

Role and Regulation of Vascularization Processes in Endochondral Bones

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ABSTRACT

Adequate vascularization is an absolute requirement for bone development, growth, homeostasis and repair. Endochondral ossification during fetal skeletogenesis is typified by the initial formation of a prefiguring cartilage template of the future bone, that itself is intrinsically avascular. When the chondrocytes reach terminal hypertrophic differentiation they become invaded by blood vessels. This neovascularization process triggers the progressive replacement of the growing cartilage by bone, in a complex multi-step process that involves the coordinated activity of chondrocytes, osteoblasts, and osteoclasts, each standing in functional interaction with the vascular system. Studies using genetically modified mice have started to shed light on the molecular regulation of the cartilage neovascularization processes that drive endochondral bone development, growth and repair, with a prime role being played by VEGF and its isoforms. The vasculature of bone remains important throughout life, as an intrinsic component of the bone and marrow environment. Bone remodeling, the continual renewal of bone by the balanced activities of osteoclasts resorbing packets of bone and osteoblast building new bone, takes place in close spatial relationship with the vascular system and depends on signals, oxygen, and cellular delivery via the blood stream. Conversely, the integrity and functionality of the vessel system, including the exchange of blood cells between the hematopoietic marrow and the circulation, relies on a delicate interplay with the cells of bone. Here, the current knowledge on the cellular relationships and molecular crosstalk that coordinate skeletal vascularization in bone development and homeostasis will be reviewed.

INTRODUCTION

During embryonic development, two distinct mechanisms are responsible for the establishment of the skeleton. The flat bones, such as those of the skull, develop via intramembranous ossification. The long bones, representing the majority of the bones of the vertebrate body including those of the axial and appendicular skeleton, develop through endochondral ossification [1,2]. In intramembranous bone formation, mesenchymal cells aggregate at the site of the future bone and differentiate directly into osteoblasts that deposit and mineralize the bone matrix. In contrast, endochondral ossification is typified by the initial formation of a cartilage template of the future bone. Hence, mesenchymal cells in the skeletal condensations first differentiate into chondrocytes depositing cartilage matrix rich in collagen type II (Col2). The prefiguring cartilaginous structure is gradually replaced by bone, involving the coordinated activity of chondrocytes, osteoblasts, osteoclasts, as well as endothelial cells. Indeed, the vasculature is crucial for the progressive conversion of the cartilage scaffolds into bones during endochondral ossification, and each of the cell types involved in the process stands in functional interaction with the vascular system and in molecular crosstalk with the endothelium.

Experiments in the 1960's that induced physical blockage of the bone's blood supply, resulting in reduced longitudinal growth, already proved the importance of the vasculature to growing bones [3,4]. More recently, researchers have used genetic mouse models involving the Cre-LoxP system, knock-in and transgenic technologies, temporal and/or tissue specific mutagenesis, to delete or activate candidate

genes of interest and define their function within the skeleton. These studies provided significant insight into the role, molecular regulation, and cellular interplay of vascularization processes associated with bone development, growth, repair, homeostasis and disease. A central role emerged for the prime angiogenic mediator Vascular Endothelial Growth Factor (VEGF); the various isoforms of VEGF altogether mediate vascularization at all stages of skeletal development, play important roles in bone homeostasis and disease, and may have potential for therapeutic enhancement of fracture healing, as summarized in this review.

MECHANISMS OF VASCULARIZATION

The formation of a mature blood vessel network during fetal development occurs in several steps. Initially, endothelial cells differentiate from their mesodermal progenitors, angioblasts or hemangioblasts, and assemble to form a primitive vascular labyrinth. This process is termed vasculogenesis. The nascent vascular bed then further expands and remodels by angiogenesis, a term designating predominantly the mechanism of sprouting from pre-existing blood vessels, to form highly branched conduits supplying oxygen and nutrients to the rapidly expanding tissues of the embryo [5,6]. According to the current model of blood vessel branching morphogenesis during angiogenesis, distinct specialized endothelial cells termed 'tip', 'stalk' and 'phalanx' cells contribute to the formation of vessel sprouts towards an angiogenic stimulus. At the forefront of the sprout, an endothelial cell is selected and induced to become a tip cell leading the sprout and extending numerous filopodia into the tissue that is being invaded. The elongation of

the sprout relies on proliferation of endothelial stalk cells, which trail behind the tip. The new branch then connects with another branch via tip cell fusion to form a vascular lumen allowing initiation of blood flow and, hence, tissue oxygenation. Further down in the branch, endothelial phalanx cells with a stable quiescent phenotype sense and regulate perfusion in the vessel. Each of these endothelial cell phenotypes has a distinct molecular signature that is transiently and plastically specified by combinatorial signals and instructive cues driving vessel branching, including signaling via the Notch and VEGF pathways (see below) [5,6].

Establishment of a functional vascular network further requires maturation of newly formed vessels into durable vessels, with distinguishable arteries, veins and capillaries. This process, termed arteriogenesis, relies on recruitment of peri-endothelial cells that become organized around the endothelial vascular wall, generally involving pericytes for smaller vessels and smooth muscle cells for larger vessels. Stabilization of the nascent plexus is completed by deposition of extracellular matrix, induction of endothelial quiescence, and tightening of cellular junctions with high amounts of VE-cadherin in the established vasculature (see [5,6]).

Angiogenesis during adulthood is mostly associated with pathological conditions, such as cancer, wound healing and regeneration processes including fracture healing [5-7]. Several of the mechanisms mediating neovascularization in these conditions largely recapitulate those that occur during embryogenesis [5,6].

Little is currently known about the basic

structural features and the mechanisms mediating the establishment of the vascular system in bone. The functional importance of the vasculature for bone development and growth, though, is well recognized, particularly regarding endochondral bones. Early experiments showed that blocking the bone's blood supply resulted in reduced longitudinal growth [3,4]. Neovascularization processes clearly represent a crucial aspect of endochondral bone development: the cartilage intermediates that prefigure the long bones are intrinsically avascular, and their conversion into bone is indiscriminately coupled to the invasion by blood vessels. In an ex vivo model, it was shown that physical blockage of blood vessel invasion into cartilaginous fetal skeletal explants completely halted their development [8]. Neovascularization of cartilage appears to determine not only the initiation but also the cessation of longitudinal bone growth, as blood vessel invasion into the epiphyseal cartilages at the ends of the bone prompts the formation of the secondary ossification center, heralding the end of the longitudinal bone growth at puberty. The vasculature remains crucial for bone health through life; a decline in the performance of the blood vessel system may contribute to osteoporosis and failure to re-establish a functional blood supply to sites of bone defects is well known to critically hamper fracture healing.

Although much remains to be learned about the mechanisms of growth of the vascular beds associated with developing and postnatal bones, studies over the last decade have started to shed light on the molecular regulation by identifying VEGF as a prime player of vascularization in bone, in line with its crucial angiogenic function in many other tissues.

THE VEGF FAMILY: PROPERTIES AND EXPRESSION IN BONE CELLS

VEGF-A (referred to as VEGF) was discovered in the 1980's [9-11] and all subsequent work has increased its recognition as one of the most powerful and critical mediators of angiogenesis and vascular function. Firstly, VEGF is mitogenic for endothelial cells derived from arteries, veins, and lymphatics in vitro, and induces a strong angiogenic response in a variety of in vivo models. Secondly, VEGF acts as a survival factor for endothelial cells. Thirdly, VEGF is chemotactic for endothelial cells and increases endothelial expression of matrix-degrading proteases, allowing migration. Lastly, VEGF is powerful in influencing hemodynamics, enhancing vascular permeability and inducing vasodilatation (see [7,12] and references therein). The essential and strictly dose-dependent function of VEGF in physiological vasculogenesis and angiogenesis was exposed by the severe vascular defects and early embryonic lethality of mice with inactivation of even a single VEGF allele. VEGF also plays a central role in pathological angiogenesis associated with various diseases, including cancer; therapeutic targeting of tumor vessels by blocking VEGF has become an established anticancer strategy [5,12,6,7].

VEGF is a 46 kDa glycoprotein that belongs to a gene family that also includes a range of homologous molecules termed VEGF-B, C, D, E, and placental growth factor (PlGF). VEGF functions as a dimer, binding to and activating two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), which regulate both physiological and pathological

angiogenesis (Fig. 1). Despite the fact that VEGF binds to VEGFR-1 with ~10-fold higher affinity than VEGFR-2, it is mainly VEGFR-2 that mediates VEGF signaling in endothelial cells [7,13]. VEGFR-2 is expressed on endothelial tip, stalk and phalanx cells, but appears to induce distinct biological effects in these cells. In tip cells, VEGFR-2 (and the co-receptor neuropilin-1 (NRP1), see further) is abundant on filopodia, which extend into the direction of a VEGF gradient such as that emanating from hypoxic cells, and induces migration and vessel branching. In stalk cells, which are exposed to lower VEGF signaling, a mitogenic response is induced. The selected induction of single tip cells in a sprout exposed to VEGF is controlled by delta-like ligand 4 (Dll4)/Notch signaling between the tip cell and neighboring cells. High VEGF levels induce strong expression of Dll4 by the tip cell; Dll4 activates Notch in the neighboring cells, which results in downregulation of VEGFR-2, NRP1 and VEGFR-3, and upregulation of VEGFR-1 and soluble VEGFR-1 that may sequester VEGF and function as decoy receptors. These cells will consequently adopt a stalk phenotype because the VEGF-induced migratory response is minimized [5,6]. Once the new vessel branch is formed and perfused, tissue oxygenation lowers VEGF; maintenance of low VEGF levels secures survival of quiescent phalanx cells and vascular homeostasis [5,6].

Besides endothelial cells that are the primary targets of VEGF, several other cell types express VEGF receptors and respond to VEGF signaling [7,13]. For instance, VEGFR-1 and VEGFR-2 are expressed in monocytes, macrophages and hematopoietic stem cells; VEGF induces migration of monocytes

(primarily via VEGFR-1) and exerts a variety of effects on bone marrow cells and hematopoiesis [7,13,14]. Overall, VEGF can promote or modulate chemotaxis, proliferation, differentiation, survival and/or activity of a range of non-endothelial cell types, including osteoblasts and osteoclasts (see below). While in most circumstances VEGF functions as a paracrine mediator, autocrine and intracrine roles have been described as well, such as in the survival of hematopoietic stem cells (HSCs) and endothelial cells [14,15], and recently also in the lineage specification and differentiation of osteoblasts [16].

The *VEGF-A* gene, located on chromosome 6, consists of 8 exons that can be subject to a variety of differential splicing combinations (Fig. 1). Hence, the gene encodes multiple isoforms with variable exon contents and differential properties and expression patterns. At least seven alternative splicing variants ranging from 121 to 206 amino acids have been identified in humans; the most frequent and best-studied ones are VEGF121, VEGF165 and VEGF189 (the number representing the number of amino-acids in the protein). Exons 1 to 5 are present in all isoforms; these contain the motifs that bind to VEGFR-1 and VEGFR-2 [17,7]. Hence, all VEGF isoforms can bind VEGFR-1 and VEGFR-2 (Fig. 1). In contrast, VEGF165 but not VEGF121 contains the domains that mediate binding to the co-receptors NRP1 and NRP2. NRP1, known also as receptors for the semaphorin family implicated in neuronal guidance, modulates the activation of VEGFR-2 mediated signal transduction. When co-expressed with VEGFR-2, NRP1 enhances the binding of VEGF165 to VEGFR-2 and

increases VEGF165-mediated chemotaxis [17,7,13].

The differential splicing of the exons 6 and 7, that encode basic domains, also determines the affinity for heparin and bioavailability of the isoforms (Fig. 1). VEGF121 lacks both these domains and fails to bind heparan sulfate-containing proteoglycans on cell surfaces and in the extracellular matrix; this renders VEGF121 freely soluble and diffusible. The longest isoforms (VEGF189 and 206) contain both exons, bind heparin with high affinity, and are retained on the cell surface or sequestered in the matrix after their secretion. VEGF165 only lacks exon 6 and is less strongly bound to the matrix, resulting in intermediate properties: part of it diffuses, but a significant fraction remains matrix-bound [17,7]. These properties are highly relevant to the angiogenic functioning of VEGF, by defining its spatial distribution and local concentration. For instance, the diffusible isoforms can radiate further from the site of secretion (e.g. ischemic tissues) to reach responsive cells (Fig. 1). On the other hand, heparin-binding VEGF isoforms provide patterning cues that control spatially restricted blood vessel branching morphogenesis, by balancing tip and stalk cell behavior [18,5]. They may also constitute a reservoir of VEGF that can be released locally as bioactive fragments by proteolytic cleavage, for instance by plasmin or matrix metalloproteinases (MMPs) [7]. Finally, heparin binding can influence receptor binding characteristics and/or growth factor stability [7]. Differential splicing of the VEGF mRNA thus enables cells to produce a combination of various isoforms, the balance of which plays a crucial role in the regulation of angiogenesis. The relative expression levels of the VEGF isoforms are

tissue-specific, possibly reflecting the relative importance of specific isoforms in different biological functions.

Several cell types in the skeleton express VEGF, including chondrocytes, osteoblasts, and osteoclasts. Studies of its expression and function have extensively used mouse as a model. The murine *VEGF* gene is quite similar in size and structure to the human gene and encodes spliced isoforms that are shorter than their human orthologue by one amino acid; the three major variants are consequently denoted as VEGF120, VEGF164 and VEGF188. In bone, the most dominant isoform appears to be VEGF164, followed by VEGF120; VEGF188 is expressed at very low levels [19]. While the prime function of VEGF is to induce angiogenesis, its actions are not exclusive to endothelial cells. In the bone environment, mesenchymal progenitor cells and osteoblasts, as well as osteoclasts and their monocyte precursors, express VEGF receptors and respond to VEGF signaling by enhanced recruitment, differentiation, activity and/or survival (reviewed in [20]). In addition, an intracrine function for VEGF in osteoblast lineage cells was recently described, determining lineage specification (osteoblastic versus adipocytic) [16]. These pleiotropic effects of VEGF may be key to its coordinative role in the orchestration of complex biological processes in skeletogenesis and bone homeostasis. Detailed elucidation of the in vivo significance of these nonvascular effects of VEGF is however difficult, given the challenges of uncoupling its angiogenic effects from direct responses of the bone cells proper.

In general as well as in the skeleton, the actions of VEGF are highly dose-dependent and its

physiological levels must be under very strict control mechanisms. Although the fine details of the regulation of VEGF expression in the skeleton in vivo are still largely unresolved and may vary depending on the developmental stage and specific location and cell type, several mechanisms have been implicated to date; these include hypoxia signaling [21] and transcriptional activity by Runx2 [22] and SP7/Osterix (Osx) [23]. Also a variety of osteomodulating hormones (e.g. parathyroid hormone (PTH), growth hormone, 1,25(OH)2D3) and locally produced growth factors (e.g. fibroblast growth factors (FGFs), transforming growth factor- β (TGF- β), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF)) can regulate VEGF expression, at least in vitro (see [20]).

The physiological importance of VEGF-mediated angiogenesis in bone growth was first shown by the partial inhibition of VEGF activity in juvenile (24-day-old) mice, via administration of a soluble VEGFR-1 chimeric protein (mFlt (1-3)-IgG or sFlt-1). Vascular invasion of the growth plate became impaired, associated with inhibition of endochondral ossification [24]. Further studies using a variety of genetically modified mice [25,19,26-29] univocally established that VEGF and its isoforms act as key inducers of the fundamental skeletal vascularization events and as direct modulators of bone development, thereby synchronizing vascular and nonvascular aspects of the complex tissue morphogenesis program. In the next few sections, the data on the role and regulation of angiogenesis over the course of bone development and growth will be reviewed.

NEOVASCULARIZATION OF CARTILAGE: KEY INDUCER OF THE CARTILAGE-TO-BONE CONVERSION IN SKELETAL DEVELOPMENT

Cartilage neovascularization processes during endochondral bone development

The conversion of avascular cartilage anlagen into highly vascularized bone and marrow tissues is initially triggered by the invasion of blood vessels into the hypertrophic chondrocyte core of fetal skeletal elements (around E14 in mouse limbs) (Fig. 2, left). These vessels come from the soft tissue surrounding the cartilage, the perichondrium, where they accumulated adjacent to the central diaphyseal portion of the bone model. Neovascularization of the cartilage intermediate is associated with its decay, by coinciding apoptosis of terminally differentiated chondrocytes, matrix digestion by proteolytic enzyme activity, and resorption of calcified cartilage spicules by invading osteoclasts or chondroclasts. These processes lead to the generation of a cavity that rapidly becomes the site of bone formation as osteoblasts populate the region: the ‘primary ossification center’ [1,2].

At the proximal and distal edges of this cavity, the chondro-osseous junctions remain sites of continual replacement of cartilage with metaphyseal bone (Fig. 2, middle), the basic cellular mechanisms of which largely reiterate those mediating the earlier events: the hypertrophic chondrocyte zone of the growth plate is invaded by capillaries progressively emanating from the metaphyseal and nutrient arteries, and are eradicated. The leftover calcified cartilage spicules function as scaffolds for osteoblasts depositing mineralized bone

matrix, establishing the spongiform trabecular bone network inside the shafts of the long bones. This ‘primary spongiosa’ first consists of woven bone and is subsequently remodeled and strengthened into the lamellar ‘secondary spongiosa’ by the actions of both osteoclasts and osteoblasts [1,2].

Thus, both the initial formation of the primary ossification center and progressive endochondral bone formation at the growth plate involve rigorous coupling of (i) chondrocyte maturation, (ii) cartilage neovascularization, (iii) osteoclast recruitment and matrix remodeling, and (iv) osteoblast differentiation and activity. Considerable progress has been made in elucidating the complex molecular crosstalk regulating these cellular interactions and their temporal-spatial sequence in skeletal development.

Matrix-binding VEGF isoforms couple cartilage neovascularization to its resorption during endochondral bone development

Different stages of cartilage neovascularization may well involve different vessel systems and mechanisms of vascular growth, but vascular invasion of cartilage is at all times preceded by chondrocyte hypertrophy. Hypertrophic chondrocytes produce several angiogenic stimulators, including VEGF [30]. VEGF inhibition in fetal limb explants developing ex vivo (under the kidney capsule of adult mice) completely blocked the initial neovascularization of the cartilaginous bone anlagen [8]. In vivo, administration of sFlt-1 to juvenile mice impaired vascular invasion of the growth plate and led to enlarged hypertrophic chondrocyte zones and reduced trabecular bone formation [24]. Thus, hypertrophic cartilage favors, in a VEGF-dependent way, its own

vascular invasion and decay and the replacement by bone. This action of VEGF, like many others, is highly dose-dependent. Deletion of a single floxed *Vegfa* allele using Col2-Cre mice led to an increased hypertrophic chondrocyte growth plate zone and reduced bone lengthening in the heterozygous conditional mutants [25], resembling the phenotype obtained by decreasing the levels of VEGF protein through sequestration [24]. While these studies provided proof that threshold levels of VEGF are essential to mediate vascular invasion of growth plate cartilage, subsequent genetic models established that specific isoforms of VEGF function in this process [19,26,28]. Mice engineered to express only a single VEGF isoform, quantitatively matching the level of the overall VEGF expression (all three isoforms) in control littermates, revealed that neither VEGF120 nor VEGF188 alone could provide the signals required for timely and adequate cartilage neovascularization and bone growth [19,26,28]. Mice expressing selectively the VEGF164 isoform (VEGF164/164 mice, carrying two alleles of a knock-in construct replacing the endogenous VEGF gene by the VEGF164 cDNA sequence) could not be discriminated from their wild-type littermates at any stage [26]. This is in line with the idea that the specific combination of properties of VEGF164 endows this isoform with the most optimal characteristics of bioavailability and biological potency [7]. In contrast, the initial neovascularization of the cartilage anlagen and establishment of the primary ossification center was delayed in VEGF120/120 embryos (expressing exclusively the soluble isoform VEGF120) and in VEGF188/188 embryos (in which only the matrix-bound isoform

VEGF188 is produced). Newborn VEGF188/188 mice showed no defects in the blood vessel invasion at the growth plate, in the resorption of the hypertrophic cartilage, or in the formation of mineralized bone in the metaphysis [26]. These features were, however, all significantly impaired in perinatal VEGF120/120 mice [19,28], similar to the models of decreased overall VEGF [24,25]. Altogether, these findings thus imply that vascular invasion of hypertrophic cartilage at both an early and later stage of endochondral ossification requires sufficiently high levels of the matrix-binding isoforms VEGF164 and VEGF188, functioning in specific ways that VEGF120 cannot compensate for.

Similar to the phenotype seen in models of partial VEGF deficiency or lack of the heparin-binding VEGF isoforms [19,28,25,24], mice lacking MMP-9 showed a lengthened hypertrophic cartilage zone, impaired vascularization at the growth plate and delayed ossification [31]. MMP-9 is produced by osteoclasts at the chondro-osseous junction and involved in the degradation of cartilage components [31,32]. The model thus appeared that upon the abundant secretion of VEGF by hypertrophic chondrocytes, the longer VEGF isoforms bind to the heparan sulfate-rich matrix and become sequestered in the cartilage. One such heparan sulfate proteoglycan may be perlecan, as blood vessel invasion was blocked in mice lacking perlecan despite upregulated expression of VEGF [33]. Trapped VEGF is released during cartilage resorption, when extracellular matrix molecules are degraded by proteases such as MMP-9, providing a system of regulated VEGF bioavailability concentrated in gradients emanating from the terminal

hypertrophic cartilage (Fig. 2). Hence, this model can explain the directional recruitment of the expanding vasculature towards the border of the growth plate, with vessels running longitudinally along the cartilage septae of the chondrocyte columns. The soluble, randomly diffusing VEGF120 isoform by itself cannot create the correct directional cues along which angiogenesis is induced; in bone and other organs, capillary growth in the presence of only the VEGF120 isoform leads to tortuous, dilated and disorganized vessels [34,19,18].

The model that mechanisms of matrix-binding and extracellular proteolysis regulate the VEGF activity reflects the tight coordination and functional interdependence of cartilage neovascularization and resorption during endochondral ossification. While MMP-9 action is required for the release of VEGF from the cartilage matrix, VEGF conversely is important to attract the osteoclastic resorptive cells that express MMP-9, both during the formation of the primary ossification center and during growth plate ossification [35,24,19]. A question thus remains as to how these cells are initially recruited. Likely, osteoclast precursors, mononucleated cells of hematopoietic origin, are brought into the region of neovascularization (the perichondrium and the chondro-osseous junction at the early and late stages, respectively) via the blood stream. The cells can be locally stimulated to differentiate to mature multinucleated osteo(chondro)clasts, possibly under the influence of RANKL produced by osteoblast lineage cells in the perichondrium and/or by hypertrophic chondrocytes in the growth cartilage [36,37], as well as via VEGF itself, as VEGF can also regulate osteoclastogenesis via nonvascular,

direct actions [38-40]. Similar to monocytes/macrophages, derived from common progenitors, osteoclasts predominantly express VEGFR-1 and are chemo-attracted to VEGF. VEGF can also directly enhance the resorption activity and survival of mature osteoclasts, and regulate osteoclastic differentiation and function [20].

Of note, other cell types than osteoclasts likely contribute to cartilage degradation during endochondral ossification. MMP-9 is not exclusively expressed by osteoclasts, and MMP-13 produced by hypertrophic chondrocytes and osteoblasts also plays a crucial role in cartilage turnover during endochondral bone growth [41,32]. The vasculature itself may be a prominent candidate to participate in the process of cartilage turnover. Endothelial cells express resorptive enzymes and have been suggested to direct apoptotic signals to terminal hypertrophic chondrocytes [7,30]. Perivascular cells termed 'septoclasts' may locate cartilage-degradative activity to the vascular invasion front [42,43]. Experiments blocking osteoclastogenesis in vitro and in vivo indicated that osteoclasts themselves are not crucially needed for the initial vascular invasion of cartilage and primary ossification cavity formation [44]. Rather, as the only cell type capable of resorbing calcified tissues, osteoclasts are necessary to remodel the nascent woven bone and remove the scaffolding calcified cartilage. During longitudinal bone growth, resorption of the transversal septae of calcified cartilage is requisite to the gradual transposing of the chondro-osseous junction, extending the length of the diaphyseal bone shaft; the vertical calcified cartilage spicules on the other hand, are incompletely resorbed, leaving remnants

onto which osteoblasts adhere and deposit bone. Several mouse models containing mutations in genes that affect osteoclast development consequently present with defective growth plate cartilage turnover and osteopetrosis [45,46] [47].

Initiation of neovascularization in early skeletogenesis

It is not fully clear what signal provides the initial trigger that launches the neovascularization of developing bones. Genetic studies in mice have revealed that several mutations affecting either fetal chondrocyte differentiation, perichondrial development and/or early osteoblastogenesis, are associated with impaired neovascularization of the cartilage anlagen and a failure to timely develop the primary ossification center. Examples include mice with either local or complete inactivation of *Ihh*, *Runx2*, *Osx*, or β -catenin (see below) [48-53]. Although it remains difficult to judge the specific contributions of each of these aspects to the vascular process – for instance given the fact that VEGF expression itself is affected in several if not all of these mutants – it does appear that the initial invasion only takes place provided that a permissive environment has been established, requiring correctly orchestrated developmental progression of chondrocyte differentiation and perichondrial osteoblast differentiation. This is regulated by a tightly coordinated molecular crosstalk between the cell types involved, the details of which are only beginning to be unraveled (Fig. 2).

A key role is played by Indian hedgehog (*Ihh*) [2,54]. *Ihh* is produced by the pre-hypertrophic chondrocytes of the fetal cartilage, and signals through the receptor Patched (*Ptc*) in adjacent

chondrocytic and perichondrial domains. Via a negative feedback signaling pathway with parathyroid hormone-related peptide (PTHrP), *Ihh* regulates the pace of cellular differentiation in the growth plate [2]. In addition, *Ihh* regulates osteoblastogenesis and bone collar formation in the perichondrium. Perichondrial cells are thought to be bipotential osteochondroprogenitors; *Runx2*, *Osx*, and canonical, β -catenin-mediated Wnt signaling are essential for the commitment of the mesenchymal precursor cells to the osteoblastic lineage [55] (Fig. 2). Committed osteoprogenitors or pre-osteoblasts differentiate into mature osteoblasts, increasingly expressing bone matrix proteins including collagen type I (*Col1*), osteopontin and osteocalcin. The mature osteoblasts deposit and mineralize a bony structure around the cartilage mold, the ‘bone collar’ (Fig. 2), which forms the initiation site of the later cortical bone. *Ihh* drives cells in the perichondrium into the osteoblast lineage by stimulating the expression of *Runx2*, the master transcriptional controller of osteoblast differentiation [56,57,53]. As such, *Ihh* coordinates the temporal-spatial coupling of hypertrophic chondrocyte maturation with perichondrial development, events that precede and are requisite for vascular invasion of the cartilage. In *Ihh*^{-/-} mice, no bone collar formed; concomitantly, invasion of the cartilaginous rudiment was impaired [53]. Conversely, experiments in which *Ihh* was genetically misexpressed in cartilage showed ectopic bone collar fragments being formed near sites of ectopic *Ihh* production, which was associated with ectopic vascular invasion at multiple sites along the immature cartilage [56,57]. Ex vivo cultured limb explants from which the perichondrium had been removed, did not develop a primary ossification center [8].

Runx2^{-/-} and Osx^{-/-} mice [50-52] and β -catenin conditional knockouts [48,49] showing a block in perichondrial osteoblastogenesis, also display a near-complete absence of vascular invasion and primary ossification. It remains however difficult to discern the specific role of correct bone collar establishment, given that these osteoblastogenic factors function in chondrocytes as well and transcriptionally regulate VEGF [22,23]. For instance, in Runx2^{-/-} mice hypertrophic chondrocyte differentiation is impaired [22] and the vascularization defect can be rescued by forced re-expression of Runx2 in cartilage [58,55].

Altogether, these studies have begun to shed light on the intertwined molecular regulation of hypertrophic chondrocyte differentiation and osteoblastogenesis, coupled to VEGF induction and cartilage resorption (Fig. 2), processes that all act in concert to guide the vasculature from the perichondrium into the nascent shaft, leading to primary ossification.

Neovascularization of endochondral bones is associated with ossification

Neovascularization of cartilage is associated with the formation of a central cavity that quickly becomes the scene for osteoblastic deposition of mineralized bone. Recent data shed light on some of the cellular aspects related to the osteogenic cell population of the primary ossification center [44,8]. Using transgenic mouse models designed for lineage tracing of stage-selective osteogenic cells, visualizing these cells in situ during skeletal development, it was found that osteoprogenitors (characterized by the early expression of Osx) formed in the perichondrium, moved into the nascent primary ossification center along with neovascularization and decay of the cartilage

template [44]. The cells subsequently differentiated inside the bone center into mature, trabecular bone-forming osteoblasts, or remained to contribute to the immature osteogenic stroma. In contrast, osteoblasts that differentiated to osteoid matrix-producing cells (characterized by Coll expression) already while residing in the perichondrium did not migrate into the bone to become trabecular osteoblasts. Instead, these cells generated the outer cortical bone. Thus, cells originating in the fetal perichondrium provide the basis for the bone-forming osteoblasts inside the bone shaft of endochondral bones; and, the capacity of osteogenic cells to translocate to (initiate novel) sites of ossification appears specific to cells in an early differentiation stage in vivo. Intriguingly, throughout the translocation of osteoprogenitors from the perichondrium into the nascent bone center, they intimately associated with the cartilage-invading blood vessels, being wrapped as pericytes around the endothelium or co-migrating along with the neovascularization front. A similar co-invasion of osteoprogenitors and angiogenic blood vessels was observed during endochondral healing of bone fractures [44]. The coinciding movement of perichondrial osteoprogenitors and blood vessels into developing bones may possibly be explained by common chemo-attractants, emanating from the degrading late-hypertrophic cartilage matrix and directionally steering the osteo-angiogenic co-invasion. Candidate secreted growth factors may include VEGF, PDGF, PlGF, IGFs, and TGF- β , all reported to be chemotactic for osteoblasts [59-61]. Alternatively and/or additionally, the key to the tight coupling of angiogenesis and osteogenesis may lie within the population of perivascular osteoprogenitors. Perivascular cells

have the potential to differentiate into functional osteoblasts and may be representatives of a skeletal stem or progenitor cell population [62-65]. Association of osteoprogenitors and blood vessels may thus offer a cellular explanation for the coupling of (neo)-vascularization and ossification, in developing bones as well as beyond the fetal stages [44].

RESISTING NEOVASCULARIZATION: IMPLICATIONS OF CARTILAGE DEVELOPMENT AND GROWTH AS AVASCULAR TISSUE

As discussed above, neovascularization of cartilage triggers the switch towards its decay and replacement by bone tissue, built onto the intermediary matrix laid down by chondrocytes. In order for the scaffolding cartilage to obtain the shape and size to outline the specific bone that it prefigures, it must therefore remain avascular during its modeling and growth. How does cartilage manage to retain its avascular status and at the same time safeguard in these challenging conditions the chondrocytes' capacity to proliferate, differentiate, and produce large amounts of matrix to drive bone growth? The regulatory mechanisms that provide answers to this question are beginning to be revealed; while perhaps seemingly contradictory, VEGF has essential roles in these aspects of endochondral bone development as well.

Vascular rearrangements in the limb bud

During the initial skeletal patterning stages in embryogenesis, the precise arrangement of the individual anatomic elements is defined. At the sites where long bones will be formed,

mesenchymal progenitors aggregate and form high cell density condensations. In the mesenchymal condensations, cells differentiate into chondrocytes under the control of the transcription factor Sox9 and start to synthesize an extracellular matrix rich in collagen type II and specific proteoglycans [1]. Vascular patterning during these early stages of limb development is characterized by the formation of a uniform vascular plexus in the limb bud mesenchyme, likely involving mechanisms of vasculogenesis and angiogenesis. This vascular plexus undergoes major rearrangements at the start of skeletogenesis, into a highly branched and patterned network that is segregated from the forming cartilaginous condensations [29,66]. Hence, the condensations emerge as avascular areas from previously vascularized regions as a result of vessel regression from the emerging cartilage anlage. Surprisingly, this process is regulated at least in part by VEGF, expressed by the developing limb bud mesenchyme in early skeletogenesis [29,66]. VEGF appears to act in this process via a long-ranging mechanism (without involvement of receptor signaling in the condensations proper), to mediate the vascular rearrangement in the surrounding tissue [29]. It is therefore conceivable that the diffusion properties of the various VEGF isoforms have an impact on the patterning of the vascular system in the developing limb.

Angiogenic inhibition in non-hypertrophic, immature cartilage

As the mesenchymal condensations develop and differentiate into cartilage, the tissue remains strictly avascular. This is inherent to cartilage, also seen in the later fetal and postnatal growth plate. The concept that explains this is that

immature chondrocytes produce angiogenic inhibitors that render them resistant to vascular invasion. Indeed, a variety of anti-angiogenic factors were found to be secreted by chondrocytes in vitro and/or in specific differentiation zones of the growth plate, including thrombospondin, troponin, chondromodulin-I (ChM-I), tenomodulin, tissue-localized inhibitors of MMPs (TIMPs), and others [67,30]. Such molecules are of substantial therapeutic interest as inhibitors of angiogenesis in cancer and other diseases. The physiological importance of these individual molecules in endochondral bone development is, however, not very clear. For instance, ChM-I deficient mice are viable and show no overt abnormalities in skeletal vascularization [68]. Possibly, redundancy among angiogenic inhibitors and compensatory mechanisms may explain why phenotypic manifestations have been minimal in loss-of-function studies to date. In addition to the expression of anti-angiogenic molecules, also (a low level of) VEGF expression was noted in the immature chondrocytes of the growth cartilage [26,27]. Likely, this complex and temporally regulated balance between angiogenic inhibition and stimulation organizes the delicate vascular rearrangements and selective spatial growth of the vascular beds in the developing endochondral skeleton.

Vascular supply of epiphyseal cartilage and invasion initiating secondary ossification

As a result of their persistent avascular status, the cartilaginous anlagen and the fetal growth plates of developing bones strictly rely on the surrounding vasculature for gas exchange, adequate nutrient supply and efficient removal of waste products. Naturally, expansion of these

growing and highly active cellular structures must be coupled to the expansion of the supplying vascular network. An elaborate epiphyseal vascular network overlaying the cartilaginous surface expands on the surface of the cartilage mass and is critically required for chondrocyte survival, proliferation and differentiation [4]. In early postnatal life, vessels derived from this vascular network invade the epiphyseal cartilage and initiate the formation of the secondary ossification center [69]. As a result, discrete layers of residual chondrocytes form true growth ‘plates’ at each end of the long bones, mediating further longitudinal bone growth until the end of adolescence, when the growth plates close and growth stops [2,1]. Epiphyseal vascularization and the establishment of the secondary centers of ossification of long bones are much less characterized than the sequence of events leading to primary ossification center formation. Soluble VEGF isoforms and membrane-type 1 (MT1)-MMP are among the few known molecular signals implicated in these processes (Fig. 2, right).

VEGF188/188 mice lacking soluble VEGF displayed impaired vascularization surrounding the epiphysis, associated with aberrant hypoxia within the cartilage and massive apoptosis of chondrocytes located in the center of the tissue, i.e. those located furthest from the blood vessels overlaying the surface of the cartilage [26]. Vascular defects and central chondrocyte death was also observed in mice lacking VEGF (all isoforms) conditionally in cartilage [70], implicating that chondrocytes themselves instruct the expansion of the epiphyseal vascular network in coordination with the growth of the avascular cartilage mass. Soluble VEGF isoforms are critically required in this

function, presumably due to their ability to diffuse to the surface of the cartilage [26]. Thus, chondrocyte-secreted VEGF stimulates vascularization in the perichondrial tissues surrounding the avascular cartilage, in order to ensure sufficient oxygen supply to support chondrocyte survival (Fig. 2). A direct survival role of VEGF in chondrocytes could furthermore contribute [26,27].

Perhaps as a result of inadequate epiphyseal vascular development, the formation of the secondary ossification centers was impaired in the VEGF188/188 mice. While matrix remodeling is a spatial prerequisite allowing the tissue-penetration of blood vessels during angiogenesis, the molecular control is region-specific. Neovascularization and turnover of cartilage is heavily dependent on MMP activity to degrade collagens, aggrecan and other matrix constituents, but different MMP family members are required at different developmental stages and locations [32]. MMP-9 and MMP-13 are of particular importance during primary ossification and bone growth (see before), whereas MT1-MMP plays a role in epiphyseal vascular invasion and formation of the secondary ossification centers [71]. MT1-MMP can be produced by osteoclasts, chondrocytes, osteoblasts and endothelial cells, and cleaves collagens type I and II, aggrecan, gelatin, fibronectin and laminin, as well as some other MMP's, activating for instance MMP-2 and MMP-13 [32]. The detailed relationship between the angiogenic and resorptive signals in secondary ossification and the precise mechanisms governing this process still need to be clarified.

Survival of chondrocytes in the hypoxic environment of cartilage

From the above, one may assume that the VEGF levels in cartilage are closely coordinated with the need for vascular expansion in the perichondrium; this appears to be regulated by cellular oxygen-sensing mechanisms. As the cartilage mass grows progressively bigger during development, oxygen diffusion fails to reach the deepest cell layers and supply the centrally located chondrocytes. Particularly these cells experience hypoxia [72]. Hypoxia is known generally as a major inducer of VEGF expression, by mechanisms involving at least in part the actions of hypoxia-inducible factor (HIF) [73]; this is also true for chondrocytes, osteoblasts, and osteoclasts [74,20,75,76]. HIF-1, a heterodimeric transcription factor consisting of an α and β subunit, is the main mediator of the cellular adaptation responses to hypoxia [73]. HIF-1 β is constitutively expressed, whereas HIF-1 α is regulated according to the oxygen tension: when sufficient oxygen is available, and the hypoxia-adaptive actions of HIF-1 are unnecessary, the HIF-1 α protein is degraded. This is mediated through the oxygen-dependent hydroxylation of HIF-1 α by a family of enzymes termed prolyl 4-hydroxylases (PHDs), which makes HIF-1 α a target for the E3 ubiquitin ligase Von Hippel-Lindau (VHL). VHL binds HIF-1 α and directs its proteasomal degradation. When oxygen is sparse, the HIF-1 α protein is not hydroxylated and thus protected from VHL-mediated degradation. As a consequence, in hypoxic conditions HIF-1 α can translocate to the nucleus, heterodimerize with HIF-1 β and associate with other co-factors to initiate its transcriptional program. This program is composed of a variety of pathways, the main ones mediating (i) the activation of anaerobic

glycolysis, an oxygen-sparing measure as compared with glucose oxidation via mitochondrial respiration, and (ii) the induction of angiogenesis and erythropoiesis (e.g. via VEGF and EPO, both direct downstream targets of HIF-1), thus enhancing the effectiveness of oxygen delivery to the hypoxic tissue [73].

In the avascular cartilage of developing endochondral bones, both these actions of HIF-1 α are of crucial importance to ensure the survival of the chondrocytes. Conditional deletion of HIF-1 α in limb bud mesenchyme or in chondrocytes was shown to lead to massive cell death of the centrally located chondrocytes [72,77]. A similar phenotype was observed upon inactivation of (diffusible) VEGF [26,27]. In each of these mutants, the areas of cell death corresponded to the inner, most hypoxic regions of the fetal growth plates. This suggested that HIF-1 α might mediate chondrocyte survival via its transcriptional effect on VEGF. Hypoxia indeed strongly increases VEGF accumulation in chondrocytes, which appears to occur primarily in a HIF-1 α -dependent manner, at least in vitro [74]. Yet, the cell death phenotype of cartilage lacking HIF-1 α could only be partially rescued by transgenic expression of VEGF [70]. The partial rescue phenotype underscored that chondrocyte-secreted VEGF primarily stimulates vascularization around the cartilage, ensuring sufficient oxygen diffusion to limit the degree of hypoxia experienced by the central chondrocytes. In addition, it showed that HIF-1 α is also required in hypoxic chondrocytes to switch on genes involved in anaerobic metabolism, independently of VEGF. In order to survive and differentiate in their avascular environment, chondrocytes need to adapt their cellular metabolism to the limited availability of oxygen; oxygen-sparing

glycolytic pathways still allow them to generate sufficient levels of energy (ATP) for survival [78,70]. Thus, the HIF-VEGF duo is vital in chondrocytes because of their roles in balancing oxygen supply and consumption (Fig. 2).

ANGIOGENIC-OSTEOGENIC- HEMATOPOIETIC COUPLING IN POSTNATAL BONE

Angiogenic-osteogenic coupling: regulation by HIF/VEGF

Adult maintenance of bone is regulated by the continual remodeling of existing bone into newer bone, through the balanced actions of osteoclasts and osteoblasts. Bone remodeling compartments exist in close physical proximity with blood vessels [79,80]. As during development, the vascular system contributes essential functions to bone homeostasis in the adult; these obviously include its intrinsic function to supply oxygen, nutrients and growth factors/hormones to the bone cells. The blood vessels likely also serve to bring in precursors of osteoclasts, to remove end products of the resorption processes, and to mediate the entry of osteoblast progenitors to bone formation sites [1,44] (Fig. 3). The close spatial and temporal association of bone formation with vascularization of the ossified tissue has been acknowledged in a wide diversity of settings – developmental, homeostatic, pathological and orthopedic – and is nowadays conceptually recognized as ‘angiogenic-osteogenic coupling’ [81,76,21].

Few molecular regulators of angiogenic-osteogenic coupling have been identified to date; the group of Thomas Clemens first exposed the prominent role played by

components of the hypoxia-signaling pathway [82]. Manipulation of HIF in fully differentiated osteoblasts (using osteocalcin-Cre-driven conditional knockout mice) caused changes in the bone volume that were directly proportional to changes in the degree of vascularization: mice with osteoblast-specific deletion of VHL, considered a model of HIF gain-of-function by inhibiting the protein's oxygen-dependent degradation, expressed high levels of VEGF and developed extremely dense, heavily vascularized long bones. Conversely, inactivation of HIF-1 α led to reductions in the bone and blood vessel volume [82]. Thus, osteoblasts emerged as important regulators of the coupling by sensing oxygen tension and directing adjustments in skeletal vascularization to meet their demands for optimal ossification [81,76].

Elucidating further the molecular regulation of angiogenic-osteogenic coupling bears therapeutic potential for bone diseases and bone regeneration. A recent study explored the consequences of increased VEGF in adult bone, using a genetic model of inducible, skeletal-specific transgenic VEGF164 overexpression [83]. Underscoring its role in angiogenic-osteogenic coupling, temporal VEGF gain-of-function induced a combined stimulatory response of hyper-vascularization and increased bone formation. However, the effects quickly turned pathological as VEGF164 induction for only two weeks resulted in severe osteosclerosis, bone marrow fibrosis and hematological anomalies including mobilization of hematopoietic cells to the circulation and spleen, altogether resembling myelofibrosis-associated bone disease [83]. These findings suggest that altering the tightly coupled processes of angiogenesis and osteogenesis can be associated

with hematologic alterations, possibly reflecting disturbances in the hematopoietic niches that exist in adult bone and marrow.

Hematopoiesis and the vascular and osteogenic niches

Like bone remodeling, also hematopoiesis takes place in the bone marrow cavity in postnatal life, standing in critical interplay with the highly vascularized local microenvironment (Fig. 3). Two specialized compartments of the bone marrow stroma have gained attention for their roles in maintaining hematopoietic homeostasis, the 'vascular and osteoblastic niches' (reviewed in [84,85]).

The vascular niche constitutes a crucial aspect of multi-lineage blood cell homeostasis by providing the principle line of exchange of hematopoietic cells between the site of their maturation in the marrow and the systemic circulation. But the bone marrow vasculature with its specific sinusoidal vessels also provides crucial support for HSCs. The most purified HSCs, fractionated as CD150(+)CD48(-)CD41(-)Lin(-) cells, were found in majority to be associated with the sinusoidal endothelium lining of the marrow vessels [86]. Co-culture studies supported a role of endothelial cells in maintaining HSCs, but endothelial cells isolated from different tissues appeared to possess differing HSC supportive abilities. The specific properties and signatures of endothelial cells in the bone marrow vasculature have only been partly characterized, but it appears that these cells display a unique spectrum of cell adhesion molecules under homeostatic conditions, including E-selectin, P-selectin, VCAM1, and ICAM1, that in other endothelial cells only appear during inflammatory responses [84,85]. In addition,

regions of the bone marrow vasculature produce factors important for mobilization, and homing of HSCs, such as CXCL12 (also known as stromal cell-derived factor (SDF)-1, ligand for the CXCR4 receptor expressed on HSCs) [87], and (Akt-activated) endothelial cells release a specific set of stem cell active paracrine growth factors, known as angiocrine factors, like FGF-2, BMP-4, and angiopoietin-1 [88]. Further research will be required to elucidate the molecular mechanisms regulating the interaction between the endothelial cells of the bone marrow vasculature and the hematopoietic cells; currently, the view is that the vascular niche promotes proliferation, differentiation of actively cycling, and short-term HSCs [84,85]. Recent studies suggest that in addition to the sinusoidal endothelium, primitive mesenchymal cells residing in perivascular locations thereof, including CXCL12-abundant reticular (CAR) cells, CD146(+) cells, and Nestin-expressing cells, may act as niches for HSCs [89,90,64]. These pericytic cells have the ability to differentiate into osteoblasts, and therefore may link the vascular and osteoblastic niches, and/or couple angiogenesis to osteoblastogenesis by providing a reserve pool of osteoprogenitors or mesenchymal stem cells associated with the vasculature.

Whereas the vascular niche provides an environment for short-term HSC proliferation and differentiation, the osteoblastic niche, anatomically positioned at the endosteal bone surface [91,92], is recognized as a reservoir for long-term storage of quiescent or slow-cycling HSCs. Correspondingly, only a small number of HSCs reside in the endosteal niche, compared with the vascular niche [85]. The functional importance of osteoblasts to form a niche for

HSCs was shown by *in vivo* studies modulating the osteoblast number, revealing a direct role in controlling the number of HSCs [93,94]. Osteoblasts express several cell-signaling molecules such as BMP-4, Jagged-1 (interacting with Notch-1 on hematopoietic precursors) and angiopoietin-1 (interacting with the receptor tyrosine kinase Tie-2, expressed by HSCs), which are important for HSC self-renewal, survival, maintenance and fate [84].

Several factors have been identified to regulate HSCs homing and lodging in the endosteal osteoblastic niche; osteoblasts influence the adhesive characteristics of the niche and interaction with HSCs via growth factor-dependent as well as contact-dependent mechanisms, including cell-cell and cell-extracellular matrix interactions (reviewed in [84]). For instance, the osteoblastic matrix glycoprotein osteopontin supports the adhesion of HSCs to the osteoblastic niche. Osteoblasts also produce membrane-bound stem cell factor (SCF), which binds and activates c-KIT that is highly expressed by HSCs and mediates adhesion, migration and differentiation of hematopoietic cells. HSCs may lodge in the endosteum by direct attachment to osteoblasts on the bone surface expressing a high level of N-Cadherin, termed 'spindle-shaped N-cadherin+CD45- osteoblastic cells' (SNO) [94], although the importance of this mechanism is still a matter of debate given that hematopoiesis appears normal in (conditional) N-cadherin-deficient mice [95] [96,97].

Despite the abundant vascularization in the medullary cavity, the Haversian canals of the cortical bone, and the periosteum, the bone environment is relatively hypoxic. The oxygen level decreases gradually towards the

endosteum, as a result of the relative distance from the central marrow vasculature or due to low blood perfusion. HSCs appear well adapted to reside and survive in a hypoxic state, particular the quiescent, long-term HSCs that generate energy mainly via anaerobic metabolism (glycolysis) [98]. Hypoxia signaling components and VEGF function in several aspects of HSC maintenance and hematopoiesis, both intrinsically and indirectly via the vascular and osteoblastic compartments in bone (see [7,13,21] and references therein); for instance, VEGF supports HSC survival via autocrine intracellular signaling [14], and HIF-1 α regulates erythropoiesis by inducing expression of EPO in osteoprogenitors [99].

Although much remains to be learned about the interplay and molecular cross talk between the vascular, osteogenic and hematopoietic compartments in adult bones, it is clear that these insights will have important therapeutic implications in several hematologic malignancies. For instance, an increase in vessel density in the marrow of patients with leukemia was reported, suggesting that activation of the vascular niche may contribute to disease progression [84]. Moreover, studies in mice indicated that altered (osteoblastic/stromal) microenvironment signaling can lead to myeloid lineage diseases: perturbations in the retinoblastoma gene induced a myeloproliferative disorder [100], inactivation of Dicer in osteoprogenitors caused myelodysplasia [101], and targeting environmental Wnt signaling affected the pathogenesis of myeloma [102]. Emerging evidence also supports the influence of the microenvironment on chemotherapy and drug resistance [84].

THERAPEUTIC POTENTIAL OF MODULATING VASCULARIZATION IN SKELETAL REGENERATION

Upon fracture of bone, the blood flow at the site of the injury is disrupted, quickly leading to blood clotting and hematoma formation. As a consequence of the vascular damage, the fracture site becomes hypoxic, likely triggering expression of VEGF and other angiogenic factors. VEGF is strongly increased locally in the fracture hematoma and is also systemically elevated in injured patients [103,104]. The reestablishment of the vascular supply of the healing tissue is crucial to the success of the repair process ([105,104]). Failure to restore the vascular network delays or impairs bone healing, as prevalently associated with advanced age and disorders such as diabetes. The presence or absence of a functional vascular bed may determine mesenchymal stem cell fate decisions, such as the differentiation along the chondrocytic or osteoblastic lineages. Moreover, the vasculature can provide a conduit for entry of osteoprogenitor cells in the callus [44,63,106].

Fracture repair in the adult closely resembles bone development and recapitulates several of the main molecular pathways operating in fetal life [107,105]. The regulatory genes controlling embryonic chondrogenesis (e.g. *Ihh*, *PTHrP*), osteoclastogenesis and matrix resorption (e.g. *MMPs*, osteoprotegerin (*OPG*), *RANKL*) and osteogenesis (e.g. *Runx2*, osteocalcin), are all re-expressed in a similar sequence during repair of bone, and instructing growth factors such as *BMPs*, *FGFs*, *PDGF*, and *IGFs*, are present in the fracture callus [108,107,105]. As during development, vascularization is a prerequisite

for ossification during bone repair and closely associated with MMP-mediated turnover of the cartilage intermediate in endochondral healing. Inhibition of endogenous VEGF activity, by sequestration or VEGF receptor blockade, inhibited bone repair and resulted in non-unions [109,110]. Disruptions in matrix remodeling due to deficiency in specific MMPs also resulted in delayed vascularization and bone repair [111-113]. For instance, MMP-9 deficient mice showed defective repair of tibia fractures with formation of non-unions, and this could be corrected by administration of exogenous VEGF [112]. Angiogenic-osteogenic coupling in repair may be similarly explained in part by provision of the required osteoprogenitor population via the vascular system. Sources of mesenchymal stem or progenitor cells can be the periosteum or the neighboring bone marrow [114]. Penetration of the cartilaginous callus by blood vessels wrapped with pericytic osteoprogenitors from the periosteum was recently visualized in a murine model of endochondral bone healing [44]. The vasculature may further bring more fracture-mobilized circulating mesenchymal stem or precursor cells with osteogenic potential to the region [63,106], and the endothelium may enhance bone formation by signaling osteogenic differentiation factors, such as BMP-2 [115].

The stimulatory effects of VEGF on angiogenesis and bone formation led to exploration of its potential use in bone repair and regeneration settings. Administration of VEGF was found to result in increased vascularity and accelerated bone healing [109,110,104,116], particularly when supplied in combination with BMPs [117,118]. VEGF is currently being tested in therapeutic ways in a

plethora of preclinical models of bone repair, and for its potential benefit in tissue engineering strategies [116]. Notably, cautiousness is enforced in the in vivo use of this potent vascular agent; for instance, mice over-expressing VEGF temporally in the skeleton (see above) revealed the risk of side effects in the bone and marrow environment, including disturbances in hematopoiesis [83]. Systemic leakage may bear risks for adverse vascular effects, as also suggested by preclinical pro-angiogenic VEGF studies in cardiovascular medicine [12]. Further in-depth analyses of the mechanisms and regulation of angiogenesis during fracture healing will undoubtedly help providing new therapies for patients with failing repair and new angles in tissue engineering applications.

OUTLOOK

While studies over the last 15 years have elucidated several functional and molecular relationships among the components that regulate vascularization during endochondral ossification, many aspects remain to be clarified. VEGF emerged as the most prominent molecular player currently known to govern the vascularization of the skeleton. However, the regulation of its expression in bone health and disease still needs to be further unraveled. Moreover, its relationship with angiogenic inhibitors such as those maintaining the avascular state of cartilage is incompletely understood, while increased insights in this area may hold clinical opportunities for anti-angiogenic treatments. From a skeletal therapeutic perspective, increased knowledge on the role of other angiogenic stimulators produced in bone (e.g. connective tissue growth

factor (CTGF), FGF, PDGF, TGF- β and angiopoietin-1) and their interplay with VEGF signaling may provide promising anabolic targets to treat metabolic bone diseases and enhance bone regeneration. The vascular anatomy and the mechanisms of the angiogenic processes in the skeleton are also as yet incompletely characterized. Studying the structural specifics of the vascular beds in bone is technically challenging, as the cortical bone shell renders the enclosed vascular system difficult to access; improved methods for visualization are emerging, for instance using high-resolution modeling of perfusion vascular casts, fluorescent and in vivo live imaging methods [79,87,91,65]. The growing arsenal of genetic tools, including inducible and site-specific mutagenesis models, will undoubtedly be instrumental in further analyses of the intrinsic molecular control of growth and maintenance of the vascular beds in the skeleton and in addressing the currently unresolved question how signals emanating from the vascular system regulate processes in the bone environment. These insights will be valuable for developing improved treatments for metabolic bone diseases, hematological disorders, bone injuries and cartilage defects.

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Figure legends

Fig. 1 Generation and characteristics of the VEGF isoforms. The gene for VEGF consists of 8 exons. Alternative exon splicing of a single pre-mRNA generates transcripts for different isoforms, the most important murine ones being VEGF₁₂₀, VEGF₁₆₄ and VEGF₁₈₈. The shortest isoform, VEGF₁₂₀, lacks the basic, heparin-binding residues encoded by exons 6 and 7, whereas VEGF₁₆₄ lacks the residues encoded by exon 6. This confers upon the secreted proteins differences in matrix binding, and creates a gradient of VEGF for directional recruitment or growth of endothelial cells during angiogenesis: VEGF₁₈₈ is mostly sequestered in the matrix, from where it can be released by proteases during matrix degradation, while VEGF₁₂₀ fails to bind heparin and can diffuse to a more distant site. VEGF₁₆₄ has intermediate matrix-association properties; VEGF₁₆₄ can furthermore bind the co-receptors NRP1 and NRP2, in addition to the main receptor tyrosine kinases (TK) VEGFR-1 and VEGFR-2

Fig. 2 Vascularization processes during endochondral bone development. (left panel) During embryogenesis, the initiation of cartilage neovascularization is coordinated with proliferation and differentiation of chondrocytes in the avascular anlagen, osteoblastogenesis and bone collar (BC) formation in the surrounding perichondrium (PCh), and the expression of high levels of VEGF by hypertrophic chondrocytes in the middle portion of the bone model. Invasion by blood vessels is accompanied by chondrocyte apoptosis, osteoclastic cartilage resorption, and co-invasion by osteoprogenitors moving to the inside of the nascent primary ossification center (POC). For details on the molecular regulators, see text. (middle panel) Longitudinal bone growth is driven by the progressive replacement of hypertrophic chondrocytes (HC) with trabecular bone, a process that relies on metaphyseal vascularization mediated by the heparin-binding VEGF isoforms, released from the matrix by MMP-9 during cartilage turnover. The VEGF gradient emanating from the hypertrophic cartilage regulates the guided attraction of blood vessels towards the terminal cartilage. VEGF stimulates the recruitment of

more osteoclast precursors that reach the region via the expanding circulation; these cells secrete more matrix-degrading MMPs, further facilitating vascular expansion by clearing the path for endothelial cell migration and angiogenesis. Hence, at the chondro-osseous junction a self-stimulatory cycle is established of progressive cartilage decay, systematic release of VEGF, and directional expansion of the vasculature. VEGF signaling also stimulates osteoblasts, completing its role as central coordinator of vascularization, cartilage turnover and ossification. (AC, RC, CC, pHC: articular, round, columnar and pre-hypertrophic chondrocytes). (right panel) When the avascular cartilage exceeds a critical size during growth, the middle of the growth plate becomes hypoxic, stabilizing HIF-1 and triggering VEGF expression in immature chondrocytes. Soluble VEGF isoforms are critical to diffuse to the surface of the cartilage, stimulating growth of the epiphyseal vascular network and tissue oxygenation. HIF-1 is additionally essential for chondrocyte survival by inducing oxygen-sparing anaerobic metabolism in the hypoxic cells. In early postnatal life, epiphyseal vessels invade the avascular cartilage and initiate a secondary center of ossification (SOC)

Fig. 3 Bone remodeling and hematopoiesis both take place in the vascularized environment of the adult bone and bone marrow. Balanced bone remodeling requires coordinated activities of osteoblasts (OB) and osteoclasts (OCL), as well as adequate vascular supply. Mesenchymal stem or progenitor cells residing in close proximity to the vascular endothelium may be key to angiogenic-osteogenic coupling and support of hematopoiesis. Hematopoietic stem cells (HSC) reside in the vascular niche near sinusoidal endothelial cells (EC) and give rise to blood cells. Quiescent, long-term HSC locate in the osteoblastic niche at the relative hypoxic endosteal bone surface; HIF-1 has roles in both HSC and in OB, i.e. to regulate HSC maintenance, bone formation and erythropoiesis. VEGF affects OB, OCL and HSC/hematopoiesis indirectly via its effects on bone vascularization and directly by signaling through VEGF receptors expressed by all these cell types, in paracrine or intracrine ways

Figure 1

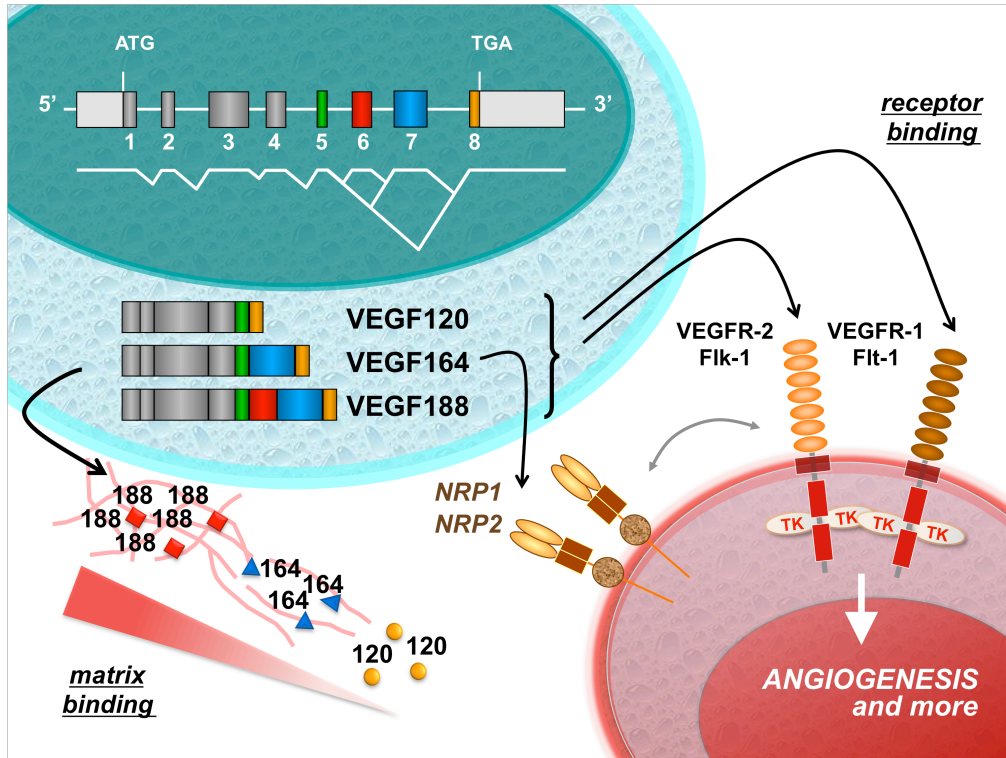


Figure 2

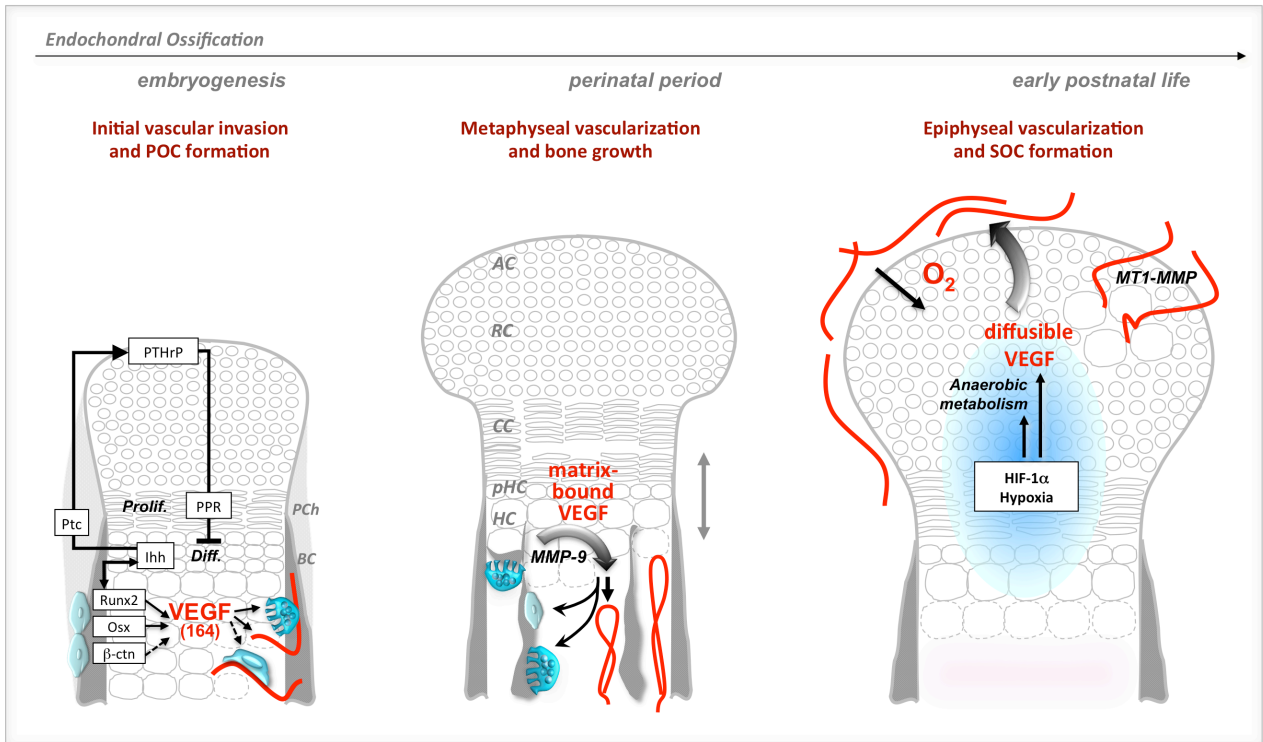


Figure 3

