Synovial Fibroblasts Exacerbate Inflammation and Peritendinous Adhesions in a Human Tendon-on-a-Chip

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INTRODUCTION: Biomedical research is increasingly prioritizing the development of new approach methodologies (NAMs), with particular emphasis on tissue chips, to offer alternative human-centric models. By generating more accurate and predictive human-specific data, these models promise to accelerate and de-risk drug development while complementing, and in some cases reducing reliance on, traditional animal models. We previously engineered a human tendon-on-a-chip (hToC) that features a 3D tendon construct and a vascular flow channel separated by an ultrathin nanomembrane.[1] The hToC recapitulates the dynamic immune cell and intrinsic tendon fibroblast interactions and demonstrates hallmarks of tendon fibrosis driven by TGF-β1, including myofibroblast differentiation and vascular activation. However, the model lacks the important contributions of synovial fibroblasts and does not model adhesions. Fibrotic adhesions are composed of disorganized matrix deposited around the tendon that can severely limit joint motion and diminish quality of life. Infiltrating immune cells and extrinsic synovial fibroblasts contribute to this fibrotic pathology by migrating to the injury site, proliferating, and secreting excess collagen and fibronectin around the tendon.[2,3] Our objective here is to model synovial fibroblast crosstalk in the hToC to determine the role of extrinsic fibroblast-like synoviocytes (FLS) in promoting fibrotic adhesions in a human-relevant model. We hypothesize that FLS promote tendon adhesion formation by promoting inflammatory signaling and matrix deposition around the tendon construct, even in the absence of exogenous TGF-β1.

METHODS: Device Setup and Culture Timeline: We engineered modular microfluidic components that are assembled with peel-and-stick adhesives. This enables efficient and reproducible assembly and allows us to mature cell types separately before combining components. Human tendon-derived fibroblasts and peripheral blood monocytes (IRB-approved protocols) were encapsulated in a type I collagen hydrogel and cultured with macrophage colony-stimulating factor for 6 days in the bottom compartment. On Day -4, the porous membrane was coated with a type I collagen hydrogel embedded with or without primary fibroblast-like synoviocytes (FLS). Human umbilical vein endothelial cells were cultured on the opposite side of the membrane in the vascular channel for 24 h. At Day 0, monocytes were added to the vascular channel, and serum-free media with or without 10 ng/ml TGF-β1 was added to the bottom compartment (Fig. 1a,b). Devices were assayed on Days 1 and 5, with n=4 replicates. Tendon Hydrogel Contraction: We imaged the tendon hydrogels every day from Day 0 to 5, and percent contraction was quantified in ImageJ. Immunofluorescence (IF) and Second Harmonic Generation (SHG) Imaging: Devices were fixed and labeled for α-SMA, a marker of myofibroblast differentiation, as well as matrix proteins collagen III and fibronectin. Devices were imaged with confocal microscopy or multiphoton microscopy to generate multiplex IF with SHG images of collagen structure. Cytokine Secretion: Media from the vascular channel was assessed using a Luminex panel for secreted TGF-β and inflammatory cytokines. Monocyte Transmigration: Circulating monocytes were labeled with CellTracker Orange, and monocyte infiltration was quantified from confocal images at Days 1, 3, and 5. Statistics: All quantitative data were analyzed using a one-way ANOVA with Tukey's post hoc test or unpaired t-tests (n = 4 biological replicates) using GraphPad Prism.

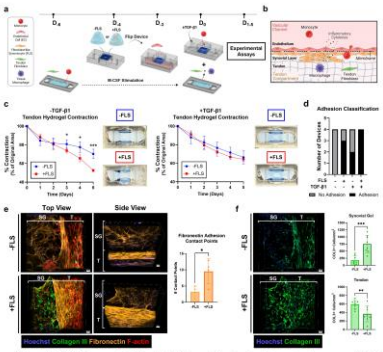

Figure 1. Modeling synovial fibroblast-driven adhesions in a human tendon-on-a-chip (hToC). **(a)** Experimental timeline for modular hToC assembly and culture with synovial fibroblasts. **(b)** Schematic of the hToC showing key cell types, including vascular, tendon, immune, and synovial compartments separated by an ultrathin, microporous nanomembrane. **(c)** Tendon hydrogel contraction, plotted as the percent of original area, with or without FLS in the absence (left) or presence (right) of TGF-β1. Insets show representative hydrogel images at Day 5. **(d)** Number of devices with and without observed adhesion formation at Day 5. **(e)** Top and side view confocal images of -FLS and +FLS devices at Day 5 stained for collagen III and fibronectin, with Hoechst and F-actin as counterstains (SG = synovial gel, T = tendon). Images demonstrate fibronectin adhesion points between synovial and tendon gels, with quantification. Scale bar = 150 μm. **(f)** Comparison of collagen III positive cells in the synovial and tendon gel with TGF-β1. (Data presented as mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

RESULTS SECTION: We first investigated the role of synovial fibroblasts (FLS) in driving adhesion-like pathology within the hToC. In the absence of exogenous TGF-β1, tendon hydrogel contraction was significantly increased in devices with FLS-laden synovial gels (+FLS) compared to acellular synovial gels (-FLS) as early as Day 3, which increased further by Day 5 (70.3 ± 5.1% vs. 52.5 ± 1.3%, n=4, $p<0.05$). Upon addition of exogenous TGF-β1, +FLS and -FLS devices exhibited comparable bulk tissue contraction (Fig. 1c). Next, we assessed whether the hToC can model adhesions. Remarkably, +FLS devices formed extended fibronectin- and COL3-rich networks connecting the vascularized synovial gel (SG) and tendon (T) construct (Fig. 1d,e). Quantification of adhesion contact points demonstrated significantly higher values in +FLS devices (n=4, $p<0.05$, Fig. 1d). COL3 expression was increased in the synovial gel of +FLS devices when supplemented with TGF-β1, while -FLS devices exhibited a higher proportion of COL3+ cells within the tendon hydrogel (n=4, $p<0.01$, Fig. 1f). In the tendon construct, the percentage of α-SMA+ cells was significantly higher in +FLS devices (n=4, $p<0.05$), with synergistic increases upon supplementing with TGF-β1 (n=4, $p<0.01$, Fig 2a). SHG imaging of the tendon construct revealed increased fibrotic collagen density in +FLS cultures at Day 5 (Fig. 2b). Monocyte infiltration was significantly elevated in +FLS devices, regardless of TGF-β1 (n=3, $p<0.01$, Fig. 2c). Cytokine analysis showed no differences in TGF-β1 in the supernatant; however, pro-inflammatory cytokines IL-6 and IL-8 were significantly elevated in +FLS devices at Day 5, with IL-6 further increased compared to -FLS devices in the absence of exogenous TGF-β1 (n=4, Fig. 2d).

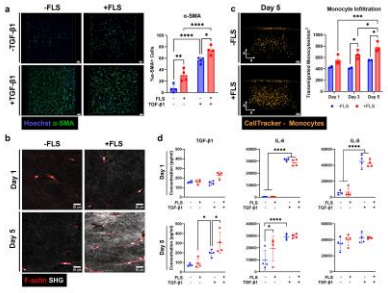

Figure 2. Synovial fibroblasts impact inflammatory and fibrotic phenotypes in the hToC tendon hydrogel. **(a)** α-SMA expression in the tendon hydrogel at Day 1, with quantification reported as % α-SMA+ cells. **(b)** Second harmonic generation images showing collagen I fibers and tendon fibroblasts stained with F-actin at Day 5. **(c)** Confocal images of monocytes labeled with CellTracker Orange in devices -TGF-β1 at Day 5. Monocyte infiltration into the tendon hydrogel quantified as the number of transmigrated monocytes/mm3. **(d)** Cytokine concentrations from sampled media as analyzed by a Luminex assay. (Data presented as mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

DISCUSSION: Our findings demonstrate, for the first time, the ability to simulate peritendinous adhesions in a tissue-on-a-chip *in vitro* model, and support the hypothesis that synovial fibroblasts drive adhesions by enhancing contraction, collagen deposition, and monocyte infiltration precipitated by increased inflammatory cytokines, including IL-6. Importantly, the impact of FLS was most pronounced in the absence of exogenous TGF-β1, further highlighting the role of FLS in promoting adhesions. The fibronectin- and COL3-rich networks connecting the tendon and synovial gels resemble nascent adhesion-like pathology, providing a mechanistic model to simulate clinically observed peritendinous adhesions and test drugs to mitigate them. Intriguingly, the increased IL-6 secretion in +FLS devices implicates a potential therapeutic target for resolving adhesions, which will be pursued in future studies. Limitations of this study include the relatively short (5-day) culture duration and the exclusion of other immune cells beyond monocytes, including T- and B-cells which drive adaptive immune responses.

SIGNIFICANCE/CLINICAL RELEVANCE: With increased emphasis on NAMs by funding and regulatory agencies, the pharmaceutical industry is poised to adopt tissue chips for testing drug candidates. Our *in vitro* platform is uniquely capable of representing not only fibrotic tendon tissue, but also the debilitating peritendinous adhesions, and could serve as a human-relevant model to accelerate and de-risk drug development.

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