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RhoA/ROCK, Cytoskeletal Dynamics, and Focal Adhesion Kinase are Required for Mechanical Stretch-Induced Tenogenic Differentiation of Human Mesenchymal Stem Cells

BAIYAO XU,[1,2] GUANBIN SONG,[2]* YANG JU,[1]** XIAN LI,[2] YUANHUI SONG,[1] AND SACHI WATANABE[1]

[1]*Department of Mechanical Science and Engineering, Nagoya University, Nagoya, Japan*

[2]*Key Laboratory of Biorheological Science and Technology, Ministry of Education. College of Bioengineering, Chongqing University, Chongqing, P. R. China*

Human bone marrow mesenchymal stem cells (hMSCs) have the potential to differentiate into tendon/ligament-like lineages when they are subjected to mechanical stretching. However, the means through which mechanical stretch regulates the tenogenic differentiation of hMSCs remains unclear. This study examined the role of RhoA/ROCK, cytoskeletal organization, and focal adhesion kinase (FAK) in mechanical stretch-induced tenogenic differentiation characterized by the up-regulation of tendon-related marker gene expression. Our findings showed that RhoA/ROCK and FAK regulated mechanical stretch-induced realignment of hMSCs by regulating cytoskeletal organization and that RhoA/ROCK and cytoskeletal organization were essential to mechanical stretch-activated FAK phosphorylation at Tyr397. We also demonstrated that this process can be blocked by Y-27632 (a specific inhibitor of RhoA/ROCK), cytochalasin D (an inhibitor of cytoskeletal organization) or PF 573228 (a specific inhibitor of FAK). The results of this study suggest that RhoA/ROCK, cytoskeletal organization, and FAK compose a "signaling network" that senses mechanical stretching and drives mechanical stretch-induced tenogenic differentiation of hMSCs. This work provides novel insights regarding the mechanisms of tenogenesis in a stretch-induced environment and supports the therapeutic potential of hMSCs.

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Tendons/ligaments are connective tissues subjected to complex mechanical microenvironments that control their growth, development, and regeneration. However, inappropriate physical training or excessive, repetitive stretching often leads to tendon/ligament overuse injuries. The management of damaged tendons/ligaments is still one of the most challenging problems in orthopedics. It has been reported that human bone marrow mesenchymal stem cells (hMSCs) were able to differentiate into tenocytes and may be used for tendon/ligament repair (Hoffmann et al., 2006). Furthermore, mechanical stimuli are able to induce tenogenic differentiation of hMSCs (Altman et al., 2002; Lee et al., 2007; Kuo and Tuan, 2008; Zhang et al., 2008). However, the mechanotransduction mechanism of this differentiation process is still unclear, and the elucidation of this mechanism is an important research goal in tendon/ligament repair.

Cell–cell and cell–extracellular matrix (ECM) adhesion sites receive a great deal of attention as putative sites of mechanotransduction. Integrins are transmembrane components of these sites, and there is ample evidence that mechanical signaling is integrin-dependent (Shyy and Chien, 1997; Geiger and Bershadsky, 2001; Vogel and Sheetz, 2006). Integrin-dependent signaling includes some important signaling events such as focal adhesion kinase (FAK), RhoA/ROCK, and cytoskeletal organization (Torsoni et al., 2005; Zhang et al., 2007; Michael et al., 2009). FAK phosphorylation at Tyr397 regulates mechanical stretch-induced tenogenic differentiation of hMSCs (Xu et al., 2011). Moreover, it plays an important role in mechanotransduction (Torsoni et al., 2005) and in guiding cell fate (Shih et al., 2011).

RhoA, as a small G-protein in the Rho family, couples with its direct effector, ROCK, in response to extracellular signals (Zhao et al., 2007). RhoA/ROCK serves as a mechanotransducer of matrix stiffness and can mediate osteogenic differentiation of hMSCs (Shih et al., 2011). RhoA/ROCK also regulates the mechanical stretch-induced β-MHC gene expression (Torsoni et al., 2005). RhoA/ROCK also

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*Correspondence to: Guanbin Song, Key Laboratory of Biorheological Science and Technology, Ministry of Education. College of Bioengineering, Chongqing University, Chongqing 400030, P. R. China. E-mail: song@cqu.edu.cn

**Correspondence to: Yang Ju, Department of Mechanical Science and Engineering, Nagoya University, Nagoya 464-8603, Japan. E-mail: ju@mech.nagoya-u.ac.jp

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regulates fluid flow-induced osteogenic differentiation, and it is a negative regulator of both adipogenic and chondrogenic differentiation (Arnsdorf et al., 2009). These studies indicate that RhoA/ROCK plays an important role in sensing mechanical stimuli and triggering cell differentiation. In addition, changes in the activation of RhoA/ROCK always result in cytoskeletal reorganization, which is essential to sensing mechanical stimuli and guiding stem cell differentiation. It has been reported that changes in RhoA/ROCK activation and in cytoskeletal tension, which controls cell shape, affect hMSC differentiation (McBeath et al., 2004). In addition, a number of studies have suggested that cytoskeletal disassembly and cell rounding promotes adipogenesis (Arnsdorf et al., 2009; Feng et al., 2010; Huang et al., 2011). Interestingly, stem cell lineage fates can be forecasted by a new approach termed cytoskeleton-based high-content imaging (Treiser et al., 2010). These studies indicate that RhoA/ROCK and cytoskeleton organization are essential to mechanical stimuli response and control cell differentiation.

Together, RhoA/ROCK, cytoskeletal organization, and FAK play important roles in sensing mechanical stimuli and regulating cell differentiation, and these factors may guide mechanical stretch-induced tenogenic differentiation of hMSCs. In this study, we characterized the expression patterns of tendon-related genes and proteins to investigate the role of RhoA/ROCK, cytoskeletal organization, and FAK in mechanical stretch-induced tenogenic differentiation of hMSCs. This study may shed light into mechanotransduction during mechanical stretch-induced tenogenic differentiation of hMSCs and tendon/ligament repair.

Materials and Methods

Cell culture

hMSCs (UE6E7T-3) were acquired from Health Science Research Resources Bank, (Osaka, Japan) and cultured in MSC growth medium (Invitrogen, Carlsbad, CA) in 25 cm^2 culture flasks (Becton Dickinson Labware, Bedford, MA) at an initial density of 1×10^4 cells/cm^2 for expansion without differentiation. Cells were kept in a humidified incubator (SANYO, Osaka, Japan) at 37°C and supplemented with 5% CO_2. 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, Otsu, Japan) and subcultured in 25 cm^2 culture flasks.

Cyclic mechanical stretching

A mechanical cell strain instrument (Model ST-140, STREX Co., LTD, Osaka, Japan), consisting of a strain unit (Fig. S1 A), a control unit (Fig. S1 B), and elastic silicone chambers (Fig. S1 C & D), was used. During stretch experiments, only the strain unit was placed in the incubator. The chambers used in the strain unit were 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).

hMSCs were plated into chambers pre-coated with human fibronectin (R & D, Minneapolis, MN) at an initial density of 1×10^4 cells/cm^2 (10 cm^2, STREX Co., Osaka, Japan) at a concentration of 1 μg/cm^2 and were allowed to grow in the incubator for 24 h. Chambers were mounted onto the strain unit and exposed to a stretch treatment at an amplitude of 10% and a frequency of 1 Hz for 48 h unless otherwise specified. As a control, static cells grew on pre-coated chambers without experiencing any stretching.

Pharmacological inhibitors

The following pharmacological inhibitors were employed: 10 μM Y-27632 (Calbiochem, San Diego, CA), 0.1 μg/ml cytochalasin D (Calbiochem) and 10 μM PF 228 (Tocris, Ellisville, MO). The efficacy and relevant concentrations of the inhibitors were cross-referenced (Fig. S2, S3 and S4). The inhibitors (or DMSO carrier for control) diluted in the same medium were added 1 h before cells were subjected to mechanical stretching at 37°C.

Measurement of cell shape index

Immediately after mechanical stimulation, cells were photographed in three regions of each membrane. Cell perimeter and area were measured with FluoView 5.0 (Olympus, Tokyo, Japan). The cell shape index (CSI) was calculated from the following formula:

$$CSI = \frac{4\pi A}{P^2} \quad (1)$$

where A and P are the area and perimeter, respectively, of the cells. CSI is a measurement of how circular or linear an object is. CSI ranges from 0 to 1, and the larger the value of CSI, the more circular an object is.

Immunofluorescence staining

At the end of the experiment, the cells were fixed in 4% paraformaldehyde for 15 min and permeablized 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, Farmingdale, New York) in primary blocking solution (1% BSA in PBS) at 4°C overnight. Then, the cells were incubated with 0.1 μg/ml DAPI (Calbiochem) in PBS for 5 min, after which 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, Tokyo, Japan).

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

Cells were lysed at the end of the stretching experiments, and total RNA was isolated using a Rneasy Mini Kit (Qiagen, Duesseldorf, Germany). 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, Carlsbad, CA). The pre-designed minor groove binder (MGB) probes of glyceraldehydes 3-phosphate dehydrogenase (GAPDH), Col I, Col III, TNC, and SCX (ABI), as well as Taqman PCR Master Mix and a Light Cycler apparatus (ABI 7300, ABI), were used to analyze the expressions of the genes of interest. The gene expression levels of Col I, Col III, TNC, and SCX were calculated using the standard curve method and normalized to GAPDH.

The 5′ and 3′ primers of epha4, eya2, six1, and β-actin (Table S1) were previously described (Chen et al., 2009). These genes were quantified by quantitative PCR using the Brilliant SYBR Green QPCR Master Mix (TakaRa, Ostu, Japan) and the Light Cycler apparatus. The gene expressions of epha4, eya2, and six1 were normalized to that of β-actin and were calculated as $-2\Delta\Delta Ct$. Each sample was repeated at least three times for the gene of interest.

Western blot

Cell lysates were collected at the end of each experiment from each culture condition. Briefly, cells were washed with PBS and then with 100 μl of detergent-based lysis buffer (M-PER Mammalian Protein Extraction Reagent, Pierce, Rockford, IL), 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. Thirty micrograms of protein from each sample were loaded onto an 8% SDS–PAGE gel for gel electrophoresis. The separated proteins were transferred to a PVDF membrane (Bio-Rad, Oakland, CA) at 80 V for 120 min. The membranes were blocked in 5% BSA/TBS-Tween 20 solution for 1 h at room temperature, followed by the application of monoclonal antibody specific for p-FAK (pY397) (Epitomics, Burlingame, CA), FAK (Epitomics), GAPDH (sc-48166, Santa Cruz, CA), collagen type I (sc-8785, Santa Cruz), collagen type III (C7805, Sigma, St. Louis,

MO), or tenascin-C (sc-9871, Santa Cruz) at 1:1,000 in 5% BSA/TBS-Tween 20. After incubation for 120 min with primary antibody at room temperature, the secondary antibody, anti-rabbit IgG-HRP (Cell Signaling, Danvers, MA), anti-goat IgG-HRP (Santa Cruz), or anti-mouse IgG-HRP (Santa Cruz) at 1:10,000 in 5% BSA/TBS-Tween 20 was applied for 1 h 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 a Fujifilm LAS-4000 (Fujifilm, Tokyo, Japan).

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

Effects of the inhibitors on mechanical stretch-induced changes in morphology

Several pharmacological inhibitors were used to test the effect of RhoA/ROCK, actin cytoskeletal organization, and FAK on mechanical stretch-induced changes in cell morphology and the actin microstructure. Specifically, cells were exposed to (1) Y-27632, which inhibits RhoA/ROCK; (2) cytochalasin D, which inhibits actin polymerization by capping the barbed end of F-actin polymers; or (3) PF 228, which inhibits FAK phosphorylation at Tyr397.

To characterize the effect of Y-27632, cytochalasin D or PF 228 on mechanical stretch-induced changes in morphology and cytoskeletal microstructure, the morphology of each group was observed by optical microscope (Fig. 1). Then, the CSI of the cells was calculated (Fig. 2), and the cytoskeletal microstructure was observed by confocal microscope (Fig. 3). Y-27632, cytochalasin D, and PF 228 appeared to alter the mechanical stretch-induced changes in morphology and cytoskeletal microstructure. Cells without exposure to any inhibitors or mechanical stretching were mostly well spread, elongated, and often triangular, with randomly distributed actin fibers. After mechanical stretching for 48 h, the cell shape was elongated, and the CSI was somewhat reduced. Moreover, the density of actin fibers was increased significantly, with more strongly stained actin fibers observed; a perpendicular alignment of cells and actin fibers with respect to the direction of stretching was also observed. Treatment with Y-27632 resulted in stellate-like cells, a decrease in CSI and a lack of actin fibers. However, exposure to mechanical stretching did not induce further changes in cell morphology, CSI or random alignment, although shorter and discontinuous actin fibers in cells and very short actin fibers in cell edges did appear. Cytochalasin D treatment resulted in a circular cell shape, an increase in CSI, and the disruption of actin fiber organization. When mechanical stretching was applied in the presence of cytochalasin D, the cells became more circular, and the actin fibers became more punctuate, with no specific alignment of cells and actin fibers with respect to the direction of stretch. PF 228 incubation for 48 h resulted in a more circular cell shape, a higher CSI and shorter actin fibers. Interestingly, PF 228 incubation for 48 h with mechanical stretching resulted in stellate-like cells, a marked decrease in CSI, shorter actin fibers and rough edges, and the combination of the inhibitor and stretching resulted in the generation of cell debris (red arrows). There was no specific alignment of cells and actin fibers with respect to the direction of stretch.

Effects of the inhibitors on mechanical stretch-activated FAK phosphorylation

The effect of Y-27632, cytochalasin D, and PF 228 on mechanical stretch-induced FAK phosphorylation at Tyr397 was investigated. Five experimental groups were designed as follows: The control group (Con) was cultured in static conditions without any inhibitors; the stretch group (S) experienced mechanical stretching only; the stretch plus Y-27632 group (S + Y) was treated with stretching and Y-27632; the stretch plus cytochalasin D group (S + C) was treated with stretching and cytochalasin D; and the stretch plus PF 228 treatment group (S + P) was treated with stretching and PF 228. Hereafter, the cells described were stretched for a period of 30 min (1 Hz and 10% strain). At the end of the experiments, the cells were lysed, and the FAK phosphorylation at Tyr397 was assessed by Western blot, as described above.

As shown in Figure 4, the inhibitors have a significantly impact on mechanical stretch-induced FAK phosphorylation at Tyr397. After being stretched for 30 min, FAK phosphorylation at Tyr397 was increased up to 1.966 ± 0.167 times ($P < 0.01$ vs. Con). This finding indicates that mechanical stretching can significantly promote FAK phosphorylation at Tyr397. When the cells were treated with Y-27632, FAK phosphorylation at Tyr397 was decreased to 1.081 ± 0.1 times ($P < 0.05$ vs. S), indicating that Y-27632 weakened mechanical stretch-induced up-regulation of FAK phosphorylation at Tyr397. When the assembly of F-actin was blocked by cytochalasin D, FAK phosphorylation at Tyr397 was decreased to 1.095 ± 0.099 times ($P < 0.05$ vs. S), suggesting that the integrity of cytoskeletal organization is essential to mechanical stretch-induced FAK phosphorylation at Tyr397. Treatment with PF 228 resulted in a dramatic decrease in FAK phosphorylation at Tyr397; the phosphorylation ratio was reduced to 0.395 ± 0.075 times ($P < 0.01$ vs. Con or S).

RhoA/ROCK, cytoskeletal dynamics and FAK regulate mechanical stretch-induced up-regulation of tenogenic gene expressions

To determine the effects of RhoA/ROCK, cytoskeletal dynamics, and FAK on mechanical stretch-induced tendon-related gene expression, cells were exposed to mechanical stretching with the incubation of Y-27632, cytochalasin D, or PF 228. Five experimental groups were designed as described above, and then the cells were subjected to mechanical stretching of 1 Hz and 10% strain for 48 h. At the end of the experiments, all cells were lysed, and the expression of the genes of interest was assessed by real time RT-PCR as described above.

As shown in Figure 5, the inhibitors have a significant influence on mechanical stretch-induced tendon-related gene expression. Mechanical stretching elicited a significant increase in the gene expressions of Col I, Col III, TNC, SCX, epha4, eya2, and six1, indicating that mechanical stretching is able to induce tenogenic differentiation of hMSCs. However, the inhibitors can attenuate the mechanical stretch-induced increase in gene expression. In the presence of Y27632, cytochalasin D, or PF228, the expression levels of the relevant mechanical stretch-induced genes were markedly reduced. These results indicate that mechanical stretch-induced tenogenic differentiation is inhibited in the presence of Y-27632, cytochalasin D, and PF 228.

RhoA/ROCK, cytoskeletal dynamics, and FAK regulate mechanical stretch-induced up-regulation of tenogenic protein expressions

The protein expression levels of the genes of interest were also investigated. As shown in Figure 6, the inhibitors have a significant influence on the expression of mechanical

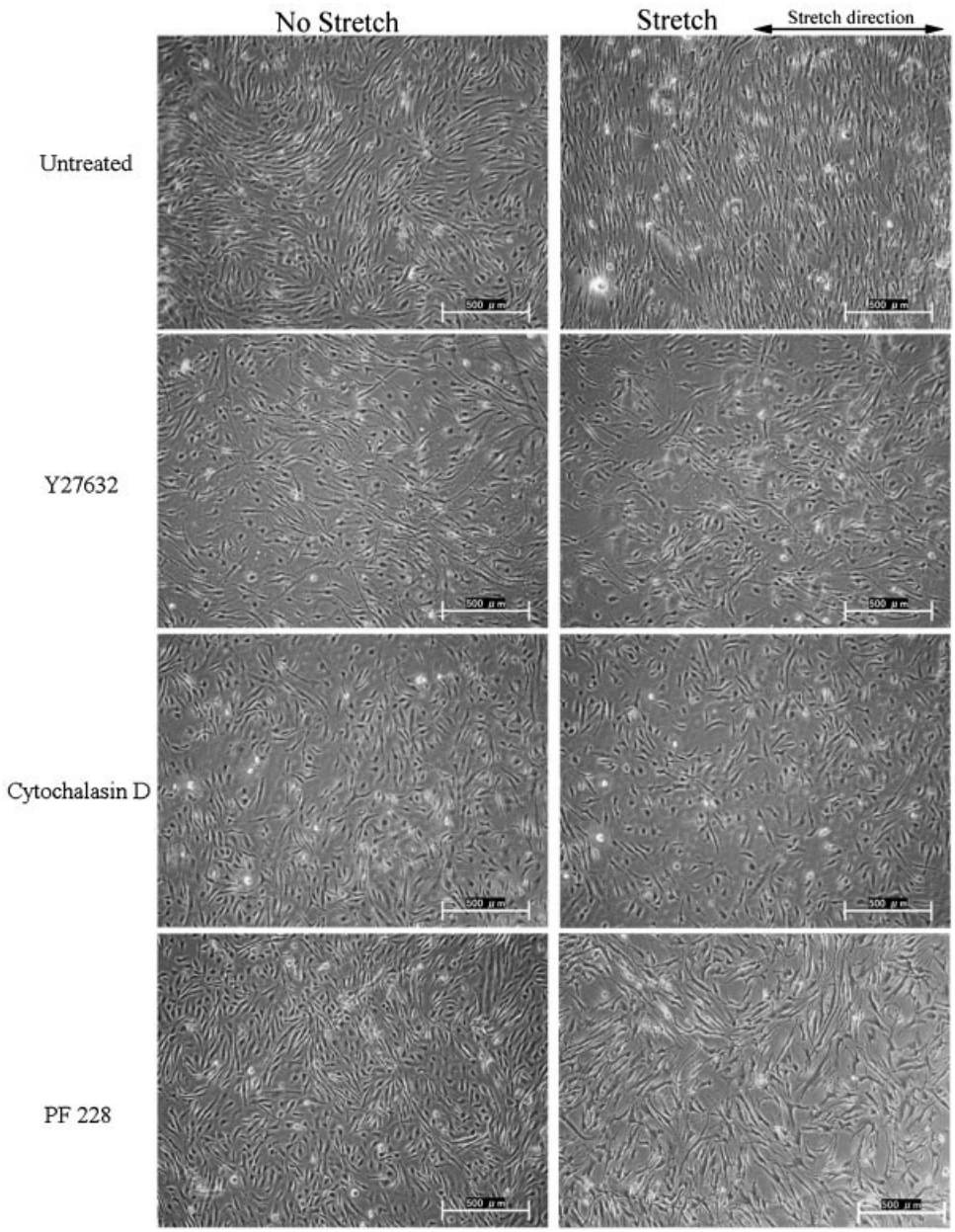

Fig. 1. Micrographs of each inhibitor treatment, with or without mechanical stretching. hMSCs without mechanical stretching exhibited long cytoplasmic processes, spindle-shaped nuclei, and random orientation. Untreated hMSCs in the presence of mechanical stretching showed an alignment perpendicular to the direction of stretching. Inhibiting actin tension with Y-27632 resulted in stellate-like cells, but there were no other gross alterations in cell shape or alignment with mechanical stretching. Inhibiting assembling of actin with cytochalasin, resulted in a more circular cell shape, but there were no other significant alterations in cell morphology or alignment with mechanical stretching. Incubation with PF 228 resulted in a more circular cell shape, and there were significant alterations, such as ruffled cell edges and random orientation, in relation to mechanical stretching.

stretch-induced tendon-related proteins. Mechanical stretching elicited a significant increase in the protein expressions of Col I, Col III, and TNC. However, the inhibitors attenuated this mechanical stretch-induced increase in protein expression. In the presnce of Y27632, the protein expressions were markedly weakened. With treatment by cytochalasin D, the protein expressions were significantly inhibited, and PF 228 incubation resulted in a reduction in the mechanical stretch-induced protein expressions.

Discussion

To develop regenerative medicine therapeutics for tendons/ligaments, it is necessary to determine whether specific

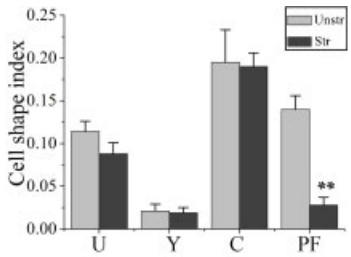

Fig. 2. Cell shape index of each inhibitor treatment, with or without mechanical stretching. For cell shape index (CSI) calculation, 20 cells were chosen randomly and analyzed. "U" indicates without treatment of inhibitor; "Y" indicates treatment with Y-27632; "C" indicates treatment with cytochalasin D; "PF" indicates treatment with PF 228; "Unstr" indicates no mechanical stretching; "Str" indicates loaded mechanical stretching. Data represent means ± standard deviations of three separate experiments (n = 3). "" means $P < 0.01$ between the stretched versus respective unstretched treatment, by Student's *t*-test.**

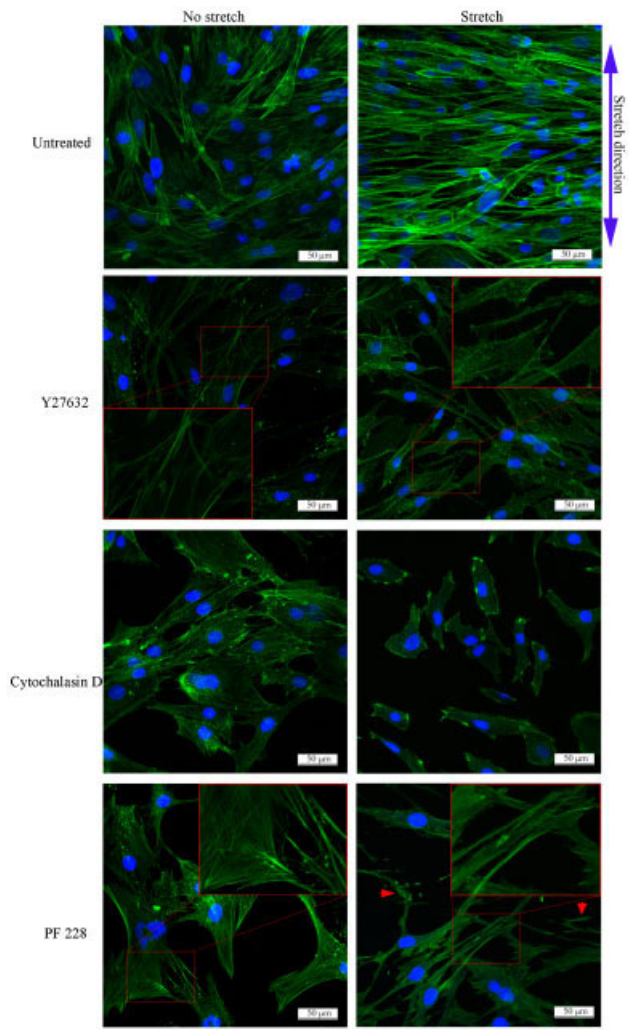

Fig. 3. Actin microstructure of each inhibitor treatment, with or without mechanical stretching. Micrographs were examined to characterize alterations in actin microstructure and cell morphology. Untreated hMSCs without mechanical stretching demonstrated a random orientation of actin fibers and contained spindle-shaped nuclei. Untreated hMSCs in the presence of mechanical stretching appeared to have a high density actin microstructure and to align perpendicularly to the direction of stretching. Inhibiting actin tension with Y-27632 resulted in a lack of actin fiber organization, ruffled cell edges and stellate-like cells; however, actin fibers became punctuate with mechanical stretching. Inhibiting actin assembly with cytochalasin D resulted in punctuate actin fibers, with neither significant alterations in cell morphology nor any realignment with mechanical stretching, although the cells were smaller. Incubation with PF 228 resulted in a more circular cell shape, and the actin fibers remained successive. However, there were significant alterations, such as destructive actin fiber organization, ruffled cell edges, and random orientation of cells, with mechanical stretching. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

mechanical signals elicit tenogenic differentiation of hMSCs and to understand the mechanotransduction mechanism of this differentiation. Our findings suggest that Y-27632, cytochalasin D, and PF 228 have a significant influence on the mechanical stretch-induced changes in cytoskeletal organization and attenuate the stretch-activated phosphorylation of FAK at Tyr397. Furthermore, our results demonstrate that RhoA/ROCK, cytoskeletal dynamics, and FAK are critical to mechanical stretch-induced tenogenic differentiation of hMSCs.

RhoA/ROCK, FAK, and mechanical stretching play important roles in cytoskeletal dynamics. Changes in the density and alignment of actin fibers are the adaptation to mechanical loads, allowing resistance to and a reduction in the experienced mechanical force. The inhibition of RhoA/ROCK results in a change from fibroblast-like cells to stellate-like cells, which is due to failure in trailing-edge retraction (Alblas et al., 2001; Worthylake et al., 2001), leaving a long tail behind cells (Kolega, 1998), discontinuous actin fibers, and no favored alignment after mechanical stretching. The alternations in the organization of actin fibers and cell shape upon treatment of PF 228 and mechanical stretching are due to stretching, which drives hMSCs to align perpendicular to the direction of stretch, while PF 228 blocks focal adhesions turnover (Sieg et al., 2000) and trailing-edge retraction (Iwanicki et al., 2008). These data suggest that mechanical stretching facilitates actin fiber formation and induces the realignment of cells and that RhoA/ROCK and FAK regulate mechanical stretch-induced cytoskeleton reorganization.

RhoA/ROCK, cytoskeleton, and FAK interact with each other through multiple signaling molecules. FAK plays a critical role as upstream effectors of RhoA/ROCK in force-induced osteopontin expression (Hong et al., 2010). The RhoA/ROCK signaling pathway also plays a crucial role in stretch-activated FAK (Torsoni et al., 2005). Our results have further shown that RhoA/ROCK is necessary for mechanical stretch-activated FAK phosphorylation at Tyr397. RhoA/ROCK and FAK may interact with each other during mechanical stretching.

FAK also regulates actin fiber organization (Iwanicki et al., 2008). Our results have demonstrated that FAK regulates stretch-induced actin polymerization and realignment and that actin fiber organization is fatal to stretch-activated FAK. These findings suggest that FAK regulates cytoskeletal organization, which in turn regulates FAK activation.

Cytoskeletal organization is regulated by RhoA/ROCK. Mechanical stimuli (Numaguchi et al., 1999; Sarasa-Renedo et al., 2006; Hirata et al., 2008) lead to RhoA/ROCK activation, which facilitates stress fiber formation. Our results have also shown that RhoA/ROCK regulates stretch-induced actin polymerization and realignment. Cytoskeletal organization may be crucial to mechanical stimuli-induced RhoA/ROCK activity, although this relationship is yet to be confirmed. From the data,

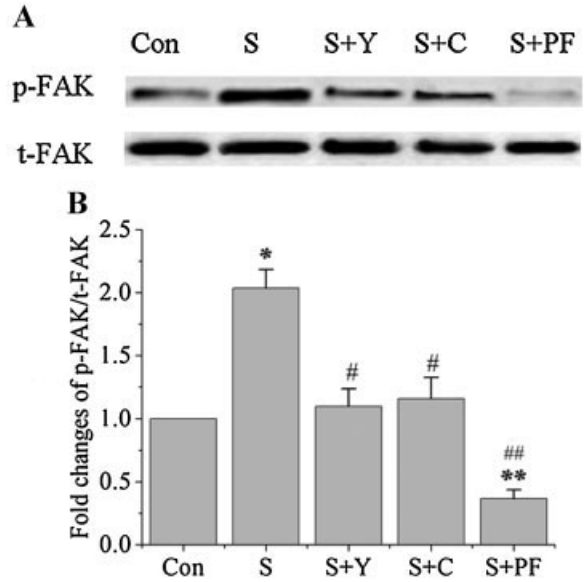

Fig. 4. Effects of the inhibitors on mechanical stretch-activated FAK phosphorylation at Tyr397. A: hMSCs were treated with or without Y-27632, cytochalasin D, or PF 228 for 1 h and then stretched for 30 min. Subsequently, the whole cell lysates were separated by SDS–PAGE and blotted with antiphospho-FAK Tyr397 (upper panel) and anti-FAK (lower panel); (B) the phosphorylation level of FAK Tyr397 was normalized to the total FAK of each group. The phosphorylation level of FAK Tyr397 of untreated, unstretched cells was normalized to 1. The control group (Con) was cultured in static conditions without any inhibitor; the stretch group (S) experienced mechanical stretching only; the stretch plus Y-27632 group (S + Y) was treated with stretch and 10 μM Y-27632; the stretch plus cytochalasin D group (S + C) was treated with stretch and 0.1 μg/ml cytochalasin D; and the stretch plus PF 228 treatment group (S + PF) was treated with stretch and 10 μM PF 228. Data represent means ± standard deviations of three separate experiments (n = 3). "" means $P < 0.01$ versus "Con". "#" and "##" mean $P < 0.05$ and $P < 0.01$, t-test, respectively, versus "S", respectively.**

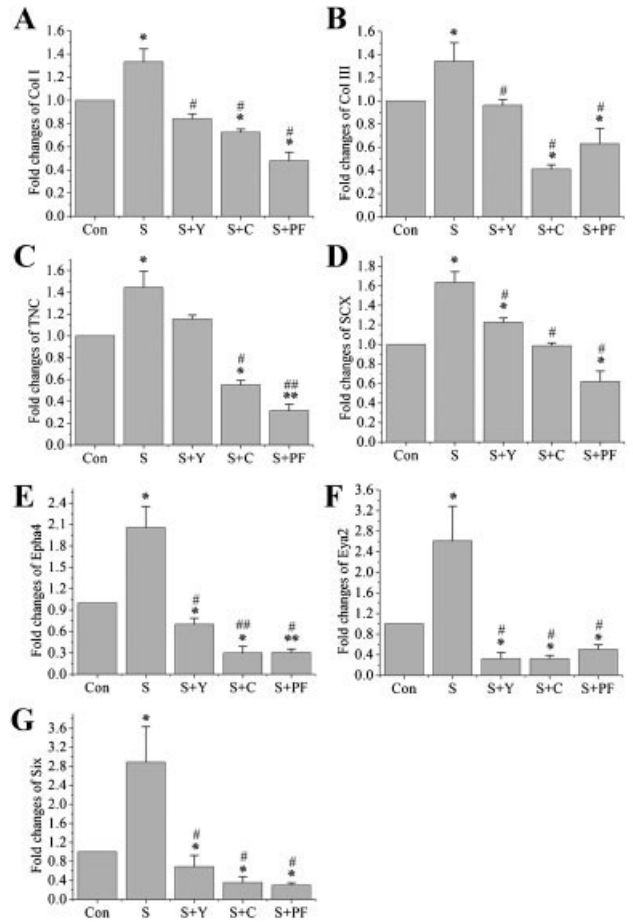

Fig. 5. Gene expression of tendon-related genes in each group. Y-27632, cytochalasin D, and PF 228 affected the mechanical stretch-induced gene expression of tendon-related genes. A: Gene expression of Col I; (B) gene expression of Col III; (C) gene expression of TNC; (D) gene expression of SCX; (E) gene expression of EphA4; (F) gene expression of Eya2, and (G) gene expression of Six1. "Con" indicates control cells without stretch, "S" indicates cells with stretch. The gene expressions of Col I, Col III, TNC, and SCX were normalized to GAPDH; the gene expressions of EphA4, Eya2, and Six1 were normalized to β-actin. The control group (Con) experienced no mechanical stretching; the stretch group (S) experienced mechanical stretching only; the stretch plus Y-27632 group (S + Y) was treated with stretch and Y-27632; the stretch plus cytochalasin D group (S + C) was treated with stretch and cytochalasin D; and the stretch plus PF 228 treatment group (S + PF) was treated with stretch and PF 228. Data represent means ± standard deviations of three separate experiments (n = 3). "*" and "" indicate $P < 0.05$ and $P < 0.01$ versus "Con", respectively. "#" and "##" indicate $P < 0.05$ and $P < 0.01$ versus "S", respectively, using Student's t-test.**

RhoA/ROCK, cytoskeletal dynamics, and FAK have reciprocal influences on one another (Fig. 7).

RhoA/ROCK, cytoskeletal organization, and FAK have important effects on cell fate. It is well established that mechanical stretching, RhoA/ROCK, the cytoskeleton, and FAK have the potential to regulate stem cell differentiation (Salasznyk et al., 2007; Ward et al., 2007; Arnsdorf et al., 2009). However, our findings have described the effect of RhoA/ROCK, cytoskeleton, and FAK on the mechanical stretch-induced tenogenic differentiation of hMSCs, which is characterized by up-regulation of tendon-related genes, including Col I, Col III, TNC, SCX, epha4, eya2, and six1. Our results have shown that mechanical stretching induces up-regulation of these, and Y-27632, cytochalasin D, and PF 228 attenuates this up-regulation. The protein expressions of Col I, Col III, and TNC were consistent with the gene expressions results. These data suggest that RhoA/ROCK, the cytoskeleton, and FAK are necessary for mechanical stretch-induced tenogenic differentiation of hMSCs. Furthermore, RhoA/ROCK, cytoskeletal dynamics, and FAK have reciprocal influences on sensing the mechanical environment and are involved in triggering stretch-induced tenogenic differentiation. Inhibition of any of these components will cause stretch-induced tenogenic differentiation to be aborted (Fig. 7).

In summary, our findings demonstrate that RhoA/ROCK, cytoskeletal organization, and FAK regulate mechanical stretch-induced realignment of hMSCs. RhoA/ROCK, the cytoskeleton, and FAK interact with each other in the regulation of mechanical stretch-induced tenogenic differentiation. Our results imply that these factors could be essential in mechanosensitivity and mechanotransduction; these findings could have important implications for research in wound healing and tendon regeneration after injury.

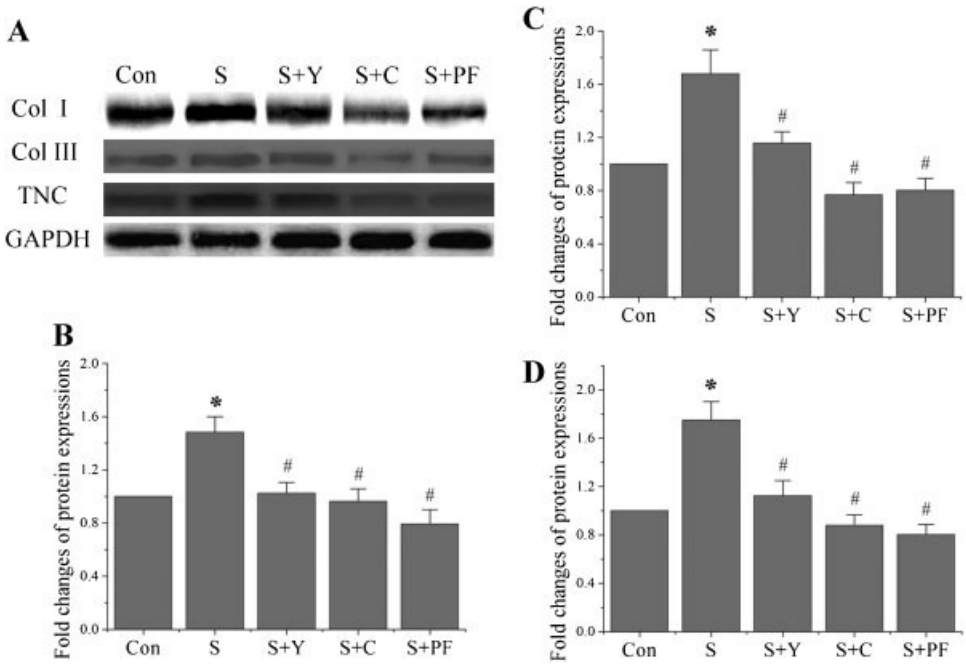

Fig. 6. Protein expressions of Col I, Col III, and TNC. A: Results of the western blot; (B) changes in the protein expression levels of Col I; (C) changes in the protein expression levels of Col III; and (D) changes in the protein expression levels of TNC. The control group (Con) experienced no mechanical stretching; the stretch group (S) experienced mechanical stretching only; the stretch plus Y-27632 group (S + Y) was treated with stretch and Y-27632; the stretch plus cytochalasin D group (S + C) was treated with stretch and cytochalasin D; and the stretch plus PF 228 treatment group (S + PF) was treated with stretch and PF 228. Data represent means ± standard deviations of three separate experiments (n = 3). "*" indicates $P < 0.05$ versus "Con", respectively. "#" indicates $P < 0.05$ versus "S", respectively, using Student's t-test.

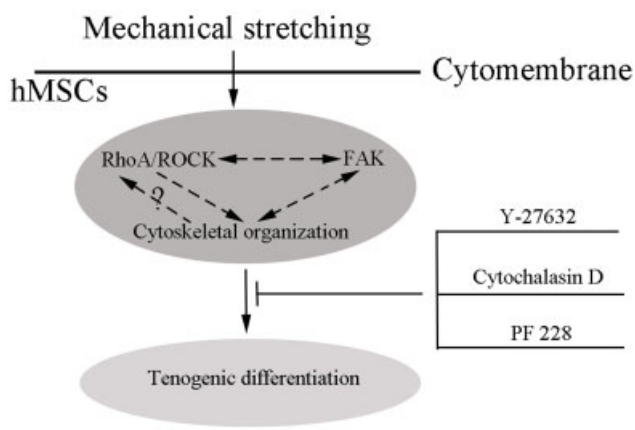

Fig. 7. Proposed schematic diagram of mechanical and biochemical regulation of tenogenic differentiation. RhoA/ROCK, cytoskeletal dynamics, and FAK interact with each other to sense the mechanical environment. They convert mechanical stretching into biochemical signaling, then trigger tenogenic differentiation. Inhibiting any component will result in the abortion of mechanical stretch-induced tenogenic differentiation.

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