Scaffold-free 3D Tendon Cell Culture Using Mouse Tendon Cells

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Disclosures

None

Introduction

Standard two-dimensional (2D) cell culture has been widely used for *in vitro* studies to understand molecular mechanisms. However, tenocyte phenotype is not well-maintained in monolayer culture and it is difficult to study extracellular matrix (ECM) organization and morphological maturation of cells without a 3-dimensional (3D) environment. To overcome this limitation, several 3D tendon cell cultures were developed by suture model[1] and Flexcell tissue culture plate system[2]. Based on these 3D tendon culture studies, we developed a scaffold-free 3D tendon culture system using mouse tenocytes, which can be used for genetic manipulation of specific target genes.

Methods

All procedures were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Tendon cells were isolated from mouse tail after one hour digestion with type I collagenase. Isolated tendon cells were grown in 20% FBS and 2mM L-glutamine in a-MEM medium. We generated growth channels with 3D-printed mold and the 2% agar in 6-well plate (Figure 1A). To enhance the attachment of the tendon cells to anchor, the anchors were surrounded by hydrophilized PCL-scaffolds. Growth area and PCL-scaffolds were coated with fibronectin. To generate the 3D tendon cell structure, tendon cells were seeded on the fibronectin-coated growth area at 2.5 x 10^6 cells/well with 20% FBS in a-MEM medium. To differentiate 3D tendon cells, TGF-β was treated every two days after seeding. Histological analysis was conducted on the 3D tendon structure at various time points (days 0, 3, 7, 14, and 21 after TGF-b treatment). qRT-PCR analysis examined tendon-related gene markers in 3D tendon structures at various time points (days 0, 3 and 7 after TGF-b treatment). To test the genetic gene manipulation by adenovirus, 3D tendon structures generated using cells from Rosa26-Ai9 mouse and $Tsc1^{f/f}$ mouse. Then, 3D tendons were infected with Ad-CMV-Cre-eGFP. All quantitative data were analyzed using student's t-test.

Results

We can generate six 3D tendons (7-8 mm length and 0.5-0.8 mm thickness) using tendon cells from one mouse tail (Figure 1B). The thickness of the 3D tendon structure was dramatically decreased without TGF-b 3D tendon cell (Figure 1C). This data suggests that TGF-b treatment is essential to maintain 3D tendon structure. Interestingly, we found that the outer layer of the 3D tendon became a tendon-like structure (Figure 1D, blue Box). This tendon-like structure also showed a maturation process similar to the one found during postnatal mouse tendon development, including decreased cell density, increased thickness, and flat cells between highly aligned extracellular matrix (Figure 1D). Consistent with histological changes, the expression of tenogenic genes are increased through time (Figure 2). These results suggest that our 3D tendon culture is a reliable *in vitro* system to study the underlying molecular and cellular mechanism regulating tendon maturation. To test the feasibility of the gene manipulation in 3D tendon culture, we infected 3D tendon with Adenovirus. We generated 3D tendon using tendon cells from R26-Ai9 mice which can express mTomato genes upon CRE recombinase expression. Adenovirus can express CRE recombinase and eGFP (Ad-CMV-Cre-eGFP). We confirmed the infection of virus by the expression of eGFP in outer layer of 3D tendon (Figure 3A, Green color). We also confirmed the CRE recombinase activity by the expression of mTomato (Figure 3A, Red color). We then used the adenovirus to activate mTORC1 signaling in 3D tendon culture by deleting Tsc1, a negative regulator of mTOC1. We generated 3D tendon using tendon cells from conditional *Tsc1* mouse line (*$Tsc1^{f/f}$*). We treated Ad-CMV-eGFP (control) or Ad-CMV-Cre-eGFP (gain-of-function) in 3D tendon generated by *$Tsc1^{f/f}$* tendon cells. Interestingly, our histologic analysis showed that the activation of mTORC1 caused increased thickness and disorganized matrix with bigger and round cells in the tendon-

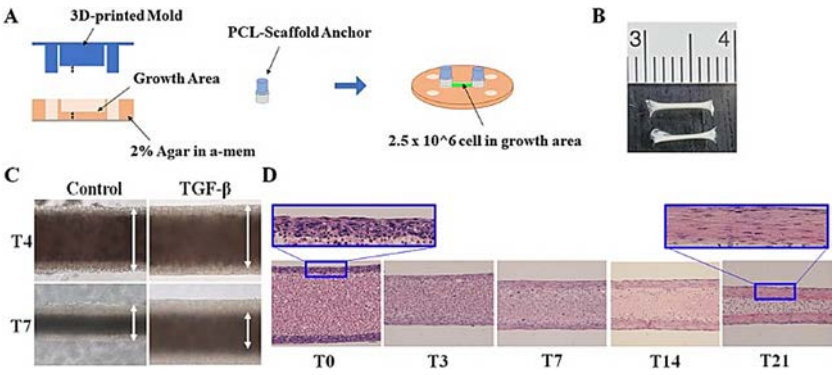

Figure 1. Method of 3D tendon cell culture system and Histology analysis of 3D tendon differentiation. The base of the 3D tendon cell culture was consisted growth area with 2% agaroses with 3D-printed-mold and hydrophilized PCL-scaffold anchor. 3D tendon cell structure was generated tendon cells at 0.5 x 10^6 in fibronectin coated growth area between PCL-scaffold anchor **(A)**. The length of 3D tendon structure is 7 to 8 mm and the thickness is 0.5 to 1 mm **(B).** 3Dtendon structure with or without TGF-b on days 4 and 7 **(C).** H&E stained 3Dtendon structure during tendon cell differentiation **(D).**

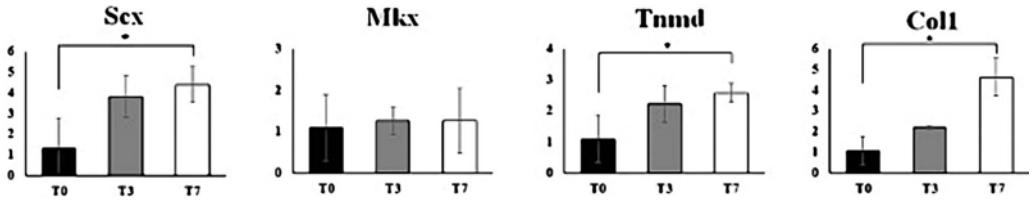

Figure 2. Transcription analysis of 3D tendon. Scleraxis, Mohawk, Tenomodulin, and Collagen type I, of tendon related genes were increased in 3D tendon differentiation.(*indicate P , 0.05 between T0 3D tendon (n 5 4).

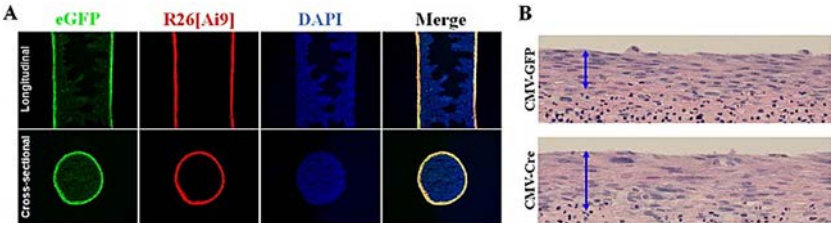

Figure 3. Infection of Adeno-virus in 3D tendon structure. CMV-eGFP and CMV-Cre-eGFP were infected 3D tendon structure from Tsc1fl/fl;R26:Ai9 mice. Green fluorescence shows the expression o feGFP and red fluorescence shows the expression of ROSA26-tdTomato, and DAPI at T7 **(A)**. H&E stained 3D tendon structure from Tsc1fl/fl mice infected CMV-GFP or CMV-Cre-eGFP at T7 **(B)**.

like structure, which is similar to the tendon phenotypes of mTORC1 gain-of-function mouse model (Figure 3B)[3].

Discussion

Our results suggest that the 3D tendon culture system using mouse tendon cells is feasible to manipulate gene expression and effective tools to investigate the molecular mechanism underlying cell maturation and ECM organization. Although 3D tendon showed tendon-like structure, the result may not fully represent an *in vivo* mechanism. The thorough comparison between *In vivo* and *in vitro* result will be necessary to increase the scientific rigor of future research using our 3D tendon culture. We also expect that this system can also be used for pharmacological screening study for tendon diseases.

Significance

This study will contribute to the understanding of cellular and molecular mechanism underlying tendon maturation in vitro using genetic manipulation.

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References

1. R Gehwolf, 2019, 2017
2. K Mubyana, 2018
3. J Lim, 2017