Histol Histopathol (2012) 27: 559-566
DOI: 10.14670/HH-27.559

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

Role of angiogenesis on bone formation

Sergio Portal-Núñez, Daniel Lozano and Pedro Esbrit
Laboratory of Mineral and Bone Metabolism, "Instituto de Investigación Sanitaria (IIS)-Fundación Jiménez Díaz", Madrid, Spain

Summary. Angiogenesis and bone formation are coupled during skeletal development and fracture healing. This relationship, although known for some time, has not been properly explored. Advances in the discovery of how angiogenesis is regulated in physiological processes like embryogenesis, endometrial regeneration and wound healing or in pathologies such as cancer have provided a deeper understanding of how angiogenic factors may interact with bone cells to improve bone formation and bone regeneration. The lack of oxygen (hypoxia) and the subsequent generation of angiogenic factors have been shown to be critical in the development of a regular skeleton and achieving successful bone regeneration and fracture healing. Given that vascular status is important for a proper bone homeostasis, defining the roles of osteoblasts, osteoclasts, endothelial cells and angiogenic factors and their interactions in bone is a key issue for the development of new strategies to manage bone pathologies and nonfused fractures.

Key words: Angiogenesis, Bone formation, Osteoblast, Osteoclast, VEGF

Introduction

Angiogenesis is the process whereby a new blood vessel is formed from a preexisting one, whereas vasculogenesis is the formation of a vascular network starting from an angioblastic stem cell (Risau, 1997; Jain, 2003). The former process is important in physiological events such as embryogenesis, organogenesis and wound healing, and also in pathological settings such as inflammation and cancer. One of the main players in angiogenesis is the endothelial cell. During the angiogenic process, endothelial cells proliferate, migrate, form tubes, and finally produce a non-leaky conduct where blood flows. This process must be properly coordinated in time and space, which implies the action of several molecules that are generally known as angiogenic factors. Different mechanisms trigger angiogenesis, perhaps the most well known is hypoxia through the stabilization of a transcription factor called hypoxia inducible factor-1 (HIF-1). This factor drives the expression of the well-characterized key factor for angiogenesis vascular endothelial growth factor (VEGF).

The relationship between angiogenesis and bone formation or fracture healing was demonstrated a long time ago (Trueta and Trias, 1961; Trueta and Buhr, 1963). However, how this relationship works is not entirely understood, although studies trying to understand the mechanisms by which angiogenesis regulates bone formation are now experiencing a new resurgence. In the present review, we will outline the principal mechanisms that govern angiogenesis, the relationship of this process with bone development and bone formation, the influence of the main molecules implicated in the angiogenic process in the biology of bone cells (namely, osteoblasts and osteoclasts), and the role of angiogenesis in bone formation and bone regeneration in physiological and pathological conditions.

HIF-1 and VEGF as master regulators of angiogenesis

Before going deeper into the understanding of the relationship between bone and angiogenesis, we will describe current knowledge about how two of the main

Offprint requests to: Pedro Esbrit Ph.D., Laboratorio de Metabolismo Mineral y Óseo, Instituto de Investigación Sanitaria (IIS)-Fundación Jiménez Díaz, Avda. Reyes Católicos, 2, 28040 Madrid, Spain. e-mail: pesbrit@fjd.es

actors (HIF-1 and VEGF) in angiogenesis regulation work.

Three different kinds of HIF (HIF-1, -2 and -3) have been characterized (Gordan and Simon, 2007). HIF-3 is not related to HIF-1 or HIF-2, and it has an unknown role in angiogenesis (Weidemann and Johnson, 2008). Although HIF-2 is also regulated under hypoxia conditions and may have overlapping functions with HIF-1 (Wang et al., 2007), we will focus on the latter. HIF-1 is a heterodimer composed of two basic helix loop helix proteins, HIF-1α and HIF-1ß, also called Arnt (from aryl hydrocarbon receptor translocator), both belonging to the Per/Arnt/Sim (PAS) protein subfamily (Wang et al., 1995). HIF-1 requires binding to two co-factors, p300 and CREB (cAMP response element binding, a transcription factor) binding protein (CBP) for its function (Kallio et al., 1998). HIF-1 protein stabilization and action is regulated by O_2 levels, so that two types of O_2 sensors have been developed in nature for HIF-1 regulation: 1) Prolyl-hydroxylase domain proteins (PHDs) that hydroxylate two residues at the oxigene-dependent degration domain (Berra et al., 2003), and asparaginyl hydroxylase, also called factor-inhibiting HIF-1 (FIH), which hydroxylates HIF-1 in an asparragine residue (N803) (Lando et al., 2002). These two enzymes are activated when the O_2 pressure in the environment is normal (depending on the particular tissue). PHD activation leads to HIF-1 ubiquitination and degradation through the action of an E3 ligase called von Hippen-Landau protein (VHL) (Kaelin, 2002); whereas hydroxylation by FIH impinges the interaction of HIF-1 with p300 and CBP, resulting in a decreased HIF-1 function. Once the cells sense hypoxia (as mentioned above, this may vary from tissue to tissue), HIF-1α is stabilized and binds to HIF-1ß forming HIF-1, and then HIF1 is translocated to the nucleus, where it exerts its function by activating gene transcription through binding to hypoxia response elements with the consensus sequence 5´-RCGTG-3´(where R is a purine residue) (Kallio et al., 1999). Nowadays, more than 100 genes have been reported to be regulated by HIF-1; these genes are related to cell survival and other metabolic functions (e.g., angiogenesis programs, apoptosis, glucose transporter expression) (Semenza, 2003; Bishop et al., 2004; Greijer et al., 2005). Among these genes, a key angiogenesis-related gene is VEGF-A (Shweiki et al., 1992; Semenza, 2009). Of note, hypoxia occurs in areas of the epiphysis of long bones where it plays a key role in promoting chondrocyte differentiation (Schipani et al., 2009), bone remodelling and bone repair after a fracture (Arnett et al., 2003; Wan et al., 2008). VEGF-A (usually known as just VEGF) was initially described as a factor that increases cellular permeability (Senger et al., 1983), and for this reason it was originally called vascular permeability factor (VPF). More recently, the angiogenic actions of VEGF were independently discovered (Leung et al., 1989), and it was demonstrated that VPF and VEGF were indeed the same molecule (Keck et al., 1989). VEGF belongs to the same family as placental growth factor and VEGF-B, -C, -D. Different VEGF isoforms have been described, the main ones being $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ in mice (Robinson and Stringer, 2001; Schipani et al., 2009).

VEGF isoforms interact with two receptors, VEGF receptor-1 (VEGFR-1), also called fms-like tyrosine kinase (Flt-1) (de Vries et al., 1992), and VEGF receptor-2 (VEGFR-2), called KDR in humans and Flk-1 in mouse (Terman et al., 1991, 1992). Both receptors belong to the receptor tyrosine kinase family and have seven immunoglobin-like domains in the extracellular region, a single pass domain, a tyrosine kinase receptor region, and a kinase-insert domain (Shibuya et al., 1990; Terman et al., 1991). Expression of VEGFR-1 is up-regulated under hypoxia conditions (Gerber et al., 1997), and this receptor also exists in a soluble form resulting from alternative splicing, which inhibits VEGF action (Kendall and Thomas, 1993). VEGFR-2 is recognized as the main mediator of signal transduction upon VEGF binding, with an approximate K_d of 75-125 pM, involving receptor dimerization and autophosphorylation in several tyrosines (Terman et al., 1992). This autophosphorylation leads to activation of different molecular pathways – namely, phospholipase C-γ, Ras GTPase, Src kinases, and extracellular signal-regulated kinases (ERKs) – in endothelial cells that trigger migration, proliferation, differentiation and pro-survival signals (Ferrara et al., 2003).

Data demonstrating that VEGF plays a key role in bone development is extensive and compelling. Thus, the embryonic avascular cartilage is not invaded by blood vessels until the bony collar (periosteal bone surrounding the initial cartilage) is already formed and the perichondrium is vascularized. In this context, it has also been demonstrated that the condensing mesenchymal tissue in the developing skeleton regulates vasculature invasion of cartilage and subsequently bone development through VEGF signaling (Eshkar-Oren et al., 2009). Furthermore, this vascular invasion can be accelerated by overexpressing different VEGF isoforms in mice (Takimoto et al., 2009).

Actions of VEGF in bone cells

Besides the angiogenic effects of VEGF on endothelial cells, VEGF exerts actions on bone cells, including osteoblasts and osteoclasts. Expression of VEGF and its receptors has been reported in both of these cell types (Tombran-Tink and Barnstable, 2004). Accordingly, it has been demonstrated that VEGF can affect osteoblast differentiation. Human-derived mesenchymal cells from trabecular bone express VEGF and VEGFR-1 during their differentiation (Mayer et al., 2005). VEGF overexpression in these cells induces mineralization, which was impaired by overexpression of the aforementioned secreted form of VEGFR-1, suggesting that VEGF acts in an autocrine fashion in this

setting (Mayer et al., 2005). Moreover, VEGF increases nodule formation and alkaline phosphatase activity, two well-known markers of osteoblast differentiation, in a dose-dependent manner in primary human osteoblastic cells (Street et al., 2002). In the latter study, it was shown that hypoxia increases the expression of VEGF, but not that of basic fibroblast growth factor (bFGF) - another major angiogenesis regulator - in these cells. These aggregated findings point to VEGF as a possible therapy in fracture healing.

Chemotaxis is one of the hallmarks of tissue formation and wound healing. Evidence that VEGF acts as a chemoattractant for endothelial cells is extensive and compelling (Grunewald et al., 2006; Roodhart et al., 2010). In addition, it has been shown that several angiogenic factors, including secreted $VEGF_{165}$, direct human osteoblastic cell migration *in vitro* (Li et al., 2005), suggesting that this might also occur *in vivo* in bone remodelling and bone fracture areas (as described below).

VEGF also promotes chondroprogenitor differentiation to chondrocytes in bone tissue. Chondrocytes originate from mesenchymal-chondroprogenitor cells in avascular bone areas and express and secrete collagen2a1 and aggrecan. Differentiation of these cells depends on a genetic program driven, at least in part, by the transcription factor Sox9 (Akiyama et al., 2002). In fact, using a mouse model expressing only the $VEGF_{188}$ isoform, it was shown that the latter is not sufficient for the development of long bones, related to a lack of proper chondrocyte function (Maes et al., 2002). However, deletion of this VEGF isoform in mice produced dwarfism, disruption of metaphyseal and secondary ossification centers development and joint dysplasia, in part due to lack of proper vascularization (Maes et al., 2004). Moreover, mice deficient in both $VEGF_{164}$ and $VEGF_{188}$ have a disturbed vascular pattern, reduced bone growth and a decrease in trabecular number, together with a reduction in the expression of bone-related genes and chondrocyte differentiation (Maes et al., 2002). In addition, in another study, the mice expressing only $VEGF_{120}$ isoform have a delayed recruitment of blood vessel in the perichodrium which hampers primary ossification in the embryo (Zelzer et al., 2002). Specific chondrocyte VEGF deletion (using col2a1 to drive the expression of Cre recombinase) in mice results in a decreased survival of chondrocytes in epiphyseal and joint areas of endochondral ossification together with a delayed vascularization (Zelzer et al., 2004).

The osteoclast as an important cell for angiogenesis

In adult life, the skeleton is in constant remodeling to repair the regular micro-cracks caused by routine activities. It is known that this remodeling takes place in a specialized structure known as "bone remodeling compartment" (BRC) (Hauge et al., 2001a). In this context, vasculature plays a major role in the maintenance of the adult bone since it supplies nutrients and O_2 and facilitates both osteoprogenitors and osteoclast precursors to reach the BRC (Eghbali-Fatourechi et al., 2005). It has been demonstrated that osteoclasts - multinucleated cells derived from hematopoietic cells of the monocytic lineage which resorb bone - have VEGF receptors (Sawano et al., 2001; Tombran-Tink and Barnstable, 2004), and VEGF acts as a chemoattractant for osteoclasts (Engsig et al., 2000). It has also been shown that VEGF may replace macrophage colony stimulating factor (M-CSF) for inducing osteoclast differentiation in rabbits and rats (Niida et al., 1999; Nakagawa et al., 2000). Moreover, injection of recombinant VEGF in op/op (osteopetrotic) mice increases the recruitment of osteoclasts, and this effect was neutralized by an anti-VEGF antibody (Niida et al., 1999). *In vitro* studies have suggested that VEGFR-1 may have a predominant role in the differentiation and function of osteoclasts (Aldridge et al., 2005).

Mice with loss of function mutation in M-CSF gene have an osteopetrotic phenotype with impaired osteoclast maturation but no angiogenic alterations in the axial skeleton, suggesting that osteoclasts are not required for angiogenesis in this setting (Marks and Lane, 1976; Deckers et al., 2002). However, various studies support the idea that osteoclasts exert an important role in angiogenesis. Thus, osteoclasts in the BRC are located close to blood vessels (Hauge et al., 2001b), and medium conditioned by osteoclasts promotes angiogenesis (Tanaka et al., 2007). Recently, Cackowski and co-workers, using an in vitro explant model of mouse metatarsals, have demonstrated that angiogenesis occurs associated with osteoclastogenesis (Cackowski et al., 2010). When these explants were treated with the N-terminal fragment of parathyroid hormone-related protein (PTHrP) - a factor which stimulates osteoclastogenesis through induction of receptor activator of nuclear factor-κB ligand (RANKL) in osteoblasts (Liao and McCauley, 2006) - angiogenesis was enhanced, and osteoprotegerin (a RANKL antagonist) abolished this response. This angiogenic effect may be mediated through the expression of matrix metaloproteinase 9 (MMP-9) and the subsequent release of VEGF from the extracellular matrix, and also by alternative mechanisms (e.g., production of ostepontin by osteoclasts) (Tanaka et al., 2007; Cackowski et al., 2010).

Taken together, these studies support the notion that osteoclasts influence angiogenesis and that a deeper understanding of the molecular mechanisms behind this relationship might help to design new approaches in the management of pathological conditions associated with a poor bone vascularization.

Vasculature and bone

The skeleton is formed by two different mechanisms: 1) intramembranous ossification; and 2) endochondral ossification. Briefly, the first mechanism takes place during the development of flat bones such as the skull and facial bones. Intramembranous ossification occurs by direct differentiation of mesenchymal cells to form new bone - a process which does not seem to depend on angiogenesis - (Wang et al., 2007). Endochondral ossification occurs in the formation of long bones (e.g., femur and tibia) during embryo development, and it is also the predominant mechanism in fracture healing by callus formation. In this type of bone formation, hypoxic areas in avascular cartilage allows HIF-1 stabilization and VEGF production, which stimulates angiogenesis; it is subsequently replaced by a network of blood vessels followed by the invasion of bone-forming cells leading to new bone formation (Gerber and Ferrara, 2000). Interestingly, the embryonic avascular cartilage is not invaded by blood vessels until the bony collar (periosteal bone surrounding the initial cartilage) is already formed and the perichondrium becomes vascularized. At this stage, overexpression of VEGF isoforms has failed to induce blood vessel formation in such avascular cartilage in mice (Takimoto et al., 2009). Thus, once the vascularized bony collar is formed, anti-angiogenic properties in the perichondrium are lost, and vascular invasion of the cartilage can be accelerated by overexpression of major VEGF isoforms (Takimoto et al., 2009).

Vascular invasion of the cartilage also depends on the action of MMPs, namely MMP-9 and MMP-13 which thus play an important role in endochondral ossification (Ortega et al., 2004). VEGF can also be provided by its release from the extracellular matrix by the action of MMPs (Bergers et al., 2000). In this scenario, blood vessels supply chondrocytes and bone cells with nutrients and mesenchymal cells (Gerber et al., 1999; Brandi and Collin-Osdoby, 2006). In fact, hypoxia - a key trigger of angiogenesis as described above - has been shown to be essential for the normal development of chondrocytes and the regular formation of the growth plate (Schipani et al., 2001). Of note, the existence of circulating osteoblast-lineage cells has been demonstrated in humans, which are abundant at puberty when bone formation is reaching its peak, correlating with the serum levels of bone formation markers (Eghbali-Fatourechi et al., 2005).

The complex mechanisms behind angiogenesis and bone development had been poorly known for a long time. However, advances in molecular and cell biology gave researchers the opportunity to characterize this link. Thus, a recent study has shed light and provided valuable data on this relationship. By using a genetic approach in mice, it has been shown that accumulation of HIF-1 (through specific osteoblast deletion of the gene that encodes VHL protein) resulted in an increased bone volume to tissue volume ratio that was concomitant with an increase in vasculature (Wang et al., 2007). The same authors also created a mouse lacking HIF-1 in osteoblasts; these mice had a reduced vasculature and, accordingly, long bones were thinner. Moreover, mice lacking both VHL and HIF-1α genes have an intermediate phenotype, which suggests that HIF-2 might be compensating for the lack of this important pathway. Consistent with what was previously mentioned about the important role of angiogenesis in endochondral bone formation, only the latter bone formation mechanism was impaired in HIF-1 knockout mice, whereas mesenchymal cell condensation in calvaria and clavicles (intramembranous bone formation) were not altered in these mice (Wang et al., 2007). *In vitro* hypoxia also inhibits the differentiation of primary rat osteoblastic cells, inducing a quiescence status without entering apoptosis (Utting et al., 2006). This might represent a mechanism to preserve osteoblast viability until bone homeostasis has been restored.

Recently, HIF-1 has been associated with activation of the canonical Wnt/ß-catenin pathway, which promotes osteoblast growth and differentiation (Khosla et al., 2008; Deschaseaux et al., 2009). Treatment of pluripotent human mesenchymal cells and the murine mesenchymal cell line C3H10T1/2 with deferroxamine (DFO), a well known hypoxia mimetic, increases the osteogenic marker alkaline phosphatase and calcium depositum (Qu et al., 2008). In addition, DFO treatment induces phosphorylation (inactivation) of glycogen synthase kinase-3-ß, which leads to an increase of ß-catenin (the main transcription factor of the canonical Wnt-pathway) (Qu et al., 2008). On the other hand, knocking down ß-catenin expression through RNA interference technology decreases alkaline phosphatase and reduces calcium depositum in this setting, supporting the dependence of these effects on the Wnt/ß-catenin pathway activation. Furthermore, it has recently been demonstrated that hypoxia decreases the expression of Sost/sclerostin - a well characterized inhibitor of this pathway- in mature osteoblasts and osteocytes in a VEGF-independent fashion, thus increasing these cells' growth and/or differentiation (Li et al., 2005b; Genetos et al., 2010).

Angiogenesis and bone regeneration

There is growing evidence that angiogenesis plays a key role in bone regeneration and fracture healing (Hunsuck, 1969; Rhinelander, 1974a,b; Colnot and Helms, 2001). However, the mechanisms by which angiogenesis and osteogenesis are coupled in this situation are not fully understood. One of the main models used to study this relationship is distraction osteogenesis (DO) in rodents. DO essentially consists of an osteotomy in which bone is separated by a device that controls the process of new bone formation. Although initially described by Codivilla (1905), years later

Ilizarov (1990) extended this practice as a regular technique in orthopedic and maxillofacial intervention (Codivilla, 1905; Ilizarov, 1990). DO occurs in three phases: latency (just after osteotomy); a distraction step that parallels bone generation; and a consolidation phase after stopping the distraction procedure (Isefuku et al., 2000; Aronson et al., 2001). This type of bone generation appears to be characterized by a major demand for blood supply.

It has been shown that angiogenic molecules, namely VEGF, angiopoetin-1 and 2 and bFGF, are overexpressed during DO (Pacicca et al., 2003). In fact, DO is impaired by the use of antibodies against VEGFR-1 and 2 (Jacobsen et al., 2008). Moreover, a general angiogenesis inhibitor such as TNP-470 (a synthetic analog of fumagillin, a well known anti-angiogenic compound) also prevents bone regeneration in a rat DO model (Fang et al., 2005). On the other hand, Wan and co-workers (Wan et al., 2008) have shown that imposing a hypoxic insult through administration of DFO into the gap of the distraction cavity in mice with knock-in VHL in osteoblasts accelerates bone regeneration concomitantly with an increased angiogenesis. Accordingly, inactivation of VEGF action (by injecting VEGFR-1 and -2 antibodies) or impairing HIF-1 activity (through genetic manipulation) leads to a failure in bone regeneration after DO in mice (Wan et al., 2008). Fracture healing, in which endochondral ossification predominates, also appears to depend on the expression of angiogenic factors, namely VEGF, and inhibition of VEGF activity abolishes callus formation and repair (Ferguson et al., 1999; Tatsuyama et al., 2000). Moreover, patients with fractures and a decrease in VEGF production might have a poor prognosis (Street et al., 2002).

Recently, we showed that the osteogenic action of PTHrP by systemic administration was associated with an increase in both the VEGF system and angiogenesis in the bone marrow-ablated tibia – another well characterized model of bone regeneration- of mice with diabetes – or glucocorticoid-induced osteopenia (Lozano et al., 2009; de Castro et al., 2010). In addition, the osteointegration of implanted silica-based mesoporous ceramics loaded with a PTHrP-derived into a cavitary defect in the rabbit femur was related to dramatic VEGF immunostaining and revascularization in the healing bone tissue surrounding the implant (Trejo et al., 2010).

Collectively, these data support the notion that improving the expression of angiogenic factors at the place of bone tissue damage would increase the chances of adequate bone regeneration. To establish this hypothesis definitely, further studies addressing the fine tuning regulation of angiogenesis in several pathophysiological conditions are needed.

Conclusions

Bone formation and angiogenesis are two processes that are intimately linked and thus cannot be independently understood (Fig. 1). A major trigger of angiogenesis in the bone microenvironment consists of hypoxia-mediated HIF-1 stabilization leading to subsequent VEGF expression and formation of a complex network of blood vessels. This process is critical for bone formation and bone regeneration and repair. Both processes (bone formation and angiogenesis) must be regulated in time and space and properly coupled in order to achieve an adequate bone homeostasis. We have advanced in our understanding of these regulations, but still many mechanisms and interactions among all of the implicated factors are hidden. How osteocytes (terminally differentiated osteoblasts that are embedded in the calcified bone matrix) may regulate angiogenesis is a new field that must be explored. How the use of angiogenic factors might help to heal non fused fractures that do not respond properly to regular therapies is another topic that should be addressed. Here, we have outlined some important aspects that influence the relationship between bone formation and angiogenesis. A deeper and better knowledge of all the underlying mechanisms in this relationship is necessary in order to improve the development of suitable therapies in bone diseases.

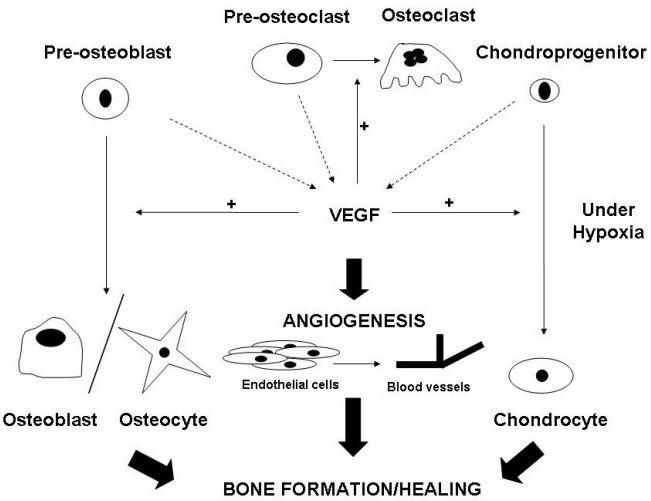

Fig. 1. Angiogenesis is intrinsically linked to bone formation and healing. Pre-osteoblasts, pre-osteoclasts and chondroprogenitors (under hypoxia) produce VEGF that promotes differentiation into osteoblasts (some of them become osteocytes), osteoclasts and chondrocytes, respectively. Collectively, these cells are the major players that govern bone maintenance and bone fracture repair. VEGF also controls endothelial migration, proliferation and tube formation. Endothelial cells will form new blood vessels which, together with the aforementioned bone cells, contribute to the process of bone formation and bone healing. Dotted arrows denote VEGF production.

Acknowledgements. Sergio Portal-Núñez is the recipient of a research contract from Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF), an Aging and Fragility Integrated Network that is supported by the Spanish Ministry of Health (RD06/0013/1002). Daniel Lozano was supported by Fundación Conchita Rábago and is currently the recipient of a research contract from Comunidad Autónoma de Madrid (CAM-FUNDAHUESO). The studies carried out in the authors' laboratory, referred to in this review, were supported by grants from Instituto de Salud Carlos III (PI050117, PI080922 and RETICEF RD06/0013/1002) of Spain, CAM (S2009/MAT-1472) and Fundación de Investigación Médica Mutua Madrileña. We are also indebted to Roger Theis for proofreading the manuscript.

References

Akiyama H., Chaboissier M.C., Martin J.F., Schedl A. and de Crombrugghe B. (2002). The transcription factor sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of sox5 and sox6. Genes Dev. 16, 2813-2828.

Aldridge S.E., Lennard T.W., Williams J.R. and Birch M.A. (2005). Vascular endothelial growth factor receptors in osteoclast differentiation and function. Biochem. Biophys. Res. Commun. 335, 793-798.

Arnett T.R., Gibbons D.C., Utting J.C., Orriss I.R., Hoebertz A., Rosendaal M. and Meghji S. (2003). Hypoxia is a major stimulator of osteoclast formation and bone resorption. J. Cell. Physiol. 196, 2-8.

Aronson J., Hogue W.R., Flahiff C.M., Gao G.G., Shen X.C., Skinner R.A., Badger T.M. and Lumpkin C.K., Jr. (2001). Development of tensile strength during distraction osteogenesis in a rat model. J. Orthop. Res. 19, 64-69.

Bergers G., Brekken R., McMahon G., Vu T.H., Itoh T., Tamaki K., Tanzawa K., Thorpe P., Itohara S., Werb Z. and Hanahan D. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat. Cell. Biol. 2, 737-744.

Berra E., Benizri E., Ginouves A., Volmat V., Roux D. and Pouyssegur J. (2003). Hif prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of hif-1alpha in normoxia. EMBO J. 22, 4082-4090.

Bishop T., Lau K.W., Epstein A.C., Kim S.K., Jiang M., O'Rourke D., Pugh C.W., Gleadle J.M., Taylor M.S., Hodgkin J. and Ratcliffe P.J. (2004). Genetic analysis of pathways regulated by the von hippel-lindau tumor suppressor in caenorhabditis elegans. PLoS Biol. 2, e289.

Brandi M.L. and Collin-Osdoby P. (2006). Vascular biology and the skeleton. J. Bone Miner. Res. 21, 183-192.

Cackowski F.C., Anderson J.L., Patrene K.D., Choksi R.J., Shapiro S.D., Windle J.J., Blair H.C. and Roodman G.D. (2010). Osteoclasts are important for bone angiogenesis. Blood 115, 140-149.

Codivilla A. (1905). On the means of lengthening in the lower limbs. Am. J. Orthop. Surg. 2, 353-369.

Colnot C.I. and Helms J.A. (2001). A molecular analysis of matrix remodeling and angiogenesis during long bone development. Mech. Dev. 100, 245-250.

de Castro L.F., Lozano D., Dapía S., Portal-Núñez S., Caeiro J.R., Gómez-Barrena E. and Esbrit P. (2010). Role of the N- and C-terminal fragments of parathyroid-hormone-related protein as putative therapies to improve bone regeneration under high glucocorticoid treatment. Tissue Eng. Part A. 16, 1157-1168.

de Vries C., Escobedo J.A., Ueno H., Houck K., Ferrara N. and Williams L.T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255, 989-991.

Deckers M.M., Van Beek E.R., Van Der Pluijm G., Wetterwald A., Van Der Wee-Pals L., Cecchini M.G., Papapoulos S.E. and Lowik C.W. (2002). Dissociation of angiogenesis and osteoclastogenesis during endochondral bone formation in neonatal mice. J. Bone Miner. Res.17, 998-1007.

Deschaseaux F., Sensebe L. and Heymann D. (2009). Mechanisms of bone repair and regeneration. Trends Mol. Med. 15, 417-429.

Eghbali-Fatourechi G.Z., Lamsam J., Fraser D., Nagel D., Riggs B.L. and Khosla S. (2005). Circulating osteoblast-lineage cells in humans. N. Engl. J. Med. 352, 1959-1966.

Engsig M.T., Chen Q.J., Vu T.H., Pedersen A.C., Therkidsen B., Lund L.R., Henriksen K., Lenhard T., Foged N.T., Werb Z. and Delaisse J.M. (2000). Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J. Cell Biol. 151, 879-889.

Eshkar-Oren I., Viukov S.V., Salameh S., Krief S., Oh C.D., Akiyama H., Gerber H.P., Ferrara N. and Zelzer E. (2009). The forming limb skeleton serves as a signaling center for limb vasculature patterning via regulation of VEGF. Development 136, 1263-1272.

Fang T.D., Salim A., Xia W., Nacamuli R.P., Guccione S., Song H.M., Carano R.A., Filvaroff E.H., Bednarski M.D., Giaccia A.J. and Longaker M.T. (2005). Angiogenesis is required for successful bone induction during distraction osteogenesis. J. Bone Miner. Res. 20, 1114-1124.

Ferguson C., Alpern E., Miclau T. and Helms J.A. (1999). Does adult fracture repair recapitulate embryonic skeletal formation? Mech. Dev. 87, 57-66.

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

Genetos D.C., Toupadakis C.A., Raheja L.F., Wong A., Papanicolaou S.E., Fyhrie D.P., Loots G.G. and Yellowley C.E. (2010). Hypoxia decreases sclerostin expression and increases Wnt signaling in osteoblasts. J. Cell. Biochem. 110, 457-467.

Gerber H.P. and Ferrara N. (2000). Angiogenesis and bone growth. Trends Cardiovasc. Med. 10, 223-228.

Gerber H.P., Condorelli F., Park J. and Ferrara N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not flk-1/kdr, is up-regulated by hypoxia. J. Biol. Chem. 272, 23659-23667.

Gerber H.P., Vu T.H., Ryan A.M., Kowalski J., Werb Z. and Ferrara N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. 5, 623-628.

Gordan J.D. and Simon M.C. (2007). Hypoxia-inducible factors: Central regulators of the tumor phenotype. Curr. Opin. Genet. Dev. 17, 71-77.

Greijer A.E., van der Groep P., Kemming D., Shvarts A., Semenza G.L., Meijer G.A., van de Wiel M.A., Belien J.A., van Diest P.J. and van der Wall E. (2005). Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J. Pathol. 206, 291-304.

Grunewald M., Avraham I., Dor Y., Bachar-Lustig E., Itin A., Jung S., Chimenti S., Landsman L., Abramovitch R. and Keshet E. (2006). VEGF-induced adult neovascularization: Recruitment, retention, and

role of accessory cells. Cell 124, 175-189.

Hauge E., Qvesel D., Eriksen E.F., Mosekilde L. and Melsen F. (2001a). Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. J. Bone Miner. Res. 16, 1575-1582.

Hauge E.M., Qvesel D., Eriksen E.F., Mosekilde L. and Melsen F. (2001b). Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. J. Bone Miner. Res. 16, 1575-1582.

Hunsuck E.E. (1969). Vascular changes in bone repair. J Oral Surg. 27, 572-574.

Ilizarov G.A. (1990). Clinical application of the tension-stress effect for limb lengthening. Clin. Orthop. Relat. Res. 8-26.

Isefuku S., Joyner C.J. and Simpson A.H. (2000). A murine model of distraction osteogenesis. Bone 27, 661-665.

Jacobsen K.A., Al-Aql Z.S., Wan C., Fitch J.L., Stapleton S.N., Mason Z.D., Cole R.M., Gilbert S.R., Clemens T.L., Morgan E.F., Einhorn T.A. and Gerstenfeld L.C. (2008). Bone formation during distraction osteogenesis is dependent on both VEGFR1 and VEGFR2 signaling. J. Bone Miner. Res. 23, 596-609.

Jain R.K. (2003). Molecular regulation of vessel maturation. Nat. Med. 9, 685-693.

Kaelin W.G. Jr. (2002). Molecular basis of the vhl hereditary cancer syndrome. Nat. Rev. Cancer 2, 673-682.

Kallio P.J., Wilson W.J., O'Brien S., Makino Y. and Poellinger L. (1999). Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J. Biol. Chem. 274, 6519-6525.

Kallio P.J., Okamoto K., O'Brien S., Carrero P., Makino Y., Tanaka H. and Poellinger L. (1998). Signal transduction in hypoxic cells: Inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. EMBO J. 17, 6573-6586.

Keck P.J., Hauser S.D., Krivi G., Sanzo K., Warren T., Feder J. and Connolly D.T. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246, 1309-1312.

Kendall R.L. and Thomas K.A. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc. Natl. Acad. Sci. USA 90, 10705-10709.

Khosla S., Westendorf J.J. and Oursler M.J. (2008). Building bone to reverse osteoporosis and repair fractures. J. Clin. Invest. 118, 421-428.

Lando D., Peet D.J., Gorman J.J., Whelan D.A., Whitelaw M.L. and Bruick R.K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. 16, 1466-1471.

Leung D.W., Cachianes G., Kuang W.J., Goeddel D.V. and Ferrara N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246, 1306-1309.

Li G., Cui Y., McIlmurray L., Allen W.E. and Wang H. (2005a). RhBMP-2, rhVEGF(165), rhPTN and thrombin-related peptide, tp508 induce chemotaxis of human osteoblasts and microvascular endothelial cells. J. Orthop. Res. 23, 680-685.

Li X., Zhang Y., Kang H., Liu W., Liu P., Zhang J., Harris S.E. and Wu D. (2005b). Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. J. Biol. Chem. 280, 19883-19887.

Liao J. and McCauley L.K. (2006). Skeletal metastasis: Established and emerging roles of parathyroid hormone related protein (pthrp). Cancer Metastasis Rev. 25, 559-571.

Lozano D., de Castro L.F., Dapia S., Andrade-Zapata I., Manzarbeitia F., Alvarez-Arroyo M.V., Gómez-Barrena E. and Esbrit P. (2009). Role of parathyroid hormone-related protein in the decreased osteoblast function in diabetes-related osteopenia. Endocrinology 150, 2027-2035.

Maes C., Carmeliet P., Moermans K., Stockmans I., Smets N., Collen D., Bouillon R. and Carmeliet G. (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., Bouillon R. and Carmeliet G. (2004). Soluble vegf isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. J. Clin. Invest. 113, 188-199.

Marks S.C. Jr. and Lane P.W. (1976). Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. J. Hered. 67, 11-18.

Mayer H., Bertram H., Lindenmaier W., Korff T., Weber H. and Weich H. (2005). Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: Autocrine and paracrine role on osteoblastic and endothelial differentiation. J. Cell. Biochem. 95, 827-839.

Nakagawa M., Kaneda T., Arakawa T., Morita S., Sato T., Yomada T., Hanada K., Kumegawa M. and Hakeda Y. (2000). Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. FEBS Lett. 473, 161-164.

Niida S., Kaku M., Amano H., Yoshida H., Kataoka H., Nishikawa S., Tanne K., Maeda N. and Kodama H. (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.

Ortega N., Behonick D.J. and Werb Z. (2004). Matrix remodeling during endochondral ossification. Trends Cell. Biol. 14, 86-93.

Pacicca D.M., Patel N., Lee C., Salisbury K., Lehmann W., Carvalho R., Gerstenfeld L.C. and Einhorn T.A. (2003). Expression of angiogenic factors during distraction osteogenesis. Bone 33, 889-898.

Qu Z.H., Zhang X.L., Tang T.T. and Dai K.R. (2008). Promotion of osteogenesis through beta-catenin signaling by desferrioxamine. Biochem. Biophys. Res. Commun. 370, 332-337.

Rhinelander F.W. (1974a). Tibial blood supply in relation to fracture healing. Clin. Orthop. Relat. Res. 34-81.

Rhinelander F.W. (1974b). The normal circulation of bone and its response to surgical intervention. J. Biomed. Mater. Res. 8, 87-90.

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

Robinson C.J. and Stringer S.E. (2001). The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J. Cell. Sci. 114, 853-865.

Roodhart J.M., Langenberg M.H., Vermaat J.S., Lolkema M.P., Baars A., Giles R.H., Witteveen E.O. and Voest E.E. (2010). Late release of circulating endothelial cells and endothelial progenitor cells after chemotherapy predicts response and survival in cancer patients. Neoplasia 12, 87-94.

Sawano A., Iwai S., Sakurai Y., Ito M., Shitara K., Nakahata T. and Shibuya M. (2001). Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood 97, 785-791.

Schipani E., Ryan H.E., Didrickson S., Kobayashi T., Knight M. and Johnson R.S. (2001). Hypoxia in cartilage: HIF-1alpha is essential

for chondrocyte growth arrest and survival. Genes Dev. 15, 2865-2876.

Schipani E., Maes C., Carmeliet G. and Semenza G.L. (2009). Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. J. Bone Miner. Res. 24, 1347-1353.

Semenza G.L. (2003). Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer. 3, 721-732.

Semenza G.L. (2009). Regulation of cancer cell metabolism by hypoxia-inducible factor 1. Semin. Cancer Biol. 19, 12-16.

Senger D.R., Galli S.J., Dvorak A.M., Perruzzi C.A., Harvey V.S. and Dvorak H.F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219, 983-985.

Shibuya M., Yamaguchi S., Yamane A., Ikeda T., Tojo A., Matsushime H. and Sato M. (1990). Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 5, 519-524.

Shweiki D., Itin A., Soffer D. and Keshet E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359, 843-845.

Street J., Bao M., deGuzman L., Bunting S., Peale F.V., Jr., Ferrara N., Steinmetz H., Hoeffel J., Cleland J.L., Daugherty A., van Bruggen N., Redmond H.P., Carano R.A. and Filvaroff E.H. (2002). Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc. Natl. Acad. Sci. USA 99, 9656-9661.

Takimoto A., Nishizaki Y., Hiraki Y. and Shukunami C. (2009). Differential actions of VEGF-A isoforms on perichondrial angiogenesis during endochondral bone formation. Dev. Biol. 332, 196-211.

Tanaka Y., Abe M., Hiasa M., Oda A., Amou H., Nakano A., Takeuchi K., Kitazoe K., Kido S., Inoue D., Moriyama K., Hashimoto T., Ozaki S. and Matsumoto T. (2007). Myeloma cell-osteoclast interaction enhances angiogenesis together with bone resorption: A role for vascular endothelial cell growth factor and osteopontin. Clin. Cancer Res. 13, 816-823.

Tatsuyama K., Maezawa Y., Baba H., Imamura Y. and Fukuda M. (2000). Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone. Eur. J. Histochem. 44, 269-278.

Terman B.I., Carrion M.E., Kovacs E., Rasmussen B.A., Eddy R.L. and Shows T.B. (1991). Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 6, 1677-1683.

Terman B.I., Dougher-Vermazen M., Carrion M.E., Dimitrov D., Armellino D.C., Gospodarowicz D. and Bohlen P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem. Biophys. Res. Commun. 187, 1579-1586.

Tombran-Tink J. and Barnstable C.J. (2004). Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: Possible mediators of angiogenesis and matrix remodeling in the bone. Biochem. Biophys. Res. Commun. 316, 573-579.

Trejo C.G., Lozano D., Manzano M., Doadrio J.C., Salinas A.J., Dapía S., Gómez-Barrena E., Vallet-Regí M., García-Honduvilla N., Buján J. and Esbrit P. (2010). The osteoinductive properties of mesoporous silicate coated with osteostatin in a rabbit femur cavity defect model. Biomaterials 31, 8564-8573.

Trueta J. and Buhr A.J. (1963). The vascular contribution to osteogenesis. V. The vasculature supplying the epiphysial cartilage in rachitic rats. J. Bone Joint Surg. Br. 45, 572-581.

Trueta J. and Trias A. (1961). The vascular contribution to osteogenesis. Iv. The effect of pressure upon the epiphysial cartilage of the rabbit. J. Bone Joint Surg. Br. 43-B, 800-813.

Utting J.C., Robins S.P., Brandao-Burch A., Orriss I.R., Behar J. and Arnett T.R. (2006). Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. Exp. Cell Res. 312, 1693-1702.

Wan C., Gilbert S.R., Wang Y., Cao X., Shen X., Ramaswamy G., Jacobsen K.A., Alaql Z.S., Eberhardt A.W., Gerstenfeld L.C., Einhorn T.A., Deng L. and Clemens T.L. (2008). Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. Proc. Natl. Acad. Sci. USA 105, 686-691.

Wang G.L., Jiang B.H., Rue E.A. and Semenza G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-pas heterodimer regulated by cellular O2 tension. Proc. Natl. Acad. Sci. USA 92, 5510-5514.

Wang Y., Wan C., Deng L., Liu X., Cao X., Gilbert S.R., Bouxsein M.L., Faugere M.C., Guldberg R.E., Gerstenfeld L.C., Haase V.H., Johnson R.S., Schipani E. and Clemens T.L. (2007). The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. J. Clin. Invest. 117, 1616-1626.

Weidemann A. and Johnson R.S. (2008). Biology of HIF-1alpha. Cell Death Differ. 15, 621-627.

Zelzer E., McLean W., Ng Y.S., Fukai N., Reginato A.M., Lovejoy S., D'Amore P.A. and Olsen B.R. (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 R.S., Schipani E. and Olsen B.R. (2004). VEGFA is necessary for chondrocyte survival during bone development. Development 131, 2161-2171.

Accepted November 30, 2011