# Collagen Organization of Mouse Tendons and Mechanotransduction Gene Expression of Mouse Tendon Fibroblasts are Dependent on HIF1-Alpha and Oxygen Tension

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**INTRODUCTION:** Tendons are essential for the transmission of muscle load from muscle to bone, yet they do not have the capability to fully regenerate post-injury.[1–3] The primary cell type in tendons are tendon fibroblasts (TFs) which contribute to formation and organization of collagen-rich extracellular matrix (ECM) necessary for mechanical function and mechanotransduction. Development and elongation of tendon depends on the transcription factor, *Scleraxis (Scx)*.[4–10] We have recently shown that mouse Achilles tendons are under hypoxic stress during embryonic development, and the maturation of tendon enthesis relies on expression of *hypoxia inducible factor-1a* (*Hif1a*). *Hif1a* also regulates expression of *Sox9* and genes associated with ECM formation, such as *Col1a1* and *Col1a2*.[11] Yet, if and how hypoxia and *Hif1a* influences tendon organization and mechanotransductive gene expression remains unknown. In this study, we tested the hypothesis that hypoxia and loss of *Hif1a* contributes to ECM organization *in vivo* and mechanotransductive gene expression *in vitro*.

**METHODS:** All work was approved by IACUC. *Hif1a*$^{fl/wt}$ wildtype (WT) mice and *Hif1a*$^{fl/fl}$; *Scx*Cre+ (*Hif1acKO)* mice were generated to study tendon ECM organization of developing (Postnatal day 14, P14) and mature (P56) Achilles tendons. Mouse hindlimbs were fixed, decalcified, paraffin sectioned, and stained with Picrosirius red to visualize collagen organization (n=3/genotype at both P14 and P56). Midsubstance Achilles tendons were imaged using brightfield and circular polarized light microscopy with a 10x objective on an epifluorescent microscope (Leica). Images were acquired using a polarization camera and ThorCam software (ThorLabs Inc.) for quantitative polarized light microscopy (qPLI). The degree of linear polarization (DoLP) and angle of linear polarization (AoLP) were analyzed using the Math and SciPy Stats libraries in Python3. Data were compared using 2-way ANOVA between genotype and age (Prism GraphPad, v10). Gene expression related to ECM (*Col1a1, Col1a2)*, progenitor markers (*Scx, Sox9)*, hypoxia (*Hif1a, Hif2a, Hilpda, Plin2),* and mechanotransduction *(Igfbp2, Igfbp3,* and *Igfbp9*) was assessed using isolated and cultured mouse tail TFs from adult *Hif1a*cKO and WT mice (n=3-4/group, age 4-6M). Cells were cultured in normoxic (20% $O_2$) or hypoxic (1% $O_2$) conditions (for 1-wk) or were treated with a HIF stabilizer Roxadustat (FG-4592) to induce 'pseudo-hypoxia' or treated with a vehicle (DMSO) for 16h. RNA was isolated after either 16h or 1-wk in culture. cDNA was synthesized for use in SYBR-based quantitative PCR (qPCR) and data were normalized to 20% $O_2$ conditions for fold change comparisons ($2^{-\Delta\Delta Cq}$; *Actb* and *Polr2a* reference genes). Statistical analysis was performed using Prism GraphPad (v10; 2-way ANOVA for genotype/hypoxia; 1-way ANOVA for FG and hypoxia experiments).

**RESULTS:** *Hif1a*cKO Achilles tendons had poor ECM organization compared to WT tendons as indicated by decreased DoLP and increased AoLP (*Fig. 1*). TFs from *Hif1acKO* mice had decreased expression of *Hif1a* compared to WT cells in both 20 and 1% $O_2$ (*Fig 2A*). For WT TFs, *Scx* and *Igfbp9* were downregulated in hypoxia compared to 20% $O_2$ conditions (Fig2A), and the effect of hypoxia on Hif1acKO cells further downregulated *Igfbp9* compared to 20% $O_2$ (*Fig 2A*). Loss of *Hif1a* reduced *Hilpda* expression compared to WT TFs in hypoxia *(Fig. 2A)*. WT TFs cultured for 16h reflect similar results as the 1-week data with hypoxia treatment *(Fig. 2B)*, and FG treatment resulted in increased *Hilpda* expression compared to hypoxia after 16h in culture (*Fig. 2B*).

**DISCUSSION:** Our findings support our hypothesis that *Hif1a* and hypoxia are regulators of ECM organization and mechanotransduction in mouse tendons. Loss of *Hif1a* in tendon progenitors resulted in a less-organized tendon during development and in adult mice. Loss of *Hif1a* may reduce sensitivity of mouse TFs to hypoxia, as indicated by damped expression of hypoxia-sensitive genes (*Hilpda*) and downregulated mechanotransductive genes (*Igfbp9*). Culture with FG may provide a more rapid response to mimicking hypoxia for *in vitro* TF culture. Future work will determine how hypoxia and HIF1a influence how cells interact with ECM substrates, and how changes in hypoxia and HIF1a expression regulates nascent ECM deposition. Additionally, the influence of Hif1a and hypoxia on TF viability remains an open question, and understanding these processes will inform the development of new therapeutics using engineered materials for guided regeneration.

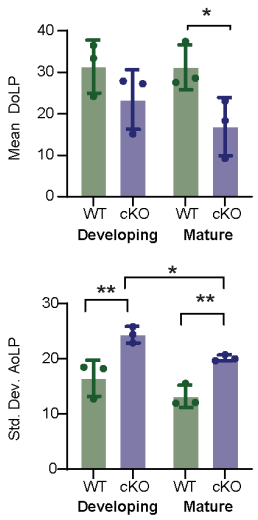


**Figure 1.** *Hif1a*cKO tendons are disorganized compared to age-matched WT tendons. *Hif1a*cKO tendons had lower degree of linear polarization (DoLP) and increased angle of linear polarization (AoLP), indicating disorganization. Dots are biological replicates.

**SIGNIFICANCE:** Surgical treatments for tendon injuries do not fully return tendon function largely due to the inability of tendon to regenerate. Identifying regulators of tendon ECM deposition and organization will lead to improved therapeutics and clinical outcomes.

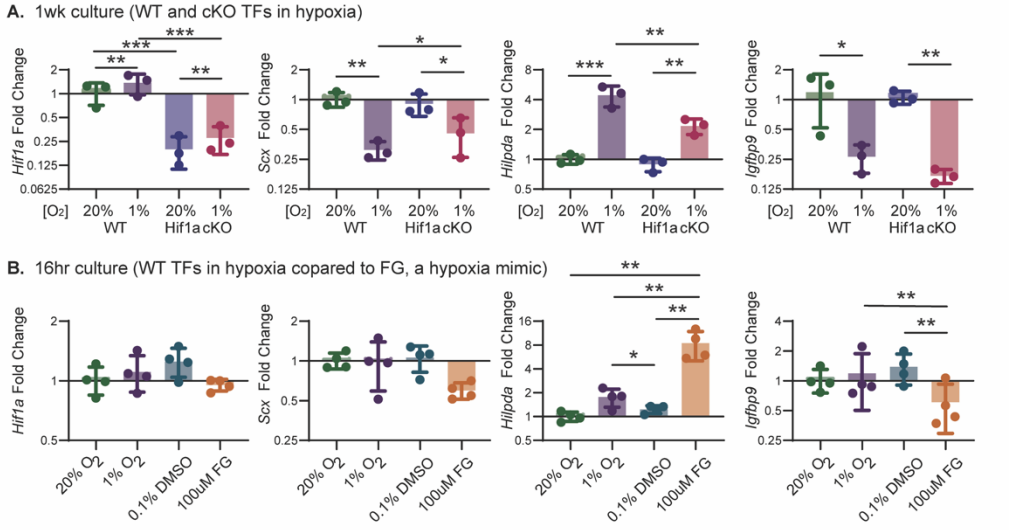


**Figure 2.** TFs from Hif1acKO mice were desensitized to hypoxia and hypoxia mimics elicited similar trends in tenogenic, hypoxia, and mechanotransductive gene expression. **(A)** TFs cultured for 1-week in hypoxia express reduced tenogenic and mechanotransductive genes as well as activated hypoxia-related genes. Loss of Hif1a desensitizes TFs to hypoxic conditions. **(B)** TFs cultured for 16h in a hypoxia mimic (FG, 100uM) show similar trends to cells cultured for 1-week in 1% oxygen. FG may act as a stronger activator of hypoxia-related genes than 1% oxygen. Each dot represents a biological replicate; Bars show mean ±SD; * p<0.05; ** p<0.01; *** p<0.001; and **** p<0.00001. (of ΔCq for gene expression).

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