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Authors

Yellowley, Clare E

Genetos, Damian C

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Hypoxia signaling in the skeleton: implications for bone health

Clare E. Yellowley, Ph.D.Damian C. Genetos, Ph.D.

Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, Davis, CA USA

Abstract

a. Purpose of Review.—We reviewed recent literature on oxygen-sensing in osteogenic cells and its contribution to development of a skeletal phenotype, the coupling of osteogenesis with angiogenesis and integration of hypoxia into canonical Wnt signaling, and opportunities to manipulate oxygen-sensing to promote skeletal repair.

b. Recent Findings: Oxygen-sensing in osteocytes can confer a high bone mass phenotype in murine models; common and unique targets of HIF-1α and HIF-2α, and lineage-specific deletion of oxygen-sensing machinery suggest differentia utilization and requirement of HIF-α proteins in the differentiation from mesenchymal stem cell to osteoblast to osteocyte; oxygen-dependent but HIF-α-independent signaling may contribute to observed skeletal phenotypes.

c. Summary: Manipulating oxygen-sensing machinery in osteogenic cells influences skeletal phenotype through angiogenesis-dependent and -independent pathways, and involves HIF-1α, HIF-2α, or both proteins. Clinically, an FDA-approved iron chelator promotes angiogenesis and osteogenesis, thereby enhancing the rate of fracture repair.

Keywords

Bone; hypoxia; Wnt; Sclerostin; HIF

1. Introduction

Oxygen bioavailability is a fundamental necessity for complex multicellular organisms; for growth beyond simple multicellular organisms to occur, it became necessary to develop internal transport and exchange mechanisms (*e.g.*, the circulatory system) that enable the bulk flow of nutrients and waste to and from, respectively, cells in the larger body [1]. The fundamental necessity of O_2 bioavailability is revealed in its absence: cellular metabolism favors anaerobic glycolysis before ultimately ceasing; thereafter by impairment of cell function and cell death; the tissue and organ death leading to death of an individual. To that end, the body has developed a variety of robust mechanisms to resolve reductions in tissue

To whom correspondence should be addressed: Damian C. Genetos, 1089 Veterinary Medicine Drive, University of California, Davis, Davis CA 95616, 530 754 0146, dgenetos@ucdavis.edu.

Conflict of Interest

Clare Yellowley and Damian Genetos declare no conflict of interest.

Compliance with Ethical Standards

Human and Animal Rights and Informed Consent

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O_2 availability (hypoxia), chiefly 1) increased cardiac output and ventilation; 2) improved vascularization; 3) enhanced blood O_2-carrying capacity; and 4) a switch from aerobic to anaerobic respiration. At the cellular level, adaptation to hypoxia— whether acute or chronic — are driven by the energy/nutrient sensor mTOR, unfolded protein responses in the endoplasmic reticulum, NF-κB-mediated transcription, or the hypoxia-inducible factor (HIF) family of transcription factors[2].

HIFs are heterodimeric proteins composed of a HIF-α and HIF-β (or ARNT) subunit. Each subunit is constitutively expressed but have opposing stability: HIF-β/ARNT subunits remain stable, whereas HIF-α subunits are degraded under conditions of sufficient O_2 bioavailability (normoxia). When cellular oxygen is sufficiently available (> ~5%), prolyl hydroxylase domain (Phd) proteins hydroxylate proline residues in HIF-α subunits. Proline hydroxylation provides a binding site for von Hippel-Landau (Vhl) tumor suppressor protein, which subsequently recruits a ubiquitin E3 ligase complex that polyubiquitinates HIF-αs and targets them for proteasomal degradation. When oxygen levels are sufficiently low, HIF-α hydroxylation by PHDs is prevented, HIF-αs accumulate, associate with HIF-β, and induce gene expression *via* binding to HIF-responsive elements (HREs) in target gene promoters. Additional regulation of HIF-a-mediated signaling involves Factor inhibiting HIF (FIH), which hydroxylates (N-terminal) asparaginyl residues, preventing association of HIF-α with transcriptional co-activators CBP/p300.

There are three HIF-α genes: *HIF1A*, *HIF2A*, and *HIF3A*. HIF-1α and HIF-2α directly induce gene transcription, whereas HIF-3α inhibits HIF-1α- or HIF-2α-mediated transcription by binding to ARNTs and sequestering their interaction with HIF-1α or HIF-2α, or preventing HIF-α and ARNT heterodimerization (reviewed in [2]). Structurally, HIF-1α and HIF-2α are composed of DNA binding and dimerization domains at their N termini, and transactivation domains at their C termini, and each gene reveals relatively high homology [3]. HIF-α/β heterodimers bind to HREs in target gene promoters, and there is evidence for both common and unique gene transcription in response to HIF-1α or HIF-2α. For example, HIF-1α induces transcription of genes involved in glycolysis (PGK, LDHA), apoptosis (BNIP3), and pH regulation (CAiX); HIF-2α induces cell proliferation (cyclin D1, TGF-alpha), de-differentiation (Oct4), and cell invasion (MMp2, Pai1); and both HIF-1α and HIF-2α induce genes involved in angiogenesis (VEGF) and glucose uptake (Glut1) [4]. Target gene specificity is not determined by specific binding of HIF-1α or HIF-2α to cognate HREs, but, rather, differences in the N-terminal transactivation domain (TAD), such that replacement of HIF-2α N-TAD with HIF-1α N-TAD is sufficient to confer HIF-1α-specific functionality to HIF-2α[3]. HIF-mediated gene transcription involves HIF-α/ARNT binding to the transcriptional coactivator CBP/p300, which provides another level of regulation of HIF-α-driven transcription. Additionally, HIF-2α is hydroxylated less efficiently by PHDs than is HIF-1α, indicating that more modest reductions in pericellular O_2 are sufficient to promote HIF-2α stabilization than HIF-1α stabilization[5,6]. Whereas HIF-1α requires very low O_2 tension or even anoxia for its stabilization, HIF-2α is active under more physiological hypoxia (~5%), indicating differential involvement of HIF-α genes under acute *vs.* chronic hypoxia[7].

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2. Hypoxia and the skeletal system.

Embryogenesis occurs in a hypoxic environment, such that HIF-α-directed signaling drives developmental morphogenesis[2]. Cells throughout the developing embryo are hypoxic and demonstrate stabilized HIF-α even after the development of a cardiovascular system. Within the skeletal system, hypoxia and HIF-α-driven signaling is involved in endochondral bone formation (see the recent review by Stegan and Carmeliet[8]).–

Approaches to measuring pO_2 tensions (wherein 100% O_2 = 760mm Hg) within bone have relied upon oxygen microelectrodes [9], bone marrow aspiration[10], or chemical surrogates for hypoxia (pimonidazole) [11]. Such approaches have established that disruption of blood vessels at a fracture site causes localized hypoxia (0.8-3% pO_2) [9], that the pO_2 of human bone marrow aspirates is 7.2%[10], or that disuse increases osteocyte hypoxia in a reversible manner[11]. Recent advances in microscopy and reporter mice have revealed two distinct types of bone capillaries—type H (high) and type L (low)— based on expression of the junctional protein CD31 (PECAM1) and the sialoglycoprotein endomucin[12]. Arteries initially flow into Type H capillaries, which are observed primarily in the growth plate, before transitioning into with type L capillaries, which branch from the metaphysis into the bone marrow within the diaphysis, prior to draining in the central vein[12,13]. Thus, perhaps paradoxically, avascular cartilage receives the most oxygenated and nutrient-rich blood, in contrast to highly vascular bone and its marrow. Using two-photon phosphorescence lifetime microscopy, Spencer *et al.* demonstrated distinct spatial heterogeneities in local pO_2 throughout bone and the bone marrow: pO_2 was greatest in the periosteum and decreased in cortical bone [14]. Despite the high vascular network within bone marrow, observed pO_2 was lower within bone marrow sinusoids compared to the endosteum, perhaps due to high metabolic demand of marrow stromal cells and hematopoietic stem cells. There is also suggestive evidence for intracortical heterogeneity in oxygen tension: osteocytes deep within cortical bone express markers of glycolysis and the oxygen-regulated protein ORP150, whereas osteocytes closer to the bone surface do not express these markers[15]. Functionally, both total and active mitochondria are greater in osteocytes near the endosteum or periosteum compared to osteocytes within cortical bone, and mid-cortical osteocytes are more resistant to ischemia-induced stress[16]

Tissue-specific deletion of *Hif1a* or *Hif2a*, or HIF-α-degrading machinery Phd2 or Vhl, reveal a variety of skeletal phenotypes that underscore the complexity of skeletal HIF-α signaling during development and disease. The tissue and developmental stage-specific *cre* drivers used to evaluate HIF-α signaling are shown in TABLE 1. Briefly, hypoxia and HIF-α promote skeletal mesenchyme condensation and limb development, promoting chondrogenesis by increasing *Sox9* and reducing osteogenesis through *Runx2* inhibition[17]. Skeletal malformations or low bone mass phenotypes are observed in *Phd2*-deficient preosteoblasts (*Col1a2-cre*; *Phd2* cKO) [18] or *Hif1a*-deficient mature osteoblasts (*Bglap-cre; Hif1a* cKO)[19]. Certain models show no skeletal effect: *Hif2a* deletion in mature osteoblasts demonstrated no overt skeletal phenotype, despite reductions in vascular density[20]; similarly, *Hif1a* deletion in osteocytes (*Dmp1-cre; Hif1a* cKO) produces mice that are phenotypically similar to wild-type controls[21]. Other animal models of altered oxygen-sensing demonstrate a high bone mass phenotype. In proliferating chondrocytes

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(*Col2a1-ERT2-cre*), deletion of either *Phd2*[22] or *Vhl*[23] produces a high bone mass phenotype involving increased bone formation relative to resorption perhaps due to the transdifferentiation of hypertrophic chondrocytes into osteoblasts[22]. Individual deletion of *Phd* genes in osteoprogenitors (*Osx-cre*) failed to produce a skeletal phenotype, whereas combined deletion of each gene (*Osx-cre; Phd1/2/3* cKO) increased HIF-α target gene expression, vascularization, and improved trabecular microarchitecture[24]. Of note, deletion of *Phd1/2* or *Phd2/3* phenocopied improvements in trabecular microarchitecture without enhancing vascularity. *Vhl* deletion in mature osteoblasts (*Bglap-cre; Vhl* cKO) causes a high bone mass phenotype associated with increased vessel density, resulting from increased bone formation rate[19]. Deletion of *Phd2* or *Vhl* in osteocytes (*Dmp1-cre; Phd2* or *Dmp1-cre; Vhl* cKO) also produces high bone mass in mice[21,25].

3. Coupling oxygen sensing to skeletal phenotypes

a. HIFs and *Vegf*.

A prevailing assumption, provided by the canonical pathway whereby PHD isoforms and Vhl regulate HIF-α stability, is that the skeletal effects observed from influencing PHD or Vhl are directly attributable to HIF-α stabilization and subsequent gene transcription, most generally *Vegf*. Indeed, *Vegf* and its various isoforms are necessary for endochondral bone formation (reviewed recently in [8]). Coupling angiogenesis to osteogenesis, and *vice versa*, involves reciprocal signaling between osteoblasts and endothelial cells: endothelial cells secrete BMP2 to promote bone formation[26], and osteoblasts secrete VEGF to enhance angiogenesis[27]. *In vivo*, manipulating *Phd*, *Vhl*, or HIFs in osteogenic cells can increase VEGF and angiogenesis[19,24]. Similar coupling involves VEGF, Notch, and Noggin[28]. Nonetheless, there is sufficient evidence that angiogenesis is insufficient to promote osteogenesis. *Osx-cre;Phd1/2* and *Osx-cre;Phd2/3* cKO mice have a high bone mass without concordant changes in *Vegf* or vascularity[24]; deleting *Hif1a* or *Hif2a* in mature osteoblasts (Bglap-cre) reduces *Vegf* expression and vessel volume equivalently, yet *Hif1a* deletion influenced Tb.BV/TV moreso than *Hif2a* deletion[20]. Further, stabilizing HIF-1α or HIF-2α comparably increases *Vegf*, but a skeletal phenotype was observed only in HIF-2α-stable mice [24]. Related observations are found in mice lacking chondrocytic *Hif1a*, whose dwarfism and cartilage destruction is not rescued by VEGF164 over-expression[29].

b. Hypoxia and Wnt signaling.

The critical requirement of Wnt/Lrp5 signaling in skeletal biology is underscored by mutations in *Lrp5* that produce reciprocal effects on the skeleton. Loss-of-function mutations in *Lrp5* cause osteoporosis pseudoglioma (OPPG), characterized by low bone mass[30], whereas mis-sense mutations in *Lrp5* are observed in individuals with high bone mass[31]. Induction of Wnt signaling occurs during load-induced bone formation[32] and is required for new bone deposition in response to external loads[33,34].

Regulation of Wnt signaling occurs *via* secreted decoy receptors (sFrps) or antagonists (*Sost*, *Dkk isoforms*) that bind to Lrp4-6 to prevent Wnt-Lrp interactions, and subsequent signal transduction[35]. Just as activating mutations in *LRP4-6* promote HBM phenotypes[30,31,36,37], complementary phenotypes emerge from deletion of Lrp4/5/6

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antagonists[38–44]: Deletion of *sFRPs* increases trabecular bone[44] and bone mineral density[43], and loss of *Dkk* isoforms or *Sost* increases markers of bone formation and bone mass[39,40]. *In vivo* and *in vitro* evidence reveal functional interaction between HIF-α and Wnt signaling without consensus: *Bglap-cre;Hif1a* cKO mice display enhanced load-induced bone formation *via* β-catenin[45], osteocyte differentiation is attenuated in *Bglap-cre; Vhl* cKO mice, and both Osterix and HIF-α cooperatively reduce Wnt signaling[46]. Alternately, other reports indicate that hypoxia or hypoxia mimetics increase Wnt signaling in bone[47,48]. In cartilage, HIF-1a binds to b-catenin to prevent transcriptional induction of Wnt target genes [49].

Although osteocytes do not directly contribute to bone formation, osteocytic β-catenin is required for the osteoanabolic effect of mechanical loading[50], and constitutively active β-catenin in osteocytes increases both cortical and trabecular bone mass through increasing both arms of remodeling[51]. Thus, regulation of β-catenin availability and transcription in osteocytes can exert profound effects on skeletal mass and microarchitecture. Two recent reports demonstrate an epistatic relationship between hypoxia and Wnt/β-catenin signaling in osteocytes to produce a high bone mass phenotype: osteocytic *Vhl* deletion decreases *Sost* and increases canonical Wnt signaling[21]. Similarly, deleting *Phd2* in osteocytes produced a high bone mass phenotype that correlated with decreased Sclerostin expression and increased canonical Wnt signaling; in this model, osteocytic *Phd2* deletion promoted Sirtuin-1-mediated deacteylation of the *Sost* promoter[25]. Whereas these reports identify an effect of hypoxia on Wnt signaling, the actual effect of hypoxia on Wnt signaling—stimulatory or inhibitory—is perhaps intrinsic to epigenetic modification or cellular bioenergetics in osteoblasts *vs.* osteocytes.

c. HIF-independent signaling by Phd and Vhl in the skeleton.

Both *Phd* genes and *Vhl* have cellular effects beyond oxygen-sensing through HIFs. In addition to HIF-a subunits, PHDs hydroxylate other substrates including IKKβ [52] and NDRG3[53], decreasing the activity or availability of each. Expressing dominant-negative IKKγ in preosteoblasts or mature osteoblasts increases bone formation [54], suggesting the skeletal phenotypes of the various *Phd2* cKO mice may be secondary to PHD2/Ikkγ-mediated phenomena. Ndrg3 contributes to cell proliferation, differentiation, development and response to stress including hypoxia[55]. Similar to HIF-α subunits, Ndrg3 is constitutively hydroxy la ted by Phd2 under normoxia prior to proteolytic degradation; under hypoxia, cellular metabolism switches from oxidative phosphorylation to anaerobic glycolysis as a source of ATP, thereby increasing lactate levels. Direct binding between lactate and Ndrg3 prevents NDRG3 association with VHL and thus its degradation. Once stabilized, NDRG3 promotes Raf/ERK signaling with resultant increases in proliferation and angiogenesis[53]. Additionally, the low bone mass phenotype of *Col1a2-ER-cre; Phd2* cKO mice is attributed not to changes in HIF-a bioavailability and gene transcription, but instead to ascorbate-regulated Osx induction[56]. If so, and to what extent, these HIF-a-independent cascades influence skeletal development and homeostasis requires careful evaluation.

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4. Osteogenic HIF signaling in skeletal pathology and pharmacology

Provided the functional and spatiotemporal linkage between angiogenesis and osteogenesis, and the uncoupling of the bone remodeling cycle favoring bone resorption with age, it is perhaps to be expected that changes in oxygen bioavailability are associated with post-menopausal and senile osteoporosis. Further, other skeletal or skeletal-associated pathologies—traumatic fracture and non—union, glucocorticoid or bisphosphonate-associated osteonecrosis, or renal phosphate wasting—implicate insufficient oxygen handling in osteogenic cells.

a. Osteoporosis and oxygen.

Bone marrow and bone perfusion is decreased in osteoporotic and aged individuals relative to estrogen complete or younger individuals [57,58]. Aging causes endothelial dysfunction, which is exacerbated by ovariectomy and improved by supplemental estrogen. The vasoactive factors released from endothelial cells (e.g., NO and PGI2) have direct effects on both osteoblasts and osteoclasts, and may influence interstitial fluid flow within osteocyte lacuna and canaliculi[59]. Serum VEGF levels positively correlate with BMD, and are decreased in post-menopausal women[60]. Further, bone from ovariectomized rodents reveal increased staining for HIF-1a and HIF-2a[48,61] suggesting that estrogen regulates HIF-α stability. This is not simply a correlative event, as pharmacologic manipulation of HIF-α isoforms, or genetic deletion of *Phd2* or *Vhl*, protects against OVX-induced bone loss in rats and mice[14,37,49,50].

b. Skeletal fracture and non-union.

Disruption of blood vessels in response to bone fracture creates a hypoxic environment. Hematoma formation isolates the site of injury from perfusion, further augmenting localized hypoxia[9]. As fracture repair closely mimics stages of embryonic bone development—in which hypoxia and HIF-a signaling plays a crucial role—it is unsurprising that trauma-induced hypoxia promotes skeletal repair involving HIF-a signaling. A number of studies have confirmed the hypoxic environment around a fracture site[62,63], suggesting that pharmacologic, biologic, or genetic manipulation of neoangiogenesis may promote callus formation and bone repair. Deferoxamine (or desferrioxamine; DFO) is an FDA-approved iron chelator used in diseases associated with iron overload, such as thalassemia syndromes[64]. DFO promotes bone consolidation in tibial[65] or mandibular distraction osteogenesis[66], increases vascularity and callus size in femoral fractures[67]and hastens bone bridging and increased vascularity in femoral segmental defects[68]. Fikewise, *Bglap-cre; Vhl* cKO mice demonstrate increased neoangiogenesis and skeletal repair compared to wild-type controls; conversely osteoblastic deletion of *Hif1a* mitigates neoangiogenesis and bone repair[65]. Local injection of DFO is also effective in promoting bone repair after glucocorticoid-associated onsteonecr

c. Chronic kidney disease-mineral bone disorder (CKD-MBD).

CKD-MBD is a systemic disorder of calcium and phosphate balance secondary to kidney dysfunction; the inability of the kidneys to adequately filter phosphate leads to secretion of the phosphaturic hormone FGF23 from bone, specifically osteocytes, to reduce expression of

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renal phosphate cotransporters in the renal proximal tubule and decrease circulating (1α,25)-$(OH)_2$-vitamin D_3. Emerging evidence indicate that FGF23 synthesis and secretion is induced by HIF-α-induced erythropoietin (EPO) produced by bone marrow cells[69–72] or osteoprogenitors/osteoblasts[69,72]; this regulation is apparent in development[69] and disease[73], but may be absent in adulthood[69]. In osteoprogenitors, a PHD-Vhl-HIF-2α axis drives EPO expression and erythropoiesis and unexpectedly, decreases renal EPO expression [69]. Provided the complex interaction between FGF23, PTH, and vitamin D_3 in calcium and phosphate homeostasis in health and disease[74], oxygen-sensing in osteogenic cells must be carefully considered as part of multi-tissue interactions and feedback mechanisms governing serum calcium and phosphate levels

d. Osteonecrosis.

Osteonecrosis, or avascular necrosis, results from disrupted blood supply to bone[75]. Ischemic cell death in bone marrow and trabeculae then contributes to subchondral bone collapse and joint arthrosis. Osteonecrosis is observed in individuals sustaining traumatic injury, or is associated with sustained or high-dose corticoid or antiresorptive therapy, but is also observed in individuals with autoimmune diseases or high alcohol consumption. Provided clinical evidence for tissue ischemia, it is not surprising that bone cell hypoxia contributes to the causation and potential resolution of osteonecrosis. Polymorphisms in *Hif1a*[76]or *Vegfc*[77] are associated with idiopathic osteonecrosis of the femoral head in Korean men, and greater VEGF-C expression is observed in the actively-repairing edematous zone immediately adjacent to necrotic tissue in the femoral head[78]. Glucocorticoid-induced osteonecrosis of the femoral head in a murine model demonstrated rapid reduction in *Hif1a* and *Vegf* expression in the femoral head, but not the distal femur, and vascular conversion to pools of edema prior to any observable effect on bone mineral density or osteocyte apoptosis[79]; *in vitro*, glucocorticoids also rapidly reduce *Hif1a* and *Vegf* expression. A small molecule PHD inhibitor, EDHB, reduced the incidence of steroid-associated osteonecrosis in rabbits, and promoted HIF-α/VEGF signaling[80]. Glucocorticoids also reduce regional matrix remodeling by osteocytes (perilacunar remodeling) which contributes to osteonecrosis and subchondral bone degeneration[81], although this study did not directly evaluate interactions between vascularity, oxygen-sensing, and perilacunar remodeling.

Antiresorptive bisphosphonate therapy is also associated with osteonecrosis of the jaw and ear canal[82,83]. Anti-angiogenic effects of aminobisphosphonates [84] is suggested as part of the multifactorial pathophysiology of bisphosphonate-related osteonecrosis of the jaw (BRONJ), though certain studies in rodents fail to support the hypothesis that bisphosphonates suppress angiogenesis. [85,86]. Decreased angiogenesis in bone would also suggest potential osteoblast- or osteocyte-intrinsic regulation of angiogenesis under disease states, yet, to date, studies designed to interrogate the connection between oxygen-sensing in osteoblasts and BRONJ are lacking; similarly, there is no current evidence of hypoxia or aberrant vascular density in another complication from prolonged bisphosphonate use, atypical femur fracture.

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e. Osteoanabolic therapies PTH and PTHrP directly and indirectly induce neoangiogenesis.

Teriparatide™ (recombinant PTH(1-34)) and abaloparatide™ (recombinant parathyroid receptor-related protein(1-34)) represent the only currently-available FDA-approved osteoanabolic agents. That these agents are osteoanabolic, in the context of angiogenesis-osteogenesis coupling, suggests that PTH may influence angiogenesis in development and repair. PTH-induced acceleration of cranial repair associates with angiopoietin-1 levels and angiogenesis and decreased arteriogenesis[87]; continuous PTH induces bone marrow vascularity independent of osteoclastogenesis[88]; PTH-related peptide (PTHrP) associates with osteolytic metastasis and VEGF expression by breast cancer cells[89]. PTH(1-84)-induced increases in trabecular bone volume fraction and *Vegf* expression in the murine tibial metaphysis are blocked by anti-VEGF antibody co-treatment[90]; similarly, angiogenesis is required for PTH(1-84)-induced increases in BMD and toughness in rats[91]. Yet, the influence of PTH on skeletal vascularization requires more thorough elucidation, as Prisby *et al.* found that PTH(1-84) reduced the number of bone marrow blood vessels, instead redistributing them closer to sites of bone formation[90]. Yet, HIF-1α may restrain osteoanabolism in response to PTH, as mice lacking *Hif1a* in mature osteoblasts reveal enhanced bone formation compared to wild-type[92].

5. Conclusions

The works reviewed above demonstrate that oxygen-sensing in bone is fundamentally necessary for skeletal development and repair, is altered in disease states, and can be pharmacologically manipulated to hasten bone repair. These works, and others, have identified causal interactions between angiogenesis and osteogenesis, as well as osteogenesis in the absence of vascular changes; in doing so, they have fundamentally established a role for oxygen-sensing in osteogenic cells as a means of driving bone formation. Yet there many questions left to be answered, and many more that are not yet considered. Among these are:

1) Which HIF-α genes are necessary throughout cellular lifespan and differentiation from a mesenchymal stem cell to osteoblast to osteocyte? Whereas *Bglap-cre; Hif1a* cKO mice have a low bone mass phenotype[19], osteocytic *Dmp1-cre; Hif1a* cKO mice do not[21], suggesting lineage-specific necessity for a particular HIF-α gene. Such lineage-specific requirements for *Hif1a vs. Hif2a* are also suggested by the work of Wu *et al.,* wherein deletion of both *Hif1a* and *Hif2a* was necessary to produce a skeletal phenotype[24].

2) Similarly, how does oxygen-sensing in osteoblasts *vs.* osteocytes influence skeletal phenotype, and how does this relate to cellular bioenergetics? Two recent reports using osteocytic *Dmp1-cre* mice reveal equivocal increases in skeletal phenotype compared to mature osteoblastic *Bglap-cre* mice[21,25], demonstrating that oxygen handling by osteocytes can have pronounced skeletal effects, but also that persistence of gene deletion, as osteoblasts differentiate into osteocytes, must be considered. Osteogenic cells are capable of both oxidative and glycolytic metabolism, using glycolysis primarily in undifferentiated mesenchymal stem cells then switching to oxidative phosphorylation in

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osteoblasts (reviewed in Riddle and Clemens[93]) as functional demand on osteoblasts to synthesize and secrete matrix increases. To date, evaluation of osteocyte bioenergetics is absent, but it is intriguing to consider the disparate skeletal phenotypes observed with osteoblast-*vs.*-osteocyte deletion of oxygen-sensing machinery as a consequence of cell-intrinsic metabolic profile.

3) To what extent do observed phenotypes in *Vhl* or *Phd* cKO animals require HIF-α signaling? Based solely on the canonical HIF signaling pathway, it is facile to assume that effects produced in such murine models are due to stabilization of HIF-1α or HIF-2α. Yet, *Col1a2; Phd2* cKO mice resemble a spontaneous fracture mouse (*sfx*) whose phenotype results from inability to correctly synthesize vitamin C[18]. Similarly, *Vhl* targets proteins beyond HIF-1α or HIF-2α for proteolytic degradation: hypoxia-induced lactate levels (themselves due to HIF-α-induced LDHA expression) bind to NDRG3 and prevent its Vhl-mediated degradation. How these HIF-independent signals contribute to skeletal development, coupling, and cellular bioenergetics is insufficiently interrogated..

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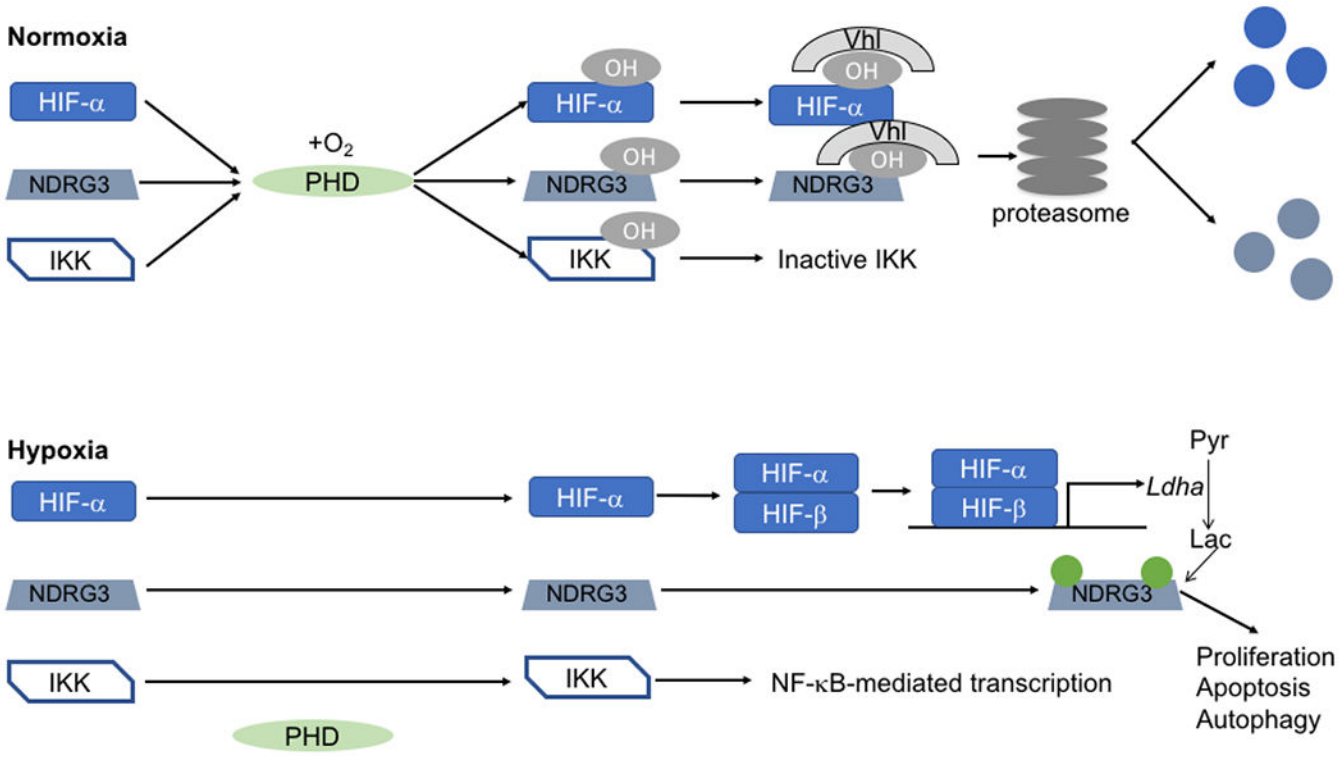

Figure 1. Canonical and non-canonical PHD/HIF/Vhl signaling.

(A) Under conditions of sufficient pericellular oxygen, PHD proteins hydroxylate proline residues in HIF-1a or HIF-2a, Ndrg3, or IKK subunits. Vhl binds to hydroxylated HIF-α and Ndrg3 and are targeted for proteasomal degradation, whereas hydroxylated IKK subunits are catalytically incapable of phosphorylating IκBα, which results in NF-κB degradation and inhibition of NF-κB-mediated gene transcription.

(B) Under conditions of reduced oxygen availability, there is insufficient oxygen available for PHD-regulated hydroxylation of HIF-α, Ndrg3, or IKK. Consequently, HIF-α binds to HIF-β to induce target gene transcription, such as lactate dehydrogenase (LDHA). LDHA subsequently converts pyruvate, generated during anaerobic glycolysis, into lactate, which binds to NDRG3 and prevents complex formation with Vhl; through currently-unknown mechanisms, Ndrg3 can influence cell proliferation, autophagy, or apoptosis. In the absence of hydroxylation, IKK promotes phosphorylation and subsequent degradation of IκBα, enabling induction of NF-κB-dependent gene transcription.

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TABLE 1.

INFLUENCE OF GENETIC DELETION OF OXYGEN-SENSING MECHANISMS IN SKELETAL DYSTEM

cre driver \ Gene	***Phd1*, *Phd2*, or *Phd3***	***Hif1a***	***Hif2a***	***Vhl***
Prx1		{Mangiavini:2014eq} • Shortened forelimbs and hindlimbs • Cell death in stylopod and zeugopod but not autopod • Delayed chondrogenic differentiation in autopod; less proteoglycan accumulation, reduced *Col2a1* mRNA		{Mangiavini:2014eq} • Shortened limbs due to reduced chondrocyte proliferation and their delayed differentiation • Delayed bone marrow cavity development • Absence of secondary spongiosa; disappearance of columnar and hypertrophic chondrocytes • Died at 4 weeks • Vhl; Hif1a double cKO phenocopied Hif1a cKO • Vhl; Hif2a cKO phenocopied Vhl cKO except for absence of bone marrow cavity
Col2a 1-ER	{Cheng:2016gn} • *Phd2* cKO • High bone mass characterized by increased Tb, Ct bone • Primary spongiosa: increased bone formation • Secondary spongiosa: reduced bone resorption • Increased transdifferentiation of hypertrophic chondrocytes to osteoblasts?			{Weng:2014jy} • High bone mass characterized by increased Tb in tibiae and lumbar vertebrae, but no effect on calvariae; cortical bone seemingly unaffected • Enhanced bone formation and response in cKO mice
Osx	{Wu:2015bs} • No skeletal effect of individual *Phd* deletion • High bone mass in triple *Phd1/Phd2/Phd*3 cKO, increased *Vegf* hypervascularization, reduced OCl number • *Phd1/2* or *PM2/3* cKO mice have Tb high bone mass without changes in vascularity • Increased *Opg*/Rankl ratio	Degradation-resistant HIF1dPA show no Tb phenotype or change in OCL number{Wu: 2015bs}	Degradation-resistant HIF2dPA increase Tb microarchitecture, reduced Tb osteoclast number{Wu: 2015bs}	
Col1a 2-ER	{Cheng:2014gm} • Decreased Tb and Ct bone mass owing to reduced BFR; bone resorption unaffected • Appendicular skeleton affected; axial unaffected • Decreased *in vitro* osteoblast differentiation			
Bglap		• Low bone mass due to decreased BFR {Wang:2007ee} • Reduced vascular density {Wang: 2007ee}{Shoment o: 2010fk} • Inability to increase *Vegf* under *in vitro* hypoxia {Shomento: 2010fk}	Reduced vascular density without overtly influencing skeletal phenotype {Shomento:2010fk}	• High bone mass in trabecular and cortical bone{Wang:2007ee} • Stabilization of HIF-1a and HIF-2a, induction of common HIF-a targets Vegf and Glut1, increased vessel volume{Wang:2007ee} • Increased bone formation rate and Ob.N {Shomento:2010fk} • No change in Ocl.N/BS or Opg{Wang:2007ee} • Appendicular skeleton affected; axial unaffected{Wang:2007ee}
Dmp1	{Stegen:2018cz} • High bone mass characterized by increased Tb, Ct • Increased bone formation relative to resorption	{Loots:2018jp} No effect on femoral cortical or trabecular phenotype.		{Loots:2018jp} • High bone mass characterized by increased Tb, Ct bone • No evidence for osteoclast defect

cre driver \ Gene	***Phd1, Phd2,* or *Phd3***	***Hif1a***	***Hif2a***	***Vhl***
	• Sirt1 deacteylation of *Sost* promoter decreases *Sost* expression, with concomitant increased Wnt signaling			• Decreased sclerostin, increased b-catenin signaling • Cortical phenotype of Vhl cKO required Wnt signaling, whereas trabecular phenotype independent of *Lrp5* and not resolved by sclerostin over-expression

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