Inhibition of Hypoxia-inducible Factor-targeting Prolyl Hydroxylase Domain-containing Protein 2 (PHD2) Enhances Matrix Synthesis by Human Chondrocytes

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Human articular cartilage is an avascular tissue, and therefore it functions in a hypoxic environment. Cartilage cells, the chondrocytes, have adapted to this and actually use hypoxia to drive tissue-specific functions. We have previously shown that human chondrocytes enhance cartilage matrix synthesis in response to hypoxia specifically through hypoxia-inducible factor 2α (HIF-2α)-mediated up-regulation of master regulator transcription factor SOX9, which in turn drives expression of the main cartilage-specific extracellular matrix genes. HIF-α isoforms are themselves regulated by specific prolyl hydroxylase domain-containing proteins, which target them for proteosomal degradation. In fact, prolyl hydroxylase domains are the direct oxygen sensors because they require molecular oxygen as a co-substrate. Here, we have identified PHD2 as the dominant isoenzyme regulating HIF-2α stability in human chondrocytes. Moreover, specific inhibition of PHD2 using RNA interference-mediated depletion caused an up-regulation of SOX9 and enhanced extracellular matrix protein production. Depletion of PHD2 resulted in greater HIF-2α levels and therefore enhanced SOX9-induced cartilage matrix production compared with the levels normally found in hypoxia (1% oxygen) implying that PHD2 inhibition offers a novel means to enhance cartilage repair in vivo. The need for HIF-specific hydroxylase inhibition was highlighted because treatment with the 2-oxoglutarate analogue dimethylmaloxalylglycine (which also inhibits the collagen prolyl 4-hydroxylases) prevented secretion of type II collagen, a critical cartilage matrix component.

Articular cartilage consists of a single cell type, the chondrocyte, which is solely responsible for the synthesis and maintenance of the extracellular matrix (1). The rigid nature of type II collagen fibrils present in the matrix confers tensile strength to the tissue, and the swelling pressure caused by water-saturated aggrecan molecules creates a compressive gel-like stiffness allowing the tissue to resist deformation and giving cartilage its ability to absorb shocks (1, 2). Due to its shock-absorbing and articulating functions, articular cartilage cannot afford a blood or nerve supply. The tissue is therefore dependent on diffusion of oxygen from the synovial fluid on one side and the vascularized underlying bone on the other. As a result, articular cartilage (at least in larger animals and man) is maintained in a low oxygen environment (1–5% oxygen tension) throughout life (3–5). There is now significant evidence that hypoxia is a critical parameter in promoting the chondrocyte phenotype. It has been shown that hypoxia up-regulates key cartilage transcription factor SOX9 and increases expression of the main extracellular matrix genes in bovine and human chondrocytes (6–10).

The response of cells to hypoxia is mediated by hypoxia-inducible factors (HIFs), heterodimeric transcription factors consisting of an α and β subunit (11). Both subunits are constitutively expressed at the mRNA level; however, the α subunit is regulated post-translationally by oxygen levels. In well oxygenated cells, the α subunit is hydroxylated on specific, conserved proline residues by HIF-targeting prolyl hydroxylase domain-containing proteins (PHD1, PHD2, and PHD3) (12–14). The PHD isoenzymes are a subfamily of dioxygenases that use molecular oxygen and 2-oxoglutarate as co-substrates to add hydroxyl groups to specific proline residues on the HIF-α subunit (15–17). The hydroxylated proline residues on HIF-α are recognized by von Hippel-Lindau protein of the E3 ubiquitin ligase complex, ubiquitinated and subsequently degraded by the 26 S proteasome (18). As intracellular oxygen concentrations are reduced, the activity of the PHDs is suppressed, and HIF-α isoforms are not hydroxylated and therefore escape proteosomal degradation and so heterodimerize with HIF-1β, translocate to the nucleus, and bind to cis-regulatory hypoxia-response elements in target genes. Previous work in our laboratory has demonstrated that hypoxic induction of the key cartilage matrix genes in human chondrocytes is mediated specifically by the HIF-2α isoform that up-regulates SOX9, leading to enhanced expression of the cartilage matrix genes (9).

Suppression of PHD enzymes leads to stabilization of HIFs and offers a potential treatment option for many ischemic disorders, such as peripheral artery occlusive disease, myocardial infarction, and stroke (19). We hypothesized that antagonizing specific PHDs could be a novel way of promoting cartilage matrix deposition from chondrocytes by increasing HIF-2α stability and up-regulating key transcription factor SOX9. We investigated the role of each PHD isoenzyme in isolated healthy
human articular chondrocytes adopting an RNAi-mediated approach. We provide strong evidence that specific inhibition of PHD2 enhances cartilage matrix production by chondrocytes, and these results thus identify PHD2-specific inhibition as a novel means to stimulate chondrocyte anabolism and therefore cartilage repair.

EXPERIMENTAL PROCEDURES

Cell Culture—Healthy human articular cartilage was obtained from patients after they provided informed consent and following local ethics committee guidelines. Samples of cartilage were obtained from patients following above knee amputations due to soft tissue sarcomas and osteosarcomas not involving the joint. Tissue was obtained from 21 patients (17 male, 4 female; aged 8–55 years, mean age 26.6 years). Cartilage was digested as described previously (9). Briefly, the cartilage was diced into small pieces and digested in collagenase overnight. Freshly isolated cells were initially seeded at a density of 8 × 10³ cells/cm² in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Chondrocytes were passaged at the time of confluency (~7 days) and re-seeded at 5 × 10³ cells/cm².

Small Interfering RNA Transfection—Primary (P0) or passaged (P1–P3) human articular chondrocytes (HACs) were seeded at 5 × 10³ cells/cm² in 6-cm dishes. Cultures were incubated at 37 °C, 20% oxygen, 5% CO₂ until 50% confluent (typically after 1 day). Lipofectamine 2000 (Invitrogen) was used to transfect cells with small interfering RNA (siRNA) at a final concentration of 10 nM in serum-free Opti-MEM I. PHD1, PHD2, and PHD3 targeting siRNAs, which were used, are listed in Table 1. A control, siRNAs against luciferase (Dharmacon, Lafayette, CO) were transfected in parallel. Four hours after transfection, the Opti-MEM was removed and replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum pre-equilibrated at the appropriate oxygen tension (20 or 1%). Cells were subsequently incubated in each oxygen tension for 3 days.

RNA Isolation, Reverse Transcription, and Real Time PCR—RNA was extracted and prepared using the RNeasy mini kit (Qiagen, Crawley, UK) following the manufacturer’s guidelines. cDNA was generated using a reverse transcription kit (Promega, Southampton, UK) and random primers from 0.5 μg of total RNA. Newly synthesized cDNA was diluted 5-fold in D2Hase-free water. Four percent of this cDNA was then used for real time PCR assays using TaqMan technology and a PCR Corbett Research thermocycler (Corbett Research, Australia). The ΔΔ threshold cycle (ΔΔCT) method of relative quantitation was used to calculate relative mRNA levels for each transcript examined. The ribosomal protein gene (RPLP0) was used to normalize the data as it was not regulated by hypoxia in our system. Pre-developed primer/probe sets for the following genes were purchased from Applied Biosystems (Foster City, CA): PHD1, PHD2, PHD3, FIH1, COL2A1, COL9A1, COL11A2, AGGRECAN, SOX9, VEGF, and RPLP0.

Protein Extraction and Western Blotting—To detect HIF-α isoforms, cells were lysed immediately after termination of the experiment in urea lysis buffer (8 M urea, 10% glycerol, 1% SDS, 5 mM dithiothreitol, 10 mM Tris-Cl); otherwise, the cells were lysed in radioimmunoprecipitation assay buffer (100 mM Trizma base, pH 7.4, 300 mM NaCl, 20 mM NaF, 2 mM EDTA, 0.2 mM β-glycerophosphate, 2%(w/v) sodium vanadate, 2% deoxycholate, 0.5% SDS, and 1% Nonidet P-40). A protease inhibitor mixture (Sigma) was added just prior to cell lysis. Cell lysates were sonicated for 5 s and spun at 12,000 rpm for 10 min at 4 °C to remove any cell debris. 20 μg of protein was resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes for Western blotting, and visualized using the enhanced chemiluminescence method. To detect PHD3 and HIF-2α, 40 μg of protein was used. Primary antibodies used were rabbit anti-SOX9 (Chemicon AB5535, 1:1000), rabbit anti-PHD2 (Abcam AB4561, 1:1000), mouse anti-PHD3 (clone 188e, a kind gift from Professor Chris Pugh, Oxford, UK), mouse anti-HIF-1α (BD Transduction Laboratories, clone 54, 1:250), mouse anti-HIF-2α (Santa Cruz Biotechnology Sc-13596, 1:250), and mouse anti-α-tubulin monoclonal antibody (Sigma, 1:4000).

For detection of secreted type II collagen, HACs were serum-starved for 2 days, 24 h following transfection. Approximately 1 ml of conditioned medium was precipitated with trichloroacetic acid, loaded on a 6% polyacrylamide gel, and blotted with anti-COL2A1 antibody (Chemicon MAB8887, 1:1000). The exact volume of media used was adjusted after standardization according to total protein content in the cell layer, measured using the Bradford assay.

Statistical Analysis—Data were compared using a one-way analysis of variance with Bonferroni post test. The software GraphPad Prism 4 (GraphPad Software, San Diego) was used to perform statistical analysis. Results are expressed as a mean ± S.E. Probability values less than 0.05 were considered significant.

RESULTS

PHD2 Is the Most Abundant PHD Isoenzyme, and PHD2 and -3 Are Hypoxia-inducible in HACs—The mRNA levels of PHD1, PHD2, and PHD3 under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions were analyzed after normalizing the data using the housekeeping gene RPLP0. The relative abundance of each gene was calculated after assigning the expression level of PHD3 at 20% oxygen to a value of 1. This method of calculating the relative abundance of the three PHD isoenzymes at the mRNA level makes the assumption that the efficiency of amplification between the different PCR primers is equal (in practice, all amplification efficiencies were similar, being over 90%). PHD2 was the most abundant transcript of the three isoenzymes in both 20 and 1% oxygen (Fig. 1A). Interestingly, although PHD3 had the most pronounced hypoxic induction (average 19.2-fold induction), it was the least abundant PHD enzyme at the mRNA level in both 20 and 1% oxygen (Fig. 1A). This is consistent with data at the protein level. PHD2 and PHD3 proteins were found to be induced by hypoxia (Fig. 1, B and C). In addition, 20 μg of PHD2 was visualized using enhanced chemiluminescence after 3 min of exposure to x-ray film, whereas 40 μg of PHD3 required 45 min of exposure to detect any protein (note, the top band in all PHD2 blots is nonspecific).

Depletion of PHDs in HACs—PHD1, PHD2, and PHD3 were specifically depleted in HACs using siRNA (Fig. 2, A–C, respec-
tively). Two different oligonucleotides were used for each gene (for sequences see Table 1). siRNA against luciferase (siLuc) was used as a control. Messenger RNA analysis by real time PCR revealed that PHD1 was depleted 72% (siPHD1 number 1) and 95% (siPHD1 number 2) on average compared with siLuc levels. PHD2 was depleted 82% (siPHD2 number 1) and 86% (siPHD2 number 2) on average, and PHD3 was depleted by 87% (siPHD3 number 1) and 84% (siPHD3 number 2) on average. Western blot confirmed the depletion of PHD2 (Fig. 2D) and PHD3 (Fig. 2E) at the protein level. Knockdown of PHD3 was performed in the presence of the iron chelator desferrioxamine (DFO) as PHD levels were easily detectable in DFO, but they were relatively low in hypoxia and were undetectable in normoxia. The oligonucleotides that produced the greatest knockdown at the mRNA level were used in further experiments.

**Depletion of PHD2 in Normoxia Stabilizes HIF-2α and Upregulates Master Chondrocyte Transcription Factor SOX9**—In normoxia, HIF-1α isoforms are rapidly degraded in the cell as there is sufficient oxygen for the HIF-targeting prolyl hydroxylases to target them for von Hippel-Lindau-mediated proteosomal degradation. However, the knockdown of PHD2 in HACs greatly stabilized both HIF-1α and HIF-2α in normoxia (20% oxygen) suggesting that PHD2 significantly contributes to HIF-α regulation (Fig. 3A). The knockdown of PHD1 also contributed to the stabilization of HIF-1α in normoxia but to a much lesser extent than PHD2 depletion, and it had no detectable effect on HIF-2α levels. PHD3 suppression had no detectable effect on levels of either HIF-α isoform in normoxia. Because depletion of PHD2 alone had such a pronounced effect on HIF-2α stabilization, and we have previously shown that HIF-2α specifically mediates hypoxic induction of the differentiated chondrocyte phenotype through up-regulation of master tissue-specific transcription factor SOX9 (9), we further explored PHD2 regulation of HIF-2α isoform stability through reoxygenation experiments. PHD2-depleted HACs were cultured in hypoxia for 3 days before being exposed to 20% oxygen for 0, 5, 10, 20, 40, and 60 min prior to lysing. Small interfering
RNA against luciferase (siLuc) was used as a control to provide accurate comparison with PHD depletion. Upon exposure to normoxia, HIF-α subunits have a very short half-life (~5 min). However, the knockdown of PHD2 greatly slowed the rate of degradation of both HIF-1α and HIF-2α (Fig. 3, B and C, respectively), thus confirming the importance of PHD2 in regulation of HIF-2α stability.

We next investigated the levels of HIF-2α-inducible cartilage-specific transcription factor SOX9 in PHD1/2/3 knockdowns in HACs in 20% oxygen. Strikingly, only depletion of PHD2 resulted in enhanced SOX9 protein levels compared with luciferase controls (Fig. 3D). This is critical because SOX9 drives expression of the cartilage-specific extracellular matrix genes that are essential for the formation of a suitable load-bearing tissue, the primary function of articular cartilage.

**PHD2 Depletion Promotes the Differentiated HAC Phenotype**

**PHD2 Depletion Promotes Matrix Production by Chondrocytes**

Levels of SOX9 were analyzed in PHD2-depleted HAC cultures from a range of patients. SOX9 protein was enhanced by PHD2 depletion in a manner very similar to that of HIF-2α, i.e. with greatest levels achieved in 1% oxygen (Fig. 4D). We next investigated type II collagen (COL2A1) as it is regulated by SOX9, and critically it provides the required tensile strength needed for cartilage to perform its load-bearing and articulating functions. Secretoles of this matrix protein were also significantly enhanced by PHD2 depletion, with highest levels occurring when PHD2 was depleted in hypoxia (Fig. 4E).

Following PHD2 depletion in HACs (Fig. 5A), SOX9 mRNA was significantly up-regulated (Fig. 5B) in a manner similar to the response observed at the protein level. In fact, the key cartilage extracellular matrix genes (which are regulated by master chondrocyte transcription factor SOX9) were up-regulated in response to PHD2 inhibition. The knockdown of PHD2 in 1% oxygen tension increased COL2A1 expression 38.5-fold, COL9A1 34.5-fold, COL11A2 58.9-fold, and AGGRECAN 6.5-fold (compared with control levels in normoxia) (Fig. 5, C–F, respectively). Expression of a nonhypoxia-regulated gene, factor inhibiting HIF (FIH), was unaltered in response to PHD2 depletion (Fig. 5G).

General 2-Oxoglutarate-dependent Hydroxylase Inhibition Up-regulates SOX9 but Inhibits Secretion of the Cartilage Collagen COL2A1—The 2-oxoglutarate analogue DMOG inhibits all members of the 2-oxoglutarate-dependent hydroxylase family, which includes not only the HIF-targeting hydroxylases, but also the collagen prolyl 4-hydroxylases. DMOG treatment increased expression of SOX9 at the gene (Fig. 6A) and protein level (Fig. 6B). *VEGF*, a classic HIF target gene was also up-regulated by DMOG (Fig. 6C). However, DMOG-induced up-regulation of SOX9 did not result in induction of the cartilage-specific collagens COL9A2, COL11A2, and COL2A1 (Fig. 6, A–D).

**Table 1**

| siRNA     | Sequence                      |
|-----------|-------------------------------|
| siPHD1 #1 | 5'- CAUGCAGGGCCAGAUAUAGUC-3' |
| siPHD1 #2 | 5'- GCAUCACCCUGAUUCAUAUAA-3' |
| siPHD2 #1 | 5'- GAAUGCUGCGACGUCAAAAT-3'  |
| siPHD2 #2 | 5'- CAAUUGGAGAUGGAAGAUGUG-3' |
| siPHD3 #1 | 5'- GGAGAGCUAACAGGCAUGT-3'   |
| siPHD3 #2 | 5'- CAGGUUAUGUUCGCCAGGGTT-3' |

**FIGURE 3. PHD2 depletion in normoxia stabilizes HIFs and up-regulates master chondrocyte transcription factor SOX9.** A, unlike PHD1 or PHD3, depletion of PHD2 resulted in significant stabilization of both HIF-1α and HIF-2α protein in HACs following 3 days of culture in 20% oxygen. Control (siLuc) shows that neither HIF isoform is normally detectable in the presence of 20% oxygen. Depletion of PHD2 significantly slows the rate of HIF-1α (B) and HIF-2α (C) degradation upon reoxygenation, i.e. switching the cells from 1 to 20% oxygen. Following siRNA transfection, HACs were incubated 3 days in 1% oxygen before being exposed to 20% oxygen for the indicated time (in minutes) prior to lysing. Control siRNA (siLuc) transfections were carried out in parallel. Note, for simplicity α-tubulin loading controls from representative experiments are shown. D, depletion of PHD2, but not PHD1 or PHD3, resulted in increased SOX9 protein in HACs after 3 days in culture in 20% oxygen.
Western blot analysis demonstrates that the addition of DMOG completely inhibits secretion of type II collagen protein into the culture medium (Fig. 6G). Therefore, DMOG, like hypoxia and PHD2 depletion, up-regulates SOX9. However, crucially, unlike hypoxia or PHD2 depletion, this does not lead to enhanced cartilage matrix gene expression, and in the case of COL2A1, it completely inhibited its secretion thus preventing elaboration of a functional cartilage matrix.

**DISCUSSION**

We have previously shown that HIF-2α (not HIF-1α) is essential for hypoxic induction of cartilage matrix gene expression in HACs (9). Furthermore, it achieves this predominantly through up-regulation of tissue-specific transcription factor SOX9, which drives cartilage matrix gene expression (20). Because HIFs themselves are regulated by specific prolyl hydroxylases (PHDs), we investigated which of these enzymes targets HIF-2α and whether their specific inhibition could promote matrix production. Using an RNAi approach, we identified PHD2 as the key hydroxylase in HACs responsible for regulating HIF-2α. Crucially, we provide clear evidence that such specific inhibition of PHD2 promotes the differentiated phenotype and enhances cartilage matrix production by HACs both in normoxia and hypoxia. The latter finding, i.e. enhanced HIF-2α stabilization (and subsequent SOX9 induction of the matrix genes) by PHD2 inhibition even in hypoxia, which is the normal condition of cartilage (3–5), suggests that PHD2 still shows significant activity in vivo. This opens up the exciting possibility of PHD2-specific inhibition as a means to enhance cartilage matrix synthesis in vivo, a fact with obvious relevance to development of novel cartilage repair therapies.

PHD1,- 2, and -3 were all detectable in normal HACs, with PHD2 being the most abundant. These hydroxylases have previously been reported in human cartilage at the mRNA and protein level (21). In addition, mRNA studies in rats reported that in all organs examined (heart, liver, kidney, brain, testis and lung) PHD2 was found to be the most commonly expressed (22, 23). Similarly to previous reports in other cell types (24, 25), our results showed that PHD2 and PHD3 are up-regulated in response to hypoxia in HACs. It has been
hypothesized that this occurs in a negative feedback mechanism ensuring the rapid degradation of HIF-α isoforms and down-regulation of hypoxia-responsive genes once the cells encounter increased oxygen levels (26). Although we observed PHD3 to be the most highly hypoxia-inducible hydroxylase (17.8-fold), it levels were relatively low, and it did not appear to significantly contribute toward HIF-α stabilization in HACs.

Koditz et al. (27) and colleagues recently demonstrated that PHD3 interacts with activating transcription factor-4 (ATF-4) suggesting it may play a role in regulating transcription factors other than HIFs.

Work in a range of cell lines has suggested differential function of the PHD enzymes in regulation of HIF-α isoforms (28). In addition, Phd2 deletion in adult mice leads to accumulation of HIF-1α but not HIF-2α in their myofibers and that HIF-1α levels were either low or undetectable. Although these studies indicate that PHDs may show differential regulation of HIF-α isoforms, at least some of the observed differences are most likely due to the relative abundance of each hydroxylase (which is dependent on cell and tissue type). In fact, the \( K_m \) values of all three PHD isoenzymes for recombinant HIF-1α and HIF-2α oxygen degradation domains are quite similar, with PHD1 having the lowest \( K_m \) value for both recombinant HIF-α oxygen degradation domains (32). All three PHD isoenzymes also have virtually identical \( K_m \) values for oxygen (21) implying that changes in oxygen tension will not affect the relative contribution of the three enzymes to HIF-α stabilization. In our experiments with HACs, only depletion of the most abundant hydroxylase (PHD2) significantly stabilized both HIF-1α and HIF-2α in 20% oxygen.

FIGURE 5. PHD2 depletion in HACs enhances expression of the key differentiated chondrocyte markers. TaqMan real time pcr data showing relative mRNA levels as follows: A, PHD2; B, SOX9; C, COL2A1; D, COL9A1; E, COL11A2; and F, AGGRECAN in control and PHD2-depleted HAC cultures. G, FIH, a known nonhypoxia inducible gene, showed no change in response to PHD2 depletion. ns, not significant; *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).
and slowed degradation of each HIF-α isoform in reoxygenation experiments. Interestingly, however, there were differences between HIF-1α and HIF-2α in regard to their stabilization by PHD2 depletion. No further increase in HIF-1α stabilization was detectable in PHD2-depleted HACs when cultured in 1% oxygen compared with that seen in 20% levels. In contrast, HIF-2α levels were further increased when PHD2-depleted cells were cultured in hypoxia implying that other factors in addition to PHD2 may contribute to HIF-2α regulation in hypoxia. Neither PHD1 nor PHD3 appear to be such factors because their depletion had little impact on HIF-2α levels. Factor inhibiting HIF (FIH) is an iron-dependent dioxygenase capable of repressing HIF-α activity (33, 34). However, depletion of FIH had no effect on SOX9 expression even when performed in combination with depletion of PHD2 (data not shown).

Despite the fact that $K_m$ values for oxygen are reported to be between 10 and 25% oxygen, i.e. higher than that encountered in vivo (21, 32), our results indicate that PHDs still display significant activity in 1% oxygen because depletion of PHD2 in hypoxia resulted in greatly increased levels of both HIF-α isoforms. To be able to enhance HIF-2α levels above those typically found in hypoxia (the physiological condition of cartilage) is potentially of great importance because we have previously shown that HIF-2α up-regulates cartilage-specific matrix expression through induction of SOX9 (9, 20). Here, we demonstrate that specific depletion of HIF-2α targeting hydroxylase PHD2 did indeed significantly up-regulate the cartilage expression of SOX9.
master regulator, transcription factor SOX9, which subsequently up-regulated expression of key matrix genes COL2A1, COL9A1, COL11A2, and AGGREGAN, crucially above the levels normally detected in hypoxia (1% oxygen). In addition, PHD2 depletion enhanced levels of secreted COL2A1 protein in HACs above those naturally occurring in hypoxia. We have thus identified PHD2-specific inhibition as a novel means to enhance cartilage matrix synthesis (pathway detailed in Fig. 7).

Experiments with the general hydroxylase inhibitor DMOG highlight the importance of development of HIF-specific hydroxylase inhibitors with regard to chondrocyte metabolism. Addition of DMOG enhanced SOX9 levels in HACs, and we have previously shown that this is dependent (like hypoxia) specifically on HIF-2α (35). However, unlike the case of hypoxia or PHD2 depletion, DMOG did not result in mRNA induction of the cartilage-specific collagen genes, COL2A1, COL9A1, and COL11A2. Furthermore, secretion of COL2A1 protein was blocked by DMOG. The latter is most likely due to the fact that DMOG also inhibits the collagen prolyl 4-hydroxylases, which are critical for post-translational processing of collagen, in particular triple helical formation (36). Similar results were reported by Gelse et al. (37) who carried out a study injecting the joints of STR/ORT mice with DMOG. These mice spontaneously develop osteoarthritis, and injection of DMOG was unable to prevent the formation of severe arthritis. However, the authors reported intracellular accumulation of type II collagen in chondrocytes presumably due to incomplete post-translational processing. Also similarly to this study, they reported a lack of Col2a1 mRNA induction by DMOG in mouse chondrocytes, despite enhanced Sox9 levels. They suggested that the increased intracellular accumulation of the collagen caused by DMOG may operate via a negative feedback mechanism to down-regulate gene expression, and counter any of the positive effects of enhanced Sox9 levels. Although this is a plausible explanation, further work is needed to clarify the issue. What these results do clearly illustrate, however, is the need for inhibitors that are specific to the HIF-targeting PHDs.

This adverse effect of general hydroxylase inhibition on collagen processing is particularly relevant to cartilage as it is a most highly collagen-rich tissue. In fact, expression of the collagen prolyl 4-hydroxylases are actually up-regulated by hypoxia (38). In addition, we have previously shown that two further enzymes involved in collagen processing LOXL3 and PLOD2 are also hypoxia-inducible (20). Therefore, inhibition of PHD2 can up-regulate not only COL2A1 transcription but also enzymes necessary for its post-translational processing, thus representing a further benefit of PHD2-specific inhibition with regard to elaboration of a functional cartilage matrix by HACs. For this approach to be applied to animal studies, and eventually clinical studies, inducible cartilage-specific PHD2 knock-out mice would be informative, in addition to development of PHD isoform-specific (or at least HIF-targeting hydroxylase-specific) small molecule inhibitors. This is currently an area of active research.

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