Oxygen-dependent regulation of tendon extracellular matrix

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INTRODUCTION: Tendons are soft, connective tissues that are essential for joint stability and mobility. Tendons are susceptible to injury, such as rupture and tendinopathy, and limited therapeutic strategies are available to restore native structure and function. The inability to restore adult tendon function is largely due to the limited capacity for tendon fibroblasts (TFs) to regenerate or remodel their surrounding extracellular matrix (ECM). Our preliminary findings show that the embryonic Achilles tendon in mice is hypoxic, suggesting low oxygen availability drives ECM deposition by TFs in the developing tendon. Postnatally, tendon structure and function rely on expression of hypoxia inducible factor-1α (HIF-1α) by TFs. Yet, overexpression of HIF-1α and poor ECM quality has been observed in tendinopathic biopsies, introducing a gap in our understanding of the role of HIF-1α and hypoxia in tendon disease. The overarching objective of our research is to identify how oxygen and HIF-1α contribute to tendon ECM deposition.

METHODS: All work was approved by IACUC. TFs were isolated from the tail tendon of mice (n=3/group, age 4-6M, mixed sex) with the following backgrounds: *Hif1a*$^{fl/fl}$; *Scx*Cre- wildtype (Ctrl), *Hif1a*$^{fl/fl}$; *Scx*Cre+ (cKO) (JAX stock 007561), and *Hif1a*$^{LSL/LSL}$ dPA; *Scx*Cre+ (dPA) (JAX stock 009673; stable expression of human HIF-1a). TFs were seeded at 2500cells/cm^2 on Collagen-I-coated glass plates and cultured in normoxic (20% O_2) or hypoxic (1% O_2) conditions for 2- (Ctrl, cKO, and dPA) or 4-days (Ctrl and cKO) in nascent metabolic protein labeling media containing 1% FBS. 15μM DBCO-488 was added to the cells to be imaged prior to fixation, CellMask Orange plasma membrane, and Hoechst nuclear staining. Cells were imaged using a 20x confocal objective on a Cytation 10 (Agilent), and Gen5 (Agilent) was used to process and analyze the images. On parallel plates, ATP availability was measured using Cell Titer Glo 2.0 (Promega), and luminescence measurements were recorded using a Cytation 10 plate reader (Agilent) then normalized to nuclear density. RNA was also isolated from parallel plates at 4-days and submitted to the University of Michigan Genomics Core for Poly-A enrichment Illumina library preparation and bulk sequencing using 151bp paired-end sequencing (Illumina NovaSeqXPlus). Differential expressed genes (DEGs) were determined from count matrices in DESeq2 in R/Bioconductor using a fold change cutoff of 1.5 and an adjusted p-value cutoff of 0.05. Database for Annotation, Visualization, and Integrated Discovery (DAVID) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to analyze biological processes and pathways. Statistical analysis was performed using Prism GraphPad (v10; 2-way ANOVA for genotype/hypoxia).

RESULTS: Both Ctrl and cKO TFs generated more nascent matrix in normoxia compared to other conditions at both the 2- and 4-day timepoints (*Fig. 1A*). cKO TFs in hypoxia deposited slightly more (but not significant) nascent matrix compared to Ctrl TFs at 1% O_2 and dPA TFs in both 20% or 1% O_2 (*Fig. 1A*). No changes in cell density were observed within timepoints across all genotypes and O_2 conditions (*Fig. 1A*). Nascent matrix deposition was lower in dPA TFs compared to other genotypes at 20% O_2 and was not influenced by O_2 (*Fig. 1A*). ATP availability was significantly reduced in 1% O_2 at both timepoints regardless of genotype (*data not shown*). In 1% O_2, Ctrl TFs were enriched for genes associated with metabolic switching, including the HIF-1 signaling pathway and the pentose phosphate pathway (*Fig. 1B and C*) compared to 20% O_2. We also identified enrichment of molecular pathways associated with ECM deposition and remodeling (*Col4a1, Col4a2, Col5a1, Mmp10, Mmp13, Mmp15, Loxl2-4, Lox*), including osteoclast differentiation (*Osr2, Runx2, Smads6-9, Bmp4, Tgfbr1-2*), proteoglycans, and regulation of the actin cytoskeleton (*Actn3, Fgf10, Dcn, Myh9, Myh10*) (*Fig. 1B and C*).

DISCUSSION: Our findings support that *Hif1a* and oxygen are regulators of ECM in TFs. Hypoxic conditions induced pathways associated with anaerobic metabolism, cell cycle, ECM and cytoskeleton organization. Lack of *Hif1a* in TFs induced pathways primarily associated with ECM & cytoskeleton organization, bone & cartilage differentiation, and cell survival (proliferation, apoptosis, cell cycle and growth, and metabolism). The knockout of *Hif1a* in hypoxia also induced autophagy and cellular senescence pathways indicating *Hif1a* is required for cell survival in hypoxia. TFs overexpressing HIF-1α deposited similar amounts of matrix at both 20% and 1% O_2 which was also comparable to the amount of matrix deposited by Ctrl TFs under 1% O_2, indicating HIF-1α regulates nascent matrix deposition in TFs in an oxygen-dependent manner. Our ongoing studies use multi-omics approaches including proteomics of the soluble ECM fractions in controlled *in vitro* model conditions to discover druggable targets. Collectively, findings from our research will identify key mechanisms responsible for the response of TFs to hypoxia that affect ECM deposition and tendon function.

SIGNIFICANCE: Surgical interventions for tendon injuries often fail to completely restore tendon function, primarily because tendons have limited regenerative capacity. Discovering factors that regulate tendon ECM deposition will enable the development of more effective treatments and better clinical outcomes.

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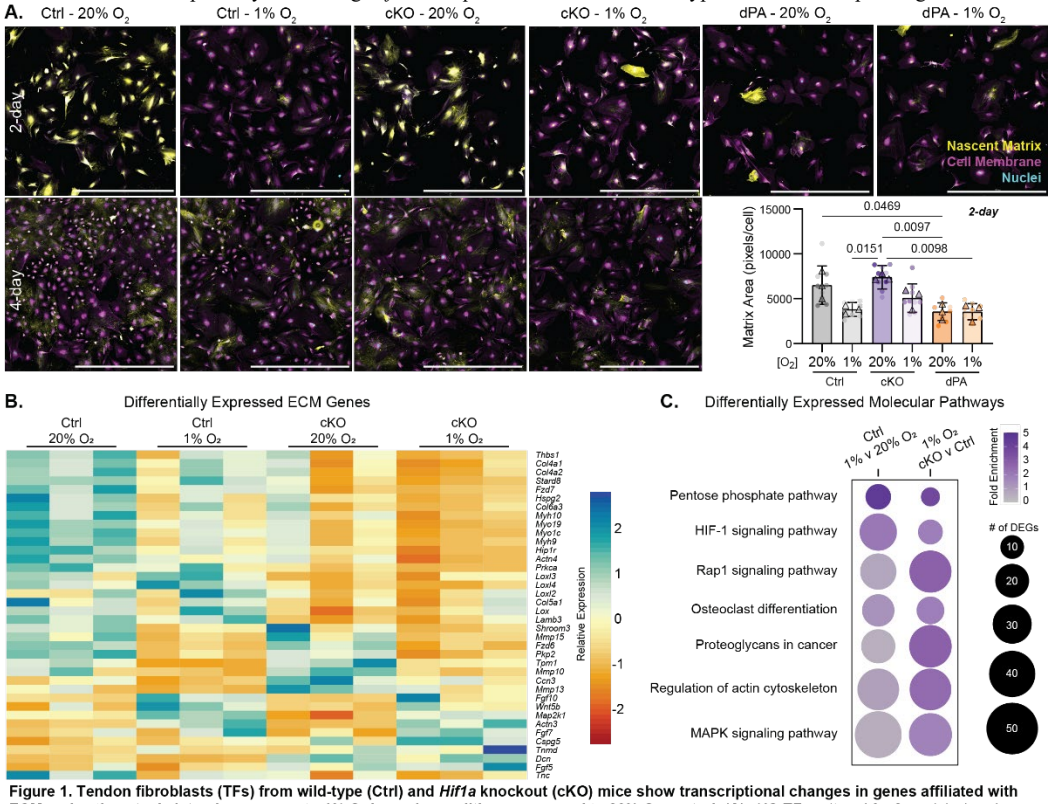

Figure 1. Tendon fibroblasts (TFs) from wild-type (Ctrl) and *Hif1a* knockout (cKO) mice show transcriptional changes in genes affiliated with ECM and actin cytoskeleton in response to 1% O_2 hypoxic conditions compared to 20% O_2 control. (A) cKO TFs cultured for 2- and 4- days in normoxia deposit more matrix per cell than cKO TFs under hypoxic conditions or Ctrl and dPA TFs. Cell nuclei are depicted in cyan, cell membrane in magenta, and nascent matrix in yellow. Scalebar is 1mm. Dots represent technical replicates (n=3); Triangles represent the mean of biological replicates (N=3); Bars show mean ± SD. **(B)** cKO TFs cultured in 1% O_2 express reduced ECM-related genes while Ctrl TFs cultured in 20% O_2 express increased ECM-related genes. **(C)** The knockout of *Hif1a* desensitizes the pentose phosphate and HIF-1 signaling pathways in response to hypoxia. Pathways associated with ECM and actin cytoskeleton show increased relative expression in cKO TFs compared to Ctrl in hypoxic conditions.