

Development of engineered tendon tissue micro-gauges (tenTuGs) for investigating tendon organization and mechanobiology

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INTRODUCTION: The mechanisms underlying tendon disease and healing are not completely understood thereby severely restricting the development of effective therapies.^{1,2} The status quo as it pertains to *in vitro* tendon biology are monolayer 2D tissue culture plastic or 3D systems with fixed geometry that typically do not provide quantitative biomechanical measures, are relatively large constructs resulting in diffusion gradients, and are incompatible with high-throughput/content microscopy.³⁻⁶ Micro-physiological systems (MPS) have emerged as an invaluable tool for disease modeling and understanding cell-extracellular matrix (ECM) interactions, which is currently not possible *in vivo*.⁷ In this study, we established a tendon specific MPS to monitor tissue contractility and compared the gene expression of tendon fibroblasts (TFs) in an engineered 3D tendon tissue micro-gauge (tenTuGs) to native mouse Achilles and tail tendons.

METHODS: The tenTuGs are generated by 3D stereolithography printing a resin mold (Phrozen Sonic Mini 8k) then casting a polydimethylsiloxane (PDMS) platform that is biocompatible.⁸ We maintain a 1:10 aspect ratio for tendon constructs, mimicking human Achilles tendon morphology while reducing diffusion gradient issues that arise in larger 3D culture systems.⁹ Construct dimensions are a 2 mm long x 0.2 mm width with 1 mm long cantilevers with tapered heads (Fig1A). Tail TFs were isolated from N=12 2-month-old C57BL/6J wildtype mice (n=6 male, n=6 female; 2 animals/device) for use in tenTuGs. From the same animals, Achilles tendons (AT) and additional tail tendons (TT) were flash frozen for tissue-level RNA isolation. Tail tendon tissue used for tenTuGs were embedded in collagen-I (PureCol®-S, Millipore) gel for expansion for 10 days. TFs were passaged once to collagen-I-coated tissue culture plastic before being embedded with collagen-I (FibriCol, Advanced BioMatrix) in a tenTuGs device. Each device included 21 micro-gauges (two cylindrical silicone pillars at 2-mm apart) in individual wells (Fig1A). TenTuGs were seeded using 4μL of 2.5mg/mL collagen-I mixed with 1.5 million TFs/mL for each gauge (Advanced DMEM/F12 containing 4% FBS, 1% PenStrep). The number of intact gauges, tissue length (distance between the center of the two pillars), and tissue width were measured on 0-, 1-, 3-, 7- and 10-days using 10x phase contrast imaging (LionHeart, Agilent). Pillar displacement was converted to contractile force using Hooke's law and the pillar bending stiffness (0.6037 μN/μm, FeBio model simulation data not shown). Statistical comparisons of survival, force, and width were performed using Prism (GraphPad v10; 2-way ANOVA for time/device). RNA was isolated from TFs after 10 days in culture or from flash-frozen tissues (AT and TT) for cDNA synthesis for SYBR-based quantitative PCR (qPCR). Expression of matrix-associated genes (*Col1a1*, *Col1a2*, *Col3a1*, *Tnc*, *Tnmd*) and progenitor markers (*Scx*, *Mkx*) were quantified from flash frozen tissues and cultured mouse tail TFs and data were normalized to AT from the same animals (Pfaffl-based; 2^{-ΔΔCq}; reference = *Rplp0* and *Polr2a*). Statistical comparisons of ΔCqs were made using Prism (GraphPad v10; two-way ANOVA for sex/tissue). Additional devices were treated with either 10ng/mL hTGFβ2 or 0.1% bovine serum albumin (BSA) control at time of seeding (supplemented media was refreshed every 48hr) and imaged at 1-, 3-, 7- and 9-days. Treated tenTuGs were imaged on day 9 using brightfield and quantitative polarized light imaging (qPLI, ThorLabs, Inc) on an epifluorescent microscope (Leica).

RESULTS: TFs survived in tenTUG constructs after 1-week in culture (Fig1B).

A preliminary study with TFs isolated from 6-month-old female mice (n=2) demonstrated long-term feasibility (4-week) of TFs in tenTuGs (Fig1C). Over a 10-day period *in vitro*, tissue tension increased and tissue width decreased (Fig2A). The majority of tenTuGs were intact after 1-day in culture and remained intact through the 10-day experiment. However, with TGFβ2 treatment, tissue tension increased rapidly and more than half of the tenTuGs failed by 7-days (Fig2B). Prior to failure, collagen alignment was increased for TGFβ2-treated tenTuGs compared to BSA-only (Fig2C). In 3D tenTuGs, *Scx* and *Tnmd* was downregulated compared to native tissues, and *Tnc* was upregulated in 3D compared to 2D culture and native tissue. Additionally, *Col1a1*, *Tnc* (Fig 3), *Col3a1* (not shown) were upregulated in 3D culture compared to 2D culture and native TT tissue.

DISCUSSION: Our findings support the utility of MPS fabrication for developing an *in vitro* platform for measuring TF contraction *in vitro* in 3D. We demonstrated the ability to generate and maintain TF viability and contractility with increased expression of matrix-associated genes. However, transcriptional markers of TF development in did not mimic that of native (adult) tissues. It is established that TGFβ2 can enhance tenogenesis and increase *Scx* expression in mesenchymal and embryonic stem cells.⁴ In agreement, when TGFβ2 was added to the tenTuGs we observed increased contractility and qPLI signal, indicating more organized collagen fiber deposition. Although several constructs were lost due to the increased contractility, our pillar geometry can be adapted to handle in the larger cellular forces induced by TGFβ2 treatment to prevent this. Future studies should compare the expression of these genes in developing (e.g., neonatal) tendon. Additional future work will examine the influence of cellular contractility on nascent matrix deposition tenTuGs.

SIGNIFICANCE: A need exists to identify mechanisms by which tendon extracellular matrix can be reorganized or regenerated in 3D, and tenTuGs provide a new platform for drug discovery of tendon therapeutics.

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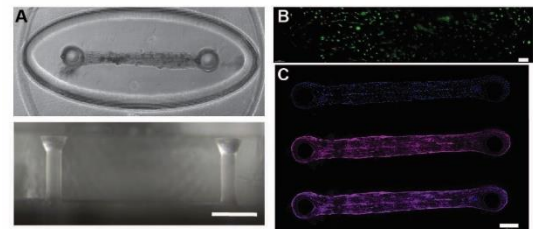


Figure 1. Mouse TFs embedded in tenTuG devices can be maintained for at least 4 weeks. (A) Visualization of the tenTuG molds from top and side. Pillars are 2mm apart; scale bar = 0.5mm. (B) Cell viability (Live, calcein in green; Dead, ethD-1 in red) staining shows high cell survival in constructs after 7 days in culture (scale bar = 0.1mm). (C) Nuclear (blue) and phalloidin (purple) staining showed dense, aligned constructs after 4 weeks in culture. scale bar = 0.2mm

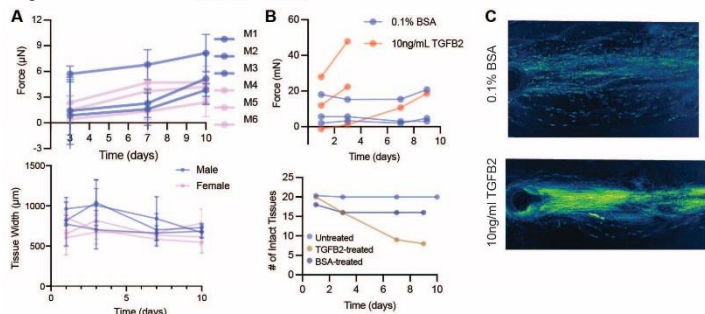


Figure 2. TFs cultured in collagen on tenTuG devices exhibit (A) increased force (decreased distance between pillars) and increased compaction (i.e., decreased tissue width) over time in culture. (B) Treatment of tenTuG constructs with TGFβ2 leads to increased contractility and premature tissue construct failure compared to untreated or BSA-treated tenTuGs. (C) Collagen organization of tenTuGs was increased with TGFβ2 treatment compared to BSA only.

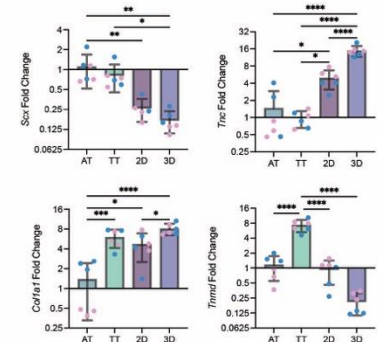


Figure 3. TFs elicit culture-dependent transcriptional changes. Gene expression of native tissues (AT and TT) compared to primary TFs isolated from TT in 2D and 3D tenTuG culture after 7 days. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 (of ΔCq for gene expression). Blue dots = male replicates (n=2 pooled per dot); pink dots = female replicates (n=2 pooled per dot).