Aging Impacts Fibrotic Markers in Achilles Tendon Tenocytes

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INTRODUCTION: The Achilles tendon is the largest and strongest tendon in the human body, relying on resident cells, mainly tenocytes, and extracellular matrix to transmit muscular forces and maintain mechanical integrity [1]. Following tendon injury, macrophages and myofibroblasts play central roles in inflammation, proliferation, and remodeling [2]. Unfortunately, with aging, the regenerative process is often compromised, leading to fibrotic scar formation and tendon fibrosis [3]. Current treatments (corticosteroids, NSAIDs) provide only temporary pain relief and do not target fibrosis. The objective of this study was to develop and evaluate an *in vitro* model mimicking tendon fibrosis using rat Achilles tendon-derived cells to induce a pro-fibrotic environment and investigate how aging influences fibrotic features. We hypothesized that an *in vitro* model could be created through TGF-β1 stimulation, (2) aging would further stimulate myofibroblast activation, and that (3) aging would modulate ECM remodeling through altered expression of *COL3A1* and *MMP2*.

METHODS: **Cell Culture:** Primary tendon-derived cells were isolated from male Fischer Brown Norway hybrid (F344xBN) rats, as males show a higher clinical susceptibility of Achilles injuries [4]. Cells were seeded and treated for 72 h with TGF-β1 (10 ng/mL) prepared in DMEM with 1% P/S. **Live/Dead Assay:** After 72 h of treatment, cells cultured were stained with calcein-AM (live) and EthD-III (dead), and imaged using EVOS M5000 fluorescence microscope. Viability was quantified with ImageJ**. Immunofluorescence:** Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA. Samples were incubated with anti-α-SMA primary and Alexa Fluor 488 secondary antibodies and imaged on EVOS M5000. Signal quantification was done with CellProfiler. **RT-qPCR:** RNA was extracted and reverse transcribed into cDNA. Gene expression was analyzed using SYBR Green chemistry on a QuantStudio 6 Flex. Relative expression was analyzed by $2^{-\Delta\Delta Ct}$ with *RPLP0* as the housekeeping gene. **Statistical Analysis:** One- or two-way ANOVA with Sidak's post hoc test in GraphPad Prism. Significance was set at $\alpha = 0.05$, data reported as mean ± SD.

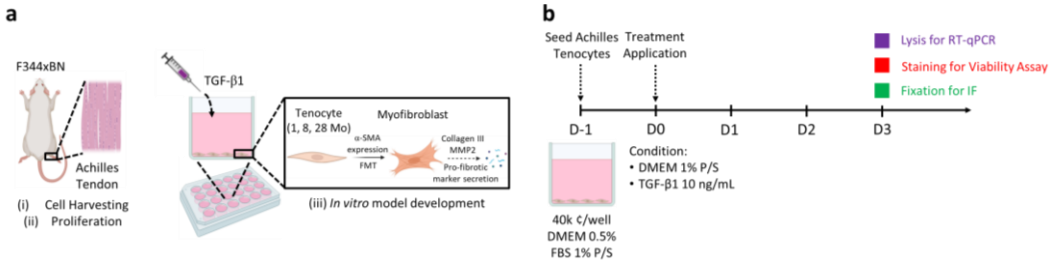

Figure 1. Development of the *in vitro* tendon fibrosis model. (a) Methodology of the fibrotic model using Achilles tendon-derived cells. (b) Experimental design.

RESULTS: The established model was cytocompatible and reproducible under serum-deprived conditions. Baseline α-SMA expression was detectable under control conditions that increased progressively with cell age. In juvenile and adult tenocytes, TGF-β1 stimulation at 10 ng/mL increased α-SMA expression and upregulation of pro-fibrotic genes (*COL3A1*, *MMP2*) across all age groups. Additionally, TGF-β1 treatment reduced the average cell area per α-SMA$^+$ cell. Aged tenocytes displayed larger basal cell areas, but following TGF-β1 stimulation, all age groups shifted toward smaller, more contractile morphologies.

DISCUSSION: Basal α-SMA expression in controls suggested intrinsic mechanosensitivity of tenocytes, increasing with animal age, possibly due to longer exposition of *in vivo* mechanical loading in older Achilles tendons. Elevated basal α-SMA expression in aged tenocytes without a contractile morphology suggests partial activation from chronic mechanical loading rather than full myofibroblast differentiation. This may contribute to the greater fibrotic susceptibility observed in aged individuals following tendon injury. TGF-β1 stimulation induced a myofibroblast-like, contractile phenotype, characterized by elevated α-SMA, *COL3A1*, and *MMP2* expression, reproducing a pro-fibrotic environment. The upregulation of *MMP2* and *COL3A1* aligns with tendon pathology, as both are hallmarks of matrix disorganization and scar formation in fibrotic tendons.

SIGNIFICANCE: Currently, there are no FDA-approved anti-fibrotic treatments for tendon fibrosis. Therefore, there is an urgent need for relevant in vitro models that will allow screening and evaluation of anti-fibrotic therapies.

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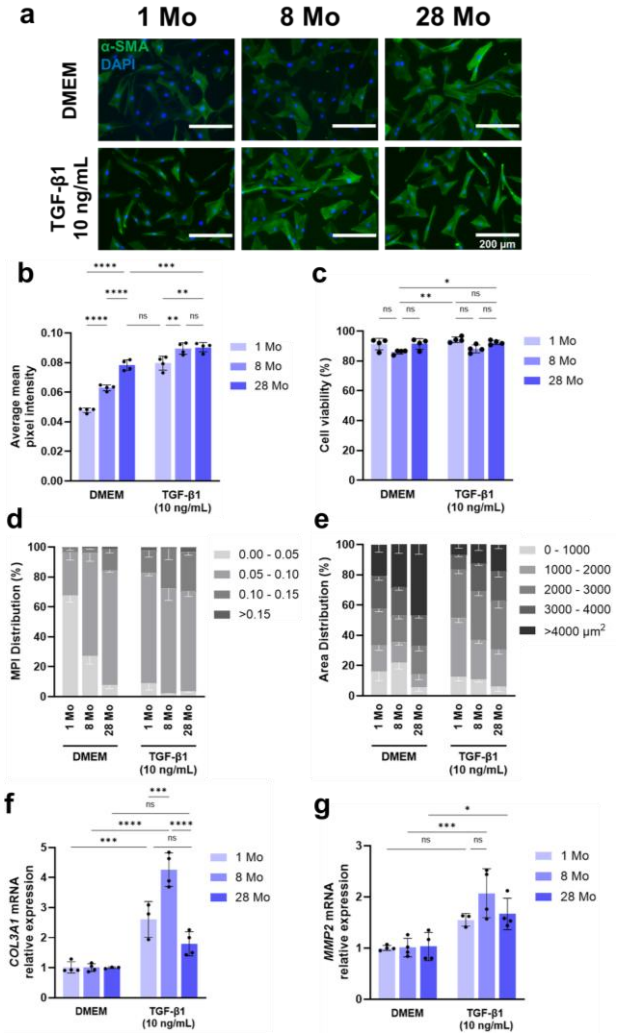

Figure 2. TGF-β1 induces a pro-fibrotic environment in a age-dependent manner. (a) IF images. (b) Absolute MPI values of α-SMA expression (n=4). (c) Cell viability (n=4). (d-e) Distribution of MPI values and α-SMA$^+$ cell area (n>150). (f-g) Gene expression.