Myosin-X Silencing in the Trabecular Meshwork Suggests a Role for Tunneling Nanotubes in Outflow Regulation

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PURPOSE. The actin cytoskeleton plays a key role in outflow regulation through the trabecular meshwork (TM). Although actin stress fibers are a target of glaucoma therapies, the role of other actin cellular structures is unclear. Myosin-X (Myo10) is an actin-binding protein that is involved in tunneling nanotube (TNT) and filopodia formation. Here, we inhibited Myo10 pharmacologically or by gene silencing to investigate the role of filopodia/TNTs in the TM.

METHODS. Short hairpin RNA interference (RNAi) silencing lentivirus targeting myosin-X (shMyo10) was generated. Human anterior segments were perfused with shMyo10 or CK-666, an Arp2/3 inhibitor. Confocal microscopy investigated the colocalization of Myo10 with matrix metalloproteinase (MMPs). Western immunoblotting investigated the protein levels of MMPs and extracellular matrix (ECM) proteins. MMP activity and phagocytosis assays were performed.

RESULTS. CK-666 and shMyo10-silencing lentivirus caused a significant reduction in outflow rates in anterior segment perfusion culture, an ex vivo method to study intraocular pressure regulation. In human TM cells, Myo10 colocalized with MMP2, MMP14, and cortactin in podosome-like structures, which function as regions of focal ECM degradation. Furthermore, MMP activity, thrombospondin-1 and SPARC protein levels were significantly reduced in the media of CK-666-treated and shMyo10-silenced TM cells. However, neither Myo10 silencing or CK-666 treatment significantly affected phagocytic uptake.

CONCLUSIONS. Inhibiting filopodia/TNTs caused opposite effects on outflow compared with inhibiting stress fibers. Moreover, Myo10 may also play a role in focal ECM degradation in TM cells. Our results provide additional insight into the function of actin supramolecular assemblies and actin-binding proteins in outflow regulation.

Keywords: trabecular meshwork, actin cytoskeleton, extracellular matrix, aqueous humor dynamics

Intraocular pressure (IOP) is established by building a resistance to aqueous humor outflow in the juxtaocular region of the trabecular meshwork (TM) and inner wall endothelium of Schlemm’s canal (SC). Dysfunction in the aqueous outflow channels causes elevated IOP, which eventually causes glaucomatous damage to the optic nerve and loss of vision. TM cells detect changes in IOP as a mechanical stretch, which activate integrins and induce several intracellular signaling pathways that result in TM cells releasing enzymes, such as matrix metalloproteinases (MMPs) to remodel the extracellular matrix (ECM). This allows greater fluid drainage from the anterior chamber to alleviate elevated IOP.

The actin cytoskeleton of TM cells is also important for IOP regulation. Activity of the small Rho GTPases, Rho, rac1, and cdc42, gives rise to stress fibers, lamellipodia, and filopodia, respectively. Several studies have demonstrated that drugs that either inhibit polymerization of actin into stress fibers or depolymerize actin stress fibers increase outflow facility ex vivo and in vivo. A Rho kinase inhibitor, Netarsudil, has recently been approved as a new drug to reduce elevated IOP in glaucoma patients. Although these studies have elegantly demonstrated the critical role of stress fibers in IOP regulation, the role of other actin-based cellular structures in IOP regulation is largely unknown. Lamellipodia are characteristic of migratory cells, such as cancer cells, and are not typically associated with sedentary TM cells. However, many dynamic filopodia decorate the surface of TM cells.

Filopodia are long, thin protrusions that emanate from the cell surface to probe their mechanical environment. Filopodia contain tight bundles of filamentous (F)-actin and arise from the Arp2/3-generated branched actin networks that lie just beneath the plasma membrane. Two filopodia arising from adjacent cells can fuse to form a connecting tube, which are called tunneling nanotubes (TNTs). These TNTs function in cellular communication and transport a variety of cargoes, including viruses, microRNAs, and cellular organelles such as endosomes, lysosomes, and mitochondria. We recently described TNT formation by TM cells. This direct method of cellular communication overcomes limitations of diffusion-based signaling in the aqueous environment of the anterior chamber. Thus, filopodia have multiple important functions, such as sensing biomechanical signals, acting as sites for signal transduction, as well as communicating signals via the formation of TNTs.

Myosin-X (Myo10) is an unconventional myosin, which functions in filopodia and TNT formation. It comprises an N-terminal head domain, which binds to F-actin and moves cargo, bound to the C-terminal tail domain, toward the tip of...
the filopodia.21 Thus, Myo10 tends to accumulate at the tips of filopodia.22 Myo10 appears to function in filopodia formation because GFP-Myo10 rapidly accumulates at sites of filopodia initiation and overexpression of Myo10 led to a massive increase in the number of dorsal filopodia.20,25 Conversely, RNA interference (RNAi) silencing or knockout of Myo10 drastically reduces the number of filopodia or shortens those filopodia that do form.19,24 Other studies show that Myo10 is essential for TNT formation.19 Myo10 localizes to other cellular structures displaying dynamic actin reorganization, such as invadopodia, podosomes, phagocytic cups, and the leading edges of lamellipodia.25–28 The role of Myo10 in the TM is unknown. However, Myo10 mRNA expression increased in cells subject to cyclical mechanical stress, which mimics IOP fluctuations.29 Myo10 appears to play a role in ECM degradation because there were lower levels of active MMP9 and MMP14 in a Myo10-silenced human breast adenocarcinoma cell line.28,30 Other reports show that Myo10 binds integrins, which may be important for phagocytosis.26,31

In this study, we investigated the function of Myo10 in the TM. We used two methods to inhibit filopodia and TNT formation: (1) CK-666, a reversible Arp2/3 inhibitor, which disrupts formation of branched actin networks from which filopodia arise15,32,33; and (2) generated short hairpin RNAi silencing lentivirus targeting MYO10 (shMyo10). The effects of CK-666 and shMyo10 on outflow rates in anterior segment perfusion culture were measured as well as the role of Myo10 in ECM remodeling and phagocytosis by TM cells.

**METHODS**

**TM Cell Culture**

Human TM cells were established from cadaver tissue and cultured following the guidelines recently described.34 Demographics of the human donors are found in Supplementary Table S1, and Western immunoblots confirming induction of myocilin following 14 days of dexamethasone treatment are shown in Supplementary Figure S1. The following experiments were performed using 3 to 5 different cell strains for each assay.

**Generation of Myo10 Silencing Lentivirus**

Short hairpin RNAi lentivirus targeting Myo10 was generated as previously described.35 Short hairpin RNAs (shRNAs) were designed using the online BLOCK-iT RNAi designer (Thermo-Fisher, Waltham, MA, USA). The 21-base pair (bp) target sequence 5′-GGTTTGTACTCACCACATT-3′ targeted the human Myo10 gene. A shRNA control (shCtrl) was previously characterized.35,36 Hairpin primers were annealed and cloned into the pENTR/U6 vector, which contained a human U6 promoter and an RNA polymerase III terminator, using T4 ligase. After DNA sequencing, shRNA cassettes were transferred into the pLenti6/BLOCK-iT DEST vector by recombination using the Gateway LR clonase II enzyme. Replication incompetent lentivirus was generated in the 293FT cell line by cotransfecting the pLenti expression plasmid with ViraPower packaging mix using Lipofectamine 2000. Lentiviral-containing supernatants were harvested 72 hours posttransfection and centrifuged at 85,000g in a L-80 ultracentrifuge (Beckman Coulter Life Sciences, Indianapolis, IN, USA) with a SW28.1 rotor for 2 hours, and viral titers were measured. Viral supernatants were then aliquoted and stored at −80°C until use.

To quantitate Myo10 gene knockdown, human TM cells or 293 cells were transduced with 105 plaque-forming units (PFUs) of shMyo10 and shCtrl lentivirus for 48 hours. Total RNA was isolated using cells-to-CDNA lysis buffer (Thermo-Fisher) and quantitative RT-PCR was performed using Myo10-specific primers (5′-CGGATGGTGTGGAAACAGTTA-3′ and 5′-CAATTACAGGGCATCCTACAG-3′). Results were normalized to 18S RNA as a housekeeping gene and expressed as fold-change relative to shCtrl-transduced cells. Significance was determined by ANOVA. To analyze Myo10 protein, RIPA lysates of shMyo10-silenced cells were subjected to Western immunoblotting with Myo10 rabbit polyclonal antibody (HPA024223; MilliporeSigma, St Louis, MO, USA). Densitometry (FIJI software; http://fiji.sc/Fiji, provided in the public domain) was used to measure band intensity and determine the percentage knockdown of shMyo10 compared to shCtrl-transduced cells.

**Anterior Segment Perfusion Culture**

The average age of human cadaver eyes used for perfusion experiments was 77.9 ± 7.9 years (range, 59–90) (Supplementary Table S1). Human eyes were dissected and anterior segments were clamped into a perfusion chamber and perfused at constant pressure (8 mm Hg) with serum-free Dulbecco’s Modified Eagles Medium.37 Anterior segments that could not be stabilized were discarded. After overnight stabilization of flow rates, 100 μM CK-666 or 0.04% dimethyl sulfoxide (DMSO) control, or 1 ml (105 PFUs) shMyo10 or shCtrl lentivirus35,36 was added as a bolus at time point 0. Flow rates were measured twice a day for a further 70 to 75 hours. For each individual perfused eye, outflow rates after treatment were normalized to the average flow rate before treatment. Data from multiple eyes were then averaged, and a standard error of the mean was calculated. ANOVA was used to determine significance.

At the end of perfusion, the anterior segments were fixed in 4% paraformaldehyde. The tissue was cut then into approximately 10 to 12 wedges and embedded into paraffin, and 5-μm radial sections were cut at the histopathology core facility (Knight Cancer Institute, Oregon Health & Science University, Portland, OR, USA). After deparaffinization and rehydration, the sections were blocked and stained with rabbit anti-Myo10 polyclonal antibodies. Primary antibodies were detected with Alexa fluor 594-conjugated goat anti-rabbit secondary antibodies (ThermoFisher). Coverslips were mounted in ProLong gold mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI), and sections were imaged on an Olympus Fluoview confocal microscope (Olympus, Waltham, MA, USA). Open-source FIJI software was used to process images postcapture.

**Western Immunoblotting**

Human TM cells (Supplementary Table S1) were grown to confluence, washed with phosphate-buffered saline (PBS), placed into serum-free medium, and treated with 100 μM CK-666 or 0.04% DMSO (vehicle) for 24 hours.32 Serum-free medium was collected, and the total amount of protein in each sample was quantitated using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Equal amounts of total protein were loaded on the gel, and proteins were separated by SDS-PAGE (BioRad Labs, Hercules, CA, USA) under reducing conditions. Primary antibodies were fibronectin (FN) mouse monoclonal (610077, clone 10; BD Biosciences, San Jose, CA, USA), tenasin C (TNC) rabbit polyclonal (AB19011; MilliporeSigma), collagen type I and collagen type IV mouse monoclonal (M-38 and M3F7; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), secreted protein acidic and rich in cysteine (SPARC) rabbit polyclonal (15274-1-AP; Proteintech Group, Rosemont, IL, USA).
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USA), and thrombospondin-1 (TSP-1) mouse monoclonal (MA1-26572, clone A6.1; ThermoFisher). Secondary antibodies were IRDye 700-conjugated anti-rabbit and IRDye 800-conjugated anti-mouse (Rockland Immunochemicals, Gilbertsville, PA, USA). Membranes were imaged on the Odyssey gel imaging system (Licor, Lincoln, NE, USA). The intensity of each band was determined by densitometry using FIJI software, and experimental data are presented as a percentage of the untreated control. Results show the average ± standard error of the mean of four technical replicates using TM cell strains derived from three biological replicates.

Immunofluorescence and Confocal Microscopy

Human TM cells were plated on collagen type I-coated Bioflex 6-well plates (FlexCell International Corp. Burlington, NC, USA) and treated for 24 hours with 100 μM CK-666 or 0.04% DMSO vehicle control.35 Immunofluorescence and confocal microscopy were performed as detailed previously.38,39 Primarily antibodies were rabbit polyclonal anti-myosin-X (PA5-55019, ThermoFisher; or AA90642C, Antibody Verify, Las Vegas, NV, USA), mouse monoclonal anti-MMP2 (MAB3308; MilliporeSigma), mouse monoclonal anti-MMP14 (MAB3319; MilliporeSigma), and mouse anti-cortactin p80/85 (05-180; MilliporeSigma). At least three TM cell strains were used.

MMP Assay

MMP activity was measured using the SensoLyte 520 generic MMP assay kit and the SensoLyte 520 Aggrecanase-1 assay kit (Anaspec, Inc., Fremont, CA, USA).30,31 Briefly, confluent human TM cells were placed in serum-free medium and treated with 100 μM CK-666 or 0.04% DMSO. For shMyo10, TM cells were transduced at the time of plating, grown for 2 hours, and then swapped into serum-free medium. Media and cell lysates were collected 24 hours later. MMPs in each sample were activated with 1 mM 4-aminophenylmercuric acetate for 90 minutes at 37°C were quenched 5-fluoroscein amidite (FAM)-labeled fluorescent substrate. Active MMPs cleaved the quencher, and 5-FAM fluorescence was measured on a plate reader (excitation/emission = 490/520 nm). Relative fluorescence units were normalized to total protein in each sample, as determined using a BCA assay. Each sample was measured in duplicate, then the data from three biological replicates were averaged, and a standard error of the mean was calculated.

Phagocytosis Assay

To measure phagocytosis, pHrodo Staphylococcus aureus fluorescent bioparticles (1 mg/ml; ThermoFisher) were resuspended in PBS and vortexed to disperse, and 50 μl of opsonizing reagent (ThermoFisher) was added. The solution was incubated at 37°C for 1 hour. After washing, the pellet was resuspended in PBS. TM cells were plated in 6-well plates (CytoOne; USA Scientific, Ocala, FL, USA) in the presence of shMyo10 silencing lentivirus for 3 days and placed into fresh medium prior to the start of the experiment. For CK-666, TM cells at 70% confluence were pretreated for 2 hours with 100 μM CK-666 in DMSO. Controls included shCtrl silencing lentivirus, 0.04% DMSO vehicle control, and pretreatment for 30 minutes with 0.78 μM cytochalasin D to disrupt the actin cytoskeleton. Opsonized pHrodo BioParticles (100 μl) were added to each well, and the plate was placed in the Incucyte ZOOM instrument (Essen Bioscience, Ann Arbor, MI, USA). Each well was imaged at 9 points, every 15 minutes for 16 hours using the phase and red fluorescence channels and the 20× objective. At the end of the experiment, the fluorescence intensity at each time point in each well was measured using FIJI. This phagocytosis assay was repeated three times using three biological replicates. Data were combined and averaged and a standard error calculated.

Statistical Analyses

The number of biological and technical replicates are stated in the figure legends. Data are presented as the average ± standard error of the mean. Significance was determined using ANOVA and when needed with Bonferroni correction. P < 0.05 was considered statistically significant.

RESULTS

Transduction of human TM cells with shMyo10 lentivirus showed a 45% reduction in MYO10 mRNA levels (Fig. 1A) and Myo10 protein levels were reduced approximately 25% (Figs. 1B, 1C). By immunofluorescence and confocal microscopy, Myo10 displayed a more punctate distribution pattern in shMyo10-silenced TM cells compared to shCtrl-transduced cells (Figs. 1D, 1E).

CK-666 is a reversible inhibitor of the Arp2/3 complex, which is important for assembly of branched actin networks from which filopodia arise.32 To test the effects of outflow, we applied CK-666 or shMyo10 lentivirus to anterior segment perfusion culture, an established ex vivo method to study IOP regulation.33 Human cadaver anterior segments were perfused with a bolus of 100 μM CK-666 (Fig. 2A). At 2 to 4 hours after CK-666 application, flow rates were significantly reduced by approximately 45% compared to DMSO vehicle controls. Flow rates then recovered toward baseline, followed by another 45% decrease in outflow between 50 and 76 hours after application (Fig. 2A) (P < 0.05). In anterior segments that were perfused with shMyo10 lentivirus, there was an approximately 40% reduction in outflow rates compared to a control lentivirus from approximately 4 hours, which was sustained throughout the perfusion experiment (Fig. 2B) (P < 0.05). Postperfusion, TM tissue sections from all quadrants of each eye were stained with hematoxylin and eosin (Figs. 2C, 2E, 2G) or immunostained with Myo10 (Figs. 2D, 2F, 2H). Hematoxylin and eosin staining showed no discernible differences in tissue architecture or cell number between CK-666-treated, shMyo10-transduced, and their controls. In Myo10-silenced TM, Myo10 protein was decreased as expected and the distribution pattern was altered. A negative control section, where the primary antibody was substituted by PBS, is shown in Supplementary Figure S2.

Studies in other cell types suggest that Myo10 may play a role in ECM degradation. Myo10 is localized to podosomes in osteoclasts and MMP9 mRNA is downregulated, and proteinase activity is reduced when Myo10 is silenced in breast cancer tumors.25,28,30 In TM cells, we previously described cellular structures called podosome and invadosome-like structures, which are regions of a TM cell important for focal ECM degradation.42 Therefore, we compared the cellular localization of Myo10 in regard to MMPs and tested the effects of shMyo10 and CK-666 on MMP activity. We localized Myo10 to ring-like structures that were similar in shape and size to podosome rosettes (5-μm diameter) and podosome clusters (10-15 μm) (Fig. 3A).42,43 To confirm this, we colocalized Myo10 with cortactin antibodies, a biomarker of podosomes (Fig. 3B). Next, we colocalized Myo10 with the MMPs MMP2 and MMP14 (Figs. 3C, 3D). In areas of presumptive podosomes, there was strong colocalization (yellow) of Myo10 with MMP2 (Fig. 3C) and MMP14 (Fig. 3D) whereas there was little colocalization in other cellular areas.
Next, we quantitated MMP activity using a fluorogenic MMP assay using a quenched peptide that generates fluorescence following cleavage of MMPs in the sample. We used a substrate specific for ADAMTS4, as well as a substrate that is cleaved by several MMPs. There was no apparent difference in MMP or ADAMTS4 activities in cell lysates from CK-666-treated or Myo10-silenced TM cells (Fig. 3E). However, MMP activity in TM cell medium was significantly reduced by 25% and 58% by CK-666 and shMyo10, respectively. We also investigated protein levels of MMP2 and MMP14 in TM cell lysates and serum-free medium from TM cells treated for 24 hours with CK-666 or transduced with shMyo10 (Fig. 3F). Densitometry showed a significant ~25% decrease in MMP14 of shMyo10-silenced TM cells and a ~15% decrease in MMP2 levels in CK-666-treated TM cells.

A critical component of ECM remodeling is the synthesis of new ECM to replace the degraded matrix. Therefore, we also investigated the levels of ECM components synthesized by TM cells in response to the treatments. We focused on ECM proteins that either have structural roles (e.g., collagen types I and IV), cell adhesion (e.g., FN), or ECM organizational roles (e.g., TNC, SPARC, and TSP-1). CK-666 was added to TM cells in culture, and serum-free medium was collected 24 hours later. Western immunoblotting and densitometry showed that there was no significant difference in the protein levels for FN, TNC, and collagens type I or type IV (Fig. 4A). However, TSP-1 protein levels were significantly reduced ($P < 0.05$), and SPARC levels were also reduced, although this just missed significance ($P = 0.065$) (Fig. 4B). SPARC was significantly reduced following shMyo10 silencing, whereas TSP-1 was reduced but not significantly (Fig. 4C). Together, these results suggest that inhibition of filopodia/TNTs decreases ECM degradation and alters the composition of the replacement ECM synthesized.

Other studies have shown that Myo10 is localized to phagocytic cups. Because TM cells are phagocytic, we investigated the effects of CK-666 and shMyo10 silencing on phagocytosis by TM cells. To measure phagocytosis, pHrodo S. aureus fluorescent bioparticles were used. These dramatically increase fluorescence when they are ingested and exposed to the intracellular acidic environment of the phagosome. Fluorescence intensity was measured over time for TM cells.
transduced with shMyo10-silencing lentivirus and for TM cells treated with CK-666. There was no significant difference in phagocytic activity of TM cells at any time point with either treatment compared to their respective controls (Figs. 5A, 5B). Representative images of the phagocytosis assay are also shown (Fig. 5C).

**DISCUSSION**

In this study, we investigated the role of the actin-binding protein Myo10 in outflow regulation. Although both CK-666 and Myo10 silencing decreased outflow in perfusion culture, there were differences in the outflow effects caused by the two
treatments. First, the outflow rates took longer to occur in Myo10-silenced cells (e.g., at 4 hours, normalized flow rates were 0.7 and 0.55 l/min in shMyo10-silenced and CK-666-treated anterior segments, respectively). This is likely due to the time that the lentivirus needs to enter the cell and cause an effect compared to the rapid uptake of the reversible CK-666 inhibitor. However, by 75 hours, both treatments led to a similar decrease of 0.6 l/min. Second, CK-666 caused a biphasic response in outflow compared to Myo10 silencing. The initial decrease (0–4 hours) is likely due to a relatively rapid effect of CK-666 on the actin cytoskeleton. Previously, we showed that actin stress fibers appeared thicker in CK-666-treated TM cells. This suggests that CK-666 caused actin to become stabilized into stress fibers, which is consistent with the observed decrease in outflow at 0 to 4 hours. CK-666 is a reversible inhibitor so, not surprisingly, the outflow rates then recovered to near baseline. We hypothesized that the second decrease in outflow was due to ECM remodeling. Alterations to actin cytoskeleton dynamics can be transmitted to the ECM via integrins, which is known as inside-out signaling or, alternatively, can activate signaling pathways in TM cells to trigger...
ECM degradation, synthesis, or reorganization of the ECM components of the outflow resistance. Thus, we tested whether CK-666 affected ECM turnover. MMP activity was significantly reduced in the medium of both shMyo10-silenced and CK-666 treated TM cells. Because TM cells continually release MMPs to remodel ECM in the outflow channels to prevent build-up of cellular debris and ECM fragments, reduced MMP activity may lead to partial obstruction of the outflow channels and the observed decrease in outflow rates in perfusion culture. Finally, we used two different methods targeting two different molecules to inhibit filopodia/TNTs, but it remains possible that Arp2/3 inhibition or Myo10-silencing may produce off-target effects that lead to a decrease in outflow.

Concomitant with altered ECM degradation, we found that SPARC and TSP-1 levels were reduced by CK-666 treatment. Both of these proteins are known to play roles in ECM structural organization. However, in knockout mice, ablation of SPARC and TSP-1 caused lower IOPs (i.e., increased outflow) compared to wild-type littermates. These apparent contradictory results may reflect transient (24 hours) versus long-term (months) effects, species differences, or experimental design. It also remains possible that additional ECM proteins are being affected. However, our results are consistent with rapid effects on the actin cytoskeleton at 0 to 4 hours, followed

**FIGURE 5.** Phagocytosis assay in shMyo-silenced and CK-666-treated TM cells. TM cells were imaged for 16 hours by the Incucyte ZOOM in the presence of opsonized pHrodo Red S. aureus bioparticles. Fluorescence intensity was measured in (A) shMyo10-silenced and (B) CK-666-treated TM cells compared to their relevant controls (n = 27 from n = 3 biological replicates). Error bars are the SEM. (C) Representative images from the phagocytosis assay at 0, 4, 12, and 16 hours after application of the pHrodo bioparticles.
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FIGURE 6. Schematic of the actin cytoskeleton of a TM cell summarizing data found in this study as well as that found in the literature. Inhibiting filopodia/TNTs by shMyo10 silencing lentivirus or CK-666 inhibition of the Arp2/3 complex decreases outflow and reduces vesicle transfer via TNTs. Conversely, relaxing actin stress fibres using Rho kinases inhibitors increases outflow and vesicle transfer via TNTs.15

by reduced ECM degradation via MMPs, and changes to the composition of the replacement ECM at >48 hours in shMyo10-silenced and CK-666-treated TM.

There are contradictory reports on the role of Myo10 in phagocytosis.24,26 The original study described that Myo10 is recruited to phagocytic cups and its inhibition resulted in reduced phagocytosis, whereas, conversely, CK-666 treatment reduced vesicle transfer via TNTs.13

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