# A Vascularized Tendon Fibrotic Organoid with Microfluidic Perfusion

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**INTRODUCTION**: Tendon microphysiological systems (MPS) are emerging as human-relevant new approach methodologies (NAMs) to accelerate the development of therapeutics for scarless healing [1]. We previously created the Human Tendon-on-a-Chip (hTOC) to model the myofibroblast microenvironment (MME), which encapsulates the cellular and molecular crosstalk influencing myofibroblast activation and inflammation [2]. These include interactions with immune cells, fibrotic extracellular matrix, and the hypervascularization present in healing tissue. Vascularization methods are well established for MPS perfusion [3], these tools present an opportunity to highlight the contribution of inflamed vascular structures in perpetuating fibrosis [4]. In this study, we describe the creation of a vascularized tendon fibrotic organoid model in a microdevice with a silicon-nitride membrane (μSiM). The μSiM facilitates vascular sprouting to the fibrotic organoid, which simulates the MME in peritendinous adhesion, while providing a microfluidic conduit for vascular and interstitial fluid flow.

**METHODS**: Fibrotic tendon organoids were made by suspending endothelial cells (ECs) (Lonza) and primary tendon-derived fibroblasts (TCs) in Matrigel (Corning) droplets [5]. Passage 3 to 6 ECs and TCs were mixed in 1:1 ratio and suspended in Matrigel at a total cell density of 6 million cells per mL. The Matrigel droplets (25 μL) were added to low adherence plates (Corning) and cultured in EGM-2 (Lonza) for 7 days +/- 10 ng/mL of TGF-β1. Day 7 organoids were fixed and stained to visualize nuclei, CD31, α-SMA, and F-actin using confocal microscopy. TC and EC were segmented using MATLAB to analyze their position in 3D space. Three organoids for each treatment condition were made and about 2000 cells from each organoid were pooled for analysis. To evaluate the spatial co-localization of ECs and myofibroblasts in the MME, the clustering of ECs around α-SMA+ TCs was plotted against normalized α-SMA intensity of the fibroblasts. To assess microvascular sprouting from fibrotic organoids, day 3 fibrotic organoids were embedded in collagen hydrogels (Advanced BioMatrix) with 80% type I and 20% type III collagen to mimic scar tissue. Organoids were cultured for 7 or 14 days after which samples were stained for CD31 and F-actin and imaged on a confocal microscope. Finally, to explore the effects of fluid flow on vascular sprouting, μSiM devices were set up with vascularized hydrogels in the bottom channel and fibrotic organoids in the top well separated by an ultrathin membrane with 20 μm diameter pores. The hydrogel contained 2 million EC per mL and 500,000 TC per mL. A syringe pump was used to draw EGM-2 media through the device at a rate of 0.2 μL per minute to mimic slow interstitial flow. After 1 week of flow, devices were fixed, stained, and imaged as before. TCs were obtained from tendon surgical specimens according to University of Rochester IRB approved protocols.

**RESULTS**: ECs and TCs cultured in Matrigel formed fibrotic organoids over 7 days as the droplets contracted into spheroids (Fig. 1A). TGF-β1 stimulation led to differences in organoid morphology (Fig. 1B). Unstimulated organoids were well organized, with smooth edges and branching vascular structures whereas stimulated organoids had rougher edges and less uniform shape. Stimulated organoids also lost the formation of vascular tubes. Higher α-SMA signal intensity in TCs was associated with closer proximity to ECs, emphasizing the role of vascular crosstalk in myofibroblast activation. In TGF-β1 stimulated organoids this pattern persisted to further distances (Fig. 1C). When embedded in high type III collagen hydrogels, fibrotic organoids demonstrated robust vascular sprouting with increased sprout length and size at 14 days compared to 7 days (Fig. 2A). The vascular sprouts from these organoids were also mature with open lumens and robust 3D branching (Fig. 2B). Slow interstitial fluid mixing was achieved in μSiM devices with fibrotic organoids using a syringe pump set-up (Fig. 3A-B). Confocal micrographs taken above and below the 20 μm pore membrane show vascular branching in both compartments that cross the membrane and form anastomoses in the pores (Fig. 3C).

**DISCUSSION**: In this study, we aimed to develop a novel tendon MPS platform for the study of the myofibroblast microenvironment centered around the vascular contribution to tendon fibrosis. Our results show that tendon fibrotic organoids can be created using primary tendon cells and ECs and that these organoids demonstrate vasculogenesis and angiogenic sprouting representative of the hypervascularization seen in peritendinous adhesions. When combined in our μSiM platform, we can add slow interstitial flow across an ultrathin, microporous membrane. Our set-up provides several advantages over existing vascular MPS models. The simple, two-compartment design provides easy access to the fibrotic organoid for sampling of the fibrotic environment or stimulation of the organoid. Furthermore, the layout and thin membrane allow for easy imaging even across a flow channel for perfusion. At present, the fluid flow in our set-up is not constrained to the open vascular networks. Future work will focus on limiting microfluidic perfusion to the vascular structures. This would then allow us to introduce circulating immune cells to the model to study mechanisms of inflammatory fibrosis and treatments to mitigate this pathology.

**SIGNIFICANCE/CLINICAL RELEVANCE**: Novel, human-relevant models are needed as new approach methodologies (NAMs) to complement, and in some instances reduce the reliance on, animal models for drug development to resolve tendon scar formation and fibrosis. Our model emphasizes the contribution of vascular pathology to peritendinous scar formation and will be a useful tool for developing new therapeutics.

**REFERENCES**: [1] Ajalik RE *et al*., *Front Bioeng Biotechnol*. 2022. [2] Ajalik RE *et al*., *Adv Healthc Mater*. 2025 [3] Korntner S *et al*., *Adv Drug Deliv Rev*. 2019 [4] Shirure VS *et al*., *Annu Rev Biomed Eng*. 2021. [5] Wei K *et al*., *Nature*. 2020.

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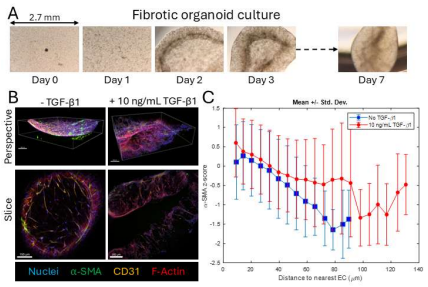


*Figure 1.* Tendon fibrotic organoid culture in Matrigel droplet. A) Phase contrast images showing fibrotic organoid formation over 7 days of static culture. B) Fibrotic organoids show formation of 3D vascular structures indicated by positive CD31 staining and varying morphology +/- TGF-β1 stimulation. Scale bars are 200 μm except for bottom left which is 150 μm . C) Distance of each fibroblast to the nearest endothelial cell plotted against normalized α-SMA intensity. Cells from 3 organoids in each condition were pooled for analysis.

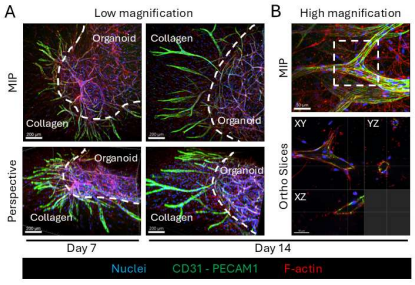


*Figure 2.* Angiogenic sprouting of fibrotic organoids into high type III collagen hydrogels. A) Organoids demonstrate angiogenic sprouting after 7 (left) and 14 (right) days of static culture shown in maximum intensity projection (top) or 3D perspective (bottom). Scale bars are 200 μm. B) High magnification confocal imaging show that vascular structures have open lumens as shown in orthogonal slices. Scale bars are 50 μm.

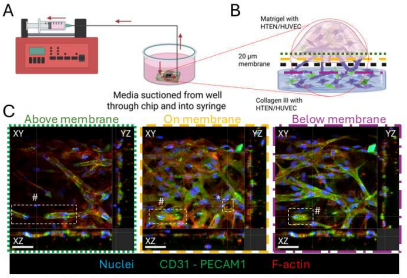


*Figure 3.* Microfluidic perfusion of fibrotic organoid and vascularized hydrogel with slow interstitial flow. A) Schematic representation of slow interstitial flow set-up utilizing a syringe pump to draw media through microfluidic device. B) Schematic representation of fibrotic organoid cultured in the top well and vascularized hydrogel in bottom channel separated by a 20 μm pore membrane. C) Confocal image slices at three levels shown in B) demonstrating vascular structures branching through the pores of the membrane (#) and an anastomosis in a pore (*). Scale bars are 50 μm.