

Determination of optimal cyclic uniaxial stretches for stem cell-to-tenocyte differentiation under a wide range of mechanical stretch conditions by evaluating gene expression and protein synthesis levels

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We examined optimal cyclic uniaxial stretches for stem cell-to-tenocyte differentiation by applying a wide range of cyclic mechanical stimuli. Human bone marrow mesenchymal stem cells (hBMSCs) were subjected to three types of cyclic elongation of 5%, 10%, or 15% at a cyclic frequency of 1 Hz for 24 h or 48 h, and differentiation into tenocytes was assessed by two methods: real-time polymerase chain reaction determination of gene expression levels and western blotting analysis of protein expression levels. The gene expression levels of the differentiation markers type I collagen (Col I), type III collagen (Col III), tenascin-C (Tnc), and scleraxis (Scx), all of which are constituents of tendon tissue, were increased when cells were exposed to 10% stretching stimulation. The levels of Col I and Tnc protein synthesis levels were also higher in the cells with 10% stretching stimulation than in those subjected to other stimuli. The results indicated that 10% stretching stimulus was efficient to induce the differentiation of hBMSCs into tenocytes. In addition, the changes in gene and protein expression levels were strongly correlated with cell orientation angle. The results presented here suggest that mesenchymal stem cell-to-tenocyte differentiation is strongly associated with cumulative elongation load on the cells. This work provides novel insights into the differentiation of tenogenesis in a strain-induced environment and supports the therapeutic potential of hBMSCs.

Key words: human bone marrow mesenchymal stem cell (hBMSC), differentiation, mechanical stretch, tenocyte

1. Introduction

Tendon tissue is a type of connective tissue that physically binds muscles to skeletal structures permitting locomotion and enhancing joint stability [1]–[3]. Many tendon injuries, ranging from repetitive strain injuries to complete ruptures, occur in athletes and other active people, and the effects of having tendon tissue with reduced functionality can be devastating to their everyday lives. Tendon injuries are difficult to manage and although spontaneous healing can occur this often results in the formation of scar tissue, which is morphologically, biochemically, and biomechanically different from healthy tendon tissue [4]. As the structure of repaired tissue differs from healthy tissue

in terms of functionality, movement, and strength [5]–[9], current conservative and surgical treatments show limited success [9].

Thus, there is a need for tendon tissue engineering. One aim of tendon tissue engineering is to produce a functional tissue replacement 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 then be implanted into the site of injury for new tissue formation to take place. This strategy relies on the availability of an appropriate cell source. The main problems are the low number of cells obtained from explanted tissue because tendons are relatively acellular and low cell density of tenocytes [10], and they are terminally differentiated with very limited

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proliferative capacity [11]. Hence, bone marrow mesenchymal stem cells (BMSCs) are commonly used in this field of study due to their high proliferative capacity and pluripotency [12]–[16].

Numerous strategies using chemical stimulation for enhancement of tendonogenesis have been reported to date. A number of growth factors (e.g., CTGF [17], GDF-5/BMP-14 [18], [19], GDF-6/BMP-13 [20], among others) induce differentiation of mesenchymal stem cells (MSCs) into tenocytes. However, mechanical stimuli are very important to the development of tendon. For functional tendon tissue engineering, chemical stimulation is not enough, and mechanical stimuli should be introduced to improve the effect of tendon repair.

During everyday movement of the body, the tendons are subjected to numerous types of mechanical strain. Tendons respond to mechanical forces by adapting their metabolism and structural and mechanical properties [2]. Mechanical stretching appears to influence human tendon fibroblast proliferation [21] and to increase the production of collagen, the primary constituent of tendon tissue [22]–[24]. Furthermore, the application of mechanical stretching appears to stimulate MSCs to proliferate and differentiate into tenocytes [25]–[29]. Therefore, mechanical stimulation is being investigated as a critical method for inducing differentiation. For example, Chen et al. evaluated MSC-to-tenocyte differentiation using quantitative reverse transcription polymerase chain reaction (qRT-PCR), analysis of mRNA expression levels, by subjecting the cells to 3% or 10% stretching mechanical stimulation at 1 Hz [28]. Kuo and Tuan reported differentiation using qRT-PCR by subjecting the cells to 1% stretching mechanical stimulation at 1 Hz [26]. Type I collagen (Col I), type III collagen (Col III), tenascin-C (Tnc), and scleraxis (Scx) were usually used to describe the differentiation into tenocyte [29], [30]. Col I molecules self-assemble into highly organized fibrils that form collagen fibers [31]. Cross-linking of these fibers in the extracellular matrix (ECM) gives them a high tensile strength and provides mechanical strength for tendon tissue [32]. Col III forms smaller, less organized fibrils [33]. 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 [34]. Scx is a transcription factor specifically expressed in tendons and ligaments, which are involved in the activation of $\text{pro}\alpha 1(\text{I})$ collagen gene expression in tendon fibroblasts [32].

Differentiation into tenocyte has not been examined in a systematic manner using cells from the same

source, and it has not yet been confirmed what degree of elongation is suitable for differentiation. Therefore, in the present study, MSC-to-tenocyte differentiation was evaluated by determining mRNA expression levels using qRT-PCR and levels of protein synthesis by western blotting. The mechanical stimulation conditions were sinusoidal 1-Hz cyclic stretching at 5%, 10%, or 15% elongation over 24 h or 48 h. No previous studies have used such a broad range of elongation and two methods of analyzing differentiation, i.e., both gene and protein expression levels, in cells from an identical source.

2. Materials and methods

2.1. Cells and culture with mechanical cyclic stretching

Human bone marrow mesenchymal stem cells (hBMSCs; cell line JCRB1136; Health Science Research Resources Bank, Tokyo, Japan) were maintained in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 0.5% GlutaMax, and 0.05% gentamicin (Invitrogen, Carlsbad, CA) at 37 °C under an atmosphere of 5% CO₂ in a humidified incubator (Sanyo, Tokyo, Japan). The hBMSCs were cultured in 25 cm² culture flasks (BD Biosciences, Franklin Lakes, NJ) at an initial density of 1.0×10^4 cells/cm² for expansion without differentiation. The medium was replaced with fresh medium every 3 days. At near-confluence, which occurred every 5–7 days, cells were detached from culture flasks with 0.25% trypsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan)/1 mM EDTA (Takara Bio, Shiga, Japan) and seeded into new culture flasks.

A simple stretching device (STB-140; Strex, Osaka, Japan), fitted with elastic silicone rubber chambers (Fig. 1a), was used to apply uniaxial cyclic deformation. The elastic chambers had a transparent bottom (32×32 mm², 100 μ m thick) and walls that were 9.9 mm in height (Fig. 1b). The chamber was deformable by up to 20% along a single direction by use of a stainless steel grappling-hook device. Uniaxial deformation of the elastic substrate was accompanied by a small degree of subsidiary deformation in the orthogonal direction because the sides of the elastic substrate were allowed to deform. Although the orthogonal deformation was suppressed by the thick walls of the elastic chamber, elongation of 10% induced up to approximately 4.3% perpendicular retraction.

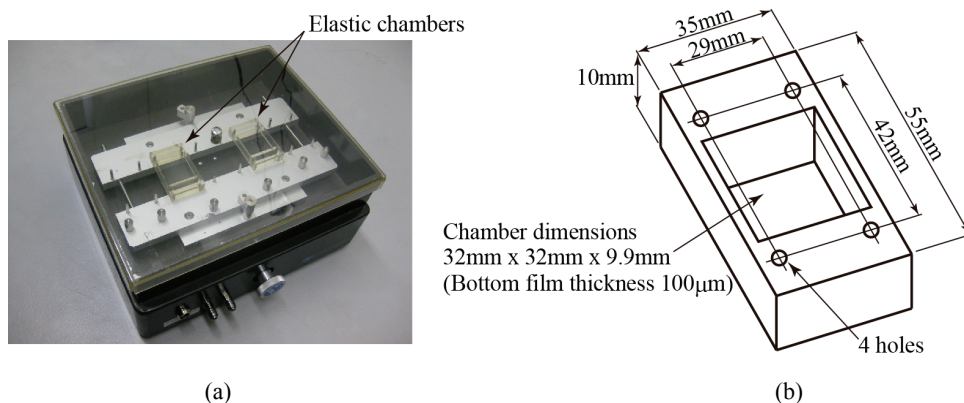


Fig. 1. System for cell culture with mechanical cyclic uniaxial stretching: (a) cell stretching device with two elastic chambers, (b) geometry of the elastic chamber

The elastic chambers were rinsed in distilled water in an ultrasonic bath for 15 min, placed in sterile phosphate-buffered saline (PBS; Cosmo Bio, Tokyo, Japan), and sterilized by exposure to ultraviolet light in a sterile hood for 30 min. Then they were coated with human fibronectin (R&D Systems, Minneapolis, MN) at a concentration of $1 \mu\text{g}/\text{cm}^2$ for 3 h. Trypsinized hBMSCs were plated onto the bottom of the chambers at a density of 1.0×10^4 cells/mL and cultured as described above for 2 days without cyclic stretching. Finally, 1-Hz uniaxial cyclic stretching of 5%, 10%, or 15% elongation was applied to the hBMSCs in the culture environment over 24 h or 48 h, because we had found that 1.0 Hz cyclic frequency is the best condition for the proliferation of hBMSCs [35], and a lot of researchers had used this frequency as a typical condition to demonstrate the differentiation behavior of hBMSCs [23], [26], [28], [36]–[38]. The experimental conditions are summarized in Table 1. Control cells were treated similarly but were not subjected to cyclic stretching.

Table 1. Experimental conditions

	Control cells group	Mechanical stimulation cells groups					
		1					
Frequency [Hz]	0	1					
Elongation [%]	0	5		10		15	
Duration time [h]	0	24	48	24	48	24	48

2.2. Measurement of cell orientation

Cellular morphology was observed using a phase-contrast microscope (IX71; Olympus, Tokyo, Japan). Photographs were taken with a CCD camera (D300s; Nikon Co, Ltd., Tokyo, Japan) and analyzed using ImageJ software (NIH) to quantify the effects of dif-

ferent types of mechanical stretching on cell alignment. Each cell in the photographs was approximated as an ellipse and the long axis was determined. The orientation angle θ of each cell with respect to the stretch axis was determined (Fig. 2). To evaluate the degree of orienting response of the cells, we constructed histograms where cell frequency was plotted against the orientation angle. If a cell was located in the conjugated position, i.e., an orientation angle of $-\theta$, the cell orientation was considered to be identical to a cell oriented at θ . Five samples ($n = 5$) were used for each experimental condition. The cell frequency of respective orientation angles was summed for each experimental condition, and the means and standard errors were calculated.

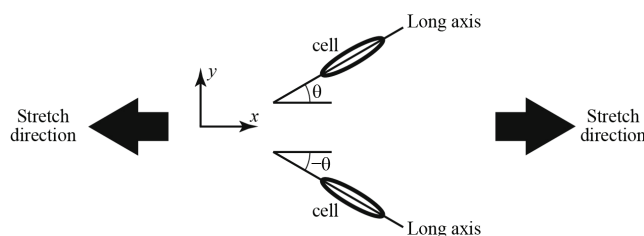


Fig. 2. Orientation angle θ definition of each cell

2.3. Quantitative RT-PCR

When the cyclic stretching period was completed, aliquots of the cells were lysed, and their total RNA was isolated using an RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). The purity and concentration of the RNA were assessed by determining the absorbance ratio at 260/280 nm. Reverse transcription was performed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control), Col I, Col III, Tnc, and Scx gene expression

levels were analyzed using pre-designed minor groove binder probes (Applied Biosystems), TaqMan PCR Master Mix (Applied Biosystems), and a Light Cycler apparatus (ABI 7300; Applied Biosystems). Gene expression levels were calculated using the standard curve method and normalized relative to the levels of GAPDH gene expression. Three samples were used for all experimental conditions.

2.4. Western blotting

Cell lysates were collected at the end of each experiment for each culture condition. Briefly, cells were washed with PBS, then 100 μ L of a detergent-based lysis buffer (M-PER Mammalian Protein Extraction Reagent; Pierce, Rockford, IL) and protease inhibitor PMSF and a cocktail of phosphatase inhibitors (1:100 dilution; Pierce) were added to each chamber for collection of total cellular proteins. Aliquots of 30 μ g protein from each sample were subjected to 8% SDS-PAGE. The separated proteins were transferred onto PVDF membranes (Bio-Rad, Hercules, CA) at 80 V for 120 min. The membranes were blocked in 5% BSA/TBS-Tween 20 solution at 4 $^{\circ}$ C overnight followed by application of monoclonal antibody specific for GAPDH (sc-48166; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2000, Col I (sc-8785; Santa Cruz Biotechnology), Tnc (sc-9871; Santa Cruz Biotechnology), and Runx2 (sc-8566; Santa Cruz Biotechnology) at 1:1000 in 5% BSA/TBS-Tween 20. After incubation for 120 min with primary antibody at room temperature, the secondary antibody, anti-goat IgG-HRP (sc-2354; Santa Cruz Biotechnology) was applied at 1:10000 in 5% BSA/TBS-Tween 20 for 1 h at room temperature. The membranes were washed three times with 0.1 TBS/Tween 20 for 10 min after each antibody application. The proteins on the PVDF

membranes were detected with the ECL detection system (Pierce), according to the manufacturer's protocol. The protein bands were quantified by volume summation of image pixels with a Fujifilm LAS-4000 (Fujifilm, Tokyo, Japan). Three samples were used for each experimental condition.

2.5. Statistical analysis

The means and standard deviations are reported for three or five repeat samples. The paired Student's *t* test was used for statistical analyses, and $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Changes in cellular morphology by mechanical stretching

Phase-contrast images showing the cellular morphologies of the control cells and those subjected to 5% (24 h), 5% (48 h), 10% (24 h), 10% (48 h), 15% (24 h), and 15% (48 h) stretching are shown in Fig. 3a–g, respectively. The application of sinusoidal cyclic stretching did not cause substantial changes in cell morphology. Stretching at 10% and 15% induced marked changes in cell orientation; the control and 5% stretched cells remained randomly oriented (Fig. 3a, b, e), whereas the cells subjected to 10% or 15% stretching began to orient almost perpendicular to the direction of stretching. The time-related changes in the orientation angle θ of each experimental condition are shown in Fig. 4. The control and 5% stretched cells did not show a distinct trend in terms of

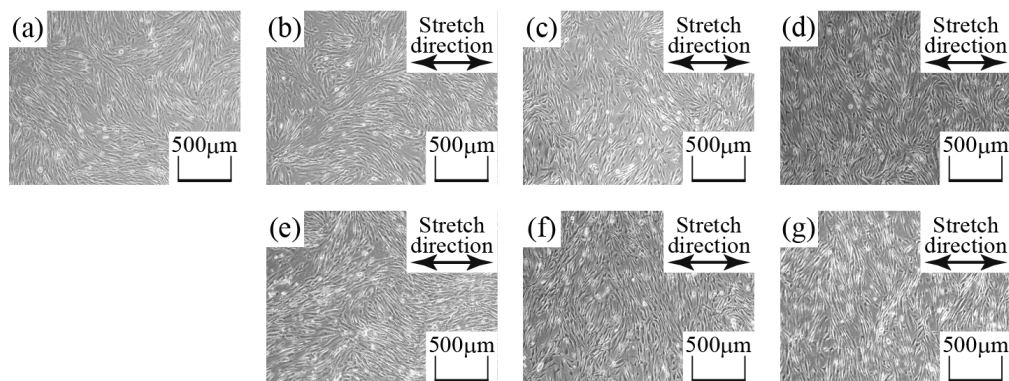


Fig. 3. Phase-contrast photomicrographs of cells subjected to cyclic uniaxial stretching: (a) control, (b) 5% stretching for 24 h, (c) 10% stretching for 24 h, (d) 15% stretching for 24 h, (e) 5% stretching for 48 h, (f) 10% stretching for 48 h, (g) 15% stretching for 48 h

orientation angle, and remained randomly oriented. The 15% stretched cells tended to be oriented in the same directions of 60–90° from 24 h. The orientation of 10% stretched cells showed the same tendency as the 15% stretched cells but took a longer time.

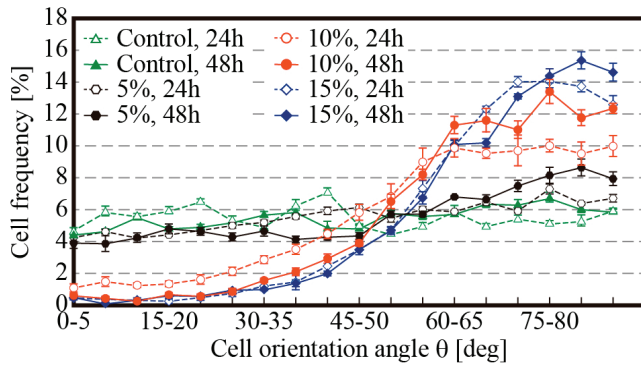


Fig. 4. Time-related changes in orientation angle θ in cells subjected to control, 5%, 10%, or 15% cyclic uniaxial stretching at 1 Hz for 24 h or 48 h as indicated

3.2. Changes in gene expressions by mechanical stretching

The time-related changes in gene expression for the MSC-to-tenocyte differentiation markers Col I,

Col III, Tnc, and Scx are shown in Fig. 5; the expression level of each gene is shown relative to that in the control cells (defined as 1.0). Almost all of the marker genes showed significant upregulation in the 10% stretched cells. In these cells, the levels of Col I and Col III gene expression remained almost constant although those of Tnc and Scx increased to 1.36–1.64 and 1.61–2.50, respectively, between 24 and 48 h. Downregulation or no significant differences in gene expression were observed for the control cells relative to the 5% and 15% stretched cells, although the level of Scx gene expression was specifically increased in the 5% stretched cells at 24 h as an exception. However, the margin of error was large and this was not statistically significant.

3.3. Changes in protein expressions by mechanical stretching

The time-related changes in Col I, Tnc, and Runx2 protein expression are shown in Fig. 6; the synthesis level of each protein is shown relative to that in the control cells (defined as 1.0) in Fig. 6b. The results were similar to those regarding mRNA expression, i.e., Col I and Tnc proteins showed significant upregulation in the 10% stretched cells. On the other

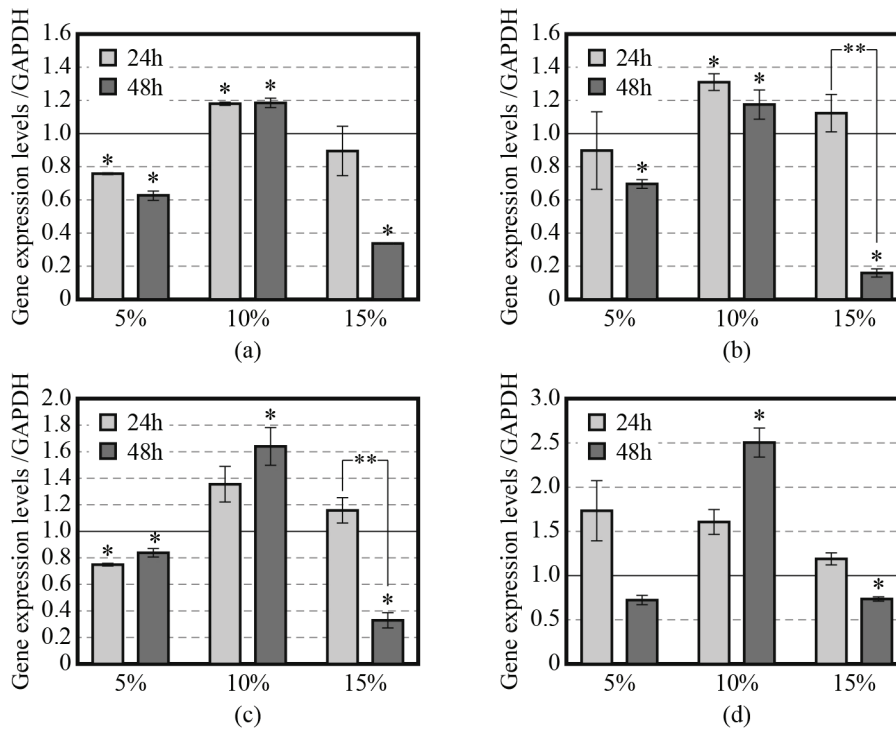


Fig. 5. Time-related changes in (a) Col I, (b) Col III, (c) Tnc, and (d) Scx mRNA levels in cells subjected to 5%, 10%, or 15% cyclic uniaxial stretching at 1 Hz for 24 h or 48 h as indicated.

Data are normalized to the corresponding mRNA levels in non-stretched cells (defined as 1).

* $P < 0.05$ relative to control; ** $P < 0.05$ relative between 24 h and 48 h

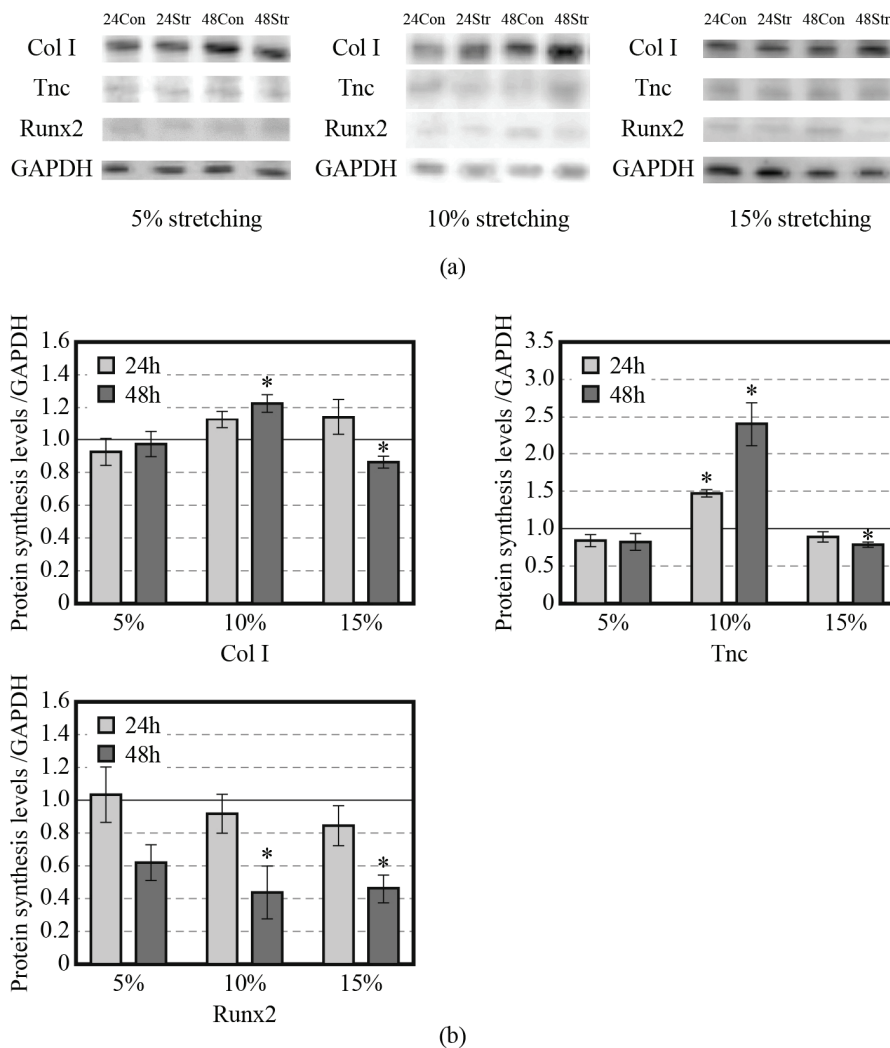


Fig. 6. Time-related changes in Col I, Tnc, and Runx2 protein levels in cells subjected to 5%, 10%, or 15% cyclic uniaxial stretching at 1 Hz for 24 h or 48 h as indicated: (a) results of the western blot. 24Con, 24Str, 48Con, and 48Str designate control for 24 h, stretching for 24 h, control for 48h, and stretching for 48 h, respectively; (b) quantitative measurements of the western blot results. Data are normalized to the corresponding protein levels in non-stretched cells (defined as 1).

* $P < 0.05$ relative to control

hand, the secretion of extracellular matrix was not active in the 5% or 15% stretched cells as the levels of Col I and Tnc protein synthesis were almost the same or decreased compared to those in the control cells. These observations indicate that such mechanical stimuli, i.e., 5% or 15% stretching, have little effect on MSC-to-tenocyte differentiation. The synthesis of Runx2 protein, which is an indicator of bone cell differentiation, did not increase under any of the experimental conditions at 24 h and showed a downward trend at 48 h.

4. Discussion

Our previous studies show that focal adhesion kinase, cytoskeleton and RhoA/ROCK play an im-

portant role in mechanical stretch-induced tenogenic differentiation of hBMSCs [36], [37]. We further investigated the effect of a wide range of mechanical stretching on differentiation of hBMSCs. From the perspective of gene expression, 10% stretching was the best condition to obtain MSC-to-tenocyte differentiation in the present study (Fig. 5). Similar results were reported previously by Chen et al. who evaluated MSC-to-tenocyte differentiation with uniaxial 3% or 10% stretching stimuli at 1 Hz [28], Zhang et al. who studied the differentiation potential of MSCs with uniaxial 10% stretching stimulation at 1 Hz [29], and Lee et al. who confirmed stem cell differentiation into ligament cells with uniaxial 10% stretching stimulation [38]. It was assumed that in 5% stretching stimulation the elongation amplitude would not be sufficient to provoke MSC-to-tenocyte differentiation. Kuo

and Tuan, however, reported that MSC-to-tenocyte differentiation was induced even with 1% stretching stimulation although the necessary duration of stimulation was as long as 7 days [26]. Conversely, Chen et al. reported that MSC-to-tenocyte differentiation was not stimulated by 3% stretching for 48 h [28]. These results suggest that the MSC-to-tenocyte differentiation by cyclic mechanical stimulation must take into account not only elongation amplitude but also the duration of stimulation. Thus, MSC-to-tenocyte differentiation may be related to the cumulative elongation load of elongation amplitude and duration of stimulation. Furthermore, a certain threshold of cumulative elongation load may exist for differentiation of MSCs into tenocytes. If so, MSC-to-tenocyte differentiation with stretching stimulation above 10% would occur earlier than with 10% stretching stimulation because the 10% stretched MSCs definitely differentiated into tenocytes (Fig. 5). However, the 15% stretched cells did not show such promotion. Rather, the gene expression levels remained constant or decreased with 15% stretching stimulation, suggesting that there is an upper limit in terms of elongation amplitude. Indeed, Costa et al. noted that a large degree of elongation (>15%) can destroy some cellular functions [39].

Axial strain of a cell, or mechanical strain along the long axis of an elliptical cell, must be discussed when investigating the relationship between differentiation and uniaxial mechanical stimulation. The cell orientation response, studied in detail by Neidlinger-Wilke et al. [40] and Wang et al. [41], is an important factor. Figure 7 shows the theoretical relationship between axial strain of cell and cell orientation angle θ when the chamber is stretched at 10% elongation [40]. Cells become oriented at an orientation angle of more than 60° to reduce axial strain of cell, as shown in this figure [29], [40]–[42]. The gene expression levels can be explained to some degree based on this relationship. The changes in gene expression levels under each experimental condition, calculated from the results shown in Fig. 5, are shown in Table 2. The cells were exposed to large axial strain loads as cell alignment had not been yet progressed sufficiently within the stimulation time of 0–24 h (Fig. 4). Thus, the changes in gene expression levels were high (Table 2). On the other hand, between 24 and 48 h, the cells experienced small axial strain loads because cell alignment had advanced sufficiently with stimulation times (Fig. 4). Therefore, the changes in expression level of each gene were relatively low compared to those at 0–24 h (Table 2). The degrees of increase in Tnc and Scx gene expression levels, however, did not

decrease markedly. This discrepancy in change rate between Col I/Col III and Tnc/Scx was attributed to the time lag of gene expression, as also suggested by Zhang et al. [29]. Thus, each gene expression profile may have a different mechanical threshold.

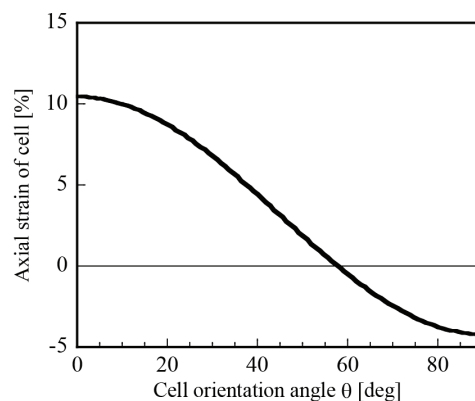


Fig. 7. Relation between axial strain of cell and cell orientation angle when the chamber is stretched at 10%

Table 2. Changes in gene expression levels in 10% stretched cells

	Col I	Col III	Tnc	Scx
0 → 24 h	1.18	1.31	1.36	1.61
24 → 48 h	1.00	-1.11	1.21	1.56

The protein synthesis levels of Col I and Tnc showed the same tendency as the respective gene expression levels (Fig. 6). The observations of protein synthesis levels also led to the same conclusion that 10% stretching stimulation was the best condition in this study in terms of differentiation and secretion of extracellular matrix, because the protein synthesis levels corresponded to secretion of extracellular matrix. These results support those reported by Zhang et al. [29], who evaluated MSC-to-tenocyte differentiation by monitoring protein synthesis levels with cyclic mechanical stimulation at 1 Hz. Several studies have indicated that uniaxial cyclic stretching induces differentiation of MSCs into bone cells [28], [43]. Differentiation into bone cells was suppressed in the present study, as indicated by Runx2, a typical osseous protein, which was downregulated over time (Fig. 6).

5. Conclusion

Cells were subjected to cyclic elongation of 5%, 10%, or 15% at a cyclic frequency of 1 Hz for 24 h or 48 h, and differentiation was assessed. This is the first study involving such a wide range of elongation levels

and evaluation of differentiation by monitoring both gene and protein expression levels. The results indicated that the gene expression levels of tendon-related markers Col I, Col III, Tnc, and Scx, were high in cells subjected to 10% stretching stimulation. The protein synthesis levels of Col I and Tnc were also higher in cells subjected to 10% stretching stimulation. Therefore, 10% stretching stimulation was the best condition for MSC differentiation into tenocytes. In addition, the changes in both gene and protein expression levels were strongly correlated with cell orientation angle. Thus, MSC-to-tenocyte differentiation is strongly associated with the cumulative elongation load of the cells, which is dependent on the elongation amplitude and duration of stimulation.

References

- [1] WOLFMAN N.M., HATTERSLEY G., COX K., CELESTE A.J., NELSON R., YAMAJI N., DUBE J.L., DiBLASIO SMITH E., NOVE J., SONG J.J., WOZNEY J.M., ROSEN V., *Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family*, J. Clin. Invest., 1997, Vol. 100(2), 321–330.
- [2] WANG J.H.C., *Mechanobiology of tendon*, J. Biomech., 2006, Vol. 39(9), 1563–1583.
- [3] GOH J.C.H., OUYANG H.W., TEOH S.H., CHAN C.K.C., LEE E.H., *Tissue-engineering approach to the repair and regeneration of tendons and ligaments*, Tissue Eng., 2003, Vol. 9(1), S31–S44.
- [4] SHARMA P., MAFFULLI N., *Biology of tendon injury: healing, modeling and remodeling*, J. Musculoskelet. Neuronal. Interact., 2006, Vol. 6(2), 181–190.
- [5] SHARMA P., MAFFULLI N., *Current concepts review tendon injury and tendinopathy: Healing and repair*, J. Bone Joint Surg. Am., 2005, Vol. 87A(1), 187–202.
- [6] MIYASHITA H., OCHI M., IKUTA Y., *Histological and biomechanical observations of the rabbit patellar tendon after removal of its central one-third*, Arch. Orthop. Traum. Su., 1997, Vol. 116(8), 454–462.
- [7] TOHYAMA H., YASUDA K., KITAMURA Y., YAMAMOTO E., HAYASHI K., *The changes in mechanical properties of regenerated and residual tissues in the patellar tendon after removal of its central portion*, Clin. Biomech., 2003, Vol. 18(8), 765–772.
- [8] CHAN B.P., FU S.C., QIN L., ROLF C., CHAN K.M., *Pyridinoline in relation to ultimate stress of the patellar tendon during healing: An animal study*, J. Orthop. Res., 1998, Vol. 16(5), 597–603.
- [9] BAGNANINCHI P.O., YANG Y., EL HAJ A.J., MAFFULLI N., *Tissue engineering for tendon repair*, Brit. J. Sport. Med., 2007, Vol. 41(8), e10.
- [10] BULLOUGH R., FINNIGAN T., KAY A., MAFFULLI N., FORSYTH N.R., *Tendon repair through stem cell intervention: Cellular and molecular approaches*, Disabil. Rehabil., 2008, Vol. 30(20–22), 1746–1751.
- [11] HAMPSON K., FORSYTH N.R., EL HAJ A., MAFFULLI N., *Tendon Tissue Engineering*, [in:] N. Ashammakhi, R. Reis, F. Chiellini (eds.), *Topics in Tissue Engineering*, Vol. 4, Chapter 3, 2008.
- [12] PITTENGER M.F., MACKAY A.M., BECK S.C., JAISWAL R.K., DOUGLAS R., MOSCA J.D., MOORMAN MA., SIMONETTI DW., CRAIG S., MARSHAK DR., *Multilineage potential of adult human mesenchymal stem cells*, Science, 1999, Vol. 284(5411), 143–147.
- [13] CAPLAN A., BRUDER S.P., *Mesenchymal stem cells: Building blocks for molecular medicine in the 21st century*, Trends Mol. Med., 2001, Vol. 7(6), 259–264.
- [14] GIOVANNINI S., BREHM W., MAINIL-VARLET P., NESIC D., *Multilineage differentiation potential of equine blood-derived fibroblast-like cells*, Differentiation, 2008, Vol. 76(2), 118–129.
- [15] AWAD H., BUTLER D.L., BOIVIN G.P., SMITH F.N.L., MALAVIYA P., HUIBREGTSE B., CAPLAN A.I., *Autologous mesenchymal stem cell-mediated repair of tendon*, Tissue Eng., 1999, Vol. 5(3), 267–277.
- [16] FRIEDENS A.J., PETRAKOV K.V., KUROLESO A.I., FROLOVA G.P., *Heterotopic transplants of bone marrow – analysis of precursor cells for osteogenic and hematopoietic tissues*, Transplantation, 1968, Vol. 6(2), 230–247.
- [17] LEE C.H., MOIOLI E.K., MAO J.J., *Fibroblastic differentiation of human mesenchymal stem cells using connective tissue growth factor*, Conf. Proc. IEEE Eng. Med. Biol. Soc., 2006, Vol. 1, 775–778.
- [18] KOCH H., JADLOWIEC J.A., FU F.H., NONN J., MERK H.R., HOLLINGER J.O., CAMPBELL P.G., *The effect of growth/differentiation factor-5 (GDF-5) on genotype and phenotype in human adult mesenchymal stem cells*, Z. Orthop. Ihre Grenz., 2004, Vol. 142(2), 248–253.
- [19] PARK A., HOGAN M.V., KESTURU G.S., JAMES R., BALIAN G., CHHABRA A.B., *Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendon-specific markers*, Tissue Eng. A, 2010, Vol. 16(9), 2941–2951.
- [20] HADDAD-WEBER M., PRAGER P., KUNZ M., SEEFRIED L., JAKOB F., MURRAY M.M., EVANS C.H., NÖTH U., STEINERT A.F., *BMP12 and BMP13 gene transfer induce ligamentogenic differentiation in mesenchymal progenitor and anterior cruciate ligament cells*, Cytotherapy, 2010, Vol. 12(4), 505–513.
- [21] ZEICHEN J., VAN GRIENSVEN M., BOSCH U., *The proliferative response of isolated human tendon fibroblasts to cyclic biaxial mechanical strain*, Am. J. Sport Med., 2000, Vol. 28(6), 888–892.
- [22] TANAKA H., MANSKE P.R., PRUITT D.L., LARSON B.J., *Effect of cyclic tension on lacerated flexor tendons in-vitro*, J. Hand Surg.-AM., 1995, Vol. 20A(3), 467–473.
- [23] NÖTH U., SCHUPP K., HEYMER A., KALL S., JAKOB F., SCHUTZE N., BAUMANN B., BARTHEL T., EULERT J., HENDRICH C., *Anterior cruciate ligament constructs fabricated from human mesenchymal stem cells in a collagen type I hydrogel*, Cytotherapy, 2005, Vol. 7(5), 447–455.
- [24] YANG G.G., CRAWFORD R.C., WANG J.H.C., *Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions*, J. Biomech., 2004, Vol. 37(10), 1543–1550.
- [25] ALTMAN G.H., HORAN R.L., MARTIN I., FARHADI J., STARK P.R.H., VOLLOCH V., RICHMOND J.C., VUNJAK-NOVAKOVIC G., KAPLAN D.L., *Cell differentiation by mechanical stress*, Faseb J., 2001, Vol. 16(2), 270–272.
- [26] KUO C.K., TUAN R.S., *Mechanoactive tenogenic differentiation of human mesenchymal stem cells*, Tissue Eng. A, 2008, Vol. 14(10), 1615–1627.

- [27] ZHANG L., TRAN N., CHEN H.Q., KAHN C.J.F., MARCHAL S., GROUBATCH F., WANG X., *Time-related changes in expression of collagen types I and III and of tenascin-C in rat bone mesenchymal stem cells under co-culture with ligament fibroblasts or uniaxial stretching*, *Cell Tissue Res.*, 2008, Vol. 332(1), 101–109.
- [28] CHEN Y.J., HUANG C.H., LEE I.C., LEE Y.T., CHEN M.H., YOUNG T.H., *Effects of cyclic mechanical stretching on the mRNA expression of tendon/ligament-related and osteoblast-specific genes in human mesenchymal stem cells*, *Connect. Tissue Res.*, 2008, Vol. 49(1), 7–14.
- [29] ZHANG L., KAHN C.J.F., CHEN H.Q., TRAN N., WANG X., *Effect of uniaxial stretching on rat bone mesenchymal stem cell: Orientation and expressions of collagen types I and III and tenascin-C*, *Cell Biol. Int.*, 2008, Vol. 32(3), 344–352.
- [30] LUI P.P.Y., RUI Y.F., NI M., CHAN K.M., *Tenogenic differentiation of stem cells for tendon repair – what is the current evidence?*, *J. Tissue Eng. Regen. M.*, 2011, Vol. 5(8), e144–e163.
- [31] EYRE D.R., PAZ M.A., GALLOP P.M., *Cross-linking in collagen and elastin*, *Annu. Rev. Biochem.*, 1984, Vol. 53, 717–748.
- [32] LEJARD V., BRIDEAU G., BLAIS F., SALINGCARNBORIBOON R., WAGNER G., ROEHL M.H.A., NODA M., DUPREZ D., HOUILLIER P., ROSSERT J., *Scleraxis and NFATc regulate the expression of the pro-alpha1(I) collagen gene in tendon fibroblasts*, *J. Biol. Chem.*, 2007, Vol. 282(24), 17665–17675.
- [33] LAPIERE C.M., NUSGENS B., PIERARD G.E., *Interaction between collagen type-1 and type-3 in conditioning bundles organization*, *Connect. Tissue Res.*, 1977, Vol. 5(1), 21–29.
- [34] ELEFTERIOU F., EXPOSITO J.Y., GARRONE R., LETHIAS C., *Binding of tenascin-X to decorin*, *FEBS Lett.*, 2001, Vol. 495(1–2), 44–47.
- [35] SONG G., JU Y., SOYAMA H., OHASHI T., SATO M., *Regulation of cyclic longitudinal mechanical stretch on proliferation of human bone marrow mesenchymal stem cells*, *Mol. Cell. Biomech.*, 2007, Vol. 4(4), 201–210.
- [36] XU B., SONG G., JU Y., *Effect of focal adhesion kinase on the regulation of realignment and tenogenic differentiation of human mesenchymal stem cells by mechanical stretch*, *Connect. Tissue Res.*, 2011, Vol. 52(5), 373–379.
- [37] XU B., SONG G., JU Y., LI X., SONG Y., WATANABE S., *RhoA/ROCK, cytoskeletal dynamics, and focal adhesion kinase are required for mechanical stretch-induced tenogenic differentiation of human mesenchymal stem cells*, *J. Cell Physiol.*, 2012, Vol. 227(6), 2722–2729.
- [38] LEE I.C., WANG J.H., LEE Y.T., YOUNG T.H., *The differentiation of mesenchymal stem cells by mechanical stress or/and co-culture system*, *Biochem. Biophys. Res. Co.*, 2007, Vol. 352(1), 147–152.
- [39] COSTA K.D., HUCKER W.J., YIN F.C.P., *Buckling of actin stress fibers: A new wrinkle in the cytoskeletal tapestry*, *Cell Motil. Cytoskel.*, 2002, Vol. 52(4), 266–274.
- [40] NEIDLINGER-WILKE C., GROOD E.S., WANG J.H.C., BRAND R.A., CLAES L., *Cell alignment is induced by cyclic changes in cell length: studies of cells grown in cyclically stretched substrates*, *J. Orthop. Res.*, 2001, Vol. 19(2), 286–293.
- [41] WANG H.C., IP W., BOISSY R., GROOD E.S., *Cell orientation response to cyclically deformed substrates: Experimental validation of a cell model*, *J. Biomech.*, 1995, Vol. 28(12), 1543–1552.
- [42] MORITA Y., MUKAI T., JU Y., WATANABE S., *Evaluation of stem cell-to-tenocyte differentiation using atomic force microscopy to measure cellular elastic moduli*, *Cell Biochem. Biophys.*, 2013, Vol. 66(1), 73–80.
- [43] KOIKE M., SHIMOKAWA H., KANNO Z., OHYA K., SOMA K., *Effects of mechanical strain on proliferation and differentiation of bone marrow stromal cell line ST2*, *J. Bone Miner. Metab.*, 2005, Vol. 23(3), 219–225.