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Optimization of differentiation time of mesenchymal-stem-cell to tenocyte under a cyclic stretching with a microgrooved culture membrane and selected measurement cells

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Purpose: There is a need for efficient stem cell-to-tenocyte differentiation techniques for tendon tissue engineering. More than 1 week is required for tenogenic differentiation with chemical stimuli, including co-culturing. Research has begun to examine the utility of mechanical stimuli, which reduces the differentiation time to several days. However, the precise length of time required to differentiate human bone marrow-derived mesenchymal stem cells (hBMSCs) into tenocytes has not been clarified. Understanding the precise time required is important for future tissue engineering projects. Therefore, in this study, a method was developed to more precisely determine the length of time required to differentiate hBMSCs into tenocytes with cyclic stretching stimulus. *Methods*: First, it had to be determined how stretching stimulation affected the cells. Microgrooved culture membranes were used to suppress cell orientation behavior. Then, only cells oriented parallel to the microgrooves were selected and evaluated for protein synthesis levels for differentiation. *Results*: The results revealed that growing cells on the microgrooved membrane and selecting optimally-oriented cells for measurement improved the accuracy of the differentiation evaluation, and that hBMSCs differentiated into tenocytes in approximately 10 h. *Conclusions*: The differentiation time corresponded to the time required for cellular cytoskeleton reorganization and cellular morphology alterations. This suggests that cells, when subjected to mechanical stimulus, secrete mRNAs and proteins for both cytoskeleton reorganization and differentiation.

Key words: *cyclic stretch, differentiation, differentiation time, human bone marrow-derived mesenchymal stem cell (hBMSC), mechanical stimulus, tenocyte*

1. Introduction

Tendon is a connective tissue that physically binds muscles to skeletal, structures, permitting locomotion and enhancing joints stability [27]. Tendon injuries, ranging from repetitive strain injuries to complete ruptures, frequently occur in athletes and active people, and the resulting reduced functionality can be devastating to their everyday lives. Tendon injuries are difficult to manage. Although spontaneous healing can occur, this often results in the formation of scar tissue, which differs morphologically, biochemically, and biomechanically from healthy tendon tissue [21]. Because the structure of the repaired tissue differs from healthy tissue in terms of functionality as well as movement and strength [2], current conservative and surgical treatments show limited success [2].

Thus, there is a pressing need for tendon tissue engineering. One of the goals of tendon tissue engineering is to produce functional replacement tissue *in vitro* that can then be implanted into the body. One strategy for tendon tissue engineering involves combining cells capable of forming tendon with a scaffold to produce a construct that can be implanted at the injury site to encourage new tissue formation. This

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strategy relies on the availability of an effective cell source. However, only a small number of cells can be obtained from explanted tissue, because tendons are relatively acellular, containing few tenocytes, which are terminally differentiated with very limited proliferative capacity [8]. Therefore, mesenchymal stem cells (MSCs) are more commonly used in this field due to their high proliferation capability and pluripotency [20].

Numerous strategies using chemical stimulus to enhance tendonogenesis, including co-culture, have been reported. A number of growth factors (e.g., connective tissue growth factor (CTGF) [11], growth/differentiation factor (GDF)-5/ bone morphogenetic protein (BMP)-14 [19], and GDF-6/BMP-13 [7]) induce differentiation of MSCs into tenocytes. However, this technique is too time-consuming (>1 week) to efficiently differentiate MSCs into mature tenocytes [7], [19], [30].

During daily movement of the body, tendons are subjected to numerous types of mechanical strain. Tendons respond to these mechanical forces by adapting their metabolism as well as structural and mechanical properties [27]. Mechanical stretching appears to influence human tendon fibroblast proliferation [28] and increase the production of collagen, the primary constituent of tendon tissue [18]. Furthermore, the application of mechanical stretching appears to stimulate MSCs to proliferate and differentiate into tenocytes [16], [17], [23]. This technique requires only several days for mesenchymal stem cells (MSCs) to differentiate into tenocytes [14], [16], [17], [29], [30]. Therefore, mechanical stimulus has been investigated as a simpler and safer differentiation-inducing technique in tendon tissue engineering.

The specific length of time required to differentiate human bone marrow-derived MSCs (hBMSCs) into tenocytes with mechanical stimulus is unknown. However, elucidating the required time is vital to support efficient regenerative medicine or tissue engineering techniques. Therefore, in this study, we developed a method to more precisely determine the length of time required to differentiate hBMSCs into tenocytes with cyclic stretching stimulus. Typically, cells seeded onto polydimethylsiloxane (PDMS) membranes are oriented randomly to the stretch direction in the initial state, which gradually align roughly perpendicularly (60–90°) to the stretch direction during cyclic stretching (Fig. 1) [16], [17]. Due to this cellular orientation behavior, it is not possible to determine how much the strain stimulation acts on the cells. To address this limitation, we used microgrooved PDMS membranes to suppress the cellular orientation behavior, making it possible to effectively provide specific constant strain stimulation to the cells. To improve the accuracy of the time required to differentiate hBMSCs into tenocytes with mechanical stimulus, only cells oriented parallel to the stretch direction were selected for evaluation of differentiation. This is the first study to determine the length of time required for MSC-to-tenocyte differentiation.

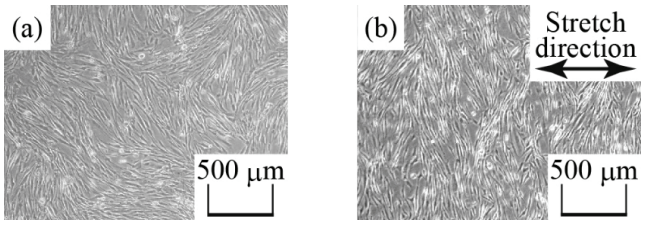

Fig. 1. Human bone marrow-derived mesenchymal stem cells (hBMSCs) seeded onto a polydimethylsiloxane (PDMS) membrane: (a) Initial state (oriented randomly), (b) Orientation after cyclic stretching (oriented specifically)

2. Materials and methods

2.1. Fabrication of cell culture chamber with aligned microgrooved membrane

A glass wafer (50.8 × 50.8 × 1.0 mm^3) was covered entirely with photoresist (AZ P4903, Micro-Chemicals GmbH, Ulm, Germany), using the spin-coating method (2000 rpm, 20 s), and dried in a thermostatic oven at 90°C for 1 h. Lines were lithographed in a 20 × 20-mm^2 central area on the coated glass wafer with a laser lithography system (μPG104-UV; Heidelberg Instruments Mikrotechnik GmbH, Heidelberg, Germany) after drying. Then, the exposed photoresist on the glass wafer was removed by submerging in developer (AZ 400K; AZ Electronic Materials USA Corp., NJ, USA) for 3 min. The wafer was dried again in a thermostatic oven at 90 °C for 1 h. This glass wafer was used as the mold to fabricate the aligned microgrooved membranes.

The glass wafer mold was covered entirely with PDMS solution (base: hardner ratio = 10:1) (SILPOT 184; Dow Corning Tray, Tokyo, Japan) using the spin-coating method (300 rpm, 1 min). The PDMS solution was cured by heating at 100 °C for 10 min, and the 300-μm-thick PDMS membrane was dismounted from the mold. The groove width and depth of the PDMS membrane was 10 and 4 μm, respectively. The grooves were separated at 100-μm intervals to enable individual measurement of cells. The microgroove dimensions were determined by the in-

vestigation of the effective size of microgrooves for tenogenic differentiation of hBMSCs. Figure 2 shows the PDMS membrane used in the present study. Finally, PDMS sidewalls were adhered to the microgrooved membrane to form a cell culture chamber. The sidewalls were sufficiently thick to minimize contraction of the microgrooved membrane due to Poisson's effect when the chamber was subjected to uniaxial stretching.

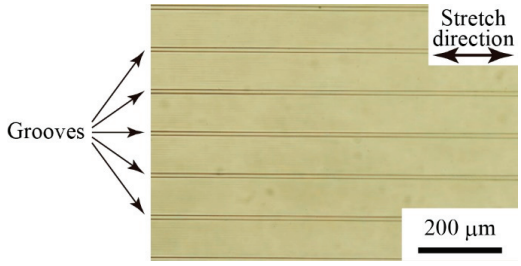

Fig. 2. Microgrooved PDMS membrane used for cell culturing. The grooves were 10 μm wide and 4 μm deep. The grooves were separated at 100-μm intervals

For cell culture application, fabricated chambers underwent ultrasonic cleaning with neutral detergent, tap water, and distilled water, subsequently. Then, they were placed in sterile phosphate buffered saline (PBS; Cosmo Bio, Tokyo, Japan) and sterilized by exposure to ultraviolet light in a laminar flow hood for 1 h. Finally, they were coated with human fibronectin (R&D Systems, Minneapolis, MN, USA) at concentration of 1 μg/cm^2 and incubated for 3 h.

2.2. Cell preparation

hBMSCs (MSC-R53-2; RIKEN BRC, Tsukuba, Japan) [25] were cultured according to the supplier's protocol, and were maintained in Dulbecco's modified Eagle medium (Wako Pure Chemical Industries Ltd., Osaka, Japan), containing 10% newborn calf serum (Life Technologies, Inc., Carlsbad, CA, USA), and 0.05% gentamicin (Life Technologies, Inc.), at 37 °C under a 5% CO_2 atmosphere in a humidified incubator. All hBMSCs used in this study were extracted from a 39-year old male and were at or before the sixth passage to ensure a high proliferative capability. The hBMSCs underwent various rigorous inspections (viability, adhesiveness, mycoplasma contamination, morphology, human individual discrimination, animal species discrimination, HBV, HCV, HIV, HTLV-1). The hBMSCs were cultured in 25-cm^2 culture flasks (BD Biosciences, Franklin Lakes, NJ, USA) at an initial density of 1.0 × 10^4 cells/cm^2 for expansion without differentiation. The medium was replaced every 3 days. At near-confluence, which occurred every 5–7 days, cells were detached from culture flasks with 0.25% w/v trypsin (Wako Pure Chemical Industries Ltd.) with 1 mM EDTA (Wako Pure Chemical Industries Ltd.) and seeded into new culture flasks. The trypsinized hBMSCs were plated in the aligned microgrooved chambers at density of 2.0 × 10^5 cells/cm^2 and cultured without cyclic stretching for 1 day. Figure 3 shows an image of most of the cells oriented along the lines of the microgrooves in the PDMS membrane under a phase-contrast microscope.

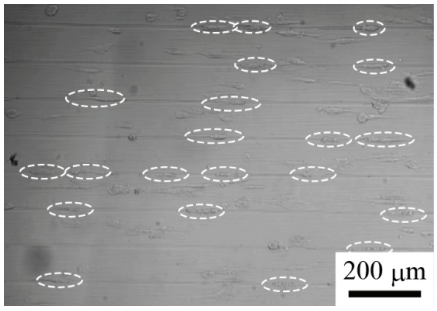

Fig. 3. Phase-contrast photomicrograph of hBMSCs on the aligned microgrooved PDMS membrane. Most of the cells are oriented parallel to the microgrooves (in white dotted ellipses)

2.3. Cyclic stretch conditions

The aligned microgrooved chambers seeded with hBMSCs were set in a mechanical cyclic uniaxial stretching system and subjected to cyclic stretching stimulus in a humidified incubator. 1 Hz and 5% as the cyclic frequency and stretch ratio, respectively, was selected, respectively. The cyclic frequency of 1 Hz was based on the physiological motions of tendon (e.g., walking is nearly 1 Hz) [22], which is also optimal for the proliferation of hBMSCs [23]. An optimal stretch ratio was preliminarily evaluated for effective

Table 1. Experimental conditions of the cyclic stretching stimulus for the tenogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs)

Stretch ratio [%]	Cyclic frequency [Hz]	Stretch duration [h]
5	1	0
		1
		3
		6
		12
		24
		48

tenogenic differentiation of hBMSCs in various stretch ratio from 0% to 10%, and the best one was 5% [9]. The stretch duration was timed precisely to investigate the time required for differentiation. The maximum duration was set to 48 h, since MSC-to-tenouste differentiation is typically completed by this time [6], [14], [17]. Table 1 lists the experimental conditions of this study.

2.4. Immunofluorescence staining

Based on our previous report [14], [17], we used type I collagen (Col I), tenascin-C (Tnc), and scleraxis (Scx) as marker proteins to evaluate hBMSC-to-tenocyte differentiation. Col I molecules self-assemble into highly organized fibrils that form collagen fibers [6]. Cross-linking of these fibers in the extracellular matrix (ECM) gives them a high tensile strength and provides mechanical strength to tendon tissue [12]. Tnc is thought to be involved in ECM formation, contributing to the mechanical stability of tendon tissue through its interactions with collagen fibrils and decorin, a proteoglycan [5]. Scx is a transcription factor specifically expressed in tendons and ligaments, involved in the activation of proα1(I) collagen gene expression in tendon fibroblasts [12], [13]. In addition, we examined the expression of runt-related transcription factor 2 (Runx2), an indicator of osteoblast differentiation, since some cyclic stretching conditions can promote such differentiation [4].

We used immunofluorescence staining to evaluate the expression levels of the marker proteins in the stretched cells according to a previously reported procedure [15]. At the end of the cyclic stretching period, the cells were fixed in 2% paraformaldehyde (Wako Pure Chemical Industries Ltd.) for 15 min and permeabilized in 0.25% Triton X-100 (Wako Pure Chemical Industries Ltd.) in PBS (Cosmo Bio) for 15 min. After removing the Triton X-100 and washing with PBS, intracellular Col I, Tnc, Scx, and Runx2 were tagged with the respective primary antibody: Polyclonal Rabbit Anti-Collagen 1 Antibody (Cosmo Bio), Scleraxis Goat Polyclonal Antibody, Tenascin-*C* Mouse Monoclonal Antibody, and Runx2 Mouse Monoclonal Antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Each antibody was diluted to 1:200 in 1% bovine serum albumin (BSA; Wako Pure Chemical Industries Ltd.) and reacted with the cells for 1 h at room temperature. After washing in PBS, the cells were incubated for 30 min at room temperature with the appropriate secondary antibody: Goat Anti-Rabbit IgG-FITC (Santa Cruz Biotechnology) for Col I, Donkey Anti-Goat IgG H&L (DyLight 405) (Rockland Immunochemicals Inc., Limerick, PA, USA) for Scx, and Goat Anti-Mouse IgG-Alexa Fluor 647 (Santa Cruz Biotechnology) for Tnc and Runx2, diluted to 1:300 in 1% BSA. The fluorescence wavelengths were 522, 420 and 665 nm for Col I, Scx, Tnc and Runx2, respectively. The PDMS chambers with the stained cells were cut into a shape similar to a glass slide. The periphery of the samples was sealed with nail polish to keep from drying out before observation. Finally, the samples were observed under a confocal microscope (A1Rsi-N; Nikon Instech Co, Ltd., Tokyo, Japan). The conjugation and affinity of the secondary antibodies, and that photobleaching did not affect the results. Runx2 staining for osteogenic differentiation was performed separately from Col I, Scx and Tnc staining for tenogenic differentiation.

Image processing was performed with ImageJ (available at http://rsb.info.nih.gov/ij/) to evaluate the fluorescence intensities of the respective cellular proteins, corresponding to synthesized protein levels. First, we selected more than 30 cells oriented parallel to the grooves of the cell culture chamber. Then, the fluorescence intensity histogram of the respective proteins in each cell was obtained. Finally, the mean fluorescence intensities (i.e., $(\bar{I}_{\text{align}})_{\text{Col I}}$, $(\bar{I}_{\text{align}})_{\text{Tnc}}$, $(\bar{I}_{\text{align}})_{\text{Scx}}$, and $(\bar{I}_{\text{align}})_{\text{Runx2}}$ were determined. After evaluation of fluctuation of the expression level of the respective proteins in each cell, we selected more than 30 cells oriented parallel to the grooves (new evaluation technique) and more than 30 cells oriented randomly (ordinary evaluation technique) in the cell culture chamber, and then calculated the mean fluorescence intensities ($\bar{I}_{\text{align}}$ and $\bar{I}_{\text{random}}$) and standard deviations (σ_{align} and σ_{random}). The coefficient of variation (*CV*), expressed as

$$CV = \frac{\sigma}{\bar{I}} \times 100 , \tag{1}$$

was obtained and compared between the new and ordinary evaluation techniques. The relative fluorescence intensity (*RFI*) of the respective proteins in the cells was determined according to equation

$$RFI = \frac{(\bar{I}_{\text{align}})_t}{(\bar{I}_{\text{align}})_{t=0}} \tag{2}$$

where t represents stretch duration (0 (control), 1, 3, 6, 12, 24, and 48 h). The *RFI* indicates the ratio of protein expression between duration t and $t = 0$, given the fluorescence intensity corresponds to the expression level of the respective proteins derived from each cell.

The fluorescence intensity of each protein at $t = 0$ was employed as the control since we confirmed that the microgrooves of the PDMS membrane didn't have an effect on the genes expression of the cells with time. The results were analyzed with a paired student's *t*-test; $p < 0.01$ was considered to be statistically significant.

3. Results

3.1. Efficacy of the fluorescence intensity evaluation in aligned cells

Figure 4 shows the CV of the fluorescence intensity of each protein between the parallel cells (new evaluation technique) and the randomly selected cells (ordinary evaluation technique). The *CV* was reduced by more than 20% for all proteins; only Col I did not show a statistically significant difference.

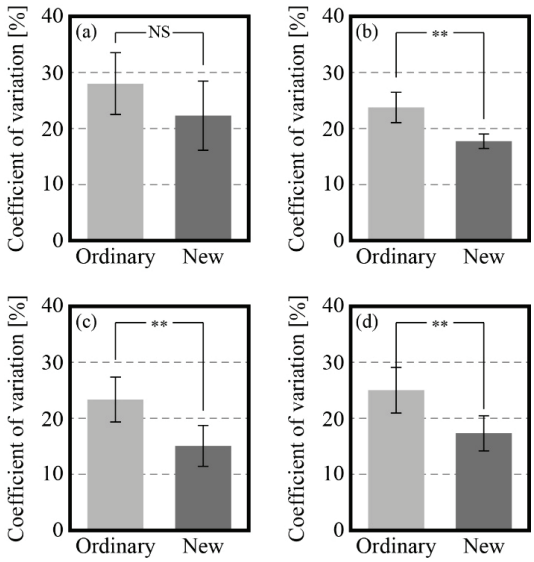

Fig. 4. Comparison of the coefficient of variation (*CV*) of the fluorescence intensities for (a) Col I, (b) Tnc, (c) Scx, and (d) Runx2 between the correctly aligned (New) and randomly selected (Ordinary) cells. Error bars indicate standard deviation. ** $p < 0.01$. NS = not significant

3.2. Differentiation of hBMSCs into tenocytes

Figure 5 exhibits some examples of the immunofluorescence micrographs showing the proteins for 48-h cyclic stretching. The *RFI*s of the tenogenic proteins are shown in Fig. 6. Cellular protein synthesis began to increase soon after applying the stretching stimulus, and gradually reached a plateau. The stretch stimulus increased the expression of Col I, Tnc, and Scx 2.2, 1.4, and 1.4 times, respectively.

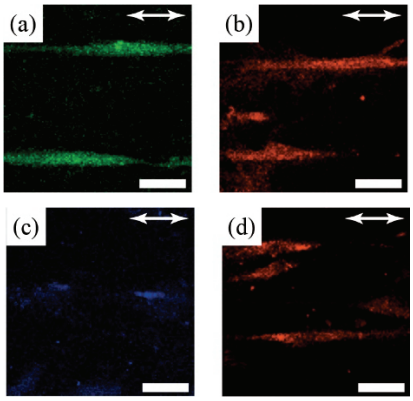

Fig. 5. Immunofluorescence micrographs showing: (a) Col I, (b) Tnc, (c) Scx, and (d) Runx2 for 48-h cyclic stretching. Direction of arrows corresponds to the cyclic stretch direction. Scale bars = 50 μm

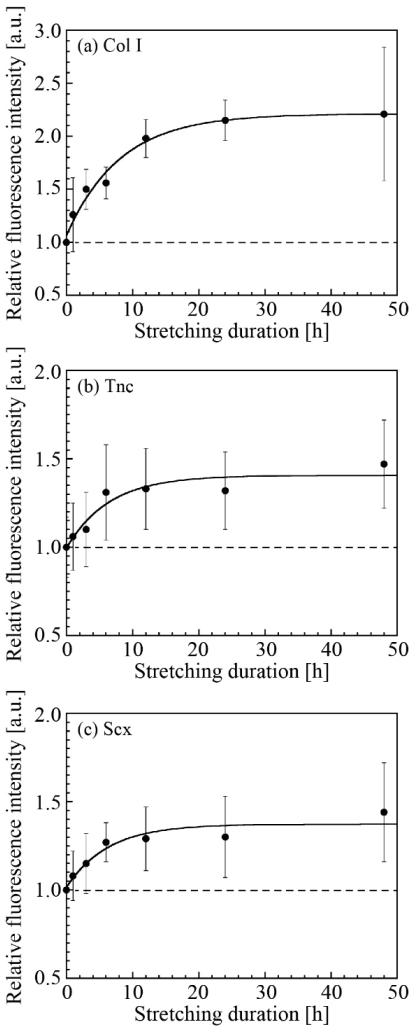

Fig. 6. Protein expression levels of: (a) Col I, (b) Tnc, and (c) Scx derived from cyclic-stretched cells over time. Error bars indicate standard deviation

3.3. Differentiation of hBMSCs into osteoblasts

Figure 7 shows the Runx2 expression levels. Based on these results, osteogenic differentiation of the hBMSCs was not promoted under the mechanical stimulus conditions used in this study, since Runx2 synthesis decreased compared with the control.

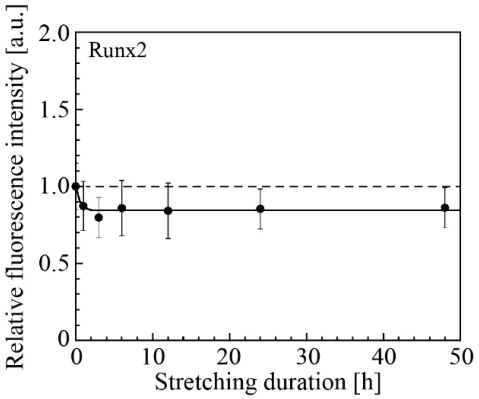

Fig. 7. Protein expression level of Runx2 derived from the cyclic-stretched cells over time

4. Discussion

The results shown in Fig. 4 suggest that selecting cells parallel to the microgrooves enables a more precise evaluation of differentiation. One might be able to point out that even the fabricated microgrooves of the PDMS membrane must involve an effect on the protein expression levels of the hBMSCs, even if the cyclic stretch stimulus isn't applied. Figure 8 shows the effect of the microgrooves on the protein expression levels of the hBMSCs. The value of the longitudinal axis is normalized by the protein expression of hBMSCs cultured on PDMS membrane without microgrooves. It can be concluded that the microgrooves of the PDMS membrane do not have any influence on the tenogenic differentiation.

There are few reports regarding the tenogenic differentiation time of hBMSCs under cyclic stretch stimulus. But typically, since cellular expression levels of mRNAs or proteins can change exponentially when cells are subjected to internal or external perturbation [1], each protein expression was fitted with an exponential function (solid lines, Fig. 6). The protein synthesis rates were obtained by differentiating the functions with respect to time (Fig. 9). The synthesis rates in Fig. 9 more clearly show that protein expression increased markedly due to mechanical stimulation. We determined the time when protein expression was nearly complete (95%), based on the expression levels (Fig. 6) and rates (Fig. 9). The lengths of time coinciding with the proteins were about 11 h for Col I and Tnc, and 10 h for Scx, respectively. We would assume 10 h as the time spent on the initial cellular differentiation since Scx is one of the most important key markers for tenogenic differentiation [13]. It suggests that half a day is enough for the induction of the differentiation, although most researchers employed more than 48 h to promote the differentiation [14], [16], [17], [29], [30]. The time for the differentiation is quite short compared with the one induced using chemical stimulus or co-culture [7], [19], [30]. The differentiation time obtained in this study is the shortest in tenogenic differentiation of hBMSCs with cyclic stretch stimulus, since we made the mechanical condition of this study similar to mechanical circumstances of *in vivo* tendon tissues. That is, actual *in vivo* tendon tissues are usually subjected to 5% elongation [3], [27] and 1 Hz cyclic frequency [22]. In addition, duration time obtained in this work corresponds to the time required for cells to reorganize their cytoskeletons and alter the morphology of their nucleus and themselves under mechanical stimulation [26]. When cells are constantly subjected to cyclic stretch stimulus, they begin to drastically remodel their cytoskeletons within several hours [10], and the process is complete in approximately 10 h [24]. This suggests that cells subjected to mechanical stimulus secrete mRNAs and proteins for both cytoskeleton reorganization and differentiation.

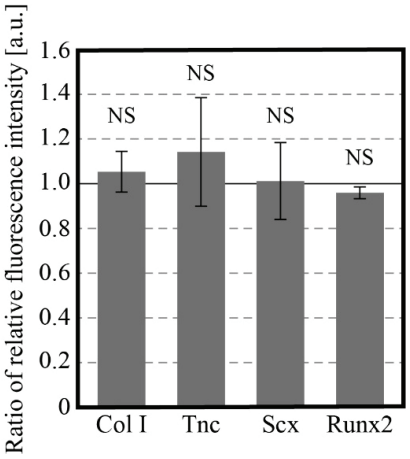

Fig. 8. Comparison of protein expression levels of hBMSCs cultured on PDMS membrane with and without the microgrooves. Both are subjected to the no-cyclic stretching. The value of longitudinal axis is normalized by the relative fluorescent intensity of hBMSCs cultured on PDMS membrane without the microgrooves. NS = not significant

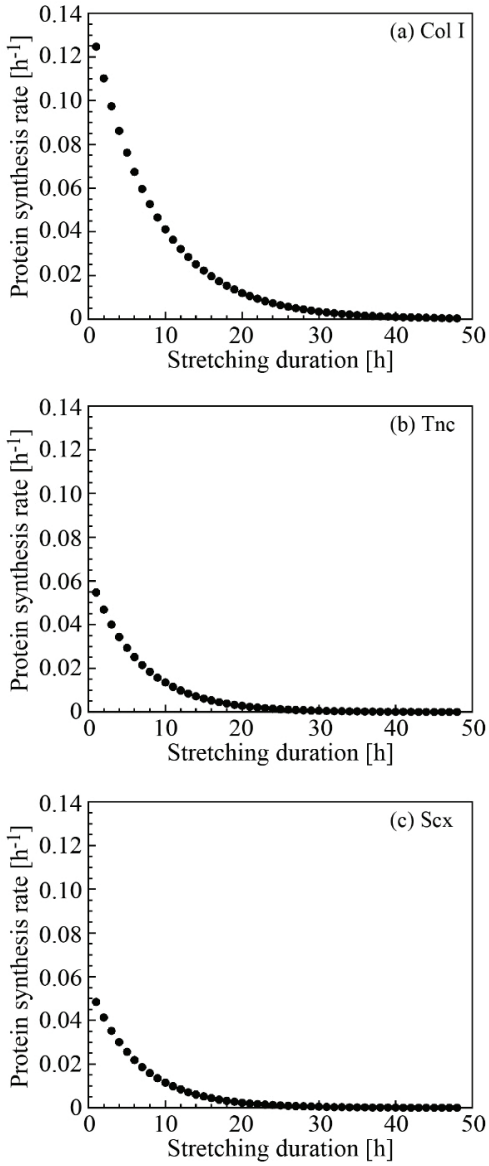

Fig. 9. Protein synthesis rate of: (a) Col I, (b) Tnc, and (c) Scx derived from the cyclic-stretched cells over time

5. Conclusions

Plating cells on a microgrooved PDMS membrane to avoid cellular alignment and selecting cells oriented parallel to the stretch direction for measuring improved the precision of the tenogenic differentiation evaluation. The hBMSCs stimulated with an optimal cyclic stretching required approximately 10 h to differentiate into tenocytes, based on the evaluation of protein levels related to differentiation. This duration coincided with the time necessary for cytoskeleton reorganization and alteration of cellular morphology.

Acknowledgements

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