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The regulation of phenotype of cultured tenocytes by microgrooved surface structure

Ji Zhu [a,1], Jie Li [a,1], Bin Wang [a], Wen Jie Zhang [a,b], Guangdong Zhou [a,b], Yilin Cao [a,b], Wei Liu [a,b,*]

[a] Department of Plastic and Reconstructive Surgery, Shanghai 9th People's Hospital, Shanghai Tissue Engineering Key Laboratory, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Rd, Shanghai, 200011, China
[b] National Tissue Engineering Center of China, Shanghai, China

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ABSTRACT

To maintain or enhance cell function by controlling its shape is an important consideration in scaffold design. Tenocyte is characterized by its unique elongated cell shape and the role remains unexplored. In this study, primary porcine tenocytes of newborn pigs were cultured respectively on culture dish (Group A), smooth (Group B) or microgroove silicone membrane (Group C, enforcing an elongated morphology) to observe the effect of cell shape on tenocyte phenotype. The results showed that elongated morphology (Group C) could help *in vitro* passaged tenocytes to retain their phenotype and function by maintaining the expression of tenomodulin (tenocyte marker) and collagen I (functional molecule). By contrast, the spread tenocytes (Groups A and B) lost or significantly reduced the expression of tenomodulin or collagen I respectively. Interestingly, the lost tenomodulin expression of Group B cells could be regained after being switched to microgroove culture condition of Group C. In addition, significantly increased RhoA-ATP level and reduced ROCK activity were found associated with elongated morphology and artificially activating RhoA or inhibiting ROCK could lead to increased tenomodulin expression in spread cells. Collectively, these results confirm that elongated morphology is essential for tenocytes to keep their phenotype and function and can redifferentiate the dedifferentiated tenocytes by the participation of RhoA/ROCK signaling, and these findings may provide insight into the design of advanced scaffold for tendon engineering.

1. Introduction

As the key component of tissue engineering, scaffold is conventionally thought to simply provide an environment allowing cells to grow, produce extracellular matrices and form tissue, in which scaffold does not play a decisive role unless chemical molecules are integrated into the scaffold to guide biological behavior of seeded cells. However, recent studies demonstrated that physical characters of a material such as matrix elasticity can actually significantly affect the biological behavior of seeded cells [1]. It was also observed that geometric cue mediated cell control is able to direct mesenchymal stem cell (MSC) differentiation [2].

The interrelationship between cell shape and its function is one of the important subjects of cell biology study [3]. In musculoskeletal and connective tissues, there are different types of cells which exhibit different cell morphologies and serve different functions. For example, osteoblasts and chondrocytes have cuboidal cell shape, while fat cells have a round cell shape. Importantly, the special cell shapes allow cells to perform special functions. It has been observed that adipogenic differentiation of MSC required spread morphology, whereas narrow cell shape is needed for MSC osteogenic differentiation [2]. Furthermore, cell function change also comes with cell morphology change. For example, bone marrow stromal cells with fibroblast-like shapes began to exhibit polygon shape after chondrogenic induction [4]. Also, endothelial progenitor cells with spindle morphology can be switched to cobblestone cell shape after endothelial differentiation [5].

In vivo niche might be an important factor that determines special cell morphology inside a specific tissue type. As an example, tendon is one type of special connective tissue that carries out the function of transmitting mechanical force form muscle to bone. To meet this functional requirement, collagen fibers are aligned longitudinally along with the axis of mechanical forces, and thus the tenocytes constrained within the narrow space between the fibers exhibit elongated morphology [6]. However, it remains unclear whether the elongated cell morphology is essential for tenocyte function and phenotype maintenance when tenocytes are deprived of their *in vivo* niche.

* Corresponding author.
E-mail address: liuwei_2000@yahoo.com (W. Liu).
[1] These authors contributed equally.

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Tenocytes or tendon fibroblasts are one type of fibroblasts, a common term for describing mesenchymal cells that mainly perform tissue supporting function in different musculoskeletal and connective tissues. It has been difficult for a long time to characterize this cell type because of no specific marker available. Fortunately, recent researches demonstrated that tenomodulin (Tnmd), a member of a new family of type II transmembrane glycoproteins, is predominantly expressed in dense connective tissue like tendon and ligament, and can serve as a differentiation marker for mature tenocytes [7,8]. In addition, deletion of tenomodulin gene could to a certain extent affect tendon development [9]. Thus, these results make a specific tenocyte marker molecule available, which can serve as a tool to investigate the interrelationship between the elongated cell morphology of tenocytes and their phenotype and function. Additionally, RhoA/ROCK signaling pathway has been shown a major signaling pathway for cell shape mediated regulation of cell function [10–12].

Based on these phenomena, we would like to hypothesize that tenocyte phenotype and function can be better maintained during *in vitro* cultured by mimicking their original *in vivo* morphology, i.e. an elongated form; and RhoA/ROCK (Rho-associated kinase) pathway is likely to play a role in regulating this process. In this study, dedifferentiation with phenotype and functional loss were observed in cultured and spread tenocytes with a worsening trend along with prolonged culture time. By contrast, forcing tenocytes in an elongated form can prevent phenotype and functional loss of cultured tenocytes or even restored the lost phenotype by switching spread to elongated morphology, and reduced ROCK activity plays an important role.

2. Materials and methods

2.1. Cell culture

Newborn pigs were purchased from Shanghai Agricultural Institute. An Institutional Review Committee of Shanghai Jiao Tong University School of Medicine approved all animal study protocols. Porcine primary tenocytes were isolated from toe flexor tendons. Briefly, flexor tendons were harvested from newborn pigs and adjacent tissues were stripped. After thorough wash in phosphate-buffered saline (PBS) and soaking in 2.5% chloramphenicol solution for 10 min, the tendons were washed again in PBS and then were minced aseptically followed by enzyme digestion with 0.2% collagenase II (Worthington, Freehold, NJ) in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C on a rotator as previously described [13,14]. The resulting cell suspension harvested at 4 h post-digestion was filtered through a sterile nylon mesh (Tetko, Elmsford, N.J.) to remove tissue residues. The filtrate was further centrifuged and cell pellets were washed with PBS and then resuspended in DMEM culture medium containing 10% fetal bovine serum (FBS), L-glutamine (292 mg/ml), penicillin (100 U/ml), streptomycin (100 mg/ml), and ascorbic acid (50 mg/ml). The cell suspension was transferred to a culture dish ($5 \times 10^5/cm^2$) and cultured at 37 °C in a humidified 5% CO_2 atmosphere. When cultured cells reached 80% confluence, they were detached with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid and subcultured at the same density ($5 \times 10^5/cm^2$) until second passages.

2.2. Microgroove membrane as a cell culture substrate

To enforce an elongated morphology of cultured tenocytes, previously reported microgroove silicone membrane, a gift kindly provided by Dr. James Wang from University of Pittsburgh [15], was employed for the study. As reported [15], the membrane surfaces were micro-fabricated to create parallel microgrooves with 10 μm ridge and groove width and 3 μm groove depth. Meanwhile, smooth silicone membranes were used as a control and both types of membranes were coated with 10 ng/ml fibronectin (Roche, Indianapolis, IN) to promote cell attachment.

2.3. Experimental design

To determine the effect of cell shape on tenomodulin and collagen I expression and its potential mechanism, regularly cultured tenocytes of the second passages were further divided into four different groups for culturing under different conditions. Group A: on regular 100-mm culture dishes for another 4 or 5 passages; Group B: on smooth silicone membrane for another 4 or 5 passages; Group C: on microgroove silicone membrane for another 4 or 5 passages; Group D: on smooth silicone membrane for 3 passages and then part of cells were transferred to microgroove membrane for another 2 passages and part of cells were cultured continuously for another 2 passages on the smooth membrane. All cells were examined for their cell shapes and structures 48 h after cell attachment with H&E staining and F-actin staining. After 4 or 5 passages of cell culture, all cells grown on different substrata were collected for RT-PCR and quantitative PCR analyses of various gene expression, and assessment of RhoA activation and ROCK activity.

2.4. RT-PCR analysis

The designed primers for PCR analysis were listed in Table 1. The cDNA was amplified by RT-PCR (Taq-polymerase, TaKaRa, Japan). PCR was performed with incubation at 95 °C for 5 min followed by 29 cycles (30 s at 95 °C, 30 s at annealing temperatures listed in Table 1 and 45 s at 72 °C) and terminated by 10 min extension at 72 °C and stored at 4 °C until analysis. PCR products were separated using a 1.5% agarose gel in TAE buffer containing ethidium bromide and subsequently photographed under UV light. β-actin or GAPDH gene was used as an internal control. Experiment was repeated in three tissue samples.

2.5. Quantitative PCR analysis

For quantitative PCR, cDNA was amplified 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). Quantitative PCRs (qPCRs) were conducted in triplicate for each sample. Gene expression was normalized to GAPDH expression by using the $2^{-\Delta\Delta C(t)}$ method [16]. Experiment was repeated in three tissue samples.

2.6. H&E staining

Porcine tenocytes were cultured on coverslips, smooth silicone membrane, microgroove silicone membrane for 48 h. The cells were fixed with 4% paraformaldehyde in PBS for 15 min and stained with H&E.

2.7. F-actin staining

To observe the effect of cell shape or drug treatment on actin organization, 48 h after incubation on three different stratum, or 24 h after treatment with lysophosphatidic acid (LPA) or Y27632, cells were fixed with 4% paraformaldehyde in phosphate buffer for 10 min, and permeabilized with 0.5% Triton X-100 in phosphate buffer for 5 min. The cells were then incubated in rhodamine phalloidin (100 nM, cytoskeleton, Denver, CO) for 30 min, and nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI) (100 nM, Sigma, St. Louis, MO) for 30 s.

2.8. RhoA activation assay and ROCK activity

Levels of GTP-bound RhoA were determined in tenocytes cultured under three different conditions for 4 passages with the G-LISA™ activation kit (Cytoskeleton, Denver, CO) according to the manufacturer's instructions as previously reported [17,18]. The signal was read by measuring absorbance at 490 nm using a microplate

Table 1
Primers used in RT-PCR and quantitative PCR analysis.

Gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)
Tenomodulin	Sense: CCAGAGGAAGAAATAGATGAGAATG Antisense: CACTGGTAGGAAAGTGAAGATCTTC	58	228
Collagen I	Sense: GATCCTGCTGACGTGGCCAT Antisense: ACTCGTGCAGCCGTCGTAGA	55	222
β-actin	Sense: CCAAGGCCAACCGTGAGAAGATGAC Antisense: AGGGTACATGGTGGTCCCGCCAGAC	58	592
GAPDH	Sense: GGTCACCAGGGCTGCTTT Antisense: CTTGACTGTGCCGTGGAAT	58	133

reader (Beckman Coulter, Fullerton, CA). Accordingly, to measure ROCK activity of porcine tenocytes cultured under the three different conditions for 4 passages, the Rho-kinase Assay Kit (MBL International, Woburn, MA) was used according to the manufacturer's instructions as previously reported [19,20]. Tetramethylbenzidine was used as a substrate, and the signal was read by measuring absorbance at 450 nm. Experiment was repeated in three tissue samples.

2.9. The effect of ROCK inhibition or RhoA activation on tenomodulin expression

For ROCK inhibition assay, cells were first grown on regular dishes, serum-starved for 24 h, then treated with ROCK inhibitor Y27632 (10 μm, sigma) [21] in serum-free condition for different times, or treated with different doses of Y27632 for 24 h followed by Real-time PCR analysis and F-actin staining. Then the cells were grown on three different substrata for 2 days and serum-starved for 24 h, treated with 10 μm Y27632 for 24 h followed by Real-time PCR assay. Similarly, for activation assay, cells were grown on three different substrata for 2 days and serum-starved for 24 h, and then treated with LPA (1 μg/ml, Sigma) [22,23] in serum-free medium for 24 h followed by Real-time PCR assay and actin staining of group A cells. Experiment was repeated in three tissue samples.

2.10. Statistical analysis

Results are presented as means ± standard deviation. The differences among different groups were analyzed using one-way ANOVA test. When the analysis of variance indicated significance in the difference among data, multiple comparisons were performed with Newman–Keuls post hoc tests. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Tenocyte cultrue and the gene expression of tenomodulin and collagen I

During *in vitro* cell culture, tenocytes lost their characteristic elongated cell morphology with cell passages and became spread in their shape (Fig. 1a). Interestingly, the mRNA expression of tenocyte marker molecule tenomodulin was markedly reduced and lost after 6 and 10 passages during *in vitro* culture as revealed by RT-PCR and Real-time PCR analyses (Fig. 1b and c). Importantly, the expression of the major functional molecule type I collagen also sharply decreased with cell passage (Fig. 1b and d), suggesting a close relationship between cell morphology and cell function.

3.2. Cell morphology and tenomodulin gene expression

To further confirm whether cell morphology could regulate tenomodulin expression, cells were incubated on three different substrata for total 4 passages. As shown in Fig. 2a, Group C cells (cultured on microgroove membrane) could maintain an elongated morphology even after 4 passages as revealed by light microscopy and H&E staining (Fig. 2a). By contrast, cells of Groups A and B (Fig. 2a) remained spread after 4 passages. It was noted that enforced elongation also reorganized the actin stress fibers longitudinally along with the cell axis (Fig. 2a). Importantly, both RT-PCR and quantitative PCR analyses revealed that the tenomodulin expression was continuously decreased in spread cells (Groups A and B) along with cell passage; however, the expression could be well maintained in the cells with enforced elongation (Group C), which was significantly higher than that of the other two groups (Fig. 2b and c). Moreover, elongated cell morphology could also maintain a higher expression level of collagen I in the cells of Group C than in the cells of Groups A and B (Fig. 2d and e) when examined at passage 4. These results indicate that elongated cell shape may play a dominant role in determining the phenotype and function of *in vitro* cultured tenocytes.

3.3. Redifferentiation of dedifferentiated tenocytes by elongated morphology

In this part of study, cells were first passaged three times on different substrata to allow for dedifferentiation of the cells in groups A and B, and then the possibility of redifferentiation was investigated by switching from Group B to Group D. As shown in Fig. 3, one passage after switching the culture condition (P3 + P1), the tenomodulin expression level of switched cells (Group D cells) remained low and equivalent to that of non-switched cells (Group B

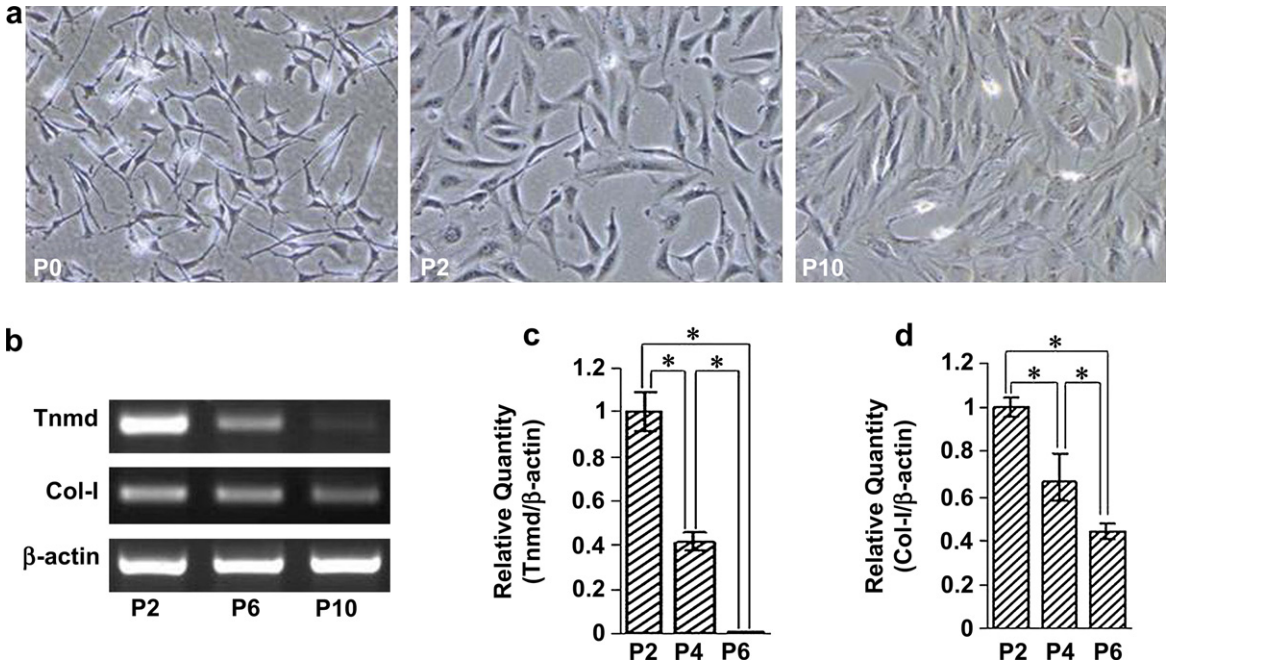

Fig. 1. In vitro cultured porcine tenocytes lost their native morphology and phenotype. Cells grown on culture dish became spread in their morphology from passage 2 to passage 10 (a) and exhibited decreased gene expression level of tenomodulin (Tnmd, b, c) and collagen I (Col-I, b, d) revealed by RT-PCR (b) and quantitative PCR (c, d). The symbol "*" indicates $p < 0.05$ and "P" represents cell passage.

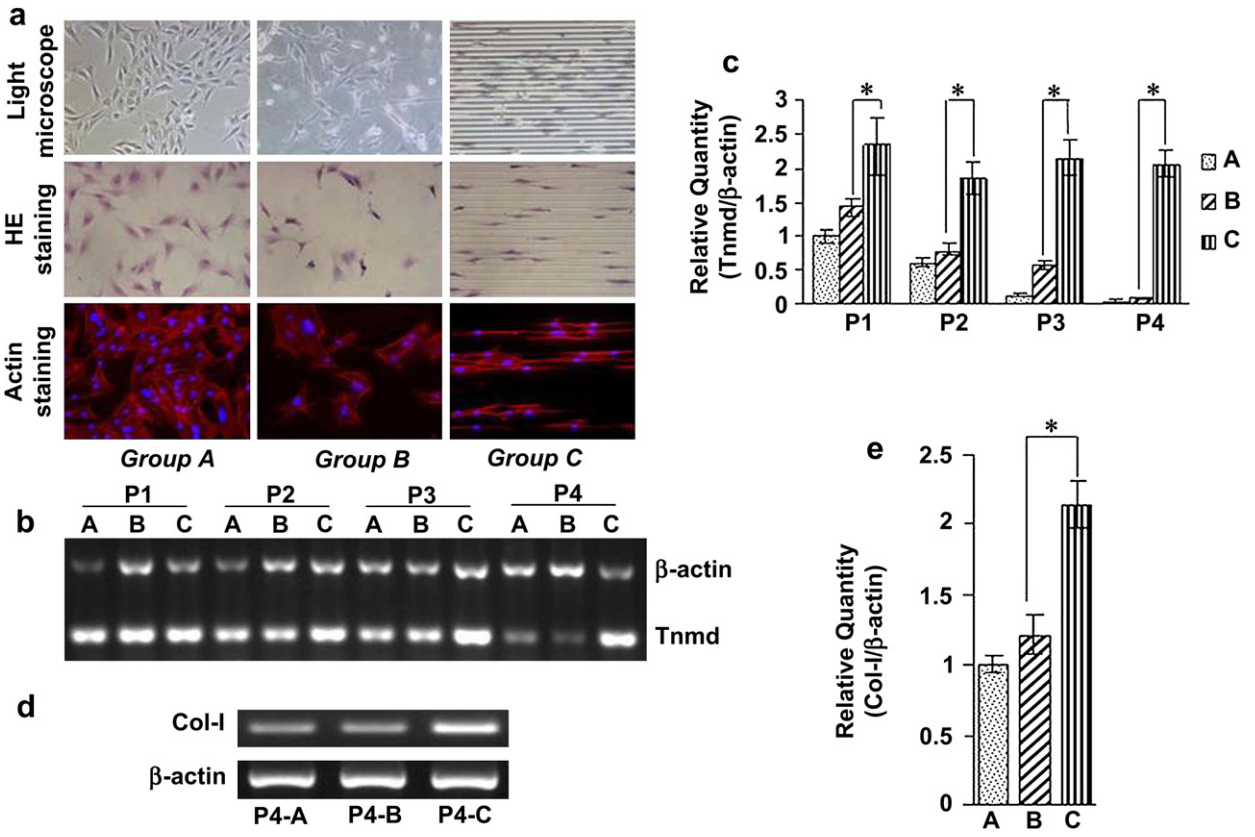

Fig. 2. Passaged tenocytes with enforced elongated morphology retained high expression level of tenomodulin (Tnmd) and collagen I (Col-1). Cells were passaged respectively on culture dish (Group A), smooth silicone membrane (Group B) and microgroove silicone membrane (Group C) to observe the morphology (a, passage 4 cell, ×100) and to examine the gene expression levels of Tnmd (b, c) and Col-1 (d, e) by RT-PCR (b, d) and quantitative PCR (c, e). P and A, B, C respectively represent cell passage and Groups A, B and C. The symbol "*" indicates $p < 0.05$.

cells) with no significant difference (Fig. 3, left). After one more passage of cell culture (P3 + P2), tenomodulin expression level continued to drop in Group B cells, whereas the expression level of Group D cells significantly increased and reached a level similar to that of Group C cells that were forced to be elongated all the time with no significant difference (Fig. 3, right). This phenomenon indicates that dedifferentiated tenocytes during *in vitro* culture could restore their phenotype after regaining their native cell morphology.

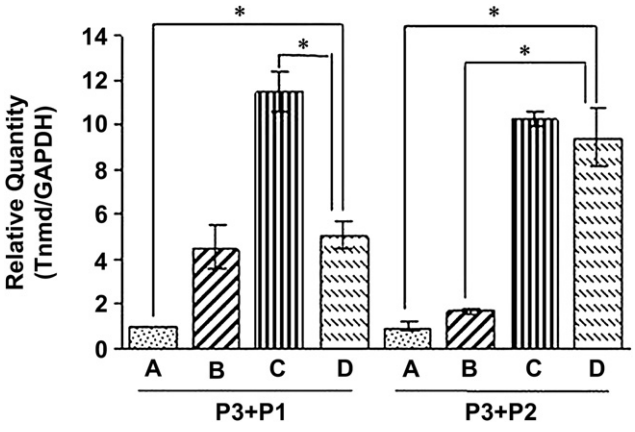

Fig. 3. Redifferentiation of dedifferentiated cultured tenocytes by morphological switch from spread to elongated shape. Group B cells were passaged on smooth silicone membrane 3 times and part of cells was then switched to microgroove membrane for another two passage (Group D). Quantitative PCR revealed the re-expression of Tnmd in Group D cells after two passages in enforced elongated morphology. P and A, B, C and D respectively represent cell passage and Groups A, B, C and D. The symbol "*" indicates $p < 0.05$.

3.4. Effect of cell morphology on RhoA/ROCK activity

To understand the potential role of RhoA/ROCK signaling in cell shape mediated tenomodulin expression, both RhoA and ROCK activities were examined for the cells that were continually grown on different substrata. As shown in Fig. 4, the level of GTP-bound RhoA was significantly increased in the cells with elongated shape (Group C) than the cells with spread morphology (Groups A and B) (Fig. 4a). By contrast, the ROCK activity was significantly decreased in the cells with elongated shape (Group C) than the cells with spread morphology (Groups A and B) (Fig. 4b), indicating that elongated cell morphology leads to enhanced RhoA activation and decreased ROCK activity.

3.5. Effects of ROCK inhibitor Y27632 on tenomodulin expression

To investigate whether manipulation of ROCK activity will affect tenomodulin expression, cells were first treated with ROCK inhibitor Y27632 to observe the effect on cell morphology. As shown in Fig. 5, cells cultured on regular culture dishes remained largely spread in their morphology after 24 h of treatment with 10 μM Y27632, although attenuated stress fiber structure was observed in treated group than in non-treated group (Fig. 5a). Interestingly, inhibition of ROCK activity could enhance tenomodulin expression

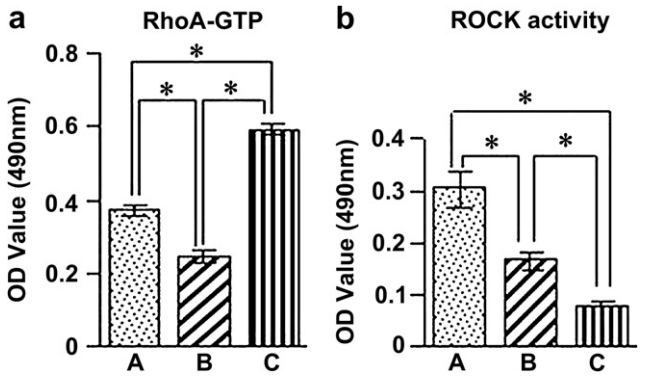

Fig. 4. Effect of cell morphology on RhoA and ROCK activity. The level of GTP-bound RhoA was significantly higher in the cells with elongated morphology than in the cells with spread morphology (a), whereas the ROCK activity was significantly lower in elongated cells than in spread cells (b). A significant difference in RhoA-GPT level and Rock activity was found as well between Groups A and B cells (both spread). A, B and C represent respectively Groups A, B and C, and the symbol "*" indicates $p < 0.05$.

with a dose-dependent and a time-dependent manner (Fig. 5b and c), indicating that ROCK activity could exert an active effect on regulating tenomodulin expression. Next, the effect was also investigated on the cells cultured on different substrata. As shown in Fig. 5d, inhibiting ROCK activity with 10 μM Y27632 treatment could significantly enhanced the tenomodulin expression in the cells of both Groups A and B ($p < 0.05$), which were in spread cell morphology. Particularly, there was a dramatic increase of the expression level in Group B cells after the treatment, leading to a level similar to that of Group C cells without significant difference ($p > 0.05$). However, after Y27632 treatment, tenomodulin expression was slightly increased in Group C cells with elongated morphology ($p < 0.05$), suggesting that the artificial inhibition with Y27632 will probably have limited effect on the elongated cells that already had a very low level of ROCK activity and thus was not able to further majorly enhance tenomodulin expression.

3.6. Effects of RhoA activator LPA on tenomodulin expression

To investigate the effect of artificial RhoA activation on tenomodulin expression, cells grown on different substrata were treated with LPA to observe the effect. As shown in Fig. 6, activation of RhoA could significantly down regulate tenomodulin expression levels of the cells (Groups A and B) with spread cell morphology, whereas no significant decrease of tenomodulin expression level was found in Group C cells grown on microgroove membrane (Fig. 6b), indicating that LPA treatment will have limited effect on the elongated cells that already had a high level of GTP-bound RhoA and thus was not able to further decrease tenomodulin expression. In addition, after LPA treatment of the cells grown on regular culture dishes, enhanced actin stress fiber formation was observed (Fig. 6a), suggesting that RhoA activity may affect tenomodulin expression that is not entirely dependent on cell morphology.

4. Discussion

Cell dedifferentiation is a common biological process when cells were deprived of their *in vivo* niche longer enough. For example, bovine articular chondrocytes became dedifferentiated after being cultured in monolayer for three weeks and lost the expression of marker molecule collagen II [24]. It has been proposed that multiple factors constitute an *in vivo* niche that maintains the phenotype of chondrocytes, such as cartilage matrices, low oxygen, growth

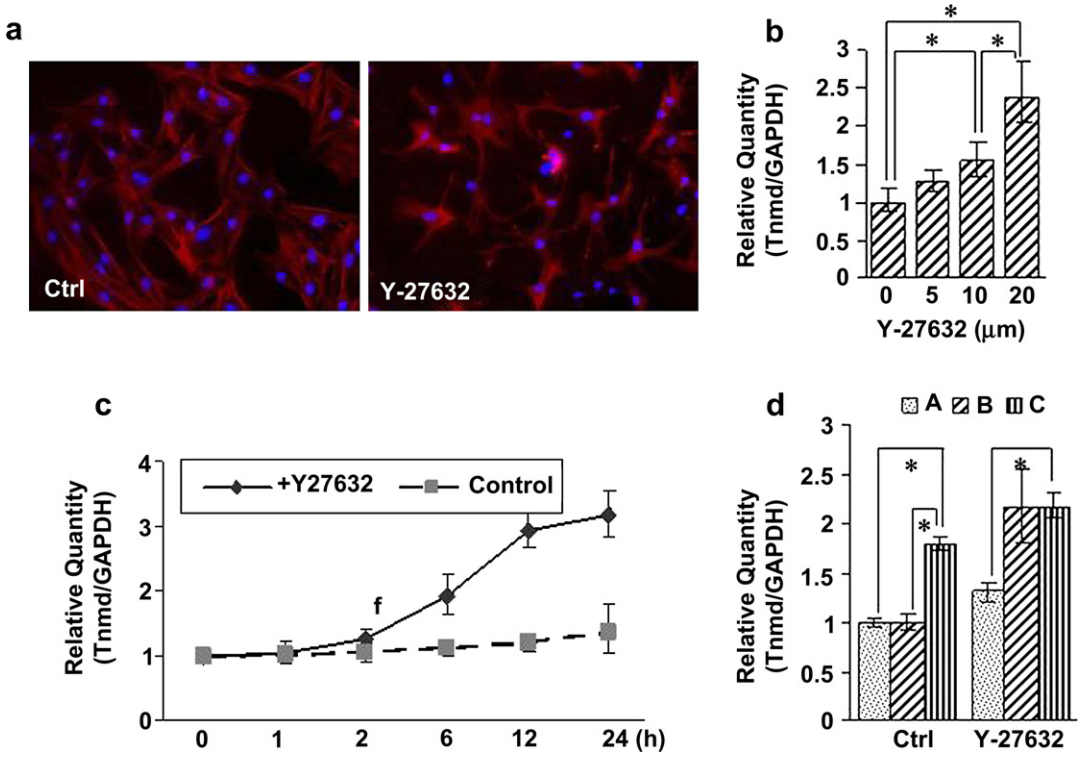

Fig. 5. Effect of ROCK inhibitor on tenomodulin (Tnmd) expression in tenocytes cultured on different substrata. Treatment of cells grown on culture dish with Y27632 led to attenuated stress fiber structure without obvious morphological change (a, ×100) and reduced Tnmd expression in dose (b) and time (c) dependent manners. Dramatic upregulation of Tnmd was observed in Group B cells comparing to control group (Ctrl) after Y27632 treatment with a level similar to that of Group C. Minor upregulation was also observed in Group C cells. A, B and C represent respectively Groups A, B and C, and the symbol "*" indicates $p < 0.05$.

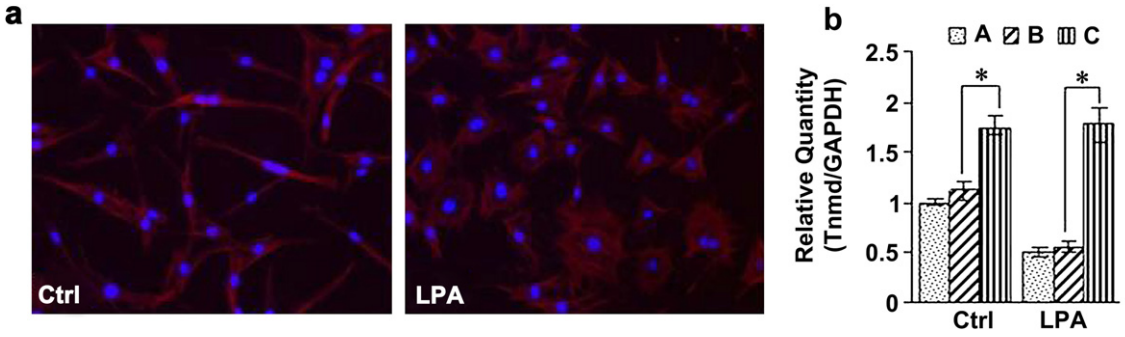

Fig. 6. Effect of RhoA activator on tenomodulin (Tnmd) expression in tenocytes cultured on different substrata. Treatment of the cells grown on culture dish with LPA did not obviously change the morphology (a, ×100), but significantly reduced Tnmd expression (b). The treatment also significantly reduced tenomodulin expression in the cells grown on smooth silicone membrane but not in the cells on microgroove membrane comparing to control groups (Ctrl) (b). A, B and C represent respectively Groups A, B and C. The symbol "*" indicates $p < 0.05$.

factors and three dimensional structure and thus are the potential factors to be used to redifferentiate the dedifferentiated condrocytes during *in vitro* culture [24–27].

Among these factors, the role of cell morphology in cell dedifferentiation might be an important subject to investigate because cell morphology change often accompanies with dedifferentiation [24] and induced differentiation of stem cell [4]. Tenocyte is one type of fibroblasts, which can be an interesting target to study the interrelationship between cell morphology and phenotype, because tenocyte exhibits a unique elongated form *in vivo*. As shown in this study, loss of this particular cell morphology during *in vitro* culture is associated with the loss of cell phenotype and function as the expression level of marker molecule (tenomodulin) and functional molecule (collagen I) significantly decreased (Fig. 1). However, the results demonstrated that simply forcing cells into an elongated morphology without other environmental alterations could prevent *in vitro* cultured kenocytes from dedifferentiation even after 4 cell passages (Fig. 2). Interestingly, simply switching the morphology from spread shape to elongated form could reverse dedifferentiation status and redifferentiate cultured tenocytes by restoring tenomodulin and collagen I expression (Fig. 3). This finding suggests that cell morphology change may not just be a passive response to cell dedifferentiation [24] or stem cell differentiation [4], rather environmentally induced cell morphology change may actually play an active role in directing the fate of cell dedifferentiation or redifferentiation for *in vitro* cultured cells.

Cytoskeleton structure is important for cell shape maintenance. As demonstrated in this study by F-actin staining, enforced elongated cell morphology could reorganize actin stress fibers from random distribution (spread cells) to longitudinally alignment along with elongated cell axis (Fig. 2a), which may lead to reduced cytoskeleton tension in narrow shaped tenocytes [28]. Furthermore, the altered cytoskeleton pattern also altered the RhoA activation level and ROCK activity. As shown in Fig. 4, elongated cell morphology led to increased level of GTP-bound RhoA and decreased ROCK activity. Although the observed decoupling of RhoA activation and the activity of its effector ROCK in elongated tenocytes was not expected, it is not unprecedented. Previous researches suggest the presence of a negative-feedback loop that occurs following Rho-dependent formation of focal adhesions [22,29]. As reported previously [28], cytoskeletal tension is required to couple RhoA to ROCK. As examples, the decoupling of RhoA and ROCK is evident in suspended, spreading-restricted or cytoskeletal tension disrupted cells, which all exhibited high levels of Rho-GTP but low ROCK activity [28,30–32]. In this case, reduced cytoskeletal tension by enforced narrow cell shape (spreading-restricted morphology) is likely to cause the decoupling.

More importantly, the decoupled RhoA/ROCK activity could actively regulated tenomodulin expression. As shown in this study, artificial inhibition of ROCK activity with Y27632 treatment could enhance tenomodulin expression even treated cells remained largely in spread morphology despite actin stress fibers were disrupted to a certain level. Reversely, artificial activation of RhoA by LPA treatment could lead to reduced tenomodulin expression with enhanced actin stress fiber structure. In contrast, for elongated cells that already exhibited highly activated RhoA or low level ROCK activity, treatment with either LPA or Y27632 could not majorly change the expression level of tenomodulin. These findings indicate that RhoA/ROCK signaling participates and play an active role in cell shape mediated tenocyte dedifferentiation and redifferentiation processes.

In this study, difference in tenomodulin expression and RhoA/ROCK activity remained observable between Group A and Group B cells, although both group cells exhibit spread cell morphology. The difference is likely to be caused by different substrata because the culture dishes may allow for better cell attachment and spreading compared to silicone membrane. In addition, Scleraxis is known important for regulating tenomodulin expression during tendon development [8,33]. However, change of cell morphology during *in vitro* culture and cell passage did not seem to affect scleraxis expression despite significantly reduced tenomodulin expression in mouse tenocytes during *in vitro* culture (data not shown), suggesting that cell shape regulated tenomodulin expression in tenocytes may not depend on scleraxis based regulation, which may play its primary role in tendon development.

Although this study dealt with basic cell biology issue, the finding of this study may provide a guide for practical application. As an example, our previous studies shown elongated tenocyte morphology in tissue engineered tendon [34,35], the design of a linear structure may help to fabricate a scaffold material that may allow seeded tenocytes to keep an elongated morphology during *in vitro* and *in vivo* tendon formation, and thus to optimize the tissue quality and function of tissue engineered tendon by well preserved phenotype and function of seeded tenocytes. Additionally, specific morphological requirement for tenocyte phenotype and function revealed by this study may provide insight into tenogenic differentiation of stem cells [2].

5. Conclusion

This study demonstrated that tenocytes underwent dedifferentiation during *in vitro* culture along with morphology change from elongated to spread and simply maintaining the cultured tenocytes in an elongated form could retain their phenotype or

even reverse dedifferentiated status towards redifferentiated status and reduced ROCK activity contribute to this unique phenomenon. In the future, defining the detailed process of RhoA/ROCK mediated tenomodulin expression such as transcriptional regulation may help to reveal the mechanism. Moreover, these findings may provide insight into the design of an advanced scaffold for tendon engineering, which is able to maintain or strengthen the function of seeded tenocytes or able to efficiently induce tenogenic differentiation of seeded mesenchymal stem cells.

Acknowledgments

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Appendix

Figures with essential colour discrimination. Figs. 1,2,5 and 6 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.05.058.

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