growth factor, a pivotal angiogenic factor, and its regulation by hypoxia signaling. We refer the reader for information on other angiogenic regulators to some excellent recent reviews [1,2]. In the second part, we discuss the importance of the vascular system during bone pathology and repair.

2. Bone development: close interaction between osteogenesis and angiogenesis

Bones are highly vascularized tissues, whether they are formed through endochondral or intramembranous ossification. Both bone-forming processes are tightly coupled to angiogenesis, the growth of new blood vessels from existing ones. During endochondral ossification, mesenchymal cells condense and form clusters within avascular regions [3,4]. Osteochondroprogenitor cells in the central part of the condensation differentiate into chondrocytes, which start to proliferate and form a cartilaginous template for future bone deposition. Chondrocytes in the center of this cartilage anlage stop to proliferate, become hypertrophic and secrete pro-angiogenic factors (Fig. 1A). Concurrently, mesenchymal cells in the outer layer of the cartilage template (*i.e.* the perichondrium) differentiate to osteoprogenitors, which also produce pro-angiogenic factors. Blood vessels are attracted to first invade the perichondrium and then the region of hypertrophic chondrocytes.

Abbreviations: BMP, bone morphogenetic protein; COL2, type II collagen; CXCL9, C-X-C motif (CXC)-chemokine; Dll4, delta-like 4; EC, endothelial cell; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; MMP, matrix metalloproteinase; mTORC1, mechanistic target of rapamycin complex 1; PHD, prolyl hydroxylase domain protein; PlGF, placental growth factor; POC, primary ossification center; Runx2, Runt-related transcription factor 2; SOC, secondary ossification center; T2DM, type 2 diabetes mellitus; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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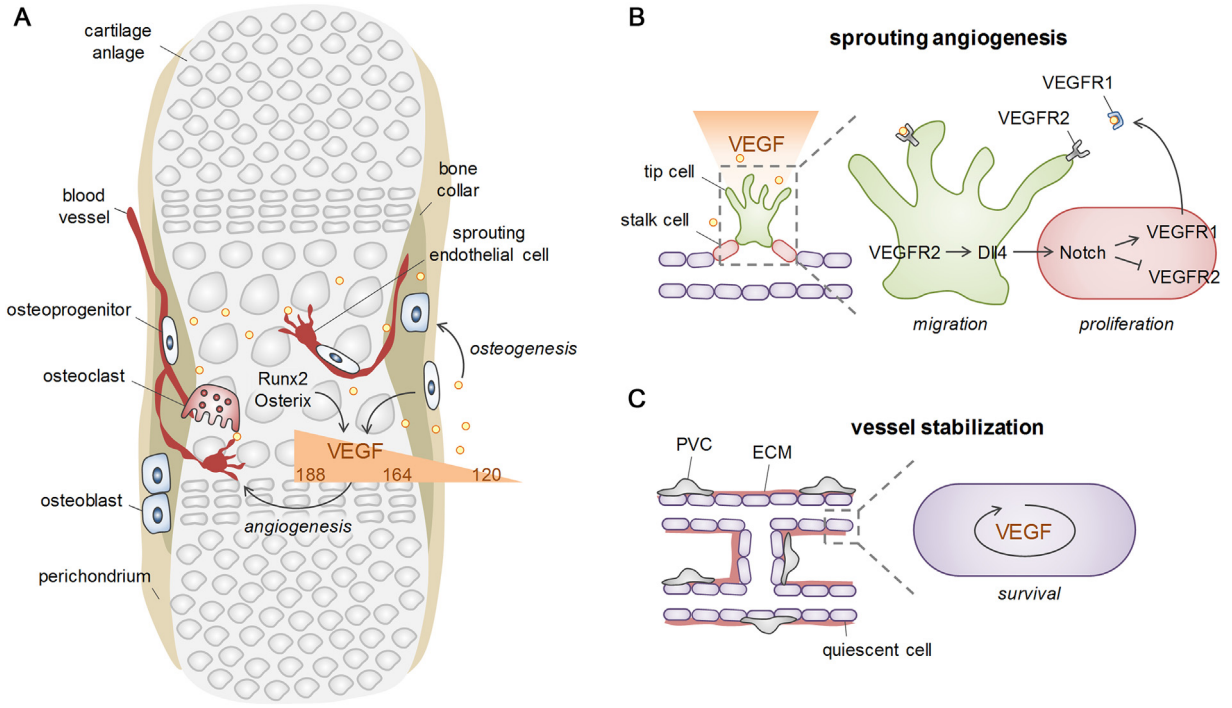

Fig. 1. Angiogenesis during early bone development. (A) During embryogenesis, the initiation of neovascularization of the cartilage anlage is coordinated by the induction of vascular endothelial growth factor (VEGF) expression in perichondrial osteochondroprogenitors and hypertrophic chondrocytes in the cartilage template, mainly by transcription factors such as Runx2 and Osterix. The combined secretion of three VEGF isoforms (VEGF120, VEGF164 and VEGF188) results in a VEGF gradient that controls guided sprouting angiogenesis. Blood vessel invasion is accompanied by osteoclastic cartilage resorption and osteoprogenitors that move to the nascent primary ossification center. (B) Sprouting angiogenesis in general is triggered by VEGF that induce sprouting and migration of a few endothelial cells, the tip cells, by activating VEGF receptor 2 (VEGFR2) signaling. Tip cell VEGFR2 activation also increases expression of the Notch ligand Delta-like 4 (Dll4) to initiate Notch signaling in neighboring endothelial cells. In these so-called stalk cells, Notch signaling regulates proliferation, but inhibits tip cell behavior by downregulating VEGFR2, while upregulating VEGFR1. (C) Vessel stabilization relies on the recruitment of perivascular cells (PVCs) and deposition of extracellular matrix (ECM) by the quiescent ECs, which depend on (autocrine) VEGF signaling for survival.

Concomitantly with the invasion of blood vessels into the cartilage template, Osterix-positive osteoprogenitors from the perichondrium move along [5]. The cartilage is degraded by the invading osteoclasts and replaced by trabecular bone, formed by osteoblasts, and a bone marrow cavity in which the hematopoietic bone marrow cells reside [3,4]. By these coordinated actions, the primary ossification center (POC) is formed. The epiphyseal growth plates that are thereby formed at both ends of the long bones further mediate longitudinal bone growth. The hypertrophic chondrocytes and cartilage matrix at the chondro-osseous junction are continuously replaced by trabecular bone, which is associated with angiogenic growth in the metaphysis and capillary invasion of the hypertrophic chondrocyte region. Simultaneously, the epiphyseal growth plate expands in size and because of its avascular nature the chondrocytes in the center become hypoxic and start to produce pro-angiogenic factors. Comparable to the formation of the POC, blood vessels, osteogenic cells and osteoclasts invade the epiphyseal cartilage and form the secondary ossification center (SOC) [3,4].

Whereas most skeletal elements are formed through endochondral ossification, most craniofacial bones and part of the clavicle are formed by intramembranous ossification, in which mesenchymal cells directly differentiate into osteoblasts [4,6]. These cells produce pro-angiogenic factors that attract blood vessels, which further promote osteogenesis. In this review, we will mainly focus on the link between endochondral ossification and angiogenesis.

During endochondral ossification, the invasion of blood vessels into the avascular cartilage template to form the POC is through a process resembling sprouting angiogenesis [7]. This highly branched vasculature further expands during the longitudinal and radial growth of the long bones. In early postnatal life, the vascular network obtains its characteristic organization, consisting of arteries, a dense capillary network and a large central draining vein. Typical for bone, two types of capillaries are present that form a single network [8]. From the arteries, the blood circulates first to capillaries present at the endosteum and in the metaphysis, close to the growth plate where they display a columnar structure, interconnected by distal loops or arches that are juxtaposed to the hypertrophic chondrocytes. These capillaries are surrounded by Osterix-positive osteoprogenitors and are also called type H vessels because of their **h**igh expression of CD31 and endomucin. The type H capillaries are connected through transition vessels to the highly branched sinusoidal vessels in the diaphysis that are surrounded by hematopoietic cells. These so-called type L vessels are characterized by **l**ow expression of CD31 and endomucin, and drain into the central vein [8]. This vascular organization has several consequences. First, it may affect the oxygen and nutrient delivery to the local microenvironment. Indeed, the metaphysis and endosteal region are considered to be less hypoxic than the diaphysis [8,9], although conflicting data exist [10]. This regional hypoxia may be due to the serial organization of the two capillary systems and thus reduced delivery of oxygen in the diaphysis. On the other hand, the high number of metabolically active hematopoietic cells in this region may lead to high oxygen consumption rate and thereby contribute to low oxygen levels. Second, the blood flow and shear rate are higher in the arteriolar capillaries than in the sinusoids [11,12]. Third, the endosteal capillaries display a tight endothelial blood-bone marrow barrier, whereas the sinusoids are more fenestrated and the vascular wall is thus more permeable [11]. The slower blood flow and higher permeability of the sinusoids promotes leucocyte trafficking primarily at this site, whereas quiescent hematopoietic stem cells (HSCs) mainly reside near endosteal capillaries, indicating the importance of vascular permeability in modulating HSC quiescence and leukocyte trafficking.

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3. Angiogenesis: a highly regulated process

A principal driver of sprouting angiogenesis is vascular endothelial growth factor (VEGF)A, usually referred to as VEGF, which is expressed by several bone cell types [13]. It mainly signals via binding to VEGF receptor (VEGFR) 2 whereas signaling via VEGR1 is less efficient. Moreover, VEGFR1, and certainly the secreted (soluble) form, also functions as a VEGF trap by preventing VEGF binding to VEGFR2 [1]. These VEGFRs are highly expressed by endothelial cells (ECs) and their activation induce migration, proliferation and survival of ECs.

VEGF exists in three major isoforms in mice, VEGF120, VEGF164 and VEGF188, which are generated by alternative mRNA splicing [14]. Due to the absence of the heparin-binding domain, VEGF120 binds little to extracellular matrix proteins and can diffuse easily. VEGF188, on the other hand, sequesters to matrix proteins and requires protease activity for its release. VEGF164 has intermediate characteristics and is in several tissues the most abundant secreted VEGF isoform. The combined secretion of these three VEGF isoforms is considered to result in a VEGF gradient that controls guided sprouting angiogenesis.

In sprouting angiogenesis (Fig. 1B), ECs with high VEGFR2 signaling become the invading tip cells that lead the sprouts towards the source of the angiogenic signal. Moreover, tip cells express high levels of the Notch ligand Delta-like 4 (Dll4) that activates Notch signaling in the neighboring ECs. Activation of NOTCH1 receptor results in suppression of VEGFR2 levels and induction of VEGFR1, thereby impairing tip cell behavior in these ECs and promoting a stalk cell phenotype. Stalk cells produce fewer filopodia, are more proliferative and form a vascular lumen [1]. Of note, dysregulation of tip-stalk cell specification by altered Dll4/Notch signaling may have paradoxical effects on vessel perfusion. Indeed, reduced Notch signaling increases vessel sprouting resulting in a dense vessel network, but the perfusion is limited and the vessel network is poorly functional. In the reverse condition with overactivation of Notch, sprouting angiogenesis is reduced but perfusion and oxygenation are improved [15]. In the next step, branches anastomose, a lumen is formed and the newly formed vessels are stabilized by recruiting perivascular cells and deposition of a basement membrane (Fig. 1C). Simultaneously, ECs stop proliferating, but VEGF and other growth factors are then needed to promote EC survival [1].

An important regulator of VEGF expression in numerous cell types is hypoxia [16]. In this stress condition, the levels of hypoxia-inducible factor (HIF) increase, and this transcription factor controls gene networks mediating not only angiogenesis, but also cell survival and metabolism. HIF activity is regulated by prolyl hydroxylase domain proteins (PHD1-3). In normoxia, PHDs use oxygen to hydroxylate HIFs, thereby targeting them for proteasomal degradation. PHDs become inactive in hypoxic conditions, resulting in stabilization of HIFs [17]. HIF signaling induces the expression of *Vegf*, one of its numerous target genes, which will in turn stimulate sprouting angiogenesis by paracrine signaling on ECs. On the other hand, VEGF required for survival of ECs in mature blood vessels is mainly produced by ECs themselves and functions in an intracrine manner [18].

4. Angiogenesis during endochondral bone development

Three different phases of angiogenesis can be distinguished during endochondral bone formation: (i) blood vessel invasion of the cartilage anlage and the formation of the POC; (ii) the longitudinal growth of the bones with blood vessel expansion at the growth plate; and (iii) blood vessel invasion of the epiphyseal growth plate and formation of the SOC.

4.1. Vascular invasion of the cartilage template

4.1.1. Sprouting angiogenesis in close interaction with osteoprogenitors

During the first phase, perichondrial osteolineage cells and hypertrophic chondrocytes express VEGF, that attracts blood vessels from the surrounding tissues to invade the perichondrium and next the cartilage template (Fig. 1A). This process resembles sprouting angiogenesis, although the contribution of the Dll4/Notch pathway is unknown. Detailed microscopic analysis recently revealed that these blood vessels lack a basement membrane, but, on the other hand, are covered by type I collagen that is likely secreted by adjacent osteoblasts [19]. This functional interaction between ECs and osteoblasts may indicate that blood vessels predict the sites of future bone formation and mineralization, but this model needs further investigation. As mentioned, the vascular invasion of the cartilage anlage is associated with entering of osteoclasts that degrade the cartilage template together with Osterix-positive osteoprogenitors that lay down the typical bone matrix [5].

4.1.2. Contribution and regulation of VEGF expression in osteolineage cells

The importance of VEGF expression by the different osteolineage cells at this stage has been shown by several mouse genetic studies (Fig. 1A). Mice with deletion of *Vegf* in Osterix-expressing perichondrial osteoprogenitors exhibit lower number of blood vessels in the perichondrium [20]. In addition, the differentiation of perichondrial osteoprogenitors into mature osteoblasts is reduced in these mutants. This impaired osteoblast differentiation results from reduced autocrine/paracrine signaling through VEGFR2 expressed on osteolineage cells [13,20], although the contribution of reduced vascularity cannot be fully excluded. Of note, Osterix positively regulates VEGF expression by binding to the VEGF promoter [21], suggesting that osteoblast differentiation itself stimulates the attraction of blood vessels, possibly in order to provide sufficient oxygen and nutrients to support their high anabolic activity.

Delayed blood vessel invasion into POC is also observed in mice with conditional deletion of *Vegf* in type II collagen (COL2)-expressing perichondrial osteochondroprogenitors and chondrocytes [22,23]. The transcriptional regulation of VEGF expression in these cells is not fully elucidated, but HIF signaling may be involved, as mice lacking *Hif-1α* expression in COL2-expressing cells show delayed POC formation [24]. The transcription factor Runt-related transcription factor 2 (Runx2; also known as core-binding factor subunit alpha-1), which is considered to be upstream of Osterix, also regulates VEGF expression in osteolineage cells, consistent with the observation that vascular invasion into POC is defective in *Runx2* null mice [25]. However, blood vessels were observed in the perichondrium, which is different from the phenotype of mice with *Vegf* deletion in Osterix-expressing cells [20]. *Vegf* deletion specifically in Runx2-expressing cells may provide further insight in the angiogenesis-osteogenesis coupling at this stage.

These studies evidently show that insufficient VEGF levels impair bone development, but also excessive VEGF levels have to be avoided, as overexpression of VEGF164 in COL2-expressing cells accelerates the angiogenic invasion and leads to excessive and aberrant bone deposition in this region, resulting in deformed limbs [26].

Concerning VEGF isoform contribution, VEGF164 expression is sufficient for POC formation, whereas delayed vascular invasion is observed when only VEGF120 or VEGF188 is expressed, likely because the VEGF gradient is not optimally formed when only the short or long VEGF isoform is expressed [27,28]. Together, these data highlight that VEGF levels and isoform types need to be tightly regulated to ensure correct formation of the POC.

4.2. Metaphyseal angiogenesis during bone growth

4.2.1. Special type of angiogenesis in bone vasculature

Vascular growth at the chondro-osseus junction of the epiphyseal growth plate differs from the typical sprouting angiogenesis in several aspects. First, some ECs within the arches have filopodia, but real tip cells are not observed [29,30]. On the other hand, vessel growth near the growth plate is mediated by bud-shaped protrusions emerging from the distal arches, followed by anastomoses between two neighboring bud structures to form new arches [30] (Fig. 2A). Second, Notch signaling in bone ECs has opposite effects on angiogenesis compared to the

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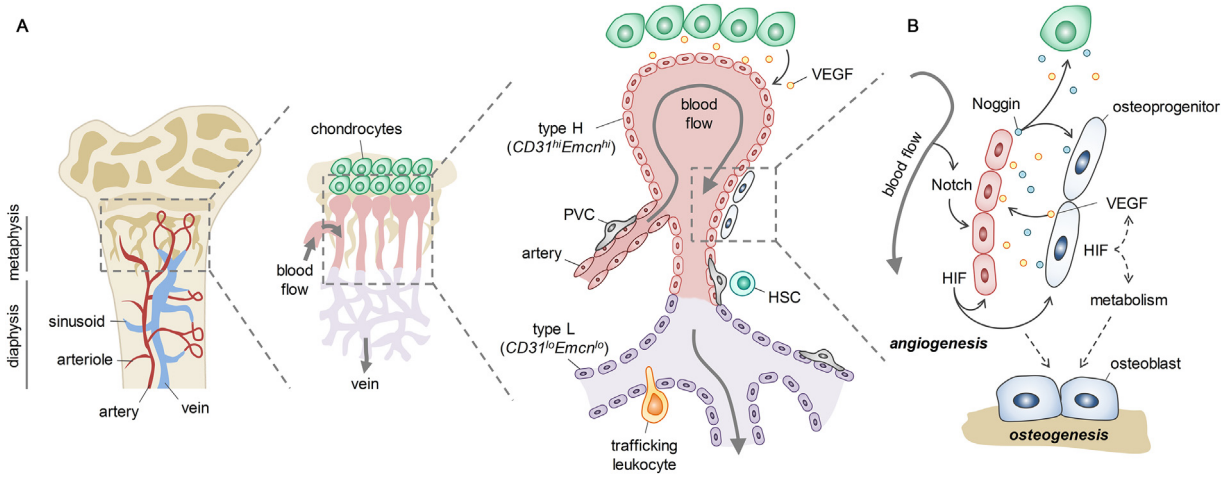

Fig. 2. Morphological and molecular characteristics of bone vasculature. (A) Blood enters the bone marrow compartment via arteries, which branch into to smaller type H vessels that are in close association with Osterix-positive osteoprogenitors and other perivascular cells (PVCs) and are localized in the metaphysis and near the endosteum. These type H vessels then connect to downstream type L sinusoids in the diaphysis that drain the blood into the central sinus and out of the bone marrow. Blood flow is higher in type H capillaries than in type L sinusoids, and they display a tighter blood-bone marrow barrier than the more permeable fenestrated type L vessels. These different characteristics may explain that quiescent hematopoietic stem cell (HSC) are found primarily near type H vessels, whereas leucocyte trafficking occurs at the type L vessels. (B) Notch signaling in bone endothelial cells, controlled by local blood flow amongst others, promotes endothelial cell proliferation and type H vessel growth in bone, which is the opposite of its role in other tissues. Notch activity in endothelial cells also increases endothelial Noggin expression, which promotes the differentiation of osteoprogenitor cells and chondrocytes and thereby regulates osteogenesis. In addition to Notch signaling, endothelial HIF also regulates bone mass by increasing type H vessels and associated osteoprogenitors. On the other hand, in early osteolineage cells, HIF controls VEGF expression and adapts cellular metabolism to support osteogenesis.

Dll4/Notch pathway in ECs in other organs. Indeed, activation of Notch signaling in bone ECs promotes EC proliferation in the columns of the type H vessels and increases the abundance of type H capillaries [29]. The exact molecular mechanism is not fully established, but may be linked to the absence of real tip cells in bone vasculature. In addition, the activation of Notch likely results in improved perfusion and oxygenation, an effect that is also observed in other tissues [15].

Notch signaling in bone ECs also controls the release of the angiocrine factor Noggin, a secreted antagonist of bone morphogenetic proteins (BMPs). Noggin stimulates the differentiation of osteoprogenitors and the maturation of chondrocytes thereby affecting trabecular bone mass, growth plate morphology and release of VEGF by hypertrophic chondrocytes [29] (Fig. 2B). The VEGF-Notch-Noggin pathway underscores the coupling that exists between angiogenesis and osteogenesis, as the effect of altered Notch signaling on bone homeostasis by modulating Noggin levels strongly emphasizes the communication of ECs to bone cells. However, the reverse link between VEGF secretion by skeletal cells and Notch signaling in bone ECs has not yet been firmly shown.

4.2.2. Chondrocytes and osteoblasts contribute to skeletal VEGF levels

VEGF is highly expressed by hypertrophic chondrocytes and is a critical factor regulating bone vasculature at this stage with consequences for growth plate morphology, chondrocyte fate and trabecular bone formation as shown by several mouse genetic studies. Indeed, blocking VEGF action by administration of a soluble recombinant VEGF receptor, which captures VEGFs, reduces vascular invasion of the growth plate and metaphyseal angiogenic growth leading to an expansion of the hypertrophic cartilage zone and reduced trabecular bone formation [31]. The VEGF signaling in bone ECs involves VEGFR2 as EC-specific deletion of VEGFR2 decreases the number of metaphyseal vessels in proximity to the growth plate [32].

Not only secreted diffusible VEGF, but likely also VEGF sequestered in the growth plate matrix contributes to optimal blood vessel growth as evidenced by the phenotype of mice deficient in matrix metalloproteinase (MMP) 9. This enzyme mediates localized proteolytic degradation of the cartilage and bone matrix. Bones from *Mmp9*$^{-/-}$ mice show decreased metaphyseal vascularization [33], a phenotype that was largely rescued by administration of exogenous VEGF [34]. This model is consistent with the observation that mice expressing only VEGF164 or VEGF188 isoforms show normal metaphyseal angiogenesis and bone formation, whereas these parameters are impaired when only the soluble VEGF120 isoform is expressed [27,28]. Likely, VEGF that is stored in the matrix and released when osteoclasts and endothelial cells invade the cartilage matrix is critical for optimal angiogenesis at the chondro-osseus junction. Of note, VEGF can promote osteoclast formation in addition to the prime pro-osteoclastogenic factor RANKL [35], indicating a feed-forward mechanism between osteoclast-mediated VEGF release from the matrix and VEGF-mediated osteoclast formation.

The control of VEGF expression in hypertrophic chondrocytes is likely mediated by osteogenic transcription factors like Osterix and Runx2 [21,25], whereas the contribution of HIF-1α is less probable. On the other hand, HIF-1α levels in bone ECs themselves modulate the metaphyseal vasculature and trabecular bone mass. HIF-1α is highly expressed in type H capillaries compared to sinusoidal L type vessels [8], despite the fact that the metaphysis is less hypoxic than the diaphysis. Moreover, stabilization of HIF-1α in ECs results in more type H vessels, increased number of Osterix-expressing cells and increased trabecular bone mass, highlighting that HIF signaling in bone ECs is critical for bone vasculature [8].

Besides VEGF, blood flow is another important regulator of angiogenic growth in bone (Fig. 2B). Blood flow is crucial for the formation of type H capillaries and angiogenic growth of the vasculature by regulating Notch signaling. In addition, blood flow also controls the number of Osterix-expressing osteoprogenitors, the expression of pro-osteogenic factors in ECs and thereby bone mass [30].

In older mice, the vasculature remains important for bone homeostasis but provides also a vascular niche for maintaining hematopoietic homeostasis. The latter topic is not discussed in this review and we refer the reader to some excellent recent reviews [12,36,37].

During adult life, VEGF is abundantly expressed by osteoblasts and its expression is then regulated by the HIF signaling pathway. Indeed, conditional deletion of *Hif-1α* in Osterix-expressing osteoprogenitors or osteocalcin-expressing mature osteoblasts results in reduced VEGF expression, decreased number of blood vessels and reduced bone mass, whereas the reverse phenotype is observed with *Hif-1α* stabilization in these osteogenic cells [10,38–41]. The close association between an increased number of blood vessels and the high bone mass

phenotype suggests that the change in vascular density leads to the increased bone mass. However, recent genetic mouse studies suggest additional explanations. First, HIF signaling in osteogenic cells may inhibit osteoclastogenesis and thereby increase bone mass. Indeed, modest accumulation of HIF-1α in Osterix-expressing osteoblasts by deletion of *Phd2* and *Phd3* increases bone mass without affecting bone vascularization, but the HIF-1α stabilization induces the expression of anti-osteoclastogenic factors resulting in reduced osteoclast number [42]. Second, HIF signaling can regulate osteoblast metabolism resulting in a bone anabolic response. As recently shown, the high bone mass phenotype induced by stabilization of HIF-1α in Osterix-expressing cells could not be blocked by conditional deletion of *Vegf* with normalization of bone vasculature, but was reversed by pharmacological inhibition of glycolysis [10], highlighting the contribution of adaptations in osteoblast metabolism to the bone phenotype.

Similar as during development, high VEGF levels should be avoided as they cause deleterious effects [26]. Indeed, brief induction of VEGF164 expression in skeletal cells results in increased blood vessel density and enhanced bone formation. However, VEGF164 overexpression for longer periods leads to osteosclerosis, bone marrow fibrosis, and hematological anomalies resembling myelofibrosis-associated bone disease.

Although there is often a good correlation between blood vessel density in bone and bone mass, the opposite condition is sometimes observed and may be linked with increased levels of angiostatic factors. Indeed, mice with activation of mTOR signaling in mature osteoblasts display a high bone mass phenotype but decreased number of type H vessels [43]. The mutant osteoblasts expressed high levels of VEGF, but its binding to VEGFR2 was inhibited by increased secretion of CXCL9, which may explain the reduced vascularity.

Together, these findings emphasize the crucial contribution of VEGF to optimal development and maintenance of bone vasculature, but also the importance of tightly controlled VEGF levels.

4.3. Vascular invasion and the formation of the secondary ossification center

The process of vascularization of the epiphyseal growth plate and formation of the secondary ossification center is less well characterized. A dense vascular network overlays the avascular epiphyseal cartilage surface until vascular canals, containing blood vessels and mesenchymal cells invade at a few sites the cartilage [44,45] (Fig. 3). Whether sprouting angiogenesis and Dll4/Notch signaling is involved is not fully established.

VEGF expression in the central region of the epiphyseal cartilage contributes to this angiogenic response as *Vegf* inactivation in COL2-expressing cells results in a less dense epiphyseal vascular network and cell death in the central region of the epiphyseal growth plate [23,24]. HIF signaling is a critical mediator of VEGF expression in epiphyseal chondrocytes. Indeed, *Hif-1α* deletion in COL2-positive chondrocytes leads to a similar phenotype with massive cell death in the central region [46]. However, the cell death phenotype could only be partially rescued by transgenic expression of VEGF [24]. These findings suggest the following model: with expansion of the epiphyseal growth plate, chondrocytes in the center become hypoxic and devoid of nutrients and this stress condition induces HIF signaling to ensure survival of the hypoxic chondrocytes. Consequently, VEGF secretion is stimulated to induce vascularization of the epiphyseal growth plate, likely associated with HIF-mediated other adaptations in chondrocytes including metabolic changes (Fig. 3). A similar phenotype with cell death in the central region is also observed when only the VEGF188 isoform is expressed, indicating that the expression of diffusible VEGF isoforms is required, likely to reach the border of the epiphyseal growth plate and there interact with the surrounding blood vessels to induce angiogenesis [28] (Fig. 3). Comparable to the role of MMP9 in metaphyseal vascularization, proteases degrading extracellular matrix proteins are also

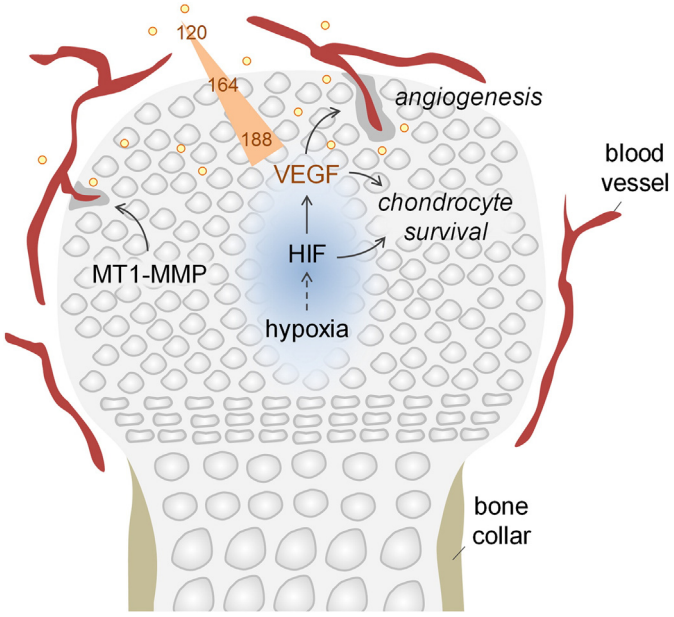

Fig. 3. Blood vessel ingrowth in the epiphyseal growth plate. Because of the lack of blood vessels, the center of the expanding growth plate becomes hypoxic, resulting in HIF stabilization and chondrocytic VEGF expression. Soluble VEGF isoforms, such as VEGF120 and VEGF164, are critical to diffuse to the epiphyseal borders to stimulate ingrowth of the surrounding epiphyseal vessels. HIF is also essential for chondrocyte survival, possibly by mediating a metabolic switch. In addition to HIF-induced angiogenesis, epiphyseal blood vessel ingrowth is facilitated by membrane type-1 matrix metalloproteinase (MT1-MMP)-mediated cartilage degradation.

critical during the formation of SOC, although they are less well characterized. Mice deficient in membrane-type matrix metalloproteinase (MT1-MMP) display delayed formation of SOC, indicating that this enzyme contributes to timely vascularization of the epiphyseal growth plate [47], but the detailed mechanism is lacking.

5. Vascular changes during aging and bone pathology

During adult life, bone mass is maintained by balancing the action of bone-forming osteoblasts versus bone-resorbing osteoclasts, which is key for maintaining strong and fully functional bones. Given the intimate relationship between angiogenesis and osteogenesis, it is not surprising that bone loss associated with aging and diseases, such as osteoporosis and diabetes, is linked to changes in the vascular system.

5.1. Aging-related changes in endothelial cell signaling contribute to decreased bone formation

Decreased bone mass has been frequently reported in humans of >60 years of age, and result from increased bone resorption and decreased bone formation [48]. Interestingly, the reduction in bone mass in elderly individuals is also associated with reduced skeletal blood flow [49]. Recent studies link these aging-related defects in skeletal blood flow and bone mass to specific changes in the phenotype and function of vessel ECs (Fig. 4). In aged (70 week-old) mice, total EC number and type L vessels remain unaltered, but the number of arteries is reduced and type H vessels are nearly absent. This structural phenotype is associated with a significant reduction in blood flow [30,50]. As mentioned, blood flow directly regulates Notch signaling in bone ECs and thereby bone formation by controlling Noggin expression. The reduced blood flow in the aging organism may therefore be a cause of reduced endothelial Notch signaling, which in turn may contribute to age-related bone loss. In support of this model, restoring Notch activity in ECs induces local growth of type H capillaries, which is associated with expansion of vessel-associated osteoprogenitors and formation of

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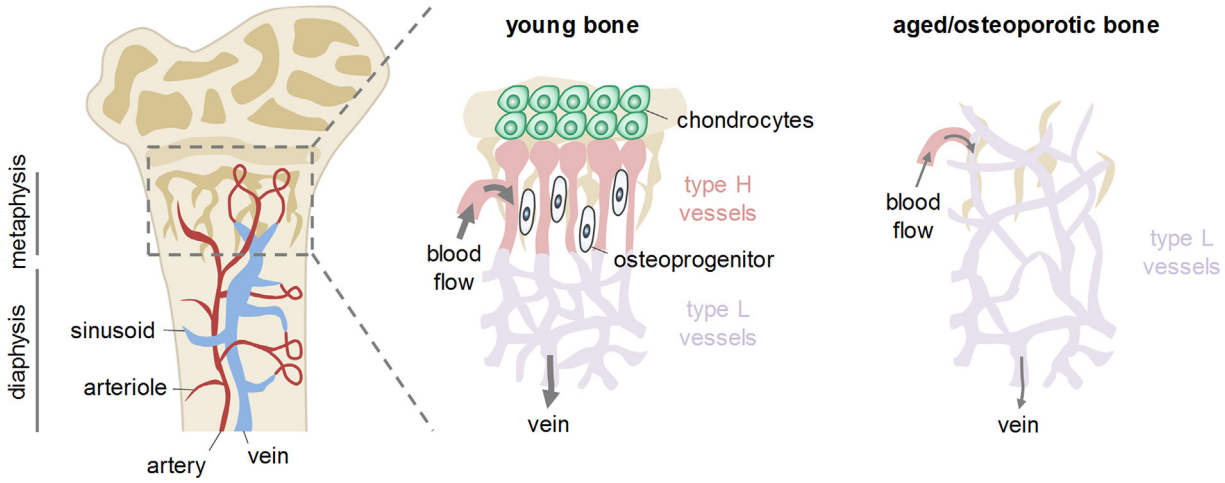

Fig. 4. Vascular changes during aging and osteoporotic bone loss. Age-related and osteoporotic bone loss is associated with decreased perfusion of the skeletal vascular system, resulting in a decrease in type H capillaries and Osterix-positive osteoprogenitor cells.

mineralized bone [30]. Whether reactivation of these developmental signaling pathways that control bone angiogenesis can be further exploited in a therapeutic setting is an interesting but outstanding question.

5.2. Malfunctioning blood vessels in skeletal and systemic diseases

Similar to aging-related dysfunction of blood vessels and bone loss, defects in the skeletal vascular system are also observed in other (experimental) models of osteoporosis. Although the primary cause of bone loss in postmenopausal women is estrogen deficiency, several clinical and preclinical animal studies report a reduced number of sinusoidal and arterial capillaries in the bone marrow, resulting in decreased bone perfusion [49,51,52]. However, a clear causal role for the vascular component in the pathogenesis of osteoporosis remains to be demonstrated. Nonetheless, activation of the HIF signaling pathway stimulates blood vessel growth together with bone formation and thereby protects mice from ovariectomy-induced bone loss [53], suggesting that pro-angiogenic strategies, combined with stimulation of a bone anabolic response, can be explored to treat osteoporotic patients.

Besides skeletal pathologies, general metabolic diseases can also negatively affect bone mass and vascularity. Obesity-induced type 2 diabetes mellitus (T2DM) is characterized by progressive development of insulin resistance in the liver and peripheral tissues accompanied by defective insulin secretion from pancreatic beta cells, leading to overt hyperglycemia [54]. Amongst many other comorbidities, T2DM patients often suffer from compromised skeletal health, which results in 40 to 70% increased fracture risk [55]. Although the underlying mechanisms are very likely multifactorial and still poorly understood, changes in the vascular system is probably an important contributing factor. Consistent with the notion that T2DM leads to the development of microangiopathy and macrovascular-induced morbidity [54], recent studies in obese Zucker diabetic fatty rats indicate that blood flow through the femoral principal nutrient artery is significantly decreased [56]. Several aspects remain however unknown, including following questions: (i) does the T2DM-induced reduction in skeletal blood flow result in similar defects in bone ECs as observed during aging, (ii) are the changes in the vascular system directly related to diabetes-induced bone loss, and (iii) can improvement of this vascular phenotype revert bone loss in diabetic patients.

6. Angiogenesis during bone repair – therapeutic implications

As discussed above, different skeletal cell types are in close association with the vascular system: chondrocytes and osteolineage cells produce angiogenic growth factors that promote vessel growth, whereas blood vessels themselves are a source of angiocrine and osteogenic signals that act on bone cells. This interplay not only exists during bone development, but also in the context of bone repair, which critically depends on optimal pro-angiogenic signaling to induce sufficient and timely blood vessel ingrowth.

6.1. Fracture healing

In the event of a fracture, bone has the unique ability to heal without the formation of scar tissue. This tremendous regenerative capacity depends on the fact that fracture repair in the adult closely resembles bone development, as it recapitulates many of the key molecular pathways during fetal life [57]. Following bone damage (Fig. 5), disruption of the local vascular system results in blood clotting and hematoma formation. The high concentration of angiogenic growth factors in the hematoma explains its strong pro-angiogenic activity [58]. Not surprisingly, removal of the hematoma attenuates repair, whereas its transplantation stimulates new bone formation [58]. Chemokines and (pro-angiogenic) growth factors released from the hematoma induce the proliferation and migration of inflammatory cells, fibroblasts and skeletal progenitor cells, together with new blood vessel ingrowth from the bone marrow, periosteum and cortical bone [57]. Depending on their relative distance to the blood vessels, these progenitor cells either differentiate into chondrocytes, which deposit a collagenous matrix that is later replaced by bone, or they directly maturate into bone-forming osteoblasts. Indeed, chondrocytes are located furthest away from the blood vessels [57,59], possibly because their metabolism is adapted to survive and function in a poorly vascularized environment. In contrast, differentiation of osteoprogenitors to mature osteoblasts during intramembranous bone repair depends more on oxidative metabolism [60] and thus requires a constant substantial supply of nutrients from neighboring blood vessels. The cartilage callus is subsequently replaced by bone tissue in a process resembling endochondral ossification. In the final phase of fracture repair, the healing bone undergoes remodeling together with the restoration of the vascular supply to the normal state [57,59].

6.2. Fracture callus-derived factors regulate blood vessel ingrowth

Many pro-angiogenic growth factors are involved in the bone repair cascade and amongst these, VEGF is the most extensively studied. During the early phases of bone healing, VEGF levels are highly elevated in the fracture hematoma but also systemically in injured patients [58,61,62]. The importance of VEGF-dependent blood vessel ingrowth at the defect site is evident, as blocking VEGF activity through treatment

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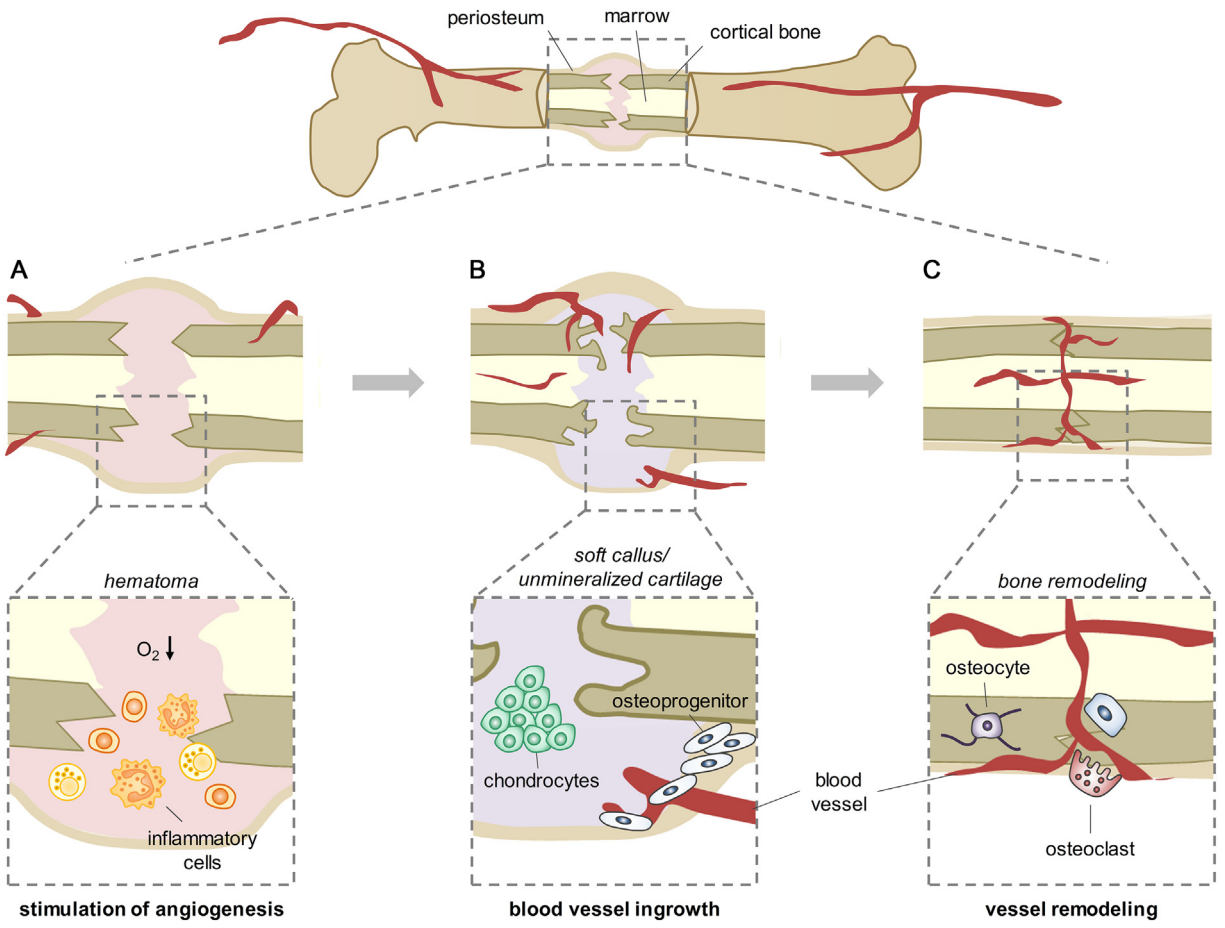

Fig. 5. An adequate angiogenic response is critical for proper fracture repair. (A) Due to the rupture of blood vessels, the fracture site becomes hypoxic (i.e. low oxygen (O_2) tension). (B) Local secretion of pro-angiogenic growth factors by several cell types present in the fracture callus stimulate new blood vessel ingrowth. Along with these blood vessels, osteoprogenitors arrive at the fracture site, where they mediate bone repair through endochondral or intramembranous ossification. (C) Finally, remodeling of both skeletal tissue and the vascular system restores the damaged bone to its original shape and function.

with VEGF antagonists or soluble VEGFR results in an impaired vascular response and, consequently, compromised bone healing [61]. Conversely, administration of recombinant VEGF accelerates blood vessel ingrowth and bone formation in preclinical models of intramembranous and endochondral fracture healing [61].

The strict spatial and temporal regulation of VEGF expression in the fracture callus suggests that changes in the local microenvironment are involved in the control of the angiogenic response. Indeed, vascular damage and hematoma formation limits blood perfusion at the fracture site, resulting in regional hypoxia. To overcome hypoxia-induced cell death, cells activate the HIF-mediated survival mechanism. Several studies have reported that HIFs are expressed in the fracture callus [16,63], suggesting that activation of HIF signaling is important for the angiogenic response during bone healing. Indeed, genetic inactivation of HIF-1α in mature osteoblasts impairs blood vessel ingrowth at the fracture site and, consequently, bone healing, whereas overexpression of HIF-1α in these cells improves angiogenesis and bone repair [64,65]. Whether other cell types of the fracture callus, including chondrocytes, also activates a vascular response via HIFs or whether HIF signaling controls other, angiogenesis-independent, steps during bone healing remains unknown.

Several other pro-angiogenic growth factors influence fracture healing, including placental growth factor (PlGF), a member of the VEGF family, fibroblast growth factor (FGF) 2 and FGF9, and DJ-1 [66]. The expression of these factors is increased in the fracture callus and systemic deletion of *Plgf*, *Fgf2*, *Fgf9* or *Dj-1* in mice show reduced bone healing together with an impaired angiogenic response. However, it is largely unknown which callus cells produce these factors and it is incompletely understood whether the defects in angiogenesis or, on the other hand, the callus cell-intrinsic effects are the primary cause of the impaired bone healing.

In addition to growth factors that directly affect the process of vessel growth, other proteins regulate angiogenesis during fracture repair in an indirect manner, such as MMPs. Similar to skeletal development, angiogenesis during endochondral bone repair is intimately linked with MMP-mediated turnover of the cartilaginous callus. Indeed, disruption of matrix remodeling caused by MMP deficiency impairs blood vessel ingrowth and bone repair, resulting in non-union [67–69]. Likely, lack of these proteases limits the release of angiogenic signaling molecules from the callus matrix, as local administration of exogenous VEGF corrects the defective bone healing in *Mmp9*$^{-/-}$ mice [34].

Thus, in addition to direct stimulation of angiogenesis by pro-angiogenic growth factors, other factors, such as MMPs, are equally crucial for proper blood vessel ingrowth during bone repair.

6.3. Therapeutic angiogenesis for enhanced bone repair

The stimulatory effect of pro-angiogenic factors on blood vessel growth during bone formation have led to the exploration of these proteins as therapeutic agents for bone repair. Administration of recombinant VEGF increases vascularization at the fracture site and accelerated bone healing in several preclinical models [66]. However, translation into the clinic is still limited, as this strategy may have some limitations due to protein instability in vivo and undesirable side effects. Indeed, as mentioned previously, mice overexpressing VEGF temporally in osteolineage cells display aberrant bone formation and

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extramedullary hematopoiesis [26]. In addition, undesirable diffusion of these potent angiogenic growth factors to other tissues may increase the risk of adverse vascular effects and development of malignancy. Current strategies are therefore focused on more localized, controlled release of these growth factors. In addition, local gene therapy using viral vectors that overexpress pro-angiogenic factors may also circumvent potential side effects. For example, injection of VEGF-overexpressing adeno-associated vectors significantly improves bone healing, particularly when supplied in combination with viral vectors overexpressing BMPs [70,71].

Another approach to avoid potential side effects caused by supraphysiological dosing of growth factors is to stimulate angiogenesis in a more physiological way by interfering with the hypoxia signaling pathway. As discussed above, activation of HIF-1α signaling enhances bone repair and vascularization, but therapeutic targeting of these transcription factors is challenging. Therefore, the PHD oxygen sensors that act upstream of HIFs are increasingly being considered as druggable targets to intervene in pathologies resulting from acute hypoxia [72,73], such as fracture repair. In general, PHD inhibitors block the catalytic activity of these enzymes or interfere with substrate binding [72]. Several of these small molecule inhibitors have reached clinical trials for ischemic diseases, although possible side effects of pan-PHD inhibitors should not be neglected, as long-term activation of HIF signaling has potential detrimental effects on bone and hematopoietic tissue [42]. Despite these caveats, several studies in preclinical animal models have shown that blocking PHD activity improves bone repair. Transient localized activation of HIF signaling using small molecule inhibitors is sufficient to increase VEGF-mediated vascularization, thereby promoting callus formation and bone healing [40,64,65]. Thus, for acute bone injuries, local and transient inhibition of PHDs might prove to be beneficial, but secondary effects at the administration site should be further investigated. Together, although stimulation of angiogenesis is an appealing strategy to accelerate fracture repair, further efforts will undoubtedly support the development of novel therapies for patients with impaired bone healing.

7. Conclusions and future perspectives

Close interaction between blood vessels and skeletal cells is observed not only during bone development, but also during bone homeostasis and pathology. Endothelial cells and osteolineage cells are often juxtaposed and they influence each other by secreting specific growth factors [2]. The prime angiogenic factor VEGF is critical for timely vascularization of bone tissue, but its therapeutic application in bone pathology and fracture repair is still limited, likely because of the narrow range of beneficial levels and the skeletal and possible systemic side effects induced by high VEGF levels. An alternative therapeutic approach may be to target the Notch signaling pathway in bone ECs, which results in increased blood vessel density in bone. Activation of this pathway also increases bone mass by stimulating Noggin secretion, thereby influencing blood vessels and osteolineage cells simultaneously [29]. More preclinical studies are however required to fully understand the underlying mechanisms and to investigate whether bone ECs can be therapeutically targeted. Another approach is to increase VEGF levels in a more physiological manner by stimulating HIF signaling. Importantly, HIF signaling not only promotes angiogenesis, but also improves cell survival. Preclinical studies evidently show that increased HIF activity in osteoblasts or ECs induces angiogenesis and stimulates bone formation [73]. Small molecules exist that inhibit PHD activity, thereby stimulating HIF activity. The outstanding challenges are to improve the specificity of these compounds and to obtain local delivery specifically in the bone environment.

Disclaimer

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