The Role of Type III Collagen in Tendon Fibrosis: Insights from Hydrogel Models for Scarless Tendon Repair

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INTRODUCTION: Scarless repair of the injured tendon remains an unsolved clinical problem with long-term detrimental effects for patients and a large burden on the healthcare system and broader economy. After injury, many tissues in the body deposit granulation and scar tissue during repair to quickly stabilize the injured area and facilitate regeneration. However, many tissues are unable to achieve full regeneration, instead experiencing the persistence of scar tissue and loss of normal function, known as fibrosis. Tendons have limited regenerative capacity and often experience this fibrotic scar formation leading to pathologic changes in the extracellular matrix (ECM) and cellular composition. Of note, there is dysregulation of the normal ECM environment and an influx of immune and vascular cells as part of the healing response. Healthy tendons are largely comprised of type I collagen. After injury, there is a rise in other collagen types in scar tissue, including type III collagen, which can rise to as much as 20% of all collagens [1]. Our prior data has shown that altering the type I to type III collagen ratio leads to a dramatic change in the hydrogel ultrastructure for *in vitro* and microphysiological models. These changes were shown to inhibit the motility of immune cells migrating through the hydrogel [2]. In the current study, we investigate the effects of type III collagen on other relevant cell types in tendon fibrosis, including endothelial cells and tenocytes. As type III collagen is expected to mimic the scar tissue environment, we hypothesize that it will facilitate angiogenesis from endothelial cells akin to the neovascularization seen after injury. Additionally, we hypothesize that type III collagen hydrogels will promote the differentiation of tenocytes to myofibroblasts seen in fibrosis.

METHODS: For these experiments, two types of collagen hydrogels were used. Commercially available collagen products (Advanced Biomatrix) were used to make hydrogels made of 95% type I and 5% type III collagen ("type I collagen") and 80% type I and 20% type III collagen ("type III collagen"). All hydrogels were mixed to 2 mg/mL of total collagen concentration. To test angiogenesis, human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured between passages 3-6 as three-dimensional spheroids using the hanging-droplet method [3]. These spheroids were then suspended in neutralized collagen solutions, and samples were placed in an incubator to allow hydrogels to form. After gel formation, EGM-2 medium (Lonza) was added on top of hydrogels, and spheroids were cultured for 24 hours. At the end of the experiments, images were taken, and sprout number and length were quantified. Human primary tenocytes, isolated from tendon tissue retrieved from hand surgery with Institutional Review Board approval, were used to assess the response to type III collagen. Tenocytes, between passages 3-6, were suspended as single cells in neutralized collagen solutions at a concentration of 500,000 cells/mL of collagen and added to a custom-made microphysiological platform for holding the hydrogels. After the polymerization of hydrogels, they were cultured in a 10% FBS-supplemented medium for 5 days. At the end of 5 days, samples were fixed and stained with an antibody targeting α-SMA (Abcam) and imaged using confocal microscopy. Contraction kinetics were compared by measuring the two-dimensional area of hydrogels in the device and normalizing to the starting area. Experimental results from type I and type III collagen groups were compared using Welch's t-test. For each condition, 19 spheroids were analyzed for angiogenesis experiments, and 5 devices per group were analyzed for the tenocyte-hydrogel remodeling experiments.

RESULTS SECTION: After 24 hours, HUVEC spheroids exhibited angiogenesis through the formation of sprouts. For each condition, 19 spheroids were analyzed across 2 separate experiments. Results indicate that spheroids cultured in type I collagen hydrogels had fewer (3.4 vs 7.4) and shorter (47 µm vs 55 µm) sprouts than their counterparts in type III collagen gels (Fig. 1). After 5 days of culture in tenocyte-collagen hydrogels, tenocytes had positive expression of α-SMA as shown in immunofluorescence imaging, regardless of the collagen type. The myofibroblasts in the type I collagen hydrogels showed alignment parallel to that of the collagen hydrogel with the device anchors. Cells in the type III collagen gels had more spread morphology with no appreciable alignment. However, type I collagen gels were contracted significantly more than type III collagen hydrogels. At the end of 5 days, type I collagen gels had contracted on average 29% of their area compared to only 10% contraction in type III collagen gels (Fig. 2).

DISCUSSION: The results in this experiment suggest that hydrogels with high type III collagen can support a more robust angiogenic response by HUVECs than gels with little type III collagen. This could be due to a direct response of the endothelial cells recognizing their biochemical environment as injury-like scar tissue. Additionally, the structural changes in hydrogels could also lead to differences in the cells' ability to migrate and sprout during phases of angiogenesis. Overall, this result supports the use of type III collagen hydrogels *in vitro* for a more accurate simulation of tendon scarring and fibrosis by recapitulating the characteristic neovascularization. Interestingly, the rapid contraction of type I collagen hydrogels and not type III gels was counter to our initial hypothesis. Our results show that cells in both hydrogels are positively expressing the α-SMA of contractile myofibroblasts, despite some differences in alignment and morphology. This suggests there is some link between cell behavior and the remodeling of the collagen fibrils in the hydrogel. We further hypothesize that the biomechanical or structural properties of the hydrogels alter the cells' ability to remodel the ECM. This phenomenon may be relevant to scar resolution as fibrosis may prevent myofibroblasts from performing their normal function of closing wounds. Future work will include active microrheology to characterize the viscoelastic properties of the hydrogels experienced by the cells. We anticipate that the insights from these experiments will help to explain the differences seen in hydrogel remodeling and inform the design of culture protocols in our human Tendon-on-a-Chip (hToC) studies.
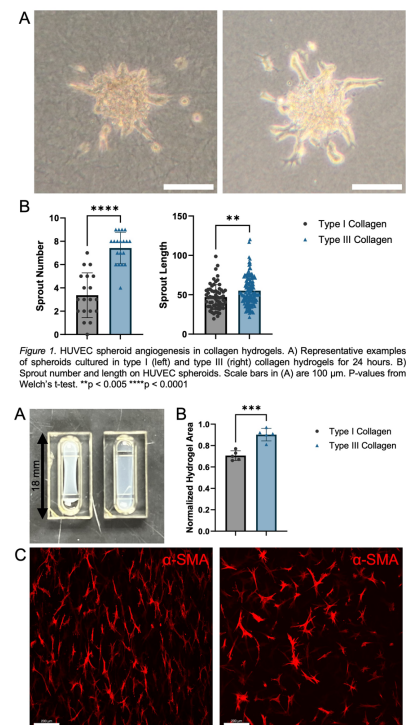

Figure 1. HUVEC spheroid angiogenesis in collagen hydrogels. A) Representative examples of spheroids cultured in type I (left) and type III (right) collagen hydrogels for 24 hours. B) Sprout number and length on HUVEC spheroids. Scale bars in (A) are 100 µm. P-values from Welch's t-test. **p < 0.005 ****p < 0.0001
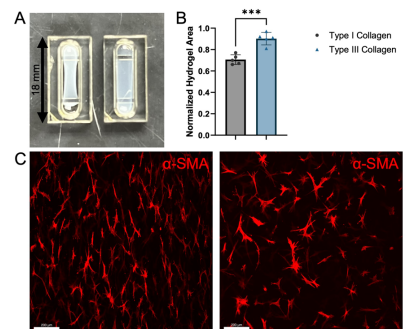

Figure 2. Collagen hydrogel remodeling by activated myofibroblasts. A) Representative examples of hydrogel remodeling by myofibroblasts in type I (left) and type III (right) collagen hydrogels after 5 days. B) Area of collagen hydrogels normalized to starting area after 5 days of culture. C) Confocal micrographs of α-SMA expressing cells in the middle of the type I (left) and type III (right) collagen hydrogels. Scale bars in (C) are 200 µm. P-values from Welch's t-test. ***p < 0.0005

SIGNIFICANCE/CLINICAL RELEVANCE: Research and development of therapies for fibrosis will ultimately benefit from more accurate modeling of scar tissue in human microphysiological systems. The work in this project provides insights into an important aspect of fibrosis in the extracellular matrix and further develops microphysiological models of the tendon for research and drug discovery.

REFERENCES: [1] Eriksen HA, et al., *J Orthop Res*. 2002 [2] Zhang VZ, et al, 2023 Annual Meeting of the Orthopedic Research Society. [3] Tetzlaff F, Fischer A, *Bio Protoc*. 2018.