CHAPTER 7

Vascular and Nonvascular Roles of VEGF in Bone Development

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Abstract

The majority of bones in the skeleton develop through the process of endochondral ossification. During this process, avascular cartilage becomes gradually replaced by highly vascularized bone tissue. VEGF is an essential mediator of all 3 key vascularization stages of endochondral bone development, and, in addition, exerts multiple nonvascular functions during each of these stages by acting directly upon the involved bone cells. In this chapter, we will discuss the various lines of evidence which demonstrate that the three major VEGF isoforms are essential to coordinate bone vascularization, cartilage morphogenesis and ossification during endochondral bone formation.

Key Messages

- VEGF is expressed by bone cells (osteoclasts, osteoblasts, and chondrocytes) involved in the process of endochondral ossification.
- VEGF receptors are expressed by endothelial cells and bone cells during endochondral ossification.
- VEGF controls the timely invasion of endothelial cells and osteoclasts/chondroclasts into developing long bones during primary ossification.
- VEGF regulates the proliferation, differentiation and/or survival of osteoclasts, osteoblasts and chondrocytes.
- The matrix-binding VEGF isoforms mediate metaphyseal angiogenesis and thereby regulate both trabecular bone formation and growth plate morphogenesis during endochondral bone formation.
- The soluble VEGF isoforms are required for epiphyseal vascularization and secondary ossification in growing long bones.

Introduction

The assembly of the skeleton during embryonic development relies on the formation of as many as 206 separate bones at sites distributed all over the body. Two distinct mechanisms are responsible for bone formation: intramembranous and endochondral ossification. During intramembranous ossification, bones develop directly from soft connective tissue. First, mesenchymal precursor cells aggregate at the site of the future bone formation, and they then differentiate into osteoblasts. The osteoblasts deposit bone matrix (osteoid) rich in type I

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collagen, which later becomes mineralized. Terminally differentiated osteoblasts become entrapped in the bone as osteocytes. This type of bone deposition occurs in close spatial interaction with vascular tissue, but little is known about the role of VEGF in this process. Truly membranous bones are the flat bones of the skull (calvarial bones and mandibles) and parts of the clavicles. The long bones of the axial and appendicular skeleton also develop from mesenchymal condensations, but here these cells differentiate into chondrocytes to form a cartilaginous model of the future bone, the cartilage anlagen. This avascular cartilage subsequently becomes replaced by highly vascularized bone tissue through the process of endochondral ossification, which encompasses 3 key vascularization stages: (i) initial vascular invasion of the cartilage anlagen to establish the primary center of ossification (diaphysis) (Fig. 1A-C,G); (ii) capillary invasion at the growth plate (metaphysis) to mediate rapid bone lengthening (Fig. 1D-F,H); and (iii) vascularization of the cartilage ends (epiphysis) to initiate secondary ossification (Fig. 1D-F,I).

In the late 1990's several in vitro experiments established that VEGF and its receptors are expressed in specific bone cell types, and a strong regulation of VEGF expression by osteo-modulators was observed. These earliest findings suggested a possible role for VEGF in bone formation in vivo. However, a generalized mouse knock-out model of VEGF could not be employed to determine the role of VEGF in bone development, due to lethality of even heterozygous VEGF knock-out embryos at a stage preceding the onset of skeletal development. Therefore, alternative approaches had to be used to explore the physiological role of VEGF in bone development. The first evidence for an important role for VEGF in postnatal metaphyseal bone development was found in juvenile mice after administration of a soluble truncated chimeric VEGF receptor, which consists of the FLT1 extracellular domain fused to an IgG-Fc domain and sequesters VEGF protein with high affinity.[1] In this model, VEGF inactivation suppressed blood vessel invasion at the growth plate and concomitantly inhibited endochondral bone formation. Another strategy to block VEGF function whilst circumventing the early embryonic lethality of VEGF null mice entailed the *Cre/LoxP*-mediated conditional inactivation of the VEGF gene (*Vegfa*) in type II collagen expressing chondrocytes.[2,3] Finally, expression of only one of the major VEGF isoforms also rescued the embryonic lethality of VEGF null mice, and was therefore able to reveal the specific contributions of these isoforms to bone development.[4-6] Altogether, these models have exposed multiple essential roles of VEGF in the sequential stages of endochondral bone formation (Fig. 1), as will be discussed.

In mice, the *Vegfa* gene encodes 3 major alternatively spliced isoforms: VEGF120, VEGF164 and VEGF188 (see Chapter 1 by Y.S. Ng). VEGF120 has a low affinity for heparin and is considered to be a freely diffusible protein. In contrast, VEGF164 and even more so VEGF188 bind heparin with high affinity; this is thought to facilitate their binding to heparan sulfate-containing proteoglycans on the cell surface and in the extracellular matrix (ECM), from which they can be released by proteolytic enzymes such as matrix metalloproteinases (MMPs). All VEGF isoforms are capable of binding the VEGF receptor tyrosine kinases FLT1 and KDR. In contrast, VEGF164, but not VEGF120 has been shown to bind to NRP1 and NRP2.

In this chapter, we will describe how VEGF expression by several different bone cell types mediates a multitude of effects during endochondral ossification. In particular, we focus on the role of VEGF as an essential mediator of all 3 key vascularization stages during endochondral bone development, and describe how VEGF exerts multiple nonvascular functions during each of these stages by acting directly upon the involved bone cells. Moreover, we will discuss how the study of bone development in transgenic mice expressing solely VEGF120 (*Vegfa120/120*), VEGF164 (*Vegfa164/164*) or VEGF188 (*Vegfa188/188*) revealed differential requirements for the VEGF isoforms at different stages of bone formation.

Expression and Regulation of VEGF and VEGF Receptors in Bone Cell Types

VEGF and its receptors are expressed by several different bone cell types involved in endochondral ossification.

Chondrocytes

Chondrocytes in the cartilage template and later in the growth plate first proliferate, and then progressively differentiate into mature hypertrophic cells. Several autocrine and/or paracrine factors have been implicated in chondrocyte development, including parathyroid hormone related protein (PTHRP; now known as parathyroid hormone like peptide, PTHLH), indian hedgehog (IHH), bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs). PTHRP and IHH form a negative feedback signaling pathway to control the pace of chondrocyte development in the growth plate. IHH also coordinates chondrocyte and osteoblast differentiation, together with the transcription factor RUNX2 (runt related transcription factor; also known as core binding factor 1, CBFA1).[7,8]

Hypertrophic chondrocytes, but not immature chondrocytes, consistently express high levels of VEGF in vivo. One factor that may control this VEGF expression is RUNX2.[9] By expressing VEGF and other angiogenic stimulators, hypertrophic cartilage becomes a target for capillary invasion and angiogenesis. In contrast, immature cartilage remains avascular due to the production of angiogenic inhibitors. As a result, the center of the developing epiphyseal growth plate becomes hypoxic,[10] but chondrocytes are well capable of surviving this challenge. For example, bovine articular chondrocytes are able to survive under oxygen tensions ranging from <0.1% to 20% for at least 7 days in vitro, with no evident differences in cell division or differentiation.[11] In response to this physiological hypoxia, immature chondrocytes in the center of the epiphysis upregulate VEGF. Accordingly, VEGF mRNA and protein levels are increased by hypoxia in cultured embryonic limbs and primary chondrocytes in vitro.[5,12] In general, hypoxia induces the expression of VEGF and other genes involved in angiogenesis and glucose metabolism via two transcriptional regulators, the hypoxia-inducible factors HIF1A and HIF2A (previously known as HIF1 alpha and HIF2 alpha; see Chapter 3 by M. Fruttiger). In agreement, inactivation of HIF1A in epiphyseal chondrocytes abolished the upregulation of VEGF in response to hypoxia in vitro.[12] Mice with inactivation of HIF1A in cartilage nevertheless showed increased VEGF expression in the epiphysis, suggesting that HIF2A and/or other factors may compensate for HIF1A loss or contribute to VEGF upregulation in vivo.[10] Studies on the VEGF receptor profile in chondrocytes showed that immature epiphyseal chondrocytes in vivo express the two VEGF isoform-specific receptors NRP1 and NRP2, but no detectable levels of FLT1 or KDR were found.[3,5] Expression of KDR has however been reported in some other cartilage types, such as the permanent thyroid cartilage of humans and cultured hypertrophic chondrocytes of chicken.[13,14]

Osteoblasts

Osteoblasts share a common mesenchymal precursor with chondrocytes, and specific regulatory factors direct the osteo-chondroprogenitors to either one of these lineages. As such, RUNX2 dominates the control of osteoblast differentiation. Mature osteoblasts produce bone matrix and abundantly express type I collagen. In a later differentiation stage, osteoblasts mineralize the osteoid and are typified by expression of osteocalcin.

Several groups reported that osteoblastic cells of mouse, rat or human origin express VEGF and its receptors, with highest expression levels being found at the late differentiation stages.[15,16] As observed in chondrocytes, VEGF production in osteoblasts is stimulated by hypoxia in a process that involves HIF1A and HIF2A.[17-19] Furthermore, VEGF expression in osteoblasts is also induced by several osteotropic factors, including BMPs, transforming growth factor beta (TGFB), prostaglandins, insulin-like growth factor 1 (IGF1), platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF2) and 1alpha,25-dihydroxyvitamin D_3, but it is inhibited by bone catabolic factors such as glucocorticoids.

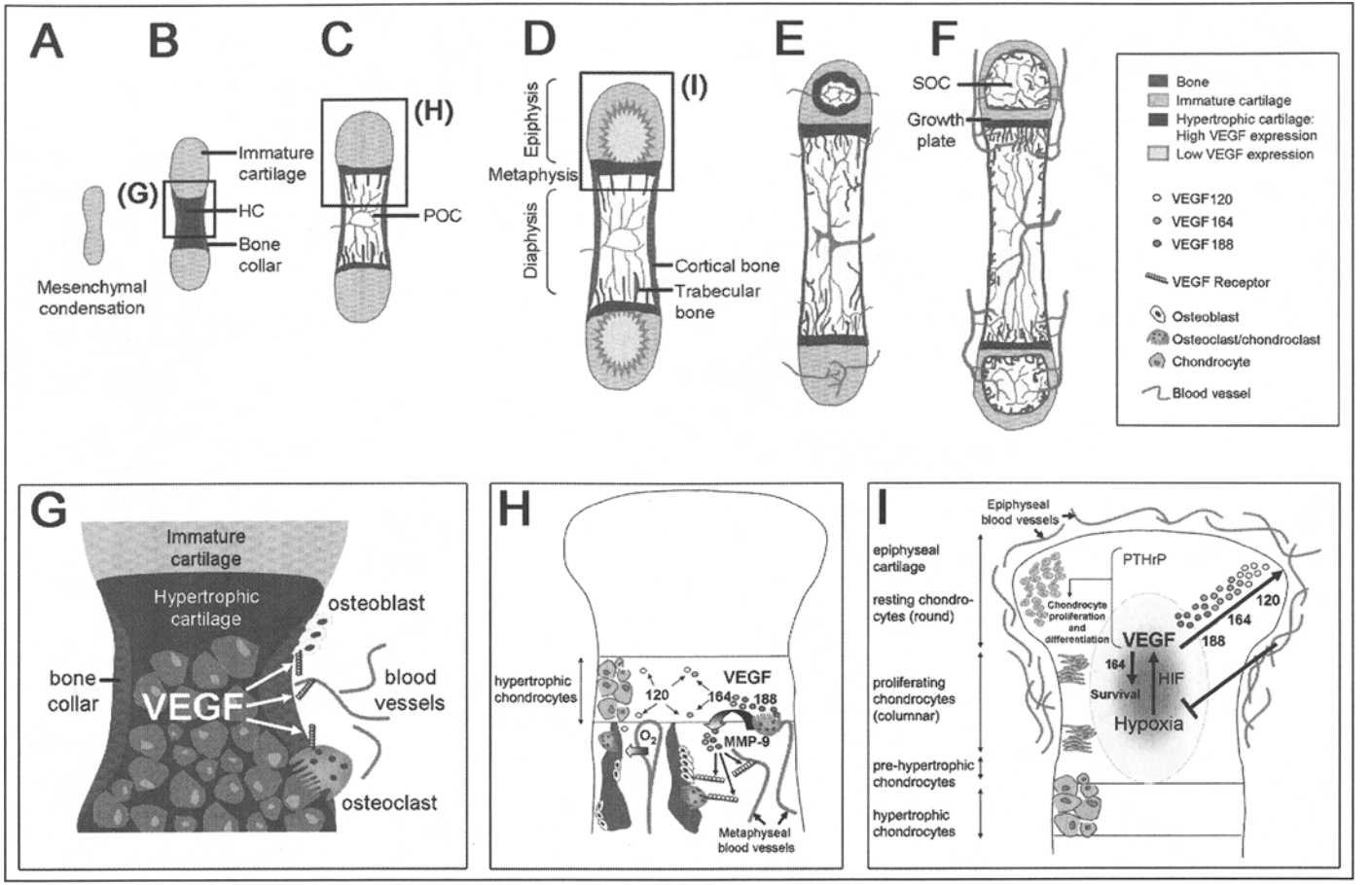

Figure 1. Legend vieewed on following page.

Figure 1, viewed on previous page. Role of VEGF in endochondral bone development. A) Around E12 in mice, mesenchymal progenitor cells condense and differentiate into chondrocytes to form the cartilage anlagen that prefigure future bones. B) Around E14, hypertrophic chondrocytes (HC) form in the cartilage center (diaphysis), while cells in the connective tissue surrounding the cartilage (perichondrium) differentiate into osteoblasts. Osteoblasts deposit a mineralized bone matrix called bone collar around the cartilage. C) The primary ossification center (POC) forms when the diaphysis becomes vascularized and is invaded by osteoclasts, which resorb the hypertrophic cartilage, and by osteoblasts, which deposit bone matrix. The net result is replacement of the avascular cartilage anlage by a vascularized long bone. In the metaphysis, hypertrophic cartilage is continually replaced with trabecular bone, a process that relies on VEGF-mediated vascularization. D) Chondrocytes in the center of the avascular termini of the long bones (epiphyses) become hypoxic and express VEGF. E) Around postnatal day 5, epiphyseal vessels are attracted into the cartilage, likely in response to VEGF signals, and this initiates formation of the secondary ossification center (SOC). F) Discrete layers of residual chondrocytes form 'growth plates' between the epiphyseal and metaphyseal bone centers to support further postnatal longitudinal bone growth. G) Role of VEGF in orchestrating the development of the primary ossification center: VEGF is produced at high levels by hypertrophic chondrocytes, likely under the control of RUNX2. Endothelial cells, osteoblasts and osteoclasts express VEGF receptors and accumulate in the perichondrium. VEGF induces vascular invasion of the cartilage and may affect the differentiation and function of osteoblasts and osteoclasts. H) Metaphyseal vascularization, cartilage resorption and bone formation are coordinated by VEGF and MMP activity: Hypertrophic chondrocytes express VEGF120, VEGF164 and VEGF188; VEGF164 and VEGF188 are sequestered in the cartilage matrix, but can be released by proteases such as MMP9 to recruit endothelial cells and act upon osteoclasts and osteoblasts; MMP9 also supports cartilage resorption. I) Role of VEGF isoforms in epiphyseal bone development: When the avascular epiphyseal cartilage exceeds a critical size, immature chondrocytes in the center become hypoxic and express VEGF. The soluble VEGF isoforms VEGF120 and VEGF164 diffuse to the periphery to stimulate expansion of the epiphyseal vascular network and its subsequent invasion into the epiphysis at the start of secondary ossification. VEGF164 also promotes survival of hypoxic chondrocytes, and, in conjunction with other factors such as PTHRP, regulates chondrocyte development.

Osteoclasts

Osteoclasts are large, multinucleated cells that are endowed with the unique capacity to degrade mineralized tissues, a process in which secreted MMPs play an important role.[20] The development of osteoclasts is a complex multi-step process that involves at least two crucial signaling molecules expressed by osteoblasts and osteoblast progenitors: macrophage-colony stimulating factor (CSF1; previously known as M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL; now also known as tumor necrosis factor superfamily member 11, TNFSF11).[21]

Osteoclasts share a common hematopoietic precursor with monocytes and macrophages, and like them express FLT1 as their main VEGF receptor.[22,23] However, expression of KDR by cultured osteoclasts has also been reported.[24,25] Primary cultures of osteoclasts prepared from murine bone marrow were found to express VEGF by RT-PCR,[25] but these results have to be confirmed by additional experimental approaches, as these cultures also contained other bone marrow derived cells.

VEGF Is Required for the Formation of the Primary Ossification Center

Embryonic long bones first develop as avascular cartilage anlagen, but following the formation of a bone collar around the cartilage, vascular invasion takes place. Concomitant with vascular invasion, the hypertrophic cartilage matrix is degraded by invading osteoclasts and/or chondroclasts, and osteoblasts and marrow cells start to populate the primary ossification center. Blocking physically the vascular invasion of hypertrophic cartilage in embryonic day (E)14 skeletal explants halts bone development, indicating that the development of cartilage anlagen into proper long bones depends on the invasion of endothelial cells.[26] The vasculature is not only critical to supply oxygen, nutrients and growth enhancing molecules, but is also considered to be a major source of progenitors for the specific cell types that form bone and marrow. Several lines of evidence indicate that the timely invasion of endothelial cells and osteoclasts/chondroclasts during early bone development is dependent on VEGF, particularly VEGF164, through its direct actions on both endothelial cells and bone cells (Fig. 1G).

VEGF Controls the Initial Vascular Invasion during Formation of the Primary Ossification Center

At the time when the primary ossification center develops, VEGF is produced by perichondrial cells, possibly osteoblasts, and by diaphyseal hypertrophic chondrocytes (see below).[4,27] *Vegfa* transcription is thought to be induced by RUNX2, given that expression of VEGF and its receptors is impaired in the bones of RUNX2-deficient mice. Moreover, these mice show no vascular invasion into any skeletal element, consistent with the idea that VEGF is a critical vascular growth factor during bone formation.[9] This idea is particularly supported by the observation that the formation of the primary ossification center is delayed in cartilage explants cultured with a VEGF-inhibiting soluble chimeric FLT1 protein.[26] The initial vascular invasion and the formation of the primary ossification center are also delayed in *Vegfa120/120* and *Vegfa188/188* mice, but not in *Vegfa164/164* mice, suggesting that specifically the VEGF164 isoform is needed for this process.[4,5] This could be due to its interaction with NRP1. Alternatively, not the VEGF164 isoform in particular, but rather a combination of soluble and bound VEGF molecules may be needed to coordinate the initial capillary invasion into the cartilage anlagen, perhaps to form a chemoattractive gradient for ingrowing vessels. A similar mechanism has been proposed for angiogenesis in other organs (see Chapter 6 by H. Gerhardt). VEGF is also a chemoattractant for osteoclasts invading into developing bones. This process involves MMPs, raising the possibility that release of matrix-bound VEGF from the hypertrophic cartilage matrix by the action of MMPs may account for the close association of vascular and osteoclastic invasion.[28-30]

Nonvascular Roles of VEGF during the Formation of the Primary Ossification Center

VEGF appears to have also nonvascular roles in the initiation of bone development (Table 1), as supported by several observations: Firstly, ossification is reduced in embryonic metatarsals cultured with a soluble FLT1 chimera that inhibits VEGF.[4] Secondly, bone collar formation and cartilage calcification are decreased in embryonic *Vegfa120/120* and *Vegfa188/188* bones at a stage preceding vascularization.[4,5] Moreover, the analysis of *Vegfa*$^{120/20}$ bones revealed retarded terminal differentiation of hypertrophic chondrocytes and reduced expression of several markers for osteoblast and chondrocyte differentiation. Thirdly, the various bone cell types involved all express VEGF receptors: Hypertrophic chondrocytes express NRP1, perichondrial cells in vivo as well as osteoblastic cells in vitro express NRP1, NRP2, FLT1 and KDR, and osteoclasts express FLT1.[23,27,31] Although the precise effect of VEGF in vivo on the cell types involved in the initial stages of bone development is not yet fully understood, these findings suggest that VEGF isoforms take part in the timely differentiation of osteoblasts and chondrocytes. Interestingly, the expression of VEGF and NRP1 is already detected in the limb bud mesenchyme at E10.5 and at the periphery of the (pre)cartilage anlagen at E12.5.[2,32] Although unresolved at present, these data do raise the possibility that VEGF may function at even earlier stages in bone development, at the time when the cartilage condensations form.

VEGF Is Required for Metaphyseal Bone Development and Longitudinal Bone Growth

Longitudinal bone growth is mediated largely by the events occurring at the metaphyseal growth plate. The tight coupling between metaphyseal vascularization and endochondral bone development may be explained by the ability of blood vessels to function as a conduit which (i) allows cell types essential for bone morphogenesis, i.e., osteoclasts and osteoblasts, to migrate to the growth plate; (ii) removes end products of the resorption process; and (iii) supplies cells in the developing bone with oxygen, nutrients and growth factors/hormones required for their activity. Metaphyseal angiogenesis is induced by the matrix-binding VEGF isoforms and is an essential prerequisite for trabecular bone formation and growth plate morphogenesis. In addition, VEGF has been shown to directly affect osteoblasts and osteoclasts (Fig. 1H; Table 1).

The Role of VEGF in Metaphyseal Vascularization

During longitudinal bone growth, it is of utmost importance that the key mechanisms of endochondral ossification are rigorously coordinated, and the analysis of several different mouse models has demonstrated that VEGF-mediated metaphyseal angiogenesis plays a critical role in this process. Disruption of VEGF function in developing bones has been achieved by injection

Table 1. VEGF effects during the three key stages of endochondral bone development

VEGF Effects	POC Development	Longitudinal Bone Growth	SOC Development
Vascular	Vascular invasion	Metaphyseal vascularization	Epiphyseal vascularization
Nonvascular	HC differentiation (*)	HC apoptosis and resorption (*)	Chondrocyte proliferation and differentiation
	OB development/ activity	OB and OC activity	
	OC recruitment/activity		Chondrocyte survival

(*) The direct effect of VEGF in these processes has not unambiguously been shown in vitro.
Abbreviations: POC: primary ossification center; SOC: secondary ossification center; HC: hypertrophic chondrocyte; OB: osteoblast; OC: osteoclast.

of a soluble truncated chimeric VEGF receptor,[1] by targeted inactivation of the VEGF-isoforms VEGF164 and VEGF188 leaving only expression of the soluble isoform VEGF120,[4,6] and by conditional deletion of a single *Vegfa* allele in cells expressing type II collagen.[2] In all three instances, (partial) loss of VEGF function impaired metaphyseal bone vascularization. Specifically, vascularization was decreased and disorganized near the growth plate and, concomitantly, trabecular bone formation and bone growth were impaired. Typically, the hypertrophic chondrocyte zone of the growth plate was enlarged, due to reduced resorption and/or apoptosis. Thus, in metaphyseal bone development, VEGF functions to attract vessels to the growth plate, which is accompanied by hypertrophic chondrocyte apoptosis, cartilage resorption by osteoclasts/chondroclasts, and trabecular bone formation by osteoblasts.

Remarkably, a similar bone phenotype characterized by an enlarged hypertrophic chondrocyte zone was observed in mice deficient in MMP9 and/or MMP13.[33-35] This led to the hypothesis that MMP9 is produced by osteoclasts/chondroclasts to release ECM-bound VEGF from the chondrocyte matrix and thereby attract blood vessels (and more resorptive cells) to the growth plate (Fig. 1H).[8] In support of this model, *Vegfa164/164* and *Vegfa188/188* mice have no enlarged hypertrophic zone, nor do they display any other metaphyseal defect.[5] Thus, expression of either of the matrix-binding isoforms, VEGF164 or VEGF188, is necessary and sufficient to provide the signals required for normal metaphyseal vessel invasion and endochondral ossification (Fig. 1H). This observation suggests that the controlled VEGF release from the cartilage matrix favors organized directional angiogenesis, most likely by creating a VEGF gradient (see Chapter 6 by H. Gerhardt).[36] Alternatively, or additionally, VEGF signaling through NRP1 may be required as the impaired metaphyseal development is seen exclusively in *Vegfa120/120* mice and VEGF120 does not bind this receptor. Mice deficient in NRP1 die before the onset of bone development due to cardiovascular defects, but the analysis of conditional knockout mice with NRP1 inactivation exclusively in cartilage or bone may reveal a role for VEGF isoform signaling through NRP1 in bone cells.

Nonvascular Roles of VEGF during Longitudinal Bone Growth

Recent studies have suggested that VEGF may influence bone formation by directly affecting osteoblasts, as VEGF stimulates osteoblast differentiation and migration in vitro.[15,31,37] Moreover, adenovirus-mediated VEGF gene transfer induces bone formation by increasing osteoblast number and osteoid forming activity in vivo.[38] VEGF signaling has also been implicated in osteoclastogenesis and subsequent cartilage/bone resorption: Firstly, VEGF directly enhances the resorption activity and survival of mature osteoclasts in vitro.[24] Secondly, RANKL induces osteoclast differentiation from spleen- or bone marrow-derived precursors in culture when provided in combination with either VEGF or CSF1. Thirdly, VEGF, like CSF1, rescues osteoclast recruitment, survival and activity in osteopetrotic *op/op* mice, which carry an inactivating point mutation in the *Csf1* gene that results in low numbers of macrophages and a complete lack of mature osteoclasts.[23] Like monocytes and macrophages, osteoclasts predominantly express FLT1 rather than KDR (see above).[22,23] Moreover, FLT1 ligands are chemoattractive for both monocytes and osteoclasts.[29,39]

VEGF Affects Epiphyseal Cartilage Development and Formation of the Secondary Ossification Center

Because developing epiphyseal cartilage is avascular, its oxygenation is critically dependent on the vascular network overlying the cartilaginous surface, which is derived mainly from the epiphyseal arteries. These peripheral vessels later invade the cartilage to initiate the development of the secondary ossification center (Fig. 1D-F).[40] Recent studies have implicated the soluble VEGF isoforms in epiphyseal vascularization and secondary ossification.[5] In addition, VEGF was shown to act as a survival factor for chondrocytes in the hypoxic epiphysis (Fig. 1I; Table 1).[3,5]

The Role of VEGF in Epiphyseal Vascularization and the Initiation of Secondary Ossification

Vegfa188/188 mice form an abnormal capillary network overlying the epiphyses, a defect that is associated with increased hypoxia and massive apoptotic cell death in the interior of the cartilage. Chondrocytes located in the adjacent peripheral areas display an imbalance in their proliferation/differentiation rate. Impaired epiphyseal vascularization and chondrocyte development are most likely the cause of the strongly reduced long bone growth in *Vegfa188/188* mice, which display a dwarfed phenotype. Thus, the soluble VEGF isoforms are essential for epiphyseal vascularization, epiphyseal cartilage development and formation of the secondary ossification center.[5] Based on these findings, we suggest a model in which the progressive growth of the avascular epiphyseal cartilage results in a state of increased hypoxia that upregulates VEGF expression (Fig. 1I); soluble VEGF isoforms then diffuse from the hypoxic center towards the periphery to induce epiphyseal vessel outgrowth and thus reduce hypoxic stress. Subsequently, VEGF induces invasion of vessels into the cartilage to initiate secondary ossification. The presence of only VEGF188 is insufficient to stimulate epiphyseal vascularization, probably because this isoform binds tightly to matrix components and cellular surfaces, thereby failing to diffuse towards the periphery. Alternatively, the phenotype could be due to reduced VEGF signaling through NRP1, as it is not presently known if VEGF188 binds NRP1. However, this latter hypothesis seems less likely, since mice expressing only VEGF120 show normal epiphyseal vascularization, even though VEGF120 does not bind NRP1.[5] Epiphyseal vascular invasion and the subsequent development of the secondary ossification center are also impaired in mice lacking MT1-MMP,[41,42] suggesting that vascular invasion depends on both the degradation of the matrix by MT1-MMP and the attraction of blood vessels by VEGF. Interestingly, MT1-MMP upregulates VEGF expression in human breast carcinoma MCF7 cells,[43] but whether it also influences VEGF expression in cartilage is currently unknown.

Nonvascular Roles of VEGF in Epiphyseal Chondrocyte Development and Survival

In addition to its effects on epiphyseal vascularization, VEGF also directly affects chondrocyte development and survival in hypoxic cartilage. Firstly, VEGF is likely to act together with other factors, such as the PTHRP pathway (see above), to regulate the balance of chondrocyte proliferation and differentiation in the epiphysis (Fig. 1I).[5] This activity may be due to VEGF isoform signaling through NRP1, as this receptor is expressed on epiphyseal chondrocytes.[3,5] Secondly, both *Vegfa188/188* mice and mice with a complete inactivation of VEGF specifically in type II collagen-expressing cells show aberrant chondrocyte death, a phenotype similar to that seen in mice lacking HIF1A in cartilage.[3,5,10] In vitro cultures of *Vegfa 188/188* embryonic limbs revealed that expression of VEGF188 is not sufficient to protect chondrocytes against hypoxia-induced apoptosis, but supplementing recombinant VEGF164 rescued this defect. Thus, VEGF164 acts as a survival factor for hypoxic chondrocytes, possibly downstream of HIF1A.[3,5,10] The role of HIF1A in cartilage survival may therefore be at least in part due to its ability to upregulate VEGF, combined with the induction of anaerobic glycolytic metabolism.[44]

Conclusions and Future Perspectives

In this chapter, we have described the multiple essential roles that VEGF fulfills to support skeletal development. However, many mechanistic aspects of VEGF function in bone development remain to be elucidated, and the potential contribution of reduced VEGF signaling in bones to human disease has not yet been examined.

Novel Mouse Models to Understand VEGF Signaling in Bone Development

Whilst it is now evident that VEGF is essential to drive vascularization during endochondral bone development, the in vivo studies performed have also underscored our limited knowledge of the bone's vascular system itself and of its role in regulating the behavior of bone cells. For example, we don't know much about the types of blood vessels involved (capillaries, venous sinusoids, arteries), nor about the presence and role of pericytes or other peri-vascular cells. It is also still largely unclear how epiphyseal vascularization and the formation of vascular canals relate to secondary ossification. Moreover, we need to understand better the resorption processes that accompany vascular invasion. For instance, the contribution of specific proteases that release VEGF from the matrix at particular stages of endochondral bone development could be further addressed by the analysis of mice with specific mutations in MMP genes. The role of the VEGF-responsive cell types involved, such as endothelial cells and (other) resorbing cells, could be addressed by specifically targeting VEGF receptors.

Importantly, it has become clear that VEGF also exerts direct effects on several key bone cell types during endochondral bone development. These direct effects are still incompletely understood, for two reasons: Firstly, in vivo models have often been difficult to analyze, as the alteration of the VEGF expression levels or the VEGF isoform balance almost inevitably causes angiogenic defects, which in turn affect bone development. Secondly, in vitro models are limited in their capacity to accurately reproduce the complex differentiation processes that occur in vivo. However, the combination of both approaches, together with the use of transgenic mice carrying cell type-specific, temporally restricted or even cellular differentiation stage-specific knockout alleles for VEGF and its receptors, including the isoform-specific VEGF receptors, will provide this critical information. Aspects to be addressed include the precise effects, mechanisms of action and regulation of VEGF in osteoblasts, osteoclasts, and chondrocytes. Particularly regarding the regulation of VEGF in cartilage, much remains to be learned about the role of the hypoxia regulatory pathway. Inactivation of von Hippel Lindau (VHL), a mediator of HIF degradation, in murine cartilage was recently shown to alter chondrocyte proliferation, further underscoring that components of the hypoxia regulatory pathway play important physiological roles in cartilage, either directly and/or by affecting VEGF levels.[45] Furthermore, it will be interesting to see whether this pathway also plays a role in other bone cells. Finally, it will be exciting to explore if VEGF contributes to the very early stages of skeletal development, prior to vascular invasion and ossification, and whether VEGF regulates the development of joints.

From Mice to Men: A Role for VEGF Misexpression in Growth Disorders?

Animal studies have shown that the precise level of VEGF is critically important for embryonic development. Interestingly, the phenotypes of *Vegfa188/188* and *Vegfa120/120* mice suggest that altering the relative levels of the VEGF isoforms—without affecting the total level of VEGF—impairs developmental processes such as vascular network formation and skeletal development and growth. In particular, normal levels of the VEGF164 isoform appear to be critical for normal bone development in mice. However, it has not yet been examined if subtle variations in VEGF or VEGF isoform expression levels affect the development of the human skeleton. For example, it is conceivable that allelic variations in the human *VEGFA* gene promoter or abnormal *VEGFA* mRNA splicing affect either VEGF expression or the production of the VEGF165 isoform (the human ortholog of murine VEGF164). Hypothetically, such changes might cause or increase the risk of growth defects, or add to the severity of skeletal disorders caused by mutation in other genes (e.g., FGF receptor 3 or PTH/PTHRP receptor). It will be particularly important to examine if *VEGFA* gene polymorphisms are linked to the pathogenesis of human dwarfing syndromes or other skeletal pathologies, because low VEGF levels are already known to predispose humans and mice to motor neuron degeneration (see Chapter 8 by J. Krum, J. Rosenstein and C. Ruhrberg), and loss of VEGF164 expression acts as a modifier of DiGeorge syndrome, a disease with vascular and craniofacial abnormalities.[46,47] Studying the role of VEGF in bone development may also provide the basis for new therapies

aimed at treating debilitating and/or unmanageable bone diseases, such as osteoporosis, bone metastases, and nonhealing fractures.

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