In Vivo Regulation of Nucleus Pulposus Cell Differentiation and Extracellular Matrix Elaboration by HIF Prolyl Hydroxylases

Lachlan J. Smith, Karthikeyan Rajagopal, Brittany Laslow, Dian Wang, Ernestina Schipani
University of Pennsylvania, Philadelphia, PA
lachlans@pennmedicine.upenn.edu

Disclosures: Lachlan J. Smith (8-JOR Spine, Connective Tissue Research; 9-ORS Spine Section, National MPS Society), Britanny Laslow (N), Karthikeyan Rajagopal (N), Dian Wang (N), Ernestina Schipani (N)

INTRODUCTION: Low back pain is the leading cause of disability worldwide and intervertebral disc degeneration is implicated as a major cause [1,2]. The earliest degenerative changes occur in the central nucleus pulposus (NP), where reductions in proteoglycan content and hydration compromise disc function [2]. Uniquely amongst skeletal cells, NP cells arise from the embryonic notochord. In humans, NP cells lose their notochordal characteristics rapidly, and by skeletal maturity have been replaced by smaller, chondrocyte-like cells [2]. We recently used single cell RNA sequencing (scRNA-Seq) to identify two distinct subpopulations in the postnatal mouse disc: progenitor NP cells and mature NP cells with distinct ECM expression profiles [3]. We also identified Cd9 as a novel marker of mature NP cells. The mechanisms that drive differentiation from progenitor to mature NP cells, however, are poorly understood. Hypoxia is a key regulator of the NP cell phenotype, including ECM expression [4]. HIF (hypoxia inducible factor) prolyl hydroxylase domain proteins (PHDs) 1, 2 and 3 perform critical roles in the adaptation of NP cells to hypoxia [4]. The objective of this study was to investigate the in vivo roles of PHD proteins in cell differentiation and ECM elaboration in the postnatal NP using conditional knockout mice.

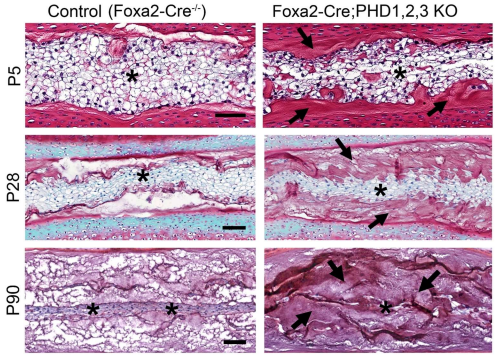

Figure 1. NP-specific knockout of PHDs 1, 2 and 3 accelerates proteoglycan-rich ECM deposition (red) in postnatal mouse lumbar disc NPs. At P90, the notochordal band* is greatly reduced. Arrows = greater proteoglycan-rich ECM in mutants; safranin-O/fast green stain; mid-coronal sections; scale = 100µm.

METHODS: *Generation of Single, Double and Triple PHD Conditional Knockout Mice:* Mice with conditional knockout of PHD proteins 1, 2, and/or 3 in the NP were generated through crossbreeding the Foxa2 promoter-driven Cre transgenic mouse with mice harboring floxed PHD alleles (PHD1$^{f/f}$; PHD2$^{f/f}$; PHD3$^{f/f}$). Controls (phenotypically normal animals) were negative for Foxa2-Cre. Mutant and control mice (n=3-4) were euthanized at 3 postnatal ages (P5, P28 and P90) for postmortem histological evaluations. *Histology and Immunohistochemistry:* Following euthanasia, lumbar spines were removed, fixed in formalin and decalcified in EDTA. Mid-coronal sections 5µm thick were double-stained with Safranin-O and fast green for assessment of proteoglycan elaboration in the NP. To evaluate impact of PHD knockout on NP cell subpopulations, sections from P28 PHD triple knockout and control mice were immunostained using a Cd9 antibody conjugated to Alexa Fluor 647, and imaged using a ZEISS Axioscan slide scanner. The numbers of progenitor (Cd9-) and mature (Cd9+) cells in the NP were quantified. Statistical differences in the number of Cd9+ cells, and the total number of NP cells between mutants and controls were established using unpaired tests ($p<0.05$).

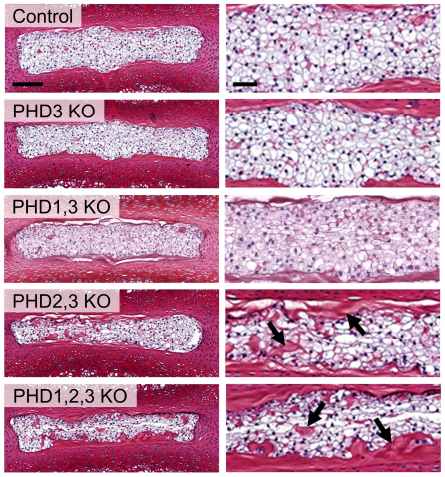

Figure 2. Histology of NPs from single, double and triple PHD conditional knockout mice at P5. Proteoglycan-rich ECM (arrows) was only observed in mutants lacking PHD2. Scales=100µm (right) and 50µm (left). Mid-coronal sections, safranin-O/fast green stain. Control is Foxa2-Cre-/-.

RESULTS: Histological assessment of lumbar discs from PHD triple knockout animals revealed striking differences compared to controls (Fig 1). Specifically, in triple PHD mutant mice NPs exhibited elevated proteoglycan-rich ECM elaboration beginning at P5, which became more pronounced at later ages. At P90, the central band of notochordal progenitor cells was fragmented and overall, greatly diminished, with almost the entirety of the NP occupied by proteoglycan-rich ECM. We also examined NP ECM elaboration in single and double mutants at P5 (Fig 2). In PHD3 single and PHD1,3 double mutants, NP ECM appeared similar to controls; however, in PHD2,3 double mutants, elevated proteoglycan-rich ECM was evident, similar to triple mutants. At P28, we observed significantly higher numbers of mature (Cd9+) NP cells in triple mutants compared to controls (Fig 3), while total NP cell numbers were not significantly different.

DISCUSSION: PHDs perform critical roles in the adaptation of NP cells to hypoxia, including promoting the degradation of hypoxia inducible transcription factors (HIFs) [4]. In vitro, PHD2 has been identified as the primary regulator of oxygen-dependent degradation of HIFs in NP cells, whereas PHD3 does not appear to participate in HIF degradation but may exert control over HIF1 transcriptional activity under hypoxic conditions [5]. A previous study showed mild degenerative changes in the discs of global PHD3 knockout mice [6]. Here, we examine how conditional knockout of PHDs 1, 2 and 3 impacts NP cellularity and ECM elaboration. Our results suggest that PHD inactivation enhances proteoglycan-rich ECM elaboration by accelerating differentiation of progenitor NP cells into mature, Cd9+ NP cells. This is supported by our prior scRNA-seq studies that showed that mature NP cells exhibit significantly higher aggrecan expression compared to progenitor NP cells. Interestingly, ECM differences were only observed in mice lacking PHD2, suggesting a key role for this specific isoform in NP ECM elaboration.

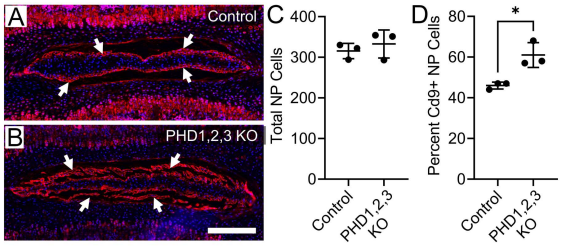

Figure 3. NP-specific triple knockout of PHDs 1, 2 and 3 accelerates emergence of Cd9+ mature NP cells in P28 mouse lumbar disc NPs. Immunofluorescent staining (red, arrows) for Cd9 in **A.** Control and **B.** PHD1,2,3 triple knockout mutants. **C.** Total NP cells. **D.** Percent Cd9+ cells in the NP. Mid coronal sections; scale = 100µm. N=3 discs; *$p<0.05$. Controls are Foxa2-Cre$^{-/-}$.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding mechanisms of NP cell differentiation will directly inform improved strategies for disc regeneration, which could significantly improve the lives of patients with disc degeneration and back pain.

REFERENCES: [1] Vos+ Lancet 2015; [2] Smith+ Dis Model Mech 2012; [3] Zhang+ BioRxiv 2023; [4] Silagi+ Nat Rev Rheumatol 2021 ; [5] Fujita+ 2012 J Biol Chem; [6] Schoepflin+ Faseb J 2017.

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