Conditional Deletion of the Phd2 Gene in Articular Chondrocytes Accelerates Differentiation and Reduces Articular Cartilage Thickness

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Based on our findings that PHD2 is a negative regulator of chondrocyte differentiation and that hypoxia signaling is implicated in the pathogenesis of osteoarthritis, we investigated the consequence of disruption of the Phd2 gene in chondrocytes on the articular cartilage phenotype in mice. Immunohistochemistry detected high expression of PHD2 in the superficial zone (SZ), while PHD3 and HIF-1α (target of PHD2) are mainly expressed in the middle-deep zone (MDZ). Conditional deletion of the Phd2 gene (cKO) in chondrocytes accelerated the transition of progenitors to hypertrophic (differentiating) chondrocytes as revealed by reduced SZ thickness, and increased MDZ thickness, as well as increased chondrocyte hypertrophy. Immunohistochemistry further revealed decreased levels of progenitor markers but increased levels of hypertrophy markers in the articular cartilage of the cKO mice. Treatment of primary articular chondrocytes, in vitro, with IOX2, a specific inhibitor of PHD2, promoted articular chondrocyte differentiation. Knockdown of Hif-1α expression in primary articular chondrocytes using lentiviral vectors containing Hif-1α shRNA resulted in reduced expression levels of Vegf, Glut1, Pgk1, and Col10 compared to control shRNA. We conclude that Phd2 is a key regulator of articular cartilage development that acts by inhibiting the differentiation of articular cartilage progenitors via modulating HIF-1α signaling.

Articular cartilage is a hyaline cartilage that provides a smooth and frictionless surface for bone movements within a joint. The thin layer of articular cartilage covers the bone surface and maintains its lubricating function for an individual’s lifetime. Damage to the articular cartilage is a significant clinical issue causing an enormous economic burden worldwide. The most common articular cartilage damage is osteoarthritis characterized by progressive degeneration of articular cartilage that leads to chronic pain and functional restrictions in the affected joints. Pathophysiological features of osteoarthritis include degeneration or loss of articular cartilage, sclerosis, an increase of subchondral bone density, and osteophyte formation1–3. The precise mechanisms that regulate the integrity of articular cartilage and the mechanisms of osteoarthritis development are still unclear and nonsurgical therapeutic interventions to articular cartilage diseases are limited to date3,4.

The articular cartilage is composed of several chondrocyte layers with unique cellular arrangements including a superficial zone (SZ), a middle zone, a deep zone, and a calcified cartilage zone. The SZ contains flat shaped articular cartilage progenitor cells robustly expressing proteoglycans such as aggrecan and lubricin5,6, while chondrocytes in the deep zone are in a columnar organization with some of them becoming hypertrophic and marked by expression of Col10α. A number of growth factors including Wnt9A, TGFβ, PTHrP, IGF-I and BMPs have been implicated in maintaining articular cartilage integrity by regulating the transit of articular cartilage progenitors towards differentiating chondrocytes6–8. It is known that increased chondrocyte hypertrophy, which leads to increased endochondral ossification, is frequently identified in early stage osteoarthritis samples9,10. Accordingly, studies have shown an inverse correlation between the onset of osteoarthritis and an increase in subchondral...
bone density. Hence, understanding the molecular mechanisms controlling the transition of articular cartilage progenitors towards hypertrophic chondrocytes is important for the prevention and treatment of articular cartilage diseases.

Prolyl Hydroxylase Domain-containing Proteins (PHDs) are negative regulators of the hypoxia-inducible factors (HIFs) which moderate chondrocyte differentiation. PHDs belong to the 2-oxoglutarate/iron-dependent dioxygenase superfamily. There are three members of Phds in mammals: Phd1 Phd2, and Phd3. All PHDs contain the highly conserved hydroxylase domain in the catalytic carboxy-terminal. PHD2 is the major regulator of HIF-1α activity. In the presence of oxygen, PHD2 hydroxylates two proline residues (Pro-402 and Pro-564) in the C-terminal of HIF-1α which leads to the ubiquitin-mediated proteasomal degradation of HIF-1α. HIF-1α is required for the maintenance and differentiation of chondrocytes in the hypoxic growth plate. Conditional deletion of HIF-1α in mesenchymal cells resulted in abnormal cartilage and joint development. On the other hand, we found that deletion of Phd2 in chondrocytes caused accelerated chondrocyte differentiation and increased endochondral bone formation in the metaphyses and epiphyses of long bones. These data clearly demonstrate that Phd2 negatively regulates chondrocyte differentiation and endochondral bone formation through the PHD/HIF regulatory pathway.

Interestingly, in addition to the growth plate chondrocytes, Phd2 and Phd3 are also highly expressed in the articular chondrocytes with distinct expression patterns. Since Phd2 is a negative regulator of chondrocyte differentiation, we hypothesize that Phd2 also inhibits the differentiation of articular cartilage progenitors, and deletion of Phd2 in chondrocytes promotes progenitors to differentiate into hypertrophic chondrocytes and thereby, reduce articular cartilage thickness. Since articular cartilage richly contains Type II collagen (Col2), we used Col2α1-Cre line to conditionally delete Phd2 gene in articular chondrocytes.

Results

PHDs and HIFs are expressed in distinct patterns in the femoral articular cartilage. To study the role of PHDs in articular cartilage development, we first evaluated the expression patterns of PHD2 and PHD3 and their targets (HIFs) using immunohistochemistry in the distal femoral articular cartilage in 2 week old mice, when articular cartilage formation is known to occur. PHD2 protein is highly expressed in the SZ of articular cartilage, but remains low in the middle-deep zone (MDZ) (Fig. 1A, blue is positive staining). By contrast, PHD3 is almost absent in the SZ, but highly expressed in the MDZ, where some of the chondrocytes are undergoing hypertrophy (Fig. 1B). HIF-1α protein, a known target of PHD2, appears mainly in the MDZ of articular cartilage, whereas PHD2 expression is low (Fig. 1C). On the other hand, HIF-2α is highly expressed in the SZ, but not in the MDZ of the articular cartilage, similar to the expression pattern of PHD2 (Fig. 1D). HIF-3α is more ubiquitous than other HIFs and is expressed throughout different zones at relatively low levels (Fig. 1E). The high expression of Phd2 and Hif-2α in the progenitor containing SZ suggests a role for Phd2 and Hif-2α in maintaining the progenitor status of articular chondrocytes. On the other hand, the predominant expression of Phd3 and Hif-1α in MDZ implicates their role in articular chondrocyte differentiation.

Conditional deletion of Phd2 in the Col2-expressing cells ablated PHD2 expression in articular cartilage. To test the hypothesis that Phd2 plays a role in maintaining the progenitor status of the cells in the SZ by preventing their differentiation towards a hypertrophic stage, we used Col2α1-Cre transgenic mice to disrupt the Phd2 gene in articular chondrocyte progenitors that express high levels of Col2α1 (Fig. 2A). In skeletal tissue, Col2α1-Cre activity was specifically detected in chondrocytes and the specificity of the Col2α1-Cre line was documented in several studies. To study Phd2 function in endochondral bone formation at the primary and secondary ossification centers. The efficiency of the Col2α1-Cre line used for disruption of the Phd2 gene in articular chondrocytes was validated by immunohistochemistry. In Fig. 2B, PHD2 protein was detected in tibial articular cartilage of 4 week old control mice, but not in the conditional knockout (cKO) mice. By contrast, PHD2 protein expressed in bone marrow was intact in the cKO mice compared to the control mice (Fig. 2B).

Deletion of Phd2 in articular cartilage reduced the thickness of the superficial zone and increased chondrocyte hypertrophy in articular cartilage. To evaluate the effect of Phd2 deletion on articular cartilage development, the articular cartilage phenotype was evaluated by histology in the long bones of 4 week old mice. We measured the thickness of the SZ and MDZ in the tibial articular cartilage. The thickness of SZ was reduced by 25% (P < 0.05) in the cKO tibia compared to control tibia (Fig. 3A,B). By contrast, the thickness of MDZ was increased by 31% (P < 0.05) in the cKO tibia compared to controls (Fig. 3A,C). The number of hypertrophic chondrocytes was increased in the articular cartilage of the cKO mice compared to controls (Fig. 3A), indicating increased chondrocyte differentiation in the articular cartilage of the cKO mice. These data suggest that loss of Phd2 promoted the transition of articular cartilage progenitors in the SZ into differentiating chondrocytes.

To examine if the histological changes of the articular cartilage in the cKO mice were maintained in adult mice, we also measured the thickness of the SZ and the MDZ in 12 week old mice. The reduction in SZ thickness in the cKO tibia was 23% (P < 0.05) compared to control tibia in 12 week old mice (Fig. 3D). The thickness of the MDZ was slightly increased in the cKO mice compared to control mice, but this change did not reach statistical significance (P = 0.19) (Fig. 3E). We further evaluated an early stage osteoarthritis phenotype in these mice. We found that the joint space was increased by 44% (P < 0.05), and the Osteoarthritis Research Society International (OARSI) score was increased by 270% (P < 0.01) in the cKO mice compared to control mice (Fig. 3F,G), suggesting that a lack of Phd2 may increase the risk of osteoarthritis development.
Deletion of Phd2 in articular cartilage reduced expression of progenitor markers, but elevated expression of hypertrophy markers. Articular cartilage progenitors in the SZ express high levels of lubricin and aggrecan\textsuperscript{5,6}. Consistent with the prediction that loss of Phd2 promoted chondrocyte differentiation, we found expression levels of both lubricin and aggrecan were markedly reduced in the cKO articular cartilage.
(Fig. 4A,B, blue is positive staining) while levels of PHD3 and HIF-1α, that are known to be highly expressed in hypertrophic chondrocytes, were elevated in the cKO articular cartilage compared to controls (Fig. 4C,D). We also examined the expression of COL10, a well-established marker of hypertrophic chondrocytes, and found that it was markedly increased in the cKO articular chondrocytes (Fig. 4E), thus suggesting elevated chondrocyte differentiation in the articular cartilage of the Phd2 cKO mice.

Inhibition of PHD2 by IOX2 promoted cell differentiation and HIF-1α signaling in primary articular chondrocytes. In order to determine if the increased differentiation of articular chondrocytes in the Phd2 cKO mice is a direct consequence of loss of PHD2 function, we treated primary articular chondrocytes with a specific inhibitor of PHD2, IOX2, for 72 hours and then measured expression levels of markers of proliferation, articular cartilage progenitors, and chondrocyte differentiation. We found that expression of proliferation markers, p57 and cyclin D1, were both reduced by 28% (P < 0.05) (Fig. 5A). Accordingly, expression levels of markers of articular cartilage progenitors, tenascin c and clusterin, were reduced by 28% and 32%, respectively (P < 0.05) (Fig. 5B). Expression of Col2 was unchanged (Fig. 5C). By contrast, expression level of Col10 was increased by 126% (P < 0.01) (Fig. 5C). Loss of PHD2 activity is known to increase protein levels of HIF-1α via inhibition of prolyl hydroxylation of HIF-1α and its subsequent proteosomal degradation. Activation of HIF-1α signaling is known to promote angiogenesis and glycolysis. Accordingly, expression levels of Vegf, an angiogenic marker and a known target of HIF-1α, were increased by 8 fold (P < 0.01) (Fig. 5D). Epo is also a target of HIF-1α. Its expression was also increased, but the increase did not reach statistical significance. Expression levels of Glut1 and Pgk1, markers of glycolysis, were increased by 154% and 170%, respectively (P < 0.05) in IOX2 treated cells (Fig. 5D).

Knockdown of Hif-1α reduced differentiation and glycolysis of primary articular chondrocytes. In order to determine the role of HIF-1α signaling in mediating Phd2 effects on chondrocyte differentiation, we evaluated the consequence of knockdown of Hif-1α in primary articular chondrocytes. We used lentiviral vectors containing shRNA specific to Hif-1α to knockdown HIF-1α. Hif-1α shRNA treatment knocked down HIF-1α mRNA expression by 92% (P < 0.01) compared to control shRNA (Fig. 6A). Expression of the HIF-1α target, Vegf, was also reduced by 75% (P < 0.01) (Fig. 6A). Furthermore, HIF-1α targets, the glycolytic enzymes Glut1 and Pgk1, were reduced by 41% and 55%, respectively (P < 0.05) (Fig. 6B). The chondrocyte hypertrophy marker Col10 was also reduced by 50% (P < 0.05), while Col2 expression was not changed (Fig. 6C). The findings that

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Figure 2. Generating the conditional Phd2 knockout mice in articular chondrocytes. (A) Conditional deletion of the Phd2 gene in Col2α1-expressing chondrocytes. (B) Immunohistochemistry analysis of PHD2 expression in tibiae of 4 week old control and cKO mice. PHD2 was expressed in the control but not in the cKO tibial articular cartilage (arrows). Expression of PHD2 in bone marrow was intact in the cKO mice (arrow heads). Blue is positive staining. BM, bone marrow.
knockdown of Hif-1α inhibited angiogenesis, glycolysis, and chondrocyte differentiation, effects that are opposite to those of IOX2 mediated inhibition of PHD2 activity, suggests that the negative effect of Phd2 on chondrocyte differentiation in articular cartilage is perhaps mediated via inhibition of the HIF-1α signaling pathway.

Discussion

In this study, we have found distinct spatial distribution of PHD2 and PHD3 in the articular cartilage, with PHD2 being mainly localized to the SZ progenitor cells while PHD3 to the differentiating chondrocytes in the MDZ. The low level of PHD2 in the MDZ was accompanied by a high level of HIF-1α in the MDZ, consistent with the well-known function of PHD2 to target HIF-1α to proteosomal-mediated degradation\(^\text{17,18}\). Conditional deletion of Phd2 in chondrocytes decreased the thickness of progenitors in the SZ and increased chondrocyte hypertrophy in the MDZ of the tibial articular cartilage, marked by down regulation of lubricin and aggrecan, and up regulation of HIF-1α and COL10. These findings suggest that high levels of Phd2 are required to maintain the

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Figure 3. Histological changes in the articular cartilage of the Phd2 cKO mice. (A) Safranin-O staining of 4 week old control and cKO tibiae. Arrows show hypertrophic chondrocytes. (B) SZ thickness in control and cKO tibiae at 4 weeks of age. (C) MDZ thickness in control and cKO tibiae from 4 week old mice. (D) SZ thickness in 12 week old control and cKO tibiae. (E) MDZ thickness in 12 week old control and cKO tibiae. (F) Thickness of joint space in 12 week old control and cKO tibiae. (G) OARSI score in 12 week old control and cKO tibiae. *\(P < 0.05\), **\(P < 0.01\), \(n = 8/\text{group}\) in (B,C), \(n = 6/\text{group}\) in (D–G). Data were presented as the mean ± SEM. Bar = 50 μm.
progenitor status of cells in the SZ. Loss of functional PHD2 in the progenitor cells of the SZ promotes articular chondrocyte differentiation. This notion is further supported by a primary articular chondrocyte study showing that inhibition of PHD2 suppressed expression of tenascin C and clusterin, but elevated expression of Col10 and HIF-1α. The Phd2 effect on articular chondrocyte differentiation appears to be mainly mediated by HIF-1α signaling as revealed from our data from experiments involving knockdown of Hif-1α and PHD2 inhibitor.

Our study demonstrated a crucial role for Phd2 in articular chondrocyte regulation. Similar to its role in endochondral bone formation, Phd2 inhibits HIF-1α signaling and hence inhibits chondrocyte differentiation. It is well established that HIF-1α is a positive regulator of chondrocyte differentiation and ossification, as conditional deletion of the von Hippel-Lindau gene (Vhl), a ubiquitin ligase that promotes proteolysis of HIFs, or overexpression of Vegf, a HIF-1α target, resulted in excessive endochondral bone formation. HIF-1α signaling also appeared to negatively affect progenitor status of the articular chondrocytes, as Phd2 deletion in chondrocytes resulted in reduced articular cartilage SZ thickness. The negative regulation of HIF-1α signaling in progenitors was also supported by the Hif-1α knockdown experiment. Hif-1α knockdown reduced expression of markers of proliferation, as well as markers of progenitors. Based on these data, one would expect a negative effect of HIF-1α on the maintainence of articular cartilage, and higher levels of HIF-1α could contribute to the pathogenesis of osteoarthritis.

In addition to its established role in regulating chondrocyte differentiation, other studies have shown that Hif-1α is also required for chondrocyte growth arrest and survival. Lack of Hif-1α resulted in chondrocyte death. Balb/C mice injected with HIF-1α inhibitor, 2-methoxyestradiol, resulted in progressive destruction of the articular cartilage, and HIF-1α inhibition in human osteoarthritic cartilage accelerates catabolic stress-induced apoptosis in vitro, thus suggesting a protective role for Hif-1α in osteoarthritis development.

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**Figure 4.** Expression of markers of articular cartilage progenitors and chondrocyte differentiation in control and cKO mice. Immunohistochemistry analysis of markers in tibial articular cartilage of 12 week old control and cKO mice. (A,B) Expression of SZ progenitor markers, lubricin and aggrecan, in articular cartilage. (C) PHD3 expression in control and cKO articular cartilage. (D) HIF-1α expression in control and cKO articular cartilage. (E) Expression of the chondrocyte hypertrophy marker COL10 in control and cKO articular cartilage. Blue is positive staining. Arrows show positive stains of each protein in the articular cartilage. Bar = 50 μm.
However, this hypothesis contradicted research showing that stabilization of HIF-1α by dimethyloxaloylglycine did not prevent osteoarthritis development in the knee joints of STR/ORT mice. Nevertheless, Hif-1α appeared to be clinically important in the development of osteoarthritis. Several studies reported elevation of HIF-1α signaling in human ostearthritic samples. The precise role of HIF-1α in articular cartilage development and the clinical significance of aberrant PHD2/HIF-1α signaling in osteoarthritis development needs to be investigated further.

Though PHD2 is the major regulator of HIF-1α, HIF-1α also interacts with PHD3. HIF-1α upregulates PHD3 through a feedback mechanism. On the other hand, PHD3 hydroxylates HIF-1α, although PHD3 is the major regulator of HIF-2α. In terms of clinical significance, HIF-2α is a catabolic factor for articular cartilage, and lack of HIF-2α is protective for osteoarthritis development. We have observed elevated expression of PHD3 in the Phd2 cKO articular cartilage. This is consistent with our previous report that disruption of Phd2 in growth plate chondrocytes elevated Phd3 expression both in vivo and in vitro. The role of Phd3 in articular cartilage development and mainanence need to be further investigated.

Based on our findings, we proposed a model for the regulation of Phd2 on articular cartilage (Fig. 7). PHD2 inhibits HIF-1α, which further inhibits PHD3. Lack of PHD2 promotes HIF-1α signaling and elevates PHD3 expression. HIF-1α translocates from the cytoplasm to the nucleus and binds to the hypoxia response element (HRE) of targeted genes including genes required for chondrocyte hypertrophy. Thus Phd2 is a key negative regulator for articular chondrocyte differentiation.

Methods

Animals. To generate Phd2 conditional knockout mice specifically in chondrocytes, Phd2 floxed mice (Phd2floxed) were first crossed with Col2α1-Cre mice to generate Cre positive, Phd2loxp-heterozygous mice (Phd2floxed;Col2α1-Cre). Phd2floxed;Col2α1-Cre mice were then backcrossed with Phd2floxed mice, to yield Cre positive, loxp-homozygous (Phd2floxed;Col2α1-Cre) conditional knockout mice and Cre negative control.
littermates (Phd2flox/flox, Phd2flox/+). The genetic background of Phd2flox/flox and Col2α1-Cre mice is C57BL/6. Animals were housed according to the approved laboratory conditions in the VMU at VA Loma Linda Healthcare System (Loma Linda, CA).

**Ethics Statement.** Animals were housed according to the approved laboratory conditions in the animal facility unit at VA Loma Linda Healthcare System (Loma Linda, CA). All experimental procedures were evaluated and carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the VA Loma Linda Healthcare System. Isoflurane was used for anesthesia, and CO2 exposure was used for euthanasia followed by cervical dislocation. All procedures performed followed the ethical guidelines for animal studies.

**Histomorphometry and immunohistochemistry.** Four and 12 week old control and cKO mice were sacrificed and the femurs were fixed in 10% formalin for 4 days, decalcified, and processed for paraffin sectioning as previously described40. Three comparable sections of the knee joint from each animal were stained with Safranin O and counter stained with Fast Green to visualize the articular cartilage. Measurements of the thickness of the SZ and the MDZ were carried out using the OsteoMeasure software (Osteometrics, Inc. Decatur,
HIF-1α 25–30% confluence and transduced with non-target shRNA were purchased from Sigma-Aldrich (St. Louis, MO). Primary chondrocytes were grown to

primer sequences are as following: p57Kip2, forward, 5'-GAAGAAGTCTGTCCATTTG-3', reverse, 5'-AGTGACCCGCATCTCATTGT-3'; cyclin D1, forward, 5'-AATGTACCTGCTTGGTCAA-3', reverse, 5'-ATGACCCATAGGGTCG-3'; tenascin C, forward, 5'-TGACTGAGAACCAGGAC-3', reverse, 5'-AGTGACCCGATCTCATTGGT-3'; clusterin, forward, 5'-AGAGGATGGATGACCCGCA-3', reverse, 5'-CTTGACTGCTTCATCGACC-3'. Other primer sequences were listed in previous publications13,14,45.

shRNA knockdown. MISSION® shRNA Lentiviral Transduction Particles against Hif-1α (CCGGTGGATAGCGATATGGTCAATGCTCGAGCATTGACCATATCGCTATCCATTTTTG) and control non-target shRNA were purchased from Sigma-Aldrich (St. Louis, MO). Primary chondrocytes were grown to 25–30% confluence and transduced with Hif-1α shRNA or scramble control lentivirus at a multiplicity of infection (MOI) of 10 by adding a pre-made viral particle stock solution (1.4 × 10^7) into 6-cm culture plates in the presence of 8 μg/ml of polybrene for 2 days, followed by puromycin selection (10 μg/ml) for 3–5 days as previously described41. The puromycin selected cells were propagated and used for gene expression experiments.

Quantitative RT-PCR. RNA was extracted from cells with Trizol reagent according to the manufacturer’s instructions (Invitrogen, Grand Island, NY). An aliquot of RNA (400 ng) from each sample was reverse-transcribed into cDNA and mRNA levels were followed by quantitative real time PCR as previously described44. Relative gene expression values were calculated using the ΔΔCT method with Ppia mRNA serving as an internal control44. Primer sequences are as following: p57Kip2, forward, 5'-GGGCACAGGAGAATCAAG-3', reverse, 5'-GGAAAGTCTGTCCATTTG-3'; cyclin D1, forward, 5'-AATGTACCTGCTTGGTCAA-3', reverse, 5'-ATGACCCATAGGGTCG-3'; tenascin C, forward, 5'-TGACTGAGAACCAGGAC-3', reverse, 5'-AGTGACCCGATCTCATTGGT-3'; clusterin, forward, 5'-AGAGGATGGATGACCCGCA-3', reverse, 5'-CTTGACTGCTTCATCGACC-3'. Other primer sequences were listed in previous publications13,14,45.

Statistics. The student’s T-test was used for statistical analysis. P < 0.05 was considered statistically significant. All data were presented as mean ± SEM (standard error of the mean).

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Abstract
Conditional deletion of the Osterix gene in articular chondrocytes increased their osteoblastic differentiation and reduced cartilage thickness.

Keywords: Osterix, Chondrocyte, Differentiation, Cartilage, Osteoblasts.

Author Contributions
S.M. designed the study, interpreted the data and wrote the manuscript. S.C. performed the described experiments, analyzed the data, and wrote the manuscript. S.P. and C.A. performed study experiments and analyzed the data.

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Additional Information

Supplementary information

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