Prolyl Hydroxylase Domain-Containing Protein 2 (Phd2) Regulates Chondrocyte Differentiation and Secondary Ossification in Mice

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Endochondral ossification plays an important role in the formation of the primary ossification centers (POCs) and secondary ossification centers (SOCs) of mammalian long bones. However, the molecular mechanisms that regulate POC and SOC formation are different. We recently demonstrated that Prolyl Hydroxylase Domain-containing Protein 2 (Phd2) is a key mediator of vitamin C effects on bone. We investigated the role of Phd2 on endochondral ossification of the epiphyses by conditionally deleting the Phd2 gene in osteoblasts and chondrocytes. We found that the deletion of Phd2 in osteoblasts did not cause changes in bone parameters in the proximal tibial epiphyses in 5 week old mice. In contrast, deletion of Phd2 in chondrocytes resulted in increased bone mass and bone formation rate (normalized to tissue volume) in long bone epiphyses, indicating that Phd2 expressed in chondrocytes, but not osteoblasts, negatively regulates secondary ossification of epiphyses. Phd2 deletion in chondrocytes elevated mRNA expression of hypoxia-inducible factor (HIF) signaling molecules including Hif-1α, Hif-2α, Vegfa, Vegfb, and Epo, as well as markers for chondrocyte hypertrophy and mineralization such as Col10, osterix, alkaline phosphatase, and bone sialoprotein. These data suggest that Phd2 expressed in chondrocytes inhibits endochondral ossification at the epiphysis by suppressing HIF signaling pathways.

While the cellular and molecular events associated with the formation of POCs have been well studied, the underlying molecular mechanisms involved in the formation of the SOCs are poorly understood. Similar to POC formation, SOC formation also involves chondrocyte hypertrophy and mineralization of the extracellular matrix of chondrocytes. However, there are several major differences between the two processes. Firstly, while the POC forms at embryonic day 15.5 (E15.5), SOC formation does not begin until postnatal days 5 to 7 in rodents⁵,⁶. Secondly, endochondral ossification in the POCs progresses in a single direction from the growth plate towards the mid-diaphysis, while endochondral ossification in the SOCs starts from the cartilaginous center of the epiphysis and radially progresses outwards until reaching the apical articular cartilage and the basal growth plate.

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Thirdly, SOC formation involves the formation of a cartilage canal to provide a cell source for the SOC development. Hence, the initial formation of SOCs, which occurs around week 1 to 3 in rodents, relies heavily on chondrocytes rather than on both osteoblasts and chondrocytes as in the case of POC formation. The molecular pathways that regulate various cellular processes that occur during SOC formation are poorly understood.

Endochondral and intramembranous ossification are regulated by a family of genes named Prolyl Hydroxylase Domain-containing Proteins (Phds) which include Phd1, Phd2, and Phd3. PHDs contain highly conserved hydroxylase domains in the catalytic carboxy-terminals and are important regulators of hypoxia-inducible factors (HIFs). We have previously discovered that Phd2 was abundantly expressed in osteoblasts and chondrocytes, and Phd2 played different roles in osteoblasts and chondrocytes during the formation of POCs. Phd2 promotes osteoblast differentiation by up-regulating osteix (Osx) expression independent of HIF signaling, and deletion of Phd2 in osteoblasts reduced size and trabecular bone mass in POCs due to a reduced bone formation rate. However, Phd2 suppresses chondrocyte differentiation by inhibiting the HIF signaling pathway, and the disruption of Phd2 in chondrocytes resulted in increased trabecular bone mass and cortical thickness in POCs. Hence, Phd2 plays distinct roles in osteoblasts versus chondrocytes.

Since SOC formation appears to differ from POC formation in several aspects and since Phd2 exerts different roles in osteoblasts versus chondrocytes during POC formation, we asked whether Phd2 also affects trabecular bone mass in the long bone epiphysis, and if so, if the effect of Phd2 on SOC formation is different in the two types of cells. To address these questions, we conditionally disrupted the Phd2 gene in osteoblasts and chondrocytes and compared bone phenotypes as well as the expression of markers for chondrocyte hypertrophy and mineralization in SOCs of epiphyses. We found interesting differences between the two conditional knockout phenotypes which are consistent with the idea that different mechanisms regulate POC and SOC formation.

Results

Conditional deletion of Phd2 in osteoblasts and chondrocytes. We used Col2a1-Cre transgenic mice to disrupt Phd2 expression in osteoblasts. We have previously used this Cre line to delete Phd2 in osteoblast lineage cells, and revealed predominant expression of iCre in bone tissues and a specific deletion of Phd2 in osteoblasts by iCre activity detected by western blot analysis. The epiphyseal bone phenotypes of the Phd2 Col2a1-Cre mice and their corresponding control littermates were analyzed. To disrupt Phd2 expression in chondrocytes, we used the Col2a1-Cre transgenic mice. Ovchinnikov et al. detected Col2a1-Cre activity in all cartilaginous primordia of the developing bones. In bones, Col2a1-Cre activity was detected specifically in chondrocytes but not in osteoblasts. We have also previously demonstrated the specific deletion of Phd2 by Col2a1-Cre in chondrocytes and the specificity of this mouse line has been well documented in many studies. The epiphyseal bone phenotypes of the Phd2 Col2a1-Cre mice and their corresponding control littermates were analyzed.

The formation of the SOC begins around postnatal day 7 in mice and the ossification of the epiphyseal cartilage is nearly complete around week 3 in mice. The vascularization of distal femoral and proximal tibial epiphyses of mice begins around week 2 to provide a source of cells for endochondral ossification. In our previous studies, we did not detect any gender-genotype interactions in the Phd2 Col2a1-Cre and Phd2 Col2a1-Cre mice for bone parameters at multiple skeletal sites including the total body, femur, tibia, and vertebral. In this study, we also tested whether a lack of Phd2 in chondrocytes affects SOC formation in a gender-dependent manner and found no gender-genotype interaction on trabecular parameters in the femoral epiphysis of the Phd2 Col2a1-Cre and control mice (data not shown). Therefore, we performed phenotypical analyses with mixed genders.

Phd2 Col2a1-Cre but not Phd2 Col2a1-Cre mice exhibited increased bone mass in SOCs of long bone epiphyses. Micro-computed tomography (μCT) analyses of proximal tibial epiphyses in 5 week old Phd2 Col2a1-Cre mice and corresponding control littermates revealed no significant differences in bone parameters between the two genotypes (Fig. 1). Figure 1A shows representative 3D images of the proximal tibial epiphyses of the control and Phd2 Col2a1-Cre mice. All bone parameters, including total volume (TV), bone volume (BV), BV/TV, trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular separation (Tb. Sp), were unaltered in the Phd2 Col2a1-Cre mice compared to control mice (Fig. 1B–G). In contrast to the lack of SOC phenotype in the epiphyseal bones of Phd2 Col2a1-Cre mice, we previously found that Phd2 expressed in osteoblasts influenced POC formation as reflected by the osteopenia phenotype in the secondary spongiosa of long bones of Phd2 Col2a1-Cre mice at 5 weeks of age.

In sharp contrast, we observed significantly increased bone mass in the Phd2 Col2a1-Cre mice at the proximal tibial epiphyses compared to littermate controls (Fig. 2). Figure 2A shows representative 3D images of the proximal tibial epiphyses of control and Phd2 Col2a1-Cre mice. While tissue volume was not changed in the Phd2 Col2a1-Cre mice (Fig. 2B), bone volume and bone volume/tissue volume were increased by 21% (P < 0.05) and 30% (P < 0.01), respectively, in the Phd2 Col2a1-Cre mice compared to controls (Fig. 2C–D). Trabecular number and thickness were increased by 33% (P < 0.05) and 24% (P < 0.01), respectively, in the Phd2 Col2a1-Cre mice compared to controls (Fig. 2E–F). In contrast, trabecular separation was decreased by 31% (P < 0.05) in the Phd2 Col2a1-Cre mice compared to controls (Fig. 2G). Increased endochondral ossification was further confirmed in the SOCs of the distal femoral epiphyses of the Phd2 Col2a1-Cre mice (Fig. 3). Similarly, increases in bone volume (17%, P < 0.05), bone volume/tissue volume (20%, P < 0.05), trabecular number (12%, P < 0.05), and thickness (21%, P < 0.01) and a decrease in trabecular separation (11%, P < 0.05) were detected in the SOCs of the distal femoral epiphyses of the Phd2 Col2a1-Cre mice compared to controls (Fig. 3).

Phd2 deletion in chondrocytes increased bone formation rate but normal resorption in SOCs of long bone epiphyses in the Phd2 Col2a1-Cre mice. Since we detected increased bone mass in SOCs of Phd2 Col2a1-Cre mice, we next examined bone formation and resorption rates in these mice. Figure 4A,B show calcein labeling and tartrate-resistant acid phosphatase (TRAP) staining in SOCs of control and Phd2 Col2a1-Cre mice (arrows show the calcein labeling and TRAP signal). Histomorphometric analyses revealed a 31% increase of bone volume...
to tissue volume in the tibial epiphyses of the Phd2Col2-Cre mice compared to control mice ($P < 0.01$, Fig. 4C), a finding consistent with the μCT data shown in Figs 2D and 3D. Bone formation rate (BFR) using different referents such as tissue volume, bone volume, and bone surface have been shown to measure different bone formation properties. While BFR adjusted for tissue volume was increased by 34% in the Phd2Col2-Cre mice ($P < 0.01$, Fig. 4D), changes in BFR using bone volume and bone surface as referents were not significant in the Phd2Col2-Cre mice compared to controls (Fig. 4E,F). Mineral apposition rate (MAR) and osteoclast surface to BS (Oc.S/BS) were also not significantly altered in the Phd2Col2-Cre mice compared to control mice (Fig. 4G,H).
Elevated marker expression for chondrocyte hypertrophy, mineralization, and HIF signaling in the epiphyses of the Phd2Col2-Cre mice. To further investigate the mechanisms for increased endochondral bone formation in the epiphyses of the Phd2Col2-Cre mice, we isolated the proximal tibial epiphyses of 4 week old control and Phd2Col2-Cre mice for RNA extraction and gene expression assessments. First, we measured expression levels of all three Phds. We detected a 44% reduction of Phd2 mRNA levels in the epiphyses of the Phd2Col2-Cre mice compared to control mice ($P < 0.05$, Fig. 5A). The deletion of Phd2 in the epiphysis was not 100% because, in addition to chondrocytes, the tibial epiphysis contains other cell types such as myelopoietic and erythropoietic cells. Furthermore, penetration of Col2α1-Cre mediated gene ablation is about 95% in chondrocytes. In contrast to the sharp reduction of the Phd2 mRNA level, Phd1 and Phd3 mRNA levels were not altered in the Phd2Col2-Cre mice compared to the controls (Fig. 5A). Next, we examined the expression levels of Col2 and Col10, markers of proliferating and differentiating chondrocytes, respectively. While expression of Col2 was not...
altered in the epiphyses of the Phd2Col2-Cre mice, Col10 expression was increased by 245% ($P < 0.05$, Fig. 5B). The increased expression of Col10 in the epiphyses of Phd2Col2-Cre mice indicates increased chondrocyte hypertrophy in the SOCs of the Phd2Col2-Cre mice. Osx expression is also stimulated during chondrocyte differentiation and expressed in pre-hypertrophic and hypertrophic chondrocytes. We observed a 43% ($P < 0.05$) increase in the Osx mRNA level in the Phd2Col2-Cre mice (Fig. 5C). We also detected increased expression of mineralization markers, alkaline phosphatase (ALP) and bone sialoprotein (BSP) by 80% and 95%, respectively, in the Phd2Col2-Cre mice compared to controls ($P < 0.05$, Fig. 5C). In contrast, expression levels of bone resorption markers, CatK and TRAP, were not altered in the Phd2Col2-Cre mice compared to controls (Fig. 5D).

Figure 3. Micro-CT analysis revealed increased bone mass in the distal femoral epiphyses of the chondrocyte-specific Phd2 knockout mice. (A) Representative 3D images of the distal femoral epiphyses of 4 week old control and Phd2Col2-Cre mice. (B–G) Quantitative data of TV, BV, BV/TV, Tb. N, Tb. Th and Tb. Sp of the distal femur epiphyses in control and Phd2Col2-Cre mice. BV and BV/TV were significantly increased in the Phd2Col2-Cre mice compared to controls. Tb. N and Tb. Th were also increased while Tb. Sp was decreased in the Phd2Col2-Cre mice compared to controls. $^*P < 0.05$, $^{**}P < 0.01$, $n = 8$/group. Data were presented as the mean ± SEM.
Figure 4. Histomorphometry analysis revealed increased bone formation rate in the epiphyses of the chondrocyte-specific Phd2 knockout mice. (A) Calcein labeling of trabeculae of the femoral epiphyses of the 4 week old control and Phd2<sup>Col2-Cre</sup> mice. Arrows show the calcein labeling. (B) TRAP staining of trabeculae of the tibial epiphyses of the 4 week old control and Phd2<sup>Col2-Cre</sup> mice. Arrows show the TRAP positive bone surface. (C–H) BV/TV, BFR/TV, BFR/BV, BFR/BS, MAR, and Oc.S/BS of the proximal tibial epiphyses in the control and Phd2<sup>Col2-Cre</sup> mice. TRAP, tartrate-resistant acid phosphatase; Tb, trabecular bone; BFR, bone formation rate; BS, bone surface; MAR, mineral apposition rate; Oc.S, osteoclast surface. *P < 0.05, **P < 0.01, n = 9/group. Data were presented as mean ± SEM. Bar = 50 μM.
We have previously reported that chondrocyte-specific deletion of Phd2 up-regulated HIF signaling in the growth plate chondrocytes. Consistently, we also found that Hif-1α and Hif-2α mRNA levels were increased by 116% (P < 0.05) and 125% (P < 0.01), respectively, in the epiphyses of the Phd2Col2-Cre mice compared to controls (Fig. 5E). The expression levels of HIF signaling targets, Vegfa, Vegfb, and Epo, were also increased in the SOCs of the Phd2Col2-Cre mice. *P < 0.05, **P < 0.01, n = 7/group. Data were normalized to controls and presented as mean ± SEM.

Elevated marker expression for chondrocyte hypertrophy and mineralization markers and HIF signaling in primary chondrocytes treated with PHD inhibitor DMOG. We further tested the regulation of HIF signaling by Phd2 in cultured primary chondrocytes isolated from SOCs of the epiphyses. The primary chondrocytes were treated with 500 μM DMOG, a PHD inhibitor, or vehicle control. We found that mRNA levels of chondrocyte markers, Col2 and Aggrecan, were reduced about 50% (P < 0.01) in cells treated the DMOG compared to vehicle controls (Fig. 6A). The mRNA level of chondrocyte hypertrophy marker Col10 was increased by 38% (P < 0.05) while MMP13 expression was unaltered in the DMOG treated cells (Fig. 6B). Treatment of DMOG also elevated HIF signaling targets. Epo mRNA level was increased by 15-fold (P < 0.05) and Vegf expression was increased by 86% (P < 0.01) in the DMOG treated SOC chondrocytes (Fig. 6C). HIF signaling also affects glycolytic metabolism. We found the mRNA levels of glycolytic enzymes, Glut1, Pdk1, and Pgk1, were also up-regulated by 184%, 115%, and 68%, respectively, in the DMOG treated cells compared to control cells (Fig. 6D). These data are consistent with the up-regulation of HIF signaling and promotion of chondrocyte hypertrophy seen in epiphyseal chondrocytes of Phd2Col2-Cre mice (Fig. 5).

Discussion
In this study, we investigated the roles of Phd2 in osteoblasts and chondrocytes in the formation of SOCs in the long bone epiphyses using cell type-specific knockout mouse models. We found that conditional deletion of Phd2 in osteoblasts using the Phd2Col1-iCre transgenic mice had no significant effect on bone parameters in the proximal tibial epiphyses at 5 weeks of age. In contrast, conditional deletion of Phd2 in chondrocytes using the Phd2Col2-Cre transgenic mice increased trabecular bone mass in the long bone epiphyses, thus demonstrating a negative role for Phd2 expressed in chondrocytes in regulating endochondral ossification. We found that the increased trabecular bone mass in SOCs of Phd2Col2-Cre mice was due to increased bone formation and not due to reduced bone resorption. Phd2 has no effect on osteoclast activity. We further demonstrated elevated HIF
signaling and expression of hypertrophy and mineralization markers including Col10, Osx, ALP, BSP in the SOCs of the Phd2Col2-Cre mice, which likely contributed to the increased ossification of the SOCs in the Phd2Col2-Cre mice. The Phd2 regulation of HIF signaling and chondrocyte hypertrophy was further confirmed in primary SOC chondrocytes by treating with PHD inhibitor.

We have previously reported the bone phenotype in the diaphyseal and metaphyseal regions of long bones in the osteoblast and chondrocyte-specific Phd2 knockout mice. The development and elongation of the diaphysis initiated from the formation of POCs in long bones. Since both osteoblasts and chondrocytes are involved in the endochondral ossification of the POC, knocking out of Phd2 in either osteoblasts or chondrocytes yielded bone phenotypes, though with distinct difference. In osteoblasts, Phd2 acts as a positive regulator for osteoblast differentiation and bone formation, while in chondrocytes, Phd2 exerts negative effects on chondrocyte differentiation and endochondral ossification. Therefore, we observed an osteopenia phenotype in the long bone diaphyses of the osteoblast-specific Phd2 knockout mice but increased bone mass in the long bone diaphyses of the chondrocyte-specific Phd2 knockout mice. This is in contrast to the phenotypes in the SOCs of long bone epiphyses in these knockout mice. These data suggest that Phd2 expressed in chondrocytes exerts an important effect on trabecular bone mass during embryonic and prepubertal growth periods in the epiphysis. However, Phd2 expressed in osteoblasts appears to be inconsequential for epiphyseal bone formation during these growth periods. The SOC is also vascularized in adult mice, thus raising the possibility of vascular supply of osteoblast and osteoclast precursors during endochondral bone formation at the epiphysis; however, at 4–5 weeks of age when the SOC is newly formed, the impact of the osteoblast precursors derived from the circulation on endochondral ossification seems minimal.

The HIF-mediated signaling pathway plays an important role in the maintenance and differentiation of chondrocytes in the hypoxic cartilage, and Phd2 is the major prolyl hydroxylase targeting HIF proteins. We have previously reported that deletion of Phd2 stimulated the HIF signaling pathway during endochondral bone formation of POCs. In this study, we have also observed elevated HIF signaling in the epiphyses of chondrocyte-specific Phd2 knockout mice as well as increased expression of hypertrophy and mineralization markers such as Col10, Osx, ALP, and BSP. Mechanistically similar regulation of chondrocyte hypertrophy and function by Phd2-HIF signaling was seen in both POC and SOC formation, further confirming an inhibitory role for Phd2 in chondrocyte differentiation and endochondral bone formation.

Figure 6. Elevated expression of chondrocyte hypertrophy marker and HIF signaling targets in SOC primary chondrocytes treated with PHD inhibitor DMOG. Primary chondrocytes isolated from SOCs were treated with 500 μM PHD inhibitor DMOG or vehicle control. Cells were extracted for real time RT-PCR analysis. (A) mRNA levels of Col2 and Aggrecan in DMOG treated chondrocytes (normalized to vehicle control). (B) mRNA levels of hypertrophy markers, Col10 and MMP13, in DMOG treated chondrocytes. (C) mRNA levels of HIF signaling targets, Epo and Vegf, in DMOG treated chondrocytes. (D) mRNA levels of HIF signaling targets, Glut1, Pdk1, and Pkg1, in DMOG treated chondrocytes. *P < 0.05, **P < 0.01, n = 4/group. Data were normalized to controls and presented as mean ± SEM.
Thus, the contrasting phenotypes in the long bone epiphyses of \textit{Phd2Col1-iCre} and \textit{Phd2Col2-Cre} mice indicate different roles for \textit{Phd2} in osteoblasts versus chondrocytes during endochondral ossification at the epiphyses. Based on our present data and previously published data, we propose a model for the role of \textit{Phd2} in the formation of SOCs (Fig. 7). Knockout of \textit{Phd2} gene in chondrocytes promotes HIF signaling and increases bone mass, demonstrating a negative role for \textit{Phd2} expressed in chondrocytes in regulating endochondral ossification at the epiphysis. The importance of HIF signaling in regulating chondrocyte differentiation has been well established. Thus, in chondrocytes, \textit{Phd2} inhibits HIF signaling including Hif-1\textsubscript{α}, Hif-2\textsubscript{α}, and HIF downstream targets, Vegfa, Vegfb, and Epo. This down-regulation of HIF signaling further inhibits chondrocyte differentiation and down-regulates expression of hypertrophy markers such as Col10, as well as mineralization markers including Osx, BSP, and ALP, thus impeding endochondral ossification at the epiphyses (Fig. 7).

In previous studies, we have determined that \textit{Phd2} is the most abundant Phd isoform expressed in bone cells\textsuperscript{37}. While DMOG has been widely used in the literature to inhibit \textit{Phd2} activity, further studies involving knockdown of \textit{Phd2} using \textit{Phd2}-specific shRNA are required to confirm our \textit{in vitro} findings.

POC formation is known to be regulated by a number of growth factors such as PTHrP, Ihh, IGF-1, Wnts and BMPs. Since SOC formation differs from POC formation, whether or not SOC formation also involves these factors needs to be elucidated. Our laboratory has recently discovered that thyroid hormone (TH) played a key role in the formation of the SOC since SOC formation coincides with the time when peak TH levels are attained. Accordingly, TH deficient mice exhibited severely comprised SOC development\textsuperscript{6}. Furthermore, TH promoted SOC ossification by activating Ihh and Osx signaling\textsuperscript{6}; however, the relationship between TH and other signaling pathways needs to be investigated.

**Methods**

**Animals.** To generate osteoblast-specific \textit{Phd2} knockout mice, \textit{Phd2} floxed mice (\textit{Phd2}\textsubscript{flox/flox}) were crossed with the \textit{Col1\textalpha2-iCre} transgenic line which expresses improved Cre recombinase (iCre) in \textit{Col1\textalpha2}-expressing cells\textsuperscript{13,14}. The \textit{Phd2}\textsubscript{flox/flox} mice were first bred to the \textit{Col1\textalpha2-iCre} mice to generate the \textit{Phd2}\textsuperscript{flox/flox};\textit{Col1\textalpha2-iCre} mice. The \textit{Phd2}\textsubscript{flox/flox};\textit{Col1\textalpha2-iCre} mice were then backcrossed with \textit{Phd2}\textsubscript{flox/flox} mice to generate \textit{Phd2}\textsuperscript{flox/flox};\textit{Col1\textalpha2-iCre}, the osteoblast-specific \textit{Phd2} knockout mice (\textit{Phd2}\textsuperscript{Col1-iCre}), and the corresponding littermate controls. Similarly, to generate chondrocyte-specific \textit{Phd2} knockout mice, the \textit{Phd2}\textsubscript{flox/flox} mice were first bred to the \textit{Col2\textalpha1-Cre} mice to generate \textit{Col2\textalpha1-Cre;Phd2}\textsuperscript{flox/flox} mice\textsuperscript{16}. The \textit{Col2\textalpha1-Cre;Phd2}\textsuperscript{flox/flox} mice were then backcrossed with \textit{Phd2}\textsubscript{flox/flox} mice to generate \textit{Phd2}\textsuperscript{flox/flox};\textit{Col2\textalpha1-Cre}, the chondrocyte-specific \textit{Phd2} knockout mice (\textit{Phd2}\textsuperscript{Col2-Cre}), and the corresponding littermate controls. The genetic background of all these mouse lines is C57BL/6.

Animals were housed in the VMU at VA Loma Linda Healthcare System (Loma Linda, CA) under standard approved laboratory conditions. Animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of the VA Loma Linda Healthcare System. Isoflurane was used for anesthesia, and CO\textsubscript{2} exposure was used for euthanasia followed by cervical dislocation.
Micro-computed tomography analysis. Conditioned knockout and control mice of 4 to 5 week old were sacrificed, and the legs were fixed for 5 days in 10% formalin before μCT analysis (VIVA CT40, SCANO Medical, Bruttisellen, Switzerland). Scanning of the epiphyses was according to previously published procedures. Microarchitecture reconstructions of the epiphyses were carried out and analyzed using the SCANCO software (SCANO Medical, Bruttisellen, Switzerland).

Dynamic calcein labeling and histomorphometry. Mice were ip injected with calcein (20 mg/kg) at postnatal day (P) 22 and P26 and euthanized at P28. Femurs were fixed and processed as previously reported. Calcein labeling was visualized with the Olympus BX60 fluorescence microscope (Olympus Corp). Bone formation and resorption parameters were measured using the OsteoMeasure software (Osteometrics Inc). BV/TV, Micro-computed tomography analysis.

Primary chondrocyte culture. Primary chondrocytes were isolated from SOCs of epiphyses and cultured as previously described. Cells were grown in ascobic acid-free αMEM medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) to 70% confluence followed by 24 hours serum free (0.1% BSA αMEM without ascobic acid) treatment. Then the cells were treated with 500 μM DMOG (Cayman Chemical, Ann Arbor, MI) or vehicle control DMSO for 72 hours. The cells were then processed for RNA extraction.

Quantitative RT-PCR. The epiphyses of tibias were dissected from 4 week old control and Phd2−/− mice and snap frozen. RNA was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen, Grand Island, NY). RNA samples were then reverse-transcribed into cDNA and followed by quantitative real time PCR as previously described. The ΔΔCT method was used to calculate relative gene expression with Ppia used as an internal control. Primer sequences are as follows: Hif-1α, forward, 5′-TGACCGGCCACATGTGTTACA-3′, reverse, 5′-AGCTCGGCTGTGTGTTTAGT-3′, Hif-2α, forward, 5′-CATTTGCTTGTTGACCAA-3′, reverse, 5′-GGTGACACGTCTTGTCTC-3′, Vegfa, forward, 5′-ATGCGGATCAAACCTCACCAA-3′, reverse, 5′-TTCTGGCTTTTGGTCTTCTTTT-3′, Vegfb, forward, 5′-ACGATGCGCTTGAAATTGTG-3′, reverse, 5′-GGTCTGATTACATTGGCGT-3′. Other primer sequences were reported in previous publications.

Statistics. Data were expressed as mean ± SEM (standard error of the mean) and were analyzed using Student’s T-test. P < 0.05 was considered statistically significant.

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**Author Contributions**

S.M. designed the study and reviewed the manuscript. S.C. performed experiments, analyzed data, and wrote the manuscript. P.A. reviewed the manuscript. S.P. and C.A. performed experiments and analyzed data.

**Additional Information**

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