

The Role of the RhoA/ROCK Signaling Pathway in Mechanical Strain-Induced Scleral Myofibroblast Differentiation

Ying Yuan,¹⁻³ Min Li,¹ Chi Ho To,⁴ Thomas Chuen Lam,⁴ Peng Wang,¹⁻³ Yunjie Yu,¹⁻³ Qingzhong Chen,¹ Xiaojun Hu,¹ and Bilian Ke¹⁻³

¹Department of Ophthalmology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

²Shanghai Key Laboratory of Fundus Disease, Shanghai, China

³Shanghai Engineering Center for Visual Science and Photomedicine, Shanghai, China

⁴Laboratory of Experimental Optometry, Centre for Myopia Research, School of Optometry, The Hong Kong Polytechnic University, Hong Kong, Hong Kong Special Administrative Region of China, China

Correspondence: Bilian Ke, Department of Ophthalmology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, 100 Haining Road, Shanghai 200080, China; kebilian@126.com.

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PURPOSE. Biomechanical properties changes and α -smooth muscle actin (α -SMA) overexpression are involved in myopia scleral remodeling. However, interactions between altered tissue biomechanics and cellular signaling that sustain scleral remodeling have not been well defined. We determine the mechanisms of mechanotransduction in the regulation of α -SMA expression during myopia scleral remodeling.

METHODS. Guinea pigs were used to establish a form-deprivation myopia (FDM) model. Protein profiles in myopic sclera were examined using tandem mass spectrometry. Ras homolog gene family member A (RhoA) and α -SMA expressions were confirmed using quantitative (q) RT-PCR and Western blotting. Scleral fibroblasts were cultured and subjected to 4% cyclic strain. Levels of RhoA, rho-associated protein kinase-2 (ROCK2), myocardin-related transcription factor-A (MRTF-A), serum response factor (SRF), and α -SMA were determined by qRT-PCR and Western blotting in groups with or without the RhoA siRNA or ROCK inhibitor Y27632. MRTF-A and α -SMA were evaluated by confocal immunofluorescent microscopy and myofibroblasts were enumerated using flow cytometry.

RESULTS. mRNA and protein levels of RhoA and α -SMA were significantly increased in the FDM eyes after 4 weeks of form-deprivation treatment. The 4% static strain increased expressions of RhoA, ROCK2, MRTF-A, SRF, and α -SMA as well as nuclear translocation of MRTF-A in scleral fibroblasts compared to those without strain stimulation. Additionally, the percentage of myofibroblasts increased after strain stimulation. Conversely, inhibition of RhoA or ROCK2 reversed the strain-induced α -SMA expression and myofibroblast ratio.

CONCLUSIONS. Mechanical strain activated RhoA signaling and scleral myofibroblast differentiation. Strain also mediated myofibroblast differentiation via the RhoA/ROCK2-MRTF-A/SRF pathway. These findings provided evidence for a mechanical strain-induced RhoA/ROCK2 pathway that may contribute to myopia scleral remodeling.

Keywords: form-deprivation myopia, scleral fibroblast, α -SMA, RhoA/ROCK2, mechanotransduction

Excessive elongation of the ocular globe, particularly in axial length, is the primary structural change in human myopia. This elongation also is observed in many experimental myopia animal models, including tree shrews, guinea pigs, and chicks.¹⁻³ The induced axial elongation is associated with remodeling of the sclera with alterations in biochemical and biomechanical properties. In myopic eyes, the content of extracellular matrix (ECM) decreases, mainly as a result of decreased glycosaminoglycan (GAG) and collagen, followed by thinning of the sclera. This thinning contributes to reduced scleral resistance to expansion in response to normal IOPs.^{4,5} Although such ECM remodeling was involved in defining scleral biomechanical properties, recent studies have reported that the scleral cells not only have secretory functions, but also contractile abilities, which may have an important role in

determining scleral matrix stress, and tissue expansion and contraction.⁶

Myofibroblasts, which typically express α -smooth muscle actin (α -SMA), are identified as contractile nonmuscle cells possessing the secretory features of fibroblasts and contractile features similar to smooth muscle. These cells were suggested to have a crucial role in wound healing and pathologic remodeling.⁷ It also is well recognized that the important stimuli driving fibroblast-to-myofibroblast differentiation are changes in the mechanical microenvironment as well as growth factor TGF- β .^{8,9} Enhanced ECM stiffness or mechanical strain promotes myofibroblast activation in organs, such as the skin, lung, and heart.^{8,10,11} In the confined ocular cavity, sclera are subjected to constant stress and strain produced by IOP, eye movement, and contraction of the periorbital muscles. During



myopia development, it was well recognized that the stress and strain on the sclera are increased as the axial elongation progresses, with corresponding thinning of the scleral tissue as a result.¹² Previous studies have indicated an increased expression of α -SMA in scleral cells under strain stimulation in the mice and tree shrew models.^{13,14} However, how this stress and strain on the sclera is converted into intracellular signals that stimulate the differentiation of fibroblasts into myofibroblasts remains unclear.

The ras homolog gene family are important regulators of a variety of cellular functions.¹⁵⁻¹⁷ Of these, RhoA is known to have a key role in actin cytoskeleton reorganization, regulation of cell shape, adhesion, and migration and transformation of cellular phenotypes.^{17,18} The Rho kinase (ROCK) family members, including ROCK1 and ROCK2, are important effectors of RhoA. Analysis of ROCK1 and ROCK2 distribution in a recent study revealed a dramatically higher expression of ROCK2 in the eye.¹⁹ Studies also have demonstrated that mechanical force activated the RhoA/ROCK2 signaling pathway, which then mediated the biochemical changes of the cells.²⁰ When cells are subjected to exogenous mechanical stress, cell-matrix adhesion complexes sense and transmit mechanical stimulation to the cytoskeleton, which further activates RhoA from the GDP- to the GTP-bound state and induces downstream molecular changes.²¹ The activity of RhoA and ROCK was altered in a cyclic stretching microenvironment in endothelial cells, smooth muscle cells, and cardiomyocytes.^{22,23} Hence, inhibition of RhoA expression could reverse stiff matrix-induced stress fiber formation in lung fibroblasts.²⁴ These findings indicated that the RhoA/ROCK signaling pathway had a crucial role in mechanical signal transduction. Serum response factor (SRF) and coactivators of the myocardin family are master regulators of the transcription of contractile and cytoskeletal genes, such as α -SMA and collagen.^{25,26} The myocardin-related transcription factors, including MRTF-A (also known as MKL1) and MRTF-B (also known as MKL2), are widely expressed in numerous tissues.²⁷ Mechanical force could activate Rho GTPases, lead to actin cytoskeleton polymerization into stress fibers, and permit nuclear translocation of MRTFs.^{28,29} Once in the nucleus, the MRTFs interact with SRF as homo- or heterodimers to drive transcription via conserved CARG box DNA elements.³⁰ Previous studies suggested that MRTF-A translocation into nucleus may contribute to myofibroblast differentiation in human lung and cardiac fibrosis.^{31,32} MRTF-A mediated gene expression has been shown to be mediated by the activation of RhoA/ROCK pathway.¹⁷

With the growing evidence available in cellular and animal models, we hypothesized that RhoA/ROCK2 constitute an intrinsic mechanotransduction pathway that could transduce and convert mechanical stimulation to a signal that promoted scleral α -SMA expression in a mammalian myopia model. Moreover, strain-induced nuclear accumulation of MRTF-A is RhoA/ROCK-dependent and required for expression of α -SMA.

MATERIALS AND METHODS

Form-Deprivation Myopia Model

All procedures conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocols were approved by the ethics committee of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine. Pigmented guinea pigs (2-week-old) were housed in the Laboratory Animal Center with a 12-hour light-dark cycle. They were randomly divided into two groups: the form-deprivation myopia (FDM, $n = 13$, randomly female and male)

treatment group and untouched age-matched animals as a normal control (NC, $n = 13$, randomly female and male) group. In the FDM group, the translucent diffuser, made from white lattices, was glued onto one randomly selected eye of each animal continuously for 4 weeks, while the other eye without any treatment served as a self-control (SC) group.

Refraction and axial length in both eyes were determined before and after FDM treatment. The refractive state was measured by an automated infrared photorefractor in a dark environment as described previously.^{33,34} Refractive error values were recorded with a custom software designed by Frank Schaeffel. For each eye, an average of 50 to 100 measurements were used for analysis. Axial length was measured using A-scan ultrasonography (Strong 6000A; Wuhan Strong Electronics Ltd., Wuhan, China) while the animals were under topical anesthesia (0.4% oxybuprocaine hydrochloride). Measurements were repeated at least eight times for each eye.

Primary Culture of Scleral Fibroblasts

Primary fibroblasts were isolated and cultured from guinea pig scleral explants. The explanted tissues were cut into 1×1 mm pieces, placed in 60-mm tissue culture dishes containing high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The medium was replenished every 3 days. After 7 to 10 days, cells grew out of the explants and then were passaged after 0.25% trypsin-EDTA (Gibco Laboratories, Gaithersburg, MD, USA) disruption. After identification with vimentin (Boster Biological Technology, Pleasanton, CA, USA), fibroblasts from the third to fifth passages were used for this experiment. RhoA specific small interfering RNA (siRNA) or ROCK inhibitor (Y-27632, 10 μ M, BD, Franklin Lakes, NJ, USA) were used to inhibit RhoA/ROCK pathway.

Mechanical Stretching Protocol

Scleral fibroblasts (2×10^5) were seeded into 6-well collagen I-coated stretch plates (Flexcell Int. Corp., Burlington, NC, USA) and incubated in FBS-supplemented DMEM for 24 hours. On reaching 70% to 80% confluency, the cells were serum-starved in FBS-free media for 24 hours and then subjected to 4% static strain for 24 hours on a Flexcell FX-5000 strain system.³⁵⁻³⁸ Cells seeded on stretch plates, but subjected to no strain served as controls.

SiRNA Transfection

SiRNA targeting guinea pig *RhoA* (sense 5'-GCUGCCAUC CGAAAGAACTT-3' and antisense 5'-GUUUCUUUCGGAUG GCAGCTT-3') and negative control (sense 5'-UUCUCCGAA CGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUC GGAGAATT-3') were designed and synthesized by GenePharma Biological Company (Shanghai, China). Briefly, the fibroblasts were transfected with siRNA using siRNA-mate (GenePharma) when the cells attained 30% to 50% confluence. The final concentrations of siRNA were 100 nM. After 6 hours of transfection, the medium was replaced with DMEM for 18 hours before strain.

Liquid Chromatography and Tandem Mass Spectrometry (LC/MS/MS) Analysis

Sclerae ($n = 3$, each group) of FDM and control eyes were collected after 4 weeks of treatment. The tissues were extracted with lysis buffer containing 4% SDS, 100 mM Tris-HCl, and 1 mM dithiothreitol (DTT). Then, proteins from each sample were reduced, alkylated, and digested using the filter-

aided sample preparation (FASP) method.³⁹ After labeling using 8-plex isobaric tags for relative and absolute quantitation (iTRAQ) reagents according to manufacturer's instructions as described previously (AB SCIEX, Framingham, MA, USA), the peptides were fractionated by strong cation exchange (SCX) chromatography (AKTA Purifier 100; GE Healthcare, Uppsala, Sweden). SCX separation was performed using buffer A (10 mM KH₂PO₄ in 25% acetonitrile [ACN], pH 3.0) and buffer B (500 mM KCl, 10 mM KH₂PO₄ in 25% ACN, pH 3.0) at a flow rate of 1 mL/min. After separation by nanoHPLC, the peptides were analyzed by the Q-Exactive system (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectra were acquired over a scan range of 300 to 1800 *m/z* with a resolution of 70,000. Protein identification and quantification were analyzed by Mascot2.2 and Proteome Discoverer1.4. A 1.2-fold cutoff was set to determine the up- and downregulated proteins with a *P* value < 0.05.

Quantitative RT-PCR (qRT-PCR) Analysis

Total RNA was extracted from scleral tissues (*n* = 5, each group) or cultured fibroblasts using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed with the Takara Reverse Transcription kit (Takara, Tokyo, Japan). qRT-PCR was performed with the Applied Biosystems (ABI; Foster City, CA). The sequences of the primers used in this study were as follows: *RhoA* forward: 5'-CGCCTTTGGATACATGGAGT-3', reverse: 5'-CAAGACAAGGCACCCAGATT-3'; *ROCK2* forward: 5'-ATCCCACAGAACCAGTCCAT-3', reverse: 5'-TCATCACCACCATCACCATC-3'; α -SMA forward: 5'-GACCGAATGCAGAAGGAGAT-3', reverse: 5'-CCACCGATCCAGACAGAGTA-3'; *MRTF-A* forward: 5'-GTGAAGCTGTGCCAATGAA-3', reverse: 5'-GCAGCACATTCTTACGCTCA-3'; *SRF* forward: 5'-CTCAACTCGCCAGACTCTCC-3', reverse: 5'-GGCTTCAGTGTGCTTGGT-3'; *COL1A1* forward: 5'-TGGGTCCTACTGGCAAACAT-3', reverse: 5'-TCA CCAACCTCTCCCTTGTC-3'; *GAPDH* forward: 5'-TCAAGAAGGTGGTGAAGCAG-3', reverse: 5'-CGTCAAAAGTGGAGAATGG-3'. Target gene expression was normalized using the 2^{- $\Delta\Delta$ Ct} method from the Ct values of the respective mRNAs relative to the housekeeping gene *GAPDH*.

Western Blotting

The proteins of scleral tissues (*n* = 5, each group) or cultured fibroblasts were extracted in RIPA lysis buffer containing protease inhibitors. A total of 30 μ g proteins were separated on SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 5% skim milk powder for 1 hour at 37°C, the membranes were incubated with primary antibody overnight at 4°C. The antibodies used included RhoA (1:1000, Cell Signaling Technology, Danvers, MA, USA), ROCK2 (1:1000, Cell Signaling Technology), SRF (1:1000, Cell Signaling Technology), α -SMA (1:200, Abcam, Cambridge, UK), MRTF-A (1:1000, Cell Signaling Technology), GAPDH (1:5000, Proteintech, Chicago, IL, USA), and Histone-H3 (1:5000, Proteintech). After rinsing three times with TBST, the membranes were incubated with rabbit/mouse secondary antibodies for 1 hour at room temperature.

Immunofluorescence and Confocal Microscopy

Scleral fibroblasts were placed on 15-mm coverslips, fixed in 4% formaldehyde for 10 minutes, and permeabilized with 0.1% Triton X-100 for 10 minutes. After blocking with 10% goat serum for 30 minutes, the cells were incubated overnight

with primary antibodies against α -SMA (1:100; Abcam) or MRTF-A (1:100, Abcam) at 4°C. Following washing three times with PBS, cells were incubated with secondary goat anti-rabbit Alexa Fluor 488 (1:500; Cell Signaling Technology) for 1 hour at room temperature. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclear material (Invitrogen-Life Technologies, Grand Island, NY, USA). Immunofluorescence was analyzed using a confocal microscope (Leica TCS SP8; Exton, PA, USA) with a \times 20 objective and Leica Application Suite X software. Images were collected at 1024- by 1024-pixel resolution.

RhoA Activation Assay

Activation of RhoA was evaluated using the RhoA Activation Assay Kit according to the manufacturer's instructions (Abcam) based on the Rhotekin pull-down assay. Briefly, after washing with PBS, the cells then were extracted using the lysis buffer. Samples then were centrifuged for 10 minutes at 14,000g and the supernatant was incubated with Rhotekin-RBD beads for 1 hour at 4°C with gentle agitation. After washing the beads with lysis buffer, proteins were removed from the beads with SDS-PAGE sample buffer. Lysates and precipitates were analyzed by Western blotting.

Nuclear/Cytosol Fractionation

To monitor the nuclear MRTF-A protein level after mechanical strain stimulation, nuclear/cytosol fractionation together with immunoblotting analysis were performed. The Nuclear/Cytosol Fractionation Kit (P0027, Beyotime Biotechnology, Jiangsu, China) was applied to isolate nucleus and cytosol according to the manufacturer's instructions.

Flow Cytometry

The α -SMA-positive cells were quantified by flow cytometric analysis. After stimulation with strain for 24 hours, cells were harvested and washed with PBS. The cells then were fixed and permeated with the fixation and permeabilization solution (BD Biosciences) for 30 minutes. After washing, cells were incubated with PE-conjugated anti- α -SMA (R&D Systems, Minneapolis, MN, USA) for 30 minutes at 4°C. Cells incubated with isotype-matched irrelevant control antibodies (R&D Systems) served as negative controls. The percentage of positive cells was detected using a flow cytometer (Beckman CytoFLEX FCM; Beckman Coulter, Pasadena, CA, USA) and analyzed using the Flowjo software (Flowjo LLC, Ashland, OR, USA).

Statistical Analysis

Student's paired *t*-test was used to compare the difference between right and left eyes. Measurements between different guinea pigs were compared by independent *t*-test. One-way ANOVA with pairwise Bonferroni post tests were used to determine the difference among different treatment groups of fibroblasts. All values were presented as mean \pm SD. *P* < 0.05 was considered statistically significant. SAS software (version 9.2; SAS Institute, Inc., Cary, NC, USA) was used to analyze statistical evaluations in this study.

RESULTS

Refraction and Axial Length Measurements

There were no significant differences (*P* > 0.05) in refraction or axial length between the right and left eyes of guinea pigs in the FDM and NC groups at the beginning of the experiments

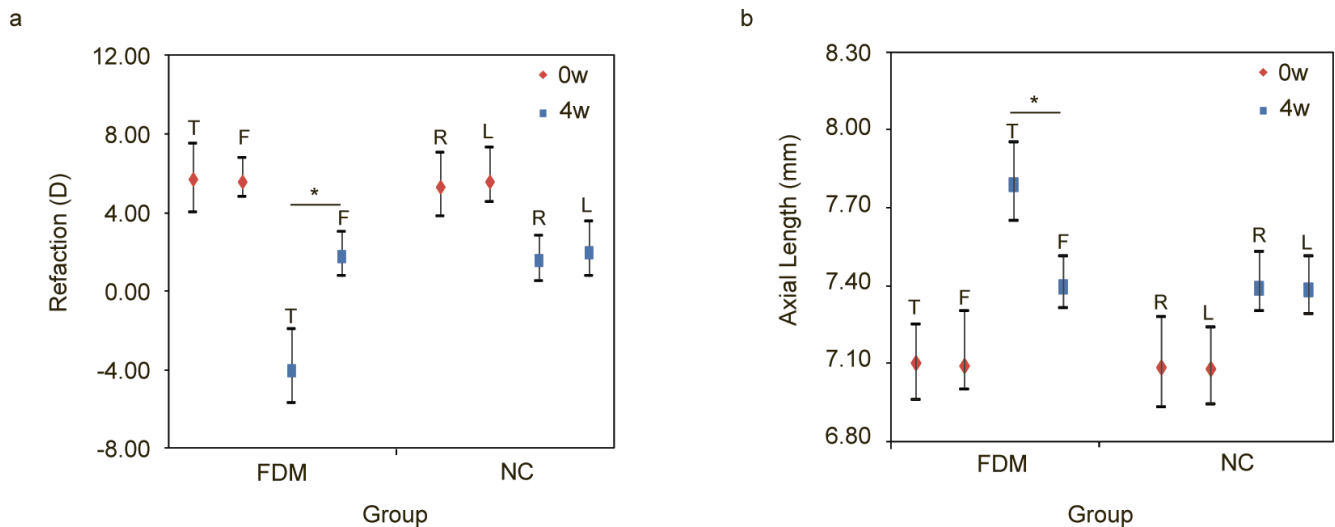


FIGURE 1. Refraction (a) and axial length (b) in NC ($n = 13$; R, right eye; L, left eye) and FDM ($n = 13$; T, treated eye; F, fellow eye) groups at the beginning and end of treatment. Data are expressed as the mean \pm SD. * $P < 0.05$.

(Fig. 1). After 4 weeks of FDM, significant myopia was induced in the treatment eye ($P < 0.05$, Fig. 1a) while the axial length was significantly increased ($P < 0.05$, Fig. 1b) when the self-control eye was compared. By contrast, no significant difference was observed in either refraction or axial length between both eyes in the NC group after the experimental period ($P > 0.05$; Figs. 1a, 1b).

Identification of Scleral RhoA and α -SMA Expression in Guinea Pig FDM Model

After FDM treatment for 4 weeks, evaluation of representative MS/MS spectra showed that scleral RhoA protein was increased in the FDM group (1.32-fold to the control group, $P < 0.05$; Fig. 2a), using the guinea pig Uniprot guinea pig database (at a false discovery rate [FDR] $< 1.0\%$). The sequence coverage of RhoA protein was 37.31%. Meanwhile, the results of qRT-PCR and Western blotting confirmed a significant increase on RhoA in the FDM eyes compared to the control eyes. ($P < 0.05$; Figs. 2b, 2c). The mRNA expression of α -SMA in the FDM eyes significantly increased compared to the control group ($P < 0.05$, Fig. 3a). Protein levels of α -SMA in the FDM eyes also were significantly higher than those of the control eyes ($P < 0.05$; Figs. 3b, 3c).

Strain Activated RhoA, ROCK2, MRTF-A and SRF Signaling Molecular and Regulated α -SMA Expression

To assess the effect of strain on the RhoA pathway and α -SMA expression, scleral fibroblasts were exposed to 4% static strain (Fig. 4a). As shown in Figure 4b, there was a significant increase in *RhoA*, *ROCK2*, *MRTF-A*, *SRF*, α -SMA, and *COL1A1* mRNA expression after 24-hour strain ($P < 0.05$, respectively). The protein levels of RhoA, ROCK2, MRTF-A, SRF, and α -SMA in the strain group also were significantly higher than those in the control group ($P < 0.05$, respectively; Figs. 4c, 4d). Additionally, activation of RhoA was increased in the strain group compared to the control group (Fig. 4e). MRTF-A was translocated to the nucleus after strain stimulus (Figs. 4f, 4g). Immunofluorescence showed that myofibroblasts in the strain group were overexpressed compared to those in the control group (Fig. 5a). From flow cytometry results showed 50.07% \pm

4.18% myofibroblasts in the strain group, which was significantly higher than those in the control group (23.43% \pm 3.23%, Fig. 5b).

Knocking-Down RhoA Leads to a Downregulation of Strain-Induced α -SMA Expression

To further assess the roles of differently expressed RhoA in strain-induced α -SMA expression of scleral fibroblasts, the expression of RhoA was knocked down by target siRNA under the control and 4% static strain conditions. As shown in Figures 5c and 5d, the expression of RhoA was significantly suppressed by siRNA transfection ($P < 0.05$). Expression of ROCK2, MRTF-A, SRF, and α -SMA was significantly decreased in the RhoA siRNA transfected fibroblasts ($P < 0.05$; Figs. 5c, 5e-h, respectively). The percentage of myofibroblasts significantly decreased to 6.49% \pm 3.30% in the siRNA transfection group compared to the control group ($P < 0.05$, Fig. 5b). In the strain groups with siRNA transfection, the expression of RhoA, ROCK2, MRTF-A, SRF, and α -SMA significantly decreased more than in fibroblasts subjected to 4% strain only ($P < 0.05$; Figs. 5c-h, respectively). The strain failed to induce expression of α -SMA in the fibroblasts subjected to RhoA siRNA transfection ($P > 0.05$, Figs. 5c, 5h). After transfection of siRNA, the percentage of myofibroblasts was 12.92% \pm 3.48% in the strain group, which was slightly higher than that in the group without strain ($P > 0.05$; Figs. 5a, 5b).

Inhibition of ROCK Regulated Strain-Induced α -SMA Expression

To further investigate the RhoA-dependent mechanism on α -SMA expression, inhibition experiments using a ROCK inhibitor Y27632 were performed. The expression of ROCK2 was decreased ($P < 0.05$, Figs. 6a, 6b). After application of 4% strain, the ROCK2 level failed to increase ($P > 0.05$, Figs. 6c, 6d). Inhibiting ROCK expression led to a significant increase in the protein level of RhoA under control and strain conditions ($P < 0.05$; Figs. 6a-d). There was a significant decrease in MRTF-A, SRF, and α -SMA expression when ROCK was inhibited ($P < 0.05$; Figs. 6a, 6b). The immunofluorescence and flow cytometry data indicated that myofibroblasts

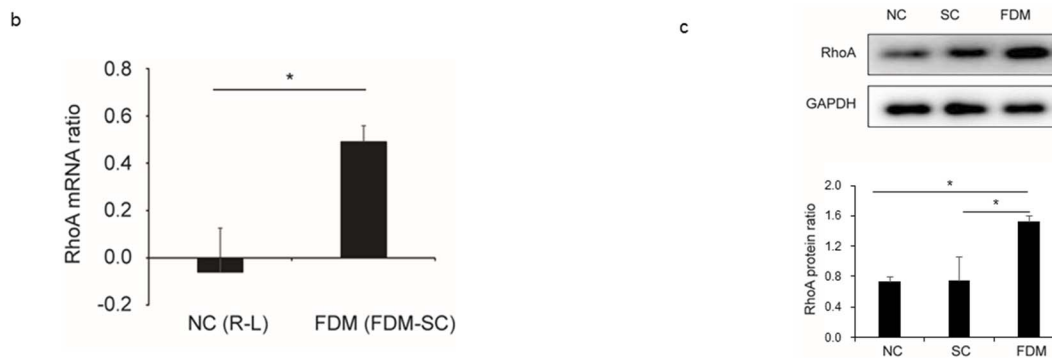
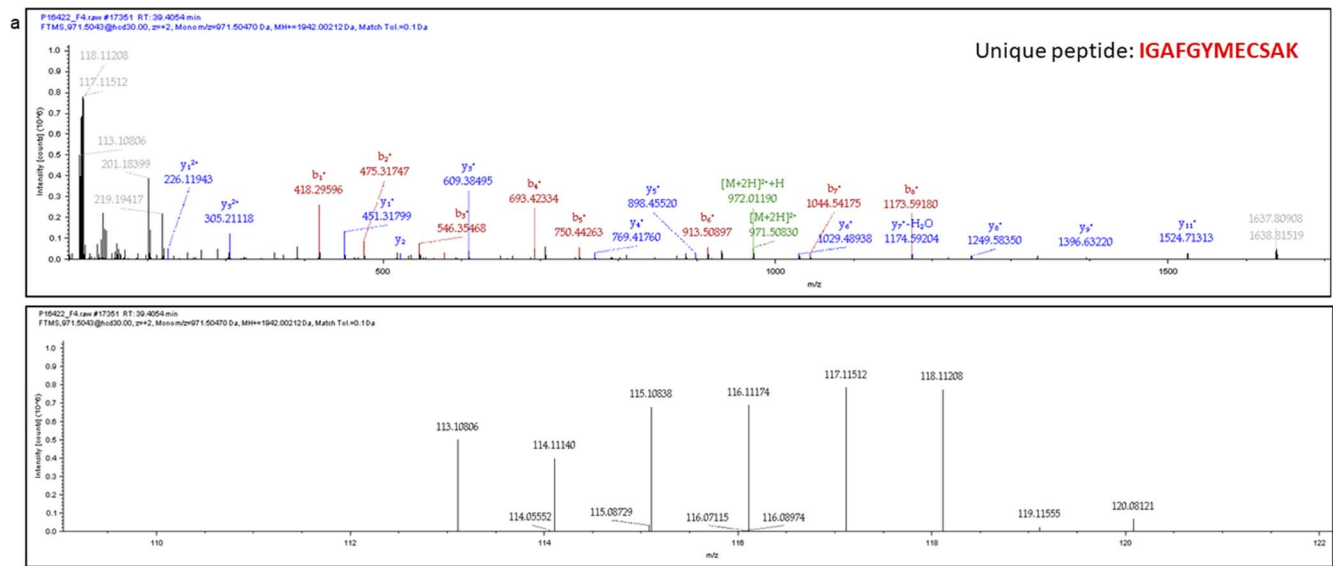


FIGURE 2. (a) MS/MS spectra of peptides of RhoA ($n = 3$, each group; upregulated by 1.32-fold; samples of control eye were labeled with iTRAQ tag 113, 114, 115; samples of FDM eye were labeled with iTRAQ tag 116, 117, 118). (b, c) QRT-PCR and Western blotting analyses of RhoA in the NC, SC, and FDM group ($n = 5$, each group). Data are expressed as the mean \pm SD. * $P < 0.05$.

significantly decreased in the ROCK-inhibited group compared to the control group ($6.13\% \pm 1.38\%$ vs. $23.43\% \pm 3.23\%$; $P < 0.05$; Figs. 5a, 5b). Strain induced an insignificant increase in MRTF-A, SRE, and α -SMA expression in the fibroblasts treated with Y27632 ($P > 0.05$; Figs. 6c, 6d). In the ROCK inhibited groups, the percentage of myofibroblasts under strain condition was $12.95\% \pm 5.16\%$, which was not significantly higher than that in the control group. ($P > 0.05$; Figs. 5a, 5b).

DISCUSSION

Mechanical force has a critical role in scleral remodeling. Our study focused on the mechanism of strain-induced scleral myofibroblast differentiation in vitro. The importance of α -SMA expression in myopia sclera was investigated in this study. Although several studies have explored the role of myofibroblasts and biomechanics in the remodeling of the sclera,^{6,14,40} none has determined how biomechanical signals are trans-

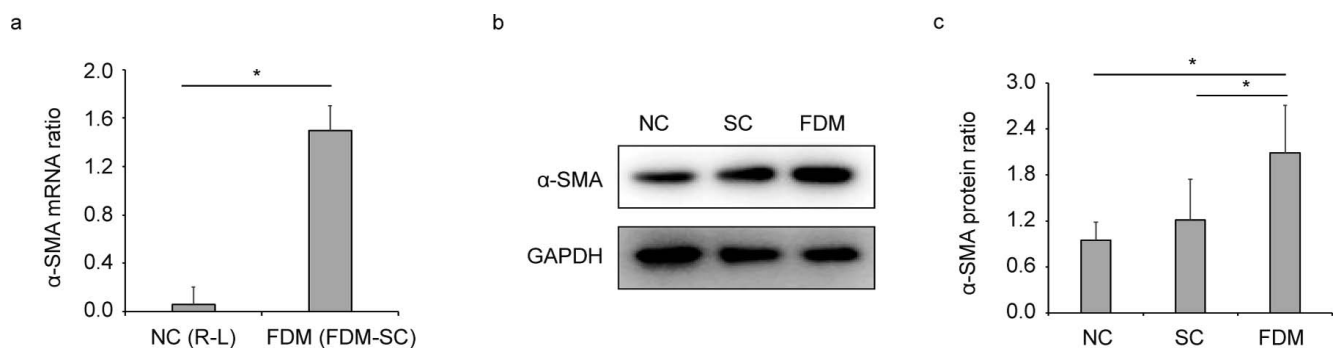


FIGURE 3. (a–c) QRT-PCR and Western blotting analyses of α -SMA in the NC, SC, and FDM groups ($n = 5$, each group). Data are expressed as the mean \pm SD. * $P < 0.05$.

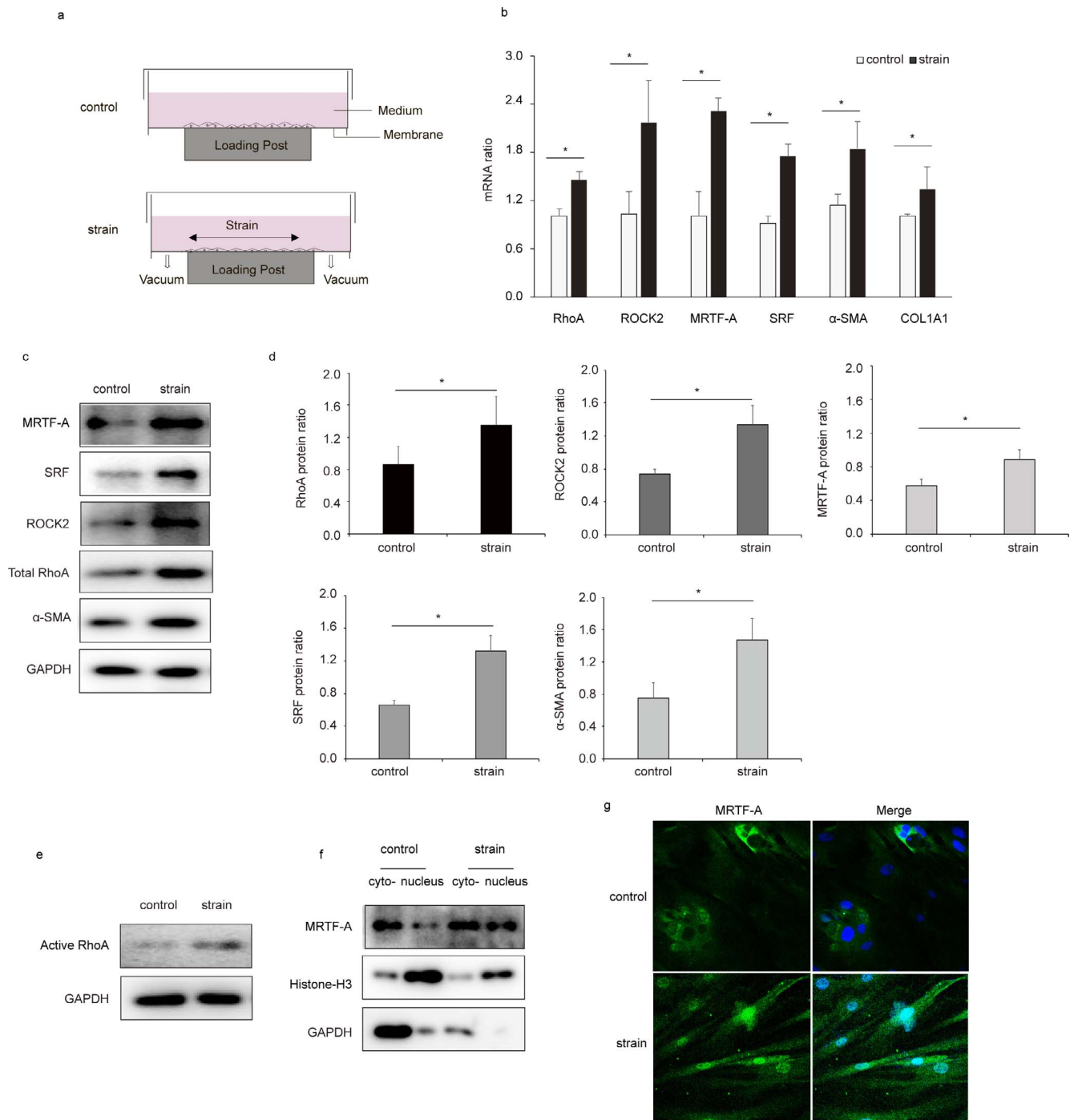


FIGURE 4. The mode of equibiaxial deformation of the Flexcell membranes over the loading posts (a). qRT-PCR analyses of *RhoA*, *ROCK2*, *MRTF-A*, *SRF*, α -*SMA*, and *COL1A1* in the scleral fibroblasts under control or 4% strain stimulation (b). Western blotting analyses of RhoA, active RhoA, ROCK2, MRTF-A, SRF and α -SMA in the scleral fibroblasts under control or 4% strain stimulation (c-f). Immunofluorescent staining of MRTF-A (g). Data are expressed as mean \pm SD of three separate experiments. * $P < 0.05$.

ferred into biochemical signals. Here, using a Flexcell system, it was possible to demonstrate that the RhoA/ROCK pathway regulated the strain-induced differentiation of scleral fibroblast to myofibroblast. To the best of our knowledge, this is the first study to identify the mechanisms of mechanotransduction pathway promoting myofibroblast differentiation we proposed (Fig. 7).

The in vivo study was able to demonstrate an increment of α -SMA expression in myopic sclera of guinea pigs. This was

consistent with the findings for myopia in tree shrews.^{14,41} According to finite element modeling, stresses and strains are positively associated with axial length and IOP, and negatively associated with scleral thickness.¹² During myopia development, scleral stresses and strains increase. The in vitro study involved application of strain to scleral fibroblasts. The results showed an increasing number of myofibroblasts. Furthermore, it was observed that strain stimulated scleral myofibroblast differentiation by promoting RhoA activation. RhoA was shown

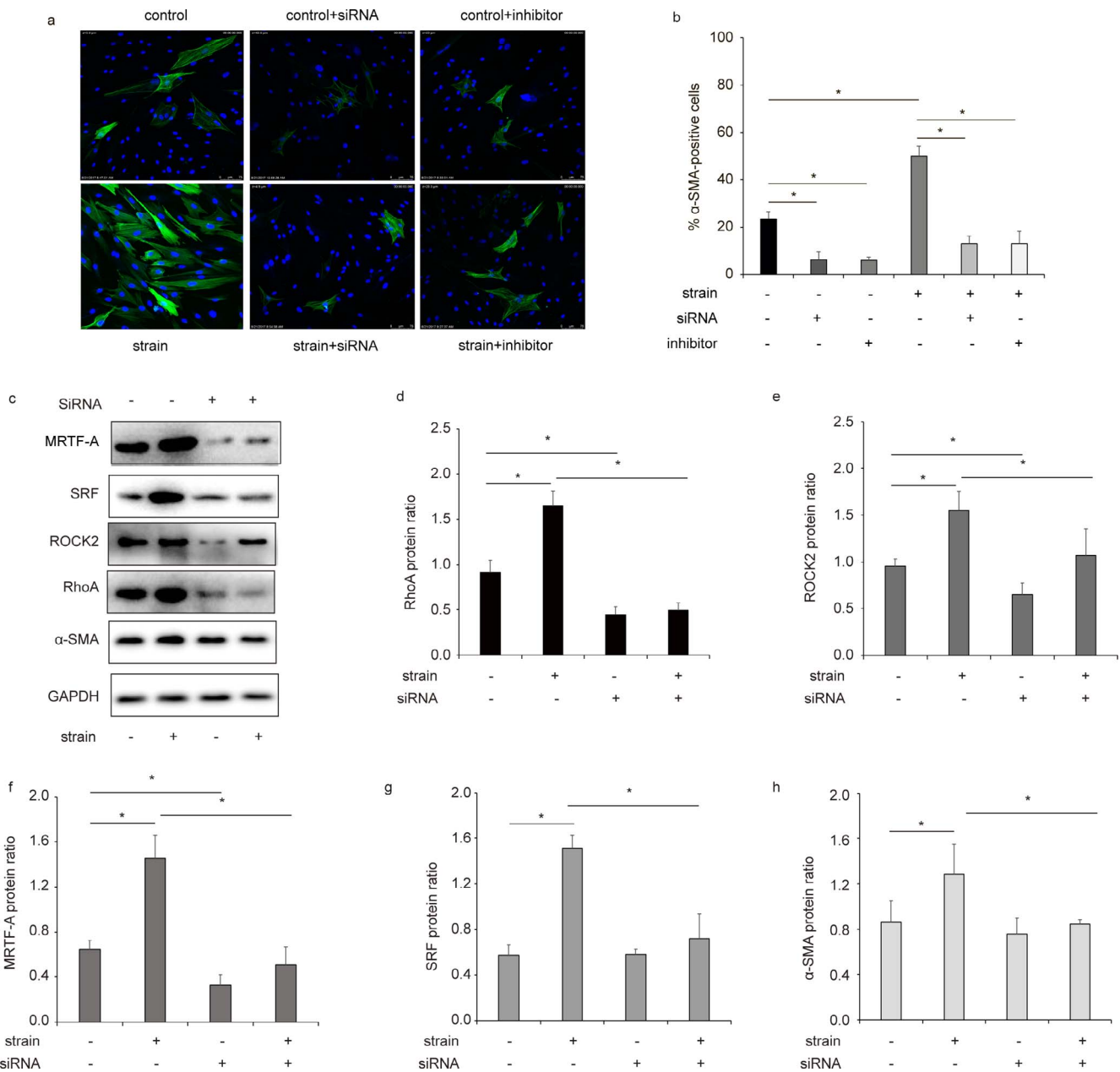


FIGURE 5. Immunofluorescent staining of myofibroblasts (a). Flow cytometry of myofibroblasts (b). Western blotting analyses of RhoA, ROCK2, α -SMA, MRTF-A, and SRF expression in RhoA specific siRNA and strain treatment groups (c-h). Data are expressed as mean \pm SD of three separate experiments. * $P < 0.05$.

to have a key role in the transduction of mechanical signals into cellular responses that increase cytoskeletal organization and contractility of airway smooth muscle cells.⁴² An increment of RhoA and myofibroblasts was observed after 4% strain application for 24 hours. When RhoA expression was knocked down, the differentiation of myofibroblast induction by strain also was suspended. This suggested that RhoA controlled strain-induced scleral myofibroblast differentiation. Previous studies have reported that RhoA was involved in smooth muscle differentiation induced by external tensile force and promoted cardiac hypertrophy.²⁶ Matrix stiffness has been shown to have an influence on myofibroblast differentiation by activation of RhoA.⁴³ Taken together, these findings indicated that activation of RhoA transformed the biomechanical

signaling to biochemical signals in response to strain to stimulate scleral myofibroblast differentiation.

Our study also investigated the downstream signaling pathway of RhoA. There was an increasing expression of ROCK2, MRTF-A, and SRF under strain conditions. If RhoA was inhibited by siRNA, mechanical strain failed to increase ROCK2, MRTF-A, and SRF expression. Overexpression of active RhoA results in the recruitment of ROCK2.⁴⁴ Inhibition of ROCK by Y27632, led to reduced expression of α -SMA, although RhoA increased. These results suggested an important role for the RhoA-ROCK2 pathway in the activation of myofibroblasts. Interestingly, RhoA increased in the presence of Y27632. Mechanotransduction depends largely on a variety of cell-matrix adhesion molecules, such as integrins, cadherins, and intercellular adhesion molecule-1.⁴⁵ During cell adhesion,

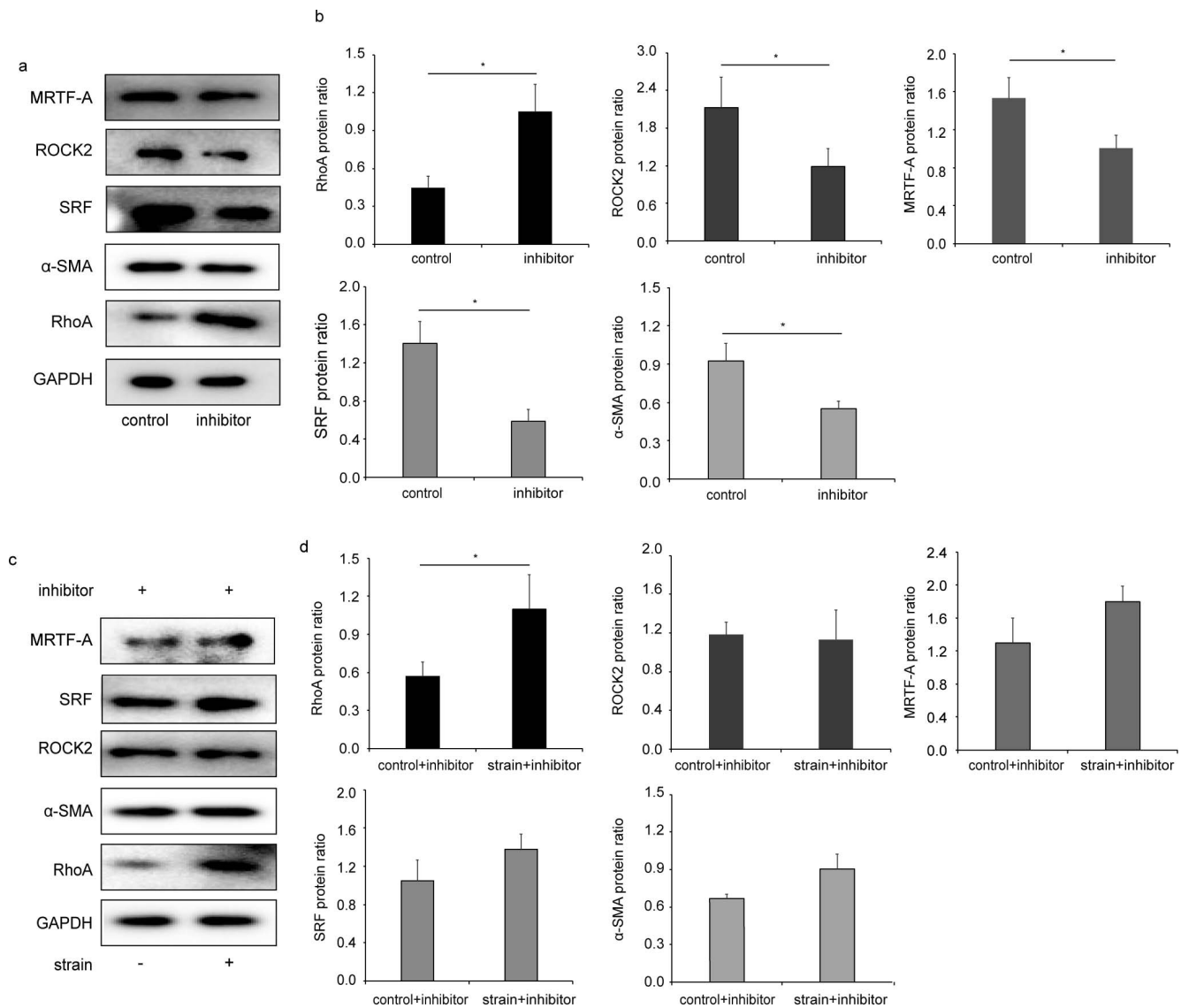


FIGURE 6. Western blotting analyses of RhoA, ROCK2, α -SMA, MRTF-A, and SRF protein expression of Y27632 treated fibroblasts in control and strain groups (a-d). Data are expressed as mean \pm SD of three separate experiments. * $P < 0.05$.

external mechanical force promotes clustering of integrins and then leads to recruitment of focal complexes at the inner side of the cell membrane.⁴⁶ Focal adhesion kinase (FAK) phosphorylation further activates RhoA and actin filaments.⁴⁷ After inhibition of ROCK activation, the mechanotransduction pathway was disrupted and the fibroblasts failed to assemble actin and exhibit stress fiber contraction in response to external strain. As a result, the mechanical force clustered more integrins and RhoA. There still was α -SMA expression under strain stimulation, although the ROCK was inhibited. This suggested that other pathways may be responsible for the myofibroblast differentiation response to mechanical strain. A previous study reported that the formin homology protein mDia1 mediated RhoA effects on matrix adhesions and the cytoskeleton, and the mDia1 and ROCK pathways mediate actin assembly concurrently.⁴⁸ Chan et al.⁴⁹ observed that knocking down mDia could partly decrease force-induced transcriptional activation of α -SMA. Therefore, the incomplete suppression of force-induced α -SMA expression after inhibition of ROCK or mDia indicated that both of these mechanotransduction pathways contribute separately, but incompletely, to

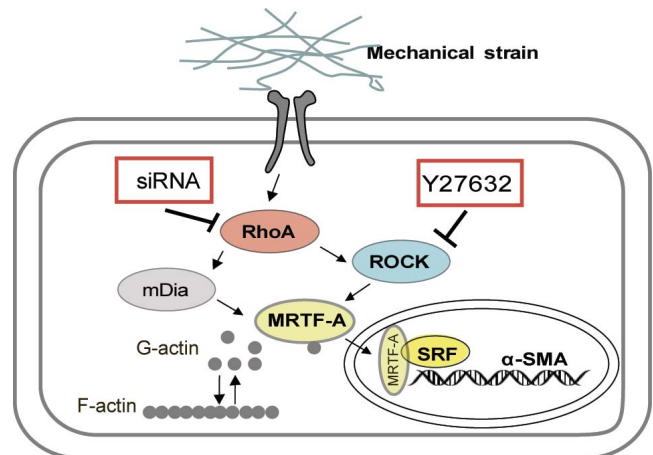


FIGURE 7. Proposed model of mechanotransduction pathway-mediated scleral myofibroblast differentiation.

myofibroblast differentiation. Overall, many signals, including ECM signals, membrane receptors, and molecules within the cell, appear to be involved in mechanotransduction.

Increased RhoA/ROCK pathways have been reported to affect the activities of transcription factors, including MRTF-A and SRF.¹⁷ We proved that RhoA activation promoted MRTF-A translocation to the nucleus under strain status and SRF expression. Moreover, we showed that RhoA silence or ROCK inhibition prevented strain-induced expression of MRTF-A and SRF, which corresponded with the absence of α -SMA, suggesting that mechanical signals through RhoA/ROCK-MRTF-A/SRF induce α -SMA expression. Matrix stiffness, tensional homeostasis, and mechanical force can induce MRTF-A translocation to the nucleus, bind and enhance SRF transcriptional activity, and subsequently induce myofibroblast differentiation.^{50,51} Previously, MRTF-A loss-of-function mice (KO) have been demonstrated to be resistant to skin, cardiac, and lung fibrosis.⁵²⁻⁵⁴ These diseases have been proved to be closely correlated with myofibroblast differentiation. Fibroblasts from such MRTF-A KO mice produced less α -SMA, which can be reversed by overexpressing MRTF-A.⁵⁵ Conversely, MRTF-A inhibitor can block matrix-stiffness and TGF β -induced α -SMA expression in colonic fibroblasts.⁵⁶ Additionally, we found a significant increase in type I collagen after mechanical strain. Similarly, Lohberger et al.⁵⁷ found a higher amount of collagen in human primary rotator cuff fibroblasts after mechanical stimulation. A previous study indicated that type I collagen synthesis depended on MRTF-A activation.⁵⁸ Therefore, MRTF-A was activated and translocated to the nucleus under 4% strain stimulation, and then induced collagen synthesis. However, the results of in vitro study were not consistent with the findings in vivo. In myopia sclera, we found a decrease in collagen content, which may be due to abnormal collagen synthesis and degradation. In myopia development, not only scleral biomechanical but also biochemical changed. It was found that matrix metalloproteinases (MMPs), a major enzyme-degrading collagen, increased. Besides, TGF- β , a cytokine inducing collagen synthesis, was proved to decrease. Therefore, it may be a complicated phenomenon for collagen deposit in vivo. Further study will be needed to investigate the competing role of biochemical and biomechanical in collagen deposit.

There are several limitations in this study. ROCK1 and ROCK2 are downstream signals of RhoA and are reported to be involved in the mechanotransduction pathway.⁵⁹ This study focused mainly on the role of ROCK2, as ROCK2 had greater expression than ROCK1 in the eye.¹⁹ Further studies are needed to explore whether ROCK1 has a role in myopia sclera remodeling. Although we found the importance of the RhoA/ROCK2-MRTF-A/SRF mechanical signaling pathway in scleral myofibroblast differentiation, other mechanisms must be involved in RhoA-dependent α -SMA expression in scleral fibroblasts. For example, it is well known that the activation of RhoA can regulate actin polymerization.⁶⁰ Studies in fibroblasts proved that MRTFs bond monomeric G-actin in the cytoplasm and translocated into the nucleus in response to signals promoting actin polymerization to constitute transcriptional complexes with SRF, then promoted the target gene expression.⁶¹ Additionally, it was found that RhoA activation also is required for YAP/TAZ activity, well-recognized mechanosensitive transcriptional regulators.⁶² Recent studies showed that RhoA regulated YAP/TAZ in a phosphorylation-dependent manner.⁶³ Collectively, these findings indicated that RhoA-dependent mechanical pathway involved in cytoskeleton, cytomolecules and transcription factors, which will need further studies.

In summary, our results demonstrated that scleral fibroblasts are mechanosensitive cells that can respond to

mechanical strain with a phenotypic transition into myofibroblasts. It also demonstrated that RhoA/ROCK2 was an important mechanotransduction pathway in the differentiation of fibroblasts to myofibroblasts modulated by strain. Strain upregulated expression of RhoA, which subsequently activated ROCK2 and mediated α -SMA expression. These findings suggested that RhoA/ROCK2 may be a potential target for prevention of strain-induced α -SMA expression involved in myopia scleral remodeling.

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