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

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Background

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 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 and Flexcell tissue culture plate system. 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.

Method

All procedures were approved by UPenn's IACUC. 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. 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 3D tendon structure at various time points (day 0, 3, 7, 14, and 21 after TGF-β treatment). qRT-PCR analysis was examined tendon related gene markers in 3D tendon structures at various time points (day 0, 3 and 7 after TGF-β treatment). To test the genetic gene manipulation by adenovirus system in 3D tendon structure, 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

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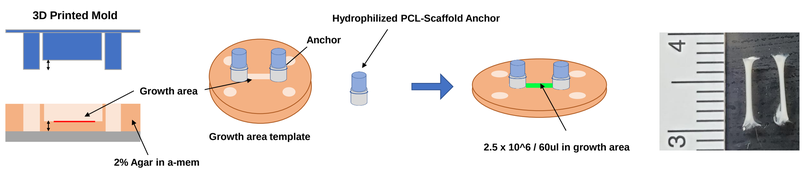
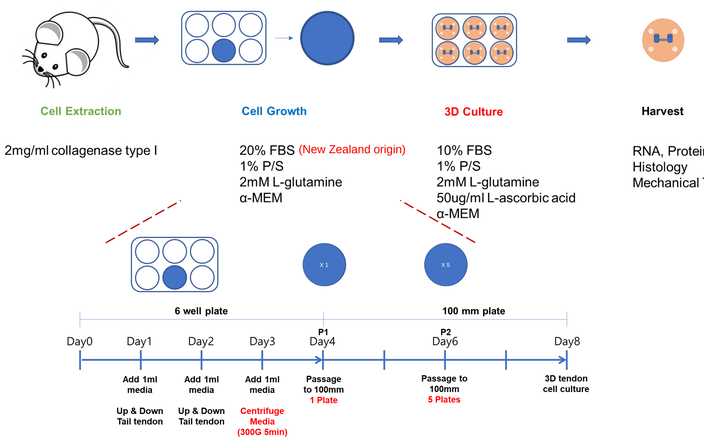
Results

1. 3D tendon cell culture system



The base of the 3D tendon cell culture was consisted growth area with 2% agaroses with 3D-printed-mold and hydrophilized PCL-scaffold anchors. 3D tendon cell structure was generated tendon cells at 2.5 x 10^6 in the fibronectin-coated growth area between PCL-scaffold anchor. The length of the 3D tendon structure is 7 to 8 mm and the thickness is 0.5 to 1 mm.

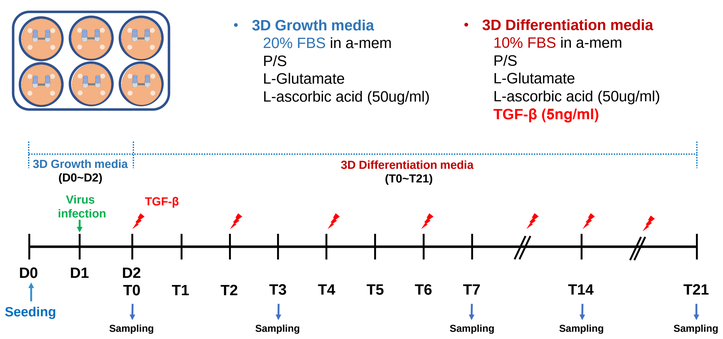
2. Overview of 3D tendon cell culture



3D tendon cell culture proceeds in 3 major steps: cell extraction, cell growth in monolayer, and 3D culture. Cell extraction. Primary tail tendon cells were extracted by collagenase type I from P25 old mice. The isolated tendon cells cultured in 6 well plates for 4days till 100% confluency. Then the cell was passaged to one 100mm dish and grow well. Consistently, These cells were split into five 100mm dishes when cells were fully confluency in a 100 mm dish. Six 3D tendon structures were generated from five fully confluency 100mm dish. Only FBS originated from New Zealand works for monolayer cell culture.

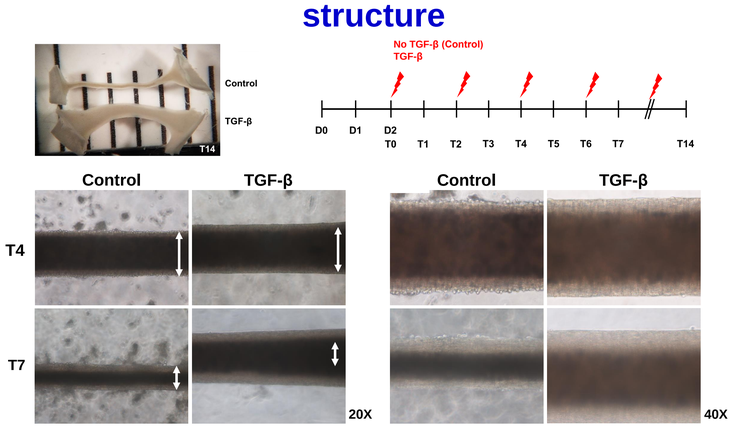
Results

3. Timeline after seeding for 3D tendon structure



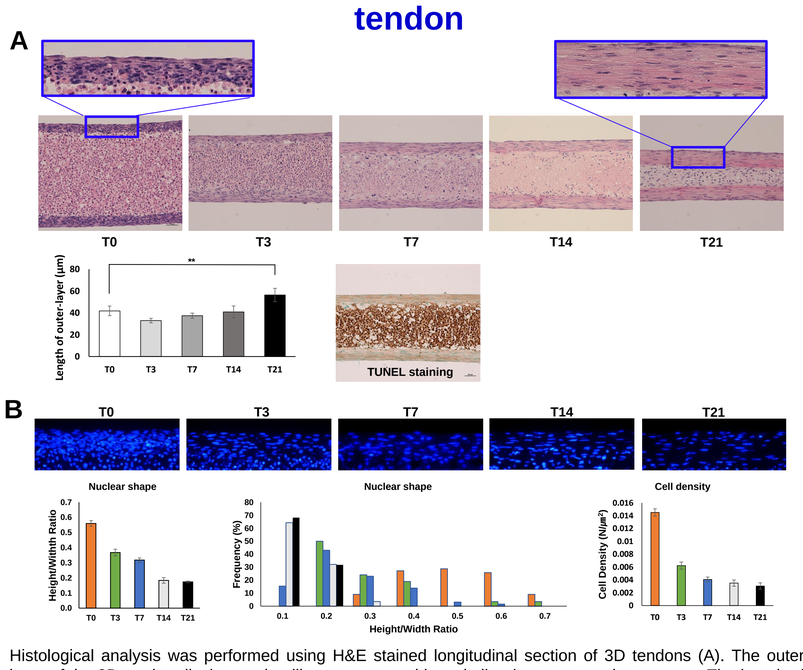
The cell was seeded on the fibronectin-coated growth area for 3D tendon culture on Day 0. On Day 2, TGF-β treatment starts processing in 3D tendon culture and it means T0. TGF-β was treated every 2 days during 3D tendon culture. Virus infection experiments were processed on Day1 and removed on Day 2.

4. TGF-β is necessary to maintain 3D tendon structure



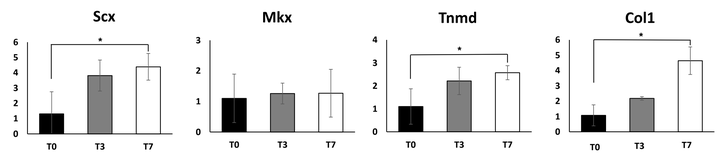
3D tendon structure was cultured with or without TGF-β for 14 days. 3D tendon structure can not maintain its thickness and dramatically decreased thickness without TGF-β compared to TGF-β-treated 3D tendon structure at both T4 and T7 stage. A lot of cells was budding out from the 3D tendon without TGF-β. The surface of outer layer is smooth in 3D tendon with TGF-β.

5. Tendon-like maturation of outer layer of 3D tendon



Histological analysis was performed using H&E stained longitudinal section of 3D tendons (A). The outer layer of the 3D tendon display tendon-like structure and is a similar tissue maturation process. The length of the outer layer was increased in the development stages. Inner cells undergo apoptosis using TUNEL staining. Nuclear shape and nuclear aspect ratio were analyzed in 3D tendon development stages (B). Tenocytes became flat and narrow at later stages.

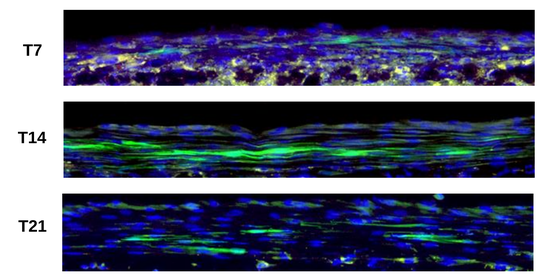
6. Transcriptional analysis of 3D tendon



RNA analysis was performed by qRT-PCR from 3D tendon during differentiation. 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=4)

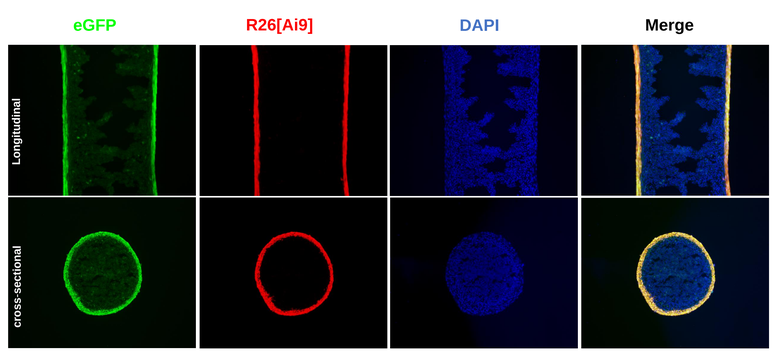
Results

7. Analysis of Col1 2.3GFP positive cell



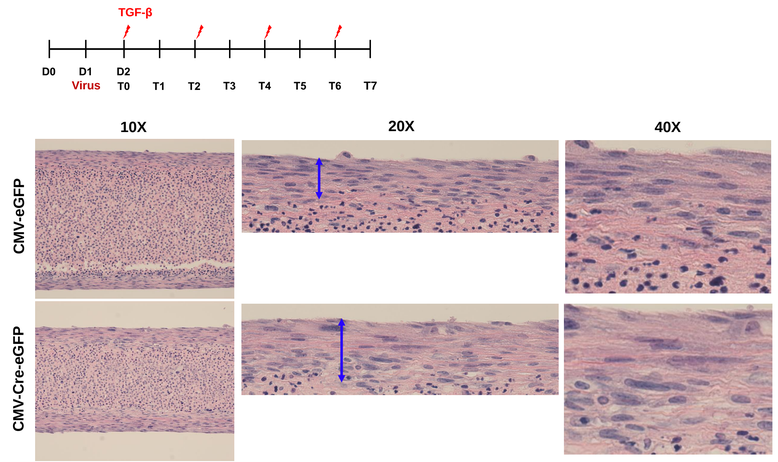
Cryohistological analysis of outer layer in 3D tendon structure from Col1 2.3GFP mice during differentiation stages. Col1 2.3-GFP cell was increased in outer layer during 3D tendon differentiation stages.

8. Effective gene manipulation using adenovirus in 3D tendon structure



3D tendon structure was generated using tendon cells from R26-Ai9 mice. CMV-eGFP was infected with 3D tendon structure. Green fluorescence shows the expression of eGFP and red fluorescence shows the expression of ROSA26-tdTomato, and DAPI at T7

9. The thickness of Tendon-like structure increased by activation of mTORC1 signaling



3D tendon structure was generated using tendon cells from Tsc1$^{fl/fl}$ mice and infected CMV-GFP or CMV-Cre on Day1. Histological analysis was performed using H&E stained 3D tendon structure infected CMV-GFP or CMV-Cre at T7. The activation of mTORc1 caused increased thickness and disorganized matrix with bigger and round cells in the tendon-like structure. The thickness changes in middle panels and cell morphology changes in the right panels.

Conclusion

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.