Regulation of Osteogenesis-Angiogenesis Coupling by HIFs and VEGF

Ernestina Schipani,1 Christa Maes,2 Geert Carmeliet,2 and Gregg L. Semenza3

ABSTRACT: Bone is a highly vascularized tissue, but the function of angiogenesis in bone modeling and remodeling is still poorly defined, and the molecular mechanisms that regulate angiogenesis in bone are only partially elucidated. Genetic manipulations in mice have recently highlighted the critical role of the hypoxia-inducible-factor/vascular endothelial growth factor pathway in coupling angiogenesis and osteogenesis. In this brief perspective, we review the current understanding of the mechanisms responsible for this coupling. Elucidation of such mechanisms will expand our knowledge of bone development and homeostasis, and it may aid in the design of new therapies for accelerating bone regeneration and repair.

J Bone Miner Res 2009;24:1347–1353. Published online on June 29, 2009; doi: 10.1359/JBMR.090602

Key words: hypoxia inducible factor, vascular endothelial growth factor, osteoblast, angiogenesis

INTRODUCTION

Bone is a highly vascularized and heterogeneous tissue that forms through at least two independent mechanisms: intramembranous and endochondral ossification.1,2 The first, in which mesenchymal cells develop directly into osteoblasts, is involved in the formation of the flat bones of the skull. The second, accounting for the development of most other bones, involves a two-stage mechanism, whereby chondrocytes form a matrix template, the growth plate, which is replaced by bone. During endochondral bone development, growth plate chondrocytes undergo well-ordered and controlled phases of cell proliferation, maturation, and death. This unique differentiation process is followed by blood vessel invasion and replacement of the cartilaginous matrix with bone.2–5

Osteoblasts or bone-forming cells are thought to originate from undifferentiated mesenchymal cells whose commitment to osteoblasts is regulated by at least two transcription factors: Runx2 and Osterix.6,7 According to the current model, committed osteoprogenitors proliferate, differentiate into postmitotic osteoblasts that synthesize and mineralize bone matrix, and finally become either terminally differentiated osteocytes encased into the bony matrix or bone-lining cells. The identification of cells that are osteoprogenitors has been difficult, but their presence in the bone marrow stroma can be confirmed by their functional capacity to divide and differentiate in vitro into bone nodule–forming osteoblasts.8

Blood vessel invasion is a critical event in the replacement of cartilage by bone and in the formation of the bone marrow cavity. Vascular endothelial growth factor (VEGF)-A is one of the critical mediators of blood vessel invasion of the cartilaginous mold. Five VEGF-A isoforms have been identified in humans, whereas there are three major isoforms in the mouse (VEGF120, VEGF164, and VEGF188). VEGF-A binds to and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flik-1), which regulate both physiological and pathological angiogenesis.9 In the embryo, VEGF signaling is essential for angiogenesis, because the deletion of even a single copy of the VEGF-A gene results in embryonic lethality because of defective vascular development.10,11 During endochondral bone development, VEGF-A is produced by both chondrocytes, particularly in their later stages of terminal differentiation, and by osteoblasts.12–14 Altering the expression or the levels of VEGF has a profound impact on vascular invasion of the cartilaginous mold. Mice expressing only the soluble form of VEGF, VEGF120, but lacking VEGF188 and VEGF164 exhibit delayed blood vessel invasion during endochondral bone development.15,16 Similarly, administration of the VEGF inhibitor mFlt(1–3)–IgG completely blocked neoangiogenesis in the growth plates of 24-day-old mice.17

Whereas cartilage is an avascular and hypoxic mesenchymal tissue,18–22 bone is highly vascularized, although the bone marrow is relatively hypoxic compared with other

1Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA; 2Laboratory of Experimental Medicine and Endocrinology, Katholieke Universiteit Leuven, Leuven, Belgium; 3Vascular Program, Institute for Cell Engineering, McKusick-Nathans Institute of Genetic Medicine, Baltimore, Maryland, USA.

The authors state that they have no conflicts of interest.
adult organs (see below).\(^{(23)}\) It is obvious to assume that blood vessels are critical in the biology of bone as providers of nutrients. However, it is also becoming progressively evident that the biological role of blood vessels in bone goes beyond being a mere source of nutrients. For example, progenitors of osteoblasts have been reported to be present in the wall of human bone marrow blood vessels.\(^{(24)}\) All in all, the function of angiogenesis in bone modeling and remodeling is still poorly defined, and the molecular mechanisms that regulate angiogenesis in bone are only partially elucidated.

In recent years, it has been shown that hypoxia is a major driving force for angiogenesis and VEGF-A expression by stabilizing the hypoxia inducible factors (HIFs) protein.\(^{(25)}\) Hypoxia is not an absolute concept, but it is rather a relative decrease of \(O_2\) availability. The definition of “physiologically” normoxic conditions for either embryonic or adult cells varies significantly. Before the circulatory system is established, mammalian development proceeds in a relatively low \(O_2\) environment of \(\sim 3\%\).\(^{(26,27)}\) Moreover, studies that have used small-molecule hypoxia markers have shown the existence of specific regions of moderate to severe hypoxia in the developing embryos.\(^{(28,29)}\) In the majority of normal adult tissues, oxygen (\(O_2\)) levels vary between 2% and 9% (compared with ambient air that contains 21% \(O_2\)).\(^{(30)}\) In contrast, \(O_2\) concentrations in regions of the bone marrow, cartilage, kidney medulla, and thymus are <1% \(O_2\).\(^{(31)}\) Hypoxia is not only a critical factor in fetal development and differentiation but is also a pathophysiological component of many human disorders, including cancer and ischemic diseases.\(^{(20,28–30)}\)

HIF-1, a ubiquitously expressed transcription factor, is a major regulator of cellular adaptation to hypoxia.\(^{(31–35)}\) It is a heterodimeric DNA-binding complex that consists of two basic helix-loop-helix (bHLH) proteins of the PER/ARNT/SIM (PAS) subfamily: HIF-1\(_{\alpha}\) and HIF-1\(_{\beta}\).\(^{(36)}\) HIF-1\(_{\alpha}\) and HIF-1\(_{\beta}\) mRNAs are ubiquitously expressed.\(^{(37)}\) In general, \(\alpha\)-class members of the PAS subfamily respond to environmental signals, whereas \(\beta\)-class molecules aid in targeting the heterodimer to their nuclear targets.\(^{(38)}\) In the HIF-1 system, HIF-1\(_{\alpha}\) levels increase exponentially as \(O_2\) levels drop below 5%.\(^{(39–44)}\) On the other hand, HIF-1\(_{\beta}\) (also known as aryl hydrocarbon nuclear translocator or ARNT) is non–oxygen responsive. On heterodimerization with HIF-1\(_{\alpha}\), the HIF-1\(_{\alpha}\cdot\)HIF-1\(_{\beta}\) complex binds to a specific sequence 5’-RCGTG-3’ (where R denotes a purine residue) termed hypoxia response element (HREs) and transactivates target genes containing HREs.\(^{(44)}\) HIF-1\(_{\alpha}\) does not directly sense variations of \(O_2\) tension,\(^{(46)}\) a class of 2-oxoglutarate–dependent and Fe\(^{2+}\)-dependent dioxygenases are the \(O_2\) sensors.\(^{(39)}\) Two types of \(O_2\) sensors are involved in HIF-1\(_{\alpha}\) action: prolyl-hydroxylase domain proteins (PHDs) and an asparaginyl hydroxylase, respectively. PHDs hydroxylate two prolyl residues (P402 and P564) in the HIF-1\(_{\alpha}\) region referred to as the \(O_2\)-dependent degradation domain (ODDD).\(^{(47)}\) This modification occurs in normoxic conditions and mediates the binding of the von Hippel-Lindau tumor suppressor protein (pVHL), which is an E3 ubiquitin ligase, to HIF-1\(_{\alpha}\). HIF-1\(_{\alpha}\) is marked with poly-ubiquitin chains and targeted for degradation by the proteasome. In well-oxygenated tissues, where \(O_2\) tension is >5%, HIF-1\(_{\alpha}\) displays one of the shortest half-lives (<5 min) among cellular proteins. Conversely, under hypoxic conditions, the activity of the PHDs is largely impaired, and proline hydroxylation cannot occur. As a result, HIF-1\(_{\alpha}\) protein accumulates, and this initiates a multistep pathway that includes nuclear translocation of HIF-1\(_{\alpha}\), dimerization with its partner HIF-1\(_{\beta}\), recruitment of transcriptional co-activators, and binding to HREs within the promoters of hypoxia-responsive genes.\(^{(48)}\) The second type of \(O_2\) sensor is an asparaginyl hydroxylase called factor inhibiting HIF-1\(_{\alpha}\) (FIH-1).\(^{(49,50)}\) This enzyme hydroxylates an asparagine residue (N803) in the carboxy-terminal transcriptional activation domain (C-TAD) of HIF-1\(_{\alpha}\). This covalent modification blocks C-TAD interaction with transcriptional co-activators, such as p300 and CBP. Thus, the two \(O_2\) sensors, PHD and FIH, by regulating the destruction and activity of HIF-1\(_{\alpha}\), respectively, ensure the repression of the HIF-1 pathway in well-oxygenated cells.

To date, >100 putative HIF-1 target genes have been identified.\(^{(51–54)}\) They are involved in a wide variety of biological processes including energy metabolism, angiogenesis, erythropoiesis, cell survival, apoptosis, redox, and pH regulation.\(^{(53,55)}\) Mouse embryos lacking HIF-1\(_{\alpha}\) exhibit multiple morphological defects as early as embryonic day E8.5 and die in utero by E10.5.\(^{(56–58)}\) Many malignant cancers contain regions of severe hypoxia, resulting in high levels of HIF-1\(_{\alpha}\) that drive tumor progression,\(^{(32,35)}\) and inhibition of HIF-1\(_{\alpha}\) has been proposed as a potentially powerful approach.\(^{(59)}\)

pVHL is expressed in most tissues and cells.\(^{(60)}\) Heterozygous germine missense mutations of the \(VHL\) gene are the cause of von Hippel Lindau syndrome,\(^{(61,62)}\) a disease characterized by a dominant predisposition to develop pheochromocytomas and highly vascular tumors of the kidney, central nervous system, and retina.\(^{(61,62)}\) Tumorigenesis results from the loss or inactivation of the wildtype allele.\(^{(61,62)}\) The importance of pVHL for proteolysis of HIF-1\(_{\alpha}\) is underscored by the finding that cells lacking functional pVHL have dramatically reduced ability to degrade this transcription factor, resulting in accumulation of high levels of HIF-1\(_{\alpha}\) under normoxic conditions.\(^{(61,62)}\)

Stimuli other than hypoxia also cause HIF-1\(_{\alpha}\) to accumulate in normoxic cells. For example, growth factors such as IGF-1 can induce HIF-1\(_{\alpha}\) synthesis through activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signal transduction pathway.\(^{(63–66)}\)

Besides HIF-1\(_{\alpha}\), two proteins with sequence similarity to HIF-1\(_{\alpha}\) have been characterized: HIF-2\(_{\alpha}\) and HIF-3\(_{\alpha}\).\(^{(67)}\) HIF-1\(_{\alpha}\) and HIF-2\(_{\alpha}\) have a similar protein structure and undergo the same oxygen-dependent proteolysis. This may indicate that they are functionally redundant, at least in some settings.\(^{(68)}\) However, the pattern of expression of HIF-2\(_{\alpha}\) is largely restricted to blood vessels, neural crest, and distinct cell populations in the brain, heart, lung, kidney, liver, pancreas, and intestine,\(^{(69)}\) whereas HIF-1\(_{\alpha}\) is expressed in all cells. Moreover, mice that are null for HIF-1\(_{\alpha}\) die at early stages of embryonic development, but mice deficient in HIF-2\(_{\alpha}\) survive until mid-to-late gestation or, depending on the strain, until birth.\(^{(56–58,70–74)}\) The two
isofoms therefore seem to have distinct developmental functions. Last, some genes are activated by either HIF-1α or HIF-2α, whereas others are only activated by one or the other. (23,75–77) HIF-3α is not closely related to HIF-1α and HIF-2α.(78) Alternative splicing of the HIF-3α primary RNA transcript produces mRNAs that encode at least six different protein isoforms,(79) one of which is an inhibitory protein that contains the N-terminal bHLH and PAS domains but lacks the C-TAD.(80) This protein acts as a negative regulator of HIF-mediated gene expression.

The next two sections of this brief perspective will summarize our current knowledge about the role of the transcription factor HIF-1α as an essential modulator of osteoblast-angiogenic coupling, particularly in the trabecular compartment of the long bones.

HIFs and Angiogenesis/Osteogenesis Coupling in Bone Development

Hypoxia is likely one of the major drivers of the tight coupling between angiogenesis and bone formation. Osteoblasts, like all other nucleated metazoan cells, express components of the HIF-1 pathway. Studies in the late 1990s have shown that hypoxia is a potent stimulator of VEGF-A mRNA expression in osteoblastic cells.(81) More recently, manipulation of the HIF-1α pathway in osteoblasts has led to altered VEGF-A levels and dramatic changes in bone mass. (82) Indeed, mutant mice that lack VHL in fully differentiated osteoblasts (ΔVHL) and thus overexpress HIFs have a strikingly increased bone volume, which was secondary, at least at early stages, to an increase of osteoblast number and of bone formation rate in absence of detectable changes in osteoclast number and/or activity. Conversely, lack of HIF-1α in osteoblasts (ΔHIF-1α) negatively impacts bone volume. The amount of bone in both ΔVHL and ΔHIF-1α mice is directly proportional to the degree of skeletal vascularization. This suggests that the regulation of bone mass in these mutants may be secondary to changes in VEGF-A levels and angiogenesis. Consistent with this idea, VEGF-A mRNA expression is upregulated in trabecular bone of ΔVHL mice. In addition, in an ex vivo assay, ΔVHL metatarsals exhibit a dramatic increase in endothelial sprouting, which is entirely reversed by preincubation with an anti-VEGF neutralizing antibody. However, the putative mechanisms responsible for coupling angiogenesis to osteogenesis physiologically, as well as in both ΔVHL and ΔHIF-1α mice, remain to be determined. It has been proposed that the bone marrow vascular setting provides a true niche for pericytic mesenchymal stem cell (MSC)-like cells and could be a source of osteoprogenitors or of MSCs with osteogenic potential.(24,83) Thus, the VEGF-dependent increase in angiogenesis observed in ΔVHL mice may lead to more bone volume by providing a larger pool of MSCs. Not mutually exclusive, HIF stabilization or inactivation may also affect osteoblasts directly and independently of angiogenesis. Although cell autonomous effects were not detected by in vitro assays of proliferation, differentiation, and apoptosis, prolonged alterations in HIF activity in vivo may modulate cellular metabolism, matrix formation, or autophagy as proposed for other cell types.(20) Moreover, VEGF-A itself has also been reported to have a direct action on osteoblast differentiation. In particular, mice that express only the VEGF120 isoform exhibit both delayed invasion of vessels into the primary ossification center and altered osteoblastic differentiation in vitro.(16) Interestingly, however, transient hypoxia has been shown to be an inhibitor of osteoblast differentiation in vitro,(84) which further suggests that the dramatic increase in bone volume in mice lacking pVHL in osteoblasts is not a cell autonomous effect but rather results from the increase in blood vessels mediated by the increased VEGF levels.

Numerous factors other than hypoxia increase HIF protein levels in osteoblasts, which consequently leads to increased VEGF-A expression; an example is IGF-1. In human osteoblast-like cells, IGF-1 induces a rapid, 3-fold increase in VEGF-A mRNA. (85) This is accompanied by an increase in HIF-2α protein without a corresponding change in HIF-2α mRNA expression. (85) IGF-1 also stimulates the phosphorylation of Akt, an effect that is abolished by pretreating the cells with the phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002. Treatment with this inhibitor also significantly reduced HIF-2α accumulation and the induction of VEGF mRNA expression by IGF-1. Thus, IGF-1 seems to induce VEGF-A expression in osteoblasts by increasing accumulation of HIF-2α protein levels in a PI3K-dependent fashion. (85) These findings highlight a potential role for HIF-2α in osteoblasts, a finding that needs to be verified in vivo.

Interestingly, manipulation of HIF levels in mature osteoblasts does not noticeably influence the formation of the flat bones of the skull. (82) The calvarial bones are formed through an intramembranous process in which mesenchymal cells differentiate directly into osteoblasts without an intermediate avascular cartilaginous template. It is possible that signals from cranial sutures and/or from the dura induce the angiogenic response necessary for intramembranous ossification or that VEGF is regulated by other factors than HIF in calvarial osteoblasts. This would explain the lack of both blood vessel and bone phenotypes in the skull of ΔVHL and ΔHIF-1α mutant mice.

HIFs and Angiogenesis/Osteogenesis Coupling in Regeneration and Repair

Angiogenesis is essential for bone repair. It has been proposed that, at fracture sites, mechanical stimuli and inflammatory signals, along with hypoxia, which results when the vascular and nutrient supply is interrupted, initiate the events that lead to bone repair. (86) When angiogenesis is delayed, chondrocytic cells rather than osteoblasts make up the healing tissue. This suggests that HIFs play a role in allocating mesenchymal lineage during repair. (87) Distraction osteogenesis (DO) is a valuable model for examining the cellular mechanisms that couple angiogenesis and bone formation during repair and regeneration. In DO, intramembranous bone formation is induced by the
application of an external fixation device that applies gradual mechanical distraction across an osteotomy. This procedure leads to a close temporal and spatial relationship between bone formation and angiogenesis. DO has also been used to investigate the role of HIF-1α in bone healing. In ΔVHL mice, DO is characterized by increases in HIF-1α protein, in VEGF-A mRNA and protein, and in a number of endothelial cells, leading to more blood vessels and more dense woven bone. At DO sites in ΔHIF-1α mice, the opposite takes place, namely deficient angiogenesis and delayed bone consolidation. Additionally, the mRNA and protein expressions of VEGF-A and of osteoblast markers (Runx2, alkaline phosphatase, and osteocalcin) are decreased in this animal model, and, conversely, increased in ΔVHL mice. Perhaps not surprisingly, desferrioxamine, a small molecule that when administered directly into the distraction gap blocks PHD activity and thus elevates HIF-1α can improve healing in a manner virtually identical with that seen when HIF-1α is activated. These studies provide proof of principle that a therapeutical approach that modulates the HIF pathway may speed bone healing.

Numerous studies have highlighted the role of VEGF-A receptor signaling in bone repair and regeneration. Both receptors, which have different affinities for the VEGF-A ligands as well as different downstream effects, are expressed by osteoblasts. During normal DO, both VEGFR1 and VEGFR2 and all three VEGF-A isoform mRNAs are induced. Moreover, inhibition of VEGF-A activity in the distraction gap by antibody blockade of VEGFR1 and VEGFR2 leads to a dramatic decrease of bone formation and a smaller number of blood vessels. Of note, the VEGF-A homolog placental growth factor (PIGF), which binds VEGFR1 as well, probably contributes, because fracture healing is impaired in mice lacking PIGF.

**CONCLUSION**

A growing body of evidence shows that angiogenesis plays a critical role in skeletal development and repair. It has been suggested that increasing numbers of blood vessels introduce more osteoblast progenitors that mature and increase bone formation (Fig. 1). It is also possible that signals emanating from vascular cells hasten osteogenesis (Fig. 1). Further elucidation of the mechanisms that are responsible for the osteoblast-angiogenesis coupling will deepen our understanding of bone development and homeostasis, and it may also aid in the design of new therapies for accelerating bone regeneration and repair.

**REFERENCES**

1. Karsenty G 2003 The complexities of skeletal biology. Nature 423:316–318.
2. Kronenberg H 2003 Developmental regulation of the growth plate. Nature 423:332–336.
3. Zelzer E, Olsen B 2003 The genetic basis for skeletal diseases. Nature 423:343–348.
4. Provot S, Schipani E 2005 Molecular mechanisms of endochondral bone development. Biochem Biophys Res Commun 328:658–665.

5. Lefebvre V, Smits P 2005 Transcriptional control of chondrocyte fate and differentiation. Birth Defects Res C Embryo Today 75:200–212.

6. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B 2002 The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

7. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G 1999 A Cbfal-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 13:1025–1036.

8. Bianco P, Robey P, Simmons P 2008 Mesenchymal stem cells: Revisiting history, concepts and assays. Cell Stem Cell 2:313–319.

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

10. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A 1996 Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439.

11. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW 1996 Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442.

12. Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Zelzer E, Olsen B 2005 Multiple roles of vascular endothelial growth factor-1alpha. J Biol Chem 280:27741–27748.

13. Zelzer E, Mamluk R, Ferrara N, Johnson R, Schipani E, Olsen B 2004 VEGF is necessary for chondrocyte survival during bone development. Development 131:2161–2171.

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

15. Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, Bouillon R, 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.

16. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor (HIF)-1alpha: Its protein stability and biological functions. Exp Mol Med 36:1–12.

17. Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Zelzer E, Olsen B 2004 Deletion of Vhlh in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development. Development 131:2497–2508.

18. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

19. Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Zelzer E, Olsen B 2004 Deletion of Vhlh in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development. Development 131:2497–2508.

20. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

21. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

22. Rodesch D, Simon P, Donner C, Jauniaux E 1992 Oxygen environment within an embryo induces apoptosis and is essential for proper morphological development. Teratology 60:215–225.

23. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

24. Rodesch D, Simon P, Donner C, Jauniaux E 1992 Oxygen environment within an embryo induces apoptosis and is essential for proper morphological development. Teratology 60:215–225.

25. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

26. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

27. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

28. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW 2004 Hypoxia-inducible factor 1: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.
CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. EMBO J 17:6573–6586.

46. Chan D, Shutpin H, Yen S, Giaccia A 2005 Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. Mol Cell Biol 25:6415–6426.

47. Berra E, Benirsi E, Ginouvès A, Valmatt V, Roux D, 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.

48. Ballou PJ, Wilson WJ, O’Brien S, Makino Y, Poellinger L 1999 Regulation of the Hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J Biol Chem 274:6519–6525.

49. Mahon PC, Hirota K, Semenza GL 2001 FIH-1: A novel protein that interacts with HIF-1alpha and VHL to modulate repression of HIF-1 transcriptional activity. Genes Dev 15:2675–2686.

50. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruck RK 2002 FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 16:1466–1471.

51. Leo C, Giaccia A, Denko N 2004 The hypoxic tumor microenvironment and gene expression. Semin Radiat Oncol 14:207–214.

52. Wykoff CE, Pugh C, Maxwell P, Harris A, Ratcliffe P 2000 Identification of novel hypoxia dependent and independent target genes of the von Hippel Lindau (VHL) tumor suppressor by mRNA differential expression profiling. Oncogene 19:2697–2705.

53. Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, van de Waal AJ, van Diest PJ, van der Wall E 2005 Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J Pathol 206:291–294.

54. Bishop T, Lau K, Epstein A, Kim S, Jiang M, O'Rourke D, Lunardi J, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU 2003 Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J 17:271–273.

55. Carmeliet P, Dor Y, Herbert J, Fukumura D, Brusselms K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshet E 1998 Role of HIF-1alpha in hypoxia mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394:485–490.

56. Tuan H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL 1998 The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev 12:3320–3324.

57. Compernolle V, Brusselms K, Acker T, Hoet P, Tjwa M, Cockman M, Wykoff C, Pugh C, Maher E, Ratcliffe P 1999 The tumor suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275.

58. Ryan HE, Lo J, Johnson RS 1998 HIF-1 alpha is required for solid tumor formation and embryonic vasculization. EMBO J 17:3005–3015.

59. Iyer N, Kotch L, Agani F, Leung S, Laughner E, Weng R, Gassmann M, Gearhart J, Lawler A, Yu A, Semenza G 1998 Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1alpha. Genes Dev 12:149–162.

60. Compernolle V, Brusselms K, Franco D, Moorman A, Dewerchin M, Collen D, Carmeliet P 2003 Cardia bifida, defective heart development and abnormal neural crest migration in embryos lacking hypoxia-inducible factor-1alpha. Cardiovasc Res 60:569–579.

61. Chan DA, Krieg AJ, Turcotte S, Giaccia AJ 2007 HIF gene expression in cancer therapy. Methods Enzymol 435:323–345.

62. Haane VH 2005 The VHL tumor suppressor in development and disease: Functional studies in mice by conditional gene targeting. Semin Cell Dev Biol 15:567–574.

63. Kaelin W, Maher E 1998 The VHL tumor suppressor gene paradigm. Trends Genet 14:423–426.

64. Ratcliffe P, Pugh C, Maxwell P 2000 Targeting tumors through the HIF system. Nat Med 12:1315–1316.

65. Zelzer E, Levy Y, Kahana C, Shilo B, Rubinstein M, Cohen B 1997 Insulin induces transcription of target genes through the hypoxia-inducible factor 1 alpha. EMBO J 17:5085–5094.

66. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL 2002 Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidyl-inositol 3-kinase signaling in colon cancer cells. J Biol Chem 277:38205–38211.

67. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL 2001 HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: Novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 21:3995–4004.

68. Zhong D, Chiles K, Felder D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL 2000 Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: Implications for tumor angiogenesis and therapeutics. Cancer Res 60:1541–1545.

69. Gordon J, Simon M 2007 Hypoxia-inducible factors: Central regulators of the tumor phenotype. Curr Opin Genet Dev 17:71–77.

70. Park SK, Dedak AM, Haase VH, Fontana L, Giaccia AJ, Johnson RS 2003 Hypoxia-induced gene expression occurs solely through the action of hypoxia-inducible factor 1alpha (HIF-1alpha): Role of cytoplasmic trapping of HIF-2alpha. Mol Cell Biol 23:4959–4967.

71. Wessner MS, Jurgesen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU 2003 Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J 17:271–273.

72. Carmeliet P, Dor Y, Herbert J, Fukumura D, Brusselms K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshet E 1998 Role of HIF-1alpha in hypoxia mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394:485–490.

73. Tuan H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL 1998 The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev 12:3320–3324.

74. Sorettegna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marek BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, Garcia JA 2003 Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1−/− mice. Nat Genet 35:331–340.

75. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC 2003 Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 23:9361–9374.

76. Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ 2005 Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol 25:5675–5686.

77. Wang V, Davis DA, Haque M, Huang LE, Yarchoan R 2005 Differential gene expression by hypoxia-inducible factor-1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 23:9361–9374.

78. Schipani ET AL.
80. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L. 2001 Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 414:550–554.

81. Steinbrech DS, Mehrara BJ, Saadeh PB, Chin G, Dudziak ME, Gerrets RP, Gittes GK, Longaker MT. 1999 Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts in vitro. Plast Reconstr Surg 104:738–747.

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

83. Maes C, Kobayashi T, Kronenberg HM. 2007 A novel transgenic mouse model to study the osteoblast lineage in vivo. Ann NY Acad Sci 1116:149–164.

84. Salim A, Nacamuli R, Morgan E, Giaccia A, Longaker M. 2004 Transient changes in oxygen tension inhibit osteogenic differentiation and Runx2 expression in osteoblasts. J Biol Chem 279:40007–40016.

85. Akeno N, Robins J, Zhang M, Czyzyk-Krzeska MF, Clemens TL. 2002 Induction of vascular endothelial growth factor by IGF-I in osteoblast-like cells is mediated by the PI3K signaling pathway through the hypoxia-inducible factor-2alpha. Endocrinology 143:420–425.

86. Danis A. 2001 [Mechanism of bone lengthening by the Ilizarov technique]. Bull Mem Acad R Med Belg 156:107–112.

87. Choi I, Ahn J, Chang C, Cho T. 2000 Vascular proliferation and blood supply during distraction osteogenesis: A scanning electron microscope observation. J Orthop Res 18:698–705.

88. Ilizarov GA. 1990 Clinical application of the tension-stress effect for limb lengthening. Clin Orthop Relat Res 8:26–28.

89. Wan C, Gilbert SR, Wang Y, Cao X, Shen X, Ramasawamy G, Jacobsen KA, Al-Aql ZS, Eberhardt AW, Gerstenfeld LC, Einhorn TA, Deng L, Clemens TL. 2008 Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. Proc Natl Acad Sci USA 105:686–691.

90. Liu XD, Deng LF, Wang J, Qi J, Zhou Q, Wang JS, Wei L, Zhu YP. 2007 Regulation of hypoxia inducible factor-1alpha on osteoblast function in osteogenesis. Zhonghua Yi Xue Za Zhi 87:3357–3361.

91. Carano RA, Filvaroff EH. 2003 Angiogenesis and bone repair. Drug Discov Today 8:980–989.

92. Street J, Bao M, deGuzman L, Bunting S, Peale FV Jr, Ferrara N, Steinmetz H, Hoeffel J, Cleland JL, Daugherty A, van Bruggen N, Redmond HP, Carano RA, Filvaroff EH. 2002 Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci USA 99:8656–8661.

93. Harper J, Gerstenfeld LC, Klagesbrun M. 2001 Neuropilin-1 expression in osteogenic cells: Down-regulation during differentiation of osteoblasts into osteocytes. J Cell Biochem 81:82–92.

94. Jacobsen KA, Al-Aql ZS, Wan C, Fitch JL, Stapleton SN, Mason ZD, Cole RM, Gilbert SR, Clemens TL, Morgan EF, Einhorn TA, Gerstenfeld LC. 2008 Bone formation during distraction osteogenesis is dependent on both VEGFR1 and VEGFR2 signaling. J Bone Miner Res 23:596–609.

95. Maes C, Coenegrachts L, Stockmans I, Daci E, Luttun A, Petryk A, Gopalakrishnan R, Moermans K, Smets N, Verfaillie CM, Carmeliet P, Bouillon R, Carmeliet G. 2006 Placental growth factor mediates mesenchymal cell development, cartilage turnover, and bone remodeling during fracture repair. J Clin Invest 116:1230–1242.

Received in original form June 11, 2009; revised form June 16, 2009; accepted June 23, 2009.