Induction of predominant tenogenic phenotype in human dermal fibroblasts via synergistic effect of TGF-β and elongated cell shape

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1Department of Plastic and Reconstructive Surgery, Shanghai Key Laboratory of Tissue Engineering, Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; 2National Tissue Engineering Center of China, Shanghai, China; 3National Chromatography R&A Centre, CAS Key Lab of Separation for Analytical Chemistry, Dalian Institute of Chemical Physics, CAS, Dalian, China; and University of Chinese Academy of Sciences, Beijing, China

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Wang W, Li J, Wang K, Zhang Z, Zhang W, Zhou G, Cao Y, Ye M, Zou H, Liu W. Induction of predominant tenogenic phenotype in human dermal fibroblasts via synergistic effect of TGF-β and elongated cell shape. Am J Physiol Cell Physiol 310: C357–C372, 2016. First published December 2, 2015; doi:10.1152/ajpcell.00300.2015.—Micropattern topography is widely investigated for its role in mediating stem cell differentiation, but remains unexplored for phenotype switch between mature cell types. This study investigated the potential of inducing tenogenic phenotype in human dermal fibroblasts (hDFs) by artificial elongation of cultured cells. Our results showed that a parallel microgrooved topography could convert spread hDFs into an artificial elongation of cultured cells. Our results showed that a parallel microgrooved topography could convert spread hDFs into an elongated shape and induce a predominant tenogenic phenotype as the expression of biomarkers was significantly enhanced, such as scleraxis, tenomodulin, collagens I, III, VI, and decorin. It also enhanced the expression of transforming growth factor (TGF)-β1, but not α-smooth muscle actin. Elongated hDFs failed to induce other phenotypes, such as adipogenic, chondrogenic, neurogenic, and myogenic lineages. By contrast, no tenogenic phenotype could be induced in elongated human chondrocytes, although chondrogenic phenotype was inhibited. Exogenous TGF-β1 could enhance the tenogenic phenotype in elongated hDFs at low dose (2 ng/ml), but promoted myofibroblast transdifferentiation of hDFs at high dose (10 ng/ml), regardless of cell shape. Elongated shape also resulted in decreased RhoA activity and increased Rho-associated protein kinase (ROCK) activity. Antagonizing TGF-β or inhibiting ROCK activity with Y27632 or depolymerizing actin with cytochalasin D could all significantly inhibit tenogenic phenotype induction, particularly in elongated hDFs. In conclusion, elongation of cultured dermal fibroblasts can induce a predominant tenogenic phenotype likely via synergistic effect of TGF-β and cytoskeletal signaling.

tenogenic phenotype; cell elongation; dermal fibroblasts; TGF-β; ROCK activity

CELL FUNCTIONS ARE HEAVILY dependent on their morphology and structures. In vivo, cells in different tissues exhibit distinctive cell morphologies. For examples, neuron has a unique structure with several dendrites to receive signals from other neurons, and with an axon to deliver signals to another neuron or to an effector, such as muscle or gland (6). Small intestinal villus epithelial cells are another example, in which a polar structure is essential for them to absorb nutritional molecules (23, 29). Regarding human mesenchymal stem cells (hMSC), there are three different morphologies in vivo, which are correlated to three different intrinsic characteristics and different biological properties (15).

In vitro, cell functions are also closely linked to their morphologies. It was observed that cell morphology change resulted from environmental change usually led to cell phenotype drift or loss of functions (30, 61). Additionally, cell function alteration induced by biophysical or biochemical signals also resulted in significant cell morphology change (18, 22).

Physical cues, which were speculated as the essential factors in tissue development over a century ago (59), are now well known as the important factors in controlling cell functions. In recent years, induced stem cell differentiation via artificial cell shape control becomes an attractive strategy in stem cell biology because it represents an important biomimetic approach via simulating native cell morphology of the in vivo environment. For examples, reported studies demonstrated that mesenchymal stem cells (MSCs) cultured on nanogratings with a certain range of widths (0.35, 1, and 10 μm) preferred to differentiate into neural-like cells in narrower groove without inducing factors (62). In another work, it was shown that hMSCs adapted to flower shape with curved edges were favorable to adipogenic differentiation, whereas hMSCs in star shape with sharp edges tended to differentiate toward osteogenic lineage (19).

In contrast to stem cells, it remains less explored if mature differentiated cells can also be induced to another phenotype via cell morphology imitation. Dermal fibroblasts and tenocytes exhibited distinctive cell morphologies in their respective niche environments. Tenocytes, which are also called tendon fibroblasts and reside within the narrow space between parallel aligned collagen (COL) fibers, exhibit particular elongated cell morphology (2). In addition, they are also constantly subject to uniaxial mechanical stretch (41). By contrast, dermal fibroblasts are typical spindle-shaped cells that randomly distribute in dermis along with COLs and other extracellular matrices (ECMs). They also bear the compress stress and multiaxial mechanical stretch (42, 45, 53, 54). However, dermal fibroblasts were employed as a feasible cell source to replace tenocytes for both in vitro and in vivo tendon engineering with tissue structure similar to tendon tissues (8, 26).
Previously, our laboratory employed a micropattern surface to artificially recapture native elongated cell morphology in vitro cultured tenocytes. This demonstrated that resimulation of native cell morphology helped to regain the normal phenotype of in vitro expanded tenocytes. This study aimed to explore the role of simulating elongated cell morphology in inducing tenogenic phenotype of vitro cultured hDFs. Cell morphology change is known to change cytoskeleton structure and lead to a series of signaling to alter the expression profile of different genes and proteins, including growth factor and ECM. Therefore, the potential mechanism of cell shape mediated phenotype switch from dermal fibroblasts to tenocytes was also investigated.

MATERIALS AND METHODS

Experimental Design

To examine the effect of micropattern-mediated cell shape control on hDF tenogenic differentiation, this study was designed as follows: 1) cells were seeded on smooth silicone membrane and kept in a spread shape (S group) for one or three passages (4 days for each passage) as an experimental group; or 2) cells were seeded on microgrooved silicone membrane and kept in an elongated shape (G group) for one passage (2,500 cells/cm² in DMEM with 4% FBS) at 2,500 cells/cm² in DMEM with 4% FBS. Cell morphology change is known to change cytoskeleton structure and lead to a series of signaling to alter the expression profile of different genes and proteins, including growth factor and ECM. Therefore, the potential mechanism of cell shape mediated phenotype switch from dermal fibroblasts to tenocytes was also investigated.

Table 1. Primers used in quantitative PCR analysis

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<th>Primer Sequence (5'-3')</th>
<th>Annealing Temperature, °C</th>
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<td>Antisense: CGACCGAGAGCCGACACAA</td>
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See text for definition of gene acronyms.
2 × 2 mm² fragments and subjected to the treatment of 0.25% trypsin plus 0.02% EDTA in PBS at 37°C for 30 min, followed by enzyme digestion with 0.15% collagenase II in serum-free DMEM at 37°C for 6–8 h. The cell pellet was then resuspended in DMEM culture medium and seeded onto 10-cm culture dish (BD FALCON) in regular density (1.5 × 10⁶ per dish). When reaching 80% confluence, cells were detached with 0.25% trypsin-EDTA and subcultured. Chondrocytes of passage 2 were used for the experiment.

Microgroove Membrane as a Cell Culture Substrate

As previously reported (46), the silicone membrane, a kind gift from Dr. James Wang of the University of Pittsburgh, was used for cell shape control. The membrane was made of polydimethylsiloxane from silicone elastomer (Dow Corning). The cells were enforced into an elongated shape on the topographical silicone membrane of parallel microgroove structure with groove width of 10 μm and groove depth of 3 μm (G group), whereas the cells were allowed to spread on plain silicone membrane (S group). To promote cell adhesion, the membrane was coated with 40 ng/ml fibronectin (Roche, Indianapolis, IN).

F-Actin Staining

To observe the effect of microgrooved structure on cell shape control, rhodamine phalloidin (Cytoskeleton, Denver, CO) was used to stain cellular actin. As previously reported (65), after being seeded on the membrane for 96 h, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and then incubated in rhodamine phalloidin (100 nM) for 30 min. Meanwhile, nuclei were counterstained with 4',6-diamidino-2-phenylindole (100 nM, Sigma, St. Louis, MO) for 30 s.

Cell Shape Analysis

Actin-stained dermal fibroblasts of both smooth and microgrooved groups were imaged on a fluorescent microscope (Nikon, Japan) and analyzed with Image-Pro Plus processing software (version 6.0, Media Cybernetics, Silver Spring, MD), as previously described (4, 47). To evaluate elongation of a cell, the cell shape was first outlined manually, the area was digitally captured, and total area was then determined. The major axis of the cell was divided by the minor axis to get the aspect ratio. To evaluate alignment, the angle of the major axis with respect to the parallel lines was taken. All measurements were performed on 25 cells per images, and a total of 4 images were measured to generate means and standard deviation.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

As previously described (5), total RNA was extracted from fibroblasts or chondrocytes using Trizol Reagent (Invitrogen, Carlsbad, CA). A total of 1.5 μg of extracted RNA were used to synthesize cDNA with avian myeloblastosis virus reverse transcriptase (Promega). Quantitative polymerase chain reaction (qPCR) was performed using a Power SYBR Green PCR master mix (2×) (Applied Biosystems, Foster City, CA) in a real-time thermal cycler (Stratagene Mx3000PTM QPCR System, La Jolla, CA) with GAPDH gene as a control. qPCR was performed with a protocol of 95°C for 10 min followed by 40 cycles (30 s at 95°C, 30 s at annealing temperature, as listed in Table 1, and 45 s at 72°C) and terminated by 5-min extension at 72°C. Each assay was performed in triplicate, and experiments were repeated in three cell samples.
Western Blot

Cultured cells were detached with 0.25% trypsin-EDTA and centrifuged for 5 min at 1,500 rpm, followed by washing in PBS. After that, cells were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) containing protease inhibitor cocktail (Calbiochem, Darmstadt, Germany). The protein content of cellular extracts was quantified by Precision Red Advanced Protein Assay (Cyto skeleton, Denver, CO), with the absorbance measurement at a wavelength of 600 nm. Total cell extracted protein was resolved by SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes. Membranes were probed with antibodies specific to tenomodulin (TNMD) (AP12686c, rabbit polyclonal antibody; Abgent, San Diego, CA), COL VI (ab92349, rabbit polyclonal antibody; Abcam, Cambridge, MA), and GAPDH (sc-365062, mouse monoclonal antibody; Santa Cruz Biotechnology, Dallas, TX). For secondary antibodies, goat anti-mouse horseradish peroxidase conjugated antibody (Cell Signaling Technology, Boston, MA) and goat anti-rabbit horseradish peroxidase conjugated antibody (Cell Signaling Technology) were used. The protein bands were developed with an enhanced ECL detection kit (Amersham, Piscataway, NJ). Three cell samples were tested.

Proteomics Analysis Procedures

Sample preparation. The collected cells were lysed in an ice-cold buffer containing 8 M urea, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100 (vol/vol), 65 mM DTT, 1 mM EDTA, 0.5 mM PMSF, and 1% protease inhibitor cocktail. The supernatant containing the total cell proteins was precipitated with five volumes of ice-cold acetonitrile-ethanol-acetic acid (50:50:0.1 vol/vol/vol) at −20°C. After being resuspended in 8 M urea and 100 mM triethylammonium bicarbonate (pH 8.0), protein concentrations were measured by Bradford assay. Then proteins derived from S and G groups were digested with trypsin and labeled with light and heavy labeling, respectively, in the same procedure as reported previously (43).

Online multidimensional reversed phase liquid chromatography-tandem mass spectrometry analysis. The two-dimensional-liquid chromatography-tandem mass spectrometry (MS/MS) system consisted of a quaternary Surveyor pump, an auto-sampler, and a LTQ-Orbitrap Velos mass spectrometer (Thermo, San Jose, CA). The LTQ-Orbitrap Velos mass spectrometer (Thermo) was operated in data-dependent mode; system controlling and data collection were carried out by Xcalibur software, version 2.1 (Thermo).

The two-dimensional-liquid chromatography-MS/MS analysis was carried out according to a reported method, with minor modifications (51). Labeled tryptic digests were loaded onto the monolith scleraxis (SCX) trapping column (7 cm × 150 μm internal diameter). After that, the peptides were eluted onto the reversed-phase (RP) column using a step gradient generated with 1,000 mM NH₄Ac (pH 2.7). An RP separation gradient with a 0.1% formic acid aqueous solution (buffer A) and acetonitrile with 0.1% formic acid (buffer B) was developed to separate the peptides retained onto the RP segment, and one separation cycle lasted 185 min (0–10% buffer B for 10 min, 10–35% for 150 min, and 35–80% for 5 min; after holding at 80% buffer B for 10 min, the entire system was reequilibrated by buffer A for 10 min). The salt concentrations of the buffer were used in seven salt steps: 50, 100, 200, 300, 400, 500, and 1,000 mM. After each elution step, a subsequent RP liquid chromatography-MS/MS was executed.

Data Analysis

The raw data files of MS/MS spectra were searched against the human Uniprot FASTA database (12/11/2013, including 88,473 entries) using the MaxQuant (version 1.3.0.5, http://www.maxquant.org/doku.php?id=maxquant:start). For peptide and protein identification, the cut off false discovery rate were both set at 0.01. Quantification mode was selected with the dimethyl Lys 0 and N-term 0 as the light labels, and dimethyl Lys 4 and N-term 4 as the heavy labels. Default settings were used for all other parameters in MaxQuant. Proteins with a change of >1.5-fold were used for further analysis.

ELISA Analysis for Transforming Growth Factor-β1 Protein Expression

After being cultured on the silicone membrane in the first passage for 3 days, two groups of hDFs were switched to serum-free DMEM for another 12 h of culture and then replaced with fresh serum-free DMEM for another 48 h of culture. After that, the culture media were...
collected, and the released transforming growth factor-β1 protein was measured using a commercial ELISA kit (Ebioscience, San Diego, CA) with absorbance measurement at a wavelength of 450 nm. All measurements were performed in triplicate and repeated in three cell samples.

The Effect of TGF-β1 on SCX and TNMD Expression

To investigate the role of TGF-β1 in mediating hDF tenogenic transdifferentiation on microgrooved silicone membrane, 3 days after the culture on the silicone membrane in serum-containing medium, the cells of the first and the third passages were switched to serum-free DMEM for 24 h. Afterwards, the cells were treated with various concentrations of recombinant human TGF-β1 (Peprotech, Rocky Hill, NJ) for another 24 h, and then RNA was extracted for qPCR analysis.

To block the effect of TGF-β, the cells were cultured on silicone membranes in the first passage for 3 days and serum-starved for 12 h. Afterwards, cells were treated with 10 μg/ml anti-TGF-β neutralization antibody (1D11, Ebioscience) or 20 μM LY-2109761 (Selleck, Houston, TX, dissolved in water) for 48 h, followed by RNA extraction and qPCR assay. The cells cultured in the third passage were also similarly treated with 20 μM LY-2109761 followed by qPCR analysis.

RhoA Activation Assay

According to the manufacturer’s instruction, levels of GTP-bound RhoA were examined in passage 3 hDFs of S group and G group using the G-LISA activation kit (cytoskeleton), and absorbance was measured at 490 nm using a microplate reader (Beckman Coulter, Fullerton, CA). Experiment was performed in triplicate and repeated in three cell samples.

ROCK Activity Assay

To examine Rho-associated protein kinase (ROCK) activity, cells were cultured in two different substrata for one and three passages, followed by cell lysis with M-PER Mammalian Protein Extraction Reagent with protease inhibitor and phosphatase inhibitor cocktail (Roche, Penzberg, Germany) and incubated for 10 min on ice. The lysates were centrifugated for 10 min at 13,000 rpm at 4°C, and the supernatants were further quantified by precision red advanced protein assay reagent. Levels of ROCK activity were measured with Rho-kinase assay kit (MBL International, Woburn, MA), as previously reported (65), and absorbance was measured at 450 nm. Experiment was performed in triplicate and repeated in three cell samples.

The Effect of ROCK Inhibitor or Cytochalasin D on Tenogenic Gene Expression

The hDFs were grown on both plain and microgrooved silicone membranes. In the first passage and the third passage, after 3 days of culture, cells were serum-starved for 24 h and then treated with Y27632 (10 μM, Sigma-Aldrich, St. Louis, MO) for 24 h, followed by qPCR assay. The third passage HDFs were also similarly treated with cytochalasin D (0.25 μg/ml, Calbiochem).

Statistical Analysis

The statistical analyses were performed using the statistical software SPSS (version 19.0, SPSS, Chicago, IL). Student’s t-test was employed to analyze the differences between two groups, and a P value < 0.05 was considered statistically significant.

Fig. 3. Effect of elongated cell morphology on dermal fibroblast transdifferentiation toward other lineages. A: qPCR analysis of chondrogenic gene expressions. B: qPCR analysis of osteogenic gene expressions. C: qPCR analysis of adipogenic gene expressions. D: qPCR analysis of neurogenic gene expressions. E: proteomic analysis of protein production of identified neuronal and myogenic markers. [No protein was up- or downregulated for more than 1.5-fold, except for Actc1 (actin, α, cardiac muscle 1)]. Values are means ± SE for A–D or SD for E. *P < 0.05 and **P < 0.01, as determined by Student’s t-test. OCN, osteocalcin; ALPL, alkaline phosphatase, liver/bone/kidney; PPAR-γ, peroxisome proliferator-activated receptor-γ; CEB/Pα, CCAAT/enhancer binding protein (CEBP); MAP2, microtubule-associated protein 2; GFAP, glial fibrillary acidic protein.
RESULTS

Microgrooved Topography Enforced Elongation of In Vitro Cultured Dermal Fibroblasts

A few days after being seeded onto a microgrooved silicone membrane with 10 μm in groove width (Fig. 1A), hDFs were forced to become elongated in contrast to spread shape of control group, as revealed by cell morphology observation and actin staining (Fig. 1B). To quantify cell elongation, individual cells of actin-stained images were outlined to analyze cell area, angle, and aspect ratio. As shown in Fig. 1, C–E, the elongated cells revealed significantly smaller cell area (3,334.08 ± 1,343.18 μm²) than that of spread cells (7,944.47 ± 2,234.79 μm², P < 0.01). Better alignment was

Fig. 4. Effect of elongated cell morphology on chondrogenic and TNMD gene expression of cultured chondrocytes. A: cultured chondrocytes exhibited elongated and spread cell shapes on microgrooved membrane (G, arrowed) and smooth membrane (S), respectively, when observed under phase-contrast microscope. B: qPCR analysis of chondrogenic gene expression. C: qPCR analysis of TNMD gene expression. Bar = 75 μm. Values are means ± SE. *P < 0.05, as evaluated by Student’s t-test.

Fig. 5. Elongated cell morphology enhanced transforming growth factor-β1 (TGF-β1) expression and the effect of exogenous TGF-β1 on the gene expression of α-SMA (α-smooth muscle actin), SCX, and TNMD in the first passage cells. A: elongated morphology enhanced gene expression of TGF-β1. B: elongated morphology increased TGF-β1 protein production. C: effect of different dosages of TGF-β1 (0, 2, and 10 ng/ml) on α-SMA gene expression. D: effect of different dosages of TGF-β1 (0, 2, 5, and 10 ng/ml) on SCX gene expression. E: effect of different dosages of TGF-β1 (0, 2, 5, and 10 ng/ml) on TNMD gene expression. Values are means ± SE. *P < 0.05, as evaluated by Student’s t-test.
also observed in elongated cells with the angle (2.82 ± 2.33°) much smaller than that of spread cells (41.43 ± 26.19°, P < 0.01), as shown in Fig. 1, F–H. In addition, the elongated cells also demonstrated significantly higher cell body aspect ratio (8.31 ± 4.04) than that of spread cells (3.30 ± 1.41, P < 0.01, Fig. 1, I–K).

**Tenogenic Transdifferentiation of Human Dermal Fibroblasts by Enforced Elongated Cell Morphology**

At the end of the third passage, these elongated cells expressed significantly higher levels of SCX (1.87 ± 0.26-fold) and TNMD (3.22 ± 0.32-fold) genes than the spread cells, which grew on the plain silicone membrane (P < 0.05, Fig. 2A). In addition, the gene expression levels of tenogenic ECM components were also much higher in elongated cells than in spread cells with significant difference (P < 0.05). These included COL I (COL1, 2.13 ± 0.17-fold), COL III (COL3, 2.79 ± 0.46-fold), COL VI (COL6, 3.69 ± 0.79-fold), and decorin (2.20 ± 0.33-fold), as shown in Fig. 2B. Western blot also showed enhanced protein production of TNMD (37 kDa) and COL VI (COL6, 147 kDa) in elongated cells compared with spread cells (Fig. 2C). Proteome analysis revealed significantly enhanced protein production of COL1A1, COL1A2, COL3A1, COL5A1, COL6A1, COL6A3, COL12A1, and decorin in elongated cells than in spread cells (Fig. 2D, P < 0.05).

**Effect of Elongated Cell Morphology on Dermal Fibroblast Transdifferentiation Toward Other Lineages**

The potentials of transdifferentiation toward other lineages were also investigated by focusing on chondrogenic, osteogenic, adipogenic, myogenic, and neurogenic lineages. qPCR analysis revealed similar gene expression levels of chondrogenic markers of COL II (COL2) and aggrecan (Fig. 3A, P > 0.05) and adipogenic markers of PPAR-γ (peroxisome proliferator-activated receptor-γ), C/EBPα (CCAAT/enhancer binding protein-α), and AP-2α (activating enhancer binding protein-2α) (Fig. 3C, P > 0.05) between elongated and spread hDFs. For osteogenic marker expression, the elongated hDFs significantly inhibited the gene expression of ALPL (alkaline phosphatase, liver/bone/kidney) and OCN (osteocalcin) compared with spread cells (P < 0.05, Fig. 3B). As shown in Fig. 3D, the gene expression level of MAP2 (microtubule-associated protein 2), a marker for neuron, was not significantly different between two groups (P > 0.05). However, the expression of GFAP (glial fibrillary acidic protein), a marker for mature astrocyte and radial glia-like neuronal stem cells in adult brain, was significantly lower in elongated cells than in spread cells (P < 0.05, Fig. 3D). In addition, proteomics analysis revealed similar protein production levels of neuronal and myogenic markers between two groups of cells [P > 0.05, except for Actc1 (actin, α, cardiac muscle 1), which was downregulated for >1.5-fold, Fig. 3E].

**Elongated Chondrocytes Failed to Induce Tenogenic Phenotype and Inhibited Their Chondrogenic Phenotype**

As shown in Fig. 4A, human chondrocytes also exhibited an elongated shape (arrowed) when grown on the microgrooved membrane. Interestingly, elongated chondrocytes decreased the gene expression levels of aggrecan and COL2 to 0.61 ± 0.12-fold and 0.29 ± 0.11-fold of their control counterparts’ level, respectively (P < 0.05, Fig. 4B). Nevertheless, elongated cells were not able to enhance the gene expression of tenogenic marker TNMD compared with control cells (P > 0.05, Fig. 4C).

![Fig. 6. Elongated cell morphology enhanced TGF-β1 gene expression and TGF-β-induced (TGFBI) production, and low-dose exogenous TGF-β1 induced tenogenic phenotype in the third passage cells. A: elongated morphology enhanced gene expression of TGF-β1. B: elongated morphology increased TGFBI protein production. C: effect of cell shape on α-SMA gene expression. D: effect of low-dose TGF-β1 (2 ng/ml) on SCX gene expression. E: effect of low-dose TGF-β1 (2 ng/ml) on TNMD gene expression. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001, as evaluated by Student’s t-test. S2, smooth silicone membrane group with 2 ng/ml TGF-β1 treatment; G2, microgrooved silicone membrane group with 2 ng/ml TGF-β1 treatment.](http://ajpcell.physiology.org/doi/10.1152/ajpcell.00300.2015)
Elongated Cell Morphology Enhanced TGF-β1 Expression

TGF-β is known as an inducer of SCX and TNMD and thus was examined. After the first passage, qPCR analysis revealed significantly increased gene expression of TGF-β1 in elongated cells with the level 1.814 ± 0.36-fold higher than that of spread cells (P < 0.05, Fig. 5A). Additionally, ELISA also showed significantly enhanced TGF-β1 protein production in elongated cells than in spread cells (1,378.61 ± 61.89 vs. 1,213.97 ± 36.77 pg/ml, P < 0.05, Fig. 5B). However, no significant difference in the gene expression level of α-SMA (α-smooth muscle actin) was observed between two groups without exogenous TGF-β1 treatment (P > 0.05, Fig. 5C).

After three passages, elongated cell morphology enhanced TGF-β1 gene expression level with 1.945 ± 0.13-fold of increase, as opposed to that of spread morphology (P < 0.05, Fig. 6A). In addition, proteomic analysis also revealed 1.770 ± 0.12-fold of increase of TGFBI (TGF-β1 protein production in Fig. 6, A). The treatment with TGF-β1 induced, which is induced by TGF-β (55), with significant difference between two groups (P < 0.05, Fig. 6B). Similar to the first passage, no significant difference in α-SMA gene expression was observed between the two groups (P > 0.05, Fig. 6C).

Low-Dose Exogenous TGF-β1 Induced Tenogenic But Not Myofibroblast Transdifferentiation

Based on the above findings, we further investigated the effect of various doses of exogenous TGF-β1 on cell morphology-mediated dermal fibroblast transdifferentiation. As shown in Fig. 5, D and E, treatment of exogenous TGF-β1 for 24 h in the first passage at a dose of 2 ng/ml could significantly enhance the gene expression of SCX and TNMD, particularly in the elongated cell group compared with nontreated cells (P < 0.05). Interestingly, at this dose, exogenous TGF-β1 failed to enhance α-SMA gene expression in the elongated cell group, rather it decreased the expression in elongated cells compared with the nontreated group (P < 0.05, Fig. 5C). With further increased dosages, TGF-β1 failed to further enhance the gene expressions of SCX and TNMD (Fig. 5, D and E). By contrast, it enhanced α-SMA gene expression in both spread and elongated cell groups (Fig. 5C).

Similarly, treatment with the dose of 2 ng/ml at passage 3 also significantly enhanced the gene expressions of SCX and TNMD in both groups compared with nontreated cells (P < 0.05), but the levels were significantly higher in elongated cells than in spread cells (Fig. 6, D and E).

Anti-TGF-β Treatment Abrogated Upregulated Gene Expression of SCX and TNMD Mediated by Elongated Cell Morphology

As shown in Fig. 7, A and B, treatment of the cells with TGF-β neutralizing antibody at the first passage could mostly abrogate upregulated expression of SCX and TNMD genes mediated by elongated cell shape (G-anti), compared with nontreated cells (G; P < 0.05), but the antibody was not able to downregulate SCX expression (P > 0.05). Rather, increased

Fig. 7. Anti-TGF-β treatment abrogated upregulated gene expression of SCX and TNMD induced by elongated cell morphology. TGF-β neutralizing antibody treatment abrogated the upregulation of SCX [microgrooved silicone membrane group treated with 10 μg/ml TGF-β neutralization antibody (G-anti); A] and TNMD gene expression (G-anti; B) in elongated cells of the first passage, but promoted TNMD gene expression in spread cells of the first passage [smooth silicone membrane group treated with 10 μg/ml TGF-β neutralization antibody (S-anti); B]. The treatment with TGF-β receptor inhibitor LY-2109761 abrogated the upregulation of SCX [microgrooved silicone membrane group treated with 20 μM LY-2109761 (GLY); C] and TNMD gene expression (GLY; D) in elongated cells of the first passage. Additionally, the same treatment abrogated the upregulation of SCX (GLY; E) and TNMD gene expression (GLY; F) in elongated cells of the third passage, but promoted SCX [smooth silicone membrane group treated with 20 μM LY-2109761 (SLY); E] and TNMD gene expression (SLY; F) in spread cells of the third passage. Values are means ± SE. *P < 0.05, as determined by Student’s t-test.
TNMD gene expression in spread cells (S-anti) compared with nontreated cells (S; \(P < 0.05\)).

In agreement with the effect of neutralizing antibody, treatment of TGF-\(\beta\) receptor inhibitor LY-2109761 at the first passage also significantly blocked the upregulated gene expression of SCX and TNMD in elongated group (GLY) compared with nontreated cells (G; \(P < 0.05\)), but, again, this treatment failed to downregulate the expression in the spread cell group (SLY vs. S), as shown in Fig. 7, C and D. At the third passages, the inhibitor treatment revealed even greater inhibitory effect on the gene expression of SCX and TNMD in elongated cells (GLY vs. G; \(P < 0.05\)), but slightly upregulated the gene expression in spread cells (SLY vs. S; \(P < 0.05\)), as shown in Fig. 7, E and F, indicating that cell shape played an important role in this phenomenon.

Elongated Cell Shape Led to Enhanced ROCK Activity, and Blocking ROCK Activity Resulted in Reduced Expression of Tenogenic Markers

It was observed that elongated cell shape led to reduced GTP-bound RhoA activity and increased ROCK activity compared with spread cells (\(P < 0.05\), Fig. 8, A and B) at the third passage. Blocking ROCK activity with specific inhibitor Y27632 at the dose of 10 \(\mu\)M could significantly downregulate the gene expressions of SCX (Fig. 8C, \(P < 0.05\)), TNMD (Fig. 8D, \(P < 0.05\)), COL1 (Fig. 8E, \(P < 0.05\)), COL3 (Fig. 8F, \(P < 0.05\)), COL6 (Fig. 8G, \(P < 0.05\)), and decorin (Fig. 8H, \(P < 0.01\)) in elongated cells (GY vs. G). However, this treatment failed to downregulate the expression of these genes (Fig. 8, C–G, \(P > 0.05\)), except for decorin (Fig. 8H, \(P < 0.05\)) in spread cells (SY vs. S).

Disruption of Cytoskeleton with Cytochalasin D Led to Reduced Expression of Tenogenic Markers

To investigate the relationship between microfilament structure and cell shape-induced tenogenic transdifferentiation, one chemical inhibitor of F-actin dynamics, cytochalasin D, was employed. As shown, cytochalasin D treatment of the third passage cells for 24 h at the concentration of 0.25 \(\mu\)g/ml led to significantly downregulated gene expression of SCX (Fig. 9A, \(P < 0.05\)), TNMD (Fig. 9B, \(P < 0.05\)), COL1 (Fig. 9C, \(P < 0.05\)), COL3 (Fig. 9D, \(P < 0.05\)), COL6 (Fig. 9E, \(P < 0.05\)), COL6 (Fig. 9F, \(P < 0.05\)), COL6 (Fig. 9G, \(P < 0.05\)), and decorin (Fig. 9H, \(P < 0.05\)) in elongated cells (GY vs. G). However, this treatment failed to downregulate the expression of these genes (Fig. 8, C–G, \(P > 0.05\)), except for decorin (Fig. 8H, \(P < 0.05\)) in spread cells (SY vs. S).

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**Fig. 8.** The effect of elongated cell morphology on GTP-bound RhoA and Rho-associated protein kinase (ROCK) activity and the effect of ROCK inhibitor on the expression of tenogenic markers in the third passage cells. A: the level of GTP-bound RhoA was significantly lower in elongated cells than in spread cells of the third passage. B: the ROCK activity was significantly higher in elongated cells than in spread cells of the third passage. The treatment of ROCK inhibitor Y27632 significantly downregulated the gene expression of SCX (GY; C), TNMD (GY; D), COL1 (GY; E), COL3 (GY; F), and COL6 (GY; G) in elongated cells of the third passage, and significantly downregulated the gene expression of decorin in both spread cells [smooth silicone membrane group treated with 10 \(\mu\)M Y27632 (SY); \(H\)] and elongated cells [microgrooved silicone membrane group treated with 10 \(\mu\)M Y27632 (GY); \(H\)] of the third passage. Values are means ± SE. *\(P < 0.05\) and **\(P < 0.01\), as determined by Student’s t-test. OD, optical density.
and decorin (Fig. 9F, P < 0.05) in elongated cells (GCD vs. G). However, this treatment could downregulate the gene expression of COL3 (Fig. 9D, P < 0.05), and at the same time upregulate the gene expression of COL6 (Fig. 9E, P < 0.05), but had little impact on other molecules in spread cells (SCD vs. S).

Dynamic Change of ROCK Activity During Culture Time Period

We also investigated the time course of cell shape caused ROCK activity variation, and the relationship between ROCK activity and the gene expression of SCX and TNMD. When seeded on microgrooved membrane, enforced elongated cell shape led to decreased ROCK activity (Fig. 10A) and increased expression of SCX and TNMD (Fig. 7, A and B) at the early time point (passage 1), which was different from the finding of later time point cells (passage 3), i.e., elongated cell shape led to increased ROCK activity (Fig. 8B), along with increased expression of SCX and TNMD (Fig. 2A).

In addition, in the first passage cells, the treatment of ROCK inhibitor Y27632 at the concentration of 10 μM for 24 h led to increased gene expression of SCX and TNMD (Fig. 10, B and C, GY vs. G), which was different from the passage 3 cells that showed decreased SCX and TNMD gene expression with Y27632 treatment (Fig. 8, C and D).

Proteomic Data Analysis: Gene Ontology Classification of the Reliably Quantified Proteins

A total of 2,116 quantified proteins were identified. Using spread group as a control level, a threshold of 1.5-fold change was applied to the selection of proteins differentially expressed in spread and elongated cells. It was found that 25 proteins...
were upregulated, whereas 29 proteins were downregulated in
elongated cells vs. spread cells (Table 2). Using the DAVID (Data for Annotation, Visualization and Integrated Discovery) Classification System (version 6.7, http://david.ncifcrf.gov/), we categorized the upregulated and downregulated
proteins into cellular component, molecular function, and bio-
ological process pertaining categories, as shown in Figs. 11–13. For both upregulated and downregulated proteins in the cellular
component (Fig. 11), most of the quantified proteins were in
the cell and organelle gene ontology (GO) category, with more
upregulated proteins locating at extracellular region. The
molecular functional category of the majority proteins were bind-
Differentially produced proteins were classified into Cellular Component and Molecular Function using Gene Ontology annotation.

**DISCUSSION**

It has been demonstrated that cell morphology and structure could determine cell functions, such as neuron and epithelial/epidermal cells (6, 17, 25, 33, 34, 50). Vice versa, forced change of cell function also led to cell morphological changes, for example, epithelial-mesenchymal transition often resulted in the loss of cell polarity of epithelial cells and the gaining of spindle shape of mesenchymal cells (49). Likewise, induced differentiation of stem cells often led to morphological change, like neuronal differentiation (18, 52), and differentiated cells also exhibit cell shape changes when they become dedifferentiated, for example, when chondrocytes were de-differentiated into fibroblasts phenotype during in vitro culture (1, 37).

Use of micropattern topography to artificially control cell morphology for directing stem cell differentiation has already been explored (22), and particularly for specific lineage differentiation of MSCs (19, 36). In fact, parallel microgroove structure is a common form of micropattern topography that has been used to induce stem cell differentiation toward neurogenic (21), myogenic (24), and tenogenic (63) lineages. However, this remains less explored for the transdifferentiation between different types of mature fibroblasts. Our laboratory previously proposed that elongated cell shape control might serve as one of the most important physical cues for inducing a tenogenic phenotype (57), based on the evidence that elongation of in vitro cultured tenocytes could prevent its phenotype loss using a microgrooved topographical membrane as a culture substrate (65).

Employing this micropattern topographical surface (46, 65), we were able to demonstrate that this particular topography could induce an elongated cell morphology, as supported by the morphological observation of actin-stained cells and the quantitative analyses of cell area, cell body major axis, and cell body aspects (Fig. 1), according to previously reported method (4, 47). Importantly, the elongated hDFs exhibited a predominant tenogenic phenotype with enhanced expression of tenogenic markers, such as SCX, TNMD, COL I, COL III, COL VI, and decorin, as revealed by qPCR, Western blot, and pro-

**Fig. 11.** The upregulated proteins (Up) [G vs. S; heavy-to-light ratio for a given protein (H/L ratio) > 1.5] and downregulated proteins (Down) [G vs. S; H/L ratio < 0.5] were classified into Cellular Component using Gene Ontology annotation.

**Fig. 12.** The upregulated proteins (G vs. S; H/L ratio > 1.5) and downregulated proteins (G vs. S; H/L ratio < 0.5) were classified into Molecular Function using Gene Ontology annotation.
Interestingly, the switched phenotype seemed to be tenogenic specific relatively, because enforced elongation failed to induce transdifferentiation toward other lineages in general, such as chondrogenic, adipogenic, myogenic, and neurogenic (Fig. 3). The induced tenogenic phenotype also seemed cell type specific, as elongated human chondrocytes failed to induce tenogenic phenotype, although the chondrogenic phenotype was inhibited to a certain extent by reducing the expression of \( \text{COL II} \) and aggrecan (Fig. 4), suggesting that the phenotype switch was much easier between different types of fibroblasts than the switch between two different types of fully mature and terminal differentiated cells with specific functions.

It was also reported that microgroove width could affect differentiation lineage, depending on the range of groove width (21, 44). We thus also tested the effect of different widths on tenogenic phenotype induction. In addition to 10-\( \mu \)m width membrane, the microgroove of 5 \( \mu \)m width was found unable to effectively induce tenogenic phenotype, whereas 20-\( \mu \)m width was able to induce, but was less optimal compared with that of 10-\( \mu \)m width (data not shown). It will be interesting to investigate whether different width micropatterns will induce transdifferentiation of hDFs toward different lineages.

It is well known that dermal fibroblasts can be transdifferentiated into myofibroblasts, given the stimulation of TGF-\( \beta \), particularly in spread cell morphology (32). Coincidently, TGF-\( \beta \) is also known for inducing tenogenic phenotype by upregulating TNMD and SCX expression (11, 27, 35). We thus investigated the role of TGF-\( \beta \) in regulating tenogenic and myofibroblast phenotypes. As shown, upregulated expression of tenogenic markers were closely associated with the upregulated expression of TGF-\( \beta 1 \) at both gene (Figs. 5A and 6A) and protein levels (Fig. 5B). TGFB1, a protein induced by TGF-\( \beta \), was also significantly enhanced for its production (Fig. 6B) in elongated cells. However, increased TGF-\( \beta 1 \) production in elongated group failed to induce \( \alpha \)-SMA expression (Figs. 5C and 6C), indicating that elongated morphology could induce tenogenic phenotype, but not myofibroblast phenotype.

In agreement with previous reports (12, 27), exogenous TGF-\( \beta 1 \) at the dosage of 2 ng/ml could significantly enhance the gene expression of TNMD (Figs. 5D and 6D) and SCX (Figs. 5E and 6E), and this enhancement was much more significant in elongated cells than in spread cells. By contrast, this dosage failed to induce \( \alpha \)-SMA expression, rather it significantly decreased its expression in elongated cells compared with nontreated cells (Fig. 5C). Furthermore, TGF-\( \beta \) neutralizing antibody (Fig. 7, A and B) and TGF-\( \beta \) signaling blocker (Fig. 7, C–F) could significantly downregulate the gene expression of tenogenic markers in elongated cells.

As expected, under the stimulation of nonphysiological dosage, exogenous TGF-\( \beta 1 \) (10 ng/ml) failed to induce a tenogenic phenotype, rather, it significantly down-regulated the gene expression of SCX and TNMD in elongated cells compared with nontreated cells (Fig. 5, D and E). When applied at this higher dosage (10 ng/ml), a concentration for myofibroblast transdifferentiation adopted by previous studies (9, 46), exogenous TGF-\( \beta 1 \) could indeed significantly upregulate the gene expression of \( \alpha \)-SMA, regardless of cell shape.

![Biological Process](http://ajpcell.physiology.org/)

**Fig. 13.** The upregulated proteins (G vs. S; H/L ratio > 1.5) and downregulated proteins (G vs. S; H/L ratio < 0.5) were classified into Biological Process using Gene Ontology annotation.
All of the evidence supports the expectation that the synergistic effect between TGF-β and cell shape-mediated other signaling plays a central role in the tenogenic phenotype switch observed in this study. This is also supported by other reports that cell shape-mediated phenotype switch is likely involved in both altered expression of TGF-β1 and cytoskeleton mediated signaling (39, 40).

Cytoskeletal tension alteration is one of the important aspects of cell shape-mediated effect (22). It has been demonstrated previously that limiting cell spreading could result in reduced cytoskeletal tension and likely the ROCK activity (3). As reported by the literature, cytoskeletal tension can regulate architecture of nucleus (20) to affect organelle and DNA organization and distribution (7, 10, 13, 16, 56), alter expression and functions of integrin, and focal adhesion kinase (14). It can also inhibit Hippo signaling (38), modulate matrix metalloproteinase-1 expression (28), determine lineage commitment of MSCs (48), etc. Therefore, cytoskeletal tension is likely to independently regulate the tenogenic phenotype, regardless of the influence derived from soluble factors. This is also supported by a previous study, which demonstrated that RhoA could be a downstream target of soluble differentiation signal, whereas cell shape control-mediated effect provided another signal for lineage commitment via ROCK-induced cytoskeletal tension (31).

In this study, enforced elongation was likely to mediate the tenogenic phenotype via TGF-β1-induced expression of SCX and TNMD on the one hand. On the other hand, the artificial restriction of cell spreading by microgroove topography would redirect actin fiber alignment and reduce cytoskeletal tension, which triggered other downstream signals to induce tenogenic marker expression. This may explain why the phenotype was more prominent in elongated cells than in spread cells when exposed to the same dose of exogenous TGF-β1 (Fig. 5, D and E). Naturally, when the particular cytoskeletal structure formed by artificial elongation was disrupted by cytochalasin D treatment, the beneficial effect was partly abrogated in most of examined molecules in elongated cells, yet the spread cells were less affected (Fig. 9).

ROCK is the downstream molecule of RhoA. In this study, ROCK activity was found decreased in elongated cells at early time point of day 4 (passage 1), along with increased SCX and TNMD expression, and the treatment of ROCK inhibitor Y27632 could enhance the gene expression of both markers (Fig. 10). However, at later time point (passage 3), ROCK activity was found increased in the elongated cell group (Fig. 8B), along with enhanced SCX and TNMD expression (Fig. 2), and ROCK inhibitor reduce their expression (Fig. 8, C and D). This apparent difference was likely caused by enhanced expression of TGF-β, which could both enhance ROCK activity (32, 60) and upregulate SCX and TNMD expression (12, 27), as previously reported. In fact, dual models of RhoA activation at early stage and downregulation at later stage were believed to be essential for TGF-β-induced epithelial-mesenchymal transition (64).

Collectively, this study demonstrated an interesting phenomenon that fibroblasts among different anatomic locations (for example, skin and tendon) were likely to be interconverted by simulating each other’s cell morphology. Despite an obvious tenogenic phenotype induced by elongation compared with spread cell group, it remained relatively weaker than primarily cultured tenocytes in terms of the expression levels of tenogenic markers (data not shown), suggesting that single-factor imitation in vitro is far from fully converting dermal fibroblasts into functional tenocytes, simply because the synergistic effect between cell shape and other factors, such as mechanical loading (57), is essential in this process. Fortunately, our laboratory’s previous in vivo study demonstrated that dermal fibroblast engineered tendon could achieve tissue structure similar to tenocyte engineered tendon and native tendon tissue under long-term mechanical loading (26), and the result of this study may partially explain the mechanism from the perspective of basic cell biology study.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: W.W., J.L., and K.W. performed experiments; W.W., Z.Z., W.Z., G.Z., M.Y., and W.L. analyzed data; W.W. prepared figures; W.W. drafted manuscript; Z.Z., W.Z., G.Z., M.Y., and W.L. approved final version of manuscript; W.L. conception and design of research; W.L. interpreted results of experiments; W.L. edited and revised manuscript.

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