Role of prolyl hydroxylase domain proteins in bone metabolism

David Wolf a, Aruljothi Muralidharan a, Subburaman Mohan a, b, c, *

a Musculoskeletal Disease Center, VA Loma Linda Healthcare System, Loma Linda, CA, 92357, USA
b Department of Medicine, Loma Linda University, Loma Linda, CA, 92354, USA
c Department Biochemistry and Orthopedic Surgery, Loma Linda University, Loma Linda, CA, 92354, USA

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A B S T R A C T
Cellular metabolism requires dissolved oxygen gas. Because evolutionary refinements have constrained mammalian dissolved oxygen levels, intracellular oxygen sensors are vital for optimizing the bioenergetic and biosynthetic use of dissolved oxygen. Prolyl hydroxylase domain (PHD) homologs 1–3 (PHD1/2/3) are molecular oxygen dependent non-heme dioxygenases whose enzymatic activity is regulated by the concentration of dissolved oxygen. PHD oxygen dependency has evolved into an important intracellular oxygen sensor. The most well studied mechanism of PHD oxygen-sensing is its regulation of the hypoxia-inducible factor (HIF) hypoxia signaling pathway. Heterodimeric HIF transcription factor activity is regulated post-translationally by selective PHD proline hydroxylation of its HIF1α subunit, accelerating HIF1α ubiquitination and proteasomal degradation, preventing HIF heterodimer assembly, nuclear accumulation, and activation of its target oxygen homeostasis genes. Phd2 has been shown to be the key isoform responsible for HIF1α subunit regulation in many cell types and accordingly disruption of the Phd2 gene results in embryonic lethality. In bone cells Phd2 is expressed in high abundance and tightly regulated. Conditional disruption of the Phd1, Phd2 and/or Phd3 gene in various bone cell types using different Cre drivers reveals a major role for Phd2 in skeletal growth and development. In this review, we will summarize the state of current knowledge on the role and mechanism of action of Phd2 as oxygen sensor in regulating bone metabolism.

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1. Introduction

It is estimated that 200 million people world-wide and 54 million people in the United States have osteoporosis. One in 2 women and 1 in 4 men after age 40 will have an osteoporosis-related fracture over their lifetime [1]. Osteoporosis is responsible for more than 2 million fractures year and this number continues to grow [2]. Two major causes are known to contribute to the pathogenesis of osteoporosis: 1) failure to achieve peak bone mass that typically occurs around the age of 30; and 2) menopause and age-induced excessive bone resorption that is not compensated by corresponding increase in bone formation. Bone loss occurs with age in part because the rate of bone resorption surpasses the rate of bone formation [3]. Many systemic and local growth factors regulate bone formation processes. At the molecular level, hypoxia signaling is identified as a key signaling pathway that plays a central role in the regulation of bone formation both during normal physiology and under disease conditions [4–6]. Hypoxia-inducible factor (HIF) activity, a heterodimeric (HIF1α/HIF2α) transcription factor for oxygen homeostasis genes, is largely regulated post-translationally by prolyl hydroxylase domain enzymes (PHD1/2/3). PHD1/2/3 were identified originally as the primary intracellular oxygen sensor [7]. PHDs, in the presence of normal dissolved oxygen levels, constitutively hydroxylate specific HIF1α subunit proline residues, accelerating HIF1α turnover through ubiquitination and proteasomal degradation, thereby preventing HIF-heterodimer assembly and its nuclear transcription activity. PHD2 has been shown to be the key isoform responsible for HIF1α regulation in many cell types and accordingly disruption of the Phd2 gene results in embryonic lethality [8,9]. Phd2 is found to be expressed in high abundance in bone cells and disruption of Phd2 gene in bone cells result in severe skeletal phenotype [10,11]. In this review article, we will discuss our current understanding of the role of PHD oxygen sensors in regulating bone metabolism.
2. Troposphere and dissolved oxygen

Cellular metabolism requires dissolved oxygen gas. Both evolutionary refinement of cellular dissolved oxygen utilization and species radiation occurred under oxygen-deplete tropospheric conditions [12–19] (Fig. 1). Consequently, these influences imbued mammalian species with unique physiological and cellular adaptations to lowered dissolved oxygen concentrations. Fig. 2 shows human dissolved oxygen concentrations differ considerably throughout the body, and their activities normally occur in dissolved oxygen environments markedly lower than atmospheric levels [20,21]. Similarly, hypoxia, a state of metabolically insufficient dissolved oxygen, differs by organ systems. To maximize intracellular dissolved oxygen utilization among widely varying levels of dissolved oxygen requirements and its availability, and to mitigate against consequential cellular hypoxia, multiple concomitant intracellular systems have evolved to optimize cellular oxygen utilization; the pairing of the 2-oxoglutarate-dependent oxygenase, prolyl hydroxylase domain (PHD) enzyme, with transcription factor, hypoxia-inducible factor alpha (HIF1α) being one such system.

3. PHDs are oxygen sensors

PHD homologs (PHD1/2/3), and factor inhibiting HIF [FIH]) are non-heme dioxygenases which sense oxygen via direct enzymatic interaction with dissolved oxygen [22–24]. PHD1/2/3 are Fe(II)-2-oxoglutarate-dependent dioxygenases (2OGD) which represent the largest group of non-heme, Fe(II)-bound, oxidizing enzymes. 2OGD enzymes are distributed throughout the bacterial, plant, animal phyla, and are engaged in a wide variety of metabolic processes including hydroxylation, demethylation, epoxidation, desaturation, and halogenation reactions. 2OGD substrates are diverse and found in proteins, nucleic acids, lipids, carbohydrates, and small molecules. For example, cytoplasmic procollagen’s vitamin C-dependent post-translational proline and lysine hydroxylation, modifications required for its tertiary folding and quaternary assembly is dependent on 2OGD processing [24,25], and nuclear epigenetic Junmji C domain containing histone lysine demethyl-lyation (KDM) enzyme and 10–11 translocation hydroxylates (TET1-3) enzyme, vital for regional context-dependent promotion or suppression of transcription, are as well 2OGD enzymes [23,24].

2OGD are non-equilibrium enzymes, favoring a forward enzymatic reaction velocity. 2OGD X-ray crystallography studies show a conserved catalytic double-stranded beta-helical core. Fe(III), possibly obtained from cytoplasmic iron chaperone poly-(26)-binding proteins (PCBP1/2) is held in a 3 amino acid histidine, aspartate/glutamate, and histidine coordinating motif, [23,27]. The oxidation state of Fe(III) increases during 2-oxoglutarate dependent catalytic conversion and returns to its ferrous state in the presence of soluble reducing agents such as vitamin C. Fe(II) also protects against intramolecular 2OGD cysteine oxidation (Fig. 3)

2OGD regulates HIF transcription activity [7,23,24,29–31]. HIF is a heterodimer transcription cofactor composed of an 2OGD substrate targeted alpha-subunit isoform (HIF1α/826aa, HIF2α/870aa, HIF3α/667aa) and a constitutively expressed beta-subunit HIFβ. The HIFα isoforms share multiple functional domains. These domains include an oxygen-dependent degradation domain (ODD), terminal trans activation domains (NTAD, CTAD), nuclear localization signal domains (NLS), a transcription activation domain (TAD), an amino-terminal basic helix-loop-helix (bHLH) DNA binding domain, and protein interaction PER-ARNT-SIM domains (PASA, PASB). The HIF3α isoform also possess a leucine zipper transactivation domain (LZIP). A heterodimer of non-hydroxylated HIFα and HiF1β in complex with other transcription factors associates with cis-hypoxia response elements (HRE; 5’-G/ACGTG-3’) within transcription regulatory regions of target genes. A multitude of HRE genes have been identified [32]. HRE genes span a range of processes, developmental to pathophysiological. HIF1β was previously described as
the aryl hydrocarbon nuclear translocator (ARNT) protein, a basic helix-loop-helix–Per-ARNT-Sim (bHLH–PAS) protein [33].

HIFs isoforms are constitutively 2OGD hydroxylated at one or both of their N-terminal ODDs (NODD, HIF1α/402aa, HIF2α/EPAS1/405aa, HIF3α/465aa), and C-terminal ODDs (CODD, HIF1α/564aa, HIF2α/531aa, HIF3α/568aa) [23,29]. Selective HIFs 2OGD hydroxylation

Fig. 3. Schematic review of PHD2 regulation of the HIF system. Regulation of hydroxylase activity by small molecules and protein factors are indicated. PHD proteins catalyze the hydroxylation of proline residues of target proteins including HIFs using oxygen (O2), iron (Fe2+), α-ketoglutarate (also known as 2-oxoglutarate, 2-OG) and ascorbic acid (vitamin C) as co-factors. Poly(rC)-binding protein (PCBP)1 and PCBP2 are iron chaperones that deliver ferrous iron via metal-mediated, protein-protein interaction. Tricarboxylic acid (TCA) cycle provides a source of α-ketoglutarate. Hydroxylation of prolyl residues within the oxygen-dependent degradation domain of HIFs in the presence of co-factors triggers recognition by the von Hippel-Lindau tumor suppressor protein (p-VHL), which is part of a E3 ubiquitin ligase complex (also containing elongin [Elo] C and B, cullin-2 [Cul-2] and RING-Box protein [Rbx] 1 to induce proteosomal degradation. In contrast, under hypoxia PHDs are unable to hydroxylase HIF1α subunits which accumulate and migrate to nucleus, subsequently forming an HIF-complex with other nuclear proteins to induce transcription of HIF1α targets.
increases binding affinity to von-Hippel Lindau tumor suppressor protein (pVHL), the E3 ubiquitin ligase substrate recognition subunit [29,33–35]. Complexed with Ring-box 1 (Rbx1), cullin 2 (Cul2), and elongin B and C, HIFα is polyubiquitinated at lysine residues, directing it to ubiquitin-dependent proteasome degradation. Constitutive 2OGD HIFα hydroxylation decreases HIFα half-life, reducing HIF1α/ HIF1β complex concentrations, and ineffective HIF1α/HIF1β HRE directed transcription activity (Fig. 3). Conversely, inhibition of 2OGD activity leads to elevated HIF1α/HIF1β complex concentrations and robust HRE targeted transcription. HRE gene elements are also located in the GLUT 1 glucose transporter, many of the glycolytic enzymes (hexose kinase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4, aldolase, phosphoglycerate kinase, enolase, and lactate dehydrogenase A), and mitochondrial Cox4-1, together capable of increasing glucose entry, glycolytic processing, and respiration [36].

Four 2OGD isoforms, prolyl hydroxylase domain-containing proteins PHD1/2/3 and factor inhibiting HIF (FIH) modify HIF homologs. PHD1/2/3 were previously designated as Egf nine homologs 1–3, and hypoxia-inducible factor prolyl hydroxylase homologs 1–3 in different numerical ordering (PHD1/EGLN2/ HPH3, PHD2/EGLN1/HPH2, PHD3/EGLN3/HPH1) [8,37]. PHD2 is also characterized as a Jumonji-C domain-containing histone demethylase transcription factor [38]. These 2OGDs utilize cofactors Fe2+, vitamin C, and co-substrates oxygen and 2-oxoglutarate to hydroxylate specific HIFα proline residues in its ODD domain (PHD1/2/3) or in the case of FIH hydroxylate asparagine residues in the HIF CTAD domain. PHD1/2/3-mediated HIFα hydroxylation increases HIFα turnover. FIH asparaginyl hydroxilation disrupts HIFα C-terminal transactivation domain (C-TAD) association with the acetyltransferase transcriptional coactivator CBP/p300 [39].

In addition to vitamin C, PHD activity requires several cofactors including alpha-ketoglutarate, an intermediate metabolite of the mitochondrial inner membrane tricarboxylic cycle (TCA, Krebs Cycle, Citric Acid Cycle) [23,36,40] Alpha-ketoglutarate is derived from isocitrate, by isocitrate dehydrogenase oxidative decarboxylation [28]. Alpha-ketoglutarate accumulates in the cytoplasm through 2 sequential mitochondrial export processes; oxoglutarate carrier (OGC, a mal99ate-a-ketoglutarate antiporter) transport across inner mitochondrial membranes, and subsequent cytosol gradient dependent diffusion through outer mitochondrial membrane channels. Accordingly, alpha-ketoglutarate is also a required co-substrate for the epigenetic 2OGD Jumonji-C domain lysine demethylases and 2OGD 10–11 translocation hydroxylases [23,41,42]. Concurrently, alpha-ketoglutarate analogues and as well other mitochondrial metabolites inhibit PHD activity, such as L, 2-hydroxyglutaric acid (L-2HGA), fumarate, succinate, and reactive oxygen species (ROS) [41]. Succinate activity has also been implicated in inflammation, immune signaling, ischemia and reperfusion injury processes, while mutations in isocitrate dehydrogenase (IDH), fumarate hydratase (FH), and succinate dehydrogenase (SDH) genes, enzymatic steps leading to alpha-ketoglutarate and analogues, are linked to cancer [32,43–46]. Other distinguishing 2OGD PHD features are a catalytic core with a slower active-site Fe2+ - O2 binding and reaction time than other 2OGDs [23]. Among the PHDs, PHD2 is the most conserved isoform [23]. Importantly, PHD2’s oxygen Km is higher than measured tissue concentrations (Fig. 2), imparting its catalytic activity responsive to changes in intracellular dissolved oxygen concentrations [47–49]. These cytosolic metabolite cofactors along with the variable cytoplasmic dissolved oxygen concentrations and significant regulatory refinement to the catalytic activity of PHDs. One vital biological process dependent upon 2OGD activity is PHD2 regulation of bone development and repair.

4. Expression of PHDs in bone cells

The 3 PHD isoforms are known to be expressed differently in various tissues [50]. Both PHD1 and PHD2 are known to be expressed widely in multiple tissues with tests showing abundant PHD1 expression and adipose and skeletal muscle tissues displaying high levels of PHD2 expression. While PHD3 mRNA levels have been reported in several tissues, its expression level is much lower than that of PHD1 or PHD2. Studies on subcellular localization of the 3 PHD isoforms also show differences in their localization pattern with PHD1 and PHD2 being primarily localized in the nucleus and cytoplasm, respectively, and PHD3 being distributed in both compartments [51]. To determine which isoforms of PHDs are expressed in bone cells, we examined the transcripts of Phd1, Phd2 and Phd3 in primary calvarial osteoblasts and MC3T3-E1 osteoblast cell line by real time RT-PCR. Our data showed that Phd2 was expressed at higher levels than Phd1 and that ascorbic acid treatment significantly inhibited Phd2 but not Phd1 expression [52]. Phd3 expression was undetectable under the serum-free culture conditions used in our study. In another study, Irwin et al [53] allowed MC3T3-E1 cells to differentiate for 14 days in the presence of ascorbic acid under normoxic (21% oxygen) conditions and then evaluated the consequence of hypoxia [25] on expression levels of all 3 PHD family members. While expression levels of Phd2 and Phd3 were increased by more than 10-fold under hypoxic conditions, Phd1 expression was not affected. In the human osteosarcoma cell line, U2OS, hypoxia-induced expression of Phd2 and Phd3 and selective suppression of HIF1α expression by RNA interference resulted in complete loss of hypoxic induction of PHD2 and PHD3 [54]. These data suggest that Phd2 appears to be the major form expressed in osteoblasts that is subject to regulation by both low oxygen and ascorbic acid treatments.

There are limited investigations evaluating expression levels of the 3 PHD proteins in various bone cell types that reside in bone during normal development and during metabolic perturbations, in vivo. Immunohistochemical staining of bone sections from 2-month-old C57BL/6J mice showed that both PHD1 and PHD2 were detected in osteoblasts that reside on the trabecular surfaces of femur. However, PHD3 expression was too weak to be detected by immunohistochemistry [55]. In our study, we evaluated the expression patterns of PHD2 and PHD3 by immunohistochemistry in the distal femoral articular cartilage in 2-week-old mice, when the articular cartilage formation is known to occur in mice [26]. While PHD2 protein was found to be expressed in high levels in the superficial zone of articular cartilage, its expression was low in the middle and deep zones. By contrast, PHD3 was undetectable in the superficial zone while it was highly expressed in the middle and deep zones where some of the chondrocytes were undergoing hypertrophy [26]. However, the issue of whether conditions that influence loss of bone or articular cartilage influence expression levels of PHDs remain to be determined.

5. Role of PHDs in skeletal development

Bone development and repair occurs throughout life. It is a coordinated and ordered process. Axial skeletal development and mineralization began during the early Cambrian to mid-Ordovician period [56]. Bone is formed primarily via 2 routes known as endochondral and intramembranous bone ossification [57]. Both of these osteogenic pathways begin with a mesenchymal tissue precursor, but how it transforms into bone differs. During endochondral ossification that occurs in the flat bones of the skull, clavicle, and most of the cranial bones, mesenchymal stem cells differentiate into osteoblasts to form bone. However, endochondral ossification occurring in the axial skeleton and the long bones
involves a more complex route with mesenchymal tissue transforming into a cartilage intermediate which is subsequently replaced by bone [58]. During intramembranous and endochondral bone formation mechanisms, mesenchymal stem cells differentiate into osteoblasts and chondrocytes, respectively, the processes that are controlled by complex transcriptional network that involves HIFs (Fig. 4).

Chondrocytes are the only resident cell types of cartilage, which are responsible for the synthesis and maintenance of the extracellular matrix. The formation and maintenance of a proper cartilage matrix is dependent on the phenotypic stability and survival of the chondrocytes [59]. Cartilage development occurs in a hypoxic environment and its proximity to vasculature appears to be a key determining factor in the formation of bone over cartilage [60–62]. Articular cartilage does not have blood vessels and is developed and maintained in a hypoxic microenvironment both during normal development as well as regeneration [63]. Chondrocytes residing in articular cartilage are, therefore, exposed to an environment with reduced oxygen tension, ranging from 7 to 10% in the superficial zone and as low as 1% in the deep zones [64,65]. Anaerobic glycolysis contributes up to 75% of their ATP requirement in the articular cartilage chondrocytes, underlining their adaptation to a low O2 environment [66]. Adaptation of chondrocytes to low oxygen tension plays an important role in both the development and maintenance of chondrocyte phenotypes and is known to be primarily mediated through the HIF signaling pathway involving many cellular targets. During degenerative joint diseases like osteoarthritis (OA), the O2 gradient across articular cartilage may be altered due to changes in articular cartilage phenotype such as cartilage thinning and erosion, changes in extracellular matrix composition, and the development of cartilage fissures [67,68]. Indeed, cultures of articular chondrocytes in a low O2 environment have been shown to lose their articular chondrocyte phenotype by their increased propensity to express markers of chondrocyte differentiation [69].

PHD2 is believed to be the crucial oxygen sensor during normoxia and mild hypoxia, which is emphasized by the fact that mice with global knockout of the Phd2 gene results in embryonic lethality by E14.5 [70]. By contrast, mice with global knockout of Phd1 or Phd3 gene develop normally [71]. Because global knockout Phd2 gene results in embryonic lethality, conditional knock out approach has been used to disrupt Phd2 gene specifically in various bone cell types. In our studies, we crossed Phd2 floxed mice with Col1a2-iCre mice in which Cre expression is under the control of the entire regulatory region of Col1α2 gene to disrupt Phd2 gene in osteoblast-lineage cells. We found that mice with conditional deletion of Phd2 gene in Col1α2 expressing osteoblasts developed short stature and died prematurely at 12–14 weeks of age [26]. Analyses of skeletal phenotype by microCT and bone histomorphometry revealed that both bone size as well as trabecular bone mass were reduced in the long bones of Phd2 conditional knockout mice that were caused by reduced osterix expression, bone formation rate and mineral apposition rate, but not due to changes in bone resorption (Table 1). The HIF hydroxylases are dependent on ascorbate, and activation of PHDs in ascorbic acid-treated cells results in ubiquitin-mediated proteosomal degradation of prolyl hydroxylated HIF1α. In our study, we found that treatment of osteoblasts with dimethyloxally glycine and ethyl 3,
4-dihydroxybenzoate, known inhibitors of PHD, completely blocked ascorbic acid effect on expression levels of markers of osteoblast differentiation. Furthermore, knockdown of Phd2 expression by lentivirus-mediated shRNA abolished ascorbic acid effects on osteoblast differentiation, thus suggesting that a role for PHD2-mediated mechanism in ascorbic acid regulation of osteoblast functions.

To examine the intrinsic effect of PHD2 in osteoblasts, Rauner et al [72] characterized the skeletal phenotype of mice with conditional loss of Phd2 expression in Osx-expressing cells and found increased bone density in both lumbar and femur of 12-week-old conditional knockout mice compared to control mice. It is known that Osx is expressed in chondrocytes besides osteoblasts and mice with conditional disruption of Osx expression in Col2a1-expressing chondrocytes died immediately after birth and exhibited severe skeletal growth retardation [73]. Thus, it remains to be determined if the increased bone density in Phd2 conditional knockout mice using Osx-Cre can be explained on the basis of loss of Phd2 expression in chondrocytes since loss of chondrocyte Phd2 expression increases bone density (see below).

In another study, Wu et al [74] have used Osx-Cre mice to disrupt all 3 Phd genes in osteoprogenitors. In this study, the authors found that selective inactivation of Phd1/2/3, Phd1/2 or Phd2/3 resulted in increased trabecular bone mass without altering cortical bone mass. Furthermore, genetic ablation of Phd2 and Phd3 gene together protected mice from ovariectomy-induced trabecular bone loss and that the increased trabecular bone mass in the Phd2/3 conditional knockout mice is predicted to involve osteoblast produced osteoprotegrin to inhibit osteoclastogenesis, and thereby, bone resorption. By contrast to the above findings, Zhu et al [55] reported that the increased trabecular bone mass in mice with deletion of Phd1/2/3 genes in Col1a1 expressing osteoblasts is mainly caused by increased osteoblast function. In this study, bone resorption and osteoclast formation were rather increased in the Phd1/2/3 conditional mice, a finding opposite to that reported by Wu et al [74]. These data suggest that Phds expressed in osteoblast lineage cells regulate osteoblast and osteoclast functions in a complex manner depending on the context of differentiation status of cells as well as the number of Phd genes disrupted.

It is known that the terminally differentiated osteoblasts, osteocytes, reside within a low oxygen microenvironment. To elucidate whether the oxygen sensor PHD2 is critical for osteocyte function, Stegen et al [23] generated osteocyte-specific Phd2 conditional knockout mice by crossing Phd2 floxed mice with Dentin Matrix Protein (Dmp1)-Cre mice and characterized their skeletal phenotype by microCT and bone histomorphometry. Disruption of Phd2 in osteocytes was shown to blunt the bone loss due to estrogen deficiency or mechanical unloading. The anabolic effects of Phd2 disruption in osteocytes is predicted to involve HIF1α-mediated Sirtulin-1-dependent deacetylation of Sost promoter, resulting in decreased sclerostin expression and enhanced Wnt signaling. Thus, oxygen sensing by PHD2 in osteocytes appear to negatively regulate bone mass via epigenetic regulation of Sost expression.

| Floxed gene | Cre line | Targeted cells | Phenotype | Mechanism | Reference |
|-------------|----------|----------------|-----------|-----------|-----------|
| Phd2        | Col2a1   | Osteoblasts    | ↓ Trabecular BMD | Bone Formation | Cheng et al., 2014 (10) |
| Phd2        | Dmp1     | Osteocytes     | ↓ Trabecular BMD | Bone Resorption | Stegen et al., 2018 (93) |
| Phd2        | Osx      | Osteoblasts    | ↑ Trabecular BMD | Bone Formation | Rauner et al., 2016 (72) |
| Phd2        | CD-68    | Hematopoietic cells | ↑ Trabecular BMD | Bone Formation | Rauner et al., 2016 (72) |
| Phd2        | Col2a1   | Chondrocytes   | ↑ Articular Cartilage | Bone Formation | Cheng et al. 2015; 2017 (11,26) |
| Phd3        | Col2a1   | Chondrocytes   | ↑ Trabecular BMD | Bone Resorption | Xing et al. 2021 (79) |
| Phd1/2/3    | Osx      | Osteoblasts    | ↑ Trabecular BMD | Bone Formation | Zhu et al., 2016 (55) |
| Phd2/3      | Osx      | Osteoblasts    | ↑ Trabecular BMD | Bone Resorption | Wu et al., 2015 (74) |
| Phd1/3      | Osx      | Osteoblasts    | ↑ Trabecular BMD | Bone Resorption | Wu et al., 2015 (74) |

Table 1: Summary of skeletal phenotypes in mice with conditional disruption of phd1/2/3 in various bone cell types (See the text for details).
ubiquitin ligase that promotes proteolysis of HIFs, or over-expression of Vegf, a HIF-1α target [75,76]. To determine the role of Phd2 expressed in chondrocytes, we generated mice with targeted deletion of Phd2 in Col2a1 expressing chondrocytes by crossing Phd2 floxed mice with Col2a1-Cre mice. Our data show that chondrocyte-specific Phd2 conditional knockout mice display a dramatic increase of bone mass in the trabeculae of long bones and spines [11,77]. In addition, loss of Phd2 expression in chondrocytes increased cortical thickness and tissue mineral density at the femoral mid-diaphysis. Disruption of Phd2 expression in chondrocytes caused increased bone formation in the primary spongiosa and reduced resorption in the secondary spongiosa. Based on the findings that expression levels of markers osteoblasts (Osx, Alp, Bsp) were increased in Phd2 knockout chondrocytes, it is speculated that the increased HIF1α signaling caused by disruption of Phd2 gene in chondrocytes promotes chondrocyte-to-osteoblast transdifferentiation and, thereby, bone formation in the chondrocyte-specific Phd2 conditional knockout mice [26].

Since changes in HIF1α signaling have been implicated in the pathogenesis of osteoarthritis [78], we investigated the consequence of disruption of the Phd2 gene in chondrocytes on articular cartilage phenotype in mice. We found that condition deletion of Phd2 gene in Col2a1 expressing chondrocytes accelerated the progression of progenitors to hypertrophic chondrocytes as revealed by increased chondrocyte hypertrophy and thickness of middle/deep zone and reduced superficial zone thickness of articular cartilage. Immunohistochemistry revealed that while protein levels of markers of articular cartilage progenitors were decreased, but protein levels of hypertrophic markers of chondrocytes were increased. Furthermore, in vitro knockdown of expression of Phd2 using lentiviral shRNA or inhibition of PHD2 activity using chemical inhibitor in articular chondrocytes revealed that PHD2 acts by inhibiting the differentiation of articular cartilage progenitors in part via modulating HIF1α signaling [26].

Loss of Phd2 expression as well as inhibition of PHD2 activity with a chemical inhibitor resulted in dramatic increases in Phd3 expression in chondrocytes. To evaluate if the increased endochondral bone formation in chondrocyte-specific Phd2 conditional knockout mice is in part caused by elevated Phd3 expression, chondrocyte-specific Phd3 conditional knockout mice were generated by crossing Phd3 floxed (Phd3fl/fl) mice with Col2a1-Cre mice. Our data show that neither trabecular bone nor articular cartilage phenotype was affected in the Phd3 conditional knockout mice, thus demonstrating that Phd2 but not Phd3 expressed in chondrocytes regulates endochondral bone formation, and the compensatory increase in Phd3 expression in the chondrocytes of Phd2 conditional knockout mice is not the cause for increased trabecular bone mass in Phd2 conditional knockout mice [79]. Consistent with the lack of skeletal phenotype in mice with disruption of Phd3 gene in chondrocytes, we found that lentiviral shRNA-mediated partial knockdown of Phd3 expression in chondrocytes did not affect expression of markers of chondrocyte differentiation (Col2, Col10, Acan, Sox9) [79]. Based on these data, we conclude that molecular oxygen, through the PHD2/HIF signaling pathway, plays a central role in bone homeostasis by controlling both angiogenesis and osteogenesis. Therefore, therapeutic targeting of PHD2 enzyme can potentially be used for the treatment of bone disorders.

6. PHD-based therapies for bone repair/regeneration

The process of bone repair and regeneration is unique and is closely associated with that of vessel ingrowth that supply oxygen and nutrients to the regenerating bone. There is now accumulating evidence in the literature that suggest a key role for HIF pathway in angiogenic-osteogenic coupling during bone repair and regeneration [80]. Therefore, upregulation of HIF1α signaling, through PHD inhibition, is considered as a viable strategy to promote vascular in growth and, thereby, fracture repair and regeneration. PHD catalytic activity can be pharmacologically inhibited by PHD-inhibitors (PHIs), such as FG-4592 (Roxadustat), the pan-hydroxylase inhibitor dimethyloxalylglycine (DMOG), or ethyl-3,4-dihydroxybenzoate (EDHB) [81]. Especially, the novel inhibitors of PHD, such as TM6008, TM6089, and FK506-binding protein 38 (FKBP38), have been found and used to activate the HIF pathway and acquire beneficial aspects of the HIF system [82,83]. Majority of the PHIs that are currently under investigation for clinical use are 2-oxoglutarate antagonists.

Based on the established role of angiogenesis in new bone formation, a number of studies have examined if fracture repair can be promoted via manipulation of HIF pathway activity. Rios et al [84] found that using siRNA against PHD2 to activate the HIF-1 pathway supports bone regeneration in an in vivo sheep model. In this study, chambers containing silk fibroin-chitosan scaffolds with siRNA against PHD2 were implanted over the periosteum. After 70 days, it was found that the mean bone volume was significantly increased in animals implanted with silk fibroin-chitosan containing Phd2 siRNA compared to control, suggesting that PHD2 inhibition supports bone regeneration. In another study, Chen et al. [85] implanted a tissue engineered compound construct consisting of the composite collagen membrane of bone marrow mesenchymal stem cells infected with lentiviral Phd2 shRNA in a rat periodontal fenestration defect model and found that Phd2 gene silencing promoted repair and reconstruction of the periodontal tissue defects. Similarly, inhibition of PHD2 function in human periodontal ligament cells via lentiviral-mediated RNA interference facilitated cell osteogenic differentiation and periodontal repair in Sprague-Dawley rats [86]. Consistent with the findings that knockdown of Phd2 expression promoted bone regeneration in various models, Shen et al [87] found that inhibition of PHD2 action by small molecule PHD inhibitors, dipheroxamine (DFO) and dimethyloxalylglycine (DMOG), at fracture sites in a stabilized murine femur fracture model not only increased vascularity at 14 days but also increased the callus size at 28 days as assessed by microCT, suggesting that HIF activation is a viable approach to increase vascularity and bone formation following skeletal trauma.

Distraction osteogenesis (DO) [88] is a surgical method of endogenous bone tissue engineering used to lengthen limbs. Based on the genetic mouse model lacking pVHL in osteoblasts to activate HIF signaling pathway promoted osteogenesis in DO, Wan et al [89] tested the effects of PHD inhibitors, DFO and l-mimosine (L-mim) in DO model and found both agents increased angiogenesis and bone formation. In a similar study, Donneys et al. [88] reported that DFO significantly increased bone structural and mechanical quality parameters compared to controls during mandible DO. While these studies are very encouraging in the use of PHD inhibitors to promote bone regeneration, one caveat is that the PHD inhibitors used are mostly nonspecific. Therefore, further research using specific PHD2 inhibitors are needed to evaluate the safety and efficiency of these inhibitors in promoting bone regeneration and repair.

7. Non-canonical roles of PHD2

The traditional well established canonical role of PHD2 is to regulate HIF protein levels and, thereby, modulate HIF signaling in target tissues. In this regard, hydroxylation of proline residues within HIF molecule leads to targeting of HIF2 to proteosomes for ubiquitination and subsequent degradation (Fig. 5). In addition to HIF proteins, PHD2 can also regulate hydroxylation of proline residues in other proteins such as and modulate their stability. In this
regard, recent studies have revealed some novel PHD2 substrates beyond HIF, thus emphasizing that PHD2 may regulate multiple signaling pathways in normal development and diseases. For example, a recent study by Fan et al \[90\] revealed that conserved proline residues within the LAP/LAP-like motifs of TET2/3 are hydroxylated by PHD2 resulting in pVHL-mediated proteosomal degradation of TET2/3 and reduced DNA hydroxymethylation. In another study, Erber et al \[91\] identified PHD2 as a key regulatory enzyme of bromodomain-containing protein-4 (BRD4) prolyl hydroxylation. Furthermore, it has been shown that PHD2-mediated prolyl hydroxylation significantly affects BRD4 interaction with key transcription factors, CDK9 and CCNT1, and thereby, regulate RNA polymerase II-mediated transcriptional activity (Fig. 5).

We and others have shown that PHD2 is localized in the nucleus, besides cytoplasm and that PHD2 has significant hydroxylase activity in the nucleus \[92\]. Ten-eleven translocation dioxygenases (TET), that also belong to the 2OGGD family as PHDs, are known to oxidize 5-methylcytosines and promote locus-specific reversal of DNA methylation and, thereby, regulate gene transcription epigenetically in various cell types. To determine if PHD2 is involved in the regulating oxidation of methyl cytosines, we determined the levels of nuclear 5-hydroxymethylcytosine (5-hmC) in osteoblasts in osteoblast cultures treated with ascorbic acid, an activator of PHD2 activity. We found that while ascorbic acid increased 5-hmC levels in osteoblast-specific genes, Alp, Ihh and Osx. By contrast, inhibition of PHD2 activity with a specific inhibitor, IOX2, decreased 5-hmC levels in the promoters of Hif1a and Vegf in chondrocytes \[92\]. Furthermore, knockdown of Phd2 expression reduced nuclear 5-hmC levels in chondrocytes. These data are consistent with the possibility that PHD2, like TETs, can regulate transcription of target genes epigenetically by its hydroxylase activity to induce demethylation (Fig. 5).

8. Future research considerations

Despite efforts to understand the role of PHD proteins in the regulation of hypoxia signaling, there are still considerable gaps (see below) in our understanding of the role and molecular mechanism of action of PHD proteins, specifically, PHD2, in regulating various cell types that contribute to development and maintenance of bone.

1) Of the 3 members of PHD family, PHD2 is expressed in more abundance than PHD1 or PHD3 in bone. Accordingly, we found that disruption of Phd2 but not Phd3 expression in chondrocytes exerted significant effect on the skeletal and articular cartilage phenotype in mice \[79\]. In addition, Wu et al \[74\] reported that while combined inactivation of Phd1/2/3, Phd2/3 or Phd1/2 in Osx expressing cells caused trabecular bone accumulation, inactivation of Phd1/3 had no effect. However, the issue of whether PHD1 and/or PHD3 expressed in osteoblasts/chondrocytes exerts significant role in bone or cartilage metabolism under conditions where bone metabolism is perturbed (e.g., estrogen deficiency, aging, glucocorticoid treatment) remains to be determined.

2) In addition to the autocrine effects of PHD2 expressed in bone cell types, there is evidence that PHD2 expressed in cells of the hematopoietic system exert an effect on the skeletal phenotype.

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**Fig. 5.** Canonical and non-canonical mechanisms of PHD2 action in target cells. In the presence of O2, Fe2+, vitamin C and α-ketoglutarate, PHD2 hydroxylates key proline residues in the oxygen-dependent degradation domain of HIF1α, and, thereby, induce degradation of HIF1α via ubiquitin proteosomal degradation pathway (canonical signaling). Besides hydroxylating HIF1α, PHD2 can also hydroxylate other targets such as 10–11 translocation dioxygenase (TET)1 and TET2 to induce their degradation (non-canonical). PHD2 hydroxylation of bromodomain-containing protein 4 (BRD4) can modify its interaction with other nuclear proteins to regulate BRD4-mediated transcriptional activity. PHD2 is also known to be localized in the nucleus and induce hydroxylation of methyl cytosine, and, thereby, regulate transcription of target genes epigenetically.
via influencing circulating levels of erythropoietin levels. In mice with conditional disruption of Phd2 in erythropoietin producing cells using CD68-Cre, there was a significant reduction in trabecular and cortical bone density due to reduction in osteoblast activity. Based on the findings of elevated serum levels of erythropoietin in the CD68-Cre/Phd2 floxed mice and that erythropoietin inhibits osteoblast functions, it is predicted that loss of Phd2 in erythropoietin producing cells results in reduced osteoblast function and diminished bone formation in part via increasing inhibitory effects of endocrine erythropoietin actions [72]. Future studies should address the issues of whether the effect of hematopoietic cell produced Phd2 on bone formation is primarily through alterations in erythropoietin signaling and if therapeutic approaches to inhibit erythropoietin signaling locally in bone can be used to correct bone formation deficit in conditions where serum levels of erythropoietin are increased.

3) Much of published work is focused on the role of Phd2 in regulating canonical hypoxia signaling in target tissues. While our published data provide experimental evidence a PHD2-mediated mechanism in regulating DNA methylation in the promoter regions of osteoblast-specific genes, further work is needed to establish that PHD2 effect on 5-hmC changes in the promoter regions of target genes are direct and the extent to which this epigenetic mechanism contributes to PHD2 effects on cartilage and bone development [92]. Furthermore, efforts on methylation sequencing and pathway analyses could provide a deeper understanding of which genes are epigenetically regulated by PHD2-mediated mechanism in osteoblasts and chondrocytes.

4) While the consequence of disruption of PHD family members in osteoblasts and chondrocytes has been determined, little is known on the expression patterns of Phd family members in osteoclast-lineage cells and their role in bone modeling and remodeling processes during normal physiological and pathological conditions.

5) Consistent with the findings that genetic ablation of Phd2 in chondrocytes and osteocytes increase bone mass, administration of pan-PHD inhibitor, dimethylxalylglycine, rescued ovariectomy-induced bone loss [93]. Future studies are warranted to determine the safety and efficacy of specific inhibitors of PHD2 over a long term to treat bone loss.

References

[1] Lindsey RC, Rundle CH, Mohan S. Role of IGFB1 and EPH signaling in skeletal metabolism. J Mol Endocrinol 2018;61:187–102.
[2] Black DM, Rosen CJ. Postmenopausal Osteoporosis. N Engl J Med 2016;374: 1969–76.
[3] Baylink DJ, Strong DD, Mohan S. The diagnosis and treatment of osteoporosis: future prospects. Mol Med Today 1999;5:131–40.
[4] Arnett TR, Gibbons DC, Utting JC, Orriss IR, Hoebertz A, Rosendaal M, et al. Hypoxia is a major stimulator of osteoclast formation and bone resorption. J Cell Physiol 2003;196:2–8.
[5] Muzylak M, Price JS, Horton MA. Hypoxia induces giant osteoclast formation and extensive bone resorption in the cat. Calcif Tissue Int 2006;79:301–9.
[6] Bozec A, Bakiri L, Hoebertz A, Eferl R, Schilling AF, Komnennov V, et al. Osteoclast size is controlled by Fra-2 through LIF/LIF-receptor signalling and hypoxia. Nature 2008;454:221–5.
[7] Kaelin Jr WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hypoxia-induction pathway. Mol Cell 2008;30:393–402.
[8] Minamishima YA, Moslehi J, Bardeesy N, Cullen D, Bronston RT, Kaelin Jr WG. Somatic inactivation of the PHD2 polyalanine causes polylysomy and congestive heart failure. Blood 2008;111:1236–44.
[9] Franke K, Kalucka J, Mamlouk S, Singh RP, Muschter A, Weidemann A, et al. HIF-1alpha is a protective factor in conditional Phd2-deficient mice suffering from severe HIF-2alpha-induced excessive erythropoiesis. Blood 2013;121: 1436–45.
[10] Cheng S, Xing W, Pourreynoo S, Mohan S. Conditional disruption of the prolyl hydroxylase domain-containing protein 2 (Phd2) gene defines its key role in skeletal development. J Bone Miner Res 2014;29:2726–8.
[11] Cheng S, Xing W, Pourreyno S, Schulte J, Mohan S. Conditional deletion of prolyl hydroxylase domain-containing protein 2 (Phd2) gene defines its essential role in chondrocyte function and endochondral bone formation. Endocrinology 2016;157:127–40.
[12] Holland HD. The oxygenation of the atmosphere and oceans. Philos Trans R Soc Lond B Biol Sci 2006;361:903–15.
[13] Kasting JF, Howard MT. Atmospheric composition and climate on the early Earth. Philos Trans R Soc Lond B Biol Sci 2006;361:1733–41. discussion 41–2.
[14] Raymond J, Segre D. The effect of oxygen on biochemical networks and the evolution of complex life. Science 2006;311:1764–7.
[15] Gregor LS. Life with oxygen. Science 2007;318:62–4.
[16] Och LM, Shields-Zhou GA. The Neoproterozoic oxygenation event: environmental perturbations and biogeochemical cycling. Earth Sci Rev 2012;110: 26–57.
[17] Lyons TW, Reinhardt CT, Planavsky NJ. The rise of oxygen in Earth’s early ocean and atmosphere. Nature 2014;506:307–15.
[18] Knoll AH, Nowak MA. The timetable of evolution. Sci Adv 2017;3:e1603076.
[19] Holdsworth MJ, Gibbs DJ. Comparative biology of oxygen sensing in plants and animals. Curr Biol 2020;30:R362–9.
[20] Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial pressure of oxygen a crucial parameter? Small molecules and hypoxia. J Cell Mol Med 2011;15:1239–53.
[21] Mckewon SR. Defining normoxia, hypoxia and hypoxia in tumours: implications for treatment response. Br J Radiol 2014;87:20130676.
[22] Markoviclovic S, Wilkins SE, Schofield CJ. Protein hydroxylation catalyzed by 2-Oxoglutarate-dependent oxygenases. J Biol Chem 2015;290:20712–22.
[23] Ugalde MS, Leising TM, Howchowdry R, Holdsworth MJ, Schofield CJ. 2-Oxoglutarate-Dependent oxygenases. Annu Rev Biochem 2018;87:585–620.
[24] Walport LJ, Schofield CJ. Adventures in defining roles of oxygenases in the regulation of protein biosynthesis. Chem Rev 2018;18:1760–81.
[25] Myllyharju J. Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and fibronectin. Curr Opin Cell Biol 2001;13:176–82.
[26] Och LM, Shields-Zhou GA. The Neoproterozoic oxygenation event: environmental perturbations and biogeochemical cycling. Earth Sci Rev 2012;110: 26–57.
[27] Lyons TW, Reinhardt CT, Planavsky NJ. The rise of oxygen in Earth’s early ocean and atmosphere. Nature 2014;506:307–15.
[28] Knoll AH, Nowak MA. The timetable of evolution. Sci Adv 2017;3:e1603076.
[29] Holdsworth MJ, Gibbs DJ. Comparative biology of oxygen sensing in plants and animals. Curr Biol 2020;30:R362–9.
[30] Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial pressure of oxygen a crucial parameter? Small molecules and hypoxia. J Cell Mol Med 2011;15:1239–53.
[31] Mckewon SR. Defining normoxia, hypoxia and hypoxia in tumours: implications for treatment response. Br J Radiol 2014;87:20130676.
[32] Markoviclovic S, Wilkins SE, Schofield CJ. Protein hydroxylation catalyzed by 2-Oxoglutarate-dependent oxygenases. J Biol Chem 2015;290:20712–22.
[33] Ugalde MS, Leising TM, Howchowdry R, Holdsworth MJ, Schofield CJ. 2-Oxoglutarate-Dependent oxygenases. Annu Rev Biochem 2018;87:585–620.
[34] Walport LJ, Schofield CJ. Adventures in defining roles of oxygenases in the regulation of protein biosynthesis. Chem Rev 2018;18:1760–81.
[35] Myllyharju J. Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and fibronectin. Curr Opin Cell Biol 2001;13:176–82.

CRediT author statement

David Wolf: Writing – Original draft. Aruljothi Muralidharan: Writing – Original draft. Subburaman Mohan: Conceptualization, Writing – Review & editing.

Conflicts of interest

The authors declare no competing interests.

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[35] Kaelin WG. Proline hydroxylation and gene expression. Annu Rev Biochem 2005;74:115–28.
[36] Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551–78.
[37] Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O’Rourke J, Mole DR, et al. Egl-9 and mammalian homologs define a family of dimers that function in oxygen sensing. Cell 2001;107:43–54.
[38] Frost J, Frost M, Batié M, Jiang H, Rocha S. Roles of HIF and 2-oxoglutarate-dependent dioxygenases in controlling gene expression in hypoxia. Cancers 2021;13:57.
[39] Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol 2004;5:343–54.
[40] Kuiper C, Vissers MC. Ascorbate as a co-factor for Fe(II) and 2-oxoglutarate-dependent dioxygenases: physiological activity in tumor growth and prolyl hydroxylase 2. Front Cell Biol 2014;4:359.
[41] Bailey PSJ, Nathan JA. Metabolic regulation of hypoxia-inducible transcription factors: the role of small molecule metabolites and iron. Biomedicines 2018;6.
[42] Martinez-Reyes I, Chandel NS. Mitochondrial TCA cycle metabolites control physiology and disease. Nat Commun 2020;11:102.
[43] Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB. Intracellular alpha-ketoglutarate maintains the pluripotency of embryonic stem cells. Nature 2015;518:413–6.
[44] Tretter L, Patkos A, Chinopoulos C, Sucanite, an intermediate in metabolism, signal transduction, ROS, hypoxia, and tumorigenesis. Biochim Biophys Acta 2016;1857:1086–101.
[45] Edsismarka B, Zorzoli E, Kandefer-Szerszen M. Alpha-ketoglutarate as a molecule with pleiotropic activity: well-known and novel possibilities of therapeutic use. Arch Immunol Ther Exp 2017;65:21–36.
[46] Vatrinet R, Leone G, De Luise M, Girolimetti G, Vidone M, Gasparre G, et al. The succinate, an intermediate in metabolism, kinetic investigations of the role of factor inhibiting hypoxia-inducible factor (FIH) as an oxygen sensor. J Biol Chem 2003;278:30772–80.
[47] Tarhonskaya H, Hardy AP, Howe EA, Loik ND, Kramer HB, McCullough JS, et al. Kinetic investigations of the role of factor inhibiting hypoxia-inducible factor (FIH) as an oxygen sensor. J Biol Chem 2013;289:19726–42.
[48] Wilson JW, Shukir D, Batié M, Frost M, Rocha S. Oxygen-sensing mechanisms in cells. FEBS J 2020;287:3888–906.
[49] Lieb ME, Menzies K, Moschella MC, Ni R, Taubman MB. Mammalian EGLN is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 2003;22:4021–33.
[50] Takeda K, Ho VC, Takeda H, Duan LJ, Nagy A, Feng G. Placental but not heart defects are associated with elevated hypoxia-inducible factor alpha levels in fetal brain. Nat Cell Biol 2006;8:1362–46.
[51] Rauner M, Franken K, Murray M, Singh RP, Hiram-Bab S, Platzbecker U, et al. Increased EPO levels are associated with bone loss in mice lacking PHD2 in EPO-producing cells. J Bone Miner Res 2016;31:1877–87.
[52] Cheng S, Xing W, Zhou X, Mohan S. Haploinsufficiency of osterix in chondrocytes impairs skeletal growth in mice. Physiol Genom 2013;45:917–23.
[53] Wu C, Rankin EB, Castellini L, Alcuda JF, LaEry CJ, Andersen R, et al. Oxygen-sensing PHDs regulate bone homeostasis during the modulation of osteo-protegerin. Genes Dev 2015;29:817–31.
[54] Maes C, Goossens S, Bartunkova S, Drobat G, Coenegrachts L, Stockmans I, et al. Increased skeletal VEGF and beta-catenin activity and results in excess ossified bones. EMBO J 2010;29:424–41.
[55] Weng T, Xie Y, Huang J, Luo F, Yu L, He Q, et al. Inactivation of Vhl in osteo-progenitor cells causes high bone mass phenotype and protects animals from osteobone-related bone cancer. J Cell Physiol 2010;226:219–29.
[56] Cheng S, Aghajanian P, Pourtemour S, Alarcon C, Mohan S. Prolyl hydroxylase domain-containing protein 2 (Phd2) regulates chondrocyte differentiation and secondary ossification in mice. Sci Rep 2016;6:35748.
[57] Fernandez-Torres J, Martinez-Nava GA, Gutierrez-Ruiz MC, Gomez-Quiroz LE, Gutierrez M. Role of HIF-1α signaling pathway in osteoarthrisit: a systematic review. Rev Bras Reumatol Epub 2017;57:162–73.
[58] Xing W, Pourtemour S, Gomez-CA, Chen Y, Mohan S. Prolyl hydroxylase domain-containing protein 3 gene expression in chondrocytes is not essential for bone development in mice. Cells 2021;10.
[59] Fan L, Li J, Yu Z, Dang X, Wang K. The hypoxia-inducible factor pathway, prolyl hydroxylase domain protein inhibitors, and their roles in bone repair and regeneration. Biomed Res Int 2014:201439156.
[60] Frasial P, Aragenes J, Carmeliet P. Inhibition of oxygen sensors as a therapeutic strategy for ischaemic and inflammatory disease. Nat Rev Drug Discov 2009;8:139–52.
[61] Nangaku M, Izuha Y, Takizawa S, Yamashita T, Fuji-Kuriyama Y, Ohneda O, et al. A novel class of prolyl hydroxylase inhibitors induces angiogenesis and exerts organ protection against ischemia. Arterioscler Thromb Vasc Biol 2007;27:2548–54.
[62] Takayama Y, Mizumachi K. Inhibitory effect of lactoferrin on hypoxic differentiation of ATDC5 mouse chondroprogenitor cells. Biomed Cell Res 2010;23:477–84.
[63] Irwin R, LaPres JK, Kinser S, McCabe LR. Prolyl-hydroxylase inhibition and HIF activation in osteoblasts promotes an adipogenic phenotype. J Cell Biochem 2007;100:762–72.
[64] Kohnen JD, Stengel P, Dooee K, Heikkinen P, Jokilehto T, Soila J, et al. Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylase. Biochem J 2004;381:761–7.
[65] Zhang G. An evo-devo view on the origin of the backbone: evolutionary development of the vertebrate. Integr Comp Biol 2009;49:178–86.
[66] Saitoh T, Miyazaki K. Inhibitory effect of lactoferrin on hypoxic differentiation of ATDC5 mouse chondroprogenitor cells. Biomed Cell Res 2010;23:477–84.
[67] Breeland G, Sinkler MA, Menezes RG. Embryology. bone ossification. StatPearls Publishing Copyright © 2021, StatPearls Publishing LLC, 2021.
[68] Kim HA, Blanco FJ. Cell death and apoptosis in osteoarthritic cartilage. Curr Drug Targets 2007;8:333–45.
[69] Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS. Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev 2001;15:2865–76.
[70] Goshima J, Goldberg VM, Caplan AL. The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. Clin Orthop Relat Res 1991;269:274–83.
[71] Pechak DG, Kujawa MJ, Caplan AI. Morphology of bone development and bone remodeling in embryonic chick limbs. Bone 1986;7:459–72.
[72] Murphy CL, HIF-2alpha expression in osteoblasts and chondrocytes: potential involvement in osteoarthritis. J Biol Chem 2003;278:30772–80.
[73] Lafont JE. Lack of oxygen in articular cartilage: consequences for chondrocyte biology. Int J Exp Pathol 2010;91:99–106.
[74] Zhou S, Cui Z, Urban JP. Factors influencing the oxygen concentration gradient from the synovial fluid of articular cartilage to the cartilage-bone interface: a modeling study. Arthritis Rheum 2004;50:3915–24.
[75] Lane JM, Brightton CT, Menkwitz BJ. Ananerobic and aerobic metabolism in articular cartilage. J Rheumatol 1977;4:343–42.