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TITLE:

Applying a Three-dimensional Uniaxial Mechanical Stimulation Bioreactor System to Induce Tenogenic Differentiation of Tendon-Derived Stem Cells

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KEYWORDS:

Mechanical loading; tendon-derived stem cell; tendinopathy; bioreactor; differentiation; tendon; stem cell

SUMMARY:

A three-dimensional uniaxial mechanical stimulation bioreactor system is an ideal bioreactor for tenogenic-specific differentiation of tendon-derived stem cells and neo-tendon formation.

ABSTRACT:

Tendinopathy is a common chronic tendon disease relating to inflammation and degeneration in an orthopaedic area. With high morbidity, limited self-repairing capacity and, most importantly, no definitive treatments, tendinopathy still influences patients' life quality negatively. Tendon-derived stem cells (TDSCs), as primary precursor cells of tendon cells, play an essential role in both the development of tendinopathy, and functional and structural restoration after tendinopathy. Thus, a method that can in vitro mimic the in vivo differentiation of TDSCs into tendon cells would be useful. Here, the present protocol describes a method based on a three-dimensional (3D) uniaxial stretching system to stimulate the TDSCs to differentiate into tendon-like tissues. There are seven stages of the present protocol: isolation of mice TDSCs, culture and expansion of mice TDSCs, preparation of

stimulation culture medium for cell sheet formation, cell sheet formation by culturing in stimulation medium, preparation of 3D tendon stem cell construct, assembly of the uniaxial-stretching mechanical stimulation complex, and evaluation of the mechanical stimulated in vitro tendon-like tissue. The effectiveness was demonstrated by histology. The entire procedure takes less than 3 weeks. To promote extracellular matrix deposition, 4.4 mg/mL ascorbic acid was used in the stimulation culture medium. A separated chamber with a linear motor provides accurate mechanical loading and is portable and easily adjusted, which is applied for the bioreactor. The loading regime in the present protocol was 6% strain, 0.25 Hz, 8 h, followed by 16 h rest for 6 days. This protocol could mimic cell differentiation in the tendon, which is helpful for the investigation of the pathological process of tendinopathy. Moreover, the tendon-like tissue is potentially used to promote tendon healing in tendon injury as an engineered autologous graft. To sum up, the present protocol is simple, economic, reproducible and valid.

INTRODUCTION:

Tendinopathy is one of the common sports injuries. It is mainly manifested by pain, local swelling, decreased muscle tension in the affected area, and dysfunction. The incidence of tendinopathy is high. The presence of Achilles tendinopathy is most common for middle- and long-distance runners (up to 29%), while the presence of patellar tendinopathy is also high in athletes of volleyball (45%), basketball (32%), track and field (23%), handball (15%), and soccer (13%)[1-5]. However, due to the limited self-healing ability of the tendon, and the lack of effective treatments, tendinopathy still influences patients' life negatively[6,7]. Moreover, the pathogenesis of tendinopathy remains unclear. There have been many investigations about its pathogenesis, mainly including "inflammation theory", "degeneration theory", "overuse theory", and so forth[8]. At present, many researchers believed that tendinopathy was due to the failed self-repair to the micro-injuries caused by excessive mechanical loading the tendon experiences[9,10].

Tendon-derived stem cells (TDSCs), as primary precursor cells of tendon cells, play an essential role in both development of tendinopathy and functional and structural restoration after tendinopathy[11-13]. It was reported that mechanical stress stimulation could cause the proliferation and differentiation of osteocytes, osteoblasts, smooth muscle cells, fibroblasts, mesenchymal stem cells and other force-sensitive cells[14-18]. Therefore, TDSCs, as one of the mechanosensitive and multipotent cells, can similarly be stimulated to differentiate by mechanical loading[19,20].

However, different mechanical loading parameters (loading strength, loading frequency, loading type and loading period) can induce TDSCs to differentiate into different cells[21]. Thus, an effective and valid mechanical loading regime is very significant for tenogenesis. Furthermore, there are different kinds of bioreactors as stimulation systems currently used for providing mechanical loading to TDSCs. The principles of each kind of bioreactor are different, so the mechanical loading parameters corresponding to different bioreactors are also different. Therefore, a simple, economic, and reproducible stimulation protocol is in demand, including the type of bioreactor, the corresponding stimulation medium, and the

mechanical loading regime.

The present article describes a method based on a three-dimensional (3D) uniaxial stretching system to stimulate the TDSCs to differentiate into tendon-like tissue. There are seven stages of the protocol: isolation of mice TDSCs, culture and expansion of mice TDSCs, preparation of stimulation culture medium for cell sheet formation, cell sheet formation by culturing in stimulation medium, preparation of 3D tendon stem cell construct, assembly of the uniaxial-stretching mechanical stimulation complex, and evaluation of the mechanical stimulated in vitro tendon-like tissue. The whole procedure takes less than 3 weeks to obtain the 3D cell construct, which is far less than some existing methods[22,23]. The present protocol has been proven to be able to induce TDSCs to differentiate into tendon tissue, and it is more reliable than the current commonly used two-dimensional (2D) stretching system[21]. The effectiveness was demonstrated by histology. In short, the present protocol is simple, economic, reproducible and valid.

PROTOCOL:

The methods described were approved and performed in accordance with the guidelines and regulations of the University of Western Australia Animal Ethics Committee.

1. **Isolation of mice TDSCs**

1.1. Euthanize the 6-8-week-old C57BL/6 mice by cervical dislocation.

1.2. Harvest patellar tendons[24] and Achilles tendons[25].

1.3. Digest tendons from one with 6 mL of type I collagenase (3 mg/mL) for 3 h.

NOTE: As the size of tendon in the mouse is small, all tendons harvested from one mouse should be used in this step.

1.4. Collect the cells and culture in complete Minimal Essential Medium (Alpha-MEM) containing 10% of fetal bovine serum (FBS) and 1% of streptomycin and penicillin mixture for 7 days.

1.5. Identify TDSCs using fluorescence-activated cell sorting by flow cytometry (expression of cell surface markers including CD44, CD90 and Sca-1; lack of the expression of CD34 and CD45).

1.6. Passage and freeze cells (passage 4 cells will be used for further steps).

2. **Culture and expansion of mice TDSCs**

NOTE: Conduct all steps in a sterile biosafety hood.

2.1. Take the warmed-up cells (mice TDSCs, 1 million cells, passage 4, to 37 °C).

2.2. Slowly add extra 4-5 mL of complete Minimal Essential Medium (Alpha-MEM).

2.3. Transfer the mixture to a 15 mL centrifuge tube with a pipette.

2.4. Top up with medium to 8 mL total with a pipette.

2.4.1. Prewarm medium in a 37 °C oven incubator.

2.5. Place the tube into centrifuge and balance.

2.6. Centrifuge and pellet cells at 347 x *g* for 5 min.

2.7. Take out after centrifuge and check the pellet at the bottom.

2.8. Decant the freezing medium, and resuspend cells gently in 1-2 mL of complete medium (avoid making too many bubbles).

2.9. Transfer resuspended cells to a T-75 flask by a pipette.

2.10. Use a pipette to add complete Alpha-MEM Medium to the flask to reach a total volume of 10 mL with final concentration of 13,000 cells/cm^2.

2.11. Place the flask into the incubator and culture at 37 °C with 5% CO_2.

2.12. Change the medium every 3 days.

2.13. Observe and monitor the cells under a microscope when changing the medium until cells are cultured to 100% confluence (about 40,000 cells/cm^2).

3. **Preparation of stimulation culture medium for cell sheet formation**

NOTE: Conduct all steps in a sterile biosafety hood.

3.1. Pour 15 mL of complete Alpha-MEM medium into a 15 mL sterile tube.

3.2. Add 6 µL of ascorbic acid (11 mg/mL) into 15 mL of medium for a final concentration of 4.4 µg/mL.

NOTE: Additionally adding 25 ng/mL connective tissue growth factor in the stimulation culture medium can accelerate the whole growth and differentiation process.

3.3. Mix gently by inverting up and down.

4. **Cell sheet formation by culturing in stimulation medium**

NOTE: Conduct all steps in a sterile biosafety hood.

4.1. Discard the normal complete alpha-MEM medium carefully, and avoid touching the cells attached to the bottom of the flask.

4.2. Add 10 mL of stimulation medium slowly and avoid causing any disturbance to the fully confluent cells.

4.3. Culture the cells in stimulation medium for 9 days (change the medium every 3 days) to sufficiently generate the cell sheet at 37 °C with 5% CO_2.

NOTE: Extracellular matrix will become thick and present to be cloudy when observed from the bottom of the flask after stimulated by ascorbic acid, which means the cell sheet is sufficiently generated.

5. **Preparation of 3D tendon stem cell construct**

NOTE: Conduct all steps in a sterile biosafety hood.

5.1. Take the flask out of the incubator.

5.2. Discard the stimulation medium completely.

5.3. Wash the monolayer cell sheet with phosphate buffer saline (PBS) by swirling the flask.

5.4. Discard the PBS completely.

5.5. Use a pipette to add 1 mL of 0.25% trypsin to the corner of the flask.

5.6. Tap the corner of the flask to de-attach the monolayer cell sheet until the corner of the cell sheet is off the bottom of the flask.

5.7. Immediately add 9 mL of complete Alpha-MEM medium to stop the trypsinization.

5.8. Continue swirling the flask to peel off the monolayer cell sheet completely.

5.9. Pour the total de-attached cell sheet into a Petri dish with medium.

5.10. Use a sterile tweezer to pick up one corner of the cell sheet and rotate in a clockwise direction for 15 times.

5.11. Pick up another end of the cell sheet and rotate in an anti-clockwise direction for 10 times to firmly generate a tendon-like in vitro construct (**Figure 1A**).

6. Assembly of the uniaxial-stretching mechanical stimulation complex in unique designed bioreactor

NOTE: Conduct all steps in a sterile biosafety hood.

6.1. Connect the hooks by the connecter and adjust to the desired distance (2 cm) between two hooks.

6.2. Gently wind the 3D TDSCs construct on the assembled hook for 3 times on each hook (**Figure 1B**).

6.3. Secure the hooks with cell construct onto the chamber of the bioreactor by tightening the screws on two ends.

NOTE: The whole chamber, including screws and hooks, is sterile (autoclave for 1.5 h at 134 °C and ultraviolet rays (UV) expose for 24 hours before use). For the metal holder of chambers, UV light expose for 48 hours before use.

6.4. Fill up the chamber with complete Alpha-MEM medium.

6.5. Connect the actuator to the culture chamber by cable.

6.6. Cut the hooks connecter by sterile scissors.

6.7. Switch on the power and corresponding channel controller to start mechanical stimulation.

6.8. Put the lid on the chamber.

6.9. Check the indicator lights and assure the bioreactor can function properly.

6.10. Put the bioreactor in the incubator and subject the 3D cell construct to mechanical stretching for 6 days (6% stretching, 0.25 Hz, 8 h, followed by 16 h rest).

7. Evaluation of the mechanical stimulated in vitro tendon-like tissue

7.1. Loosen the screws by a screwdriver and take the assembly off carefully.

7.2. Put the tendon-like tissue into 4% paraformaldehyde for fixation for 15 min.

7.3. Place the fixed tendon-like tissue into a biopsy cassette with biopsy foam pads.

7.4. Process to dehydrate the sample and finally evaluate by H&E histological staining.

7.5. Repeat the whole protocol and extract RNA from the tendon-like tissue for evaluating the expression of tenogenic markers by quantitative PCR (qPCR). Primers for the selected genes are listed in **Table 1**.

NOTE: Histology protocol and qPCR protocol are standard and following a previous study[21].

REPRESENTATIVE RESULTS:

Before mechanical stimulation, TDSCs were grown to 100% confluence in complete medium and displayed a disorganized ultrastructural morphology (**Figure 2A**). After 6 days of uniaxial stretching mechanical loading, extracellular matrix (ECM) and cell alignments were well orientated (**Figure 2B**). Cells were well populated and well enveloped in ECM after mechanical loading. Cell morphology was presented to be elongated and was more similar to normal tendon cell compared to the one without stretching (**Figure 2C**). Cell density in cell construct with loading was higher than in the one without loading. QPCR results showed that the present method increased the expression of the tenogenic markers including Scleraxis, Mohawk, Tenomodulin, and COL1A1 (**Figure 3**) compared to the ones treated by static culture.

FIGURE AND TABLE LEGENDS:

Figure 1: Assembly of three-dimensional (3D) cell construct over the hooks of the bioreactor. (**A**) General view of the 3D cell construct over the hooks. (**B**) The diagram to assemble the 3D cell construct over the hooks

Figure 2: Cell morphology before and after mechanical stimulation. (**A**) Tendon-derived stem cells were grown to 100% confluence in complete medium and displayed a disorganized ultrastructural morphology before mechanical stimulation. (**B**) Histologic images showed H&E staining of cell construct after mechanical stimulation. (**C**) Histologic images showed H&E staining of a control cell construct cultured in bioreactor for 6 days without mechanical stimulation.

Figure 3: Expression level of tenogenesis markers. Individual gene-expression levels were first normalized against the internal control, 36B4, and then normalized against gene-expression levels from static cultures. Results from 3 experiments are shown. The primer sequences used for qPCR analysis are provided in **Table 1**.

Figure 4: The three-dimensional uniaxial mechanical stimulation bioreactor system

Table 1: Primer sequences used for qPCR analysis

DISCUSSION:

The tendon is a mechanosensitive fibrous connective tissue. According to previous research, excess mechanical loading could lead to osteogenic differentiation of tendon stem cells,

whereas insufficient loading would lead to disordered collagen fiber structure during tendon differentiation[21].

A common view is that the key to an ideal bioreactor is the ability to simulate the in vitro cellular microenvironment that cells in vivo undergo. Therefore, mimicking the in vivo normal stress environment in vitro is the key of stimulating the single-lineage differentiation of TDSCs into tendon cells. In the present protocol, the width of the TDSC construct was 2 cm, and the movement range of the hook was 0.12 cm, which means the strain was 0.12/2 (%). In conclusion, TDSCs were mechanically stimulated in a bioreactor for 6 days (6%, 0.25 Hz, 8 h, followed by 16 h rest) in the present protocol. After evaluation, the mechanical loading parameters used in this protocol have been proven to induce TDSCs to generate tendon-like tissue. It should be noted that the bioreactor parameters should specifically match the corresponding type of stretching system. For example, the proper parameters for monolayered cell stretching are different from the present 3D cell sheet stretching[26,27]. A possible reason is the different force modes[21]. In 2D monolayer stretching system, cells attach to the culture substrate by focal adhesions at the bottom and connect to each other via cell–cell junctions. However, in a 3D cell sheet, there are also many more cell–ECM connections formed as a result of the 3D niche, which means the cells are under pressure in addition to stretching.

The effectiveness of the present protocol was demonstrated by morphology and the expression level of tenogenic markers. In terms of morphology, apart from histology, using confocal immunofluorescence microscopy to detect collagen I is a common way to characterize the collagen organization and then evaluate the ECM alignment. Previous study confirmed the well-aligned collagen type I bundle formation in the TDSC construct with 6% uniaxial mechanical stimulation for 6 days but not in the one without loading[21]. Tenogenic markers were used to identify tendon cells, including Scleraxis (SCX), Mohawk (MKX), Tenomodulin (TNMD) and COL1A1. Only TNMD was a non-transcription factor, and the rest of them are transcription factors[28]. They were all well recognized. SCX could regulate tendon development in the embryonic stage[29], while MKX could regulate tendon differentiation and maturation in postnatal stage[30]. Moreover, the mechanical properties (max load and stiffness) of the tendon-like tissue have been evaluated in previous study as well, and it showed the mechanical properties of TDSC construct with loading were better than in the one without loading[21].

The medium can affect cell growth and differentiation. In the present protocol, in order to form the cell sheet in a proper way, TDSCs were grown to 100% confluence in standard uncoated plastic flasks in complete medium, and then TDSCs were stimulated to promote ECM deposition by adding 4.4 mg/mL ascorbic acid for 6 d. Several growth factors, including insulin-like growth factor I, vascular endothelial growth factor, platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor, and growth differentiation factor 5, played an important role in tendon formation and healing[31-34]. Additionally adding 25 ng/mL connective tissue growth factor in stimulation culture medium as Ni et al. reported could accelerate the whole growth and differentiation process[35].

At present, the 2D cell sheet, also known as the monolayered cell, is the most common system used for mechanical investigations. Biaxial stretch provides multidirectional tension, both longitudinally and laterally, as the uniaxial stretch only provides longitudinal tension[36]. Thus, monolayered cell culture with biaxial stretch could mimic the growth environment of some types of cells, such as cardiomyocytes and epidermal cells. However, the 3D cell construct is more similar to the real shape of a tendon, and uniaxial stretch is more similar to the stress characteristics of tendon cells, compared with 2D cell sheet and biaxial stretch. Thus, the combination of the 3D cell construct and the uniaxial stretching is more competent for better understanding of the tendon and its mechanical micro-environment. This provides a theoretical basis for using a 3D uniaxial stretching system to stimulate the differentiation of TDSCs. Thus, the 2D cell sheet was finally rolled into a 3D cell construct and underwent a uniaxial stretch in the present protocol.

The bioreactor used in the present protocol consisted of actuator and culture chamber (**Figure 4**). The details of the present bioreactor are available in a previous study[37]. Currently, the most common actuators used for tendon included pneumatic actuators, linear motors, and step motorball screws[38-40]. Chambers included integrated and separated chambers[41,42]. Among them, a separated chamber with a linear motor provided accurate mechanical loading and was portable and easy to be adjusted[43].

Except TDSCs, other types of stem cells might be a potential cell source to be used in the present method. Previous research has shown that other types of stem cells might have potential therapeutic ability and might improve tendon regeneration. Compared to injured equine tendons treated with conventional noncellular based management, the re-injury rate of the tendons treated by BMSCs was lower[44,45]. Moreover, intratendinous injection of BMSCs into a tendinopathy model could effectively induce tendon regeneration[46]. Adipose derived stem cells could effectively treat equine tendinopathies leading to complete recovery and return to normal activity in horses[47]. Together, there was enough evidence to suggest that stem cells, except TDSCs, could treat tendon degeneration. However, as a cautionary note, transplantation of BMSCs into injured tendons has been shown to induce ectopic osteogenic differentiation in a rabbit model, suggesting that stem cells from a specific tissue might have a tendency to differentiate into undesired cells of their tissue of origin[48]. In previous research, loading-deprived treatment to TDSCs could result in osteogenesis as well[21], which led to the proposal that the present method has the potential to induce tenogenesis of other stem cells and avoid osteogenesis with specific loading regime. Further research is necessary to explore these specific loading regimes for other types of stem cells. If this scenario holds, more cell source to treat tendinopathy will be available.

There are still some limitations to be acknowledged. First, although a noncorrosive and autoclavable material, stainless steel, was used to build the bioreactor system, the culturing conditions and the culture medium still corrode the chamber including the screw and the hook. Thus, routine inspection of the chamber and timely rust removal are necessary. Second, in order to make the present bioreactor economic and portable, there is not an environmental

control and medium circulation system in it. Thus, the operator needs to transport the bioreactor and open the chamber for medium exchange every 3 days, which increases the risk of contamination. The operator must always pay attention to keep the TDSCs environment sterile .

For the treatment of tendinopathy, it is vital to clarify the pathological process from a non-surgical perspective. In terms of surgical treatment, an effective method is to use engineered autologous grafts. The process of stimulating TDSCs to mimic differentiation in vivo is helpful for the investigation of pathological process of tendinopathy. The engineered scaffold-free tendon tissue has the ability to promote tendon healing in a rat patellar tendon window injury model. Besides, previous work proved the mechanical loading could improve the mechanical properties of cultured tendon. Thus, this protocol provides a reproducible method for directed differentiation of TDSCs into tendon-like tissue so that it can be used simply and feasibly for potential engineered tendon culture.

DISCLOSURES:

The authors have nothing to disclose.

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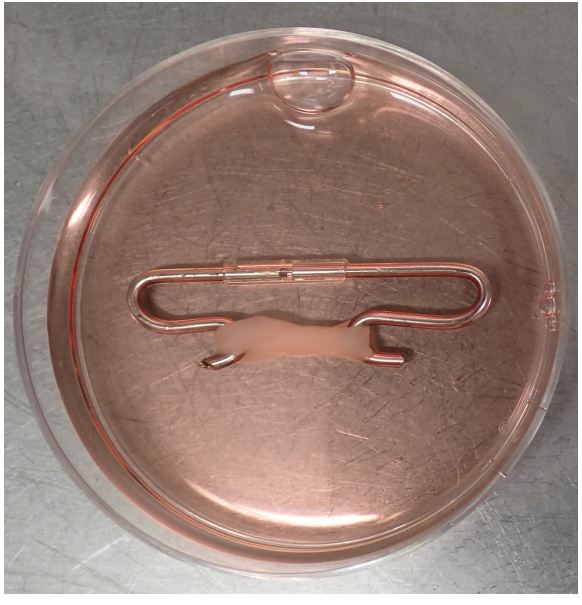
Figure

A



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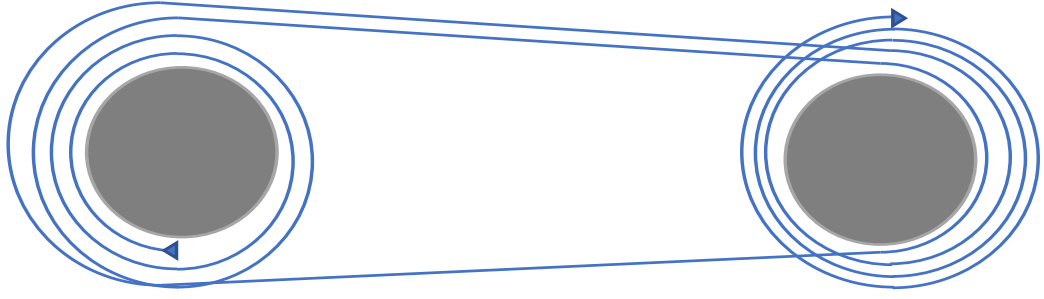

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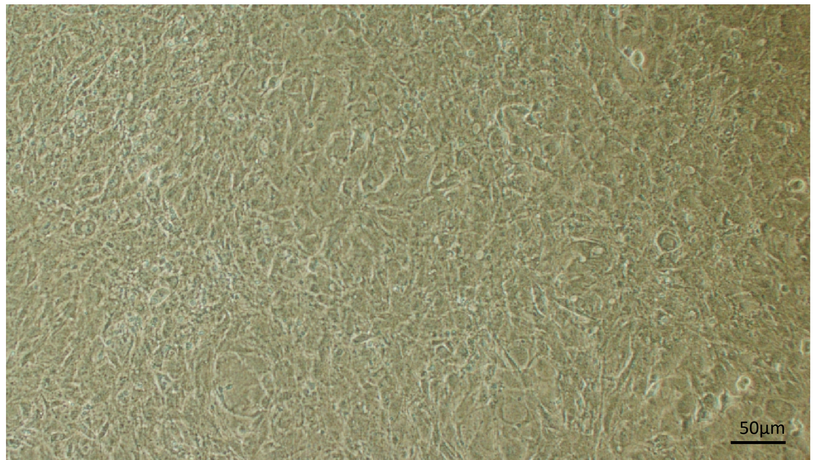

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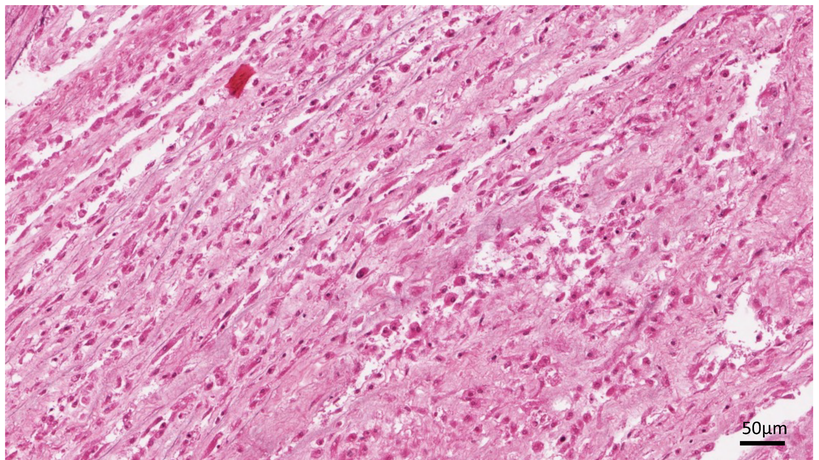

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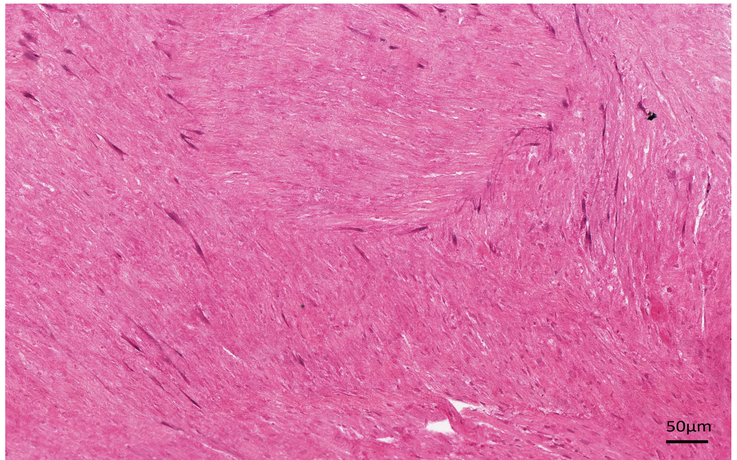
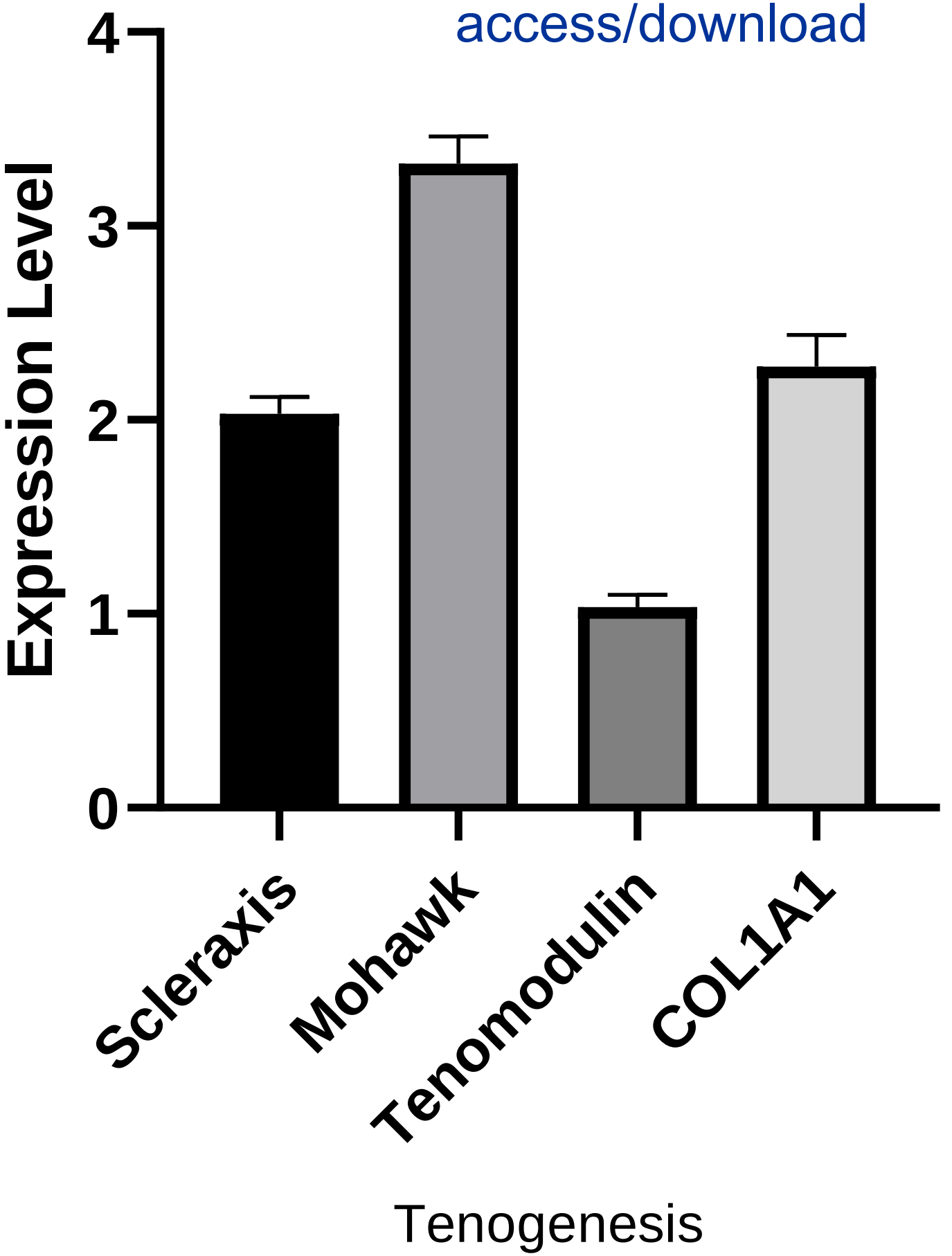


Figure



Figure

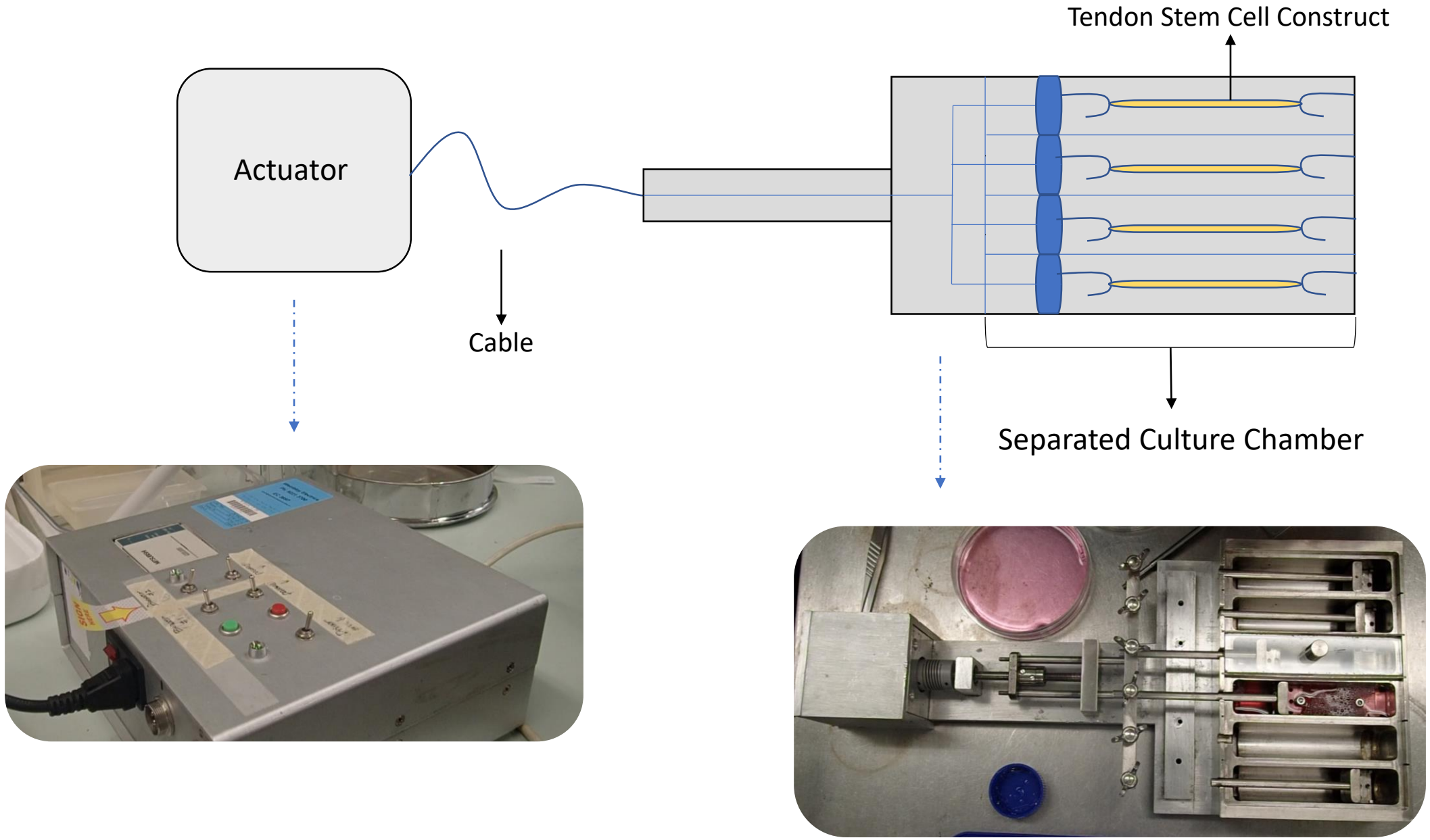


Table 1. Primer sequences used for qPCR analysis

Gene	Primer sequence	
	Forward 5'->3'	Reverse 5'->3'
COL1A1	TGACTGGAAGAGCGGAGAGT	GTTCGGGCTGATGTACCAGT
Scleraxis	CCCAAACAGATCTGCACCTT	GGCTCTCCGTGACTCTTCAG
Mohawk	GTCCGGCAGCCAGATTTAAG	TCGCTGAGCTTTCCCCTTTA
Tenomodulin	CCGCAGAAAAGCCTATTGAA	GACCACCCATTGCTCATTCT
36B4	CTTCCCACTTGCTGAAAAGG	CGAAGAGACCGAATCCCATA

Name of Material/ Equipment	Company	Catalog Number
Ascorbic acid	Sigma-aldrich	PHR1008-2G
Fetal bovine serum (FBS)	Gibcoä by Life Technologies	1908361
Histology processor	Leica	TP 1020
Minimal Essential Medium (Alpha-MEM	Gibcoä by Life Technologies	2003802
Mouse Tendon Derived Stem Cell		
Paraformaldehyde	Sigma-aldrich	441244
Streptomycin and penicillin mixture	Gibcoä by Life Technologies	15140122
Three-dimensional Uniaxial Mechanical Stimulation Bioreactor System	Centre of Orthopaedic Translational Research, Medical School, University of Western Australia	

Trypsin	Gibcoä by Life Technologies	1858331

Comments/Description

Isolated from Achilles tendons of 6- to 8-wk-old C57BL/6 mice. Then digested with type I collagenase (3 mg/ml; MilliporeSigma, Burlington, MA, USA) for 3 h and passed through a 70 mmcell strainer to yield single-cell suspensions.

Available from the corresponding author upon request. Or make it according to our design* *Wang T, Lin Z, Day RE, et al. Programmable mechanical stimulation influences tendon homeostasis in a bioreactor system. Biotechnol Bioeng. 2013;110(5):1495–1507. doi:10.1002/bit.24809

Dear Dr. Nguyen,

Thank you and thank the editors and reviewers very much for the advice and comments on our manuscript *Apply a Three-dimensional Uniaxial Mechanical Stimulation Bioreactor System to Induce Tenogenic Differentiation of Tendon-Derived Stem Cells*. In the current form, we have modified our manuscript and our video according to the editorial comments.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please upload Table 1 as a separate xls/xlsx file.

Answer: As suggested, Table 1 is uploaded as a separate xls file.

2. Additional comments are in the attached manuscript.

Answer: Line 113. The citations corresponding to harvest patellar tendons and Achilles tendons are provided in the current version (ref.24 and ref.25).

Line 115. As the size of tendon in the mouse is small, all tendons harvested from one mouse should be used in step 1.3. We add this statement in the current version and the change is marked in yellow shadow.

Changes to be made by the Author(s) regarding the video:

1. On-Screen Text:

• For the Introduction and Discussion, consider showing the name caption for Ruan Rui only long enough for the audience to read and then fade it out. It is obstructing Ruan's face during some parts.

Answer: As suggested, we shorten the name caption and then fade it out. Thank you for your suggestion.

2. Retimed Shots:

• 05:00 For this shot of looking at the culture under the microscope, there seems to be a very slow playback here of three frames. If you don't have enough footage for this shot, then a freeze frame would work better instead of the somewhat jerky slow motion currently in the video.

Answer: As suggested, we show a freeze frame in the current version.

3. Audio:

• Ensure that all audio peaks occur between -12 and -6 dB. This may require some light adjusting from section to section. The Introduction and Conclusion are generally okay, the protocol narration is a little quiet and volume needs to be increased.

Answer: As suggested, we adjust the audio of every section to the level of the Introduction and Conclusion part. We hope that the revised audio will meet with approval.

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Author(s): Ziming Chen, Peilin Chen, Chris Ruan, Lianzhi Chen, Jun Yuan, David Wood, Tao Wang, Ming Hao Zheng

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