Mechanistic Basis of Rho GTPase-Induced Extracellular Matrix Synthesis in Trabecular Meshwork Cells

Padmanabhan P. Pattabiraman¹ and Ponugoti Vasantha Rao ¹, ²

Departments of Ophthalmology, Pharmacology and Cancer Biology, Duke University School of Medicine, Durham. NC 27710

Running head: Rho GTPase-induced cellular contraction and ECM synthesis

Correspondence:
P. Vasantha Rao, Ph.D
Department of Ophthalmology
AERI building, Room 4010
Duke University School of Medicine
Durham, NC 27710
Phone: 919-681-5883
Fax: 919-684-8983
Email: rao00011@mc.duke.edu
ABSTRACT

Elevated intraocular pressure arising from impaired aqueous humor drainage through the trabecular pathway is a major risk factor for glaucoma. To understand the molecular basis for Rho GTPase-mediated resistance to aqueous humor drainage, we investigated the possible interrelationship between actomyosin contractile properties and extracellular matrix (ECM) synthesis in human trabecular meshwork (TM) cells expressing a constitutively active form of RhoA (RhoAV14). TM cells expressing RhoAV14 exhibited significant increase in fibronectin, Tenascin C, laminin, α-smooth muscle actin (αSMA) levels, and in matrix assembly in association with increased actin stress fibers and myosin light chain phosphorylation. The RhoAV14-induced changes in ECM synthesis and actin cytoskeletal reorganization were mimicked by lysophosphatidic acid (LPA) and TGF-β2, which are known to increase resistance to aqueous humor outflow and activate Rho/Rho kinase signaling. RhoAV14, LPA, and TGF-β2 stimulated significant increases in extracellular signal-regulated kinase (Erk1/2) phosphorylation, paralleled by a profound increase in the expression of fibronectin, serum response factor (SRF), and αSMA. Treatment of RhoA activated TM cells with inhibitors of Rho kinase or Erk on the other hand, decreased fibronectin and αSMA. While suppression of SRF expression (both endogenous and RhoA, TGF-β2–stimulated) via the use of short hairpin RNA decreased the levels of αSMA, fibronectin was unaffected. Conversely, fibronectin was found to induce αSMA expression in an SRF-dependent manner. Collectively, these data on RhoA-induced changes in actomyosin contractile activity, ECM synthesis/assembly, and Erk activation, along with fibronectin-induced αSMA expression in TM cells, reveal a potential molecular interplay between actomyosin cytoskeletal tension and ECM synthesis/assembly. This interaction could
be significant for the homeostasis of aqueous humor drainage through the pressure sensitive trabecular pathway.

**Keywords:** Fibronectin; actomyosin; extracellular signal-regulated kinases; serum response factor
INTRODUCTION

Glaucoma is a second major cause of blindness in the United States. A major risk factor for primary open angle glaucoma (POAG) is elevated intraocular pressure (IOP) caused by increased resistance to aqueous humor outflow localized within the trabecular pathway (15, 56). Abnormal accumulation of extracellular matrix/material, which increases resistance to drainage of aqueous humor through the trabecular pathway, is believed to be partly responsible for the elevated IOP and POAG (24, 28, 49). The cellular mechanisms that regulate the production and turnover of extracellular matrix (ECM) within the outflow pathway, and how ECM turnover is linked to regulation of aqueous humor outflow through the TM, are far from clearly understood (13, 24, 27, 49). Therefore, it would seem both necessary and critical to understand the molecular basis of resistance to aqueous humor outflow. This knowledge may provide important insights into the etiology of glaucoma and support development of effective therapies.

There is now widely documented evidence in support of a link between cytoskeletal integrity within the cells of trabecular pathway consisting of the trabecular meshwork (TM), the juxtacanalicular connective tissue (JCT), and the endothelial lining of Schlemm’s canal (SC) and aqueous humor outflow through the trabecular pathway. Supportive evidence for this link comes from perfusion studies using cytoskeletal modulating agents, such as actin depolymerizing agents, inhibitors of myosin light chain kinase, myosin II, protein kinase C, Rho GTPase, and Rho kinase, and from both, in vitro and in vivo model systems (11, 19, 41, 51). These studies suggest that agents which increase actin depolymerization, and decrease cell-ECM interactions and myosin II phosphorylation within the cells of trabecular pathway, increase aqueous humor outflow presumably by causing cellular relaxation, and by altering the geometry and stiffness of the outflow pathway tissues, and fluid flow through the inner wall of SC (41, 51). Conversely,
agents that activate Rho GTPase and myosin II activity including LPA, sphingosine-1-phosphate, TGF-β2 and endothelin-1 decrease aqueous humor outflow facility concomitant with increased contractile activity of the TM cells, indicating a potential importance of actomyosin organization and the contractile force generated by the actomyosin system in the regulation of aqueous humor drainage (16, 33, 39, 57, 63).

Organization of the cellular actomyosin-based cytoskeletal network is dynamically regulated under physiological conditions. Extracellular signals serve as inputs to drive changes in cell morphology, cell adhesion, contractile/mechanical properties and gene expression via actin-myosin cross-bridging and mechanotransduction (17, 20, 21). Alterations in cytoskeletal isometric tension generated through actomyosin contraction in turn activates signaling pathways which transduce intracellular events into outputs that determine how the cell interacts with its environment, as exemplified by cell:ECM adhesive interactions and ECM assembly and rigidity (4, 20, 42, 53, 65). TM cells exhibit smooth muscle-like physiology based on their electromechanical properties (58) and the expression profile of various smooth muscle specific proteins, including α-smooth muscle actin (αSMA) and CPI-17 (11, 39, 51, 58). Regulation of mechanical and contractile properties of the pressure-sensitive TM cells is recognized to play a significant role in modulation of aqueous humor outflow and ocular pressure homeostasis (22, 29, 41, 58, 60). While there is a substantial amount of phenomenological data in the literature regarding the possible role of the ECM and cytoskeletal integrity in modulation of aqueous humor outflow, the mechanistic understanding for these interactions has lagged behind (24, 41, 49, 51). Thus, identifying the molecular basis by which the ECM, cytoskeletal integrity, cellular tension and mechanotransduction modulate outflow facility through the TM is vitally important for understanding of homeostasis of intraocular pressure.
In our recent study, we reported increased resistance to aqueous humor outflow facility in organ cultured eye anterior segments expressing a constitutively active RhoA GTPase (63). This effect of RhoA was found to be associated with altered gene expression including genes encoding ECM components and various cytokines, and with increased contractile activity in TM cells (63). To understand the molecular basis for a potential interaction between the contractile force generated by actomyosin assembly and ECM synthesis and organization in TM cells (63), here we investigated the involvement of different MAP kinases and transcription factors in Rho GTPase-mediated activation of ECM protein synthesis. This study reveals a critical role for Erk and SRF in the regulation of expression of ECM and αSMA, respectively, in Rho GTPase activated and ECM treated TM cells. The data from this study also uncover the importance of potential molecular interactions between actomyosin-based contractile activity and ECM-based mechanotransduction in regulation of ECM synthesis and contractile activity within the aqueous humor drainage pathway and in homeostasis of aqueous humor outflow.
MATERIAL AND METHODS

Materials

Human recombinant TGF-β2, mouse monoclonal anti-vinculin, anti-tubulin, anti-α smooth muscle actin antibodies, TRITC – Phalloidin and fibronectin from bovine plasma were from Sigma-Aldrich (St Louis, MO). Rabbit anti phospho-SAPK/JNK, anti phospho-p38 MAPK, anti phospho p44/42 MAPK, Erk1/2 and phospho-specific anti-myosin light chain (Thr18/Ser19) polyclonal antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit anti-SRF (SC335) was from Santa Cruz Biotechnology (Santa Cruz, CA). Cell permeable C3 transferase-CT04 (Rho GTPase Inhibitor) was purchased from Cytoskeleton, Inc (Denver, CO). Protease inhibitor cocktail tablets (complete, Mini, EDTA-free) and phosphatase inhibitor cocktail tablets (phosphostop) were from Roche (Basel, Switzerland). MEK inhibitor U0126 was from Promega Corp (Madison, WI). Rabbit anti-fibronectin, anti-Tenascin C and anti-Laminin antibodies were a generous gift from Harold P. Erickson, Department of Cell Biology, Duke University, Durham, NC. Y-27632 and SB220025 were from Welfide Corporation (Japan) and Calbiochem (Gibbstown, NJ), respectively.

TM Cell Cultures

Human primary TM cells were cultured from the TM tissue isolated from the freshly obtained corneal rings given away after they have been used for the corneal transplantation at the Duke Ophthalmology clinical service. Initially, the extracted TM tissue was chopped into small pieces in serum, and then the tissue slices were placed under a glass coverslip in a six-well plastic culture plates and cultured in Dulbecco’s modified Eagle Medium (DMEM) containing 20% FBS and Penicillin –Streptomycin-Glutamine. Expanded population of TM cells was sub-
cultured after 4-5 days, resulting in the development of a homogenous TM population. All experiments were conducted using confluent cultures of between 4-6 passages and cells were cultured at 37°C under 5% CO₂, in DMEM containing 10% FBS and Penicillin–Streptomycin–Glutamine. All experiments were done after serum starvation for at least 24 hours unless mentioned.

**Adenovirus-mediated Gene Transduction**

Replication defective recombinant adenoviral vectors encoding either GFP alone or constitutively active RhoA (RhoAV14) and GFP were provided by Patrick Casey, Department of Pharmacology and Cancer Biology, Duke University School of Medicine.

For shRNA experiments, replication defective recombinant adenoviral vectors expressing short hairpin RNA against SRF (Ad-shSRF) or the control adenovirus expressing shRNA against GFP (Ad-shGFP) were kindly provided by Joseph Miano (University of Rochester School of Medicine) and were described previously (8). The viral vectors were amplified as we described earlier (63). Human TM cells grown either on gelatin-coated glass coverslips or in plastic petridishes were infected with adenoviral vectors for respective experiments at around 50 MOI (multiplicity of infection). When cells showed adequate transfection (>80%, as assessed based on GFP fluorescence) usually after nearly 24-36 hours, cells were serum starved for 24 hours to be used in the experiments.

**Rho Inhibition Assays**
Serum starved human TM cells (24 hours) were treated with 1.0 µg/ml of cell permeable C3 transferase (Rho GTPase inhibitor) for 6 hours. This time was enough to induce a robust change in cell morphology. For the rescue experiments, human TM cells treated initially with C3 transferase for 4 hours were stimulated with LPA (5 µg/ml) or TGF-β2 (4 ng/ml) for 2 hours.

**Myosin Light Chain Phosphorylation Assay**

The effect of RhoAV14 and TGF-β2 on phosphorylation status of MLC on confluent human TM cell cultures (serum starved for 48 hours) was determined by urea-glycerol gel electrophoresis as we described earlier (63). After the treatments, the confluent cell cultures were extracted with cold 10% trichloroacetic acid and cell precipitates collected after centrifugation at 13,000 rpm were washed and finally dissolved by sonicating in 8M Urea buffer. The protein was separated on urea-glycerol gels and transferred onto nitrocellulose filters. The filters were then subjected to immunoblot using anti-phospho MLC2 antibody and blots were developed by enhanced chemiluminescence (ECL) detection system.

**Immunoblotting**

Total protein and membrane rich fractions (40,000 RPM insoluble pellet) were isolated from serum starved confluent cultures of human TM cells with or without treatments. Bio-Rad protein assay reagent (Cat. No. 500-0006) was used to estimate protein concentration. Samples containing equal amounts of protein were mixed with Laemmli buffer and separated by SDS polyacrylamide gel electrophoresis (either 10% or 5.5% acrylamide), followed by transfer of resolved proteins to nitrocellulose membranes. Membranes were then blocked for 2 hours at room temperature in Tris-buffered saline containing 0.1% Tween-20 and 5% (w/v) nonfat dry
milk. Membranes were then probed with primary antibodies in conjunction with a horse radish peroxidase (HRP) conjugated secondary antibodies. Detection of immunoreactivity was performed by enhanced chemiluminescence (ECL). Densitometry of scanned films was performed using NIH Image software. Data were normalized to the loading controls (β-tubulin).

**Immunofluorescence Staining and Microscopy**

TM cells were grown on gelatin-coated glass coverslips until attaining confluency. After appropriate treatments, cells were washed in PBS twice and then fixed in 4% paraformaldehyde for 15 minutes. After fixing, washing, permeabilizing, and blocking, cells were incubated with the respective primary and Alexa-fluor conjugated-secondary antibodies for 2 hours each at room temperature as we described earlier (40). Later the coverslips were washed and mounted on to glass slide with Aqua Mount (Lerner Laboratories, Pittsburg, PA). The slides were observed under a Nikon Confocal system (C1 Digital Eclipse) and z-stack images were collected and processed.

**Fibronectin coating**

Fibronectin-coated plastic plates were prepared as we described earlier (63), by coating 100mm x 20mm Corning cell culture dishes overnight with 20μg/ml of fibronectin in sterile PBS. For the controls, the plates were treated with PBS alone. Excess ECM was removed by rinsing the plates three times with PBS. HTM cells or HTM cells expressing shGFP or shSRF in serum free medium were directly plated onto either the ECM-coated plates or PBS treated plates and incubated for 8 hours at 37 °C. After 8 hours, the cells were scraped, lysed, and the total protein was immunoblotted.
RT-PCR

Total RNA was extracted using RNeasy Micro Kit (Qiagen, Valencia, CA) as per the user manual from HTM cells treated with shSRF or the control shRNA (shGFP). Five micrograms of total RNA was used for first-strand synthesis using SUPERSCRIPT First strand synthesis system for RT-PCR (Life Technologies, Carlsbad, CA). The cDNA was amplified in a total volume of 50 µl with 1.5 mM MgCl₂, 0.2 mM dNTPs, 2U Taq polymerase (Life Technologies) and 0.5 µM of sense and antisense primers for SRF. The control reaction was done with GAPDH primers separately. The primer sequences are as follows:

hSRF forward: 5' GCC ACT GGC TTT GAA GAG AC 3'
hSRF Reverse: 5' CCA GAT GAT GCT GTC AGG AA 3'

GAPDH Forward: 5'TGCACCACCAACTGCTTAGC 3'
GAPDH Reverse: 5' GGCATGGACTGTGGTCATGAG 3'

Amplification was done using the primers mentioned above with the following PCR program: Initial denaturizing step at 94°C for 4 min then followed by: 94°C for 1 min, 58°C for 30 sec and 72°C for 30 sec for 30 cycles and a final step at 72°C for 10 min. The PCR products were separated by electrophoresis on 1% agarose gel and visualized by staining with ethidium bromide.

Statistical Analysis
All data represent the average results of at least 3 independent experiments. Quantitative data were analyzed by the Student’s t-test and minimum values of $p<0.05$ were considered as statistically significant.
RESULTS

*RhoAV14–induced changes in actin cytoskeletal organization and ECM synthesis/assembly in TM cells.*

To understand the effects of Rho GTPase on the cytoskeletal architecture, ECM synthesis, and matrix assembly in TM cells, we expressed a constitutively active RhoA (RhoAV14/GFP) or GFP alone in human TM (HTM) cells using recombinant adenoviral vectors. After 24 hours of viral vector infection (>80% infection efficiency based on GFP expression), the TM cells were serum starved for 24 hours and monitored for changes in cell morphology using a phase contrast microscope. The RhoAV14 expressing TM cells exhibited stiffened and contractile morphology with obvious cell retraction compared to GFP expressing control cells (Fig 1A). This change in cell morphology was associated with a robust increase in actin stress fibers (phalloidin staining) and focal adhesion formation (vinculin staining) in RhoAV14 expressing TM cells (Fig.1B). Additionally, immunoblot analysis of the membrane rich fraction of the RhoAV14 expressing TM cells showed significantly increased levels of vinculin compared to GFP expressing cells (Fig. 1C). Similarly, analysis of myosin light chain (MLC) phosphorylation in the RhoAV14 expressing H TM cells showed a marked increase compared to control cells (Fig. 1D). Moreover, quantitative immunoblot analysis of cell lysates derived from the RhoAV14 expressing TM cells showed significantly increased levels of fibronectin, laminin, Tenascin C, and αSMA proteins compared to control cells expressing GFP alone (Fig. 1E). The RhoAV14 expressing TM cells also revealed increased filamentous and bundled organization (fibrils) of the fibronectin compared to the untreated control cells (Fig. 1F).
LPA and TGF-β2–induced changes in actin cytoskeletal organization and ECM synthesis/assembly in TM cells

In addition to assessing direct effects of activated RhoA on TM cell actomyosin and ECM organization, we also evaluated the effects of TGF-β2 and LPA on these cellular events. The reason for choosing these two physiological agonists was that these agents have been shown to increase the resistance to aqueous humor outflow in perfusion models (16, 33), and they activate Rho/Rho kinase signaling in various cell types including TM cells (37, 39). Serum starved HTM cells treated with LPA (5 μg/ml) and TGF-β2 (10 ng/ml) for two hours exhibited increased actin stress fibers and focal adhesion formations (Fig. 2A). Further, similar to the RhoAV14, these changes were associated with increased MLC phosphorylation and increased levels of vinculin in the membrane-enriched fraction (Fig. 2B and C). Moreover, both LPA and TGF-β2 treated TM cells also revealed increased levels of fibronectin, laminin, and αSMA compared to untreated control cells (Fig. 2D). Again as in the case of RhoAV14 expressing cells, TGF-β2 and LPA treated HTM cells exhibited increased and compact assembly of fibronectin compared to untreated control cells (Fig. 2E). Moreover, pretreatment of HTM cells with Rho GTPase inhibitor (C3 transferase; 1.0μg/ml) for six hours suppressed the TGF-β2 and LPA induced increases in the levels of fibronectin, laminin, and αSMA, (Fig. 3A) in association with decreased actin stress fibers and focal adhesions (Fig. 3B) indicating the significance of Rho GTPase activity in the regulation of ECM synthesis and assembly, and expression of αSMA (contractile activity regulating protein) mediated by LPA and TGF-β2. As well, treatment of HTM cells with an inhibitor of Rho kinase (10 μM Y-27632 for two hours), a downstream kinase effector of Rho GTPase (14), led to decreases in the levels of fibronectin, laminin, and αSMA in association with decreased actin stress fibers, focal adhesions, and MLC
phosphorylation as compared to untreated control cells (Fig. 4A and B), further supporting a definite role for Rho/Rho kinase pathway and actomyosin in ECM synthesis and assembly.

**RhoA, TGF-β2 and LPA induced Erk activation in TM cells**

After noting the effects of RhoAV14, LPA and TGF-β2 on ECM synthesis and assembly in association with an actomyosin assembly-mediated increase in contractile activity in TM cells, we looked further into the identity of downstream signaling events that may aid Rho GTPase-induced ECM synthesis. For this, we assessed the effects of RhoAV14, LPA and TGF-β2 on the activation status of the mitogen-activated protein (MAP) kinases - p38, JNK and ERK kinases (Erk1/2), in TM cells. HTM cells either transfected with RhoAV14 for 24 hours and serum starved for 24 hours, or serum starved HTM cells stimulated with TGF-β2 or LPA for two hours exhibited significant increases in Erk1/2 phosphorylation (activation) compared to untreated control cells (Fig. 5A and B). On the other hand, both p38 MAPK and JNK did not reveal any changes in phosphorylation status under similar conditions (data on JNK not shown). The protein levels of total Erk1/2 did not change in response to any of the above described treatments (Fig. 5A-C). Further, the levels of phospho–Erk1/2 were found to be markedly decreased in wild type HTM cells treated either with Rho kinase inhibitor (Y-27632 for 2 hours) or Rho GTPase inhibitor (C3 transferase for 4 hours), indicating the importance of the Rho/Rho kinase pathway in regulation of Erk activation in TM cells (Fig. 5C).

**Effects of Erk inhibition on ECM synthesis and αSMA in TM cells**

Wild type HTM cells treated for four hours with U0126, a highly selective inhibitor of Erk1/2 (12), exhibited marked decreases in phospho- Erk 1/2 levels (Fig. 6A). Importantly,
treatment with U0126 also resulted in significant reduction in the levels of both fibronectin and αSMA proteins in wild type HTM cells compared to untreated controls (Fig. 6A). Further, the inhibition of fibronectin or αSMA expression by Erk inhibitor was not rescued by LPA, TGF-β2 or RhoAV14 (Fig. 6A and B). On the other hand, a selective inhibitor of p38 MAPK (SB220025) showed no effects on the expression of fibronectin or αSMA (Fig. 6B) in normal HTM cells. Interestingly, the U0126 induced changes in fibronectin and αSMA expression were also associated with decreases in MLC phosphorylation (Fig. 6C) and in actin stress fibers (not shown) in normal HTM cells. Collectively these observations imply a critical role for Erk activation in fibronectin and αSMA expression in TM cells under both normal and TGF-β2, LPA, and RhoA stimulated conditions.

_RhoA and TGF-β2 regulated ECM and αSMA expression via SRF expression_

In addition to a direct role in regulation of cytoskeletal organization and function, Rho GTPase controls the activity of the myocardin-related transcription factors (MRTF-A and MRTF-B), which are transcriptional co-activators of the transcription factor- SRF (35, 54). SRF has been recognized to serve as a master control element in the expression of various cytoskeletal proteins including αSMA (34). To obtain further insight into the regulation of fibronectin and αSMA expression by Rho GTPase and TGF-β2, we determined the effects of both RhoAV14 and TGF-β2 on SRF expression in HTM cells. HTM cells expressing the constitutively active RhoAV14 exhibited a significant increase in SRF protein levels compared to GFP expressing controls (Fig 7A). Similarly, serum starved wild type HTM cells treated with TGF-β2 for two hours revealed significant increases in SRF expression (Fig. 7A).
Additionally, to understand whether there exist a sequential and kinetic relationship between the TGF-β2-induced expression of fibronectin, SRF, αSMA, and Erk activation in HTM cells, serum starved wild type HTM cells were stimulated with TGF-β2 and monitored for time-dependent changes in the levels of fibronectin, SRF, αSMA, and Erk activation. Interestingly, both SRF expression and Erk activation exhibited an early and parallel response that is evident at 30 minutes following stimulation and continuing over a 24 hour-period (Fig. 7B). On the other hand, the expression of fibronectin and αSMA exhibited a lag time of 2 to 4 hours after which there was a significant induction of expression (Fig. 7C). However, unlike the expression profile of αSMA whose expression continued to increase up to 24 hours following an initial lag, fibronectin expression peaked between 4 to 8 hours of TGF-β2 stimulation and plateaued thereafter (Fig. 7C). These data indicate that the expression of SRF and Erk activation precedes and may be a prerequisite for the induction of expression of αSMA and fibronectin in TGF-β2 stimulated HTM cells.

**SRF and regulation of αSMA and fibronectin in HTM cells**

To further investigate the potential role of SRF in regulation of αSMA and fibronectin expression, we utilized a knockdown approach based on adenovirus-mediated expression of short hairpin RNA against SRF (Ad-shSRF). Treatment of wild type HTM cells with Ad-shSRF for 24 hours significantly knocked down the expression of SRF mRNA (Fig. 8A upper panel) and protein (Fig. 8A, third panel) compared to control cells treated with short hairpin RNA against GFP (Ad-shGFP). As shown in Figure 8B, knocking down of SRF in HTM cells led to significant decrease in the levels of αSMA. Importantly, the shSRF induced suppression of αSMA expression was not rescued by either TGF-β2 or RhoAV14 indicating a critical role for
SRF in the regulation of TGF-β and RhoAV14-induced expression of αSMA in HTM cells (Fig. 8B). TGF-β2 and RhoAV14 induced expression of fibronectin in HTM cells on the other hand, appears not to be dependent on SRF expression (Fig. 8B). Interestingly, inhibition of Erk activation in HTM cells decreased SRF protein levels, an effect that was not rescued by TGF-β2 indicating a requirement for Erk activity in the TGF-β2-induced expression of SRF (Fig. 8C).

**Effects of fibronectin on αSMA, SRF expression, and Erk activation in HTM cells**

To determine whether the ECM exerts a possible feedback effect on the expression of SRF, αSMA, or Erk activation, control HTM cells or HTM cells expressing shGFP or shSRF suspended in serum free medium were plated onto either the fibronectin-coated or control (PBS treated) plates. As reported by us in earlier studies (63), serum starved HTM cells cultured on fibronectin-coated plates exhibited a spread out morphology, while control cells cultured on PBS treated plates showed rounded morphology (data not shown). Immunoblot analyses of fibronectin-stimulated HTM cells or HTM cells expressing shGFP revealed significant increases in levels of αSMA, SRF, and phospho-Erk as compared to control cells cultured on PBS treated plates (Fig. 9A & B). Contrarily, shSRF expressing cells plated on fibronectin-coated plates showed little changes in levels of αSMA, SRF, and phospho-Erk levels (Fig. 9A &B). Further, suppression of SRF expression in control TM cells reduced the basal levels of αSMA and phospho-Erk (Fig. 9A & B).
DISCUSSION

The primary goal of this study was to understand the mechanistic basis by which Rho GTPase controlled actomyosin contractile activity regulates ECM synthesis and assembly in the context of homeostasis of aqueous humor resistance in the trabecular pathway. The major findings of this study include 1. Rho GTPase induced actomyosin assembly and contractile activity stimulates ECM synthesis and assembly, and αSMA expression, in TM cells; 2. Erk kinase activation appears to be critical for the RhoA, LPA and TGF-β2-induced ECM, and αSMA expression; 3. While RhoA, LPA and TGF-β2-induced SRF expression downstream to Erk appears to be critical for the regulation of αSMA, Erk activation alone induced by RhoA, LPA and TGF-β2 is sufficient for the regulation of fibronectin expression in TM cells; and 4. Fibronectin via feedback activation induces αSMA expression in association with increased SRF expression and Erk activation in TM cells. Collectively, these observations support existence of a potential bidirectional interplay and balance between Rho GTPase regulated contractile force and ECM derived mechanical tension in the pressure sensitive TM cells, and suggest that this interplay between contractile activity and ECM synthesis/assembly in the cells of aqueous humor outflow pathway including TM, SC and JCT represents a crucial regulatory component in the homeostasis of aqueous humor outflow resistance, as depicted schematically in Figure 10.

Aqueous humor drainage via a pressure sensitive trabecular pathway is believed to be a major route of total outflow in a normal eye (15, 48). Among the various factors that influence the resistance to aqueous humor outflow, ECM accumulation is considered to play a predominant role (1, 24, 28, 49). Alterations in ECM content and organization have been found to be associated with increased resistance in the outflow pathway of human glaucomatous eyes (24, 28, 49, 62). Further, perfusion of ECM degrading matrix metalloproteinases (MMPs) has been
shown to increase aqueous humor outflow facility through the trabecular pathway indicating the importance of ECM turnover in the homeostasis of aqueous humor outflow resistance (5, 24). Similarly, the integrity of actin cytoskeletal organization and the contractile characteristics of trabecular outflow pathway have been shown to modulate aqueous humor outflow facility (41, 51, 58). Of particular note, agents that affect myosin II activity and actin polymerization have been reported to influence aqueous humor outflow through the trabecular pathway implying an important role for TM, JCT, and SC tissue contractile and relaxation properties in the regulation of aqueous humor outflow resistance (11, 41, 51, 64). To this end, activators and inhibitors of the Rho/Rho kinase pathway, one of the predominant signaling pathways that regulate cellular contraction (47), have been shown to exert contrasting effects on aqueous humor outflow, with activators increasing and inhibitors decreasing outflow resistance (41). Importantly, in our recent study, expression of constitutively active RhoA in the trabecular outflow pathway was found to increase outflow resistance in a response associated with increased actomyosin assembly and with increased expression of various ECM related genes and cytokines such as TGF-β, interleukin-1 and connective tissue growth factor (CTGF) in TM cells, indicating a potential interaction between Rho GTPase-induced actomyosin assembly and ECM synthesis (63). The mechanistic underpinnings of this association between the activity of Rho/Rho kinase pathway and ECM synthesis in outflow pathway cells, however, have not been unraveled, and in this study, we attempted to explore the potential molecular interplay between actomyosin contractile activity and ECM turnover in TM cells.

Human TM cells expressing a constitutively activated form of RhoA (RhoAV14) which exhibited a visually notable contractile morphology with increased actin stress fibers, vinculin membrane targeting, and MLC phosphorylation, demonstrated increased levels of fibronectin,
fibronectin fibril formation, laminin, Tenascin C, and αSMA. Further, the morphological changes and changes in expression of ECM proteins were suppressed by the Rho GTPase inhibitor (C3 transferase) and Rho kinase inhibitor (Y-27632), in association with decreased MLC phosphorylation, actin stress fibers, focal adhesions and fibronectin fibrils. These observations suggest that TM cells sense RhoAV14-induced contractile activity perhaps cytoskeletal tension and that this in turn induces ECM synthesis and assembly, which can in effect serve to increase mechanical force to counteract the actomyosin derived contractile tension and cell retraction (7, 42). αSMA has been thought to serve as a mechanotransducer, based on its ability to physically link mechanosensory elements and to enhance its own, force-induced expression (55). Rho GTPase activation is known to induce αSMA expression and assembly in other cell types as well (30, 55). As in the case of TM cells (63), the Rho/Rho kinase pathway has been shown to induce ECM synthesis in other mechanosensing cell types including vascular endothelial cells and fibroblasts (2, 6, 43). As well, mechanical stress, shear stress, and ECM stiffness have been recognized to influence Rho GTPase activity in different cell types indicating an existence of a potential feed forward and back interplay between Rho-regulated myosin II contractile tension and ECM-derived mechanical force/rigidity (3, 4, 20, 23, 46, 52). The feedback response from ECM to Rho GTPase activation and contractile activity was supported partly by our previous data (63) on ECM induced Rho GTPase activation and myosin II phosphorylation, along with data presented in this study on fibronectin-induced αSMA expression in TM cells.

In addition to the direct effects of RhoAV14, stimulation of TM cells with physiological agonists such as LPA and TGF-β2 that are known to induce Rho GTPase activation and MLC phosphorylation in TM and other cell types (18, 33, 36-38), leads to an increase in levels of
fibronectin, fibronectin fibrils, laminin, and αSMA in RhoA and Rho kinase dependent manner. Pretreatment of TM cells with Rho GTPase inhibitor (C3 transferase) suppressed the LPA and TGF-β2-induced increase in expression of fibronectin, laminin, and αSMA and caused decreases in actin stress fibers, focal adhesions and MLC phosphorylation, further confirming the participation of Rho GTPase activity in LPA and TGF-β2 induced changes in ECM and αSMA.

Interestingly, perfusion with both LPA and TGF-β2 has been reported to decrease aqueous humor outflow facility (16, 33) in enucleated eyes. In the case of TGF-β2, increased resistance to aqueous humor outflow is reported to be associated with increased levels of synthesis of ECM components (13, 27, 49). Our observations regarding the direct effect of RhoAV14 and those of Rho GTPase activating agonists on expression of fibronectin, fibronectin assembly, αSMA expression, actin stress fibers and myosin II activity in TM cells collectively uncovers the significance of Rho/Rho kinase signaling, cell contractile activity, and mechanotransduction in homeostasis of ECM synthesis, and αSMA expression.

To obtain further insight into the downstream molecular basis for RhoA induced ECM synthesis and αSMA expression in TM cells, we explored the possible involvement of the mitogen–activated protein kinases and transcriptional factors by examining the activation status of the Erk, JNK and p38 kinases and expression levels of the transcriptional factor- SRF. Rho GTPase, in addition to its well recognized role in actin dynamics and cell adhesion, participates in numerous cellular processes including gene expression and transcriptional regulation via signaling pathways involving the mitogen-activated protein kinases, and serum response factor and NFκB (21). TM cells treated with RhoAV14, TGF-β2 or LPA exhibited increases in Erk 1/2 activation and SRF expression. Moreover, inhibitors of both Rho GTPase (C3 transferase) and Rho kinase (Y-27632) suppressed Erk activation in TM cells revealing the significance of
Rho/Rho kinase regulated actin dynamics and contractile activity in Erk activation. Although, the Rho GTPases are also known to regulate p38 and JNK activation under different conditions, these kinases are preferential targets of the Rac and Cdc42 GTPases (21). Rho/Rho kinase induced fibronectin and Tenascin C synthesis has been documented to depend on the Erk activity in other cell types (3, 6).

To understand the significance of Erk activation and SRF expression for regulation of ECM synthesis and αSMA expression in TM cells, Erk activity was targeted using a pharmacological inhibitor, while SRF expression was suppressed using shRNA. Treatment of wild-type HTM cells with Erk inhibitor led to decreases in the basal levels of fibronectin and αSMA. Further, the Erk inhibitor-induced decreases in basal levels of expression of fibronectin and αSMA levels were not rescued by either RhoAV14, TGF-β2 or LPA, indicating a critical role for Erk activity in regulating fibronectin and αSMA expression in HTM cells. Similar to the effects of the Rho kinase inhibitor Y-27632, the Erk inhibitor U0126 also caused cell shape changes in HTM cells in association with decreased actin stress fibers (data not shown) and MLC phosphorylation. This is consistent with the documented effects of Erk inhibitor on MLC phosphorylation and actin cytoskeletal organization in other cell types (9, 25, 61). The similar but independent effects of inhibitors of Rho kinase and Erk on the expression of fibronectin and αSMA in association with decreased actin stress fibers and cell shape changes points out the significance of myosin II derived contractile force in the regulation of fibronectin and αSMA expression. Our observations regarding the role of Erk activity in the regulation of expression of fibronectin and other ECM molecules in TM cells are consistent with published observations in which TGF-β and other cytokines have been shown to stimulate fibronectin expression in an Erk-dependent manner in different cell types (31, 44, 59). Very recently, ECM rigidity has been
reported to increase fibronectin fibril formation, Erk activation, focal adhesion kinase activity, 
αSMA and actin stress fibers in TM cells further reiterating the significance of 
mechanotransduction in the aqueous humor outflow pathway (45).

In addition to involvement of Erk activation in TM cell ECM synthesis and αSMA 
expression, SRF expression was noted to be increased in RhoAV14, and TGF-β2 stimulated TM 
cells indicating a potential role for SRF in this response. SRF, a transcription factor whose 
activity depends on the ratio of filamentous actin to monomeric G-actin and on Rho GTPase 
activity, regulates expression of various cytoskeletal proteins including actin and immediate 
early genes in different cell types (26, 32, 34). When Rho GTPase is activated, the G-actin levels 
are reduced and under this condition, myocardin, the co-activator of SRF translocate to the 
nucleus and activate the SRF (32). Reduction of SRF expression in TM cells via shRNA leads to 
significant decreases in the levels of αSMA. Further, both RhoAV14 and TGF-β2 failed to 
rescue the expression of αSMA when SRF levels were reduced in TM cells, indicating a critical 
requirement for SRF in the expression of αSMA. On the other hand, RhoAV14 and TGF-β2-
induced expression of fibronectin was found to be unaffected by the decreased levels of SRF 
indicating that SRF is not an obligatory factor in the regulation of fibronectin expression in TM 
cells. Activation of Erk and increased SRF expression appear to precede the expression of 
fibronectin and αSMA based on the kinetics of these events in TM cells. Additionally, Erk seems 
to act upstream of SRF in regulating expression of αSMA and fibronectin in TM cells possibly 
through different downstream mechanisms. For example, Erk has been shown to phosphorylate 
myocardin, a co-activator of SRF (50), and Elk-1, a transcription factor involved in ECM 
synthesis and turnover (10).
In conclusion, the results of this study reveal that TM cells appear to sense the actomyosin derived contractile force and induce ECM synthesis/assembly, and conversely, ECM assembly/rigidity influences actomyosin contraction via Rho GTPase activation (63) and αSMA expression to maintain a bidirectional balance between cell-induced contractile tension and ECM mechanical force. This molecular interplay between contractile activity and ECM synthesis could play a significant role in homeostasis of aqueous humor drainage by influencing the cell and tissue stiffness within the outflow pathway. Importantly, regulation of Rho/Rho kinase signaling activity appears to play a crucial role in cells of the aqueous humor outflow pathway by linking the actomyosin-regulated contractile activity with expression of ECM proteins, and αSMA, in addition to its well-recognized role in actin cytoskeletal organization, cell shape, and cell adhesive interactions.
GRANTS

This work was supported by National Institutes of Health R01 Grants EY018590 and EY12201 (to P. V. Rao) and P30EY005722.

ACKNOWLEDGEMENTS

We thank Drs. Patrick Casey and Harold Erickson for proving the adenoviral vectors expressing RhoAV14 and GFP, and ECM (Fibronectin, Tenascin C and laminin) antibodies, respectively. We also thank Dr. Joseph Miano for providing the Adeno-shSRF and Adeno-shGFP, and Dr. David Epstein for his insightful discussion during these studies.
REFERENCES


FIGURE LEGENDS

**Figure 1.** RhoAV14 induced changes in actin cytoskeletal organization and ECM synthesis/asemly in human TM cells.

Serum starved HTM cells transduced with an adenoviral vector expressing RhoAV14/GFP exhibit contractile cell morphology (A), increased actin stress fibers (B) and focal adhesions (B), increased vinculin membrane localization (C), increased myosin light chain phosphorylation (D), increased levels of αSMA, ECM proteins (fibronectin, laminin, Tenascin C) (E), and increased ECM assembly or fibril formation (F) compared to cells expressing GFP alone. Representative images drawn from multiple independent analyses are shown here. Histograms show significant differences between treated and untreated cells at a minimum of P<0.05 based on triplicate analyses. Asterisks indicate statistical significance between the control and RhoAV14 expressing cells. Bars (Fig. 1 A & B: 50 μm; 1F: 10 μm), indicate image magnification. β-tubulin levels were assessed to control for loading uniformity.

**Figure 2.** LPA and TGF-β2-induced changes in human TM cell actin cytoskeletal organization, and ECM synthesis.

Serum starved human TM cells were treated with LPA (5 μg/ml) or TGF-β2 (4 ng/ml) for 2 hours and examined for changes in F-actin distribution (Phallodin staining), focal adhesion formation (vinculin staining), MLC phosphorylation, and levels of fibronectin, laminin, and αSMA. Both LPA and TGF-β2 induced formation of actin stress fibers and focal adhesions (A). Both agonists also increased vinculin membrane localization (B), myosin light chain phosphorylation (C), protein levels of fibronectin, laminin, and αSMA (D) compared to untreated control cells. Additionally, immunostaining of fibronectin and laminin was found to be much
more intense in LPA and TGF-β2 treated TM cells compared to control cells (E). Histograms depict quantitative changes in indicated proteins based on triplicate analyses. Asterisks indicate significant differences (at a minimum of P<0.05) between treated and untreated cells. Bars (10 μm) represent image magnification. β-tubulin was immunoblotted for loading uniformity.

**Figure 3.** Effects of RhoA inhibition on LPA and TGF-β2-induced changes in actin cytoskeletal organization, and ECM and αSMA expression in human TM cells.

Serum starved human TM cells were treated with cell permeable C3 transferase (inhibitor of Rho GTPase, 1 μg/ml) for 6 hours and evaluated for actin cytoskeleton reorganization, focal adhesions formation, changes in protein levels of fibronectin and αSMA, and levels of membrane associated vinculin. As shown in the figure, C3 transferase treatment reduced actin stress fibers (B), focal adhesions (B), and vinculin membrane localization (A). The levels of both fibronectin and αSMA were reduced significantly (A). Additionally, the effects of C3 transferase on actin cytoskeleton, focal adhesions, fibronectin and αSMA were not rescued by the addition of LPA or TGF-β2 (A & B). Quantitative changes (histograms) were based on triplicate analyses. Bars (10 μm) indicate magnification. Asterisks indicate significant differences (at a minimum of P<0.05) between treated and untreated cells. β-tubulin was immunoblotted for loading uniformity.

**Figure 4.** Effects of Rho kinase inhibitor on actin cytoskeletal organization, MLC phosphorylation, and ECM synthesis/organization in human TM cells.

Human TM cells treated with Rho kinase inhibitor (10 μM Y-27632 for 2 hours)
exhibited a marked decrease in actin stress fibers, focal adhesions and fibronectin (A). Additionally, the levels of phosphorylated myosin light chain, αSMA, fibronectin and laminin were reduced dramatically in Y-27632 treated HTM cells as compared to control cells (B). β-tubulin was probed as a loading control. Bar (10 μm) indicates image magnification.

**Figure 5.** RhoA, LPA and TGF-β2-induced Erk activation in human TM cells.

Serum starved human TM cells expressing RhoAV14 for 24 hours or stimulated either with LPA (5 μg/ml for 2 hours) or with TGF-β2 (4 ng/ml for 2 hours) exhibited significant increases in the levels of phospho-Erk1/2 compared to untreated controls. Total Erk1/2 protein levels were similar between the treated and untreated control cells. Additionally, inhibition of RhoA (C3 transferase, 1 μg/ml for 4 hours) and Rho kinase (Y-27632, 10 μM for 2 hours) led to a decrease in phospho-Erk protein levels in human TM cells. As shown in the figure, the levels of phospho-p38 MAPK and JNK were unaltered by RhoA, LPA and TGF-β2 in serum starved TM cells. Histograms derived from triplicate analyses show that RhoAV14, LPA and TGF-β2 induce quantitative changes in Erk activation. Asterisks indicate significant difference (at minimum of P<0.05) between the treated and untreated cells. β-tubulin levels were assessed for loading uniformity.

**Figure 6.** Effects of Erk inhibition on the expression of fibronectin and αSMA in human TM cells.

Serum starved human TM cells treated with Erk inhibitor (U0126; 25 μM for 4 hours) revealed dramatic reduction in Erk phosphorylation (A). Protein levels of both fibronectin and αSMA were found to be significantly decreased in U0126 treated TM cells (A). Further,
stimulation of TM cells with TGF-β2 (4 ng/ml) or LPA (5 μg/ml) for 2 hours (A) or trasfection with RhoAV14 (B) failed to reverse the effects of Erk inhibitor on fibronectin and αSMA expression. Further, Erk inhibitor (U0126) alone was found to decrease the levels of phospho-myosin light chain in TM cells (C). Histograms show significant changes (with minimum of P<0.05) between treated and untreated cells based on triplicate analyses. β-tubulin was probed as a loading control.

**Figure 7.** Effects of RhoA and TGF-β2 on SRF expression in human TM cells.

Serum starved TM cells either expressing RhoAV14 or treated with TGF-β2 (4 μg/ml for 2 hours) revealed significant increases in the levels of SRF (A). Panel B shows the time-dependent response of TM cells to TGF-β2 (2 ng/ml) as assessed by Erk activation and SRF expression. Panel C depicts the time-dependent effect of TGF-β2 on fibronectin and αSMA expression. Unlike Erk activation and SRF expression, the protein levels of fibronectin and αSMA were found to exhibit a lag time of 2 hours before increasing significantly. Histograms represent mean of triplicate analyses. Asterisks indicate a significant difference (at minimum of P<0.05) between the treated and untreated cells. β-tubulin was blotted as a loading control.

**Figure 8.** Effects of SRF suppression on the expression of αSMA and fibronectin in human TM cells.

To determine the role of SRF in regulation of expression of αSMA and fibronectin in TM cells, TM cells were transfected with adenoviral vectors expressing SRF shRNA or GFP shRNA for 24 hours and serum starved, prior to analyzing cell lysates for changes in levels of SRF, αSMA, and fibronectin by immunoblot analysis. Panel A shows a dramatic decrease (>90%) in
both SRF expression (RT-PCR analysis-based quantitation of mRNA) and SRF protein levels (immunoblot analysis) in TM cells treated with SRF-specific shRNA compared to a GFP-specific shRNA control. Additionally, the levels of αSMA were found to be significantly reduced in the SRF shRNA treated cells compared to those treated with the GFP shRNA control (B). However, fibronectin protein levels were found to be similar between the SRF shRNA and GFP shRNA treated cells (B). Interestingly, while treating the SRF shRNA expressing cells with either RhoAV14 or with TGF-β2 (4 ng/ml for 2 hours) failed to rescue the decreases in αSMA protein levels, suppressed expression of SRF did not influence fibronectin protein levels induced by both RhoAV14 and TGF-β2 (B). These observations indicate that while the expression of SRF is obligatory for the regulation of αSMA expression, fibronectin expression appears to be regulated independent of SRF. Panel C depicts the effects of Erk inhibition on SRF expression in TGF-β2 treated and untreated TM cells. Human TM cells treated with Erk kinase inhibitor (25 μM U0126 for 4 hours) exhibited significant reductions in SRF protein levels (C), and TGF-β2 (4 ng/ml for 2 hours) failed to rescue the Erk inhibitor-induced decrease in SRF expression indicating that Erk acts upstream of SRF and regulates SRF expression in TM cells. Histograms show significant differences (at minimum of P<0.05) between the treated and untreated controls based on triplicate analyses. β-tubulin was probed to confirm equal loading of protein between the treated and untreated specimens for the immunoblots.

Figure 9. Fibronectin-induced αSMA, SRF expression and Erk activation in HTM cells.

To determine the effects of ECM on the expression of αSMA, HTM cells or HTM cells expressing shGFP or shSRF in serum free medium were plated onto either fibronectin-coated plates or PBS treated plates, and after 8 hours, cell lysates were prepared and examined for
changes in the levels of αSMA, SRF, and phospho-Erk1/2 by immunoblot analyses. A) Showing a representative immunoblot for changes in αSMA, phospho-Erk, and SRF. B) Quantitative immunoblot analysis showing significant increases in the levels of αSMA, which was paralleled by induction of Erk and SRF expression in fibronectin-stimulated HTM cells. shSRF expressing cells either plated on PBS treated or fibronectin-coated plates showed little changes in αSMA, p-Erk and SRF levels. Histograms show significant differences (at minimum of P<0.05) between the fibronectin-treated and PBS-treated controls based on triplicate analyses. β-tubulin was probed to confirm equal loading of protein between the treated and untreated specimens for the immunoblots.

**Figure 10.** Schematic illustration of a potential bidirectional molecular interplay between Rho GTPase regulated contractile activity and ECM-derived mechanical force in trabecular meshwork cells. Rho/Rho kinase signaling and ECM appear to regulate ECM synthesis/assembly and contractile activity through feed forward and back mechanisms, respectively.
Fig. 3

Pattabiraman et al.

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>CTL</th>
<th>C3</th>
<th>C3+LPA</th>
<th>C3+TGFβ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinculin (M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Image of immunofluorescence staining](image)
Fig. 4

A

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>Y27632</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pattabiraman et al.

B

CTRL

Actin/DAPI

Vinculin/DAPI

Y27632

FN/DAPI

CTRL

Y27632
Fig. 5

Pattabiraman et al.

A

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>Rho AV</th>
</tr>
</thead>
<tbody>
<tr>
<td>pErk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Erk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp38 MAPK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSAPK/JNK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>LPA</th>
<th>TGFβ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pErk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Erk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp38 MAPK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>C3</th>
<th>Y27632</th>
</tr>
</thead>
<tbody>
<tr>
<td>pErk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Erk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Fig. 6**

Pattabiraman et al.

**A**

<table>
<thead>
<tr>
<th></th>
<th>CT1</th>
<th>U0126</th>
<th>U0126</th>
<th>U0126</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+LPA</td>
<td>+TGFβ2</td>
<td></td>
</tr>
</tbody>
</table>

- pErk
- Total Erk
- SMA
- FN
- β-Tubulin

**B**

<table>
<thead>
<tr>
<th></th>
<th>CT1</th>
<th>SB220025</th>
<th>GFP + U0126</th>
<th>RhoAV + U0126</th>
</tr>
</thead>
</table>

- SMA
- FN
- β-Tubulin

**C**

<table>
<thead>
<tr>
<th></th>
<th>CT1</th>
<th>U0126</th>
</tr>
</thead>
</table>

- pMLC
- β-Tubulin
Fig. 7  

A

B

C

Pattabiraman et al.
Fig. 9  

Pattabiraman et al.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>shGFP</th>
<th>shSRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pErk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Graph showing relative units for different conditions](graph.png)
Fig. 10  Pattabiraman et al.

![Graph showing signaling pathways and increased ECM and stiffness](image-url)