

Mechanical stretch-induced f-actin reorganization and tenogenic differentiation of human mesenchymal stem cells

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Human bone marrow mesenchymal stem cells (hMSCs) are multipotent adult stem cells which are capable of diverse lineages commitment and are considered as a promising cell source for various tissue repair and regeneration. It is well known that mechanical stimuli regulate the biological functions of hMSCs. The purpose of this paper is to investigate the effect of mechanical stretch on cytoskeleton reorganization, gene and protein expressions of differentiation-related markers. Our findings showed that mechanical stretch induced f-actin reorganization, promoted the gene expressions of tendon-related markers including collagen type I (Col I), collagen type III (Col III), tenascin-C (TNC) and scleraxis (SCX), and decreased the gene expressions of collagen type II (Col II) and MSC-protein (MSC-p). Our studies also manifested that the protein expressions of Col I and TNC were increased, and the protein expression of Runx2 was inhibited by mechanical stretch. These results indicate that mechanical stretch may trigger the differentiation of hMSCs into tenocytes. This work provides novel insights into the differentiation of tenogenesis in a strain-induced environment and supports the therapeutic potential of hMSCs.

Key words: human mesenchymal stem cells, mechanical stretch, f-actin, tenocytes, differentiation.

INTRODUCTION

Tendon is fibrous connective tissue which connects muscle to bone, and transmits the forces for body movement by change of its structure, mechanical properties and form (Goh *et al.*, 2003). However, tendon injuries from overuse or degeneration due to aging are common clinical problems because injured tendon has poor healing ability (Sharma & Maffulli, 2006). Traditionally, surgical technique and graft are used to bridge the gap of ruptured tendon or replace the missing tendon (Vunjak-Novakovic *et al.*, 2004). However, the healing effects of these methods are not always efficacious and there are many unsolved problems, such as reducing curing time etc. Therefore, tendon tissue engineering utilizing mesenchymal stem cells (MSCs) as seed cells is under develop-

ing as a more powerful healing strategy.

MSCs are multipotent adult stem cells which are capable of diverse lineages commitment (Pittenger *et al.*, 1999), and are considered to be a promising cell source for various tissue repair and regeneration. More interestingly, under mechanical stimuli, MSCs could realign vertically to the direction of mechanical force (Kurpinski *et al.*, 2006), and differentiate into osteoblasts (Jagodzynski *et al.*, 2004), chondrocytes (Huang *et al.*, 2004), smooth muscle cells (Kurpinski *et al.*, 2006) and tenocytes (Altman *et al.*, 2002). Our previous study also showed that focal adhesion kinase regulates mechanical stretch-induced tenogenic differentiation of MSCs (Xu *et al.*, 2011). The ability of mechanoactive tenogenic differentiation of MSCs is conducive to tendon repair and regeneration (Hoffmann *et al.*, 2006; Lee *et al.*, 2007; Kuo & Tuan, 2008; Zhang *et al.*, 2008). Moreover, an early moderate exercise was found having significant favorable effects on tendon healing, and indicating that mechanical

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stretch and MSCs have positive effect on tendon repair (Krampera *et al.*, 2006). However, during mechanical stretch-induced tenogenesis of MSCs, the alteration of cell orientation and cytoskeletal organization, and whether mechanical stimuli induces differentiation commitment of other cell lines are still not well understood.

This study focused on the changes in cytoskeletal organization, and the inadequate differentiation in the process of mechanical stretch-induced tenogenic differentiation of human bone marrow mesenchymal stem cells (hMSCs). To describe the tenogenic differentiation, the following genes were chosen to be investigated. Collagen type I (Col I) and collagen type III (Col III) are the main extracellular matrixes of tendon (Wang, 2006). Tenascin-C (TNC) is a multi-functional extracellular matrix glycoprotein during tendon development (Mehr *et al.*, 2000). Recently, scleraxis (SCX), a basic helix-loop-helix transcription factor, was associated to the development of tendon (Schweitzer *et al.*, 2001; Espira *et al.*, 2009). Therefore, Col I, Col III, TNC and SCX are commonly used to describe tenogenic differentiation. Collagen type II (Col II), MSC-protein (MSC-p) and Runx2 were selected as marker genes of chondrocytes (Mackay *et al.*, 1998), osteocytes (Salasnyk *et al.*, 2004) and MSCs (Chen *et al.*, 2008), respectively. This study may shed light into the mechanical stretch-induced tenogenic differentiation of hMSCs and the application of hMSCs for tendon tissue engineering.

MATERIALS AND METHODS

Cell culture

hMSCs (UE6E7T-3) were acquired from Health Science Research Resources Bank, and cultured in MSC

growth medium (Invitrogen) in 25 cm² culture flasks (Becton Dickinson Labware) at an initial density of 1×10^4 cells cm⁻² for expansion without differentiation. The phenotype, purity and differentiation potential of the commercial hMSCs have been identified by the Health Science Research Resources Bank. The cells were kept in a humidified incubator (Sanyo) at 37°C and supplemented with 5% CO₂. The culture medium was changed every 3 days. After reaching confluence (usually about 5-7 days) cells were detached with 0.25% trypsin/1 mM EDTA (Takara) and subcultured in 25 cm² culture flasks.

Cyclic mechanical stretch

A mechanical cell strain instrument (Model ST-140, STREX Co.), consisting of a control unit, a strain unit and rectangular silicone chambers, was used (Fig. 1A, B & C). During stretch experiments, only the strain unit was placed in the incubator. The chambers were used in the strain unit driven by an eccentric motor that allowed variation in the magnitude (2~20%) and frequency (0.01-1.5 Hz) of the applied strain (Song *et al.*, 2007). The mechanical stretch mode is shown in Figure 1D.

hMSCs were plated into chambers (10 cm², STREX Co.) pre-coated with human fibronectin (R & D) at an initial density of 1×10^4 cells cm⁻² at a concentration of 1 µg cm⁻², and were allowed to grow in the incubator for 24 hrs. Chambers were mounted onto the strain unit and exposed to a stretch treatment at amplitude of 10% strain and a frequency of 1 Hz for 24 hrs and 48 hrs (the following mechanical stretch in this study is under the same condition). As control, static cells were cultured in fibronectin pre-coated chambers without experiencing any stretch.

For cytoskeleton disruption studies, cytochalasin D (Calbiochem), an inhibitor of microfilament was

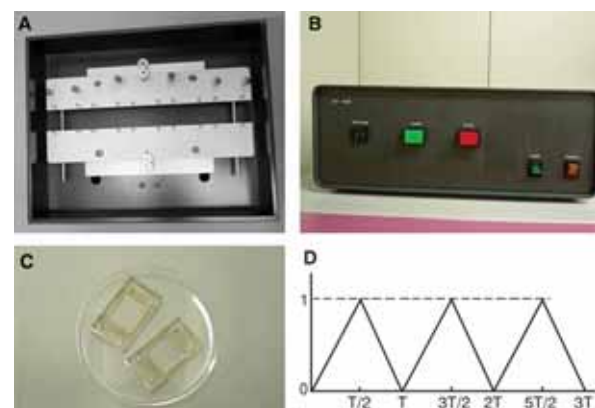


FIG. 1. Cell cyclical mechanical stretching device. A) Photograph of the strain unit of the mechanical strain instrument (Model ST-140, STREX Co., LTD, Japan); B) Photograph of the control unit of the instrument; C) Photograph of the silicon chambers for cell culture; D) The fashion of the stretch: 0 indicates free situation and 1 indicates stretched situation; stretch T/2, contract T/2 – repeat.

employed to block f-actin polymerization. Cytochalasin D was solubilized in 0.1% dimethylsulfoxide (DMSO) carrier, then the cells were pretreated with cytochalasin D ($0.1 \mu\text{g ml}^{-1}$) and DMSO carrier for 1 hr before exposure to mechanical stretch.

Cell orientation

Immediately after mechanical stimulation, we photographed cells on three regions of each membrane. The orientation of 20 cells in each cell group were determined using Image J. Cell orientation was defined as the angle (θ) between the long axis of the cell and the stretching direction (Fig. 2). The angle (θ) is acute angled and the value of θ belongs to the set of $[0^\circ, 90^\circ]$. The range 0° - 90° was divided into 9 groups, each encompassing a range of 10° . The percentage of cells in each group was used to express the cell orientation distribution.

Immunofluorescence staining

At the end of the experiment, the cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton-X-100 in PBS for 10 min. Cells were pre-incubated in 200 μl of 0.1 μM FITC conjugated phalloidin (Enzo Life Science) in primary blocking solution (1% BSA in PBS) at 4°C over-night. Then, the cells were incubated 0.1 mg ml^{-1} propidium iodide (PI, Enzo Life Science) in PBS for 5 min. After that, mounting medium (10 μl) was dispensed on the cells. The cells were washed three times with PBS for 5 min after each step. A glass coverslip was placed on the slide and sealed with nail polish before observation. Finally, the slides were visualized with a confocal microscope (Nikon A1Rsi, Nikon).

RNA isolation and real-time reverse transcription quantitative polymerase chain reaction (RT-PCR)

Cells were lysed at the end of the experiments, and total RNA was isolated using a Rneasy Mini Kit (Qiagen). The 260/280 absorbance ratio was measured for verification of the purity and concentration of the RNA. Reverse transcription was completed using a High Capacity RNA-to-cDNA Kit (ABI). Analysis by real-time RT-PCR was conducted using Taqman PCR Master Mix and predesigned minor groove binder (MGB) probes of glyceraldehydes 3-phosphate dehydrogenase (GAPDH, Hs99999905_ml). Collagen type I (Col I, Hs01028956_ml), collagen type II (Col II, Hs00156568_ml), collagen type III (Col III,

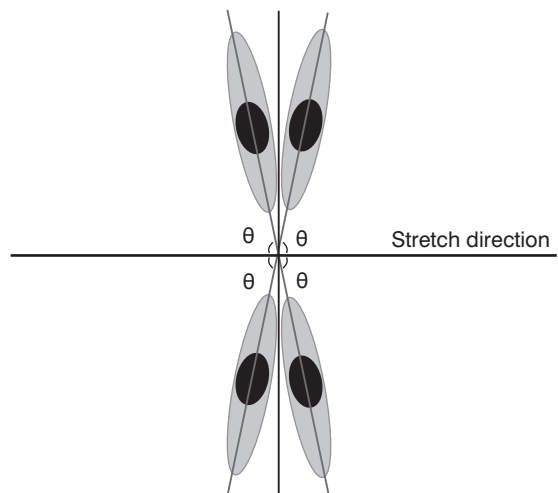


FIG. 2. Cell orientation. Cell orientation was defined as the angle θ ($\theta \in [0^\circ, 90^\circ]$) between the long axis of hMSC and the stretching direction. Four kinds of orientation of hMSCs subjected to uniaxial stretching in the x-direction are shown.

Hs00164103_ml), MSC-protein (MSC-p, Hs00414261_ml), tenascin-C (TNC, Hs00233648_ml), and scleraxis (SCX, Hs03054634_gl) (ABI) were used to detect the expressions of the genes of interest. The gene expression levels were calculated using the standard curve method and normalized to the expression of GAPDH.

Western blotting

Cell lysates were collected at the end of each experiment from each culture condition. Briefly, cells were washed with PBS, and then washed with 100 μl of detergent-based lysis buffer (M-PER Mammalian Protein Extraction Reagent, Pierce), and protease inhibitor PMSF and a cocktail of phosphatase inhibitors (Pierce, 1:100 dilution) were added to each chamber for collection of total cellular proteins. Equal amounts of proteins (5-15 μg) from each sample were loaded onto a 8% SDS-PAGE gel for electrophoresis. The separated proteins were transferred to a PVDF membrane (Bio-rad). The membrane was blocked in 5% BSA/TBS-Tween 20 solution at 4°C overnight followed by the application of primary monoclonal antibody specific for GAPDH (I-19: sc-48166, goat IgG), pro-Col1A2 (N-18: sc-8785, goat IgG), tenascin-C (N-19: sc-9871, goat IgG) and Runx2 (C-1: sc-8566, goat IgG) (all primary antibodies from Santa Cruz Biotechnology Inc.) at 1:1000 in 5% BSA/TBS-Tween 20. After overnight with primary antibody at 4°C , the secondary antibody, anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc.) at 1:10000 in 5% BSA/TBS-Tween 20 was applied for 1 hr at room

temperature. The membrane was washed three times with 0.1% TBS/Tween 20 for 10 min after each antibody application. The proteins on the PVDF membrane 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 Fujifilm LAS-4000 (Fujifilm).

Statistical analysis

The means and standard deviations were reported for three single repeat samples. A paired Student's *t*-test was performed, and a *p* value of less than 0.05 was considered to be statistically significant.

RESULTS

Cell orientation influenced by mechanical stretch

The alignments of the hMSCs were changed after stretch for 24 hrs and 48 hrs. The percentage of cells oriented at angles close to the stretch direction was significantly reduced. Most of the cells oriented to angles of 60°-90°. It was observed that about 70% of cells aligned in the range of 80°-90° after stretch for 24 hrs, and about 80% of cells aligned in the range of 80°-90° after stretch for 48 hrs. The percentage of cells oriented to angle of 80°-90° after stretch for 48 hrs was more than that of stretch for 24 hrs. In con-

trast, cells in the control group showed random orientation with no favoured direction (Fig. 3).

Influence of mechanical stretch on actin microstructure

To characterize mechanical stretch-induced alterations in cytoskeleton microstructure, actin staining with FITC-phalloidin and PI was conducted after with and without mechanical stretch for 24 hrs and 48 hrs (Fig. 4). It was observed that mechanical stretch altered cytoskeleton density and organization after stretch for 24 hrs and 48 hrs. As shown in Figure 4A & C, in the cases of 24 hrs and 48 hrs without stretch, the actin fibers were observed distributing in the edge of cell. Most of them aligned in the long axis direction of hMSCs. Moreover, some actin fiber networks were observed in the cytoplasm and only a few of actin fibers were observed near nucleus. However, as shown in Figure 4B & D, after stretch for 24 hrs and 48 hrs, the actin fiber density was increased and there was specific alignment of the fibers which was almost vertical to the direction of the stretch. The distribution of actin fiber after stretch for 24 hrs and 48 hrs was more homogeneous than that of the control. Many actin fibers were observed near nucleus besides in the edge of hMSCs. Moreover, most of the actin fibers aligned parallel to each other and there was almost no actin fiber network.

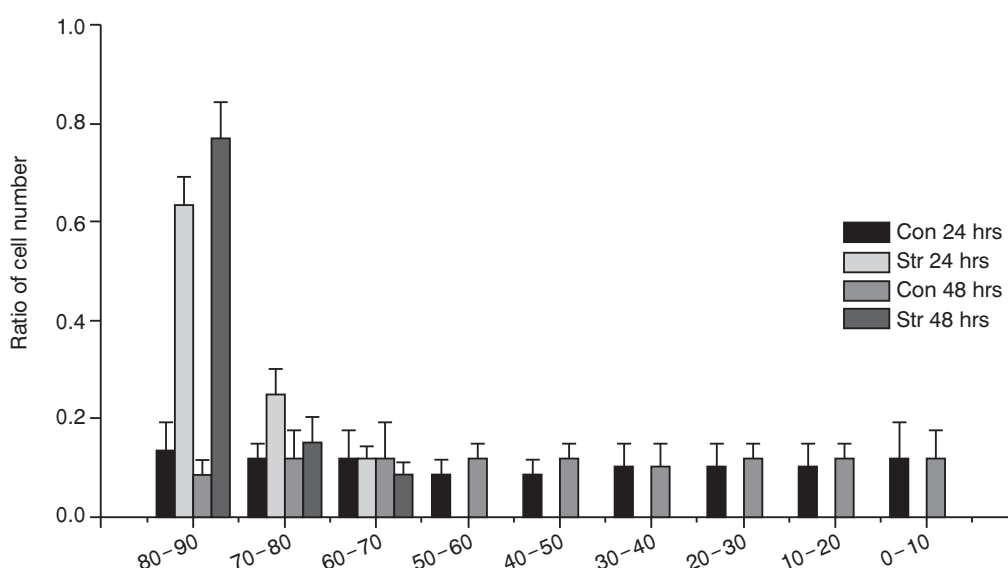


FIG. 3. Distribution of cell orientation. Mechanical stretch had significant influence on cell alignment. 20 cells were chosen randomly and analyzed. After stretch for 24 hrs or 48 hrs, the percentage of cells oriented at angles close to the stretch direction was significantly reduced. Most of the cells oriented to angles of 60°-90°.

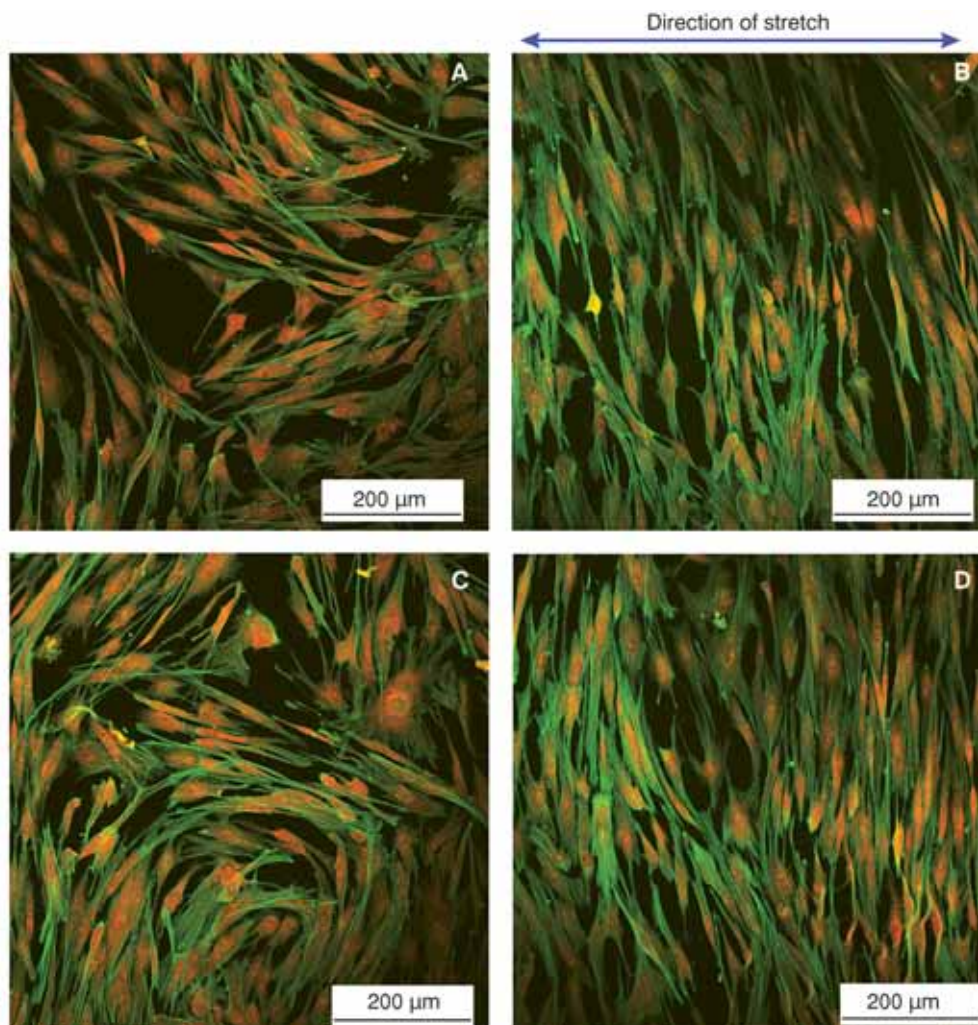


FIG. 4. Staining with FITC-phalloidin and PI after with and without mechanical stretch for 24 hrs and 48 hrs. A) Control group of 24 hrs showed mostly well spread, elongated, often triangular with randomly distributed actin fibers; B) Stretch group of 24 hrs showed higher density, strongly stained and homogeneous distribution actin fibers which perpendicularly align with respect to the direction of stretch; C) Control group of 48 hrs showed mostly well spread, elongated, often triangular with randomly distributed actin fibers; D) Stretch group of 48 hrs showed higher density, strongly stained and homogeneous distribution actin fibers which perpendicularly align with respect to the direction of stretch.

Influence of cytochalasin D on mechanical stretch-induced cell realignment

To characterize the effect of cytochalasin on mechanical stretch-induced changes in cell alignment, the morphology of each group was observed by optical microscope (Fig. 5). Cells without exposure to cytochalasin D or mechanical stretching were mostly well spread and elongated, with random orientation. After mechanical stretch for 48 hrs, a perpendicular alignment of cells with respect to the direction of stretch was observed. Cytochalasin D treatment resulted in a circular cell shape. When mechanical stretch was applied in the presence of cytochalasin D, there

was no specific alignment of cells and actin fibers with respect to the direction of stretch.

Influence of mechanical stretch on gene expressions

To characterize mechanical stretch-induced tenogenic differentiation of hMSCs, Col I, Col III, TNC, SCX, Col II and MSC-p were measured by real time RT-PCR. The gene expression levels were quantified for Col I, Col II, Col III, MSC-p, TNC and SCX normalized to the expression of endogenous control gene, GAPDH at each termination of experiment. After stretch for 24 hrs and 48 hrs, gene expressions of matrix molecules, such as Col I (up to 1.220 ± 0.057

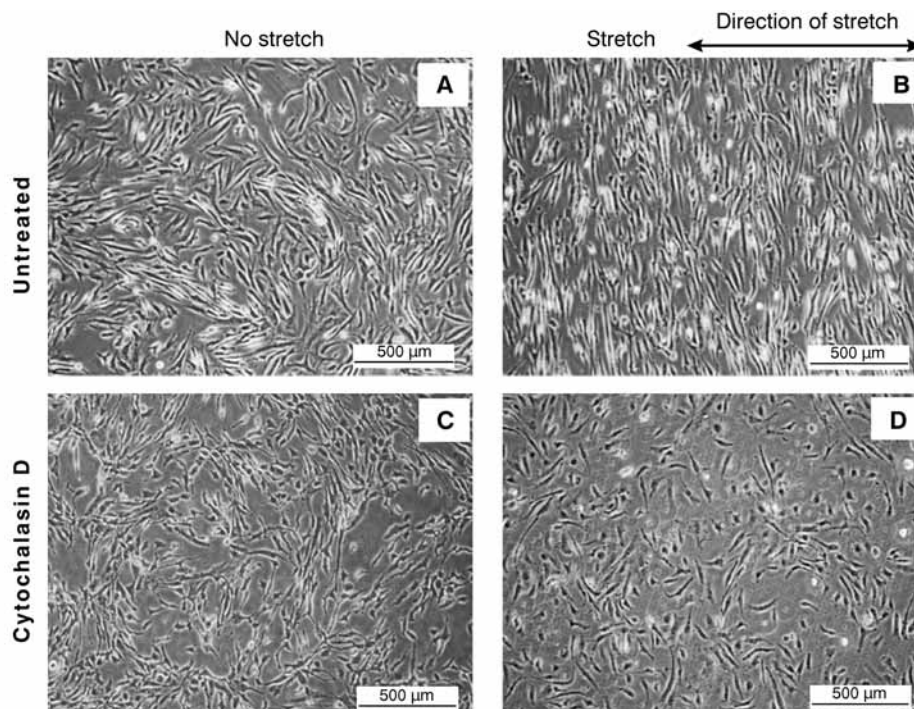


FIG. 5. Influence of cytochalasin D on mechanical stretch-induced cell realignment. A) hMSCs without mechanical stretch exhibited long cytoplasmic processes, spindle-shaped nuclei and random orientation; B) Cytochalasin D untreated hMSCs in the presence of mechanical stretching, showed an alignment perpendicular to the direction of stretching; C) Inhibiting assembling of actin with cytochalasin D, resulted in a more circular cell shape; D) Treatment of cytochalasin D blocked mechanical stretch-induced hMSCs alignment. The cells showed no particular orientation but were randomly oriented.

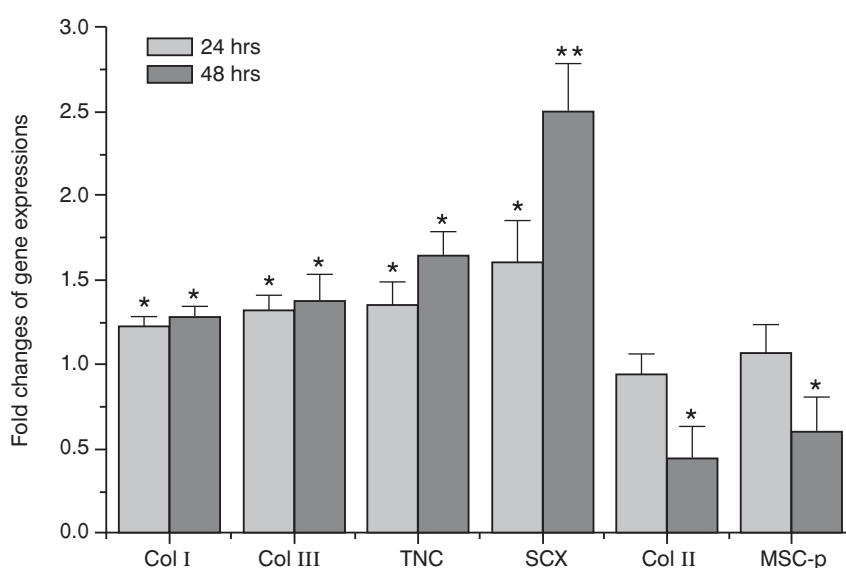


FIG. 6. Fold changes of genes expressions. All transcript levels were normalized to GAPDH. Gene expression level of each gene of control group was normalized to 1. Proportional fold changes in transcript levels in stretch group (Str) were expressed relative to control group according culture hours. “*” and “**” mean $p < 0.05$ and $p < 0.01$ compared to control group, respectively (t -test).

after stretch for 24 hrs, and up to 1.285 ± 0.049 after stretch for 48 hrs), Col III (up to 1.310 ± 0.087 after stretch for 24 hrs, and up to 1.375 ± 0.152 after stretch for 48 hrs), TNC (up to 1.355 ± 0.133 after stretch for 24 hrs, and up to 1.64 ± 0.146 after stretch for 48 hrs) and SCX (up to 1.605 ± 0.244 after stretch for 24 hrs, and up to 2.503 ± 0.285 after stretch for 48 hrs) were up regulated by mechanical stretch. Although the gene expression of Col II was very low, it was still observed to down-regulate a little after stretch for 24 hrs, and significantly down-regulate to 0.433 ± 0.186 after stretch for 48 hrs. MSC-p was observed to up-regulate to 1.055 ± 0.183 after stretch for 24 hrs, and significantly down-regulate to 0.587 ± 0.212 after stretch for 48 hrs (Fig. 6).

Influence of mechanical stretch on protein expressions

To evaluate the influence of mechanical stretch on protein level, the protein expressions of Col I, TNC and Runx2 were investigated by western blot (Fig. 7). The results showed that the protein expression of Col I was increased to 1.191 ± 0.042 after stretch for 24 hrs, and increased to 1.258 ± 0.093 after stretch for 48 hrs; protein expression of TNC was increased to 1.47 ± 0.047 after stretch for 24 hrs, and increased to 1.941 ± 0.160 after stretch for 48 hrs. Although the gray value of the Runx2 band is very low due to low protein expression of Runx2 in hMSCs, the protein expression of Runx2 was still observed to decrease to 0.977 ± 0.086 after stretch for 24 hrs and decrease to 0.419 ± 0.128 after stretch for 48 hrs.

DISCUSSION

Our findings showed that mechanical stretch induced the realignment of hMSCs which showed perpendicular orientation with respect to the direction of stretch. Several studies have reported that mechanical stretch causes cells to orientate away from the direction of stretch and the favoured cell orientation direction is about 60° (Yamada *et al.*, 2000; Neidlinger-Wilke *et al.*, 2001). Our results showed that hMSCs changed their orientation close to 80° after stretch for 24 hrs and 48 hrs, and the favoured cell orientation direction was about $70\text{--}90^\circ$ in response to mechanical stretch. The difference of the angles is due to the difference of cell type, applied stretch and the value of Poisson's ratio of the substrate (Wang *et al.*, 1995).

Except for the changes in cell orientation, mechanical stretch also altered the cytoskeleton organization of hMSCs. Our results showed that the density of actin fibers was significantly increased. It suggests

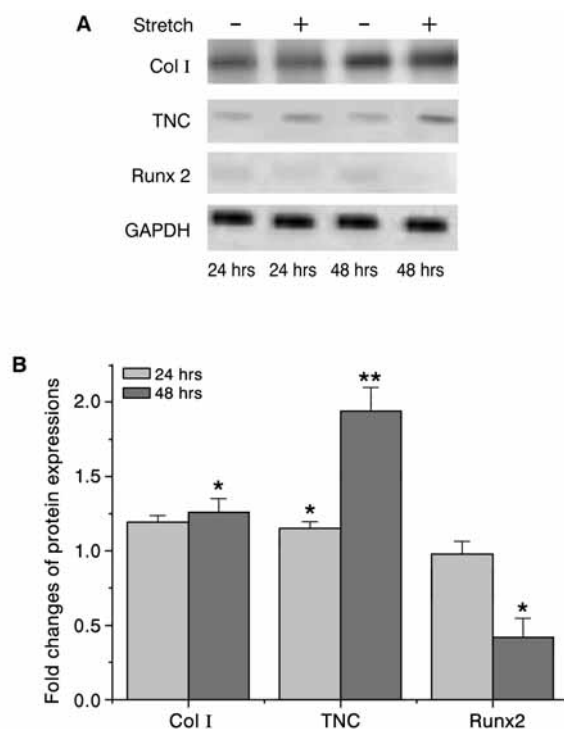


FIG. 7. Protein expressions of Col I, TNC and Runx2. A) Protein expressions of Col I, TNC and Runx2 analyzed by western blot; B) statistical analysis of relative protein expressions of Col I, TNC, and Runx2 which were normalized to GAPDH. Protein expression level of each protein of control group was normalized to 1. “*” and “**” mean $p < 0.05$ and $p < 0.01$ compared to control group, respectively (*t*-test).

that mechanical stretch facilitates the actin fibers formation. The perpendicular alignment of the more strongly stained actin fibers with respect to the direction of stretch was also observed. However, the mechanical stretch-induced alignment was completely blocked by the treatment of cytochalasin D (Fig. 5). It indicates that stretch-induced cell alignment depends on the arrangement of stress fiber, and cells resist and adapt to the force balance by changing their actin orientation (Wang & Grood, 2000).

Similar to chemical factors, mechanical stretch is an important factor which regulates hMSCs differentiation. Although there is no specific marker for the differentiation of hMSCs into tenocytes, SCX, TNC, Col I and Col III are commonly used to describe the process of tenogenic differentiation of hMSCs (Chen *et al.*, 2008). Consistent with the other studies (Lee *et al.*, 2007; Kuo & Tuan, 2008; Zhang *et al.*, 2008), mechanical stretch could up-regulate the gene expressions of Col I, Col III, TNC and SCX after stretch for 24 hrs and 48 hrs. 10% order strains occur normally in a tendon/ligament *in vivo*, and proper strains are

helpful for tendon repair (Herrick *et al.*, 1978). The tenogenic differentiation of hMSCs by mechanical stretch (10% strain and 1Hz) may explain why proper strains can promote tendon repair. In addition, mechanical stretch inhibited the gene expressions of MSC-p and Col II. MSC-p is an adjunctive indicator for assessing the differentiation of MSCs, and the decrease of MSC-p indicates that MSCs are in the process of differentiation. Although the Col II gene expression is very low in hMSCs, a decrease of expression of Col II was still detected by real-time RT-PCR. It indicates that the mechanical stretch in our study may not induce hMSCs differentiation into chondrocytes while promote commitment of hMSCs into tenocytes.

Moreover, the protein expressions of Col I and TNC were increased after stretch for 24 hrs and 48 hrs. The results certify that the mechanical stretch promotes the synthesis of tendon-related extracellular matrixes (Col I and TNC). At the same time, the protein expression of Runx2 was inhibited by mechanical stretch. It suggests that this mechanical stretch may not lead to osteoblast differentiation commitment. In contrast to our results, other recent studies showed the differentiation of MSCs to osteoblasts or chondrocytes by mechanical stimulation. Naito *et al.* (2011) constructed ring-shaped bone-like tissues using MSCs in the hydrogel cultures and found that collagen hydrogel-based ring-shaped bone-like tissues conditioned with osteoinductive supplements developed enhanced biomechanical properties. Sarraf *et al.* (2011) seeded mouse bone marrow-derived MSCs in type I collagen gels and incubated the cells in a tensioning force bioreactor, and demonstrated that cell fate choice depends on minute, cell-derived forces. Qi *et al.* (2008) showed that mechanical strain (40 min and 2000 microstrains) may act as a stimulator to induce differentiation of rat MSCs into osteoblasts. Nguyen *et al.* (2011) found electrospun poly(L-Lactic Acid) nanofibres loaded with dexamethasone induced osteogenic differentiation of hMSCs. Our results together with these findings showed that the cellular reactions of mechanical stimulation depend on the loading fashion and the cell types, and so on. This might be one reason why different research groups obtain various results using different mechanical stimulations or stretch parameters or cell types.

During the process of differentiation, cells undergo changes in actin organization, while changes in actin organization in turn regulate cellular differentiation (McBeath *et al.*, 2004; Arnsdorf *et al.*, 2009; Trei-

ser *et al.*, 2010). The changes in actin organization are important to the mechanical stretch-induced tenogenic differentiation. Depletion of changes in actin organization with treatment of cytochalasin D blocks the mechanical stretch-induced tenogenic differentiation.

In summary, mechanical stretch leads to the increase of actin fiber density and specific vertical alignment of the fibers with respect to the direction of stretch. Furthermore, mechanical stretch may trigger the differentiation of hMSCs into tenocytes, and may not induce the osteoblast/chondroblast differentiation commitment. These findings will serve as a basis for understanding molecular mechanisms of tendon differentiation and may provide novel view into the tendon tissue repair and regeneration.

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