Unraveling the Role of mTOR in Tendon Fibrosis:
Implications for Targeted Therapies and Scar-Free Healing

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INTRODUCTION: Currently, the incomplete understanding of the complex cellular and molecular processes involved in the fibrotic healing process has hindered the development of biological therapies for true regenerative tendon repair. A common driver of these fibrotic pathologies is Transforming Growth Factor-beta 1 (TGF-β1), which drives matrix turnover, cell proliferation, α-SMA activation, and the upregulation of the putative senescence marker, Plasminogen Activator Inhibitor 1 (PAI-1) [1]. RNA-Seq data from our lab found PTEN pathways to be significantly enriched in scarless healing in PAI-1 KO mice [2]. PTEN is a master regulator of mTOR signaling and has been associated with the regulation of cellular proliferation and senescence as precipitating factors in fibrosis. Furthermore, our *in vitro* data has established how TGF-β1, PAI-1, and mTOR interact in tendon fibroblasts to regulate the fibrotic phenotype, α-SMA, Ki67, and γ-H2AX [3, 4, 5]. With these *in vitro* findings in hand, our objective is **to understand the role of mTOR in various cell types involved in tendon repair and evaluate mTOR inhibition as a disease-modifying therapy for injured flexor tendons.**

METHODS: All animal use was performed in accordance with protocols approved by the University of Rochester's Committee on Animal Resources (UCAR). The murine injury model encompassed a partial laceration of the deep digital flexor tendon in the middle digit of the hind paw of C57Bl6/J mice. Subsequently, the mice were allowed to heal for up to 28 days following the surgical procedure. For **flow cytometry** analysis, we enzymatically digested the tendon injury milieu at D7 & D14 post-surgery (n=4 per time point) to release the cells and probed for cell specific markers (Scleraxis, CD31, CD45), mTORC1 signaling proteins (SMAD2/3, PTEN, Akt, pS6 and p4E-BP1), and fibrosis markers (α-SMA, Ki67, and γ-H2AX). For **histology and Nanostring GeoMX spatial proteomic profiling** at day 7 post-injury (n=2-3 per group), 5-μm serial sections of formalin-fixed, paraffin-embedded tissues were affixed to charged slides and stained with immunofluorescent markers for PI3K/AKT Signaling, Cell Death, and Immune Cell Typing. To evaluate the effects of mTOR inhibition on tendon injury, 4 mg/kg of Rapamycin, a selective mTORC1 inhibitor, or DMSO vehicle, was administered IP daily starting at D7, the peak of the inflammatory phase, over a span of ten consecutive days. The animals were then sacrificed at day 28 post-injury, followed by tissue harvest for **Biomechanical tensile testing** (n=9-13 per treatment). In all cases, uninjured tendons were used as controls. Statistic: Welch ANOVA and Dunnett's T3 multiple comparisons with p<0.05 to declare significant differences between group means.

RESULTS SECTION: Using flow cytometry, we isolated viable cells from the tendon injury site and categorized the cells using specific markers, including CD31 for endothelial cells and CD45 for leukocytes (Fig. 1A). At 7- and 14-days post-injury, while the number of endothelial cells did not change over time, the number of CD45^{+} leukocytes increased significantly compared to uninjured tendons while the CD31⁻Cd45⁻ cells decreased (Fig. 1B). Furthermore, we observed α-SMA and γ-H2AX to be upregulated with injury, which was associated with increased phosphorylated mTOR proteins, pS6 and p4E-BP1, within the CD31⁻CD45⁻ cell population (Fig. 1C). To specifically probe tendon cells, we gated for CD31⁻CD45⁻SCX⁺ cells and observed a sustained increase in α-SMA activity up to 2-weeks post-injury (Fig. 1D). To better understand the spatial interactions of these cells and molecular pathways, we used the NanoString GeoMx Digital Spatial Proteomics to investigate the peritendinous microenvironment at 7-days post tendon injury compared to uninjured tendon. Collectively, our observations revealed an upregulation in mTOR-associated signaling proteins, phosphorylated S6, total S6, MET, and pan-AKT, with downregulation of PRAS40, a negative regulator of mTOR (Fig. 2B). We also observed increases in pro-inflammatory and macrophage markers including CD68, CD163, CD11b, Ly6C, CD14, CD45, and the dendritic cell marker CD11c (Fig. 2D). However, the notable decline in the CD31 and CD39 suggests changes to the vascular microenvironment at D7 post-injury. The injured tissue also displayed a substantial enrichment in apoptotic markers, including BIM, BCLXL, Caspase 3, BAD, Perforin, and PARP, with a notable reduction in DNA damage response with γ-H2AX and P53 (Fig. 2C). Given the observed association between the mTOR activation and tendon injury, we postulated that rapamycin might

Figure 1: Flow cytometry analysis of the injury milieu of wildtype mice (**A**) identifying cell subsets, endothelial, leukocyte, and CD31⁻CD45⁻, using CD31 and CD45 markers at 1- and 2-weeks post-injury and (**B**) graphed as subpopulation proportions pre- and post-injury. Shifts in expression of fibrotic markers α-SMA, γH2AX, and Ki-67 upon injury (day 7) in association with mTOR proteins p4EBP1 or pS6, specifically within the CD31⁻CD45⁻ subpopulation (**C**), and within the within the CD31⁻CD45⁻SCX⁺ tendon fibroblast subpopulation.

improve the tendon healing response. *In vitro,* tenocytes treated with Rapamycin exhibited suppression of αSMA activity in association with suppression of pS6 and p4E-BP1 activity (Fig. 3A,B). In vivo, injured tendons treated with Rapamycin showed accelerated increases in tendon tensile stiffness and strength compared to DMSO-treated controls (Fig. 3C).
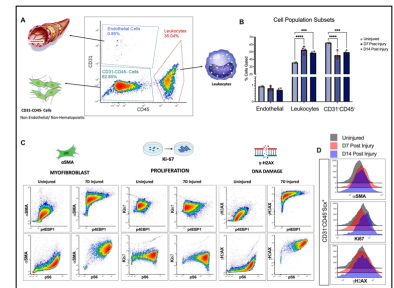
DISCUSSION: In summary, our study has elucidated a pivotal role mTOR signaling holds in driving myofibroblast differentiation *in vitro* and *in vivo* in injured tendon fibroblasts, thereby orchestrating fibrotic tendon healing. This corroborates similar observations in other fibrotic conditions in major organs. The identification of mTOR activation, which seems associated with inflammation, is significant because it is a druggable target. Our results indicate that rapamycin treatment accelerates increases in tendon tensile stiffness and strength, likely through the suppression of immune and α-SMA activity. These findings not only underscore the pivotal role of mTOR signaling in tendon repair but also highlight the potential of mTOR inhibitors like Rapamycin as a disease-modifying therapeutic strategy for tendon injuries. Future research should focus on optimizing the dosage and timing of mTOR inhibitors to fully restore the biomechanical properties of injured tendons.
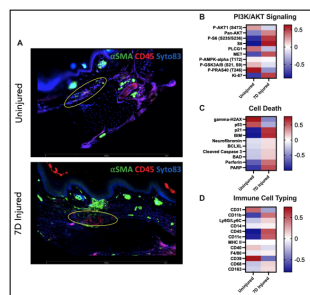

Figure 2: NanoString GeoMx Spatial Proteomics of uninjured versus day 7 post-injury mouse tendons. (**A**) Morphological staining to visualize α-SMA, CD45 and Syto83 with yellow ovals of the sampled region. Heatmaps assessing the protein expression of panels for (**B**) PI3K/AKT signaling (**C**) Cell death (**D**) Immune cell typing all normalized to housekeeping protein Histone3. (N=2-3 per group)
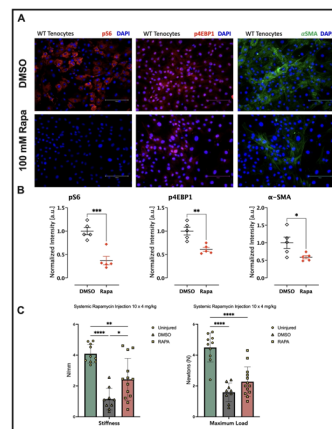

Figure 3: Rapamycin inhibition of mTOR signaling (**A**) *in vitro* immunofluorescence staining on flexor tenocytes at 100nM stained for pS6, p4EBP1, and α-SMA then (**B**) quantified. N=5. Unpaired t test with Welch's correction with p<0.05. (**C**) At 28 days post-injury, stiffness and maximum load data of mice treated with daily intraperitoneal injections between Day 7 and Day 16 post-injury. N=9-13. Welch ANOVA with Dunnett's T3 multiple comparisons with p<0.05.

SIGNIFICANCE/CLINICAL RELEVANCE: Since mTOR inhibitors are in clinical trials as disease-modifying agents for pulmonary fibrosis, the association between mTOR signaling and poor outcomes of tendon injury makes mTOR a novel and promising therapeutic target for fibrotic peritendinous adhesions.

REFERENCES: [1] Farhat, Y., et al., PLoS One, 2012. [2] Freeberg, M.A.T., et al., Journal of Orthopaedic Research, 2020. [3] Alenchery RG., et al., J Orthop Res. 2023. [4] Alenchery et al. ORS 2022 Annual Meeting Paper No. 1161. [5] Alenchery et al. ORS 2023 Annual Meeting Paper No. 1959.