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CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

VEGF: an Essential Mediator of Both Angiogenesis and Endochondral Ossification

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ABSTRACT

During bone growth, development, and remodeling, angiogenesis as well as osteogenesis are closely associated processes, sharing some essential mediators. Vascular endothelial growth factor (VEGF) was initially recognized as the best-characterized endothelial-specific growth factor, which increased vascular permeability and angiogenesis, and it is now apparent that this cytokine regulates multiple biological functions in the endochondral ossification of mandibular condylar growth, as well as long bone formation. The complexity of VEGF biology is paralleled by the emerging complexity of interactions between VEGF ligands and their receptors. This narrative review summarizes the family of VEGF-related molecules, including 7 mammalian members, namely, VEGF, placenta growth factor (PLGF), and VEGF-B, -C, -D, -E, and -F. The biological functions of VEGF are mediated by at least 3 corresponding receptors: VEGFR-1/Flt-1, VEGFR-2/Flk-1, VEGFR-3/Flt-4 and 2 co-receptors of neuropilin (NRP) and heparan sulfate proteoglycans (HSPGs). Current findings on endochondral ossification are also discussed, with emphasis on VEGF-A action in osteoblasts, chondroblasts, and chondroclasts/osteoclasts and regulatory mechanisms involving oxygen tension, and some growth factors and hormones. Furthermore, the therapeutic implications of recombinant VEGF-A protein therapy and VEGF-A gene therapy are evaluated. Abbreviations used: VEGF, Vascular endothelial growth factor; PLGF, placenta growth factor; NRP, neuropilin; HSPGs, heparan sulfate proteoglycans; FGF, fibroblast growth factor; TGF, transforming growth factor; HGF, hepatocyte growth factor; TNF, tumor necrosis factor; ECM, extracellular matrix; RTKs, receptor tyrosine kinases; ERK, extracellular signal kinases; HIF, hypoxia-inducible factor

KEY WORDS: angiogenesis; bone, cartilage, endochondral ossification; neovascularization, vascularity, recombinant protein; gene therapy; VEGF; VEGF receptor.

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INTRODUCTION

During embryogenesis, blood vessels are formed by two processes, vasculogenesis and angiogenesis. Vasculogenesis is the formation of new blood vessels involving *de novo* differentiation of endothelial cells from mesoderm-derived precursor cells (angioblasts) (Gonzalez-Crussi, 1971). In contrast, angiogenesis, termed 'neovascularization', is the formation of new blood vessels from a pre-existing vascular network, with continued expansion of a vascular tree in response to an increase in tissue mass (Risau, 1997; Carmeliet, 2005). Almost all tissues develop a functional circulatory system by sprouting or non-sprouting angiogenesis to form a primary capillary plexus. The metabolism and survival of normal tissue depend on the adequate supply of oxygen and nutrients from the vasculature. During adulthood, however, cells of most blood vessels are quiescent, except for those which respond to physiological processes such as wound healing, tissue remodeling, and the female reproductive cycle and pathological processes such as tumor expansion and metastasis (Folkman and Shing, 1992; Folkman, 1995). However, dysregulated and excessive vessel growth has a significant impact on health, and contributes to various diseases. Insufficient vessel growth or abnormal vessel regression causes stroke, Alzheimer's disease, amyotrophic lateral sclerosis, hypertension, osteoporosis, respiratory distress, and other disorders. In contrast, cancer, rheumatoid arthritis, psoriasis, and diabetic retinopathy are the best-known diseases resulting from excessive or abnormal angiogenesis (Carmeliet, 2005).

The final event in endochondral ossification of bone growth and development is the replacement of the avascular cartilage template by highly vascularized bone tissue (Fig. 1). In this process, chondrocytes become hypertrophic and then produce a calcified cartilaginous extracellular matrix (ECM) and angiogenic stimulators, thus providing a target for capillary invasion and angiogenesis (Alini *et al.*, 1996). The new vasculature supplies a conduit for the recruitment of the cell types involved in cartilage resorption and bone deposition, while providing the signals necessary for normal morphogenesis (Harper and Klagsbrun, 1999). Histological findings suggest that osteoblasts and osteoprogenitor cells always develop concomitantly with endothelial cells in the newly formed blood vessels at sites where new bone is formed (Deckers *et al.*, 2000). Capillary endothelial cells provide microvasculature during bone remodeling (Erlebacher *et al.*, 1995), and this vascular invasion is a prerequisite for bone formation (Guenther *et al.*, 1986). Moreover, in the mandibular condyle, the terminal ends of the penetrating capillaries closely follow the outline of the previously eroded chondrocytic capsule, and the process of erosion is closely related to and dependent on osteoclastic activity (Durkin *et al.*, 1973). Certainly, angiogenesis is of critical importance for the growth and development of long bones and the mandibular condyle, and these two biological events share a common mediator.

Angiogenesis is thought to depend on a delicate balance between endogenous stimulators and inhibitors (Polverini, 1995). Many

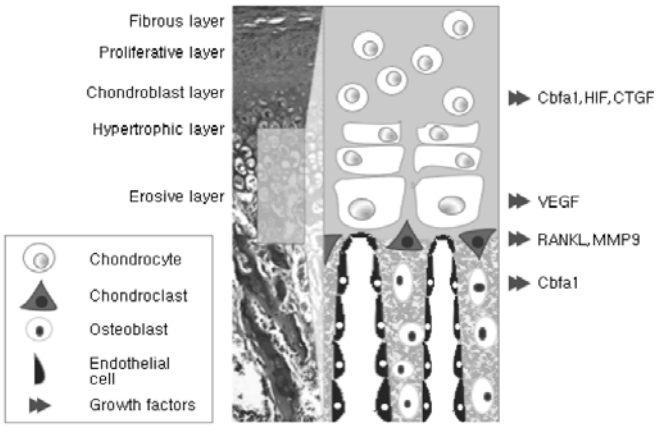

Figure 1. Schematic representation of the role of VEGF in mandibular condylar growth. The mandibular condyle is histologically distinguishable as 5 different layers: the fibrus, proliferative, chondroblast, hypertrophic, and erosive layers. Mesenchymal stem cells differentiate into chondroblasts, which form the framework of the cartilage matrix. VEGF, released from hypertrophic chondrocytes, induces endothelial cell invasion, which in turn induces vascular channel formation in the terminal layer of hypertrophic cartilage. The invading blood vessels bring progenitor mesenchymal cells, which later differentiate into the osteoblasts and chondroclasts/osteoclasts involved in endochondral ossification. Some growth factors regulate VEGF expression and participate in different stages of endochondral ossification.

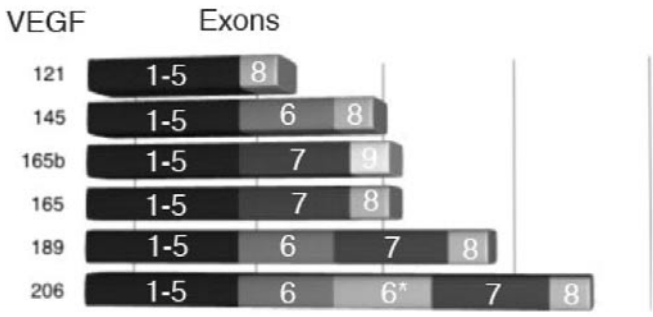

Figure 2. VEGF-A isoforms. There are at least 6 different isoforms of VEGF-A that arise by alternative exon splicing. $VEGF_{165}$ is the predominant molecular species among the isoforms.

molecules have been implicated as positive regulators of angiogenesis, including members of the vascular endothelial growth factor (VEGF) family, fibroblast growth factor (FGF), transforming growth factor (TGF)-α, TGF-β, hepatocyte growth factor (HGF), tumor necrosis factor (TNF)-α, angiogenin, interleukin (IL)-8, and the angiopoietins (Folkman and Shing, 1992; Yancopoulos *et al.*, 2000). Among these pro-angiogenic factors, VEGF, a homodimeric protein of 34 to 42 kDa, is a fundamental and potent regulator of normal and abnormal angiogenesis (Senger *et al.*, 1993). It was first identified by Ferrara and Henzel (1989) as an endothelial-specific growth factor from bovine pituitary follicular cells, and is produced by many types of cells, including fibroblasts, smooth-muscle cells, hypertrophic chondrocytes, and osteoblasts (Gerber *et al.*, 1999; Ferrara and Gerber, 2001; Gerber and Ferrara, 2003). Furthermore, VEGF-mediated angiogenesis is pivotal in growth of the long bones and the mandibular condyle (Gerber *et al.*, 1999; Rabie and Hagg, 2002; Rabie *et al.*, 2002a; Leung *et al.*, 2004). Recent studies have shown that VEGF may act as an essential mediator during these processes and that it has multiple functions, not only in bone angiogenesis, but also in different aspects of bone development, including chondrocyte differentiation, osteoblast differentiation, and osteoclast recruitment (Zelzer *et al.*, 2004; Zelzer and Olsen, 2005).

This review summarizes the ongoing efforts made in: (1) characterizing VEGF family members, especially VEGF-A, and their receptors; (2) investigating the regulatory system of VEGF-A expression; (3) exploring the multiple roles of VEGF-A in endochondral ossification of long bones, as well as in mandibular condylar growth; and (4) developing therapeutic applications for recombinant protein and gene therapy.

THE VEGF FAMILY AND ITS CHARACTERISTICS

VEGF is now understood to be one of a family of VEGFs that includes VEGF, placenta growth factor (PLGF), and VEGF-B, -C, -D, -E, and -F (Veikkola and Alitalo, 1999; Ferrara *et al.*, 2003; Roy *et al.*, 2006). They all share a common structure of 8 characteristically spaced cysteine residues in a VEGF homology domain.

VEGF-A

VEGF, generally referred to as VEGF-A, is a fundamental mediator of physiologic and pathophysiologic angiogenesis. It is also called vascular permeability factor (VPF) and is produced by cultured vascular smooth-muscle cells (Tischer *et al.*, 1991). Structurally, VEGF-A is an antiparallel dimer that has receptor-binding sites at each pole of the dimer. The VEGF-A gene has been mapped to human chromosomes 6p21.3 (Vincenti *et al.*, 1996), which can be alternatively spliced to generate at least 6 distinct isoforms of 121, 145, 165, 189, and 206 amino acids (termed $VEGF\text{-}A_{121}$, $VEGF\text{-}A_{145}$, $VEGF\text{-}A_{165}$, $VEGF\text{-}A_{189}$, and $VEGF\text{-}A_{206}$, respectively); a variant of $VEGF\text{-}A_{165}$, namely, $VEGF\text{-}A_{165b}$, has also been identified (Tischer *et al.*, 1991) (Fig. 2, Table 1). Murine VEGF-As are shorter than the corresponding human isoforms by 1 amino acid, and they are denoted $VEGF\text{-}A_{120}$, $VEGF\text{-}A_{144}$, $VEGF\text{-}A_{164}$, and $VEGF\text{-}A_{188}$ (Tamayose *et al.*, 1996). The VEGF-A isoforms have distinct biological activities, such as differential interaction with heparan sulfate proteoglycans (HSPGs) and neuropilin (NRP). The amino acids that are encoded by exons 1-5 are conserved in all isoforms, but alternative splicing can occur in exons 6 and 7 (Houck *et al.*, 1991).

$VEGF\text{-}A_{121}$, the shortest splice form, is acidic and is more freely diffusible. It lacks exons 6 and 7, which encode the binding domains of HSPGs and NRPs. Consequently, $VEGF\text{-}A_{121}$ does not bind either HSPGs or NRPs. The decreased retention of $VEGF\text{-}A_{121}$ on the cell surface and in the ECM renders this isoform highly diffusible, which acts as a mitogenic and chemoattractant for endothelial cells during angiogenesis and vasculogenesis (Houck *et al.*, 1992).

$VEGF\text{-}A_{145}$, an isoform lacking exons 6b and 7, appears in an intermediary manner, since it is secreted, but a significant segment remains bound to the cell surface and ECM (Ferrara and Davis-Smyth, 1997). $VEGF\text{-}A_{145}$ binds to VEGFR-1, VEGFR-2, HSPG, and NRP-2, but not to NRP-1 (Poltorak *et al.*, 1997; Gluzman-Poltorak *et al.*, 2000). Binding to HSPG, $VEGF\text{-}A_{145}$ was found to induce endothelial cell proliferation and *in vivo* angiogenesis (Poltorak *et al.*, 1997). However, the

Table 1. Properties of the VEGF-A Isoforms

Isoforms	Structure	Binding Ability	Functions	References
VEGF-A_{121}	Lacks exons 6 &7	VEGFR-1,-2	↑* EC proliferation, migration, osteoclastogenesis	Houck *et al.*, 1992; Wise and Yao, 2003
VEGF-A_{145}	Lacks exons 6b &7	VEGFR-1,-2, HSPG, NRP-2	↑ EC proliferation	Poltorak *et al.*, 1997
VEGF-A_{165}	Lacks exons 6a & 6b	VEGFR-1,-2, HSPG, NRP-1,-2	↑ EC proliferation, permeabilization, osteoclastogenesis	Wise and Yao, 2003; Cramer *et al.*, 2004
VEGF-A_{165b}	Lacks exons 6a & 6b	VEGFR-1,-2, HSPG, NRP-1,-2	↓ EC proliferation, migration	Cui *et al.*, 2004
VEGF-A_{189}	Contains all the exons	HSPG, NRP-1,-2	↑ EC proliferation, migration	Herve *et al.*, 2005
VEGF-A_{206}	Contains all the exons	HSPG, NRP-1,-2	ND	Ferrara and Davis-Smyth, 1997

* ↑ induced, ↓ inhibited. EC, endothelial cells; ND, not determined.

actual physiological roles of VEGF-A_{145}'s affinity to NRP-2 remain unclear.

VEGF-A_{165}, lacking exons 6a and 6b, is physiologically the most abundant isoform of VEGF-A (Tischer *et al.*, 1991). It binds to all of the receptors, including VEGFR-1, VEGFR-2, HSPGs, NRP-1, and NRP-2. Moreover, the affinity to VEGFR-1 is 10-fold higher than that to VEGFR-2 (Waltenberger *et al.*, 1994). VEGF-A_{165} and VEGF-A_{121} are highly expressed in muscle, brain, kidney, eye, and chondrocytes (Gengrinovitch *et al.*, 1995). However, mice expressing only the VEGF-A_{120} isoform (VEGF-$A^{120/120}$) presented with delayed recruitment of blood vessels into the perichondrium and delayed invasion of vessels into the primary ossification center (Zelzer *et al.*, 2002), whereas VEGF-$A^{164/164}$ mice showed normal vascular development. In contrast, VEGF-A_{121} was reported to have 10- to 100-fold less endothelial cell mitogenic activity than VEGF-A_{165}, due to lack of a HSPG-binding basic region (Gitay-Goren *et al.*, 1996). Moreover, when chondrocytes are cultured in low oxygen conditions, they secrete VEGF-A_{165}. Both VEGF-A_{165} and VEGF-A_{121} might promote angiogenesis contributing to long bone development, cause permeabilization of blood vessels, induce proliferation of vascular endothelial cells, and stimulate osteoclastogenesis for tooth eruption (Ng *et al.*, 2001; Wise and Yao, 2003; Cramer *et al.*, 2004). The splice variant VEGF-A_{165b} contains the same quantity of amino acids as VEGF-A_{165}, except for 6 amino acids in the C-terminal region, denoted exon 9 (Bates *et al.*, 2002; Woolard *et al.*, 2004). The C-terminal is critical for mitogenic signaling; hence, the differentiation in the VEGF-A_{165b} variant is likely to affect its biological activity (Cui *et al.*, 2004; Woolard *et al.*, 2004; Byrne *et al.*, 2005). Although VEGF-A_{165b} can bind VEGFR-2, its binding leads to neither phosphorylation of the receptor nor activation of the downstream signal pathway. VEGF-A_{165b} is an endogenously inhibitory form of VEGF-A and reduces VEGF-A-activated proliferation and migration of endothelial cells (Cui *et al.*, 2004; Woolard *et al.*, 2004).

VEGF-A_{189} and VEGF-A_{206} contain all the exons. VEGF-A_{189} has an insertion of 24 amino acids that are highly enriched in basic residues, and VEGF-A_{206} has an additional insertion of 17 amino acids. These features mean that VEGF-A_{189} and VEGF-A_{206} are not secreted from cell surfaces, but are tightly bound to HSPGs and NRP, allowing for the sequestration of VEGF-A_{189} and VEGF-A_{206} in the ECM and on cell surfaces (Houck *et al.*, 1992; Park *et al.*, 2003). VEGF-A_{189} is usually present in low amounts, and VEGF-A_{206} expression is restricted to embryonic tissues (Ferrara and Davis-Smyth, 1997). Hence, they are less active than other isoforms secreted *in vivo*, such as VEGF-A_{121}, VEGF-A_{145}, and VEGF-A_{165}. Protease cleavage of matrix-bound VEGF-A_{189} allows for the release of an active, freely diffusible 110-amino-acid fragment (Lee *et al.*, 2005). A recent study demonstrated that VEGF-A_{189} could promote endothelial cell proliferation and migration *in vitro*, and angiogenesis in the Matrigel plug assay *in vivo* (Herve *et al.*, 2005). However, VEGF-$A^{188/188}$ mice showed dwarfism, impaired development of growth plates and secondary ossification centers, and knee joint dysplasia. This phenotype was at least partly due to defective vascularization surrounding the epiphysis, leading to ectopically increased hypoxia and massive chondrocyte apoptosis in the interior of the epiphyseal cartilage. In addition, an *in vitro* study showed that the VEGF-A_{188} isoform alone is also insufficient to regulate chondrocyte proliferation and survival responses to hypoxia (Maes *et al.*, 2004).

PLGF

The first member of the VEGF family to be discovered, PLGF, shares 53% identity with the platelet-derived growth factor (PDGF)-like region of VEGF (Nissen *et al.*, 1998). The human PLGF gene has been mapped to chromosome 14q24 (Mattei *et al.*, 1996). PLGF is anticipated to have 149 amino acids and is encoded by 7 exons that span 800 kb (Roy *et al.*, 2006). By alternative splicing, 4 forms of PLGF protein are generated: PLGF-1, PLGF-2, PLGF-3, and PLGF-4 (Maglione *et al.*, 2000). Only PLGF-2 is capable of binding heparin (Hauser and Weich, 1993). Overexpression of PLGF-2 in perivascular tissue raises VEGF-A_{165} and VEGF-A_{121} levels and produces significant angiogenesis (Roy *et al.*, 2006). PLGF has a powerful chemotactic effect on monocytes, since injection of PLGF protein and adenovirus-mediated PLGF gene transfer increases macrophage accumulation (Roy *et al.*, 2005). Furthermore, PLGF deficiency impairs proliferation and differentiation of osteochondroprogenitors during bone repair (Maes *et al.*, 2006).

VEGF-B

VEGF-B, which is also called VEGF-related factor (VRF), encodes 188 amino acids, consists of 8 exons and 6 introns, and is located on chromosome 11q13 (Paavonen *et al.*, 1996). VEGF-B transcripts can be alternatively spliced into two different variants encoding proteins VEGF-B_{167} and VEGF-B_{186} (Li *et al.*, 2001). The promoter region of VEGF-B is

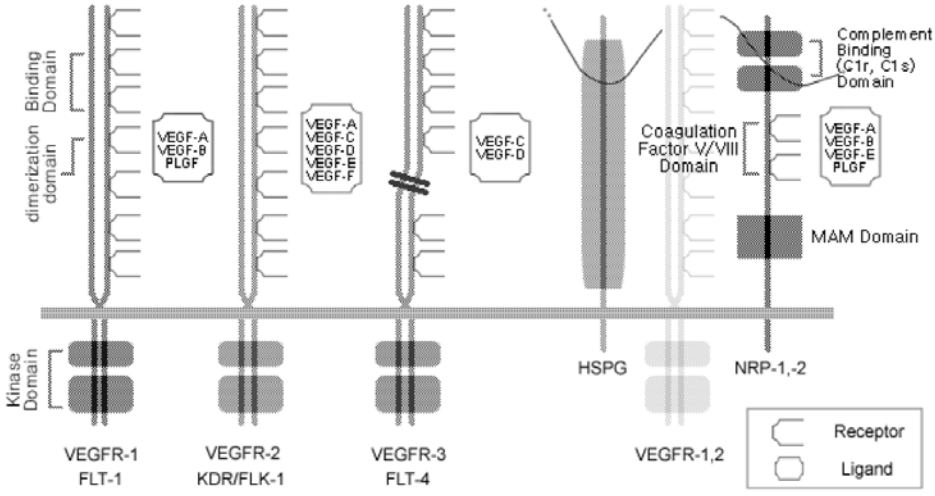

Figure 3. Schematic representation of the VEGF receptors and their ligands. The tyrosine kinase receptors (VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, VEGFR-3/Flt-4) are organized into 7 extracellular immunoglobulin (Ig)-like domains. VEGF binding has been localized to the second and third Ig-like domains. The fourth Ig-like domains are thought to interact directly with each other in a ligand-induced receptor dimer. In VEGFR-3, the fifth Ig domain is replaced by a disulfide bridge. The extracellular domain is followed by a single transmembrane region, a split tyrosine-kinase domain. The interaction of VEGFR-1, -2 with either NRP-1, -2, or HSPG may facilitate the binding of VEGF to its receptor.

similar to that of VEGF-A, in that both promoters are associated with a CpG island and contain transcription-factor-binding sites for Sp1 and AP-2 (Silins *et al.*, 1997). However, the VEGF-B promoter includes Egr-1 sites, not hypoxia-inducible factor-1 and AP-1 sites. Consequently, stimuli such as hypoxia, which can regulate VEGF-A expression, appear to have no effect on the expression of VEGF-B (Enholm *et al.*, 1997; Roy *et al.*, 2006). VEGF-B is highly abundant in the heart, skeletal muscle, and pancreas and is required in vascularization of skeletal muscle (Enholm *et al.*, 1997). Silvestre *et al.* (2003) demonstrated that VEGF-B, partly through its receptor VEGFR-1, induces angiogenesis associated with an activation of Akt and eNOS-related pathways.

VEGF-C

VEGF-C, which is also referred to as VEGF-related protein (VRP), is located on chromosome 4q34 (Paavonen *et al.*, 1996). The VEGF-C gene contains more than 40 kb of genomic DNA and consists of 7 exons (Roy *et al.*, 2006). VEGF-C is a secreted protein that consists of 399 amino acids and was identified as a ligand for the tyrosine kinase receptor VEGFR-3, which is associated with the lymphatic vasculature (Karkkainen *et al.*, 2004). Structurally, VEGF-C shares approximately 30% sequence homology with the central core of VEGF-A. *In vivo,* VEGF-C can stimulate angiogenesis in a rabbit ischemic hind-limb model, rabbit cornea assay, and early chick chorio-allantoic membrane. *In vitro,* VEGF-C can stimulate endothelial cell proliferation and migration, but it is less potent than VEGF-A. Gene transfer of VEGF-C produced moderate angiogenesis in rabbit skeletal as well as perivascular tissue (Bhardwaj *et al.*, 2003). VEGF-C is overexpressed in rheumatoid arthritis (RA) synovial tissues, when compared with osteoarthritis (OA) or normal synovial tissues (Wauke *et al.*, 2002). In addition, tumor necrosis factor (TNF)-α has been recently identified to induce VEGF-C expression significantly in rheumatoid synoviocytes in a dose-dependent manner. It suggests that VEGF-C may be indispensable for the pathogenesis of RA through contributing to local lymphangiogenesis and tumor angiogenesis (Cha *et al.*, 2007).

VEGF-D

VEGF-D, also called c-fos-induced growth factor (FIGF), can activate the receptors VEGFR-2 and VEGFR-3 in humans, but only VEGFR-3 in mice (Baldwin *et al.*, 2001). It is expressed in many adult tissues, including the limb buds, teeth, liver, and heart as well as the lung and kidney mesenchyme and periosteum of the vertebral column (Avantaggiato *et al.*, 1998). The human VEGF-D gene is 2.0 kb in size and is located on chromosome Xp22.31 (Yamada *et al.*, 1997). VEGF-D is mitogenic for endothelial cells *in vitro* (Bhardwaj *et al.*, 2003), angiogenic *in vivo* and *in vitro* (Marconcini *et al.*, 1999), and lymphangiogenic in tumors (Miyata *et al.*, 2006). Compared with other human VEGFs, VEGF-D is thought to be the most potent angiogenic and lymphangiogenic factor when delivered into rabbit hindlimb skeletal muscle through adenoviral vector-mediated gene transfer (Rissanen *et al.*, 2003).

VEGF-E

VEGF-E was first identified in the genome of NZ-7, NZ-2, and D1701 strains of the Orf virus, a parapoxvirus that infects goats, sheep, and occasionally humans (Ferrara and Davis-Smyth, 1997; Ogawa *et al.*, 1998). VEGF-E exclusively binds to VEGFR-1 with high affinity, similar to VEGF-A_{165}, resulting in receptor autophosphorylation and a biphasic rise in the intracellular concentration of free calcium ions (Meyer *et al.*, 1999). Although VEGF-E does not bear a heparin-binding basic region, structurally similar to VEGF-A_{121}, its biological activities for endothelial cell growth and vascular permeability are almost equal to those of the potent angiogenesis and vascular permeability factor, VEGF-A_{165} (Ogawa *et al.*, 1998). This VEGF homolog is a potent angiogenesis stimulator and can induce the proliferation, migration, sprouting, and mitotic activity of vascular endothelial cells. More recently, Inoue *et al.* (2006) reported that plasmid-mediated VEGF-E/PLGF chimera gene therapy significantly promoted angiogenesis in a rat model of hindlimb ischemia without stimulating inflammatory cell infiltration.

VEGF-F

The seventh member of the VEGF family, VEGF-F, was recently identified from snake (viper) venom (Suto *et al.*, 2005). VEGF-F consists of 2 VEGF-related proteins, designated vammin (110 residues) and VR-1 (109 residues), both of which have a 50% primary structural identity with VEGF-A_{165} and bind selectively to VEGFR-2 (Suto *et al.*, 2005). VEGF-F contains a short C-terminal heparin-binding region; the C-terminus of VEGF-F specifically blocks VEGF-A_{165} activity both *in vitro* and *in vivo* (Yamazaki *et al.*, 2005).

VEGF RECEPTORS

The main receptors involved in VEGF signal transduction are the PDGF receptor subfamily of receptor tyrosine kinases (RTKs)—namely, VEGFR-1, VEGFR-2, VEGFR-3—as well as co-receptors, such as NRP-1 and -2 and HSPGs (Ferrara *et al.*, 2003) (Fig. 3). More than one type of VEGF receptor contributes to the biological responses to VEGF, thereby ensuring balanced signaling.

VEGFR-1

VEGFR-1, or fms-like tyrosine kinase receptor 1 (flt-1), is a 180-kDa transmembrane protein that is considered to be integral to vascular maintenance and the recruitment of endothelial precursor cells during vasculogenesis (Shibuya, 2006). It binds to all the isoforms of VEGF-A, VEGF-B, and PLGF, and transduces the distinct biological responses (Park *et al.*, 1994; Olofsson *et al.*, 1998). Autiero *et al.* (2003) suggested that the mechanism underlying the different responses of VEGF and PLGF might be the induction of different phosphorylation patterns in VEGFR-1. Another important feature of VEGFR-1 is that the soluble VEGFR-1 can function as a natural VEGF-A inhibitor (a decoy receptor). These unique characteristics of VEGFR1 suggest that it acts as both a negative regulator, *via* its ligand-binding domain mediated by VEGFR-2, and a positive regulator, *via* its tyrosine kinase (Gille *et al.*, 2001). Moreover, VEGFR1 might be influenced through interaction with VEGFR-2. VEGFR-$1^{-/-}$ embryonic stem cells resulted in the increased differentiation of endothelial cells, but assembly of these cells into abnormal vascular channels mediated by the increased phosphorylation of VEGFR-2 (Fong *et al.*, 1995; Roberts *et al.*, 2004). In contrast, VEGFR-2-mediated proliferation of endothelial cells can be suppressed by VEGFR-1, and this effect is dependent on PI3K (Zeng *et al.*, 2001). More recently, mice that were deficient in flt-1 signaling (*op/opFlt1*$^{TK-/-}$) were shown to have very depleted numbers of osteoclasts and osteoblasts, and, consequently, also a deficiency in bone marrow cavity formation. The fibrous tissue gradually filled in the narrow bone marrow cavity, resulting in severe marrow hypoplasia (Niida *et al.*, 2005). These findings suggested that VEGFR-1 signaling is critical for osteoblast activity during bone formation through participation in osteoclastogenesis, as well as in the maintenance of a bone marrow hematopoiesis-supportive microenvironment.

VEGFR-2

VEGFR-2, or kinase insert domain-containing receptor (KDR)/fetal liver kinase 1 (flk-1), is a 230-kDa glycoprotein that is thought to mediate almost all of the observed endothelial cell responses to VEGF (Neufeld *et al.*, 1999). It binds to all the isoforms of VEGF-A, VEGF-C, VEGF-D, and VEGF-F, but not VEGF-B_{167} and PLGF (Ferrara and Davis-Smyth, 1997). VEGFR-2 is predominantly located on the surfaces of endothelial cells and is thought to initiate intracellular signal transduction, which is associated with the integrin-dependent migration of endothelial cells, since it can form a complex with integrin $\alpha V\beta_3$ and the induction of endothelial cell proliferation, migration, and *in vivo* angiogenesis (Hutchings *et al.*, 2003). It has been reported that VEGFR-2-mediated cell migration induced by VEGF-A_{165}, but not by VEGF-A_{121}, is strongly enhanced in the presence of NRP-1 receptors (Shraga-Heled *et al.*, 2007). VEGFR-2 knockout mice showed severely impaired vasculogenesis and hematopoiesis in the embryo, leading to embryonic death (Shalaby *et al.*, 1995). Moreover, expression of VEGFR-2 occurs in a differentiation-dependent manner and declines post-natally (Millauer *et al.*, 1993). In contrast, the binding of VEGF to VEGFR-1 does not seem to result in the induction of cell proliferation, and it is believed that its role is mainly inhibitory (Shraga-Heled *et al.*, 2007).

VEGFR-3

VEGFR-3 or flt-4, a 170-kDa glycosylated protein, is a receptor for VEGF-C and VEGF-D, but not for VEGF-A. It is required for the development of blood as well as lymphatic vessels and is thought to have a general function in blood vascularization during early development (Lohela *et al.*, 2003); later, its role becomes restricted mostly to the development of the lymphatic vascular system (Dayoub *et al.*, 2003). Furthermore, expression of VEGFR-3 has been reported to be higher in human osteoarthritic chondrocytes than in persons without arthritis (Shakibaei *et al.*, 2003).

NRPs

NRPs were recently identified to be VEGF co-receptors and belong to a family of non-tyrosine kinase transmembrane receptors that have a small cytoplasmic domain and multiple extracellular domains (Soker *et al.*, 1998). NRP-1 lacks an intracellular tyrosine kinase domain and therefore must act in conjunction with other receptors to mediate VEGF signaling. It can associate with both VEGFR-1 and VEGFR-2 by specifically binding VEGF-A_{165} and PLGF, VEGF-B, and VEGF-E, but not VEGF-A_{121} (Zelzer *et al.*, 2001). NRP-2 also lacks a cytoplasmic signaling domain and can bind to VEGF-A_{165}, VEGF-A_{145}, and PLGF and can interact with VEGFR-1 (Gluzman-Poltorak *et al.*, 2000, 2001). NRPs act as active mediators in immune responses, neuronal development, and angiogenesis (Klagsbrun *et al.*, 2002). Deletion of the NRP-2 gene impairs formation of small lymphatic vessels, suggesting that NRP-2 may act as a co-receptor for VEGFR-3 (Yuan *et al.*, 2002). Both NRP-1 and NRP-2 can enhance VEGF-A_{121}-induced phosphorylation of VEGFR-2 and VEGF-A_{121}-induced proliferation of endothelial cells. The enhancement of VEGF-A121 activity by NRP-1 has been reported to be accompanied by a significant increase in the binding affinity of VEGFR-2 to VEGF-A_{121} and was not associated with the formation of new VEGFR-2/NRP-1 complexes (Shraga-Heled *et al.*, 2007). NRP-1 expression in cultured MC3T3-E1 osteoblasts was down-regulated in a differentiation-dependent manner and bound to VEGF-A_{165} (Deckers *et al.*, 2000). Consistent with the *in vitro* studies, NRP1 is expressed *in vivo* by osteoblasts, but not osteocytes, in the metaphysis and trabeculae of growing mouse bones and embryonic chick bones (Harper *et al.*, 2001). The overexpression of NRP-1 showed extra digits, suggesting that limb morphogenesis is very sensitive to exogenous neuropilin expression (Kitsukawa *et al.*, 1995). In addition, the binding of semaphorin-3A (an axonal chemorepellent) to NRP-1 may compete with VEGF-A_{165} for an overlapping binding site on NRP-1, and may inhibit the migration of endothelial cells and the outgrowth of capillary tubes from rat aortic segments (Narazaki and Tosato, 2006). Thus, NRP-1 appears to be a novel regulator of osteoblast activity in development of the skeletal system, and semaphorin-3A might affect its affinity to VEGF-A_{165}. However, the contribution of NRP-2 to bone development is still unclear.

Table 2. Regulation of VEGF Gene Expression

Factors	Functions	Role in VEGF Expression	References
HIF1a	Hypoxic mediator and regulates chondrocyte metabolism	up-regulation	Dibbens *et al.*, 1999
Cbfa1	Regulates osteoblast differentiation and chondrocyte maturation	up-regulation	Zelzer *et al.*, 2001
BMPs	Regulate chondrocyte and osteoblast differentiation	up-regulation	Yeh and Lee, 1999
TGF-β1	Regulates osteoblast differentiation	up-regulation	Spector *et al.*, 2000
CTGF	Regulates chondrocyte proliferation and ECM production	up-regulation	Ivkovic *et al.*, 2003
MMP-9	Regulates ECM degradation	up-regulation	Vu *et al.*, 1998
TNF-α	Regulates osteoclast recruitment and differentiation	up-regulation	Wise and Yao, 2003

HSPGs

HSPGs are transmembrane, glycosylphosphatidylinositol-anchored or secreted proteins that contain covalently linked heparan sulfate chains that modulate the activity of a large number of secreted signaling molecules. HSPGs might regulate the binding of VEGF-A_{165} to its receptors *via* an accessory low-affinity binding site; in this way, HSPGs promote VEGF-A_{165} signaling in transit from the surface of an adjacent cell (Houck *et al.*, 1992; Selleck, 2006). Such ligand-receptor interactions stimulate VEGFR-2 turnover and cause a marked increase in duration and amplitude of the VEGFR signal (Jakobsson *et al.*, 2006; Selleck, 2006). HSPGs also have profound effects on the bioactivity of VEGF-A_{165}, but not VEGF-A_{121}: The high-affinity binding to VEGF-A_{165} affects its diffusion, half-life, and interaction through VEGFR-2 (Larrivee and Karsan, 2000; Stringer, 2006). HSPGs are considered to be both important co-receptors and important regulators of VEGF-A gradients. Exogenous heparin is known to inhibit both binding ability between VEGFR-2 and VEGF-A_{165} and autophosphorylation of VEGFR-2 induced by VEGF-A_{165}, but it has no effect on the interaction of VEGFR-2 with VEGF-A_{121} (Tessler *et al.*, 1994). In contrast, exogenous heparin significantly inhibits the affinity for VEGFR-1 with both VEGF-A_{165}- and VEGF-A_{121} (Cohen *et al.*, 1995). These observations suggest that the C-terminal region of VEGF-A_{165} is important in the interaction of cell-associated VEGFR-2, while heparin affects the activity role of VEGFR-1 independently of the heparin-binding ability of the ligands (Yamazaki and Morita, 2006).

REGULATION OF VEGF-A GENE EXPRESSION

Several mechanisms have been demonstrated to be involved in the regulation of VEGF expression, including hypoxia and several growth factors and hormones. An overview of the regulation factors appears in Table 2, and these will be discussed in the following sections.

Hypoxia

Oxygen plays a critical role, both *in vitro* and *in vivo*, in the regulation of VEGF-A expression. Hypoxic induction of VEGF-A appears to be a ubiquitous response, in which the 28-base consensus sequence in the 5′ VEGF promoter is augmented by a 3′ enhancer (Levy *et al.*, 1995). Under hypoxic conditions, hypoxia-inducible factor-1α (HIF-1α) binds to the 5′ consensus sequence in the VEGF-A promoter, termed the 'hypoxia responsive element', which in turn increases VEGF-A transcription (Madan and Curtin, 1993). Hypoxia-induced VEGF-A up-regulation is controlled at both the transcriptional and post-transcriptional levels (Dibbens *et al.*, 1999). A study of human osteoblast-like MG63 cells revealed that transcriptional regulation of VEGF-A mRNA expression under hypoxic conditions is mediated through increased levels of the basic-helix-loop-helix transcription factor Hif-2α, but not Hif-1α (Akeno *et al.*, 2001). The induction of VEGF-A by Hif-2α is likely to represent a physiologically relevant response to hypoxia in osteoblasts. However, HIF-1α-null chondrocytes were unable to maintain ATP levels in hypoxic microenvironments, indicating a fundamental requirement for HIF-1α in the regulation of chondrocyte metabolism, and hence vascularization during long-bone development (Pfander *et al.*, 2003; Cramer *et al.*, 2004).

Cbfa1

Cbfa1 (core-binding factor a1, also called runx2, Osf2, or AML3), a homolog of the *Drosophila* Runt protein, is a transcription factor that is required for endochondral and intramembranous bone formation. It is expressed in osteoblasts, pre-chondrogenic mesenchymal condensations, and hypertrophic chondrocytes, where it serves as the earliest transcriptional regulator of osteoblast differentiation (Rabie *et al.*, 2004; Tang and Rabie, 2005). Cbfa1 is sufficient to induce premature and ectopic chondrocyte hypertrophy (Rabie *et al.*, 2004). In Cbfa1-deficient mice, VEGF-A cannot be up-regulated, chondrocyte differentiation to hypertrophy is impaired, and cartilage angiogenesis does not occur. These findings demonstrate that Cbfa1 is an essential factor for mediating VEGF-A functions during endochondral ossification (Zelzer *et al.*, 2001). Whether Cbfa1 acts as a direct transcriptional regulator of the VEGF-A gene is unclear. However, the VEGF-A promoter contains Cbfa1 binding sites, and over-expression of Cbfa1 in cultured fibroblasts increases both mRNA and protein expression levels of VEGF (Zelzer *et al.*, 2001).

BMPs

Bone morphogenetic proteins (BMP) are multi-functional growth factors that belong to the TGF-β superfamily. The addition of BMP-2, -4, and -6 to the murine osteoblast-like cell line KS483 led to an augmentation of calcium deposition and VEGF-A production in a dose-dependent manner (Deckers *et al.*, 2000). Osteogenic protein-1 (OP-1 or BMP-7) increased the steady-state level of VEGF-A mRNA by about three-fold in an OP-1 concentration- and time-dependent manner in primary cultures of fetal rat calvaria cells. The increase in VEGF-A

mRNA level depended on transcription and was sensitive to cell replication (Yeh and Lee, 1999). The mRNA levels for VEGFR-1 and VEGFR-2 in the fetal rat calvaria cells were low but detectable by RT-PCR, and were not changed by OP-1 (Yeh and Lee, 1999). Kakudo *et al.* (2006) investigated whether recombinant human BMP-2 could cause undifferentiated mesenchymal cells to differentiate into chondrocytes and osteoblasts, which then expressed VEGF, thereby creating an advantageous environment for vascularization in bony tissue. In addition to increased VEGF-A expression, BMP-2 is able to up-regulate VEGF-B and VEGF-C expression and synthesis, as well as increase VEGFR-2 levels (Bluteau *et al.*, 2007).

TGF-β1

At least part of the osteogenic activity of TGF-β1 may be attributed to the production of VEGF-A (Chang *et al.*, 2003). The addition of TGF-β1 to MC3T3-E1 cells induced VEGF-A mRNA production, and this induced expression was superinduced by cycloheximide and blocked by actinomycin D and Ro 31-8220, a protein kinase C (PKC) inhibitor. Curcumin, an inhibitor for transcription factor AP-1, also blocked the induction (Spector *et al.*, 2000). Moreover, phorbolmyristate acetate (PMA), a PKC activator, can enhance VEGF expression in cultured dental follicle cells (Wise and Yao, 2003). Further research has shown that both p44/p42 MAP kinase and p38 MAP kinase contribute to TGF-β-stimulated VEGF-A synthesis in osteoblasts, which suggests a positive feedback loop for osteoblast differentiation (Tokuda *et al.*, 2003a).

CTGF

Connective tissue growth factor (CTGF) is a crucial regulator of cartilage ECM (Perbal, 2004). *Ctgf* deficiency in Ctgf(-/-) mice leads to skeletal dysmorphisms, as a result of impaired endochondral ossification (Ivkovic *et al.*, 2003). These defects are linked to decreased expression of VEGF-A mRNA and protein levels in the expanded hypertrophic zone in newborn *Ctgf* mutants. Thus, VEGF-A is probably a target of CTGF action in hypertrophic cartilage (Ivkovic *et al.*, 2003). Moreover, this decrease is not the result of reduced Cbfa1 expression, suggesting that CTGF is either downstream of, or acts in parallel with, Cbfa1 in regulating VEGF-A expression (Ivkovic *et al.*, 2003). VEGF-A_{165}-induced angiogenesis was reported to be inhibited selectively and strongly by complexing with CTGF. This complex formation interrupts the binding of VEGF-A_{165} to VEGFR-2 (Inoki *et al.*, 2002).

MMP9

Reduced expression of the osteoclastic protease MMP9 affects angiogenesis-inducing activity in growth plates (Vu *et al.*, 1998), and MMP9-null growth plates share several characteristics with mouse growth plates in which VEGF-A activities have been blocked, such as expansion of the hypertrophic layer and reduction in both vascularization and ossification (Vu *et al.*, 1998). Hence, it has been suggested that MMP9 regulates vascular invasion by releasing the VEGF-A that is bound to the hypertrophic cartilage matrix. Once released, VEGF-A can bind to its receptors on endothelial cells, osteoclasts, and osteoblasts, and, in so doing, can stimulate their migration and activity at the fracture site (Nakagawa *et al.*, 2000).

TNF-α

TNF- α is a pro-inflammatory cytokine that enhances VEGF-A expression in follicle cells and periodontal ligament cells (Oyama *et al.*, 2000; Wise and Yao, 2003). The enhanced expression of VEGF-A in the dental follicle cells by TNF-α is considerable and could be the main avenue by which TNF-α expression leads to the recruitment and differentiation of osteoclasts (Wise and Yao, 2003).

Hormones

It is widely recognized that the role of thyroid hormone in bone metabolism is mainly ascribed to the regulation of osteoblast activities. Triiodothyronine (T_3) alone could stimulate VEGF-A release in MC3T3-E1 cells, so it is likely that thyroid hormone stimulates bone metabolism partially *via* the up-regulation of VEGF-A release (Tokuda *et al.*, 2003b). In human osteoblast-like cells, a steady expression of VEGF-A is induced by 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (Wang *et al.*, 1996). Furthermore, when alkaline phosphatase(ALP)-positive human osteoblast-like cells are co-cultured with ALP-negative human umbilical vein endothelial cells, the addition of 1,25-$(OH)_2D_3$ stimulates ALP activity in the osteoblast-like cells, and increases the expression of VEGF mRNA, followed by increased secretion of VEGF-A (Petit *et al.*, 1997). More recently, ED-71, a novel vitamin D[1,25-$(OH)_2D_3$] analog, has been demonstrated to improve angiogenesis within the bone marrow cavity through the increased expression of VEGF-A_{120} (Okuda *et al.*, 2006).

ANGIOGENESIS AND ENDOCHONDRAL OSSIFICATION

VEGF-A is predominantly produced in tissues that acquire new capillary networks (Shweiki *et al.*, 1993), and the importance of VEGF-A during angiogenesis has been demonstrated in gene knockout studies. Homozygous *Vegfa* knockout mice die at embryonic days E8-E9, and mice lacking a single *Vegfa* allele die at days E11-E12, due to deficient endothelial cell development and lack of blood vessels (Carmeliet *et al.*, 1996; Tammela *et al.*, 2005). Moreover, inducible deletion of VEGF-A in neonatal mice results in stunted growth and increased apoptosis of endothelial cells (Gerber *et al.*, 1999). The knockout of one allele of VEGFR-2 in mice also resulted in lack of normal blood vessel development and embryonic lethality (Shalaby *et al.*, 1995). The failure of vasculogenesis leads to growth retardation, developmental anomalies, and, ultimately, embryonic lethality (Carmeliet *et al.*, 1996). Using a VEGF-A120/120 mouse model created by *Cre-loxP*-mediated removal of VEGF-A exons 6 and 7, researchers showed that VEGF-A_{164} and/or VEGF-A_{188} is important in both mediating vascularization and ensuring the normal differentiation of progenitors into hypertrophic chondrocytes, osteoblasts, endothelial cells, and osteoclasts (Maes *et al.*, 2002, 2004).

Hence, VEGF-A is not only essential for normal angiogenesis, but it is also involved in endochondral ossification (Midy and Plouet, 1994; Gerber *et al.*, 1999; Rabie *et al.*, 2002a; Aldridge *et al.*, 2005) (Fig. 1). Cells in the upper zone of the hypertrophic cartilage, especially in the mandibular condyle, secrete VEGF-A, which then regulates the invasion of new blood vessels from the perichondrium and influences the removal of the cartilage matrix (Rabie and Hagg, 2002; Rabie *et al.*, 2002a). The invading blood vessels bring progenitor mesenchymal cells to the mineralization front, and the cells later differentiate into osteoblasts and chondroclasts/osteoclasts involved in endochondral ossification (Rabie and Hagg, 2002;

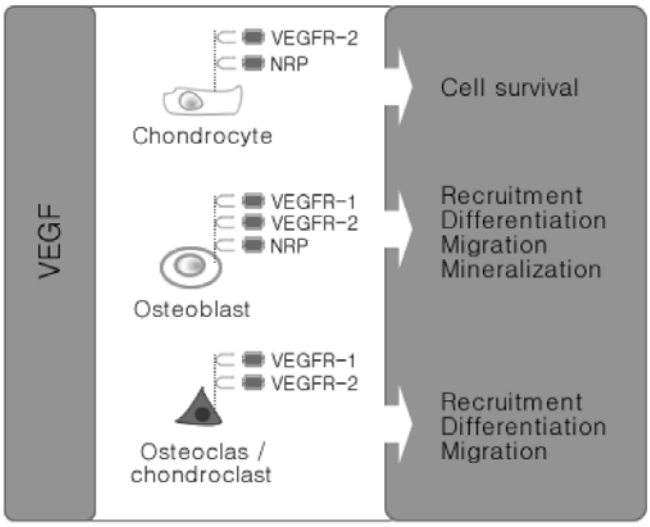

Figure 4. Schematic representation of the role of VEGF and the corresponding receptors on osteoblasts, chondrocytes, and chondroclasts/osteoclasts.

Leung *et al.*, 2004). In 24-day-old mice, inactivation of VEGF-A through the systemic administration of a soluble VEGF-A receptor protein, Flt-(1-3)-IgG, suppresses blood vessel invasion, impairs trabecular bone formation, and expands the hypertrophic zone in the growth plate (Gerber *et al.*, 1999). The chondrocyte-specific inactivation of VEGF-A results in aberrant endochondral bone formation, lack of normal blood vessel development, and embryonic lethality in transgenic mice (Haigh *et al.*, 2000). Therefore, VEGF-A seems to play a key role in the highly regulated process of endochondral ossification, which depends on the interplay of chondrocytes, osteoblasts, and osteoclasts/chondroclasts (Fig. 4).

VEGF-A Regulates Chondrocyte Activity

In rats, VEGF-A protein is expressed at low levels in maturing chondrocytes; however, it is expressed at high levels in hypertrophic chondrocytes and in mineralized regions of the cartilage of the growth plate in neonatal wild-type and in the mandibular condyle of growing and adult rats (Carlevaro *et al.*, 2000; Rabie and Hagg, 2002; Henriksen *et al.*, 2003; Leung *et al.*, 2004; Xiong *et al.*, 2005). With the C28/12 cell line (immortalized chondrocytes), the proliferation of chondrocytes can be induced by the addition of VEGF in a dose-dependent manner (Pufe *et al.*, 2004). In addition, VEGF-A, VEGF-B, VEGF-C, and VEGF-D were detectable and up-regulated at the mRNA and protein levels during chondrogenic differentiation of primary chondrocytes in the ATDC5 chondrogenic cell line (Bluteau *et al.*, 2007). More recently, some studies have demonstrated that VEGF-A secretion by hypertrophic chondrocytes is not only a paracrine process that attracts and stimulates proliferation of endothelial cells, but is also an autocrine process that is needed for chondrocyte survival (Maes *et al.*, 2004; Zelzer *et al.*, 2004; Zelzer and Olsen, 2005). Moreover, culture of chondrocytes under hypoxic conditions up-regulates VEGF-A expression *via* H1F-1a (Pfander *et al.*, 2003). Thus, VEGF-A signaling appears to occur downstream of HIF-la in the chondrocyte survival pathway. Recently, VEGFR-2 was revealed to co-localize with VEGF-A in the hypertrophic layer, whereas VEGFR-1 and VEGFR-3 were rarely detectable (Carlevaro *et al.*, 2000). Similarly, NRP-1 and NRP-2 expression by chondrocytes may be an autocrine/paracrine process that maintains chondrocyte survival and controls cell differentiation and proliferation (Maes *et al.*, 2004; Zelzer *et al.*, 2004; Zelzer and Olsen, 2005; Bluteau *et al.*, 2007).

VEGF-A Regulates Chondroclast/Osteoclast Activity

It seems that VEGF-A is involved in controlling endothelial cell activities, as well as in osteoclastic differentiation, migration, and activity (Zelzer and Olsen, 2005). In addition, cartilage and bone resorption by chondroclasts and osteoclasts regulates vascular invasion and relies on degradation of the cartilaginous matrix, which occurs mainly *via* the release of VEGF-A (Rabie *et al.*, 2002a,b). In fact, osteoclasts express VEGF receptor 1, which can bind VEGF-A to induce osteoclast recruitment and bone-resorption activity (Niida *et al.*, 2005). A single injection of recombinant human VEGF-A (rhVEGF-A) into osteopetrotic op/op mice (lack of CSF-1) induced osteoclast recruitment and survival, while stimulating osteoclastic bone resorption (Niida *et al.*, 1999). In contrast, the inhibition of VEGF-A resulted in impaired angiogenesis and a decrease of chondroclast and osteoblast numbers in growth plates (Dass *et al.,* 2007). It has been suggested that VEGF-A is involved not only in osteoclastic recruitment and differentiation, but also in enhancing osteoclastic bone-resorbing activity in cultured rabbit mature osteoclasts (Nakagawa *et al.*, 2000). A recent study demonstrated that VEGF-A-elicited pathways are involved in the induction of receptor of NF-κB ligand (RANKL) expression, suggesting that VEGF-A plays an important role in modulating the angiogenic action of RANKL under physiological or pathological conditions (Henriksen *et al.*, 2003). In the process of monocytic precursor cell differentiation, Niida *et al.* (1999) demonstrated that VEGF-A can substitute for CSF-1 in the presence of RANKL to promote osteoclastogenesis. Semi-quantitative RT-PCR and fluorescence-activated cell-sorter analysis revealed that VEGF-A can significantly increase both mRNA expression and surface protein expression of receptor activator of NF-κB (RANK) in human endothelial cells, thereby increasing the angiogenic responses of endothelial cells to RANKL (Min *et al.*, 2003). This up-regulation takes place mainly through the Flk-1/KDR-PKC-ERK signaling pathway (Min *et al.*, 2003). VEGF-A can also up-regulate RANKL expression in osteoclast precursors, but it cannot fully substitute for CSF-1 to promote proliferation and osteoclastogenesis (Yao *et al.*, 2006). Furthermore, according to a review by Zelzer and Olsen (2005), VEGF-A is produced by hypertrophic chondrocytes, induces osteoclastogenesis in the perichondrium, and stimulates migration of the osteoclasts into hypertrophic cartilage through the extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway (Henriksen *et al.*, 2003; Min *et al.*, 2003).

VEGF-A Regulates Osteoblastic Activity

Although the exact mechanism of VEGF-A production and secretion in osteoblasts has not yet been fully clarified, VEGFs have been implicated in various aspects of osteoblast function. *In vitro,* VEGF-A mRNA was found to be expressed in rat calvaria-derived osteoblast-enriched cells (Harada *et al.*, 1994). During calvaria organ culture, treatment with $VEGF\text{-}A_{164}$ led to

Table 3. Effects of VEGF Protein and Gene Therapy on Endochondral Ossification

Growth Factor	Delivery Method	Animal Model	Effects	References
rhVEGF VEGFR-2/Fc	Microsphere injection	Rabbit tibia distraction osteogenesis	VEGF increased the blood flow in distracted bone, and VEGF-inhibitor decreased bone blood flow, but failed to influence bone mineral content during bone regeneration	Eckardt *et al.*, 2003
rhVEGF	PLGA scaffolds	Rat radiated calvarial defects	Blood vessel formation, bone coverage, and bone mineral density were induced.	Orlandini *et al.*, 2006
rhVEGF	Bioactive glass	Rat cranial bone defects	Blood vessel density and bone mineral density were improved.	Leach *et al.*, 2006
rhVEGF	Demineralized bone matrix	Rabbit parietal bone defects	New bone formation was enhanced.	Rabie *et al.*, 1996
rhVEGF	Local injection	Rat experimental tooth movement	The number of osteoclasts during experimental tooth movement and the amount of tooth movement were improved.	Kaku *et al.*, 2001; Kohno *et al.*, 2003
rhVEGF	Collagen type I carrier	Rabbit mandibular defect	More intensive angiogenesis and increased bone regeneration	Kleinheinz *et al.*, 2005
VEGF	Gene-activated matrix	Rabbit bone defects	Vascularization and bone regeneration were improved.	Geiger *et al.*, 2005
VEGF BMP-4	Retrovirus transferred MDSCs*	Mouse calvarial defects	VEGF had a synergistic effect with BMP_4 to recruit more mesenchymal stem cells, and to induce cartilage formation and remodeling during endochondral ossification.	Peng *et al.*, 2002
VEGF-A	Adenovirus local injection	Rat femur defect	Shortened endochondral phase and enhanced angiogenesis and osteogenesis	Tarkka *et al.*, 2003
VEGF-A	Adenovirus local injection	Rabbit femur	Stimulated osteoblast activity and more bone formation	Hiltunen *et al.*, 2003
$VEGF_{164}$ RANKL	AAV** local injection	Rat mandibular condyle	Increased size of mandibular condyle	Rabie *et al.*, 2007
VEGF	AAV-coated allografts	Mouse femoral defect	Increased bone remodeling and vascularization, which led to a new bone collar around the graft.	Ito *et al.*, 2005

* MDSC, muscle-derived stem cells.
** AAV, adeno-associated virus.

a significant increase in the thickness of parietal bone, demonstrating a stimulatory effect of VEGF-A on bone formation (Zelzer *et al.*, 2002). Several mechanisms for controlling osteoblast activity have been proposed. First, VEGF-A could couple angiogenesis and osteogenesis by manipulating the angiogenic response to osteoblastic activity. Second, VEGF-A could act as an autocrine regulator of osteoblastic differentiation and activity. Third, by expressing VEGF-A, osteoblasts could induce cells in the vicinity to express factors that, in turn, regulate osteoblastic activity (Zelzer and Olsen, 2005). The following studies support all three possibilities.

In 24-day-old mice, the inhibition of VEGF-A by soluble Flt-1 decreased angiogenesis, reduced cartilage formation, and delayed cartilage resorption (Gerber *et al.*, 1999). VEGF-A can also directly promote differentiation of human primary cultured osteoblasts (Street *et al.*, 2002). Deckers *et al.* (2000) showed that a low level of VEGF-A expression occurs at the early stage of osteoblast differentiation, before the detection of mRNAs encoding bone sialoprotein and osteocalcin. Only during the terminal differentiation of osteoblasts is their expression greatly increased, achieving a maximum level during the period of mineralization (Deckers *et al.*, 2000). This finding suggests that VEGFs are indispensable in the regulation of bone remodeling, because they stimulate osteoblast differentiation. Moreover, the amount of increase in VEGF expression during osteoblastogenesis depends on the maturity of the osteoblastic cells (Furumatsu *et al.*, 2003). Finally, VEGF-A acts as a potent chemoattractant for rat and human osteoblasts, as well as bone-marrow-derived mesenchymal progenitor cells (Nakagawa *et al.*, 2000; Mayr-Wohlfart *et al.*, 2002; Fiedler *et al.*, 2005). VEGF treatment induces VEGF-D expression in osteoblasts, and the inactivation of VEGF-D activity by neutralizing antibodies or VEGFR-3 silencing inhibits both VEGF-A- and VEGF-D-dependent nodule formation in osteoblasts, suggesting that VEGF-D is a downstream effector of VEGF-A in osteogenesis (Orlandini *et al.*, 2006).

THERAPEUTIC IMPLICATIONS IN ENDOCHONDRAL OSSIFICATION AND PERSPECTIVES

Several experiments with recombinant VEGF proteins or genes have demonstrated that these treatment regimes seem to be safe, and the results have been encouraging for the treatment of coronary and limb ischemia (Street *et al.*, 2002; Kastrup, 2003) and burn wounds (Galeano *et al.*, 2003). However, the VEGF-A that was locally applied to rabbit tibia during distraction osteogenesis increased the blood flow in the distracted limb, but failed to influence bone mineral content and histomorphometric indices of bone regeneration (Eckardt *et al.*, 2003). A possible explanation is that, during distraction osteogenesis, a high level of endogenous VEGF-A has already been secreted, and therefore the additional delivery of VEGF-A has little or no effect, owing to optimal endogenous VEGF-A signaling. Similar results were obtained in *ex vivo* gene therapy based on retrovirus-transferred muscle-derived stem cells (MDSCs): Supplying VEGF-A alone was not sufficient to initiate the cascade of bone regeneration in the critical-sized calvarial defects (Peng *et al.*, 2002). However, VEGF-A acts synergistically with BMP_4 to recruit mesenchymal stem cells and induce cartilage formation and remodeling during endochondral ossification (Peng *et al.*, 2002).

Gain-of-function studies with recombinant VEGF-A proteins or genes have increasingly found significant increases

in vascularization and bone regeneration in defects (Table 3). For example, when poly(lactic-co-glycolic acid) (PLGA) scaffolds that have been incorporated with rhVEGF-A were loaded into radiated calvarial defects of Fisher rats, there were significant increases in blood vessel formation, bone coverage, and bone mineral density when compared with defects loaded with only PLGA scaffolds (Orlandini *et al.*, 2006). Scaffolds coated with a rhVEGF-A-releasing layer (bioactive glass) also demonstrated significant improvements in blood vessel density and bone mineral density in rat cranial bone defects (Leach *et al.*, 2006). The combination of rhVEGF-A and demineralized bone matrix grafts (Rabie, 1997) significantly increased new bone formation in rabbit parietal bone defects (Bassem *et al.*, 2006). Local administration of rhVEGF-A exhibited an increased number of osteoclasts during experimental tooth movement, as well as increases in the amount of tooth movement (Kaku *et al.*, 2001; Kohno *et al.*, 2003). The use of rhVEGF-A in bone defect models has shown that new blood vessel formation preceded the osteogenic front, and that increased angiogenesis corresponded to increased bone formation (Kleinheinz *et al.*, 2005). Although these findings are promising, the possible applications of these growth factors are limited by the need for repeated and expensive dosages, and by their short biological half-life (Zhang *et al.*, 2002).

Recent advances in molecular biology have given promise for the development of novel approaches, and gene therapy for the maximal stimulation of osteogenesis ultimately overcomes conventional growth factor delivery limitations, which act as a one-time bolus (Chen *et al.*, 2002). Using a gene-activated matrix (GAM) to deliver the VEGF-A gene, Geiger *et al.* (2005) demonstrated a significant increase in vascularization and bone regeneration in rabbit bone defects. *In vivo* gene therapy with adenoviral VEGF-A has been proven to be able to modify bone defect healing in the rat femur (Tarkka *et al.*, 2003). Similarly, using a rabbit model, Hiltunen *et al.* (2003) concluded that VEGF-A gene transfer delivered by an adenovirus vector resulted in increased bone formation by stimulating osteoblast activity. However, a major disadvantage of current adenoviral vectors is the induction of a significant host immune response (Jiang *et al.*, 2001). They are therefore inappropriate for the treatment of non-fatal craniofacial anomalies. Among the viral and non-viral vectors, the recombinant adeno-associated virus (rAAV) vector is an efficient and safe procedure to facilitate gene induction in the skeletal system. Ito *et al.* (2005) established an unconventional means of evaluating cortical bone healing with femoral allografts coated with freeze-dried rAAV encoding RANKL and VEGF-A, and identified a continuing active resorption of the dead cortical bone with new bone formation. This approach markedly altered allograft healing and generated a live, vascularized, remodeling, bony union. Local injection of rAAV mediated VEGF-A expression in the condylar cartilage, demonstrating a significantly increased size of the mandibular condyle (Rabie *et al.*, 2007). Therefore, VEGF-A has possible clinical applications for achieving both angiogenesis and bone formation. Further proof-of-concept advances are needed to bring this approach to fruition, and to stimulate maximum osteogenesis.

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REFERENCES

Akeno N, Czyzyk-Krzeska MF, Gross TS, Clemens TL (2001). Hypoxia induces vascular endothelial growth factor gene transcription in human osteoblast-like cells through the hypoxia-inducible factor-2alpha. *Endocrinology* 142:959-962.

Aldridge SE, Lennard TW, Williams JR, Birch MA (2005). Vascular endothelial growth factor receptors in osteoclast differentiation and function. *Biochem Biophys Res Commun* 335:793-798.

Alini M, Marriott A, Chen T, Abe S, Poole AR (1996). A novel angiogenic molecule produced at the time of chondrocyte hypertrophy during endochondral bone formation. *Dev Biol* 176:124-132.

Autiero M, Luttun A, Tjwa M, Carmeliet P (2003). Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J Thromb Haemost* 1:1356-1370.

Avantaggiato V, Orlandini M, Acampora D, Oliviero S, Simeone A (1998). Embryonic expression pattern of the murine figf gene, a growth factor belonging to platelet-derived growth factor/vascular endothelial growth factor family. *Mech Dev* 73:221-224.

Baldwin ME, Catimel B, Nice EC, Roufail S, Hall NE, Stenvers KL, *et al.* (2001). The specificity of receptor binding by vascular endothelial growth factor-d is different in mouse and man. *J Biol Chem* 276:19166-19171.

Bassem E, El-Mofty S, Basma GM, Wong RW, Bendeus M, Rabie AB (2006). Vascular endothelial growth factor augments the healing of demineralized bone matrix grafts. *Int J Surg* 4:160-166.

Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, *et al.* (2002). VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 62:4123-4131.

Bhardwaj S, Roy H, Gruchala M, Viita H, Kholova I, Kokina I, *et al.* (2003). Angiogenic responses of vascular endothelial growth factors in periadventitial tissue. *Hum Gene Ther* 14:1451-1462.

Bluteau G, Julien M, Magne D, Mallein-Gerin F, Weiss P, Daculsi G, *et al.* (2007). VEGF and VEGF receptors are differentially expressed in chondrocytes. *Bone* 40:568-576.

Byrne AM, Bouchier-Hayes DJ, Harmey JH (2005). Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). *J Cell Mol Med* 9:777-794.

Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F (2000). Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *J Cell Sci* 113(Pt 1):59-69.

Carmeliet P (2005). Angiogenesis in life, disease and medicine. *Nature* 438:932-936.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, *et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380:435-439.

Cha HS, Bae EK, Koh JH, Chai JY, Jeon CH, Ahn KS, *et al.* (2007). Tumor necrosis factor-alpha induces vascular endothelial growth factor-C expression in rheumatoid synoviocytes. *J Rheumatol* 34:16-19.

Chang SC, Chuang HL, Chen YR, Chen JK, Chung HY, Lu YL, *et al.* (2003). Ex vivo gene therapy in autologous bone marrow stromal stem cells for tissue-engineered maxillofacial bone regeneration. *Gene Ther* 10:2013-2019.

Chen Y, Cheung KM, Kung HF, Leong JC, Lu WW, Luk KD (2002). In vivo new bone formation by direct transfer of adenoviral-mediated bone morphogenetic protein-4 gene. *Biochem Biophys Res Commun* 298:121-127.

Cohen T, Gitay-Goren H, Sharon R, Shibuya M, Halaban R, Levi BZ, *et al.* (1995). VEGF121, a vascular endothelial growth factor (VEGF)

isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. *J Biol Chem* 270:11322-11326.

Cramer T, Schipani E, Johnson RS, Swoboda B, Pfander D (2004). Expression of VEGF isoforms by epiphyseal chondrocytes during low-oxygen tension is HIF-1 alpha dependent. *Osteoarthritis Cartilage* 12:433-439.

Cui TG, Foster RR, Saleem M, Mathieson PW, Gillatt DA, Bates DO, *et al.* (2004). Differentiated human podocytes endogenously express an inhibitory isoform of vascular endothelial growth factor (VEGF165b) mRNA and protein. *Am J Physiol Renal Physiol* 286:F767-F773.

Dass CR, Tran TMN, Choong PFM (2007). Angiogenesis inhibitors and the need for anti-angiogenic therapeutics. *J Dent Res* 86: 927-936.

Dayoub H, Dumont RJ, Li JZ, Dumont AS, Hankins GR, Kallmes DF, *et al.* (2003). Human mesenchymal stem cells transduced with recombinant bone morphogenetic protein-9 adenovirus promote osteogenesis in rodents. *Tissue Eng* 9:347-356.

Deckers MM, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Lowik CW (2000). Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. *Endocrinology* 141:1667-1674.

Dibbens JA, Miller DL, Damert A, Risau W, Vadas MA, Goodall GJ (1999). Hypoxic regulation of vascular endothelial growth factor mRNA stability requires the cooperation of multiple RNA elements. *Mol Biol Cell* 10:907-919.

Durkin JF, Heeley JD, Irving JT (1973). The cartilage of the mandibular condyle. *Oral Sci Rev* 2:29-99.

Eckardt H, Bundgaard KG, Christensen KS, Lind M, Hansen ES, Hvid I (2003). Effects of locally applied vascular endothelial growth factor (VEGF) and VEGF-inhibitor to the rabbit tibia during distraction osteogenesis. *J Orthop Res* 21:335-340.

Enholm B, Paavonen K, Ristimaki A, Kumar V, Gunji Y, Klefstrom J, *et al.* (1997). Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene* 14:2475-2483.

Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R (1995). Toward a molecular understanding of skeletal development. *Cell* 80:371-378.

Ferrara N, Davis-Smyth T (1997). The biology of vascular endothelial growth factor. *Endocr Rev* 18:4-25.

Ferrara N, Gerber HP (2001). The role of vascular endothelial growth factor in angiogenesis. *Acta Haematol* 106:148-156.

Ferrara N, Henzel WJ (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161:851-858.

Ferrara N, Gerber HP, LeCouter J (2003). The biology of VEGF and its receptors. *Nat Med* 9:669-676.

Fiedler J, Leucht F, Waltenberger J, Dehio C, Brenner RE (2005). VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. *Biochem Biophys Res Commun* 334:561-568.

Folkman J (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1:27-31.

Folkman J, Shing Y (1992). Angiogenesis. *J Biol Chem* 267:10931-10934.

Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376:66-70.

Furumatsu T, Shen ZN, Kawai A, Nishida K, Manabe H, Oohashi T, *et al.* (2003). Vascular endothelial growth factor principally acts as the main angiogenic factor in the early stage of human osteoblastogenesis. *J Biochem (Tokyo)* 133:633-639.

Galeano M, Deodato B, Altavilla D, Squadrito G, Seminara P, Marini H, *et al.* (2003). Effect of recombinant adeno-associated virus vector-mediated vascular endothelial growth factor gene transfer on wound healing after burn injury. *Crit Care Med* 31:1017-1025.

Geiger F, Bertram H, Berger I, Lorenz H, Wall O, Eckhardt C, *et al.* (2005). Vascular endothelial growth factor gene-activated matrix (VEGF(165)-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. *J Bone Miner Res* 20:2028-2035.

Gengrinovitch S, Greenberg SM, Cohen T, Gitay-Goren H, Rockwell P, Maione TE, *et al.* (1995). Platelet factor-4 inhibits the mitogenic activity of VEGF121 and VEGF165 using several concurrent mechanisms. *J Biol Chem* 270:15059-15065.

Gerber HP, Ferrara N (2003). The role of VEGF in normal and neoplastic hematopoiesis. *J Mol Med* 81:20-31.

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 5:623-628.

Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, *et al.* (2001). Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J Biol Chem* 276:3222-3230.

Gitay-Goren H, Cohen T, Tessler S, Soker S, Gengrinovitch S, Rockwell P, *et al.* (1996). Selective binding of VEGF121 to one of the three vascular endothelial growth factor receptors of vascular endothelial cells. *J Biol Chem* 271:5519-5523.

Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G (2000). Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165. *J Biol Chem* 275:18040-18045; *erratum* in 275:29922.

Gluzman-Poltorak Z, Cohen T, Shibuya M, Neufeld G (2001). Vascular endothelial growth factor receptor-1 and neuropilin-2 form complexes. *J Biol Chem* 276:18688-18694.

Gonzalez-Crussi F (1971). Vasculogenesis in the chick embryo. An ultrastructural study. *Am J Anat* 130:441-460.

Guenther HL, Fleisch H, Sorgente N (1986). Endothelial cells in culture synthesize a potent bone cell active mitogen. *Endocrinology* 119:193-201.

Haigh JJ, Gerber HP, Ferrara N, Wagner EF (2000). Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. *Development* 127:1445-1453.

Harada S, Nagy JA, Sullivan KA, Thomas KA, Endo N, Rodan GA, *et al.* (1994). Induction of vascular endothelial growth factor expression by prostaglandin E2 and E1 in osteoblasts. *J Clin Invest* 93:2490-2496.

Harper J, Klagsbrun M (1999). Cartilage to bone—angiogenesis leads the way. *Nat Med* 5:617-618.

Harper J, Gerstenfeld LC, Klagsbrun M (2001). Neuropilin-1 expression in osteogenic cells: down-regulation during differentiation of osteoblasts into osteocytes. *J Cell Biochem* 81:82-92.

Hauser S, Weich HA (1993). A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* 9:259-268.

Henriksen K, Karsdal M, Delaisse JM, Engsig MT (2003). RANKL and vascular endothelial growth factor (VEGF) induce osteoclast chemotaxis through an ERK1/2-dependent mechanism. *J Biol Chem* 278:48745-48753.

Herve MA, Buteau-Lozano H, Mourah S, Calvo F, Perrot-Applanat M (2005). VEGF189 stimulates endothelial cells proliferation and migration in vitro and up-regulates the expression of Flk-1/KDR mRNA. *Exp Cell Res* 309:24-31.

Hiltunen MO, Ruuskanen M, Huuskonen J, Mahonen AJ, Ahonen M, Rutanen J, *et al.* (2003). Adenovirus-mediated VEGF-A gene transfer induces bone formation in vivo. *FASEB J* 17:1147-1149.

Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW (1991). The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 5:1806-1814.

Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 267:26031-26037.

Hutchings H, Ortega N, Plouet J (2003). Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. *FASEB J* 17:1520-1522.

Inoki I, Shiomi T, Hashimoto G, Enomoto H, Nakamura H, Makino K, *et al.* (2002). Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *FASEB J* 16:219-221.

Inoue N, Kondo T, Kobayashi K, Aoki M, Numaguchi Y, Shibuya M, *et al.* (2006). Therapeutic angiogenesis using novel vascular endothelial growth factor-E/human placental growth factor chimera genes. *Arterioscler Thromb Vasc Biol* 27:99-105.

Ito H, Koefoed M, Tiyapatanaputi P, Gromov K, Goater JJ, Carmouche J, *et al.* (2005). Remodeling of cortical bone allografts mediated by adherent rAAV-RANKL and VEGF gene therapy. *Nat Med* 11:291-297.

Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, Stephenson RC, *et al.* (2003). Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* 130:2779-2791.

Jakobsson L, Kreuger J, Holmborn K, Lundin L, Eriksson I, Kjellen L, *et al.* (2006). Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Dev Cell* 10:625-634.

Jiang ZL, Reay D, Kreppel F, Gambotto A, Feingold E, Kochanek S, *et al.* (2001). Local high-capacity adenovirus-mediated mCTLA4Ig and mCD40Ig expression prolongs recombinant gene expression in skeletal muscle. *Mol Ther* 3:892-900.

Kaku M, Kohno S, Kawata T, Fujita I, Tokimasa C, Tsutsui K, *et al.* (2001). Effects of vascular endothelial growth factor on osteoclast induction during tooth movement in mice. *J Dent Res* 80:1880-1883.

Kakudo N, Kusumoto K, Wang YB, Iguchi Y, Ogawa Y (2006). Immunolocalization of vascular endothelial growth factor on intramuscular ectopic osteoinduction by bone morphogenetic protein-2. *Life Sci* 79:1847-1855.

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, *et al.* (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 5:74-80.

Kastrup J (2003). Therapeutic angiogenesis in ischemic heart disease: gene or recombinant vascular growth factor protein therapy? *Curr Gene Ther* 3:197-206.

Kitsukawa T, Shimono A, Kawakami A, Kondoh H, Fujisawa H (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* 121:4309-4318.

Klagsbrun M, Takashima S, Mamluk R (2002). The role of neuropilin in vascular and tumor biology. *Adv Exp Med Biol* 515:33-48.

Kleinheinz J, Stratmann U, Joos U, Wiesmann HP (2005). VEGF-activated angiogenesis during bone regeneration. *J Oral Maxillofac Surg* 63:1310-1316.

Kohno S, Kaku M, Tsutsui K, Motokawa M, Ohtani J, Tenjo K, *et al.* (2003). Expression of vascular endothelial growth factor and the effects on bone remodeling during experimental tooth movement. *J Dent Res* 82:177-182.

Larrivee B, Karsan A (2000). Signaling pathways induced by vascular endothelial growth factor (review). *Int J Mol Med* 5:447-456.

Leach JK, Kaigler D, Wang Z, Krebsbach PH, Mooney DJ (2006). Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration. *Biomaterials* 27:3249-3255.

Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML (2005). Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol* 169:681-691.

Leung FY, Rabie AB, Hagg U (2004). Neovascularization and bone formation in the condyle during stepwise mandibular advancement. *Eur J Orthod* 26:137-141.

Levy AP, Levy NS, Wegner S, Goldberg MA (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 270:13333-13340.

Li X, Aase K, Li H, von Euler G, Eriksson U (2001). Isoform-specific expression of VEGF-B in normal tissues and tumors. *Growth Factors* 19:49-59.

Lohela M, Saaristo A, Veikkola T, Alitalo K (2003). Lymphangiogenic growth factors, receptors and therapies. *Thromb Haemost* 90:167-184.

Madan A, Curtin PT (1993). A 24-base-pair sequence 3′ to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer. *Proc Natl Acad Sci USA* 90:3928-3932.

Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, *et al.* (2002). Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech Dev* 111:61-73.

Maes C, Stockmans I, Moermans K, Van Looveren R, Smets N, Carmeliet P, *et al.* (2004). Soluble VEGF isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. *J Clin Invest* 113:188-199.

Maes C, Coenegrachts L, Stockmans I, Daci E, Luttun A, Petryk A, *et al.* (2006). Placental growth factor mediates mesenchymal cell development, cartilage turnover, and bone remodeling during fracture repair. *J Clin Invest* 116:1230-1242.

Maglione D, Battisti M, Tucci M (2000). Recombinant production of PIGF-1 and its activity in animal models. *Farmaco* 55:165-167.

Marconcini L, Marchio S, Morbidelli L, Cartocci E, Albini A, Ziche M, *et al.* (1999). c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. *Proc Natl Acad Sci USA* 96:9671-9676.

Mattei MG, Borg JP, Rosnet O, Marme D, Birnbaum D (1996). Assignment of vascular endothelial growth factor (VEGF) and placenta growth factor (PLGF) genes to human chromosome 6p12-p21 and 14q24-q31 regions, respectively. *Genomics* 32:168-169.

Mayr-Wohlfart U, Waltenberger J, Hausser H, Kessler S, Gunther KP, Dehio C, *et al.* (2002). Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. *Bone* 30:472-477.

Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M, *et al.* (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J* 18:363-374.

Midy V, Plouet J (1994). Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. *Biochem Biophys Res Commun* 199:380-386.

Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, *et al.* (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72:835-846.

Min JK, Kim YM, Kim YM, Kim EC, Gho YS, Kang IJ, *et al.* (2003). Vascular endothelial growth factor up-regulates expression of receptor activator of NF-kappa B (RANK) in endothelial cells. Concomitant increase of angiogenic responses to RANK ligand. *J Biol Chem* 278:39548-39557.

Miyata Y, Kanda S, Ohba K, Nomata K, Hayashida Y, Eguchi J, *et al.* (2006). Lymphangiogenesis and angiogenesis in bladder cancer: prognostic implications and regulation by vascular endothelial growth factors-A, -C, and -D. *Clin Cancer Res* 12(3 Pt 1):800-806.

Nakagawa M, Kaneda T, Arakawa T, Morita S, Sato T, Yomada T, *et al.* (2000). Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. *FEBS Lett* 473:161-164.

Narazaki M, Tosato G (2006). Ligand-induced internalization selects use of common receptor neuropilin-1 by VEGF165 and semaphorin3A. *Blood* 107:3892-3901.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 13:9-22.

Ng YS, Rohan R, Sunday ME, Demello DE, D'Amore PA (2001). Differential expression of VEGF isoforms in mouse during development and in the adult. *Dev Dyn* 220:112-121.

Niida S, Kaku M, Amano H, Yoshida H, Kataoka H, Nishikawa S, *et al.* (1999). Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. *J Exp Med* 190:293-298.

Niida S, Kondo T, Hiratsuka S, Hayashi S, Amizuka N, Noda T, *et al.* (2005). VEGF receptor 1 signaling is essential for osteoclast development and bone marrow formation in colony-stimulating factor 1-deficient mice. *Proc Natl Acad Sci USA* 102:14016-14021.

Nissen NN, Polverini PJ, Koch AE, Volin MV, Gamelli RL, DiPietro LA (1998). Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *Am J Pathol* 152:1445-1452.

Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M (1998). A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. *J Biol Chem* 273:31273-31282.

Okuda N, Takeda S, Shinomiya K, Muneta T, Itoh S, Noda M, *et al.* (2006). ED-71, a novel vitamin D analog, promotes bone formation and angiogenesis and inhibits bone resorption after bone marrow ablation.

Bone 40:281-292.

Olofsson B, Korpelainen E, Pepper MS, Mandriota SJ, Aase K, Kumar V, *et al.* (1998). Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. *Proc Natl Acad Sci USA* 95:11709-11714.

Orlandini M, Spreafico A, Bardelli M, Rocchigiani M, Salameh A, Nucciotti S, *et al.* (2006). Vascular endothelial growth factor-D activates VEGFR-3 expressed in osteoblasts inducing their differentiation. *J Biol Chem* 281:17961-17967.

Oyama T, Sakuta T, Matsushita K, Maruyama I, Nagaoka S, Torii M (2000). Effects of roxithromycin on tumor necrosis factor-alpha-induced vascular endothelial growth factor expression in human periodontal ligament cells in culture. *J Periodontol* 71:1546-1553.

Paavonen K, Horelli-Kuitunen N, Chilov D, Kukk E, Pennanen S, Kallioniemi OP, *et al.* (1996). Novel human vascular endothelial growth factor genes VEGF-B and VEGF-C localize to chromosomes 11q13 and 4q34, respectively. *Circulation* 93:1079-1082.

Park J, Ries J, Gelse K, Kloss F, von der Mark K, Wiltfang J, *et al.* (2003). Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. *Gene Ther* 10:1089-1098.

Park JE, Chen HH, Winer J, Houck KA, Ferrara N (1994). Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 269:25646-25654.

Peng H, Wright V, Usas A, Gearhart B, Shen HC, Cummins J, *et al.* (2002). Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest* 110:751-759.

Perbal B (2004). CCN proteins: multifunctional signalling regulators. *Lancet* 363:62-64.

Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, *et al.* (1997). Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 151:1523-1530.

Pfander D, Cramer T, Schipani E, Johnson RS (2003). HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes. *J Cell Sci* 116(Pt 9):1819-1826.

Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, *et al.* (1997). VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. *J Biol Chem* 272:7151-7158.

Polverini PJ (1995). The pathophysiology of angiogenesis. *Crit Rev Oral Biol Med* 6:230-247.

Pufe T, Harde V, Petersen W, Goldring MB, Tillmann B, Mentlein R (2004). Vascular endothelial growth factor (VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. *J Pathol* 202:367-374.

Rabie AB (1997). Vascular endothelial growth pattern during demineralized bone matrix induced osteogenesis. *Connect Tissue Res* 36:337-345.

Rabie AB, Hagg U (2002). Factors regulating mandibular condylar growth. *Am J Orthod Dentofacial Orthop* 122:401-409.

Rabie AB, Deng YM, Samman N, Hagg U (1996). The effect of demineralized bone matrix on the healing of intramembranous bone grafts in rabbit skull defects. *J Dent Res* 75:1045-1051.

Rabie AB, Leung FY, Chayanupatkul A, Hagg U (2002a). The correlation between neovascularization and bone formation in the condyle during forward mandibular positioning. *Angle Orthod* 72:431-438.

Rabie AB, Shum L, Chayanupatkul A (2002b). VEGF and bone formation in the glenoid fossa during forward mandibular positioning. *Am J Orthod Dentofacial Orthop* 122:202-209.

Rabie AB, Tang GH, Hagg U (2004). Cbfa1 couples chondrocytes maturation and endochondral ossification in rat mandibular condylar cartilage. *Arch Oral Biol* 49:109-118.

Rabie AB, Dai J, Xu R (2007). Recombinant AAV mediated VEGF gene therapy induces mandibular condylar growth. *Gene Ther* 14:972-980.

Risau W (1997). Mechanisms of angiogenesis. *Nature* 386:671-674.

Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, *et al.* (2003). VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ Res* 92:1098-1106.

Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, Bautch VL (2004). The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. *Am J Pathol* 164:1531-1535.

Roy H, Bhardwaj S, Babu M, Jauhiainen S, Herzig KH, Bellu AR, *et al.* (2005). Adenovirus-mediated gene transfer of placental growth factor to perivascular tissue induces angiogenesis via upregulation of the expression of endogenous vascular endothelial growth factor-A. *Hum Gene Ther* 16:1422-1428.

Roy H, Bhardwaj S, Yla-Herttuala S (2006). Biology of vascular endothelial growth factors. *FEBS Lett* 580:2879-2887.

Selleck SB (2006). Signaling from across the way: transactivation of VEGF receptors by HSPGs. *Mol Cell* 22:431-432.

Senger DR, Van de Water L, Brown LF, Nagy JA, Yeo KT, Yeo TK, *et al.* (1993). Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev* 12:303-324.

Shakibaei M, Schulze-Tanzil G, Mobasheri A, Beichler T, Dressler J, Schwab W (2003). Expression of the VEGF receptor-3 in osteoarthritic chondrocytes: stimulation by interleukin-1beta and association with beta(1)-integrins. *Histochem Cell Biol* 120:235-241.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, *et al.* (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62-66.

Shibuya M (2006). Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J Biochem Mol Biol* 39:469-478.

Shraga-Heled N, Kessler O, Prahst C, Kroll J, Augustin H, Neufeld G (2007). Neuropilin-1 and neuropilin-2 enhance VEGF121 stimulated signal transduction by the VEGFR-2 receptor. *FASEB J* 21:915-926.

Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E (1993). Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest* 91:2235-2243.

Silins G, Grimmond S, Egerton M, Hayward N (1997). Analysis of the promoter region of the human VEGF-related factor gene. *Biochem Biophys Res Commun* 230:413-418.

Silvestre JS, Tamarat R, Ebrahimian TG, Le-Roux A, Clergue M, Emmanuel F, *et al.* (2003). Vascular endothelial growth factor-B promotes in vivo angiogenesis. *Circ Res* 93:114-123.

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 92:735-745.

Spector JA, Mehrara BJ, Luchs JS, Greenwald JA, Fagenholz PJ, Saadeh PB, *et al.* (2000). Expression of adenovirally delivered gene products in healing osseous tissues. *Ann Plast Surg* 44:522-528.

Street J, Bao M, deGuzman L, Bunting S, Peale FV, Ferrara N, *et al.* (2002). Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci USA* 99:9656-9661.

Stringer SE (2006). The role of heparan sulphate proteoglycans in angiogenesis. *Biochem Soc Trans* 34(Pt 3):451-453.

Suto K, Yamazaki Y, Morita T, Mizuno H (2005). Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms: insight into selective VEGF binding to kinase insert domain-containing receptor but not to fms-like tyrosine kinase-1. *J Biol Chem* 280:2126-2131.

Tamayose K, Hirai Y, Shimada T (1996). A new strategy for large-scale preparation of high-titer recombinant adeno-associated virus vectors by using packaging cell lines and sulfonated cellulose column chromatography. *Hum Gene Ther* 7:507-513.

Tammela T, Enholm B, Alitalo K, Paavonen K (2005). The biology of vascular endothelial growth factors. *Cardiovasc Res* 65:550-563.

Tang GH, Rabie AB (2005). Runx2 regulates endochondral ossification in condyle during mandibular advancement. *J Dent Res* 84:166-171.

Tarkka T, Sipola A, Jamsa T, Soini Y, Yla-Herttuala S, Tuukkanen J, *et al.* (2003). Adenoviral VEGF-A gene transfer induces angiogenesis and promotes bone formation in healing osseous tissues. *J Gene Med* 5:560-566.

Tessler S, Rockwell P, Hicklin D, Cohen T, Levi BZ, Witte L, *et al.* (1994).

Heparin modulates the interaction of VEGF165 with soluble and cell associated flk-1 receptors. *J Biol Chem* 269:12456-12461.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, *et al.* (1991). The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266:11947-11954.

Tokuda H, Hatakeyama D, Akamatsu S, Tanabe K, Yoshida M, Shibata T, *et al.* (2003a). Involvement of MAP kinases in TGF-beta-stimulated vascular endothelial growth factor synthesis in osteoblasts. *Arch Biochem Biophys* 415:117-125.

Tokuda H, Kato K, Oiso Y, Kozawa O (2003b). Contrasting effects of triiodothyronine on heat shock protein 27 induction and vascular endothelial growth factor synthesis stimulated by TGF-beta in osteoblasts. *Mol Cell Endocrinol* 201:33-38.

Veikkola T, Alitalo K (1999). VEGFs, receptors and angiogenesis. *Semin Cancer Biol* 9:211-220.

Vincenti V, Cassano C, Rocchi M, Persico G (1996). Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. *Circulation* 93:1493-1495.

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, *et al.* (1998). MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 93:411-422.

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269:26988-26995.

Wang DS, Yamazaki K, Nohtomi K, Shizume K, Ohsumi K, Shibuya M, *et al.* (1996). Increase of vascular endothelial growth factor mRNA expression by 1,25-dihydroxyvitamin D3 in human osteoblast-like cells. *J Bone Miner Res* 11:472-479.

Wauke K, Nagashima M, Ishiwata T, Asano G, Yoshino S (2002). Expression and localization of vascular endothelial growth factor-C in rheumatoid arthritis synovial tissue. *J Rheumatol* 29:34-48.

Wise GE, Yao S (2003). Expression of vascular endothelial growth factor in the dental follicle. *Crit Rev Eukaryot Gene Expr* 13(2-4):173-180.

Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, *et al.* (2004). VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. *Cancer Res* 64:7822-7835.

Xiong H, Rabie AB, Hagg U (2005). Neovascularization and mandibular condylar bone remodeling in adult rats under mechanical strain. *Front Biosci* 10:74-82.

Yamada Y, Nezu J, Shimane M, Hirata Y (1997). Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics* 42:483-488.

Yamazaki Y, Morita T (2006). Molecular and functional diversity of vascular endothelial growth factors. *Mol Divers* 10:515-527.

Yamazaki Y, Tokunaga Y, Takani K, Morita T (2005). Identification of the heparin-binding region of snake venom vascular endothelial growth factor (VEGF-F) and its blocking of VEGF-A165. *Biochemistry* 44:8858-8864.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000). Vascular-specific growth factors and blood vessel formation. *Nature* 407:242-248.

Yao S, Liu D, Pan F, Wise GE (2006). Effect of vascular endothelial growth factor on RANK gene expression in osteoclast precursors and on osteoclastogenesis. *Arch Oral Biol* 51:596-602.

Yeh LC, Lee JC (1999). Osteogenic protein-1 increases gene expression of vascular endothelial growth factor in primary cultures of fetal rat calvaria cells. *Mol Cell Endocrinol* 153:113-124.

Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen MJ, Alitalo K, *et al.* (2002). Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 129:4797-4806.

Zelzer E, Olsen BR (2005). Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. *Curr Top Dev Biol* 65:169-187.

Zelzer E, Glotzer DJ, Hartmann C, Thomas D, Fukai N, Soker S, *et al.* (2001). Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. *Mech Dev* 106:97-106.

Zelzer E, McLean W, Ng YS, Fukai N, Reginato AM, Lovejoy S, *et al.* (2002). Skeletal defects in VEGF(120/120) mice reveal multiple roles for VEGF in skeletogenesis. *Development* 129:1893-1904.

Zelzer E, Mamluk R, Ferrara N, Johnson RS, Schipani E, Olsen BR (2004). VEGFA is necessary for chondrocyte survival during bone development. *Development* 131:2161-2171.

Zeng H, Dvorak HF, Mukhopadhyay D (2001). Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem* 276:26969-26979.

Zhang F, Fischer K, Lineaweaver WC (2002). DNA strand gene transfer and bone healing. *J Long Term Eff Med Implants* 12:113-123.