HIF Prolyl Hydroxylase Inhibition Enhances Healthy Extracellular Matrix Production by Nucleus Pulposus Cells

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Introduction: Intervertebral disc degeneration, a major contributor to low back pain, is associated with progressive loss of healthy extracellular matrix (ECM) components including aggrecan and collagen II from the central nucleus pulposus (NP) [1]. This in turn leads to decreased disc height, altered mechanical properties, and structural failure. Prolyl hydroxylase domain (PHD) enzymes are essential components of the oxygen-sensing machinery within NP cells, and perform critical roles in their adaptation to the hypoxic local tissue microenvironment by regulating the protein stability of hypoxia-inducible transcription factors (HIFs) and downstream target genes required for survival, apoptosis, autophagy, angiogenesis and ECM synthesis [2,3]. Previously, we showed that genetic deletion of PHDs in the mouse NP accelerates healthy ECM production during postnatal growth [4]. Roxadustat, a pan-PHD inhibitor used clinically to treat chronic kidney disease-associated anemia, stabilizes HIFs [5]; however, its effects on the intervertebral disc are unknown. The goal of this study was to investigate how pharmacological inhibition of PHDs using roxadustat impacts ECM synthesis by NP cells. We hypothesized that roxadustat treatment would enhance expression of healthy ECM and suppress expression of fibrotic ECM in adult NP cells in a dose-dependent manner.

Methods: NP Cell Isolation and Pellet Culture: Cells were isolated enzymatically from the NPs of 3 adult bovine tails (commercially sourced; mixed sex, sex not specified) and expanded once in monolayer in complete medium (DMEM supplemented with sodium pyruvate, vitamin C, FBS, and gentamicin). Donors (n=3) were maintained separately for all experiments. NP cells were centrifuged to form 3D cell pellets ($2x10^5$ cells per pellet), then cultured in complete medium supplemented with vehicle (dimethyl sulfoxide) or roxadustat at concentrations of 5 or 20 μM. Pellets were harvested at 7, 14, and 21 days for analysis. Histology: Pellets were fixed in formalin and embedded in paraffin. Sections 8 μm thick were obtained from the pellet mid-plane and stained with Alcian blue and picrosirius red to demonstrate glycosaminoglycans (GAGs) and collagen, respectively. Biochemical Analysis: Pellets were digested with proteinase K and total sulfated GAG per pellet was quantified using the dimethylmethylene blue (DMMB) assay. Gene Expression: Three pellets per donor for each condition were pooled, total RNA was extracted, and qPCR was performed to measure relative expression of ECM genes including collagens I (COL1A1) and II (COL2A1), aggrecan (ACAN), and the hypoxia marker phosphoglycerate kinase 1 (PGK1). Ribosomal protein L13 (RPL13) was used as the housekeeping gene. Statistical Analysis: Significant ($p<0.05$) differences were established using 2-way ANOVA and Tukey's post hoc tests.

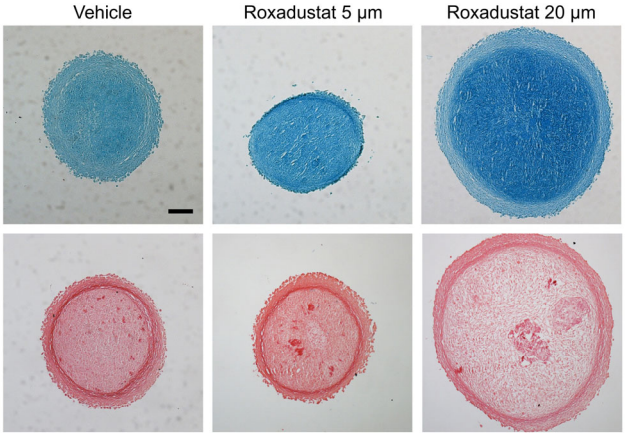

Figure 1: Representative Alcian blue (top) and picrosirius red (bottom) stained histological sections from pellets after 21 days of culture showing GAG and collagen, respectively. Scale = 100μm.

Results: After 21 days of culture, pellets treated with 20 μM roxadustat were subjectively larger than those treated with 5 μM roxadustat or vehicle, and demonstrated increased GAG centrally (Figure 1). GAG content measured using the DMMB assay was 1.4-fold higher in 20 μM roxadustat treated pellets after 21 days compared to vehicle ($p=0.034$, Figure 2). With respect to gene expression (Figure 3), compared to vehicle, COL1A1 expression was 2.2-fold lower at 7 days ($p=0.009$), and COL2A1 expression was 8.8-fold higher at 21 days ($p=0.008$) for pellets treated with 20 μM roxadustat. ACAN expression was higher in the 20 μM roxadustat group compared to both 5 μM roxadustat and vehicle at 21 days but only reached significance versus 5 μM roxadustat (4.4-fold higher, $p=0.010$). PGK1 expression was significantly upregulated by roxadustat treatment at all time points.

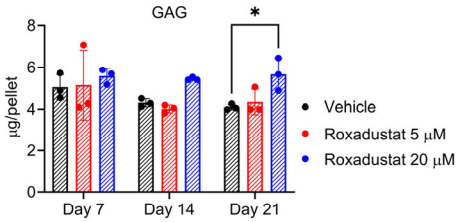

Figure 2: Glycosaminoglycan (GAG) content per pellet at each timepoint. *p<0.05; n=3.

Discussion: In this study we demonstrated using an in vitro pellet culture model that pharmacological inhibition of PHDs using roxadustat increases expression of healthy ECM components, including collagen II and aggrecan, by adult NP cells in a dose-dependent manner. Specifically, roxadustat treatment at a concentration of 20 μM increased pellet size, GAG content, and collagen II and aggrecan gene expression after 21 days, while suppressing collagen I expression. Furthermore, increased PGK1 expression in treated pellets confirmed activation of HIF signaling. Collectively, these results support pharmacological PHD inhibition as a potential treatment strategy for disc degeneration, specifically by enhancing expression of healthy ECM components by NP cells. These findings are consistent with prior reports showing that activation of HIF signaling regulates downstream ECM production [6]. As cells were commercially sourced, we were not able to assess sex-dependent effects in this initial study, however this will be the subject of future studies. Ongoing work is assessing the therapeutic effects of roxadustat treatment on disc degeneration in vivo.

Significance: Findings suggest that the PHD inhibition may hold promise as an anabolic therapy for disc degeneration and associated low back pain by stimulating healthy ECM production by NP cells.

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References: [1] Freemont Rheumatology 2009; [2] Fong+ Cell Death Differ 2008; [3] Fujita+ J Biol Chem 2012; [4] Smith+ Orthop Res Soc Trans 2024; [5] Jatho+ Cells 2022; [6] Thoms+ J Biol Chem 2010.

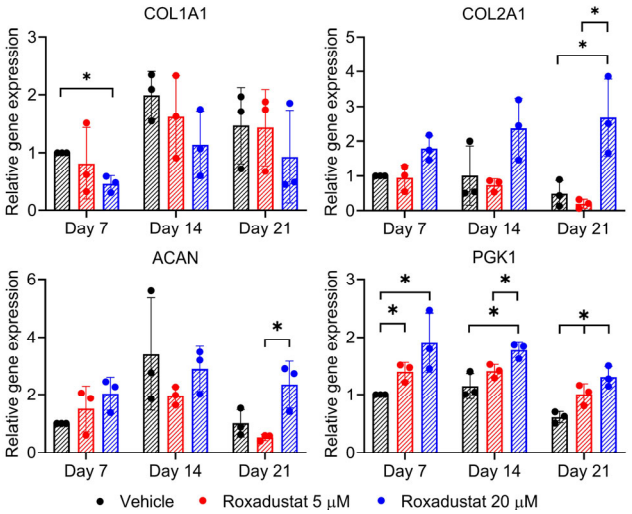

Figure 3: Relative gene expression at each timepoint normalized to RPL13 and day 7 vehicle-treated pellets. *p<0.05; n=3.