The TGFβ-Signaling enhances Cellular and Matrix Maturation in Scaffold-free Three-Dimensional (3D) Tendon Construct

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INTRODUCTION: Tendons are connective tissues transmitting mechanical forces between muscles and bones. Given its characteristics of strength and elasticity, tendon can prevent muscle injury by absorbing the mechanical impacts [1]. In spite of its notable tensile strength, overuse of tendons in sports or daily activity causes tendon injury, and injured tendon barely restores original structural and mechanical integrity [2]. Therefore, tendons are targets for tissue engineering and regenerative medicine. However, the biological mechanisms regulating molecular and cellular maturation are poorly understood partly due to the lack of a reliable *in vitro* system, which hinders advancing tendon tissue engineering and regenerative medicine [3]. To overcome this limitation, we previously developed a scaffold-free 3-dimensional (3D) tendon culture system and tested tenogenic maturation and mechanical properties [4]. Our previous study showed that TGFβ (Transforming growth factor β) treatment is necessary to maintain the 3D tendon constructs, but the precise function of TGFβ in 3D tendon culture was not established. In the current study, we investigated the function of TGFβ in molecular and cellular maturation in Scaffold-free 3D tendon culture.

METHODS: All procedures were approved by UPenn's IACUC. Both male and female mice were used in this study. We isolated mouse tail tendons at 28 days of age. These tail tendons were digested with type I collagenase for 1 hour. The tendon cells from mouse tail tendons were initially cultured in a 6-well plate with growth media (20% FBS, 2 mM L-glutamine in a-MEM medium). After 90-100 % confluency, tendon cells were passaged to 100 mm plate for further amplification. For 3D tendon culture, the amplified cells (2.0 X 10⁶ cells) in monolayer culture were seeded in the fibronectin-coated growth channel and cultured with differentiation media (20% FBS, 2 mM L-glutamine in a-MEM medium). After generating 3D tendon structures, we treated vehicle or TGFβ every two days until harvested. H&E staining was conducted at T14 (T0 is the initiation day of TFGβ treatment). BrdU and TUNEL assay were conducted at T3, 7, and 14. The measurement of the nucleus aspect ratio was performed by the Image J software program. We measured both height and width of the nucleus in the peripheral layer of 3D tendons and calculated the ratio at the T0, 3, 7, and 14. qRT-PCR analysis was performed to assess the expression of tendon-related gene markers in 3D tendon structures at T0, T3, T7, and T14. All quantitative data were analyzed using the student's t-test.

RESULTS: We previously found that the peripheral layer of 3D tendon constructs undergoes tendon-like tissue maturation. Therefore, we acquired H&E-stained images of the peripheral layer after TGFβ treatment to test the effects of TGFβ on tissue maturation, such as tissue thickness and cell density (Fig 1A). As expected, TGFβ treatment enhanced thickness and decreased cell density of the peripheral layer compared to vehicle treatment at T14 (Fig 1B). The decreased cell density in the peripheral layer of 3D tendon constructs prompted us to investigate cellular proliferation and apoptosis. We analyzed the cell proliferation and apoptosis ratio by BrdU staining and TUNEL assay in the 3D tendons at various stages (T3, 7, 14) (Fig 2A, B). The level of cell proliferation in each group decreased as the stage progressed in both layers. However, TGFβ treated groups exhibited increased cell proliferation compared to control groups in both layers as the stage progressed (Fig 2C, D). TGFβ-treated groups showed a lower apoptosis rate than those of control groups at an early stage (T3) only in the peripheral layer (Fig 2E). However, no difference was observed in apoptosis level between vehicle- and TGFβ-treated groups in the inner layer at all stages (Fig 2F). To investigate cellular phenotypes in 3D tendon constructs, we first examined the morphological changes of cells in the 3D tendon construct (Fig 3A-D). We measured the ratio of the nuclear transverse length to its length in the direction of the channel length. In this measurement, values closer to 1.0 indicates a rounder cell shape, and values closer to 0 indicate a more elongated cell shape. Gradual cell elongation was observed in the peripheral layer of both vehicle- and TGFβ-treated constructs, but TGFβ-treated constructs had more elongated cells than vehicle-treated constructs throughout the stages. To test the effect of TGFβ on tenogenic differentiation, we examined the expression of tenogenic gene markers such as Scleraxis (Scx), Tenomodulin (Tnmd), and Collagen type 1 (Col1a1) (Fig 3E-G). TGFβ treatment significantly enhanced the expression of Scleraxis, but there was no significant effect on the expression of Tnmd and Col1a1.

DISCUSSION: Our data suggest that TGFβ treatment increased cellular proliferation in both peripheral as well as inner layers and prevented cellular apoptosis in the peripheral layer at early stages, which could explain the increased thickness of TGFβ-treated tendon constructs. TGFβ dramatically induced the expression of Scleraxis but not the expression of Col1a1 and Tenomodulin. These data suggest that cells undergo early tenogenic differentiation without further molecular maturation with TGFβ treatment.

SIGNIFICANCE/CLINICAL RELEVANCE: This study provides a reliable *in vitro* tendon cell culture model which can be used to screen various biological molecules that is essential for tendon regeneration.

REFERENCES: [1] T Roberts 2014, [2] L Galatz 2015, [3] Nichols 2019 [4] L Yeon-Ju 2022.
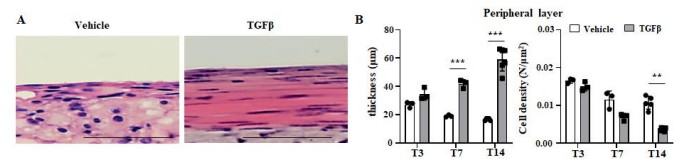

Figure 1. TGFβ induces tendon-like structures in the peripheral layer of the 3D tendon. (A) H&E-stained longitudinal section of peripheral layer at T14. Scale bars indicate 100 μm. (B) Quantification results of thickness and cell density of peripheral layer. ** indicates P<0.01 and *** indicates P<0.001, n=3
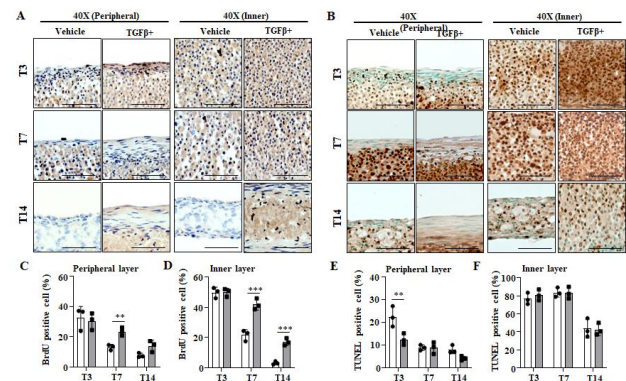

Figure 2. Histological analysis of layer of 3D tendon constructs. (A) BrdU-stained longitudinal sections of 3D tendon structure in both control and TGFβ+ at T3, 7, and 14 stages. (B) TUNEL assay longitudinal images of 3D tendon structure. (C, D) The quantification results of BrdU positive cells of the control and TGFβ+ groups at T3, 7, 14 stages in inner- and peripheral-layer. (E, F) The quantification results of TUNEL positive cells of the control and TGFβ+ groups at T3, 7, 14 stages in inner- and peripheral-layer. Scale bars indicate 100 μm. ** indicates P<0.01 and *** indicates P<0.001, n=3
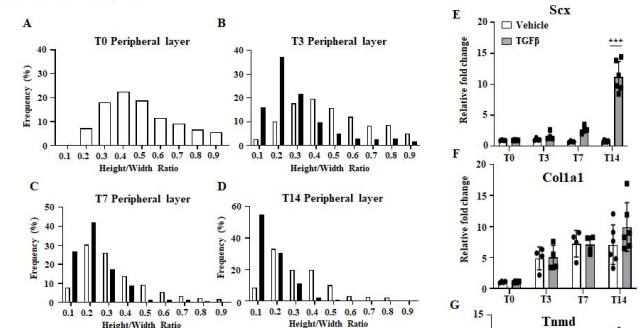

Figure 3. Morphological and molecular changes of cells in the peripheral layer of 3D tendon constructs. (A) The Frequency of nucleus height/width ratio in peripheral-layer of 3D tendon at T0 stage, (B) T3 stage, (C) T7 stage, (D) T14 stage both control and TGFβ+, respectively. (E) Quantitative real-time PCR (qRT-PCR) results for tenogenic makers as Scleraxix (Scx), Tenomodulin (Tnmd), and Collagen type I (Col1a1) in 3D tendon constructs. *** indicates P<0.001, n=4~6