Elasticity-Dependent Modulation of TGF-β Responses in Human Trabecular Meshwork Cells

Hong Han, Thomas Wecker, Franz Grehn, and Günther Schlunck

PURPOSE. To gain further insight into a possible role of biomechanical cues in glaucoma, the authors assessed the influence of extracellular matrix (ECM) elasticity on TGF-β2-induced changes in trabecular meshwork (TM) cells.

METHODS. Human TM cells derived from donor cornea rings were plated on rigid collagen-coated tissue culture plastic or polyacrylamide gels of different elasticity and treated with vehicle or TGF-β2. Activation of Smad-2/3, ERK, and AKT signaling and expression of α-SMA and PAI-1 proteins were assessed by Western blot analysis. Subcellular localization of α-SMA was determined by confocal immunofluorescence microscopy. Transcription of collagen-I, -IV, and -VI, α-SMA, PAI-1, fibronectin, fibrillin-1, cochlín, and MGP-1 was studied by RT-qPCR. The contribution of non-Smad signaling pathways to TGF-β-induced expression of α-SMA and PAI-1 expression was assessed using the small molecule inhibitors U0126 and LY294002.

RESULTS. TGF-β2-induced activation of Smad-2/3, ERK, and AKT signaling as well as expression of collagen-I, α-SMA, fibronectin, and MGP-1 were attenuated with increasing elasticity. In contrast, TGF-β2-induced collagen-6, fibronectin, PAI-1, and cochlín expression were enhanced on elastic substrates. The MEK-inhibitor U0126 blocked TGF-β2-induced α-SMA expression, whereas α-SMA expression was enhanced. PI3K inhibition with LY294002 reduced α-SMA expression.

CONCLUSIONS. ECM elasticity modulates TGF-β-induced signaling and protein expression in human TM cells. Non-Smad signaling contributes to TGF-β-induced alterations. Increasing ECM elasticity in vitro promotes protein expression patterns reminiscent of patterns reported in primary open-angle glaucoma. Therefore, changes in TM elasticity and mechanical load may have a significant role in primary open-angle glaucoma.

The trabecular meshwork (TM) constitutes the major regulated ocular outflow pathway in adult human eyes. It is a mechanosensitive structure subjected to dynamic strain by the ciliary muscle, intraocular pressure, and intrinsic contractile elements. Primary open-angle glaucoma (POAG) and aging are associated with structural changes in the TM, which may alter mechanical tissue characteristics and a concomitant increase of aqueous humor outflow resistance. In POAG patients, the severity of optic nerve damage correlates with the extent of extracellular matrix (ECM) deposition in the juxtaocular levels of TGF-β2 have been detected in POAG patients and TGF-β2 induced ECM deposition and increased outflow resistance in human anterior chamber perfusion studies and rodent overexpression models. TGF-β2 was shown to induce TM cell transdifferentiation, as indicated by the robust expression of the myofibroblast marker α-smooth muscle actin and an increased expression of several ECM-related genes. Intraglobular, α-SMA expression is lost with age and in POAG in vivo, despite the presence of active TGF-β2.

Besides cytokines and growth factors, biomechanical cues are increasingly recognized as essential modulators of cell differentiation and function, with decisive roles in development and disease. Furthermore, mounting evidence suggests an essential role of mechanical stimuli acting on TM cells in IOP homeostasis. On the cellular level, mechanical stimuli are translated into biochemical signals in a process termed mechanotransduction, with integrin-dependent cell adhesions and the actin cytoskeleton serving as major force transducers. Substrate elasticity limits the forces exerted by adherent cells in a dynamic equilibrium. Given that focal adhesions increase with mechanical load, substrate elasticity controls adhesion-dependent signals. Thus, ECM elasticity is a basic biomechanical feature that is detected by most cell types, differs from brain to bone, varies with age and disease, and has a strong impact on the actin cytoskeleton. Seminal studies revealed that ECM elasticity governs tissue formation and is sufficient to guide mesenchymal stem cell differentiation. As a consequence, changes in cell and tissue elasticity may be of pathophysiological relevance in POAG.

Here, we studied the impact of ECM elasticity on TGF-β2-induced effects because both appear to have a role in POAG. Our data indicate that ECM elasticity modulates TGF-β2-induced signaling, protein expression, and transcription patterns in human TM cells. In vitro, elastic rather than rigid substrates evoke expression patterns reminiscent of changes described in POAG, suggesting tissue hypercompliance as a contributing factor in POAG pathogenesis.

MATERIALS AND METHODS

Reagents

Antibodies raised against the following proteins were used: α-SMA (Sigma, Taufkirchen, Germany), PAI-1 (R&D Systems, Wiesbaden, Germany), p-AKT (Cell Signaling Technology, Danvers, MA), p-ERK (Promega, Mannheim, Germany), GAPDH (Chemicon/Millipore, Temecula, CA), and p-Smad2/3 (Zymed, South San Francisco, CA). Alexa-488-conjugated goat anti-mouse (Molecular Probes, Eugene, OR), and HRP-
conjugated secondary antibodies (Jackson/Dianova, Hamburg, Germany) were used. Phalloidin-TRITC (Sigma) was used to stain filamentous actin. TGF-β2 was obtained from PeproTech (Rocky Hill, NJ) as a mammalian cell–expressed recombinant human protein and was used at 2 ng/mL in all experiments.

Cell Culture

Human TM tissue was derived from donor corneal rings, and cells were cultivated according to methods published earlier,30,31 with slight modifications. The tenets of the Declaration of Helsinki were followed in all procedures. In brief, donor rings were transferred from the storage medium and kept in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biochrom, Berlin, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from PAA) for 24 hours. Under microscopic guidance, anterior and posterior incisions were placed to isolate the TM, which was then removed using forceps and cut into smaller sections. The tissue sections were placed in 24-well plates, covered with a glass coverslip to avoid floating, and incubated in growth medium (as described). Confluent cell layers were passaged by trypsinization. From the second passage, FCS concentration was lowered to 3%. Cells were characterized by assessing baseline α-B-crystallin expression and increased myocilin expression after 7 days of dexamethasone treatment. The myocilin response to dexamethasone treatment has been reported as specific for TM cells.52-55 Cells from three different donors were used from passages 3 to 10. All experiments were performed at least three times with similar results.

Flexible Substrates

Polyacrylamide substrates were prepared as described.74-75 The gels were composed (final concentrations) of 7% acrylamide, 3% acrylamidodipropyltrimethylammonium-chloride, 0.2% or 0.8% bisacrylamide to achieve high or moderate gel elasticity, respectively, 0.1% ammonium-umlersulfate, and 0.2% N,N,N',N'-Tetramethylmethylendiamine (all Sigma) in PBS. The charged trimethylammonium-chloride compound allows for ECM binding. For immunofluorescent stains, 24-mm round coverslips were coated with amino-silane (Sigma) for 2 minutes, air-dried, and washed in water, activated with 0.5% glutaraldehyde (Roth, Karlsruhe, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from PAA) for 24 hours. Under microscopic guidance, anterior and posterior incisions were placed to isolate the TM, which was then removed using forceps and cut into smaller sections. The tissue sections were placed in 24-well plates, covered with a glass coverslip to avoid floating, and incubated in growth medium (as described). Confluent cell layers were passaged by trypsinization. From the second passage, FCS concentration was lowered to 3%. Cells were characterized by assessing baseline α-B-crystallin expression and increased myocilin expression after 7 days of dexamethasone treatment. The myocilin response to dexamethasone treatment has been reported as specific for TM cells.52-55 Cells from three different donors were used from passages 3 to 10. All experiments were performed at least three times with similar results.

Western Blot Analysis

Cells plated on collagen-coated tissue plastic or polyacrylamide gels (as described) were used for Western blot analysis. Cells were rinsed with ice-cold PBS, and total cell protein extracts were prepared using a RIPA lysis buffer (20 mM Tris, 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing phosphatase and protease inhibitors (Phosphatase Inhibitor Cocktail III [Calbiochem/Merck, Bad Soden, Germany] and Complete Protease Inhibitor [Roche, Mannheim, Germany]). Protein extracts were boiled in Laemmli sample buffer and subjected to SDS-PAGE. Proteins were transferred onto a PVDF membrane (Amersham, Braunschweig, Germany) using a Bio-Rad gel-blotting apparatus. Membranes were blocked in 3% BSA in TBST (10 mM Tris HCl, 150 mM NaCl, 0.1% Tween 20) for 1 hour. Membranes were incubated with primary antibody overnight at 4°C and with a peroxidase-conjugated secondary antibody for 60 minutes at room temperature. After each incubation step, membranes were washed in TBST for 30 minutes. Peroxidase was visualized by enhanced chemiluminescence and exposure to enhanced chemiluminescence film (Hyperfilm ECL; both Amersham, Braunschweig, Germany) for appropriate times.

Immunofluorescence Confocal Microscopy

Cells plated on collagen-coated glass coverslips or polyacrylamide gels were fixed in 2% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked in 2% normal goat serum (Jackson-Immuno, Hamburg, Germany), labeled with primary antibodies against α-SMA (1:500) in blocking buffer at 4°C overnight, and washed in PBS. Alexa-488–conjugated secondary antibodies against mouse IgG were used at a dilution of 1:500 for 1 hour at room temperature in blocking buffer. Phalloidin-TRITC (Sigma) was used to counterstain the F-actin cytoskeleton (1 hour, room temperature), and, after washing in PBS, the samples were mounted in mounting medium containing DAPI (Vectorshield; Vector Laboratories, Burlingame, CA). Cells were viewed with a laser scanning confocal microscope (TCS SP-2, Leica Microsystems, Bensheim, Germany).

RT-qPCR

Cells plated on collagen-coated tissue plastic or polyacrylamide gels (as described) were rinsed with PBS, gently scraped from the substrate, and collected by centrifugation. The cell pellet was then processed using the RNeasy kit (Qiagen, Hilden, Germany), as recommended by the manufacturer. Two micrograms of extracted RNA were reverse transcribed (Superscript II; Qiagen) using oligo-dT primers. cDNA was processed using the RNeasy kit (Qiagen, Hilden, Germany), as recommended by the manufacturer. Two micrograms of extracted RNA were reverse transcribed (Superscript II; Qiagen) using oligo-dT primers (Promega). Primer pairs (Table 1) were designed using Primer 3 software26 (http://frodo.wi.mit.edu/primer3/input.htm). A commercially available kit (SYBR Premix Ex Taq II; Takara Bio Inc., Otsu, Japan) was used for SYBR green–monitored real-time PCR amplification performed in triplicate (Step One Plus; Applied Biosystems, Foster City, CA). Enzyme activation (95°C, 20 seconds) was followed by 40 cycles of denaturation (95°C, 5 seconds), primer annealing (Table 1, 10 sec-

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<th>Transcript</th>
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<th>Annealing Temperature (°C)</th>
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Table 1. Primer Sequences

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Matrix Elasticity Modulates TGF-β Responses

Extracellular Matrix Elasticity Modulates TGF-β–Induced Protein Expression

Previous experiments revealed an elasticity dependence of baseline protein expression in TM cells.29 Because substrate elasticity altered TGF-β-induced signals, we were compelled to study its influence on TGF-β–induced protein expression. TM cells were plated on regular tissue culture plastic (TCP), a more rigid (0.8% bis-acrylamide) or elastic (0.2% bis-acrylamide) polyacrylamide gel, allowed to adjust for 7 days, treated with vehicle or TGF-β2 for 2 days, harvested, and assessed by Western blot analysis. TGF-β2–induced expression of PAI-1 was elasticity dependent, revealing the strongest signal on the more elastic 0.2% bisacrylamide gel (Fig. 2), whereas α-SMA expression was weakest on this substrate. For both proteins, less elastic 0.8% polyacrylamide gels revealed intermediate responses compared with either highly elastic 0.2% polyacrylamide gels or rigid tissue culture plastic.

Extracellular Matrix Elasticity Modulates TGF-β–Induced Cytoskeletal Incorporation of α-SMA

TGF-β has been shown to enhance actin stress fibers and to induce α-SMA expression.13 To gain insight into the influence of ECM elasticity on these TGF-β–induced morphologic changes, TM cells were plated on elastic substrates or glass coverslips, allowed to adjust for 7 days, treated with TGF-β2 or vehicle control for 3 days, and processed for confocal immunofluorescence microscopy. As reported previously, baseline actin stress fibers were abundant on rigid substrates and declined with increasing elasticity.29 (Fig. 3). On rigid glass cov-

RESULTS

Extracellular Matrix Elasticity Modulates TGF-β Signaling

To assess the effect of ECM elasticity on TGF-β–induced signal transduction, TM cells were plated on more rigid (0.8% bis-acrylamide) or elastic (0.2% bis-acrylamide) polyacrylamide gels, allowed to adjust for 7 days, and subsequently challenged with TGF-β2 for different times. On both substrates, TGF-β2 activated Smad2/3, ERK, and AKT signaling (Fig. 1). Activation onset times and peak phosphorylation levels were rather similar on both substrates, but the signals were more persistent on the substrate of higher rigidity (0.8% bis-acrylamide; Fig. 1). A strong Smad activation at 1 hour preceded ERK and AKT signaling, which were both detected from 6 hours and peaked at 12 hours after stimulation but wore off thereafter on the 0.2% bis-acrylamide gel, whereas signals remained strong on the 0.8% bis-acrylamide gel.
erslips, TGF-β increased actin stress fibers and induced abundant incorporation of α-SMA. Elastic substrates blocked TGF-β-induced α-SMA expression and incorporation into stress fibers in an elasticity-dependent fashion. The smallest effect of TGF-β on α-SMA expression was seen on the most elastic 0.2% bis-acrylamide polyacrylamide gels.

**Extracellular Matrix Elasticity Modulates TGF-β–Induced Transcription Patterns**

Next, we were interested in studying the effect of ECM elasticity on the gene transcription of proteins whose expression levels were reported to change with POAG. TGF-β induced the transcription of α-SMA, PAI-1, fibronectin, fibulin-1, cochlin, and collagens-1, -4, and -6, whereas MGP-1 was suppressed (Fig. 4). Maximal transcript levels for PAI-1, fibronectin, cochlin, and collagen-6 were induced on the most elastic substrate (0.2% bis-acrylamide). In contrast, the transcription of α-SMA, fibulin, MGP-1, and collagen-1 genes was strongest on the most rigid substrate (tissue culture plastic). Except for collagen-4 transcripts, which were most abundant with intermediate substrate elasticity, all others showed linear elasticity dose dependence.

**Non-Smad Signaling Mediates TGF-β–Induced Protein Expression**

To explore the contribution of non-Smad signaling to the expression of the TGF-β-induced proteins α-SMA and PAI-1, we performed stimulation experiments in the presence or absence of kinase inhibitors. Baseline activation levels of AKT and ERK were lower on highly elastic (0.2% bis-acrylamide) than on rigid (0.8% bis-acrylamide) polyacrylamide gels (Fig. 5). Inhibition of MEK-1 and -2 using U0126 (10 μM) blocked ERK phosphorylation, enhanced α-SMA protein expression, and slightly inhibited PAI-1 expression. Inhibition of PI3K by LY294002 (20 μM) sufficiently blocked AKT phosphorylation and abrogated α-SMA expression with little impact on PAI-1 (Fig. 5B).

**DISCUSSION**

Biomechanical cues and tissue compliance gain increasing attention as possible modifiers of POAG pathogenesis. However, a general concept is not yet established. TM cells interact with the ECM to maintain tissue structure and aqueous outflow. Several studies suggest that TGF-β2 modifies TM cell-matrix interactions in POAG, leading to increased ECM deposition and elevated outflow resistance. TGF-β-induced remodeling may alter tissue rigidity with functional implications for the TM as a mechanosensitive structure. In previous experiments, we had observed a strong impact of ECM elasticity on TM cell protein expression and function. We now provide evidence for ECM elasticity-dependent modulation of TGF-β2-induced effects in TM cells. Furthermore, an increase in substratum compliance appears to support shifts in protein expression patterns reminiscent of changes observed in POAG.
TGF-β2 activated the classical Smad-2/3 and non-Smad signaling pathways in TM cells. When cells were plated on highly elastic (0.2% bis-acrylamide cross-linker) or more rigid (0.8% bis-acrylamide) polyacrylamide gels rather than on rigid tissue culture plastic, the persistence of TGF-β-induced Smad-2/3, ERK, and AKT phosphorylation was dependent on ECM elasticity and more sustained on the rigid substrate (Fig. 1). Cells sense ECM using integrins, which also serve as mechanotransducers, and it has been shown that integrin-dependent cell adhesion governs growth factor-induced ERK signal persistence to modulate subsequent responses. Integrins also link matrix elasticity to ERK activation, further supporting our results.

Next, we studied the expression of PAI-1 and α-SMA to assess whether ECM elasticity-dependent changes in TGF-β-induced signals are associated with altered protein expression patterns. PAI-1 mitigates protease activation and may contribute to aberrant ECM deposition in glaucomatous TM. α-SMA increases cellular contractile force generation and is expressed in juvenile TM. Our experiments revealed that expression of both proteins is modulated by matrix elasticity. TGF-β-induced PAI-1 expression increased with substrate elasticity, whereas α-SMA expression declined (Fig. 2). In support of these data, TGF-β-induced cell transdifferentiation and PAI-1 expression were shown to be dependent on integrin-mediated cell adhesion, suggesting a mechanism for elasticity-dependent modulation. We had previously observed an effect of ECM elasticity on baseline α-SMA expression, but no respective data had been available on TGF-β-induced changes or PAI-1 expression. TGF-β2 is known to induce α-SMA in TM cells and the fact that TM α-SMA expression is lost with age and in POAG despite the presence of active TGF-β posed an intriguing conundrum. Our data suggest the possibility that changes in TM elasticity could account for these observations. By the same token, increased tissue elasticity might enhance the expression of PAI-1, which has been detected in increased levels in the aqueous humor of POAG patients.

As another important feature, heterogeneous alterations of the TM cell actin cytoskeleton have been reported in POAG with tangled cross-linked actin networks and punctate actin concentrations. To examine the influence of ECM elasticity on TGF-β-induced changes in cytoskeletal morphology, cells were plated on glass coverslips or polyacrylamide gels of different elasticity, stimulated with vehicle or TGF-β2, and processed for confocal immunofluorescence microscopy. TGF-β-induced α-SMA expression and its incorporation into stress fibers were attenuated with increasing substrate elasticity (Fig. 3), but an increase in F-actin stress fibers from baseline was detected on all substrates. This result is in line with the observation that α-SMA expression is regulated in a mechanosensitive manner. Our data indicate that TGF-β can induce actin tangles even on soft substrates, albeit to a lesser extent than on rigid glass coverslips. Therefore, a soft matrix would allow for...
TGF-β-induced changes in F-actin with little concomitant α-SMA expression, similar to the findings in POAG eyes.\textsuperscript{15,28}

Several changes in protein expression and transcription have been characterized in the TM of POAG patients and in POAG models. Collagen-VI was found associated with sheath-derived plaques in the cribriform meshwork, which are enhanced with age and even more so in POAG.\textsuperscript{4,49} In normal eyes, collagen-I was detected in trabecular core ground substance and collagen-IV in TM cell basement membranes and in the juxtacanalicular region close to Schlemm’s canal endothelial cells.\textsuperscript{50,51} Similarly, increased deposition of collagen-VI and -IV was detected in the lamina cribrosa of POAG eyes.\textsuperscript{52} TGF-β2-induced collagen-I and -IV expression has been reported in TM cells.\textsuperscript{53} Our data indicate that the collagen transcription pattern in TM cells depends on matrix elasticity (Fig. 4A). Compliant substrates strongly promoted baseline and TGF-β-induced transcription of collagen-VI and, to a lesser extent, collagen-IV but diminished TGF-β-induced collagen-I transcription. Thus, elastic ECM induces a collagen expression pattern in vitro that is reminiscent of changes reported in POAG.

TGF-β-induced transcription of PAI-1 and fibronectin was also enhanced on soft polyacrylamide gels (Fig. 4B). Both proteins were associated with glaucomatous changes in earlier studies.\textsuperscript{54,55}

Cochlin is an ECM protein expressed mainly in the inner ear but also in the eye, which modulates cell adhesion. It is induced by TGF-β2 in an anterior chamber perfusion model, it increases outflow resistance when overexpressed,\textsuperscript{56–58} and cochlin deposits were found in glaucomatous TM.\textsuperscript{59} TM cells plated on elastic substrates revealed increased baseline and TGF-β-induced cochlin transcription (Fig. 4B), again pointing toward matrix elasticity as an essential modulator.

However, increased transcription was not a general phenomenon in TM cells plated on elastic substrates. Transcription levels of α-SMA, fibulin-1, and matrix-GLA protein-1 (MGP-1) were reduced with increasing elasticity (Fig. 4B). This corroborated our findings for α-SMA protein expression (Fig. 2). Moreover, decreased levels of fibulin-1 and MGP-1 have been found in the context of glaucoma. Fibulin-1 was shown to suppress fibronectin-regulated cell adhesion, migration,\textsuperscript{58} and cell-mediated contraction.\textsuperscript{59} In lamina cribrosa cells derived from POAG patients, fibulin-1 transcription was downregulated compared with cells from healthy donors.\textsuperscript{60} MGP-1 inhibits tissue calcification and is highly expressed in the TM of healthy eyes, induced by IOP elevation, and downregulated by TGF-β1.\textsuperscript{61} In glaucomatous tissue, MGP-1 gene expression is reduced.\textsuperscript{62} In line with these observations, our data indicate that a loss of matrix rigidity attenuates MGP-1 transcription. Interestingly, ECM compliance was recently shown to regulate osteogenesis by modulating ERK activity,\textsuperscript{63} further supporting our results.

To examine the contribution of non-Smad signaling pathways to TGF-β-induced protein expression, we used specific inhibitors for MEK1/2 (U0126) or PI3K (LY294002). Inhibition of the PI3K-AKT pathway blocked α-SMA expression with little impact on PAI-1 (Figs. 5A, 5B). In contrast, inhibition of the MEK-ERK pathway enhanced baseline and TGF-β-induced α-SMA expression but attenuated PAI-1 expression (Figs. 5A, 5B). This indicated that TGF-β-induced PAI-1 expression requires ERK signaling, which is supported by earlier studies in renal epithelial cells.\textsuperscript{46} Regulation of α-SMA appears to be more complex. Isolated inhibition of MEK-ERK signaling with preserved AKT activation boosted α-SMA. Attenuation of both signaling pathways by increased substrate elasticity led to a decline in α-SMA levels. These data suggest that α-SMA expression may depend on the balance of countering AKT and ERK signals. Thus, non-Smad signaling clearly has a role in TGF-β-induced TM cell alterations.

In summary, our data establish that an increase in substratum compliance supports protein expression patterns reminiscent of glaucoma in TM cells. This finding is unexpected given reports of increasing scleral rigidity with age.\textsuperscript{64,65} However, the multitude of proteins affected and the fact that distinct proteins were upregulated or downregulated in a consistent manner suggest a valid regulatory system. Interestingly, not all ocular tissues change elasticity with age in a similar fashion.\textsuperscript{46} and recent observations support the idea that tissue hypercompliance may occur in POAG: Corneal hysteresis is lower in glaucomatous eyes than in healthy eyes\textsuperscript{66–68} and even negatively correlates with disease progression.\textsuperscript{69} Furthermore, reduced scleral rigidity is predicted to significantly increase the IOP-dependent strain at the optic nerve head,\textsuperscript{70} and lamina cribrosa hypercompliance was detected in an early glaucoma animal model.\textsuperscript{71}

Trabecular meshwork compliance depends on tissue composition and structure as well as on prestress exerted by the ciliary muscle. As prestress is lost in excised tissue, ex vivo compliance measurements deserve cautious consideration. The effect of ciliary muscle contraction on tissue shape and outflow resistance declines with age and presbyopia.\textsuperscript{58} These facts and our findings suggest the hypothesis that TM hypercompliance due to a loss of prestress and a subsequent TGF-β-driven repair response may have a role in POAG.

### References

14. Zhao X, Ramsey KE, Stephan DA, Russell P. Gene and protein expression changes in human trabecular meshwork cells treated...


