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A Central Role for Hypoxic Signaling in Cartilage, Bone, and Hematopoiesis

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Abstract

Hypoxic signaling plays an essential role in maintaining oxygen homeostasis and cell survival. Hypoxia-inducible transcription factors HIF-1 and HIF-2 are central mediators of the cellular response to hypoxia by regulating the expression of genes controlling metabolic adaptation, oxygen delivery, and survival in response to oxygen deprivation. Recent studies have identified an important role for HIF-1 and HIF-2 in the regulation of skeletal development, bone formation, and regeneration, as well as joint formation and homeostasis. In addition, overexpression of HIF-1 and HIF-2 is clinically associated with osteosarcoma and osteoarthritis. Together, these findings implicate hypoxic signaling as a central regulator of bone biology and disease.

Keywords

Hypoxia; Bone; VHL; HIF-1; HIF-2; Cartilage; Hematopoiesis; Osteoarthritis; Osteosarcoma; Osteogenesis; Angiogenesis; Osteoblast; Chondrocyte; Limb bud mesenchyme; Joint

Introduction

Oxygen is an essential biological molecule required for oxidative metabolism, the generation of adenosine 5'-triphosphate, and survival. To maintain oxygen homeostasis, metazoan organisms have adapted the hypoxic signaling pathway to facilitate oxygen delivery and cellular adaptation to oxygen deprivation. As a result, hypoxic signaling plays

an important role in development, tissue homeostasis, and pathologic conditions. The bone is a physiologically hypoxic tissue, and a central role for the hypoxic signaling pathway is emerging in bone biology and disease. This review will summarize our current knowledge regarding hypoxic signaling in bone and will highlight recent advances within this field.

Hypoxia-Inducible Factors (HIFs)

The transcription factors HIF-1 and HIF-2 are key effectors of the cellular response to hypoxia. In response to low oxygen tensions, HIF-1 and HIF-2 activate gene expression programs controlling glucose uptake, metabolism, angiogenesis, erythropoiesis, cell proliferation, differentiation, and apoptosis [1].

HIF activity is regulated by oxygen through two distinct mechanisms. First, oxygen negatively regulates HIF protein stability. Under normoxic conditions, prolyl hydroxylase enzymes (PHDs 1–3) use oxygen as a substrate to hydroxylate key proline residues within the HIF-1 and HIF-2 α subunits [2–4]. This hydroxylation event triggers the proteasomal degradation of HIF mediated by the VHL (von Hippel-Lindau disease tumor suppressor gene) E3 ubiquitin ligase complex [3, 5, 6]. Under hypoxic conditions (< 5% partial pressure of oxygen [pO_2]), low oxygen availability and multiple mitochondrial products including tricarboxylic acid cycle intermediates and ROS inhibit PHD activity resulting in HIF- α stabilization and translocation to the nucleus [7]. In the nucleus, HIF- α subunits dimerize with ARNT, bind to hypoxia-responsive elements within regulatory elements of target genes, and recruit transcriptional coactivators including p300/CBP to activate transcription. Although HIF-1 and HIF-2 bind similar target DNA sequences, they have both overlapping and distinct functions in the cellular response to hypoxia [8, 9].

In addition to regulating HIF protein stability, oxygen also modulates HIF activity by controlling its ability to activate transcription. Similar to the PHDs, factor-inhibiting HIF-1 (FIH-1) belongs to the 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily that uses oxygen as a substrate to hydroxylate the HIF- α subunits [10]. FIH-1-mediated hydroxylation occurs on particular asparagine residues within the HIF-1 α and HIF-2 α C-terminal transactivation domains and directly interferes with the ability of HIF to recruit the coactivators p300/CBP to target genes [11]. Thus, full activation of HIF transcriptional activity requires both HIF- α stabilization and C-terminal activation domain activation [11]. Both of these events are regulated by hydroxylase enzymes that require molecular oxygen for their activity.

Hypoxia and Bone Development

During development, the hypoxic signaling pathway plays an essential role in coordinating organogenesis and angiogenesis. As the developing embryo grows, tissues quickly deplete oxygen and nutrient supplies available by simple diffusion [12]. Hypoxic gradients are established and promote the formation of new blood vessels that deliver oxygen and nutrients to the growing tissue. The importance of the hypoxic signaling pathway in development is underscored by the finding that germline inactivation of HIF-1, HIF-2, and ARNT results in embryonic lethality. Inactivation of HIF-1 results in embryonic lethality at E10 due to severe vascular and central nervous system (CNS) defects [13]. Similarly,

ARNT-deficient mice die by embryonic day 10.5 from defective angiogenesis of the yolk sac [14, 15]. Interestingly, inactivation of HIF-2 in different genetic backgrounds results in distinct developmental defects. One group observed that HIF-2-deficient embryos die by day 16.5 due to insufficient catecholamine synthesis and bradycardia [16]. Another group found that HIF-2-deficient embryos die by day 13.5 due to severe vascular defects in the yolk sac and embryo proper [17]. A third group observed that HIF-2-deficient mice die shortly after birth due to insufficient surfactant production from type II alveolar cells [18]. Finally, inactivation of HIF-2 in a mixed 129/C57 genetic background results in viable mice that develop multiple-organ pathology associated with mitochondrial dysfunction [19]. Collectively, these findings demonstrate that HIF family members are essential yet play distinct roles in embryonic development.

In bone, hypoxia plays an important role in endochondral ossification. This process occurs in two stages whereby chondrocytes initially form a matrix template at the avascular growth plate, followed by blood vessel invasion and the replacement of the cartilaginous matrix by bone through the actions of osteoblasts and osteoclasts. Recent studies targeting the HIF pathway in chondrocytes and osteoblasts have demonstrated that the HIF pathway influences both stages of endochondral ossification. Conditional ablation of HIF-1 in growth plate chondrocytes and the limb bud mesenchyme results in defective skeletal development associated with massive cell death in the inner chondrocyte layer of the growth plate [20–22]. A role for hypoxia and HIF-1 in chondrocyte survival, proliferation, and differentiation has been observed and may contribute to the skeletal defects observed in HIF-1-deficient mice [23]. Differently from HIF-1, lack of HIF-2 causes only a modest and transient delay of endochondral bone development. Heterozygous HIF-2 embryos develop mild and transient dwarfism that resolves within 2 weeks following birth [24•]. Similarly, inactivation of HIF-2 in the limb bud mesenchyme results in a mild and transient delay in endochondral bone development at E17.5 [25•]. The molecular mechanisms responsible for this phenotype are still largely unknown, but may involve an impairment of the latest stage of chondrocyte hypertrophy in the fetal growth plate [24•] [25•].

Paradoxically, loss of VHL in chondrocytes also results in severe dwarfism. Similar to HIF-1-deficient growth plates, VHL-deficient growth plates displayed severe hypocellularity. However, the hypocellularity of VHL-deficient growth plates was associated with decreased chondrocyte proliferation rather than chondrocyte death [26]. Whether this phenotype is HIF dependent remains unknown. Conditional inactivation of VHL and HIF-1 in chondrocytes resulted in chondrocyte death and a phenotype that resembled HIF-1-deficient mice [26]. With the recent generation of HIF-2 conditional knockout mice, we are now able to investigate the contribution of HIF-2 to VHL-associated phenotypes in cartilage [27]. Loss of VHL is also associated with decreased proliferation and senescence in mouse embryo fibroblasts, indicating that VHL is an important factor for cell cycle progression under certain cellular contexts [28, 29].

Manipulation of the HIF pathway in osteoblasts revealed that the hypoxic signaling pathway has an important role in coupling osteogenesis to angiogenesis both in bone development and in postnatal bone homeostasis. Blood vessel invasion of the cartilaginous template is a critical event in bone formation. In addition to delivering oxygen and nutrients to the

avascular bone collar, blood vessels deliver both osteoclasts that degrade the matrix template as well as osteoblast precursors needed for subsequent bone formation [30, 31•]. Conditional deletion of HIF-1 or HIF-2 in osteoblasts results in a significant decrease in bone volume, whereas HIF-1 and HIF-2 overstabilization through loss of VHL leads to an increase in bone mass [32•, 33, 34]. The changes in bone volume were associated with modulation of bone vascularization and vascular endothelial growth factor (VEGF)-A expression [32•, 33, 34].

VEGF-A is one of the critical mediators of blood vessel invasion of the cartilaginous mold, and it is a classical downstream target of HIF. Blocking VEGF activity either genetically or pharmacologically has a profound impact on vascular invasion of the cartilaginous mold. Mice lacking VEGF-A isoforms 164 and 188 exhibit delayed blood vessel invasion during endochondral bone development associated with decreased trabecular bone volume [35, 36]. Similarly, administration of soluble VEGF receptors to 24-day-old mice completely blocked neoangiogenesis in the growth plates and significantly impaired trabecular bone formation [37]. During endochondral bone development, VEGF is produced by both chondrocytes and by osteoblasts [26, 38, 39].

Collectively, these data support a model by which the HIF pathway influences bone formation through the regulation of angiogenesis. Whether the HIF pathway plays additional roles in endochondral ossification remains to be determined. A recent report by Maes et al. [31•] identified a specific role for osterix-expressing osteoprogenitor cells in the formation of trabecular osteoblasts, osteocytes, and stromal cells inside the developing bone. Most strikingly, they observed that osteoprogenitors were found in direct contact with invading blood vessels and appeared to invade the cartilage matrix alongside blood vessels [31•]. Although the exact mechanisms governing the interaction between blood vessel invasion and osteoprogenitor function in the developing bone remain unclear, it will be important to determine the role of the HIF pathway in osteoprogenitor cells and bone formation.

Interestingly, manipulation of the HIF pathway in mature osteoblasts does not appear to affect the formation of flat bones in the skull [32•]. Calvarial bones are formed through an intramembranous process in which mesenchymal cells differentiate directly into osteoblasts without an intermediate cartilaginous template [23]. Although this process is also coupled to angiogenesis, it is perhaps more likely that signals from cranial sutures and/or from the dura induce the angiogenic response or that VEGF is regulated by other factors than HIF in calvarial osteoblasts. In support of this notion, a blood vessel phenotype was not found in the skull of VHL or HIF-1 α -deficient mice [32•].

Hypoxia and Physiologic Activities in Bone

In addition to regulating organogenesis and development, hypoxia plays an important role in maintaining tissue homeostasis. In most adult tissues, oxygen tensions range from 2% to 9% [40]. Physiologic tissue oxygen tensions are significantly lower than ambient oxygen tensions of 21% oxygen because the pO₂ in blood dramatically decreases as it travels from the lungs throughout the body [40]. Thus, hypoxic gradients and the HIF signaling pathway control normal tissue function. For example, oxygen gradients in the liver control metabolic zonation by regulating the expression of genes for carbohydrate metabolism [41].

Relative to most other tissues, the bone is a particularly hypoxic tissue with pO_2 levels below 1% in hypoxic regions and up to 6% in sinusoidal cavities [40]. Low oxygen tensions in the bone are likely a result of low oxygen levels in sinusoidal blood and high levels of oxygen consumption by hematopoietic cells. Blood flow in the bone travels from the central artery into arterioles, capillaries, and finally into sinusoids accompanied with a progressive drop in blood velocity and oxygenation [42]. It has been estimated that the blood flow rate in sinusoids is approximately 1/10 to 1/20 of that found in arterioles [43]. In addition to low blood flow and oxygen tension within sinusoids, mathematical models predict that a layer of three myeloid progenitors is sufficient to consume most oxygen provided by a nearby sinusoid [42, 44].

Hypoxia controls numerous activities in the adult bone including angiogenesis [32•, 33], bone repair [45, 46], osteoblast function [32•, 33], metabolism, as well as osteoclast size and activity [47, 48]. In particular, the HIF signaling pathway plays an important role in bone formation during fracture repair. The processes that drive bone formation during fracture repair recapitulate skeletal development [49]. Similar to endochondral bone formation, the HIF pathway plays an important role in coupling angiogenesis to bone formation at fracture sites. Notably, genetic inactivation of HIF-1 in osteoblasts leads to impaired vascularization and bone regeneration in distraction osteogenesis models, whereas inactivation of VHL enhances vascularization and bone regeneration [34]. Of clinical importance, pharmacologic stabilization of HIF using local injection of prolyl hydroxylase inhibitors increases angiogenesis and callus formation during fracture repair [50]. However, the systemic administration of prolyl hydroxylase inhibitors for bone repair has to be carefully considered, because partial HIF-1 deficiency in mice (HIF-1 heterozygous mice) was reported to enhance bone regeneration following fracture repair despite decreased angiogenesis [46]. This report suggests the mechanism involves HIF-1 effects on apoptosis, suggesting that HIF-1 regulates cell number at both the proliferation and apoptosis stages of cell life span.

Recent studies have also highlighted the role of hypoxia in the bone in the regulation of hematopoietic stem cells (HSCs) and hematopoiesis. In adult mammals, hematopoiesis occurs almost exclusively in the bone suggesting that factors within bone may regulate hematopoiesis. Evidence to support this concept was first obtained from RUNX2-deficient mice that lack a mineralized skeleton and fail to establish hematopoiesis in the bone marrow [51, 52]. Moreover, multiple cell types within the bone marrow have been reported to influence hematopoiesis including osteoblasts, endothelial cells, and mesenchymal stem cells [53, 54]. The majority of studies thus far support a role for osteoblasts in the regulation of HSCs. Multiple groups have reported a direct correlation between osteoblast and HSC numbers in the bone marrow as well as at ectopic sites [55–56, 57•].

Tracking HSCs in transplantation assays revealed that HSCs localize to the most hypoxic regions of the bone marrow at the endosteal bone surface [58]. Studies using hypoxic cell markers confirmed that HSCs are hypoxic in vivo [42, 59]. Ex vivo HSC cultures supported a functional role for hypoxia in maintaining HSC cell cycle quiescence and a dedifferentiated state [60, 61]. Recent studies have demonstrated that hypoxia and the HIF signaling pathway are critical factors regulating HSC function in vivo. Simsek et al. [62•]

found that long-term HSC metabolism is dependent on glycolysis instead of mitochondrial respiration and this process is regulated through the transcriptional activation of Meis1 by HIF-1. Takubo et al. [63•] showed that dysregulation of HIF-1 levels affects HSC cell cycle quiescence and numbers. Deletion of HIF-1 in HSCs resulted in a loss of HSC cell cycle quiescence and HSC numbers during stress conditions [63•]. Together these studies demonstrate an important role for the hypoxic microenvironment in bone in the regulation of HSCs. Future studies will address whether hypoxic signaling in bone cells influences hematopoiesis.

Hypoxia and Pathologic Conditions in Bone

Hypoxia and activation of the HIF pathway are associated with a variety of pathologic conditions. Most notably, activation of the HIF pathway plays a significant role in tumor progression. VHL, the primary negative regulator of HIF protein stability, was initially cloned as the von Hippel-Lindau disease tumor suppressor gene [64]. This familial tumor syndrome is characterized by the development of benign vascular tumors in the CNS and retina as well as malignant clear cell tumors in the kidney [65]. The importance of VHL as a tumor suppressor is underscored by the finding that VHL is also inactivated in the majority of sporadic renal cell carcinomas and hemangioblastomas [64, 66]. Analysis of VHL renal tumors demonstrated that loss of VHL function results in the constitutive activation of HIF and consequently its target genes, irrespective of oxygen concentrations. HIF activation induces the expression of genes involved in the regulation of angiogenesis, erythropoiesis, glucose uptake and metabolism, cellular proliferation, and metastasis; all of which are associated with tumorigenesis and are postulated to promote VHL disease-associated manifestations.

In addition to genetic activation of the HIF pathway through VHL mutations, the HIF pathway is commonly induced during tumor growth through hypoxia. Similar to embryonic development, a growing tumor quickly depletes oxygen and nutrient supplies obtained by diffusion from nearby blood vessels. As a result, tumor cells become hypoxic and use the HIF pathway to facilitate angiogenesis, survival, and growth [12]. Immunohistochemical analysis of human cancer specimens indicates that the majority of primary human tumors and metastases have increased HIF-1 and/or HIF-2 protein compared with the surrounding normal tissue [67]. Moreover, HIF overexpression in tumors is often associated with increased patient mortality [67]. Experimental studies have demonstrated that HIF activity is required for tumor growth and metastasis in a number of tumor models [67]. Thus, targeting HIF and its targets for cancer therapy is an attractive strategy for the treatment of cancer [68].

In bone, osteosarcoma is the most common primary malignant neoplasm. Immunohistochemical analysis of human osteosarcoma specimens revealed that HIF-1 is significantly increased in tumor cells compared with normal adjacent cells. In addition, HIF-1 expression correlates with metastasis, poor patient survival, and overall survival [69, 70]. In vitro studies have suggested that HIF activity controls osteosarcoma angiogenesis and proliferation [69, 71]. However, further studies are needed to assess the role of HIF osteosarcoma progression in vivo.

In addition to bone cancer, a role for the HIF pathway in osteoarthritis has recently been discovered. Saito et al. [24•] and Yang et al. [72] found that HIF-2 expression was significantly increased in human osteoarthritis specimens. Furthermore, a single nucleotide polymorphism within the HIF-2 promoter was identified that correlates with the development of osteoarthritis in human patients. Interestingly, this mutation conferred increased HIF-2 promoter activity in chondrogenic, but not nonchondrogenic cells [24•]. Using both loss-of-function and gain-of-function approaches, it was found that HIF-2 plays a role in cartilage destruction associated with osteoarthritis. This phenotype was associated with HIF-2 control of catabolic genes associated with cartilage matrix degradation including metalloproteinases as well as VEGF expression [24•, 72].

Conclusions

Hypoxic signaling plays an important role in the cellular adaptation to low oxygen. Increasing evidence supports a central role for the HIF signaling pathway in bone biology and disease. Mouse models in which key components of the HIF signaling pathway have been genetically manipulated in the limb bud mesenchyme, chondrocytes, and osteoblasts have demonstrated a critical role for this pathway in skeletal development, trabecular and cortical bone formation, bone regeneration, as well as joint formation and homeostasis (Table 1).

Hypoxia is undoubtedly a major regulator of HIFs; however, a function of HIFs in normoxic conditions has also been reported. Thus, it will be important to perform studies targeting prolyl hydroxylase family members (ie, the “oxygen sensors”) in bone and cartilage to provide the missing direct link between hypoxia and HIFs in bone biology and disease.

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Table 1
Bone, cartilage, and joint phenotypes generated by genetic manipulation of the hypoxic signaling pathway in mice

Hypoxic signaling factor	Cell type	Cre transgenic promoter	Phenotype	References
Bone				
VHL Loss-of-function	Osteoblast	Osteocalcin	Increased trabecular and cortical bone in long bones associated with increased angiogenesis and VEGF production. Enhanced bone regeneration in distraction osteogenesis models	Wang et al. [32•], Wan et al. [34]
VHL/HIF-1 Loss-of-function	Osteoblast	Osteocalcin	Increased trabecular and cortical bone and vascularization in long bones relative to control mice. However, decreased trabecular and cortical bone relative to osteocalcin-VHL mice	Wang et al. [32•]
HIF-1 Loss-of-function	Osteoblast	Osteocalcin	Decreased trabecular and cortical bone and vascularity in long bones. Impaired bone regeneration in distraction osteogenesis models	Wang et al. [32•], Shomento et al. [33], Wan et al. [34]
HIF-2 Loss-of-function	Osteoblast	Osteocalcin	Decreased bone volume and vascularity	Shomento et al. [33]
HIF-1 Loss-of-function	Germline heterozygous	N/A	Enhanced bone regeneration following femoral fracture associated with decreased apoptosis	Komatsu et al. [46]
Cartilage				
VHL Loss-of-function	Chondrocytes	Col2a1	Severe dwarfism with shortening of the limb skeleton. VHL null growth plate displayed severe hypocellularity, increased matrix, and a delay in the development of the secondary ossification center. This phenotype was associated with a decrease in chondrocyte proliferation	Pfander et al. [26]
VHL/HIF-1 Loss-of-function	Chondrocytes	Col2a1	Postnatal death with deformation and shortening of the limbs. Hypocellular growth plate associated with massive cell death. These mice phenocopy Col2a1-HIF-1 null mice	Pfander et al. [26]
HIF-1 Loss-of-function	Chondrocytes	Col2a1	Shortening of the limbs and skeletal defects associated with massive chondrocyte cell death in the fetal growth plate. Postnatal death within hours of birth due to tracheal abnormalities and cleft palate	Schipani et al. [20], Unpublished
HIF-1 Loss-of-function	Limb bud mesenchyme	Prx-1	Severe retardation of limb skeletal formation with impaired cartilage and joint formation. Extensive chondrocyte cell death was observed	Amarilio et al. [21], Provot et al. [22]

Hypoxic signaling factor	Cell type	Cre transgenic promoter	Phenotype	References
HIF-2 Loss-of-function	Limb bud mesenchyme	Prx-1	Mild and transient decrease in E17.5 tibia length associated with a modest delay in late hypertrophy at the fetal growth plate	Araldi et al. [25•]
HIF-2 Loss-of-function	Germline knockout	N/A	Perinatal death associated with multiorgan pathology including severe dwarfism, skeletal myopathy, retinopathy, hepatic steatosis, hypocellular bone marrow, and cardiac hypertrophy.	Scortegagna et al. [19]
HIF-2 Loss-of-function	Germline heterozygous	N/A	Mild and transient dwarfism in embryos with normal organ pathology. A mild delay in chondrocyte hypertrophy in fetal growth plates was reported	Saito et al. [24•]
Joint				
HIF-2 Loss-of-function	Germline heterozygous	N/A	Decreased cartilage degradation in a surgical and collagenase-induced osteoarthritis associated with decreased Col10a1, MMP-13, and VEGF expression	Saito et al. [24•], Yang et al. [72]
HIF-2 Gain-of-function	Adenoviral delivery of HIF-2 to joint	N/A	Severe cartilage destruction in the joint resembling osteoarthritis associated with increased expression of catabolic genes	Yang et al. [72]

HIF hypoxia-inducible factor; *MMP* matrix metalloproteinase; *N/A* not applicable; *VEGF* vascular endothelial growth factor; *VHL* von Hippel-Lindau