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PERSPECTIVES

Hypoxia and the Hypoxia-Inducible Factors in the Skeleton

Ernestina Schipani[1] and Thomas L. Clemens[2]

[1]Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA
[2]Department of Pathology, University of Alabama and the Veterans Administration Medical Center, Birmingham, Alabama, USA

Abstract

In recent years, the transcription factor Hif-1α has emerged as the central factor controlling the cellular response to reduced oxygen. The purpose of this *Perspective* is to briefly summarize our current knowledge about hypoxia and Hif-1α in the cells that compose the skeleton. Advances in understanding the molecular mechanisms that mediate the complex and multifaceted action of this transcription factor both in chondrocytes, osteoblasts and osteoclasts open the way to novel therapies to improve bone accretion and repair. *IBMS BoneKEy*. 2008 Aug;5(8):275-284.

Introduction

The ability of mammalian cells to sense and respond to changes in oxygen tension is fundamental for many physiological and pathophysiological processes (1;2). The transcription factor Hif-1α has emerged as the central regulator of the hypoxic response in mammals (3-7). Two other family members, Hif-2α and Hif-3α, have also been characterized (8). Hif-1α is activated when oxygen (O_2) levels drop below 5%, and its activity progressively increases with a decrease in O_2 gradient (9). Hetero-dimerization of Hif-1α with Hif-1β (also known as aryl hydrocarbon nuclear translocator, ARNT), another basic-helix-loop-helix Per/Arnt/Sim (PAS) domain protein (4), allows binding to a hypoxia response element (HRE) and transactivation of its target genes (10). The mRNAs encoding Hif-1α and -β are widely expressed and detectable in all assayed human and rodent tissues (11). Whereas Hif-1β protein is constitutively expressed, Hif-1α protein is subject to rapid degradation by O_2-dependent proteolysis (12-16).

Hif-1α does not directly sense variations in O_2 tension; the key regulation is orchestrated by a class of 2-oxoglutarate-dependent and Fe^{2+}-dependent dioxygenases, which are the O_2 sensors (9). Two types of O_2 sensors control Hif-1α action. The first type, prolyl-hydroxylase domain (PHD) proteins, hydroxylate Hif-1α at two prolyl residues (P402 and/or P564) within a region referred to as the O_2-dependent degradation domain (ODDD) (17). This modification allows binding of the von Hippel-Lindau tumor suppressor protein (VHL), a component of an E3 ubiquitin ligase complex, to Hif-1α. Hif-1α is then marked with polyubiquitin chains and degraded by the proteosome. In well-oxygenated tissues, Hif-1α displays one of the shortest half-lives (<5 min) among cellular proteins. Under hypoxic conditions, proline hydroxylation cannot occur, and Hif-1α protein is stabilized (Fig. 1) (10). The PHDs are distinct from the family of collagen prolyl-hydroxylases. The second type of O_2 sensor is an asparaginyl hydroxylase, referred to as factor inhibiting Hif (FIH) (18). This enzyme hydroxylates an asparagine residue (N803) in the carboxy-terminal transcriptional activation domain (C-TAD). This covalent modification abrogates C-TAD interaction with transcriptional co-activators, such as p300 and CBP. Thus, the two O_2 sensors, PHD and FIH, by controlling destruction and activation of Hif-1α, respect-

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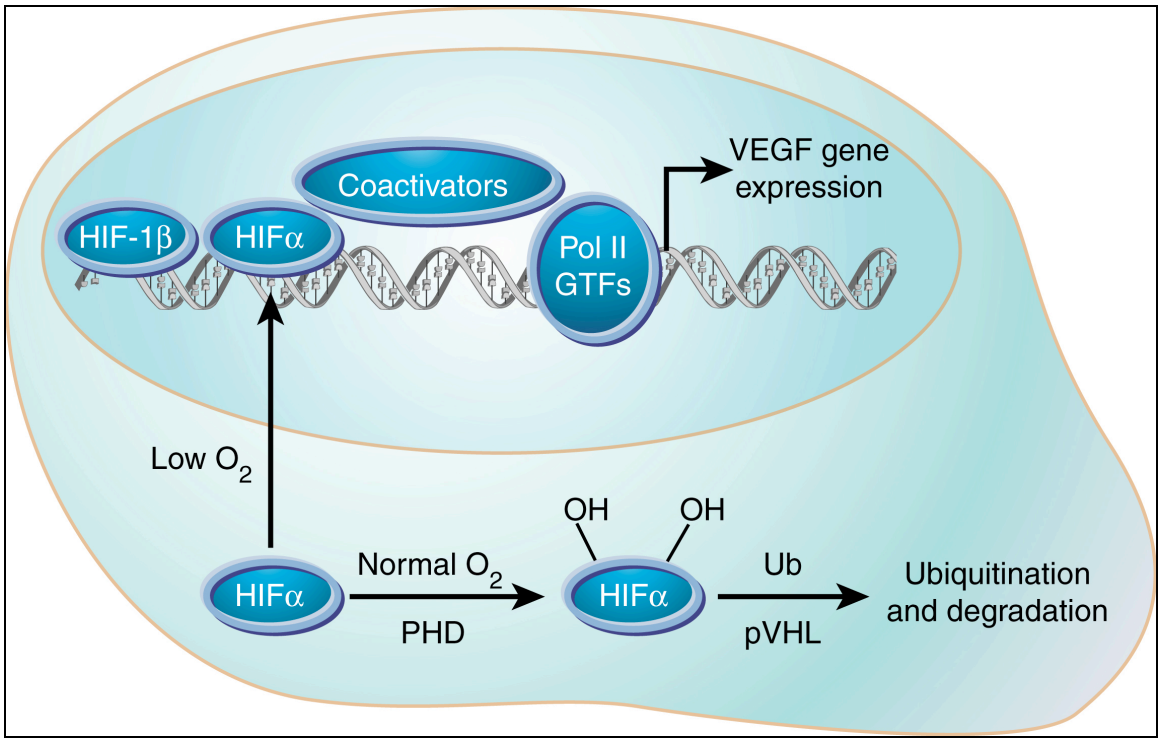

Fig. 1. The hypoxia-inducible factor pathway.

ively, ensure the repression of the Hif-1α pathway in well-oxygenated cells.

To date, more than one hundred putative Hif-1α target genes have been identified (19-22), which are involved in a variety of biological processes including energy metabolism, angiogenesis, survival and regulation of pH. The importance of Hif-1α transcriptional activity in embryonic development is emphasized by the observation that mouse embryos lacking Hif-1α begin to exhibit multiple morphological defects as early as E8.5 and die *in utero* by E11 (23-25). However, the early lethality makes it difficult to determine the direct developmental role of Hif-1α in response to hypoxia. Additionally, Hif-1α is recognized as a strong promoter of tumor growth. Malignant tumors are often highly hypoxic, and pharmacological inhibition of Hif-1α is currently being explored as an anticancer therapy (5). Genes leading to cell death can also be induced by this transcription factor (26). However, an increasing amount of evidence supports the notion that Hif-1α promotes "survival" of hypoxic cells. This appears to be the case for growth plate chondrocytes.

Hypoxia and Hif-1α in Chondrocytes and Joints

Recent studies from a number of laboratories including our own provide *in vivo* evidence for a pro-survival function of this transcription factor, at least in a developmental setting (27). The developing fetal growth plate presents with a typical in-out gradient of oxygenation and a hypoxic central region. Hif-1α is essential for survival of hypoxic chondrocytes, particularly those in the center of the developing growth plate (27).

While it is likely that HIF-1α is a "survival" factor for hypoxic chondrocytes, more uncertain are the downstream mediators of HIF-1α survival function. Hif-1α may use a variety of mechanisms to promote cell survival in hypoxic conditions. Some of them involve regulation of glucose metabolism. Louis Pasteur was the first to notice that O_2-deprived cells exhibit increased conversion of glucose to lactate, the so-called "Pasteur

effect." Activation of the Pasteur effect in mammalian hypoxic cells is Hif-1α-dependent, since Hif-1α up-regulates glucose transporters such as Glut1, glycolytic enzymes that support an increase in ATP production by anaerobic glycolysis, and the enzyme lactic dehydrogenase (LDH) that converts pyruvate to lactate (23;28). It has also been recently reported that Hif-1α inhibits mitochondrial oxidative phosphorylation by negatively modulating the entry of pyruvate into the tricarboxylic acid (TCA) cycle (29;30). Paradoxically, hypoxia causes oxidative stress and release of reactive oxygen species (ROS) (31). By inhibiting the entry of pyruvate into the mitochondria, Hif-1α attenuates not only mitochondrial respiration but also production of ROS in hypoxic cells (31). Therefore, a lack of Hif-1α in hypoxic tissue could result in decreased accumulation of ATP, increased production of ROS, and increased hypoxia secondary to augmented consumption of O_2. Each of these metabolic changes could significantly affect the ability of cells to survive in a hypoxic environment. Consistent with this model and with the hypoxic status of the fetal growth plate, mRNA encoding phosphoglycerokinase-1 (PGK), a key enzyme of anaerobic glycolysis, is strikingly up-regulated in the fetal growth plate, in comparison with the surrounding soft tissue (27), and we have demonstrated that disruption of HIF-1α expression alters this expression pattern (27). These findings, which are supported by our *in vitro* data in primary monolayer cultures (32), indicate that in the absence of HIF-1α, hypoxic chondrocytes may not be able to maintain ATP levels. Therefore, impaired anaerobic glycolysis could be one of the causes of the central cell death phenotype observed in HIF-1α-deficient growth plates.

The survival action of Hif-1α in chondrocytes is likely to be mediated in part by the actions of VEGF, a 45kDA homodimeric glycoprotein that belongs to the dimeric cysteine-knot growth factor super-family (33;34). The mouse VEGF gene encodes at least three isoforms (VEGF120, VEGF164, and VEGF188) that arise via alternative splicing (35;36). While VEGF164 and VEGF188 bind the extracellular matrix component heparan sulfate, VEGF120 lacks the binding motif necessary for this interaction (37;38). VEGF is a principal regulator of blood vessel formation and hematopoiesis (33), but the mechanism by which VEGF regulates these processes has been elusive, perhaps as a result of the expression of multiple isoforms and then the presence of more than one receptor. Recently, an internal autocrine loop mechanism by which VEGF controls survival of hematopoietic stem cells has been proposed (39), but the role of VEGF as a critical mediator of the survival action of Hif-1α is still controversial. Two independent reports have shown that in Hif-1α null embryos, VEGF expression is increased, not reduced, and vascular regression appears to be secondary to mesenchymal cell death rather than to VEGF deficiency (25;40). Our studies and those of others have demonstrated that in the fetal growth plate, VEGF is expressed not only in late hypertrophic chondrocytes, where it is critical for blood vessel invasion and replacement of cartilage by bone (41-46), but also, even if at a considerably lower level, in the center of the proliferative and upper hypertrophic layers (*i.e.*, in the hypoxic zones of the growth plate) (45;47). Numerous lines of evidence indicate that expression of VEGF in the "hypoxic" domain of the growth plate is Hif-1α-dependent. First, in fetal growth plates in which HIF-1α transcriptional activity is up-regulated as a result of VHL conditional knockout, the pattern of expression of VEGF is perturbed, particularly in the hypoxic domain (47). Second, hypoxia increases VEGF accumulation in proliferative chondrocytes *in vitro* in a HIF-1α-dependent manner (32;48). Collectively, these observations raise the possibility that VEGF could be a downstream mediator of Hif-1α survival function. Consistent with this conclusion, both the universal knockout of VEGF164 and VEGF120, and the conditional knockout of all three VEGF isoforms in chondrocytes, led to a cell death phenotype in the center of the proliferative layer and in the upper hypertrophic zone of the fetal growth plate that mimicked what was observed in the HIF-1α-deficient growth plates, though it was considerably milder (45;49). Surprisingly, VEGF expression appeared to

be up-regulated in the viable chondrocytes adjacent to the area of cell death in the Hif-1α-deficient growth plate (27). In light of this paradoxical finding and of the phenotypical differences between the VEGF-deficient and Hif-1α-deficient growth plates, it is still unresolved whether VEGF is a critical downstream effector of Hif-1α survival function in growth plate chondrocytes. Addressing this question will provide important mechanistic insights into the biological role of Hif-1α.

Numerous lines of evidence suggest that autophagy is a survival mechanism downstream of hypoxia and Hif-1α (50). A role for the autophagic pathway downstream of Hif-1α in chondrocytes has also been proposed (51). It is thus possible that autophagy is an additional mechanism adopted by this transcription factor to mediate chondrocyte adaptation to hypoxia.

In addition to its effects on chondrocyte survival and proliferation, Hif-1α also influences chondrocyte differentiation and matrix accumulation. The issue of the possible roles of hypoxia and Hif-1α in cell differentiation is another highly debated topic (52-59). We have shown that Hif-1α is required in early chondrogenesis for a timely differentiation of mesenchymal cells into chondrocytes (60). This finding provided the first *in vivo* model of a positive function of Hif-1α in differentiation during development, and further establishes the essential role of this transcription factor in the biology of chondrocytes during bone growth. It will now be necessary to unequivocally confirm the important and surprising role of hypoxia and Hif-1α in differentiation, and then identify the molecular mechanism(s) downstream of Hif-1α as a differentiation factor in cartilage. More importantly, it will be necessary to establish whether hypoxia *per se* is a requirement for a timely chondrocyte differentiation *in vivo*, or whether the role of Hif-1α in chondrogenesis is essentially homeostatic (*i.e.,* to overcome the putative negative effect of hypoxia *per se*). Notably, hypoxia has been reported to be a positive regulator of Sox-9 mRNA expression (61;62).

Lack of Hif-1α in limb bud mesenchyme also severely affects joint development, which was secondary to a delay of joint specification (60). As chondrogenesis and joint formation are tightly coupled, it is possible that the impairment of joint formation in the absence of Hif-1α is secondary to the delay of early chondrogenesis.

Hypoxia and Hif-1α in Osteoblasts

Like other oxygen-sensitive cells, osteoblasts also express components of the HIF-1 pathway. Recent studies show that manipulation of the HIF-1α pathway in osteoblasts, with consequent overproduction of VEGF and other angiogenic factors, stimulated angiogenesis in the long bones that was associated with robust bone formation at the sites of vessel in-growth (64). Mice overexpressing HIFs by disrupting VHL in osteoblasts showed striking and progressive increases in bone volume, whereas the diameter of the ΔHIF-1 mutant bones, which were isolated from mice lacking Hif-1α in osteoblasts, was reduced relative to the controls. Importantly, the amount of bone in the axial skeleton of these two mutants was directly proportional to the amount of skeletal vasculature. These observations suggested the possibility that loss of pVHL with consequent upregulation of HIFs in osteoblasts increased the production of angiogenic factors, which then promoted bone formation secondarily to increasing angiogenesis. Consistent with this idea, the expression of VEGF mRNA was upregulated in the trabecular bone of ΔVHL femurs. However, the precise mechanisms responsible for coupling angiogenesis to osteogenesis remain to be determined. Interestingly, manipulation of HIF levels in osteoblasts did not noticeably influence the formation of the flat bones of the skull. The calvarial bones are formed through an intramembranous process which involves condensing mesenchymal stem cells that derive from the neural crest. This type of bone forms without replacement of a precedent, avascular cartilaginous template by bone and marrow as is required for endochondral ossification. It is possible that other signals such as cranial suture and dural mechanical tensions organize angiogenesis necessary for intramembranous ossification.

Angiogenesis is also essential for bone repair. It has been proposed that the abrupt interruption of vascular and nutrient supply, together with mechanical loading stimuli, initiate the sequence of events that lead to new bone formation (65). In this environment, the osteoblast is particularly well-situated to sense the initial signals emanating from inflammatory and mechanical signals generated in the tissue and the abrupt interruption of oxygen and nutrients in this zone. If angiogenesis is delayed, the healing tissue tends to be made up of chondrocytic cells rather than osteoblasts (66), suggesting a role for HIF-1α in mesenchymal lineage allocation. Using a distraction osteogenesis model (DO), Wan *et al*. investigated the role of HIF-1α in bone healing using the two mutant mouse models described above, namely mice lacking VHL and Hif-1α in osteoblasts. As expected, loss of VHL was accompanied by increased HIF-1α protein, increased VEGF mRNA and protein and increased CD31 immunostaining (67). Significant increases in both vessel volume per total volume (VV/TV) and vessel number were observed in the ΔVHL mutants compared to controls, and this was accompanied by the formation of more dense woven bone in the distraction gap (67). The precise opposite phenotype was observed in the HIF-1-deficient mutants. These animals demonstrated deficient angiogenesis and bone consolidation following DO (67). Perhaps not surprisingly, small molecules, which block the activity of prolyl hydroxylases (PHDs) and thereby elevate Hif-1α when administered directly into the distraction gap, can improve healing in a manner virtually identical to that seen in the genetic model of Hif-1α activation. These studies provide proof of principal that pharmaceuticals can be developed to speed bone healing.

Hypoxia and Hif-1α in Osteoclasts

Hif-1α is also likely to be an important survival factor for osteoclasts. Mice lacking Fra2 or LIF 1 have giant osteoclasts, and the bone marrow from either mutant is highly hypoxic with significant stabilization of Hif-1α protein (68). The increased levels of hypoxia in the marrow of the mutant mice appears to be a consequence of a placental defect (68). Moreover, the formation of TRAP positive cells from fetal liver progenitors is significantly augmented in hypoxic *in vitro* conditions (68). Collectively, these exciting findings strongly suggest that hypoxia and Hif-1α could also play an important role in osteoclast biology. Along these lines, there is evidence that osteoclasts *per se* could stimulate angiogenesis by producing a variety of pro-angiogenic factors (69;70). These findings are in line with the well-known anti-angiogenic properties of anti-resorptive agents such as bisphosphonates (70). Notably, macrophages, which share with osteoclasts the same lineage origin, also express numerous pro-angiogenic factors (71). Moreover, Hif-1α has been reported to be crucial for macrophage survival in a disease setting (71;72). In light of these findings, it will be interesting to explore the role of hypoxia and Hif-1α in osteoclast recruitment, survival and function *in vivo*.

Summary

In this brief *Perspective*, we have highlighted the critical role of Hif-1α in cartilage development as well as in bone modeling, remodeling and regeneration. It will now be important to identify the molecular mechanisms that mediate the complex and multifaceted action of this transcription factor both in chondrocytes, osteoblasts and osteoclasts. Moreover, it is possible that stimuli other than hypoxia could be upstream of Hif-1α; alternatively, hypoxia could have effects that are independent of the Hif family of transcription factors. The dissection of these different possibilities will be both challenging and exciting.

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