Elevated Hydrostatic Pressure Selectively Enhances Matricellular Gene Expression in Human Trabecular Meshwork Cells

Jonathan Lautz
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Part of the Neurosciences Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu. Copyright © 2017 Jonathan Lautz
LOYOLA UNIVERSITY CHICAGO

ELEVATED HYDROSTATIC PRESSURE SELECTIVELY ENHANCES MATRICELLULAR GENE EXPRESSION IN HUMAN TRABECULAR MESHWORK CELLS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

THE DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

JONATHAN D. LAUTZ

CHICAGO, IL

DECEMBER 2017
© Copyright by Jonathan Lautz, 2017

All rights reserved.
ACKNOWLEDGMENTS

I would first and foremost like to thank my mentor, Dr. Evan Stubbs Jr. You taught me the right way to ask questions, gave me the freedom to explore my scientific curiosity and challenged me to become a better scientist. You have been a fantastic mentor and I have truly enjoyed my time in your laboratory. I would also like to thank Dr. Langert for her daily advice and keeping me sane throughout grad school. My thanks to Drs. Ton, Shephard, and Vaagenes. Daily coffee kept me awake, productive, and laughing. I would also like to thank Dr. Kartje for the opportunity to contribute to work from her lab as well as her time, effort, and mentorship as a member of my committee. To my entire committee, thank you for your guidance, time, and most of all, patience throughout this process.

I would also like to thank my family for the enormous amount of support they have provided throughout the last 6 years. I would especially like to thank both of my parents. Dad, I love our random lunches. When I’m willing to listen, you always teach me so much. You are a fantastic father and role model. Mom, thank you for always pushing me, especially when I don’t want you to. You have taught me how to be a strong, compassionate and kind person. I know I would not be where I am today without both of you. Finally, I would like to than my amazing girlfriend Kate. Thank you for spending so much time this last year on airplanes for me. Thank you for all of the encouragement and thank you for always be so sweet and thoughtful.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................... iii

LIST OF TABLES .................................................................................................................. vii

LIST OF FIGURES ............................................................................................................... viii

LIST OF ABBREVIATIONS ................................................................................................... x

ABSTRACT ............................................................................................................................... xiv

## CHAPTER 1: LITERATURE REVIEW

- Primary Open-Angle Glaucoma ......................................................................................... 1
- The Visual Pathway .............................................................................................................. 2
- Trabecular Meshwork and Intraocular Pressure ................................................................. 4
- Therapeutic Management of POAG ................................................................................... 7
- The Extracellular Matrix of the TM .................................................................................. 8
- Transforming Growth Factor-β2 and POAG ..................................................................... 10
- Non-Canonical Rho GTPase Signaling in POAG ............................................................... 14
- Endothelin-1 Mediated Vasoconstriction in POAG ............................................................ 15
- Connective Tissue Growth Factor Mediates TGF-β2 Remodeling of the ECM .......... 17
- TGF-β2 Mediated Perturbation of the Kallikrein-Kinin System in POAG .......... 19
- Mechatosensation within the TM .................................................................................... 23

## CHAPTER 2: MATERIALS AND METHODS

- Human Trabecular Meshwork Culture ........................................................................... 30
- Experimental Conditions ................................................................................................. 31
- siRNA-Targeted Knockdown ............................................................................................ 33
- Real-Time PCR ................................................................................................................ 33
- Western Immunoblot ........................................................................................................ 34
- Cell-Based ELISA ............................................................................................................ 35
- Enzyme Linked Immunosorbent Assay .......................................................................... 36
- Lactate Dehydrogenase Assay ......................................................................................... 36
- Cell Viability .................................................................................................................... 37
- Immunocytochemistry ..................................................................................................... 37
- Porcine Anterior Segment Perfusion .............................................................................. 38

## CHAPTER 3: RESULTS

- TGF-β2 Selectively Alters Matricellular and ECM Gene Expression in Primary and Transformed Human TM Cells .................................................................................. 40
- TGF-β2 Attenuates Constitutive Bradykinin Receptor Expression in Primary and Transformed Human TM Cells ................................................................................... 42
- TGF-β2 Utilizes the Canonical Smad3-dependent Pathway to Attenuate B2 Receptor Expression .............................................................................................................. 44
- Elevated Hydrostatic Pressure Induces Cell Stress in Human TM Cells .................. 48
Elevated Hydrostatic Pressure Alters the Actin Cytoskeleton in Human TM Cells...51
Elevated Hydrostatic Pressure Selectively Alters Gene Expression in Human TM Cells ................................................................................................................................................53
Exposure to Clinically Relevant Hydrostatic Pressure Loads Increase ET-1, TGF-β2, and CTGF Expression as well as ET-1 Release.................................................................................................................................62
Inhibition of ETβ Receptor Activation Prevents Elevated Hydrostatic Pressure Mediated Increases in TGF-β2 and ET-1, but not CTGF, mRNA Expression.....65
Canonical TGF-β2 Signaling is not Altered by Elevated Hydrostatic Pressure ........67
Elevated Hydrostatic Pressure Increases CTGF, TGF-β2, and ppET-1 mRNA Expression Independent of TRPV Mechanosensitive Ion Channels .........................69
Elevated Hydrostatic Pressure Induces Cell Stress Independent of Oxidative Stress..................................................................................................................................................70
Physiological Consequences of Increased Perfusion Flow Rate on TM Tissue in situ.........................................................................................................................................................74

CHAPTER 4: DISCUSSION
TGF-β2 Alters Gene Expression in TM Cells .................................................................78
The Therapeutic Potential of Targeting TGF-β2-mediated Signaling .........................82
Elevated Hydrostatic Pressure Induces Cell Stress and Apoptosis in TM Cells ..........84
Elevated Hydrostatic Pressure Alters the Actin Cytoskeleton in TM Cells ...............85
Elevated Hydrostatic Pressure Elicits Selective Changes in Gene Expression in TM cells ..................................................................................................................................................87
Elevated Hydrostatic Pressure Elicits a Sustained Increase in CTGF mRNA Content and Protein Secretion ...........................................................................................................................................89
Elevated Hydrostatic Pressure Transiently Increases TGF-β2 Content..................90
ET-1 Synthesis and Secretion is Enhanced in Response to Elevated Hydrostatic Pressure ............................................................................................................................................90
Elevated Hydrostatic Pressure Elicits Altered Expression of Proteases in Primary TM Cells ...........................................................................................................................................91
TM Cells Respond to Elevated Hydrostatic Pressure by Increasing Bradykinin B1 Receptor mRNA Expression ............................................................................................................................................92
TM Cells are Sensitive to Clinically Relevant Changes in Hydrostatic Pressure......93
The Role of the ETβ Receptor in Elevated Hydrostatic Pressure Mediated Changes in Gene Transcription.............................................................................................................................................94
Elevated Hydrostatic Pressure Does not alter TGF-β Signaling in TM Cells ........96
The Role of Reactive Oxygen Species in Cellular Responses to Elevated Hydrostatic Pressure .............................................................................................................................................97
TM Cells Respond to Elevated Hydrostatic Pressure by a Mechanism Independent of TRPV Channel Activation.............................................................................................................................................98
Incorporating Hydrostatic Pressure into the Tensegrity Model of Mechanosensation............................................................................................................................................99
The Physiological Consequences of Increased Perfusion Flow Rate on the TM .....101
Summary and Conclusion ..................................................................................................................102

BIBLIOGRAPHY .................................................................................................................................105
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>PCR Primers</td>
</tr>
<tr>
<td>Table 2.</td>
<td>Primary Antibodies</td>
</tr>
<tr>
<td>Table 3.</td>
<td>TGF-β2 alters gene expression in human TM cells</td>
</tr>
<tr>
<td>Table 4.</td>
<td>TGF-β2 attenuates B2 receptor expression independently of Rho GTPase signaling</td>
</tr>
<tr>
<td>Table 5.</td>
<td>Elevated hydrostatic pressure elicits selective changes in the expression of genes relevant to ECM remodeling</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>The Prevalence of Glaucoma</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Schematic of the visual pathway</td>
<td>3</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Aqueous Humor Outflow</td>
<td>5</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Canonical TGF-β2 Signaling</td>
<td>12</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Rho Mediated Signaling</td>
<td>15</td>
</tr>
<tr>
<td>Figure 6</td>
<td>The Kallikrein-Kinin System</td>
<td>20</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Hydrostatic Pressure Chamber</td>
<td>32</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Porcine Anterior Segment Perfusion</td>
<td>39</td>
</tr>
<tr>
<td>Figure 9</td>
<td>TGF-β2 decreases constitutive expression of B2 receptor protein</td>
<td>43</td>
</tr>
<tr>
<td>Figure 10</td>
<td>TGF-β2 mediated attenuation of the B2 receptor selectively involves the</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>canonical Smad3-dependent pathway</td>
<td></td>
</tr>
<tr>
<td>Figure 11</td>
<td>TGF-β2 attenuates receptor B2 mRNA independently of mRNA stability</td>
<td>48</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Elevated hydrostatic pressure induces cell stress in transformed TM cells</td>
<td>50</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Elevated hydrostatic pressure alters the actin cytoskeleton in transformed</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>TM cells</td>
<td></td>
</tr>
<tr>
<td>Figure 14</td>
<td>Elevated hydrostatic pressure elicits selective changes in gene</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>expression</td>
<td></td>
</tr>
<tr>
<td>Figure 15</td>
<td>Elevated hydrostatic pressure increases CTGF mRNA content and</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Protein secretion</td>
<td></td>
</tr>
<tr>
<td>Figure 16</td>
<td>Elevated hydrostatic pressure increases TGF-β2 mRNA content and</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Protein secretion</td>
<td></td>
</tr>
</tbody>
</table>
Figure 17. Elevated hydrostatic pressure transiently enhances ppET-1 mRNA and secreted ET-1 content.................................................................57

Figure 18. Elevated hydrostatic pressure induces time-dependent changes in gene expression.................................................................59

Figure 19. Extracellular matrix mRNA expression is altered in response to elevated hydrostatic pressure .........................................................61

Figure 20. Clinically relevant changes in hydrostatic pressures alter matricellular expression and ET-1 release .................................................................64

Figure 21. Inhibition of ET_B receptor activation prevents hydrostatic pressure mediated changes in TGF-β2 and ppET-1 mRNA content........66

Figure 22. Elevated hydrostatic pressure does not alter canonical TGF-β2 signaling in human TM cells.................................................................68

Figure 23. Elevated hydrostatic pressure increases CTGF, TGF-β2, and ppET-1 mRNA expression independent of TRPV channel activation............71

Figure 24. Elevated hydrostatic pressure induces cell stress independent of oxidative stress.................................................................73

Figure 25. Physiological consequences of increased perfusion flow rate on TM tissue in situ .................................................................75

Figure 26. Mechanism by which TGF-β2 elevates IOP and subsequent TM cellular responses .................................................................103
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH</td>
<td>Aqueous humor</td>
</tr>
<tr>
<td>AKT</td>
<td>Activated protein kinase</td>
</tr>
<tr>
<td>B2M</td>
<td>Beta-2 microglobulin</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCN</td>
<td>CTGF, CYR61, Nov</td>
</tr>
<tr>
<td>CDKN</td>
<td>Cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>Col</td>
<td>Collagen</td>
</tr>
<tr>
<td>CLANs</td>
<td>Cross-linked actin networks</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ECE</td>
<td>Endothelin converting enzyme</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ET&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Endothelin A</td>
</tr>
<tr>
<td>ET&lt;sub&gt;B&lt;/sub&gt;</td>
<td>Endothelin B</td>
</tr>
<tr>
<td>EZR</td>
<td>Ezrin</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
</tbody>
</table>
FBN  Fibrillin
FN   Fibronectin
G-actin Globular actin
GAGs Glycosaminoglycans
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GDP  Guanine diphosphate
GTM3 Glaucomatous trabecular meshwork (cell line) 3
GTP  Guanine triphosphate
HB-EGF Heparin binding EGF-like growth factor
HDAC Histone deacetylase
ICAM-1 Intercellular adhesion molecule 1
ICP  Intracranial pressure
IGF  Insulin growth factor
IGFR Insulin growth factor receptor
IL   Interleukin
IOP  Intraocular pressure
IP$_3$ inositol 1,4,5-trisphosphate
ITGB Integrin
JCT  Juxtacanicular tissue
JNK  c-jun n-terminal
KLK  Kallikrein
LAMC2 Laminin, subunit gamma 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRTF</td>
<td>Myocardin-related transcription factor</td>
</tr>
<tr>
<td>MSN</td>
<td>Moesin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NTG</td>
<td>Normal-tension glaucoma</td>
</tr>
<tr>
<td>NTM5</td>
<td>Normal trabecular meshwork (cell line) 5</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy</td>
</tr>
<tr>
<td>PA</td>
<td>Plasminogen activator</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PFX</td>
<td>Pseudoexfoliation</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphoinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PLAT</td>
<td>Plasminogen activator tissue type</td>
</tr>
<tr>
<td>PLAU</td>
<td>Plasminogen activator urokinase</td>
</tr>
<tr>
<td>PLAUR</td>
<td>Plasminogen activator urokinase receptor</td>
</tr>
<tr>
<td>PLG</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>POAG</td>
<td>Primary open-angle glaucoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>POSTN</td>
<td>Periostin</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>R-smad</td>
<td>Receptor associated smad</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal ganglion cell</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBEs</td>
<td>Smad binding elements</td>
</tr>
<tr>
<td>SC</td>
<td>Schlemm’s canal</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and cysteine rich</td>
</tr>
<tr>
<td>TβR</td>
<td>Transforming growth factor-β receptor</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>THBS</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TM</td>
<td>Trabecular meshwork</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid</td>
</tr>
<tr>
<td>TPG</td>
<td>Translaminar pressure gradient</td>
</tr>
<tr>
<td>VCAN</td>
<td>Versican</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor-A</td>
</tr>
<tr>
<td>VTN</td>
<td>Vitronectin</td>
</tr>
</tbody>
</table>
ABSTRACT

Primary open-angle glaucoma (POAG) is a progressive optic neuropathy characterized by loss of peripheral vision secondarily associated with elevated intraocular pressure (IOP). Transforming growth factor (TGF)-β2 is markedly elevated in the aqueous humor (AH) of patients with POAG. It has been previously shown that TGF-β2 increases IOP, in part, by inducing expression and release of endothelin-1 (ET-1) and connective tissue growth factor (CTGF) within the trabecular meshwork (TM). ET-1 and CTGF may ultimately lead to increases in IOP by enhancing TM cell contractility and extracellular matrix (ECM) deposition. Here we confirm that TGF-β2 enhances mRNA content of ET-1, CTGF, and various ECM components in cultured human TM cells. In addition, we demonstrate a dose- and time-dependent decrease in the bradykinin B2 receptor expression in response to exogenous TGF-β2. This finding represents a novel mechanism by which TGF-β2 may elevate IOP through perturbation of the kallikrein-kinin system. Despite these advancements, the direct effect of elevated IOP on TM cell responsiveness remains unknown.

To accurately model and characterize the effect of elevated IOP on the TM in vitro, we designed and built a custom hydrostatic pressure chamber. Cultured human TM cells exposed to elevated hydrostatic pressure exhibit decreased viability, accumulation of F-actin, and selective changes in the expression of matricellular and structural ECM genes associated with the pathogenesis of POAG including TGF-β2, ET-1, and CTGF.
Furthermore, secreted ET-1, in response to elevated hydrostatic pressure, acts in an autocrine manner through the ET_{B} receptor to enhance TGF-β2 and ppET-1 mRNA, but not CTGF content. The mechanism(s) by which TM cells sense and respond to elevated hydrostatic pressure remain unknown, but findings presented here suggest that neither activation of mechanosensitive TRPV channels nor oxidative stress is involved. Finally, we demonstrate that a second form of mechanical stimuli, fluid sheer stress as a result of increased perfusion flow rate in the anterior segment perfusion assay, can also influence both CTGF and ppET-1 mRNA content in the TM in situ.

Taken together, these results support the hypothesis that pressure-dependent changes in TM cell gene expression represent a feed-forward mechanism that exacerbates TGF-β2 associated increases in TM cell contractility and altered ECM synthesis and deposition in affected POAG patients.
CHAPTER 1
LITERATURE REVIEW

Primary Open-Angle Glaucoma.

Glaucoma is a leading cause of irreversible blindness, affecting nearly 65 million people worldwide. This number is expected to rise to 112 million by the year 2050.\textsuperscript{1,2} In the US alone, glaucoma healthcare expenses exceed 1.9 billion annually.\textsuperscript{3} This number likely underestimates the true socioeconomic impact of this disease as many Americans in the early, asymptomatic stages of glaucoma remain undiagnosed.\textsuperscript{5} Glaucoma can be broadly subdivided into angle-closure and open-angle pathologies. In patients with angle-closure glaucoma, the angle between the iris and cornea is constricted as a result of trauma or inflammation. This produces a marked and rapid increase in intraocular pressure (IOP) leading to blurred vision and acute pain. Angle-closure glaucoma is less prevalent, effecting only 26% of patients and is often asymmetric.\textsuperscript{2} In contrast, primary open angle glaucoma (POAG) is a slowly progressing form of glaucoma that is far more common (prevalence of 1.86% in individuals over the age of 45 in the United States alone),\textsuperscript{6} causes very little or no pain, and is typically bilateral. POAG begins with loss of peripheral vision and can culminate in irreversible blindness if left untreated. Currently, over 2.7 million Americans suffer from POAG with this number expected to more than
double to 6.3 million by 2050, highlighting the importance of advancing patient care (Figure 1). Regrettably, in patients with POAG the gradual loss of vision is often the first recognizable symptom. By the time visual deficits are perceived, however, extensive and profound injury to the optic nerve has occurred. Despite its overwhelming prevalence and socioeconomic impact, the treatment of patients with POAG remains limited and palliative.

![Figure 1. The Prevalence of Glaucoma.](https://nei.nih.gov/eyedata/glaucoma)

**The Visual Pathway.**

Perception of our visual world begins with the capture of a single photon of light (390-700 nm) by rod and/or cone photoreceptors, highly specialized neuroepithelial cells localized to the outer layers of the retina. Electrochemical changes initiated by light-hyperpolarized photoreceptors are subsequently “processed” by second (bipolar, horizontal, amacrine) and third-order (retinal ganglion cell) neurons of the retina to yield discriminatory spatial and temporal contextual information about the visual field perceived. The unmyelinated axons of retinal ganglion cell neurons carrying “processed” information exit the eye through the optic disc at the lamina cribrosa, a discrete
anatomical pinch-point where axons become centrally myelinated forming cranial nerve 2 (optic nerve). Within the eye, the unmyelinated axons of retinal ganglion cells are exposed to IOP. Upon exiting the eye however, the optic nerve is subjected to intracranial pressure (ICP) derived from cerebrospinal fluid in the subarachnoid area of the retrobulbar space. The difference between these two pressurized zones, known as the translaminar pressure gradient (TPG), is typically quite small (≈2.6 mmHg).\textsuperscript{7,8}

![Figure 2. Schematic of the visual pathway.](reviewsOptometry.com)

Myelinated axons of the optic nerve collectively transfer visual information from each visual field in a topographically preserved orientation. On its way to the primary visual cortex, the optic nerve partially decussates at the optic chiasm (Figure 2) maintaining a precise topographic orientation of each point in our visual fields. The majority of decussated nerve fibers proceeds through the optic tract and terminate in one of six discrete layers within the lateral geniculate nucleus of the thalamus. From there,
topographically preserved visual information travels along the optic radiations either below (Meyer’s loop) or above the lateral ventricles and terminate medially within the occipital lobe (primary visual cortex, Broadman’s area 17) of each hemisphere.

**Trabecular Meshwork and Intraocular Pressure.**

The pathophysiology of optic nerve injury in POAG is unclear, but most likely is associated with the abnormal buildup of pressure within the eye. Whereas elevated IOP (>21 mmHg) is a recognized major risk factor for the initiation and progression of glaucomatous neuropathy, the initial cause of elevated IOP remains a poorly-understood hallmark of this disease.\(^9\),\(^{10}\) IOP is maintained by a balance between the production and outflow from the eye of aqueous humor (AH), a clear CSF-like fluid that provides nutrients to the avascular tissues of the anterior and posterior chambers of the eye. The rate of AH turnover varies in a diurnal manner, but is estimated to average around 2.4 \(\mu\)l/min.\(^{11}\) AH is actively secreted from the non-pigmented epithelium of the ciliary body into the posterior chamber, flows through the pupil into the anterior chamber before draining from the eye by either the conventional or uveoscleral pathway. In the uveoscleral pathway (Figure 3A), AH passively seeps between tissue spaces in the ciliary muscle into supraciliary and suprachoroidal spaces before finally draining through the sclera to episcleral tissue.\(^{12}\) The rate of AH drainage through the uveoscleral pathway is controlled by the contraction or relaxation of the ciliary body, and thus is relatively insensitive to pressure.\(^{12}\)
At the junction where the iris meets the cornea (iridocorneal angle) there is a unique anatomical structure composed of endothelial and smooth-muscle-like contractile cells resting on a series of fenestrated beams and sheets of extracellular matrix (ECM) called the trabecular meshwork (TM, Figure 3B).\textsuperscript{13, 14}

In the conventional outflow pathway, AH passes through the TM in a \textit{pressure-dependent} manner, enters Schlemm’s canal (SC), where it runs through a series of collector ducts and eventually rejoins the vasculature at the episcleral vein. The conventional outflow pathway can be subdivided into three distinct anatomical regions (the uveal, corneoscleral and juxtacanicular (JCT) meshwork), each with unique morphology and function. Outer TM cells, near the uveal and corneoscleral regions, are phagocytic, acting as a sort of filter of AH.\textsuperscript{15} Due to their large intercellular spaces, these layers offer relatively little resistance to AH outflow. TM cells of the JCT region, however, are embedded in a dense layer of ECM proteins,\textsuperscript{16} and the majority of outflow resistance through the TM is

\textbf{Figure 3. Aqueous Humor Outflow.} (A) Diagram of AH outflow pathways. Adapted from touchophthalmology.com (B) Scanning electron micrograph of the trabecular meshwork adapted from Histology of the Human Eye, An Atlas and Textbook.
attributed to the increased organization of ECM and decreased intercellular space in this area. The innermost cells of the JCT are directly adjacent to SC. The endothelial cells of SC form a monolayer with tight junctions generating additional resistance to AH outflow (an estimated 10%). AH is thought to enter the lumen of SC through trans- or paracellular pores that form in the endothelium of SC. Impaired formation of these components can result in increased resistance to AH outflow.

Maintenance of IOP at healthy levels is of critical importance to preserving the overall function and health of the retina and optic nerve. AH production is relatively insensitive to changes in pressure; thus, IOP is primarily regulated by resistance to AH outflow. Total AH outflow through the pressure-independent uveoscleral pathway decreases with age and has been reported to account for as little as 10% of total outflow in elderly (>60 years of age) patients. Consequently, only the conventional pathway is considered relevant to normal IOP maintenance. Elevated IOP, due to enhanced resistance to AH outflow through the TM, is transduced to the posterior segment of the eye eliciting optic nerve damage, retinal ganglion cell death, and visual deficits. A small but significant percentage of patients with POAG (15-25%) do not develop elevated IOP. This form, termed normal-tension glaucoma (NTG), may have a unique pathophysiology independent of elevated IOP. Both POAG and NTG patients, however, tend to exhibit ICPs that are 3-4 mmHg lower than healthy control subjects as well as similar morphologic changes in the optic nerve head, suggesting that the imbalance between IOP and ICP may play an important role in glaucomatous damage. Supporting this hypothesis, reduction of IOP in NTG patients has been shown to decrease the risk of long-term progression.
Therapeutic Management of POAG.

Current therapeutic options for the management of patients with POAG include both surgical and pharmacological strategies consisting of non-specific forms of intervention designed to lower IOP.\textsuperscript{26, 27} Carbonic anhydrase inhibitors, such as the acetazolamide Diamox, reduce IOP by attenuating AH production from the epithelium of the ciliary body processes.\textsuperscript{28} Beta-adrenergic antagonists, such as Timolol, inhibit AH secretion by limiting the synthesis of cyclic adenosine monophosphate (cAMP).\textsuperscript{29} Latanoprost, a prostaglandin F\textsubscript{2a} analog, increases uveoscleral outflow by decreasing the expression of connective tissue within the ciliary body and enhancing the secretion of matrix metalloproteinase (MMPs) leading to remodeling of the extracellular matrix.\textsuperscript{30-32}

Regrettably, to date, there is no FDA-approved therapeutic strategy that primarily targets the conventional outflow pathway. In many cases, patient IOP cannot be controlled by a single medication and often requires the use of 3 or more different classes of drugs. Reported limitations of pharmacological options include decreased efficacy over time, limited bio-availability, and the need for frequent dosing. Surgical intervention is often required, including selective laser trabeculoplasty or trabeculectomy, to clear portions of obstructed TM. While this intervention does produce an immediate and marked drop in IOP, intraocular pressure often rebounds to pathological levels. Thus, while non-specific therapies that lower IOP do show limited success in slowing the progression of POAG, they are not sufficient to completely halt its advancement. \textit{The development of therapeutic strategies that specifically target the pathogenic causes of elevated IOP are critically needed to advance the care of affected patients.}
The Extracellular Matrix of the TM.

The ECM of the TM is a complex, yet highly organized, scaffold of glycosaminoglycan’s (GAGs), proteoglycans, basement membrane proteins (collagen, laminin, fibronectin), matricellular proteins, and cell-surface ECM receptors. GAGs are negatively charged mucopolysaccharides primarily found bound to ECM that can be divided into 4 subclasses: heparan sulfate, chondroitin sulfate, keratan sulfate, and hyaluronic acid. The role of GAGs in the pathogenesis of POAG is still emerging, but evidence suggests they may play a role in regulating AH outflow resistance through the TM. Collagens type I, III, IV, and VI are expressed in the TM and form evenly-spaced fibrils that are essential for providing support for cell attachments and tensile strength to the TM. Type VI collagens, in particular, have been observed to be a key component of fibrils attaching cells of SC to the JCT. Non-fibrillar collagens (type VII, VIII, XI, XV, and XXII) are also expressed by the TM, though their specific function is not well understood. The TM also contains extensive microfibril networks, often referred to as sheath-derived plaques, composed of elastin, fibronectin, versican, fibrillin, and decorin. These microfibrils are known to bind to numerous cell surface receptors. The TM also expresses a number of cell-surface receptors enabling attachment of ECM to TM cells including integrins, syndecans, and CD44. The role of these receptors in IOP regulation is unclear, but may involve the formation of cross-linked actin networks (CLANs) and binding of cells to fibronectin containing microfibrils.

Degradation and biosynthesis of new ECM components are essential to normal TM maintenance. In order to tightly regulate ECM turnover, the TM expresses and regulates a variety of proteinases. MMPs, plasminogen activator (PA), tissue inhibitors
of metalloproteinases (TIMPS), and PA inhibitor (PAIs) have all been localized to the TM.\textsuperscript{42,43} MMPs are a family of calcium-dependent endopeptidases that degrade collagens, fibronectin, decorin, versican and elastin.\textsuperscript{44,45} Whereas MMP-2, -14, -15, and -16 are constitutively expressed by the TM,\textsuperscript{43,46} MMP-1, -3, and -9 are inducible and upregulated in response to various cytokines or growth factors.\textsuperscript{47} PAs have also been demonstrated to activate proMMPs, while PAIs inhibit MMP activity.\textsuperscript{48} Addition of purified MMPs to perfused human anterior segments elicits marked increases in outflow facility while the addition of TIMPs significantly decrease outflow facility.\textsuperscript{49} These data strongly suggest that MMPs are constitutively active within the TM and that MMP activity modulates AH outflow through the TM. MMPs may contribute to IOP maintenance by a mechanism involving more than simple ECM degradation, as they are also involved in cell-matrix interactions, activating latent proteins, and providing clues for cell proliferation and survival.\textsuperscript{44} Altered ECM composition or increased ECM content may elicit aberrant elevation of IOP by increasing cell-protein interactions and ECM cross-linking. This in turn would increase TM rigidity and interfere with the TM’s ability to sense changes in pressure and adjust ECM turnover.

Patients with POAG display a characteristic accumulation of ECM components in the TM. Post mortem studies have demonstrated increased expression of fibrillin-1 and collagen VI containing sheath-derived plaques in JCT region of the TM when compared to age-matched healthy controls.\textsuperscript{16,50} While the full composition and function of these plaques remains unclear, it is likely that these plaques do play an integral role in the aberrant increase in AH outflow resistance. The TM of glaucomatous patients is also stiffer than healthy individuals when measured by atomic force microscopy.\textsuperscript{51}
Interestingly, increased tissue stiffness has also been linked to reduced pore formation in SC.\(^{52}\)

There are also marked differences in the gene expression profiles of various ECM components between glaucomatous and healthy eyes. Liton et al. found marked increases in the expression of fibronectin, collagen (types III, V, VI, XI, and XIV), laminin, and thrombospondin-3 mRNA in glaucomatous TM cells, compared to TM cells harvested from healthy eyes.\(^{53}\) In this same study, integrin\(\alpha9\), MMP3, and MMP10 were significantly decreased. Diskin et al. demonstrated marked increases in the levels of syndecan 4, intercellular adhesion molecule (ICAM)-1 and several other ECM components in post-mortem glaucomatous tissue.\(^{54}\) While its mechanism is not yet fully understood, it is likely that ECM accumulation or remodeling plays a causal role in IOP elevation in patients with POAG. Consequently, manipulation of ECM turnover remains an important area of interest for the development of new therapeutic strategies.

**Transforming Growth Factor-\(\beta2\) and POAG.**

We and others have shown that AH collected from some POAG patients prior to phacoemulsification contains aberrantly elevated levels of the pro-fibrotic cytokine transforming growth factor (TGF)-\(\beta2\).\(^{55-59}\) Perfusing biologically active TGF-\(\beta2\) (10 ng/ml) through human or porcine anterior segments elicits a marked and sustained decrease in outflow facility.\(^{60-63}\) Similarly, adenoviral gene transfer of TGF-\(\beta2\) into rodent eyes in vivo significantly increases IOP within days of injection.\(^{64,65}\) Collectively, these data suggest a causative role for biologically active TGF-\(\beta2\) in the pathogenesis of POAG.
TGF-β2 is a member of the TGF-β superfamily involved in the regulation of proliferation, differentiation, embryonic development, and wound healing. Early studies report expression and localization of all three isoforms of TGF-β in multiple ocular tissues including the lens epithelial cells, conjunctival stroma, and the ciliary body. The TM is no exception, and in vitro studies by our laboratory clearly demonstrate constitutive expression and secretion of TGF-β2 from cultured primary and transformed human TM cells. Interestingly, all three TGF-β isoforms are remarkably similar, sharing over 80% sequence homology and activating the same signaling pathways. Only TGF-β2, however, has been shown to be elevated in the AH of affected patients, and consequently, considered relevant to the pathogenesis of POAG. TGF-β2 is secreted as an inactive latent propeptide, bound to one of four latent TGF-β binding proteins. Following proteolytic processing by either thrombospondin-1 or plasmin mediated cleavage; a homodimer of the now biologically active TGF-β2 binds to a subtype of the transforming growth factor receptor (TβR) subfamily, TBRII (Figure 4). Activated, ligand-bound, TβRII monomers then dimerize to enable recruitment and transphosphorylation of a distinctly separate (type I; TβRI) subtype of the TGF-β receptor. A third TGF-β receptor (TβRIII) has been reported, but evidence suggests that this receptor serves as a non-essential accessory role at presenting TGF-β2 to TβRII. Following transphosphorylation, TβRI undergoes a conformational change which elicits recruitment, and phosphorylation, of canonical receptor-associated smad signaling proteins (R-smad2 and R-smad3).
Dimers of phosphorylated R-smad2 or R-smad3 passively associate with a third unphosphorylated smad protein (smad4). The newly formed smad heterotrimer enters the nucleus by an unknown mechanism where it initiates transcriptional events by complexing with smad binding elements (SBEs) present within the genome. In addition to this well-characterized canonical signaling pathway, TGF-β2 also activates several signaling pathways that are independent of the smad proteins, including the mitogen-activated protein (MAP) kinase, phosphoinositide (PI)-3 kinase/activated protein kinase (AKT), c-jun n-terminal (JNK), and Rho GTPase/Rho kinase pathways. Cross-talk between these non-canonical pathways and smad proteins are essential for downstream regulation and modulation of TGF-β mediated signaling.

TGF-β2 activated signaling pathways play a vital role in the regulation of cell proliferation, inflammation, and ECM production. As ECM dysregulation is critical
component of POAG, it is plausible that elevated levels of TGF-β2 in AH of POAG patients induces increased ECM production/remodeling contributing to aberrant increases in AH outflow resistance. *In vitro* studies have established pro-fibrotic TGF-β2 as a key mediator of ECM protein expression and regulation in human TM cell, including collagens (I, III, IV, and VI), laminin, fibronectin, secreted protein acidic and cysteine rich (SPARC), PAI-1, and TIMP-1. TGF-β2 has also been shown to increase expression of ECM cross-linking genes transglutaminase-2 and lysyl oxidase, two enzymes which contribute to TM tissue stiffness. Experimentally altering TM elasticity, *in vitro*, modulates TGF-β2 mediated signaling and thereby promotes a phenotype similar to that of glaucomatous TM. *Ex vivo*, TGF-β2 promotes the accumulation of fibrillary matter and fibronectin in perfused anterior segments. Collectively, these studies suggest that the profibrotic cytokine TGF-β2 is an important initiator of fibrosis within the TM, eliciting increased resistance to AH drainage and elevated IOP.

While much is known about TGF-β signaling in the eye, our current knowledge of the mechanisms involved in the aberrant elevation of TGF-β2 in patient AH remains limited. Recent evidence suggests TGF-β2 expression levels within the TM may be influenced, in part, by epigenetic dysregulation. Overexpression of miR-29b, a micro RNA important in the regulation of DNA methyltransferases and histone deacetylase (HDAC)s, markedly decreases TGF-β2 mRNA content. The amount of secreted biologically active TGF-β2 is, however, unaltered by miR-29b. By comparison, hyperacetylation of histones associated with the TGF-β2 promoter markedly increases TGF-β2 expression in human TM cells. Additionally, perfusion of bovine anterior segments with a histone deacetylase inhibitor concomitantly increases TGF-β2
expression and decreased outflow facility. Alternatively, inhibition of Rho GTPases decreases constitutive synthesis and secretion of TGF-β2 from TM cells. Therefore it is likely that both increased RhoA activity, as well histone hyperacetylation, play an important role in the pathogenesis of POAG by increasing TGF-β2 expression and release by the TM.

**Non-Canonical Rho GTPase Signaling in POAG.**

Included among the Ras superfamily of small monomeric GTPases is a family of Rho GTPases (Rho, Rac1, Cdc42) that regulate a diverse array of intracellular signaling pathways which affect vesicle transport/trafficking, endocytosis, cell cycle progression, cell contractility, stress fiber or focal adhesion formation. Of these, the Rho subfamily consists of RhoA, RhoB, and RhoC. Following activation by guanine nucleotide factor-mediated GDP: GTP nucleotide exchange, GTP-bound Rho activates Rho-associated protein kinases (ROCK1 & ROCK2). Once activated, these kinases phosphorylate and activate LIM kinase which in turn stabilizes filamentous actin networks by inactivating coflin, an actin depolymerase (Figure 5).

In parallel, activated Rho kinases also phosphorylates myosin light chain (MLC) while inhibiting MLC phosphatase, indirectly facilitating contraction of polymerized actin fibers. Studies from our laboratory have shown that in human TM cells, TGF-β2 mediated activation of Rho GTPase signaling elevates IOP, in part, by modifying F-actin stress fiber organization and promoting cell contractility. TGF-β2 treated TM cells have also been reported to exhibit a modest increase in Rac1-mediated formation of cross-linked actin networks, which may contribute to elevated IOP. Interestingly,
expression of constitutively active RhoA significantly increases expression of fibronectin, tenascin, laminin, and alpha-smooth muscle actin.\textsuperscript{89}

![Diagram of the Rho GTPase mediated signaling pathway](image)

\textit{In vivo}, constitutively active RhoA induces elevated IOP and an associated increase in F-actin, both of which are ameliorated by Rho kinase inhibitors.\textsuperscript{90} Perfusion of porcine anterior segments with the ROCK inhibitor Y-27632 decreases outflow resistance in a dose dependent manner.\textsuperscript{91} \textit{In vivo}, topical administration of ROCK inhibitors to both normotensive and ocular hypertensive rabbits results in a significant decrease in IOP, further solidifying the link between TGF-β2 mediated increases in Rho GTPase activity and the pathophysiology of POAG.\textsuperscript{92}

**Endothelin-1 Mediated Vasoconstriction in POAG.**

Endothelin-1 (ET-1) is a potent vasoactive peptide that is also elevated in the AH of patients with POAG.\textsuperscript{93-96} In this regard, our laboratory observed a modest increase in ET-1 content in AH samples from Veterans with POAG (40 ± 20 fg/µl; n = 6) as
compared to healthy age-matched subjects (20 ± 10 fg/µl; n= 10). POAG patients with progressive visual loss have elevated plasma levels of ET-1 compared to POAG patients with stable visual function. 97 Plasma levels of ET-1 in patients with NTG are similarly increased, compared to healthy age-matched controls, indicating that this peptide may also be involved in the pathogenesis of NTG. 98

Of the three endothelin isoforms (ET-1, ET-2, ET-3) reported, only ppET-1 is thought to modulate outflow facility by binding to one of two major metabotropic G-protein coupled receptor subtypes (ET_A or ET_B) expressed within TM. Secreted as a prepropeptide from human TM cells, ppET-1 undergoes proteolytic processing to its mature 21 amino acid ET-1 form by endothelin converting enzymes (ECE). While ET-1 is best known as a vasoactive peptide responsible for controlling vascular tone, it also stimulates cell migration, ECM deposition, and the secretion of inflammatory mediators and growth factors. Activation of ET_A receptors elicits pronounced vasoconstriction by stimulating phospholipase Cβ, liberating inositol 1,4,5-trisphosphate from phosphatidylinositol 4,5-bisphosphate, mobilizing intracellular calcium, and ultimately eliciting a marked contraction of the actin cytoskeleton. ET_A also stimulates the activation of small monomeric Rac1 and RhoA GTPases. 100, 101 In contrast, activation of ET_B receptors facilitates vasodilation by eliciting nuclear factor-κB (NF-κB)-mediated synthesis and release of cyclins and nitric oxide. 102 Activation of ET_B receptors is also reported to promote retinal ganglion cell death in POAG. 103, 104 An additional receptor subtype (ET_C) has been identified, although its physiological significance is uncertain.

The corneal epithelium, iris, ciliary body, choroid, retinal vessels, and optic nerves constitutively express and release ET-1. 105, 106 While quiescent TM cells do not
express measurable levels of ET-1, challenging cultured primary human TM cells with TGF-β2 robustly increases both the synthesis and secretion of ET-1. Both ET_A and ET_B receptors are constitutively expressed by human TM cells, and treatment with exogenous ET-1 induces RhoA GTPase dependent contraction of the actin cytoskeleton, supporting a physiologic role for ET-1 in the TM. ET-1 is also pro-fibrotic mediator, known to induce accumulation of collagens type I and VI in optic nerve head astrocytes. Administration of ET-1 \textit{ex vivo} acutely increases outflow resistance in perfused bovine anterior segments, possibly by remodeling the ECM. Interestingly, \textit{intravitreal} injection of ET-1 produces a rapid, but transient, increase in IOP in rabbits that can be inhibited by selective ET_A receptor antagonist. It remains quite plausible that elevated content of TGF-β2 in AH of affected patients elevates IOP by a mechanism involving ET-1 mediated contraction of the actin cytoskeleton and remodeling of the ECM in the TM.

**Connective Tissue Growth Factor Mediates TGF-β2 Remodeling of the ECM.**

Connective tissue growth factor (CTGF) is a secreted, multi-modular member of the CCN (CTGF, Cyr61, Nov) family of regulatory proteins essential in the regulation of angiogenesis, wound healing, and fibrosis. The many diverse biological functions of CTGF are mediated by its four unique domains: insulin-like growth factor binding (IGFB) domain, chordin-like cysteine-rich domain, thrombospondin type-1 repeat domain, and a C-terminal cysteine-knot. CTGF expression is primarily regulated at the transcriptional level and is induced by hypoxia, mechanical deformation, sheer stress, and various growth factors.
Whereas CTGF is one of the most highly expressed genes in the TM, other have found TGF-β2 markedly increases CTGF expression in primary and transformed human TM cells. ET-1 has also been shown to increase CTGF expression in fibroblasts although this finding has not been repeated in TM cells. While a specific CTGF receptor has not been reported, CTGF itself binds with integrins, tyrosine kinase receptors, and epidermal growth factor receptors. These findings suggest that CTGF acts a molecular bridge, integrating extracellular proteins and intracellular signaling networks. Regions within the cysteine-rich domain of CTGF interact with TGF-β to prolong TGF-β mediated signaling.

Several in vitro studies have now shown that CTGF serves as a critical downstream mediator of pro-fibrotic TGF-β2 signaling. In CTGF null embryonic fibroblast, TGF-β2 elicits potent smad3 phosphorylation but fails to increase expression of α-smooth muscle actin. In TM cells, siRNA mediated knockdown of CTGF prevents TGF-β2 mediated increases in fibronectin, and actin stress fiber formation. Alternatively, CTGF can mimic many of the molecular mechanisms and structural aspects of POAG seemingly independent of TGF-β2 signaling. In vivo, adenoviral overexpression of CTGF in the anterior chamber produces a glaucomatous phenotype, with mice displaying increased fibronectin expression in the TM, elevated IOP, and optic nerve damage. In vitro, addition of CTGF to human TM cell cultures induces increased actin stress fiber formation as well as deposition of fibronectin and collagens I, II, IV, and VI. CTGF has no effect, however, on the activity of MMP-2, MMP-9, or PA-1. In contrast, TGF-β2 markedly enhances PA-1 activity in human TM cells. Thus,
while TGF-β2 and CTGF tend to elicit similar effects, these results show that each can induce distinct and independent outcomes from one another.\textsuperscript{120}

While patients with pseudoexfoliation (PFX) glaucoma, a secondary form of glaucoma caused by increased deposition of white, dandruff-like material in the anterior segment, do exhibit an elevated content of CTGF in their AH, patients with POAG were found to have levels similar to those of age-matched healthy controls.\textsuperscript{127} While it is premature to implicate CTGF as causative in the pathogenesis of POAG, an association between TGF-β2 and CTGF appears likely as a critical mediator of fibrosis in the TM of patients with POAG. In separate studies examining systemic sclerosis, TGF-β injections into the dermis of mice resulted in only a transient dermal fibrosis. TGF-β injections followed by serial injections of CTGF, however, led to a sustained fibrotic phenotype.\textsuperscript{128} We propose that elevated levels of TGF-β2 in the AH of patients with POAG initiates remodeling of ECM that is subsequently sustained, or perhaps exacerbated, through the actions of CTGF.

\textbf{TGF-β2 Mediated Perturbation of the Kallikrein-Kinin System in POAG.}

An equally engaging mechanism by which TGF-β2 may promote pathological elevation of IOP includes perturbation of the Kallikrein-Kinin system. Early studies reported that bradykinin (BK), a potent vasodilating kinin peptide, was capable of lowering IOP. Perfusion of bovine or human anterior segments with bradykinin elicited a marked, but transient, decrease in IOP.\textsuperscript{129,130} Intravitreal injection of BK induced a significant and sustained (~8h) decrease in IOP in ocular hypertensive Dutch-belted
rabbits. Topically applied bradykinin, however, was without effect on IOP in either Dutch-belted rabbits or cynomolgus monkeys, possibly due to cornea restricted bioavailability. By comparison, topical application of FR-190997, a hydrophobic bradykinin analog, reduced IOP in ocular hypertensive monkeys. Collectively, these data support the use of BK analogues as novel, potentially viable, regulators of AH outflow. Their development and application as a first-line therapeutic strategy for the management of POAG, however, awaits clinical phase trial evaluation and refinement.

The kallikrein-kinin system (Figure 6) consists of specific serine proteases (kallikreins) that act upon kinin peptide precursor proteins (kininogens) to generate biologically active nonapeptide bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) and decapeptide kallidin. Kallidin can subsequently be cleaved by aminopeptidases to form bradykinin. Importantly, the biochemical machinery responsible for synthesizing bradykinin has been detected in trace amounts in AH from human and porcine. Kallikreins and kininogens have also been localized to the human retina, choroid, ciliary body, optic nerve, and TM.

![Figure 6. The Kallikrein-kinin System. Diagram of the components of the kallikrein-kinin system adapted from Heitsch 2000.](image)
Intracellular signaling events initiated by the kinins are governed by two selective subtypes of metabotropic G-protein coupled receptors (B1 and B2). The B1 receptor is an inducible receptor and is typically expressed only in response to a traumatic or inflammatory challenge. Although B1 receptor mRNA has been localized to ocular tissues, this receptor has not been detected at the protein level within the eye. Moreover, B1 receptor agonists, including Des-Arg⁹-BK (an active metabolite of BK), fail to elicit any measurable responses, in vitro, from TM, ciliary body, or non-pigmented epithelial cells. By comparison, the B2 receptor is constitutively expressed in a variety of tissues including brain stem, cerebral cortex, thalamus, and the ependyma of the lateral and third ventricle. In the eye, the B2 receptor is expressed in the TM, ciliary muscle, nonpigmented epithelium, optic nerve, and retina.

Given its constitutive expression and localization within the eye, activation of the B2 receptor, rather than the B1 receptor, is believed to largely mediate kinin-dependent ocular hypotension. Perfusing anterior segments with HOE-140, a B2 receptor antagonist, prevents bradykinin dependent lowering of IOP. Furthermore, bradykinin exhibits a much higher (>1000 fold) affinity for the B2 receptor compared to the B1 receptor. Bradykinin, however, is an extremely labile peptide (half-life in plasma of 15s). Both bradykinin and kallidin are quickly metabolized by kininases that include carboxypeptidases, aminopeptidases, angiotensin converting enzymes, and neutral endopeptidases. While the use of kininase inhibitors to extend the therapeutic effect of bradykinin seems logical, this avenue may produce limited results as the B2 receptor also exhibits rapid agonist-dependent desensitization. The effect of long-term repeated
exposure to bradykinin on B2 receptor localization and function in the eye remains unknown.

While the B2 receptor is typically coupled to G-protein effector $\text{G}_\alpha_q$, this receptor also couples with $\text{G}_\alpha_i$, $\text{G}_\alpha_s$, and $\text{G}_{12/13}$.\textsuperscript{143-145} Binding of bradykinin to the B2 receptor stimulates $\text{G}_\alpha_q$ mediated activation of phospholipase C$\beta$, thereby catalyzing the breakdown of phosphoinositol-4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG).\textsuperscript{146} IP$_3$ subsequently binds to and opens a calcium-selective ligand-gated ion channel expressed on a subdomain of the endoplasmic reticulum, resulting in the mobilization of intracellular calcium. Cultured TM cells exposed to bradykinin exhibit a rapid (40s) increase in intracellular calcium, which subsequently activates a plethora of calcium-dependent enzymatic pathways, including phospholipase (PL) A2.\textsuperscript{147,148} Cyclooxygenases expressed in TM cells metabolize PLA$_2$-catalyzed release of arachidonic acid to prostaglandins and thromboxanes. Ciliary muscle cells, non-pigmented ciliary epithelial cells, and cells of the TM respond rather robustly to bradykinin by increasing prostaglandin secretion.\textsuperscript{130,131} The ocular hypotensive effects of bradykinin can thus be attributed, at least in part, to local stimulation of prostaglandin secretion from the ciliary body leading to increased uveoscleral outflow. Perfusing human anterior segments with a prostaglandin F$_{2\alpha}$ analog (Latanoprost) also lowers IOP (increases outflow facility), suggesting that increased production of prostaglandins by the TM may also act locally to increase AH outflow through the conventional pathway.\textsuperscript{149} This possibility, however, remains contentious. An alternative mechanism by which BK may influence conventional outflow involves production of MMPs, including MMP-9. In vitro BK induces a rapid but transient phosphorylation of the MAP kinase cascade
leading to increased MMP-9 secretion.\textsuperscript{140,150} Inhibition of this proteinase prevents bradykinin mediated changes in outflow facility in bovine anterior segment perfusion studies.\textsuperscript{129} These results suggest that bradykinin may elicit matrix metalloproteinases mediated ECM turnover within the TM. We are unaware of any studies to date, however, that have demonstrated bradykinin mediated changes in ECM content within the TM.\textsuperscript{130}

The use of bradykinin as a potential therapeutic warrants further examination. However, it is worth noting that no previous study has accounted for how elevated levels of TGF-β2 in patient AH may affect bradykinin mediated signaling. Airway smooth muscle cells respond to a TGF-β2 challenge in a dose- and time-dependent manner by increasing B2 receptor mRNA and receptor protein content leading to a potentiation of bradykinin mediated increases in intracellular calcium.\textsuperscript{151} In marked contrast, we have recently demonstrated that TGF-β2 markedly decreases B2 receptor mRNA content in human TM cells within 24h.\textsuperscript{152} While changes in mRNA levels do not always correspond to protein content, or even the functional state of the receptor, further studies are warranted to determine the effect TGF-β2 on the kallikrein-kinin system within the TM.

**Mechanosensation within the TM.**

IOP is not static, but rather dynamic, constantly fluctuating due to blinking, eye movement, or oscillations associated with ocular pulse. Within the TM, there exist regulatory mechanisms designed to maintain IOP at a healthy level. Anterior segment perfusion studies demonstrate that a rapid rise in IOP elicits compensatory changes in outflow resistance that quickly return IOP to near baseline pressures, demonstrating the TM’s ability to dynamically respond to and regulate IOP homeostasis.\textsuperscript{19,153,154} The
hydraulic conductivity of AH through the TM is primarily determined by TM cell contractility and extracellular environment.\textsuperscript{14,155} Consequently, the homeostatic response of the TM to an increased pressure load must ultimately include compensatory alterations in ECM turnover and modification of the cytoskeleton leading to a collective decrease AH outflow resistance. The effect of chronic over activation of this response by sustained elevated IOP is not well understood, but most likely leads to a permanent remodeling of the ECM and change in cell morphology.

Elevated IOP is most often modelled \textit{in vitro}, by the culture of TM cells on a flexible membrane followed by uni- or biaxial stretch resulting in distortion of the plasma membrane. In studies utilizing cultured human and bovine TM cells, application of either cyclical or static stretch increases the expression and secretion of active MMP-2.\textsuperscript{156,157} By comparison, the effect of stretch on the expression of TIMPs is unclear. Cyclical stretch of bovine TM cells results in increased TIMP-1 expression, while static stretch has no effect upon the levels either of TIMP-1 or TIMP-2.\textsuperscript{158} This lack of consistency highlights the importance of utilizing multiple systems to model the mechanical stress experienced by the TM. To date, three independent studies have examined changes in the gene expression profile in response to mechanical stress, each utilizing a distinct model. Gonzalez \textit{et al.} investigated the effects of high perfusion pressure (50 mmHg x 6h) on TM cells in perfused human anterior segments.\textsuperscript{159} In this study, eleven genes involved in ECM remodeling, cytoskeleton reorganization, and reactive oxygen species (ROS) were consistently upregulated.\textsuperscript{159} Vittitow and Boras identified 40 upregulated and 14 downregulated genes in response high perfusion rate (10 µl/min) over 2-4 days in perfused human anterior segments.\textsuperscript{160} Interestingly, MMP-2 expression was significantly
increased in this model, similar to *in vitro* studies. Finally, Vittal et al. characterized the effect of static stretch (up to 48h) in porcine TM cells.\(^{46}\) In this assay, 126 genes were upregulated including CTGF, fibronectin, and collagen XIV. Cyclical stretch of human TM cells *in vitro* has also been demonstrated to increase TGF-\(\beta\)1, CTGF, and MMP-2 content.\(^{161}\) These changes in gene expression were prevented by the addition of a TGF-\(\beta\)1 neutralizing antibody. While TGF-\(\beta\)1 content was increased in this experiment, the effect of cyclical stretch on TGF-\(\beta\)2 was not determined. In addition to inducing changes in gene expression, mechanical stress may lead to alternative mRNA splicing. Mechanical stretch of porcine TM cells induces both increased content and a novel isoform with deletion of two exons and a premature stop codon, of collagen XII mRNA.\(^{162}\) Changes such as these could very likely affect ECM stiffness or ECM-cell interactions interfering with healthy IOP homeostasis.

The mechanisms by which TM cells sense and respond to mechanical stretch have yet to be elucidated, but may involve the mechanosensitive receptor transient receptor potential vanilloid (TRPV) 4.\(^{163}\) TRPV4 is a nonselective cation channel that is activated by a disparate variety of stimuli including hypotonicity, mechanical deformation, heat, and pH.\(^{164}\) TRPV4 is constitutively expressed *in vivo* in mouse and human TM tissues as well as endothelial cells of SC and non-pigmented epithelial cells.\(^{165},^{166}\) Pharmacological activation of TRPV4 *in vitro* increases fibronectin secretion and induces a marked increase in intracellular calcium in both primary and immortalized TM cells.\(^{165}\) Furthermore, the formation of actin stress fibers and focal adhesion complexes induced by mechanical stretch of TM cells is completely abrogated by the TRPV4 inhibitor HC-06.\(^{165}\) Interestingly, inhibition of TRPV4 in human bronchial cells prevents Rho GTPase-
mediated ATP release induced by hypotonicity implying that TRPV 4 may regulate the activity of this small monomeric G-protein. The role of TRPV4 in the regulation Rho GTPase activity in the TM, however, remains unknown. When the TRPV4 antagonist HC-06 was administered intraperitoneally in the mouse microbead occlusion model of POAG, however, inhibition of TRPV4 concomitantly decreased IOP and enhanced RGC survival. Collectively, these results strongly suggest that TRPV4 is a critical mediator of mechanosensation within the TM. Moreover, elevated IOP may chronically over activate TRPV4 channels, leading to a permanent increase in fibronectin deposition, RhoA GTPase activation, actin stress fiber formation, and retinal ganglion cell death.

Of the various types of mechanical stimuli experienced by the TM, increased hydrostatic pressure is the least often studied form and consequently the least well understood. Hydrostatic pressure is unique in that it is a non-deforming, isotropic mode of stress. Consider that mammalian cells are essentially fluid filled “sacs” that exhibit very little compressibility. An increase in 10 MPA (75,006 mmHg, upper range of hydrostatic pressure experienced in vivo) would result in only a 0.4% change in cell volume. Thus the mechanism by which cells sense elevated hydrostatic pressure and downstream signaling cascades coupled to this stimuli are most likely quite distinct when compared to other mechanical stressors (fluid sheer stress, physical stretch). Mechanical distension through biaxial stretch therefore, is most likely not a suitable in vitro model to mimic elevated hydrostatic pressure.

A more appropriate method to study the effects of elevated hydrostatic pressure involves injection of gas (95% air/5% CO2) into a sealed, humidified, and temperature controlled chamber with appropriate equipment to monitor and maintain pressure.
Numerous studies have utilized this model in a variety of cell types to examine the effect of elevated hydrostatic pressure on ocular tissues. Exposure of optic nerve head astrocytes identified 744 differentially expressed genes in response to elevated hydrostatic pressure (60 mmHg) over time.\textsuperscript{171,172} Interestingly, among the most responsive genes were Rho specific guanine nucleotide exchange factors, inducible nitric oxide synthase, and R-smad3.\textsuperscript{173} Additionally, elevated hydrostatic pressure (70 mmHg x 24h) inhibits secretion of interleukin (IL)-6 from rat retinal astrocytes.\textsuperscript{174} In rat retinal microglia, however, IL-6 secretion is markedly enhanced in response to elevated hydrostatic pressure demonstrating that responses to changes in hydrostatic pressure are cell type specific. Human TM cells respond to elevated hydrostatic pressure by concomitantly increasing adenylyl cyclase activity and altering their actin cytoskeleton.\textsuperscript{175,176} The effect of elevated hydrostatic pressure on TM cell gene expression and ECM turnover however, has yet to be elucidated.

Multiple studies have demonstrated that mitochondrial dysfunction and oxidative stress are early mediators of elevated hydrostatic pressure. When exposed to elevated hydrostatic pressure (60 or 100 mmHg), transformed RGC-5 cells increase expression of the antioxidant heme oxidase (HO)-1 in a dose dependent manner.\textsuperscript{177} Short-term exposure of RGC-5 cells to elevated hydrostatic pressure (100 mmHg) increases intracellular calcium followed by activation of caspase 3, leading to apoptosis.\textsuperscript{178} Similarly, culturing PC-12 cells (70 mmHg x 24h), glaucomatous TM cells (80 mmHg x 24h), or RGC-5 cells (30 mmHg x 72h) elicits a marked decrease in cellular ATP and a collapse of mitochondrial membrane potential while inducing apoptosis.\textsuperscript{179-181} Elevated hydrostatic
pressure most likely induces apoptosis through abnormal mitochondrial fission initiating increased release of optic atrophy type I (OPA1) and cytochrome c into the cytoplasm. In vitro studies demonstrating increased oxidative stress in response to elevated hydrostatic pressure parallel observations of oxidative damage found in glaucomatous eyes. Early reports found increased content of endothelial leukocyte adhesion molecule-1, a known cellular response to oxidative stress, in glaucomatous TM. More recently, an increase in end-product lipid peroxidation was found in patient SC and TM when compared to healthy controls. A separate study demonstrated increased oxidative DNA damage that positively correlated with increased IOP and visual field loss. Glaucomatous TM also exhibit increased deletion of mitochondrial DNA, indicative of mitochondrial failure. Finally, AH from glaucomatous patients contains increased levels of malondialdehyde (a marker of oxidative stress) and enzymatic antioxidants, super oxide dismutase and glutathione peroxidase. The levels of malondialdehyde in patient AH also correlated positively with severe visual loss. It is clear that patients with POAG experience increased oxidative stress, most likely as a result of elevated IOP. However, the effect of increased oxidative stress on the conventional outflow pathway has yet to be elucidated. Due to diminished antioxidant defenses, the TM is highly sensitive to ROS. Thus, the TM may be uniquely susceptible to oxidative damage. In this regard, short-term (30 min) oxidative insult has been demonstrated to reduce cell binding to ECM components fibronectin collagen (types I and 4) and laminin leading to a reversible rearrangement of the actin cytoskeleton. Application of chronic oxidative insult inhibits the proteasome, promotes TM senescence, and reduces TM cell viability. Of particular relevance, TGF-β stimulates ROS production while simultaneously
decreasing antioxidant expression in various tissues. Elevated content of TGF-β2 in the AH therefore may very well contribute to the pathogenesis of POAG by increasing ROS in the TM. Further studies are currently being conducted by this laboratory to specifically determine the effect of elevated hydrostatic pressure and TGF-β2 on oxidative stress in the TM.
CHAPTER 2
MATERIALS AND METHODS

Human Trabecular Meshwork Cell Culture.

The use of human cadaver material by this laboratory is approved by the Edward Hines Jr. VA and Loyola University Chicago institutional review boards in compliance with the tenets of the Declaration of Helsinki. Fresh cadaver corneoscleral rims from healthy individuals were obtained (Eversight Illinois, Chicago, IL) at the time of corneal transplant and primary human TM cells are prepared using a collagenase-free procedure as described previously. Briefly, TM cells were cultured on Falcon Primaria flasks in Eagle’s Minimum Essential Medium containing 2 mM L-glutamine supplemented with 5% adult bovine serum, 10% fetal bovine serum, 0.1% gentamycin, 1% amphotericin B, and a mixture of essential and non-essential amino acids. The purity of primary human TM cell cultures typically exceeds 95%, as routinely determined by cell morphology (adherent flattened non-spindle shaped soma with prominent lamellipodia). Currently, there are no known cytochemical markers of TM cells. TM cell viability is typically >90% as determined by trypan blue (0.2%) dye exclusion. Primary TM cell cultures exhibiting more than 5% spindle-shaped morphologic characteristics are considered contaminated with fibroblasts and discarded. Individual TM cell lines were restricted to a maximum of 6 passages. Simian virus 40-transformed human TM cells
from a healthy donor (NTM5) and a glaucomatous donor (GTM3) were acquired from Alcon Laboratories (Fort Worth, TX) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM GlutaMAX-I supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, and 1% amphotericin B. TM cultures were maintained at 37°C under an atmosphere of 95% air/5% CO₂.

**Experimental Conditions.**

Upon reaching confluence, cells were cultured x 24h in fresh serum-free DMEM media supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin, and 1% amphotericin B, and subsequently challenged with TGF-β2 (Cell signaling: 1-5 ng/ml) for up to 48h. In some experiments, cells were pretreated with C3 exoenzyme (Cytoskeleton, 10 µg/ml) or with actinomycin D (Sigma-Aldrich, 1 µg/ml) x 30 min as indicated. To determine the effect elevated hydrostatic pressure elicits on gene expression in TM cells, confluent serum-starved TM cells were placed in a custom made humidified chamber (Figure 7A) pre-warmed to, and maintained at, 37°C and pre-charged with an atmospheric mixture of 95% air/5% CO₂. Once the chamber was sealed, the hydrostatic pressure within the chamber was elevated by allowing a small amount of additional compressed air mixture to enter the sealed chamber. To measure hydrostatic pressure in real-time, one end of communicating polyethylene tubing (0.034 x0.050 in) was fastened to an exposed (no lid), mid-level positioned, 100 mm petri dish containing an equivalent volume of sterile saline. The other end of the tubing was fastened to an internal stainless steel communicating port that traversed the chamber wall. An additional piece of polyethylene tubing (0.034 x 0.50 in) was fastened to the external end of the stainless
steel communicating port and the remaining open end attached to an APT300 pressure transducer (Harvard Apparatus). Hydrostatic pressure was continuously monitored in real-time and recorded (Figure 7B) every 30s with an available PowerLab 8/35 high-performance data acquisition system supplied with LabChart Pro software. Cells were cultured at elevated hydrostatic pressures of 15, 30, or 45 mmHg above ambient (1 atmosphere/760 mmHg) pressure x 1.5h, 3h, 6h, or 48h. For comparison, control cells were cultured in an identical manner in a tissue culture incubator at 37°C (ambient pressure) under a humidified atmosphere of 95% air/5% CO₂. No significant change in media pH was observed between elevated and ambient controls. In some experiments, cells were pretreated with various receptor antagonists AMG-9810 (Sigma-Aldrich, 1 µM), BQ-123 (Sigma-Aldrich, 1 µM), BQ-188 (Sigma-Aldrich, 1 µM), HC-06747 (Sigma-Aldrich, 1 µM), or SB-431542 (Sigma-Aldrich, 1 µM) x 30 min as indicated. Alternatively, cells were pretreated with antioxidants trolox (Sigma-Aldrich, 100 µM) or resveratrol (Cayman Chemical, 20 µM) x 24h prior to treatment. Higher doses of resveratrol were noted to be toxic to GTM3 cells as indicated by MTT and LDH assays.

![Figure 7. Hydrostatic Pressure Chamber.](image)

(A) Custom built chamber used to expose culture cells to elevated hydrostatic pressure. (B) Representative sustainable pressure load on culture GTM3 cells as a function of time.
siRNA-Targeted Knockdown.

Semi-confluent cultures of human TM cells were transfected x 24h with commercially available silencer siRNA (Ambion/ThermoFisher Scientific) directed against R-Smad2 (25 nM), R-Smad3 (10nM), or a scrambled sequence (25 nM; negative control) using lipofectamine RNAiMAX reagent in a 1:1 mixture of Opti-MEM and DMEM lacking serum, antibiotics, and antimycotics. Alternatively, primary human TM cell cultures were reverse-transfected x 8h in the same manner. Following transfection, culture media was replaced with serum-free DMEM containing 100 U/ml penicillin, and 100 µg/ml streptomycin, and 1% amphotericin B, and cells were incubated in the absence or presence of TGF-β2, as indicated.

Real-Time RT-PCR.

Total RNA was extracted from human TM cells with TRIzol reagent and 1 µg was reverse-transcribed using iScript Reverse Transcription Supermix (BioRad) as previously described. Human specific oligonucleotide primers (Table 1) were amplified by real-time PCR using either a Mini-Opticon or CFX Connect Real-Time PCR detection system. For each sample, the specificity of the real-time reaction product was determined by melt curve analysis. Reaction efficiencies for each transcript were between 90 and 110%. Relative fold-changes in mRNA expression were normalized to GAPDH. The expression profile of GTM3 cells exposed to hydrostatic pressure was measured using a predesigned “POAG” microplate array (Bio-Rad) according to the manufacturer’s instructions.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Protocol</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK B1R</td>
<td>5’-ATCTGGGTGTTTGCTTGGGCCG-3’, 3’-AGGCCAGGATGCTGTAGTTG-5’</td>
<td>94°C x 5 min, (94°C x 60 s, 62°C x 45 s, 72°C x 10 s) x 35</td>
<td>435</td>
</tr>
<tr>
<td>BK B2R</td>
<td>5’-ACACAGGGCCAGTCATCAGCAC-3’, 3’-CATGCAATGGCCGCTCTTGC-5’</td>
<td>95°C x 5 min, (95°C x 30 s, 60°C x 60 s, 72°C x 30 s) x 30</td>
<td>143</td>
</tr>
<tr>
<td>Coll1a</td>
<td>5’-CTAAAGGCCAACCTTGGTGT-3’, 3’-TCCAGGACACAACTATTAC-5’</td>
<td>95°C x 5 min, (95°C x 30 s, 60°C x 45 s, 72°C x 30 s) x 40</td>
<td>107</td>
</tr>
<tr>
<td>Coll1a2</td>
<td>5’-GAAGGAAAGAGAGGCCCATC-3’, 3’-TCCAGGACAGACATTAC-5’</td>
<td>95°C x 5 min, (95°C x 30 s, 60°C x 45 s, 72°C x 30 s) x 40</td>
<td>96</td>
</tr>
<tr>
<td>Coll2a</td>
<td>5’-CAGGATGGGCGAGTTATAATG-3’, 3’-GAGGCGACACCTTGCATT-5’</td>
<td>95°C x 5 min, (95°C x 30 s, 60°C x 45 s, 72°C x 30 s) x 40</td>
<td>885</td>
</tr>
<tr>
<td>Coll4a</td>
<td>5’-CAGGCGCTAAAGGAGTATAATG-3’, 3’-GAGGCGACACCTTGCATT-5’</td>
<td>95°C x 5 min, (95°C x 30 s, 60°C x 45 s, 72°C x 30 s) x 40</td>
<td>115</td>
</tr>
<tr>
<td>Coll6a</td>
<td>5’-GAAGGAAAGAGAGGCCCATC-3’, 3’-TCCAGGACAGACATTAC-5’</td>
<td>95°C x 5 min, (95°C x 30 s, 60°C x 45 s, 72°C x 30 s) x 40</td>
<td>110</td>
</tr>
<tr>
<td>CTGF</td>
<td>5’-CTCTCGAGCGTCTAGAAGGC-3’, 3’-GATGCACCTTGGCTCT-5’</td>
<td>94°C x 5 min, (94°C x 10 s, 60°C x 40 s) x 40</td>
<td>94</td>
</tr>
<tr>
<td>ET A</td>
<td>5’-GTGGAACAGAAAGAGGACGC-3’, 3’-ATTCACTTGGCTCTGTC-5’</td>
<td>95°C x 5 min, (95°C x 10 s, 60°C x 60 s) x 40</td>
<td>181</td>
</tr>
<tr>
<td>ET B</td>
<td>5’-TCACTGGCTGAGCTTATTGCT-3’, 3’-AGCAATGGTCCAGATAACTCCA-5’</td>
<td>95°C x 5 min, (95°C x 10 s, 60°C x 60 s) x 40</td>
<td>206</td>
</tr>
<tr>
<td>FN</td>
<td>5’-AGCGGACCTACCTAGGCAAT-3’, 3’-GGTGTTCGATGGTCACT-5’</td>
<td>95°C x 5 min, (95°C x 30 s, 60°C x 45 s, 72°C x 45 s) x 40</td>
<td>222</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-TCCACACCTGTTGCTTAA-3’, 3’-ACCACAGCTGACATC-5’</td>
<td>94°C x 5 min, (94°C x 30 s, 62°C x 30 s, 72°C x 30 s) x 30</td>
<td>452</td>
</tr>
<tr>
<td>ppET-1</td>
<td>5’-TATCAGCAAGTGATGAGG-3’, 3’-CGAAGGCTGTCACAAATGTC-5’</td>
<td>94°C x 5 min, (94°C x 30 s, 58°C x 45 s, 72°C x 30 s) x 40</td>
<td>181</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>5’-GGACTTTATAGGTTCCTGTA-3’, 3’-GCCCCACCTACAGACCC-5’</td>
<td>94°C x 5 min, (94°C x 30 s, 59°C x 45 s, 72°C x 45 s) x 40</td>
<td>451</td>
</tr>
<tr>
<td>VEGF A</td>
<td>5’-TGCAATTGCTTGCTGTAGA-3’, 3’-TGCATTCAAGCTTGGCT-5’</td>
<td>95°C x 5 min, (95°C x 15 s, 60°C x 60 s) x 35</td>
<td>81</td>
</tr>
</tbody>
</table>

**Table 1. PCR Primers.** PCR primer sequences with corresponding protocols and amplicon product sizes.

**Western Immunoblot.**

Human TM cells were lysed in 2x Laemmli's sample buffer and lysates stored at -80°C until use. Cell culture media was collected and concentrated 2-fold by vacuum centrifugation and stored at -80°C until use. Proteins (10-30 µg per lane) in cell lysates or media were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto
nitrocellulose membranes as previously described. Membranes were blocked (5% BSA or 5% Blotto) and incubated overnight at 4°C in the presence of primary antibodies (Table 2). Washed (TBS supplemented with 0.05% Tween-20) membranes were incubated x 1h at 23°C in a 1:10,000 dilution of peroxidase-conjugated donkey anti-goat, goat anti-mouse, or goat anti-rabbit IgG secondary antibody, respectively. Following a final washing step, immunostained proteins were visualized by picoECL.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK B2</td>
<td>BD Biosciences</td>
<td>1:1000</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>CTGF</td>
<td>Santa Cruz</td>
<td>1:500</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Cell Signaling</td>
<td>1:10,000</td>
</tr>
<tr>
<td>pSmad2</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Smad2</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>pSmad3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Smad3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2. Primary Antibodies. List of primary antibodies, source, and dilutions used for Western Immunoblot.

Cell-Based ELISA:

The content of B2 receptor expressed by human TM cells was quantified using a cell-based ELISA as described previously. Confluent cultures of human TM cells were fixed with 4% paraformaldehyde at 23°C x 10 minutes. Fixed cells were subsequently permeabilized with 0.1% Triton X-100 and blocked x 1h at 23°C with 1% BSA in PBS and subsequently incubated overnight at 4°C in the presence of mouse anti-human B2 receptor (BD Bioscience, 1/50 dilution) primary antibody. Immunostained cells were incubated x 1h at 23°C with a 1/1000 dilution of horseradish peroxidase-
conjugated goat-anti mouse secondary antibody. Color development was initiated with SigmaFast OPD substrate solution x 30 min at 23°C. Color development was stopped with the addition of 3N HCl and quantified at 492 nm. Relative expression of B2 receptor was normalized to cell density. Cell density was determined by colorimetric staining with Janus Green. Briefly, washed cells were treated with 0.5 N HCl x 10 min at 23°C and optical density was measured at 595 nm. All groups were normalized to vehicle controls.

**Enzyme Linked Immunosorbent Assay.**

The content of active or total TGF-β2 or total ET-1 released into cell culture media were quantified using commercially-available ELISA kits (R&D Systems) as previously described.\(^3,60\) Cell culture media was cleared by centrifugation (700g x 5 min) and assayed according to manufacturer’s instruction. Latent (total) TGF-β2 was activated to by the addition of 1N HCL and subsequently neutralized with 1.2N NaOH/0.5M HEPES. The optical density was read at 450 nm with a 540nm correction.

**Lactate Dehydrogenase Assay.**

Lactate dehydrogenase released by human TM cells into the culture media was quantified as previously described.\(^196\) Aliquots (50 µl) of collected media were added to 96 well plates and incubated with an equal volume of assay buffer (2 mM iodonitrotetrazolium chlorium, 3.2 mM β-nicotinamide adenine dinucleotide sodium salt, 160 mM lithium lactate, 15 µM 1-methoxyphenazine methosulfate in 0.2M Tris-HCl) x 1h at 23°C in the dark. 1 N acetic acid (50 µl) was added and formation of the colored
formazan product was measured spectrophotometrically by measuring absorbance at 490 nm.

**Cell Viability.**

Human TM cell viability was assessed as a function of mitochondrial redox potential using an MTT cell proliferation assay. Confluent human TM cells were incubated x 2h at 37°C in 96-well plates containing 100 µl per well of a 1.2 mM water soluble solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) prepared in HEPES-buffered (10 mM, pH 7.4) Hank’s balanced salt solution (HBSS) containing calcium and magnesium. Following incubation, supernatant was removed and insoluble formazan product was solubilized by the addition of dimethyl sulfoxide (DMSO). Absorbance was read at 570 nM.

**Immunocytochemistry.**

Hydrostatic pressure-mediated changes in F-actin formation and stabilization were visualized as previously described. Human TM cells were fixed in freshly prepared phosphate-buffered (10 mM, pH 7.4) 4% paraformaldehyde (PFA) x 10 min at 23°C followed by incubation x 20 min with 165 nM phalloidin 488 (ThermoFisher Scientific). Cells were counterstained with 4’,6-diamidino-2-phenyindole (DAPI) nuclear acidic stain and imaged utilizing a Leica TCS SPE confocal microscope and imaging suite.
Porcine Anterior Segment Perfusion.

Fresh porcine globes were procured from a local abattoir (Park Packing, Chicago, IL) and anterior segments prepared within 4-6h as described previously. Whole globes were briefly immersed in 5% povidone-iodine solution and subsequently dipped in sterile PBS containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% amphotericin B. Aseptic globes were bisected at the equator into anterior and posterior segments. The posterior segment containing the vitreous body and the retina was discarded. The iris, lens and adjacent ciliary bodies were carefully dissected away from the anterior segment and the remaining segment containing the TM, cornea, and surrounding sclera were mounted onto a custom-made stage forming a sealed-circuit chamber (Figure 8), with the TM serving as the sole outflow facility. Two cannulas communicate within the chamber, one for media exchange and a second for real-time monitoring of intraocular pressure (IOP). Pairs of prepared and mounted anterior segments were continuously perfused at a constant flow rate of either 4.5 or 9 µl/min using DMEM supplemented with 100 u/ml penicillin, 100 µg/ml streptomycin, and 1% amphotericin B according to previously reported methods. Segments were allowed to stabilize during the first 24h and cultured for an additional 24h. IOP is monitored and recorded in real-time using an available PowerLab 8/35 high-performance data acquisition system supplied with LabChart Pro software. IOP data are reported as mean mmHg ± SEM. Following perfusion, porcine TM was harvested and changes in gene expression were analyzed by qRT-PCR as described above.
Figure 8. Porcine Anterior Segment Perfusion (A) Schematic representation of porcine anterior segment perfusion apparatus. (B) Custom made stage prior to, and after (C) mounting of porcine segment.
CHAPTER THREE

RESULTS

TGF-β2 Selectively Alters Matricellular and ECM Gene Expression in Primary and Transformed Human TM Cells.

Previously published studies from our laboratory confirm a causal role of biologically active TGF-β2 in elevating IOP in patients with POAG.\(^{60,86,99}\) While not fully understood, the mechanism by which TGF-β2 elevates IOP is thought to involve altered expression and secretion of matricellular and structural ECM proteins. Challenging transformed (GTM3, NTM5) or primary human TM cells with biologically active TGF-β2 (5 ng/ml, x 24h) markedly increased (2-6 fold) the mRNA content of the CTGF (Table 3) when compared with vehicle (200 nM HCl) treated controls, consistent with previously published studies.\(^{47}\) Given that CTGF is a critical downstream regulator of TGF-β2 mediated signaling, this matricellular protein has also been strongly implicated in the pathogenesis of various fibrotic diseases, including POAG.\(^{120,121}\) In addition, exogenous TGF-β2 also markedly and selectively enhanced (2-12 fold) the mRNA expression of several structural ECM protein subunits, including Col1α1, Col1α2, and Col4, in both transformed and primary human TM cells (Table 3). Not all structural ECM protein subunits, however, were altered by TGF-β2 treatment. While human TM
cells expressed measurable levels of Col6, this subtype was unaffected by TGF-β2 treatment. By comparison, primary human TM cells responded to TGF-β2 treatment by notably increasing (2.25-fold) fibronectin (FN) mRNA content (Table 3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA content (% veh)</th>
<th>hTM N</th>
<th>mRNA content (% veh)</th>
<th>NTM5 N</th>
<th>mRNA content (% veh)</th>
<th>GTM3 N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ECM Component</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col1α1</td>
<td>290 ± 19%*</td>
<td>4</td>
<td>268 ± 4%*</td>
<td>3</td>
<td>1219 ± 30%*</td>
<td>6</td>
</tr>
<tr>
<td>Col1α2</td>
<td>238 ± 14%*</td>
<td>4</td>
<td>234 ± 16%*</td>
<td>3</td>
<td>400 ± 64%*</td>
<td>6</td>
</tr>
<tr>
<td>Col2α1</td>
<td>135 ± 50%</td>
<td>4</td>
<td>51 ± 21%*</td>
<td>3</td>
<td>74 ± 10%</td>
<td>6</td>
</tr>
<tr>
<td>Col4</td>
<td>190 ± 17%*</td>
<td>4</td>
<td>341 ± 67%*</td>
<td>3</td>
<td>1043 ± 163%*</td>
<td>6</td>
</tr>
<tr>
<td>Col6</td>
<td>83 ± 25%</td>
<td>4</td>
<td>100 ± 12%</td>
<td>3</td>
<td>91 ± 17%</td>
<td>6</td>
</tr>
<tr>
<td>FN</td>
<td>225 ± 35%*</td>
<td>4</td>
<td>60 ± 21%</td>
<td>3</td>
<td>133 ± 4%*</td>
<td>6</td>
</tr>
<tr>
<td><strong>Matricellular Proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTGF</td>
<td>383 ± 27%*</td>
<td>4</td>
<td>194 ± 25%*</td>
<td>3</td>
<td>586 ± 75%*</td>
<td>6</td>
</tr>
<tr>
<td>ppET-1</td>
<td>238 ± 23%*</td>
<td>4</td>
<td>852 ± 103%*</td>
<td>3</td>
<td>1184 ± 26%*</td>
<td>6</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>130 ± 21%</td>
<td>4</td>
<td>116 ± 15%*</td>
<td>3</td>
<td>130 ± 35%</td>
<td>6</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>107 ± 26%</td>
<td>4</td>
<td>134 ± 23%</td>
<td>3</td>
<td>90 ± 11%</td>
<td>6</td>
</tr>
<tr>
<td><strong>Receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK B1</td>
<td>43 ± 9%*</td>
<td>5</td>
<td>48 ± 7%*</td>
<td>5</td>
<td>36 ± 14%*</td>
<td>5</td>
</tr>
<tr>
<td>BK B2</td>
<td>24 ± 15%*</td>
<td>5</td>
<td>29 ± 27%*</td>
<td>5</td>
<td>40 ± 14%*</td>
<td>5</td>
</tr>
<tr>
<td>ET A</td>
<td>17 ± 13%*</td>
<td>4</td>
<td>43 ± 6%*</td>
<td>3</td>
<td>61 ± 13%*</td>
<td>3</td>
</tr>
<tr>
<td>ET B</td>
<td>nd</td>
<td>4</td>
<td>nd</td>
<td>3</td>
<td>nd</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3. TGF-β2 alters gene expression in human TM cells. Primary (hTM) or transformed (GTM3, NTM5) TM cells were conditioned in serum-free media (x24h), and subsequently cultured in the absence (200 mM HCl) or presence of TGF-β2 (5 ng/ml, x24h). Relative mRNA content was quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes expressed as the mean ± SEM (N=3-6). *p <0.05 compared to vehicle control, unpaired Student’s t-test.

nd: not detectable

Transformed TM cells, however, only marginally (or not all) altered FN mRNA content following treatment with exogenous TGF-β2. The expression of the structural ECM protein subunit Col2α1 and matricellular proteins TGF-β2 and vascular endothelial
growth factor (VEGF)-A was not appreciably affected by TGF-β2 treatment in primary or transformed human TM cells.

Given that AH harvested from some POAG patients also contains elevated levels of ET-1,94,96 we investigated a possible mechanistic link exists between TGF-β2 signaling and ET-1 expression. Primary human TM cells challenged with TGF-β2 exhibited a 2.4-fold increase in ppET-1 mRNA content that was even more robustly (8-12 fold) observed in transformed human TM cell lines. While TGF-β2 mediated increases in secreted ET-1 were not quantified here, previously studies from our laboratory have demonstrated that TGF-β2 increases both the synthesis and secretion of ET-1 from human TM cells.99 Concomitantly, TGF-β2 treatment decreased, by as much as 80%, ET_A receptor mRNA content (Table 3) in primary and transformed human TM cells. ET_B receptor mRNA, however, was not appreciably expressed in human TM cells consistent with previous reports.108 The expression of the ET_B receptor protein by cultured human TM cells, however, has been reported.107,108

TGF-β2 Attenuates Constitutive Bradykinin Receptor Expression in Primary and Transformed Human TM Cells.

BK, a short-lived nonapeptide known for its potent vasoactive properties, is a well-documented regulator of IOP whose activity may very well be influenced by TGF-β2 signaling. Activation of the B2 receptor lowers IOP in a number of experimental models of glaucoma.29,130,131 Given the hypotensive efficacy of BK and its importance as a potentially valuable therapeutic option, we investigated the effect TGF-β2 signaling elicits on kallikrein-kinin system in human TM cells. Recently, Sharif et al. reported that
TGF-β2 reduces the expression of B2 receptor mRNA in cultured human TM cells. In our hands, primary and transformed human TM cells similarly responded to TGF-β2 (5 ng/ml, x24h) by reducing the constitutive expression of both B1 and B2 receptor mRNA by as much as 75% (Table 3). Importantly, TGF-β2 (5 ng/ml, x24h) also elicited a 60% reduction in constitutive expression of B2 receptor protein content in human TM cells, when compared to vehicle controls (Figure 9C, D). The effect of TGF-β2 was both dose- and time-dependent. Whereas relatively low doses of TGF-β2 (1-2.5 ng/ml, x24h) were without effect on B2 receptor content, a higher dose (5 ng/ml) was seen to significantly attenuate B2 receptor protein expression (Figure 9A).

**Figure 9. TGF-β2 reduces constitutive expression of B2 receptor protein.** Transformed (GTM3) TM cells were conditioned in serum-free media (x24h), and subsequently cultured in the absence (200 nM HCl) or presence of TGF-β2 (1-5 ng/ml, 0-48h) as indicated. (A, B) Relative expression of the B2 receptor in GTM3 cells treated with TGF-β2 (0-5 ng/ml, x0-48h) as indicated. (C) Western Immunoblot and (D) quantitative comparison of B2 receptor in cell lysates from both GTM3 and NTM5 cell lines. Data shown are normalized changes from 2 independent experiments and expressed as the mean ± SEM (N=5-8). * p <0.05 relative to vehicle control, One-way ANOVA with a Bonferroni’s post hoc analysis; ** p <0.05 relative to vehicle control, Student’s t-test.
The effect of TGF-β2 (5 ng/ml) on B2 receptor protein expression was not immediate, taking 24h to first manifest itself and persisting through 48h (Figure 9B). Unlike the constitutively expressed B2 receptor, the B1 receptor is inducible, typically expressed at the protein level in response to trauma or inflammation. In vitro, treating human TM cells with B1 receptor specific agonists fails to elicit a functional response and, to date, the B1 receptor has not been localized, at the protein level, to any ocular tissues. In our hands, B1 receptor protein was not expressed to a measurable extent in either primary or transformed human TM cells.

**TGF-β2 Utilizes the Canonical Smad3-dependent Pathway to Attenuate B2 Receptor Expression.**

Previous studies from our lab, and others, have identified a unique non-canonical role for the Rho subfamily of small monomeric GTPases in mediating TGF-β2 downstream signaling events involved in regulating cytoskeletal organization. In addition, RhoA has also been shown to play a role in regulating gene transcription, although the mechanism of this regulation is not fully understood. To determine if the Rho GTPase subfamily of proteins play a role in TGF-β2 mediated decreases in B2 receptor content, primary or transformed TM cells were pretreated (x1h) with vehicle (dH2O) or C3 exoenzyme (10 µM) and subsequently cultured in the absence or presence of TGF-β2 (5 ng/ml, x24h). As we previously observed, TGF-β2 markedly attenuated B2 receptor mRNA content by as much as 90% in primary or transformed GTM3 cells (Table 4). Pre-treating cells with C3 exoenzyme (a cell permeable pan inhibitor of the Rho GTPases) did not, however, prevent TGF-β2 from attenuating B2 receptor mRNA
content in human TM cells. These findings suggest that TGF-β2 elicits a decrease in B2 receptor expression by a mechanism that occurs independently of non-canonical Rho GTPase signaling.

<table>
<thead>
<tr>
<th>TM Cell Type</th>
<th>Treatment</th>
<th>BK B2 receptor mRNA content</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTM (primary)</td>
<td>Vehicle</td>
<td>100 ± 27%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>TGF-β2 (5 ng/ml)</td>
<td>8 ± 2%*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C3 + TGF-β2 (5ng/ml)</td>
<td>10 ± 3%*</td>
<td>3</td>
</tr>
<tr>
<td>GTM3 (transformed)</td>
<td>Vehicle</td>
<td>100 ± 24%</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>TGF-β2 (5 ng/ml)</td>
<td>58 ± 25%*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C3 + TGF-β2 (5ng/ml)</td>
<td>43 ± 8%*</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 4. TGF-β2 attenuates B2 receptor expression independently of Rho GTPase signaling.

Targeted, siRNA mediated knockdown of Smad2 or Smad3 expression was next utilized to determine the role of the canonical R-Smad dependent signaling pathway in mediating TGF-β2 decreases in B2 receptor content. Primary or transformed human TM cells were transfected with either Smad2 (25 nM), Smad3 (10 nM), or control scrambled (25 nM) siRNA and subsequently cultured in the absence or presence of TGF-β2 (5 ng/ml, x24h). In the presence of control scrambled siRNA, TGF-β2 reproducibly and significantly attenuated constitutive B2 receptor mRNA and protein expression (Figure 10A, B). In contrast, transfecting TM cells with Smad3 siRNA completely prevented TGF-β2 mediated decreases in both B2 receptor mRNA and protein content (Figure 10A, B). Unexpectedly, transfecting TM cells with Smad2 slightly attenuated TGF-β2
mediated decreases in B2 mRNA, although not to a statistically significant level. By comparison, selective knockdown of Smad2, however, had no effect on TGF-β2 mediated decreases in B2 receptor protein expression. (Figure 10A, B).

**Figure 10. TGF-β2 mediated attenuation of the B2 receptor selectively involves the canonical Smad3-dependent pathway.** Primary (hTM) or transformed (GTM3) TM cells were transfected (x24h) in serum-free media with control scrambled (25 nM), Smad2 (25 nM) or Smad3 siRNA (10 nM), and subsequently cultured in the absence (200 nM HCl) or presence of TGF-β2 (5 ng/ml, 24h), as indicated. Relative content of B2 receptor (A) mRNA and (B) protein was quantified by qRT-PCR and cell based ELISA, respectively. (C) siRNA knockdown of Smad2 and Smad3 was confirmed via Western immunoblot. Data shown are GAPDH-normalized fold changes from 1-2 experiments and expressed as the mean ± SEM (N=2-6). * p <0.05, One-way ANOVA with a Bonferroni’s post hoc analysis.
As shown in Figure 10C, transfecting human TM cells with Smad2 or Smad3 siRNA nearly abolished the expression of total and phosphorylated Smad2 or total and phosphorylated Smad3 proteins, respectively. Moreover, knockdown of either Smad2 or Smad3 did not elicit a compensatory change in the expression level of the other R-Smad (Figure 10C). Selective knockdown of either R-smad in the absence of TGF-β2 elicited similar results (data not shown). Collectively, these findings demonstrate that TGF-β2 elicits a decrease in the expression of the B2 receptor in human TM cells by selectively activating the canonical Smad3 dependent pathway. Whereas both Smad2 and Smad3 are measurably expressed within human TM cells, the mechanism by which TGF-β2 selectively recruits Smad3 in this process remains unclear.

TGF-β2 may selectively degrade B2 receptor expression in human TM cells by invoking one of many possible pathways, including altered expression of the B2 receptor gene, altered stability of B2 receptor mRNA, or even facilitation of B2 receptor protein sequestration and degradation. To determine which, if any, of these possible mechanisms are responsible, transformed human TM cells were conditioned in serum free media (x24h), pre-treated with the transcription inhibitor actinomycin D (1 µg/ml, x1h), and subsequently cultured in the absence (200 nM HCl) or presence of TGF-β2 (5 ng/ml) for up to 24h. Pre-treatment with actinomycin D elicited a time-dependent decrease in endogenous B2 receptor mRNA content with a qualitative half-life of approximately 3h (Figure 11). Challenging cells with TGF-β2 did not appreciably alter degradation of B2 receptor mRNA content, suggesting that TGF-β2 does not reduce B2 receptor protein levels by affecting B2 receptor mRNA stability. From these findings, we speculate that TGF-β2 most likely attenuates B2 receptor expression in human TM cells either by
inhibiting gene transcription or by facilitating the degradation of the B2 receptor protein. On-going studies using a variety of biochemical inhibitors including the protein synthesis inhibitor cycloheximide, autophagosomal/lysosomal proteolytic pathway inhibitors (ammonium chloride, chloroquine, 3-methyl adenine, wortmannin), and proteasomal inhibitors (lactacystin, epoxomicin) should help elucidate this mechanism (Von Zee and Stubbs, 2011). The functional consequence of decreased B2 receptor expression in the human TM has yet to be explored.

![Graph showing B2 receptor mRNA levels over time with and without TGF-β2]

**Figure 11.** TGF-β2 attenuates B2 receptor mRNA independently of mRNA stability. Transformed (GTM3) TM cells were conditioned in serum-free media (x24h), pretreated with actinomycin D (1 µg/ml, x30m), and subsequently cultured in the absence (200 nM HCl) or presence of TGF-β2 (5 ng/ml, x24h). Relative content of B2 receptor mRNA was quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes from 2 experiments and expressed as the mean ± SEM (N=6). * p <0.05, unpaired Student’s t-test.

**Elevated Hydrostatic Pressure Induces Cell Stress in Human TM Cells.**

The TM maintains IOP within physiologic levels (~15 mmHg) by dynamically responding to constantly changing hydrostatic pressures. Mechanisms that govern these responses directly influence AH outflow resistance through the TM by altering the actin cytoskeleton and changing ECM expression level or composition. While the
mechanism by which TGF-β2 increases AH outflow resistance through the TM has been well studied, the effect of sustained mechanical stress on the TM in response to elevated IOP is not as well understood. Chronic over activation of homeostatic mechanisms designed to maintain IOP elicits a permanent change in the morphology and extracellular milieu of the TM. Previous studies examining TM cell responses to mechanical stress have largely focused on the effect of physical stretch or fluid sheer stress on TM cells, while neglecting the role of elevated hydrostatic pressure in POAG. To better understand the effect elevated hydrostatic pressure may elicit on the conventional outflow pathway, human TM cells were conditioned in serum free media (x24h), and subsequently cultured at ambient (atmospheric 760 mmHg) or elevated (+30 mmHg above atmospheric) hydrostatic pressures.

Previous reports, conducted in other ocular cell types, showed that short-term (<24h) exposure to elevated hydrostatic pressure induces mitochondrial dysfunction, while more prolonged exposures (≥ 24h) often induce apoptosis. By comparison, culturing human TM cells under a controlled sealed atmosphere of 95% air / 5% CO₂ at elevated hydrostatic pressure (+30 mmHg, up to 12h) only marginally diminished mitochondrial function as quantified by MTT reduction (Figure 12A). In contrast, prolonged exposure of human TM cells to elevated hydrostatic pressure (24h) elicited a marked decrease in MTT reduction (51%), suggestive of mitochondrial dysfunction. Elevated hydrostatic pressure also elicited an initial modest increase in LDH secretion (up to 12h) from human TM cells that was similarly enhanced during prolonged (24h) exposure (Figure 12B), consistent with increased cell stress.
Figure 12. Elevated hydrostatic pressure induces cellular stress in transformed TM cells. GTM3 cells were conditioned in serum-free media (x24h) and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, 1.5-24h). Mitochondrial function and membrane permeability were assessed by (A) MTT reduction and (B) LDH release, respectively. In both cases, relative values were normalized to ambient pressure controls. Data shown are normalized changes expressed as the mean ± SEM (N=8). * p <0.05, Two-way ANOVA with a Bonferroni’s post hoc. (C) Western Immunoblot of total and cleaved Caspase 3. Levels of GAPDH are shown for comparison as loading controls.
MTT reduction and LDH release are often used as measures of cell viability. More accurately, however, these assays quantify NAD(P)H-dependent cellular oxidoreductase (mitochondrial metabolism) and plasma membrane integrity/permeability, respectively. To directly assess whether elevated hydrostatic pressure effects human TM cell viability, caspase 3 cleavage (an indicator of caspase 3 activation and apoptosis) was quantified by Western immunoblot. Levels of total caspase 3 in human TM cells exposed to elevated hydrostatic pressure (+ 30 mmHg, x1.5-24h) remained constant with no evidence of cleavage/activation (Figure 12C). In addition, no significant changes in cell densities were observed in response to elevated hydrostatic pressure at these time points (data not shown). Following prolonged (48h) exposure to elevated hydrostatic pressure, however, human TM cells exhibited a marked increase in the levels of cleaved caspase 3 (Figure 12C). Collectively, these findings suggest that exposing human TM cells to pathophysiological levels of elevated hydrostatic pressures time-dependently elicits cellular (possibly oxidative) stress leading to apoptosis. Further studies were limited to 24h hours in order to characterize TM cell responses to elevated hydrostatic pressure prior to the onset of apoptosis.

**Elevated Hydrostatic Pressure Alters the Actin Cytoskeleton in Human TM Cells.**

Elevated IOP is strongly associated with the enhanced formation of actin stress fibers, increasing rigidity and stiffness of the TM, ultimately leading to greater resistance to AH outflow.\(^8^8,\,90,\,99\) *In vitro*, either mechanical stretch or treatment with TGF-β2 increases actin stress fiber formation in human TM cells.\(^99,\,165\) The effect of elevated hydrostatic pressure on the actin cytoskeleton, however, has not been previously reported.
Exposing transformed human TM cells to elevated hydrostatic pressure (+30 mmHg, 3h or 24h) markedly increased phalloidin positive (filamentous-actin, F-actin) staining when compared to ambient pressure controls (Figure 13A-D). By comparison, the re-organization of F-actin elicited by exposure to elevated hydrostatic pressure was distinctly unique (appearing more punctate and nuclear) from that induced by exogenous TGF-β2 (Figure 13E, F). Human TM cells treated with TGF-β2 (5 ng/ml) at ambient pressures exhibited increased formation of actin stress fibers, consistent with previously published studies.99, 199

<table>
<thead>
<tr>
<th>Vehicle (ambient pressure)</th>
<th>Elevated Pressure (+30 mmHg)</th>
<th>TGF-β2 (5 ng/ml) (ambient pressure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>C.</td>
<td>E.</td>
</tr>
<tr>
<td>3h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.</td>
<td>D.</td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 13. Elevated hydrostatic pressure alters the actin cytoskeleton in transformed TM cells.](image)

Transformed (GTM3) TM cells were conditioned in serum-free media (x24h) and subsequently cultured at (A, B) ambient or (C, D) elevated pressure (+30 mmHg) for 3 or 24h, as indicated. (E, F) Shown for comparison are GTM3 cells treated with TGF-β2 (5 ng/ml, 3 or 24h) at ambient pressure. Filamentous actin was visualized with phalloidin (green) and counterstained with DAPI (blue).
Elevated Hydrostatic Pressure Selectively Alters Gene Expression in Human TM Cells.

In addition to remodeling the actin cytoskeleton, exposing human TM cells to elevated hydrostatic pressure may also elicit changes in gene transcription. To determine whether exposing TM cells to elevated hydrostatic pressure affects gene expression, transformed TM cells were conditioned in serum free media (x24h) and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, x6h). Using a commercially available pre-designed “POAG” nanoarray, we simultaneously measured changes in the expression of 16 different genes previously reported to be involved in the pathogenesis of POAG. The expression of 12 of 16 genes measured, including TGF-β1, was not significantly affected by exposure to elevated hydrostatic pressure (Figure 14).

Figure 14. Elevated hydrostatic pressure elicits selective changes in gene expression. Transformed (GTM3) TM cells were conditioned in serum-free media (x 24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, x 6h). Relative changes in mRNA content were analyzed by qRT-PCR utilizing a commercially available, pre-designed “POAG” nanoarray. Data shown are the mean fold-changes ± SEM (n=4) from two independent experiments. * p<0.05 relative to ambient pressure control, Student’s t-test.
Periostin (POSTN), a matricellular ECM protein involved in ECM remodeling, was below the level of detection in transformed TM cells cultured under either condition. By comparison, exposing TM cells to elevated hydrostatic pressure (+30 mmHg, x 6h) elicited statistically significant increases in cyclin dependent kinase inhibitor (CDKN) 1a (2.5-fold), VEGF- A (5.9-fold) and CTGF (9.9-fold), when compared to ambient pressure controls. These results demonstrate for the first time that elevated hydrostatic pressure selectively induces changes in gene expression in human TM cells.

Novel findings from this study demonstrate that TM cells respond to elevated hydrostatic pressure by upregulating CTGF, a known downstream regulator of TGF-β2 mediated gene expression signaling events (Table 3, Figure 14). To better understand the sequence of molecular events, the time course by which elevated hydrostatic pressure elicits selective changes in gene expression was characterized (Figure 15). When compared to ambient pressure controls, both primary and transformed human TM cells responded to elevated hydrostatic pressure by rapidly (<1.5h) increasing CTGF mRNA expression (Figure 15A, B) and by releasing (~6h) measurable levels of CTGF protein into the culture media both of which were sustained for 24h. By comparison, the enhanced expression of endogenous TGF-β2 mRNA induced by elevated hydrostatic pressure lagged behind that of CTGF mRNA expression, being first detectable at ~3h, peaking at 6h, and returning to baseline levels by 24h (Figure 16A). Release of total or active TGF-β2 into the culture media, however, continued to increase throughout the 24h assayed (Figure 16 B, C). Secreted TGF-β2 from primary human TM cells in response to elevated hydrostatic pressure was below the level of detection at all time points tested.
Figure 15. Elevated hydrostatic pressure increases CTGF mRNA content and protein secretion. Primary (hTM) or transformed (GTM3) human TM cells were conditioned in serum-free media (x24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, 1.5-24h). (A) Relative GAPDH-normalized changes in CTGF mRNA content were quantified by qRT-PCR. (B) Secreted CTGF was visualized by Western immunoblot. Data shown are from 1-2 separate experiments and expressed as the mean ± SEM (N=3-6). * p <0.05 relative to ambient pressure control. Two-way ANOVA with a Bonferroni’s post hoc analysis.
**Figure 16. Elevated hydrostatic pressure transiently increases TGF-β2 mRNA content and protein secretion.** Primary (hTM) or transformed (GTM3) cells were conditioned in serum-free media (x24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, 1.5-24h). (A) Relative GAPDH-normalized fold changes in TGF-β2 mRNA content was quantified by qRT-PCR. (B) Secreted total TGF-β2 and (C) active TGF-β2 were quantified by ELISA. Data shown are from 1-2 separate experiments and expressed as the mean ± SEM (N=3-6). * p <0.05 relative to ambient pressure control, Two-way ANOVA with a Bonferroni’s post hoc analysis.
Both primary and transformed TM cells also responded to elevated hydrostatic pressure by rapidly (<1.5h) increasing ppET-1 mRNA expression (Fig. 17A). Unlike CTGF expression, however, changes in ppET-1 mRNA levels were transient, returning to control levels by 24h. Secreted ET-1 levels peaked at ~6h before returning to the levels of ambient pressure control by 24h in both primary or transformed TM cells (Fig. 17B).

Figure 17. Elevated hydrostatic pressure transiently enhances ppET-1 mRNA and secreted ET-1 content. Primary (hTM) or transformed (GTM3) human TM cells were conditioned in serum-free media (x24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, 1.5-24h). (A) Relative GAPDH-normalized-fold changes in ppET-1 mRNA content were quantified by qRT-PCR. (B) Secreted ET-1 was quantified by ELISA. Data shown are from 1-2 separate experiments and expressed as the mean ± SEM (N=3-6). * p <0.05 relative to ambient pressure control, Two-way ANOVA with a Bonferroni’s post hoc analysis.
We also examined the effect of elevated hydrostatic pressure on the expression of bradykinin B1 or B2 receptors and endothelin A receptors, of which all three are constitutively expressed by human TM cells at the mRNA level.\textsuperscript{108, 130} Both primary and transformed human TM cells exposed to elevated hydrostatic pressure (+30 mmHg) exhibit a rapid (<1.5h) increase in B1 receptor mRNA content (Figure 18A). This effect was sustained in primary TM cells but transient in transformed cells. By comparison, a differential effect was observed for B2 receptor mRNA content. Primary TM cells responded to elevated hydrostatic pressure by inducing a rapid (<1.5h), yet transient, increase in B2 receptor mRNA expression (Figure 18B). Elevated hydrostatic pressure had no effect on B2 receptor expression in transformed TM cells, highlighting unique differences between primary and transformed human TM cells.

In contrast to changes in bradykinin receptor mRNA expression, both primary and transformed TM cells exposed to elevated hydrostatic pressure exhibit a time-dependent sustainable decrease in ET\textsubscript{A} receptor mRNA expression (Figure 18C). Endogenous expression of the ET\textsubscript{B} mRNA in primary or transformed TM cells, however, was unaffected by elevated hydrostatic pressure, remaining below the level of detection in either cell type.

Consistent with changes seen on our “POAG” nanoarray (Figure 14), elevated hydrostatic pressure also induced a transient increase in VEGF-A mRNA content, with this response being more robust in transformed TM cells (Fig. 18 D). Interestingly, the time course for pressure-induced changes in VEGF-A expression was qualitatively similar with that observed for TGF-β2. Earlier studies support a role for VEGF-A in the regulation of IOP\textsuperscript{200} as well as in the pathophysiology neovascular glaucoma, a secondary
glaucoma associated with neovascularization of the iris. Our findings are consistent with these reports. In addition, these findings suggest that elevated hydrostatic pressure may initiate neovascular changes within the TM, and particularly Schlemm’s canal, which may hinder AH outflow and contribute to elevated IOP in affected patients.

![Graphs showing time-dependent changes in gene expression](image)

Figure 18. Elevated hydrostatic pressure induces time-dependent changes in gene expression. Primary (hTM) or transformed (GTM3) human TM cells were conditioned in serum-free media (x24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, 1.5-24h). (A) Relative GAPDH-normalized fold changes in mRNA content was quantified by qRT-PCR (B) Secreted ET-1 was quantified by ELISA. Data shown are from 1-2 separate experiments and expressed as the mean ± SEM (N=3-6). * p <0.05 relative to ambient pressure control, Two-way ANOVA with a Bonferroni’s post hoc analysis.
As previously noted, human TM cells also exhibit marked changes in the expression of several ECM related genes following a TGF-β2 challenge (Table 3) or within TM tissue harvested from POAG patients. In vitro studies strongly support a causal role for both TGF-β2 and mechanical stretch in eliciting altered ECM content. We found that exposing primary or transformed human TM cells to elevated hydrostatic pressure differentially alters ECM mRNA content in a time-dependent manner (Figure 1). Primary TM cells exposed to elevated hydrostatic pressure exhibit rapid (<1.5h), yet transient, increases in Col1α1, Col2α1, Col4, and FN (Figure 19A, C, D, F) mRNA content. By comparison, expression of Col1α1, Col2α1, Col4, or fibronectin in transformed TM cells was largely unaffected by exposure (1.5h-24h) to elevated hydrostatic pressure. The expression of Col1α2 in both primary and transformed TM cells responding to elevated hydrostatic pressure was uniquely different, being reduced over the time-course of 24h (Figure 19B). Col6 expression remained unaltered in primary or transformed TM cells by exposure to elevated hydrostatic pressure for up to 12h However, prolonged exposure (x24h) of transformed TM cells to elevated hydrostatic pressure elicited a marked increase in Col6 mRNA expression (Figure 19E).

To gain a more complete understanding of the effect of elevated hydrostatic pressure on gene expression relevant to ECM remodeling, we utilized a commercially available, predesigned “ECM remodeling” nanoarray. Notably, in primary TM cells, elevated hydrostatic pressure (+30 mmHg, x 6h) elicited increased expression in only 3 of the 38 genes measured (Table 5). These genes included structural growth factors (Heparin-binding EGF-like growth factor, Insulin-like growth factor 2), proteases (MMP3), and inhibitors of proteases (SERPINE1) gene expression by human TM cells,
consistent with remodeling of the ECM in POAG. The expression of 24 of the genes measured was not significantly altered by exposure to elevated hydrostatic pressure while the remaining 11 were below the level of detection under either condition.

**Figure 19. Extracellular matrix mRNA expression is altered in response to elevated hydrostatic pressure.** Primary (hTM) or transformed GTM3 cells were conditioned in serum-free media (×24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, ×1.5-24h) as indicated. Relative mRNA content was quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes from 1-2 independent experiments and expressed as the mean ± SEM (N=3-6), *p <0.05 relative to ambient pressure control, Two-way ANOVA with a Bonferroni’s post hoc analysis.
Exposure to Clinically Relevant Hydrostatic Pressure Loads Increases ET-1, TGF-β2 and CTGF Expression as well as ET-1 Release.

Human TM cells respond to elevated hydrostatic pressure by altering their actin cytoskeleton and selectively and differentially changing their matricellular/ECM mRNA expression/stability (Figures 15-19, Table 5). The relationship between a given

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA content (x±SEM)</th>
<th>N</th>
<th>mRNA content (x±SEM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>100 ± 15%</td>
<td>3</td>
<td>86% ± 15%</td>
<td>3</td>
</tr>
<tr>
<td>ColIa1</td>
<td>100 ± 25%</td>
<td>3</td>
<td>134 ± 34%</td>
<td>3</td>
</tr>
<tr>
<td>ColIa2</td>
<td>100 ± 13%*</td>
<td>3</td>
<td>131 ± 13%</td>
<td>3</td>
</tr>
<tr>
<td>ColIa3</td>
<td>100 ± 15%</td>
<td>3</td>
<td>105 ± 16%</td>
<td>3</td>
</tr>
<tr>
<td>ColIa1</td>
<td>100 ± 24%</td>
<td>3</td>
<td>130 ± 28%</td>
<td>3</td>
</tr>
<tr>
<td>ColIa3</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>EGFR</td>
<td>100 ± 10%</td>
<td>3</td>
<td>100 ± 10%</td>
<td>3</td>
</tr>
<tr>
<td>ERBB</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>EZR</td>
<td>100 ± 31%</td>
<td>3</td>
<td>111 ± 34%</td>
<td>3</td>
</tr>
<tr>
<td>FN1</td>
<td>100 ± 18%</td>
<td>3</td>
<td>61 ± 11%</td>
<td>3</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>100 ± 18%</td>
<td>3</td>
<td>839 ± 153%*</td>
<td>3</td>
</tr>
<tr>
<td>IGFR1</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>IGFR2</td>
<td>100 ± 33%</td>
<td>3</td>
<td>123 ± 40%</td>
<td>3</td>
</tr>
<tr>
<td>IL6</td>
<td>100 ± 12%</td>
<td>3</td>
<td>513 ± 60%*</td>
<td>3</td>
</tr>
<tr>
<td>ITGB1</td>
<td>100 ± 17%</td>
<td>3</td>
<td>136 ± 23%</td>
<td>3</td>
</tr>
<tr>
<td>KLK3</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>LAMC2</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>MMP1</td>
<td>100 ± 16%</td>
<td>3</td>
<td>111 ± 18%</td>
<td>3</td>
</tr>
<tr>
<td>MMP2</td>
<td>100 ± 16%</td>
<td>3</td>
<td>63 ± 10%</td>
<td>3</td>
</tr>
<tr>
<td>MMP3</td>
<td>100 ± 18%</td>
<td>3</td>
<td>651 ± 116%*</td>
<td>3</td>
</tr>
<tr>
<td>MMP7</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>MMP9</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>MMP13</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>MMP14</td>
<td>100 ± 22%</td>
<td>3</td>
<td>119 ± 26%</td>
<td>3</td>
</tr>
<tr>
<td>MSN</td>
<td>100 ± 22%</td>
<td>3</td>
<td>88 ± 19%</td>
<td>3</td>
</tr>
<tr>
<td>PAI-4</td>
<td>100 ± 24%</td>
<td>3</td>
<td>293 ± 61%</td>
<td>3</td>
</tr>
<tr>
<td>PAI-2</td>
<td>100 ± 18%</td>
<td>3</td>
<td>145 ± 27%</td>
<td>3</td>
</tr>
<tr>
<td>PLAT</td>
<td>100 ± 35%</td>
<td>3</td>
<td>190 ± 65%</td>
<td>3</td>
</tr>
<tr>
<td>PLAU</td>
<td>100 ± 49%</td>
<td>3</td>
<td>139 ± 67%</td>
<td>3</td>
</tr>
<tr>
<td>PLAUR</td>
<td>100 ± 11%</td>
<td>3</td>
<td>110 ± 12%</td>
<td>3</td>
</tr>
<tr>
<td>PLG</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>SPARC</td>
<td>100 ± 15%</td>
<td>3</td>
<td>98 ± 16%</td>
<td>3</td>
</tr>
<tr>
<td>TIMP1</td>
<td>100 ± 15%</td>
<td>3</td>
<td>103 ± 21%</td>
<td>3</td>
</tr>
<tr>
<td>TIMP2</td>
<td>100 ± 15%</td>
<td>3</td>
<td>123 ± 19%</td>
<td>3</td>
</tr>
<tr>
<td>VCAN</td>
<td>100 ± 32%</td>
<td>3</td>
<td>71 ± 22%</td>
<td>3</td>
</tr>
<tr>
<td>VTN</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5. Elevated hydrostatic pressure elicits selective changes in the expression of genes relevant to ECM remodeling. Primary (hTM) cells were conditioned in serum-free media (x 24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, x6h). Relative changes in mRNA content were analyzed by qRT-PCR utilizing a commercially available, predesigned “ECM remodeling” nanoarray Data shown are the mean fold-changes ± SEM (N=3). * p<0.05 relative to ambient pressure control, Student’s t-test

ND = not detected
hydrostatic pressure load and the resulting cellular responses to such a challenge \textit{in vitro} must, however, be carefully considered when interpreting these findings. For example, previous studies have shown that exposing chondrocytes to low levels (2.5 MPa) of hydrostatic pressure elicits an increase in proteoglycan synthesis, while higher levels (7.5 MPa) of hydrostatic pressure were found to inhibit gene transcription.\textsuperscript{203, 204} \textit{In vivo}, human TM cells constantly experience small diurnal and pulsatile fluctuations in hydrostatic pressures much smaller than hydrostatic pressures typically applied in an experimental setting.\textsuperscript{205} Together, these reports raise important questions regarding whether human TM cells will respond to a more clinically relevant change in hydrostatic pressure and if various pressure loads/levels will elicit a differential response, similar to a pharmacological dose response to a given ligand.

To determine whether human TM cells respond differentially to clinically relevant and increasing levels of elevated hydrostatic pressure, cells were conditioned in serum free media and exposed (x 6h) to ambient or low (+15 mmHg), elevated (+30 mmHg), or high (+45 mmHg) hydrostatic pressures. Compared to ambient controls, exposing TM cells to changes in hydrostatic pressure low as 15 mmHg significantly upregulated TGF-β2, CTGF, and ppET-1 mRNA content (Figure 20 A-C) and ET-1 release (Figure 20E), consistent with previously observed changes (Figures 15-17). Expression and release of these matricellular proteins in response to hydrostatic pressure was maximal at 15 mmHg, with no further increase seen up to 45 mmHg tested (Figure 20 D, E). These cellular responses appeared to be selective, as release of total TGF-β2 was not significantly altered by exposure (x6h) to any level of increased hydrostatic pressure (Figure 20D) as previously observed (Figure 16).
Figure 20. Clinically relevant changes in hydrostatic pressure alter matricellular mRNA expression and ET-1 release. Transformed (GTM3) TM cells were conditioned in serum-free media (x24h), and subsequently cultured at ambient or elevated hydrostatic pressure (+15-45 mmHg, x6h) as indicated. (A-C) Relative mRNA content was quantified by qRT-PCR and normalized to GAPDH. (D) Total TGF-β2 and (E) secreted ET-1 were quantified by ELISA. Data shown are from 2 independent experiments and expressed as the mean ± SEM (N=6). * p <0.05 relative to ambient pressure control, Two-way ANOVA with a Bonferroni’s post hoc analysis.
Inhibition of ET<sub>B</sub> Receptor Activation Prevents Elevated Hydrostatic Pressure Mediated Increases in TGF-β2 and ET-1, but not CTGF, mRNA Expression.

Previously, we have reported that human TM cells respond to exogenous TGF-β2 (5 ng/ml, x24h) by robustly increasing both ppET-1 and CTGF mRNA expression.<sup>47,99</sup> These findings are consistent with CTGF being known as a key intermediate regulator of TGF-β2 dependent gene expression signaling pathways.<sup>124</sup> The mechanism(s) by which physiological/elevated hydrostatic pressure elicits selective increases in expression of these matricellular genes remains, however, remains unclear. Findings from our time-dependent expression and release studies (Figures 15-17) demonstrate that elevated hydrostatic pressure mediated increases in CTGF and ppET-1 mRNA expression and protein release occur prior changes in TGF-β2 content. Thus, the possibility that elevated hydrostatic pressure increases ppET-1 and CTGF mRNA expression due to TGF-β2 autocrine signaling appears unlikely. To confirm this supposition, TM cells were pre-treated (30 min) with 1 µM SB-431542, a TGF-βRII antagonist, and subsequently exposed to elevated hydrostatic pressure. Inhibition of TGF-βRII had no effect on pressure-induced changes in TGF-β2, CTGF, or ppET-1 mRNA expression (Figure 21).

An alternative mechanism may involve autocrine signaling by ET-1. Though we have yet to examine the effects of ET-1 stimulation on matricellular gene expression in quiescent human TM cells, previous findings demonstrate that pressure-induced increases in mRNA expression and release of ET-1 occur prior to changes in TGF-β2 content (Figures 15, 16). In addition, treatment of vascular smooth muscle cells with exogenous ET-1 has been shown to markedly enhance CTGF expression.<sup>206</sup>
Figure 21. Inhibition of ET<sub>B</sub> receptor activation prevents hydrostatic pressure mediated changes in TGF-β2 and ppET-1 mRNA content. Transformed (GTM3) TM cells were pretreated (x30min) with either vehicle (0.1% DMSO) or selective receptor antagonists of ET<sub>A</sub> (BQ-123), ET<sub>B</sub> (BQ-788), or TβRI (SB-431542), as indicated, and subsequently cultured at ambient or elevated pressure (+30 mmHg, x6h). Relative mRNA content was quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes from a representative experiment, repeated twice, and expressed as the mean ± SEM (N=3). * p <0.05, Student’s t-test relative to ambient pressure control. ** p<0.05, Student’s t-test relative to elevated hydrostatic pressure.
As such, it is plausible that ET-1 may very well play a role in elevating CTGF mRNA and protein content. To determine if ET-1 is acting in such an autocrine manner, transformed TM cells were cultured in serum free conditions (x24h), pretreated with vehicle or specific ET receptor antagonists (x30min), and subsequently exposed to ambient or elevated hydrostatic pressures (+30mmHg, x6h), as indicated (Figure 21). As previously demonstrated (Figures 15-17), elevated hydrostatic pressure markedly enhanced TGF-β2, CTGF, and ppET-1 mRNA expression (Figure 21 A-C). Pretreatment with an ET_A (BQ-123) receptor antagonist had no effect on elevated hydrostatic pressure mediated increases in TGF-β2, CTGF, or ppET-1 mRNA content. In contrast, pretreatment with an ET_B (BQ-188) receptor antagonist, or the combination of BQ-123 plus BQ-188, markedly attenuated elevated hydrostatic pressure mediated increases in both TGF-β2 and, surprisingly, ppET-1 mRNA (Figure 21 B, C). By comparison, BQ-188 had no effect on elevated hydrostatic pressure mediated increases in CTGF mRNA expression. These findings support a role for ET-1 autocrine signaling in elevated hydrostatic pressure mediated increases in TGF-β2 and ET-1, but not CTGF, mRNA expression. These observations, however, await confirmation using primary human TM cells.

**Canonical TGF-β2 Signaling is not Altered by Elevated Hydrostatic Pressure.**

It is a prevailing theory that increased levels of TGF-β2 in aqueous humor of some POAG patients aberrantly elevates IOP by acting upon the TM to increase actin stress fiber formation and altering ECM gene expression and protein remodeling.\(^{55, 58, 78}\) As shown in Figs. 15 and 16, elevated hydrostatic pressure, *in vitro*, robustly
increases the time-dependent expression and release of both TGF-β2 and CTGF. As a known regulator of TGF-β2 signaling, CTGF facilitates binding of TGF-β2 to TGF-βRII, promoting smad3 phosphorylation. \(^{210}\) Whereas autocrine signaling of endogenous TGF-β2 does not play a role in elevated hydrostatic pressure induced changes in CTGF or ppET-1 mRNA expression (Figure 21), we considered the possibility that elevated hydrostatic pressure itself may potentiate TM cell responses to exogenous TGF-β2 by enhancing canonical signaling. To determine if elevated hydrostatic pressure alters TGF-β2 signaling, transformed TM cells were serum starved (x24h), and treated with vehicle or TGF-β2 (5 ng/ml) at ambient or elevated hydrostatic pressure (+30 mmHg, x6h).

A.

![Western immunoblot](image)

**Figure 22. Elevated hydrostatic pressure does not alter canonical TGF-β2 signaling in human TM cells.** Transformed (GTM3) human TM cells were serum starved (x24h), and subsequently treated with vehicle (200 nM HCl) or TGF-β2 (5 ng/ml) at ambient or elevated pressure (+30 mmHg, x6h), as indicated. (A) TGF-β2 mediated smad3 phosphorylation was measured by Western immunoblot. (B) Relative changes in mRNA content were quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes expressed as the mean ± SEM (N =3) *p<0.05, Two-way ANOVA with a Bonferroni’s post hoc analysis.
When treated at ambient pressures, transformed TM cells responded to exogenous TGF-β2 by modestly increasing smad3 phosphorylation (Figure 22A) without measurably altering TGF-β2, CTGF, or ppET-1 mRNA content (Figure 22B). By comparison, while exposing TM cells to elevated hydrostatic pressure alone did not initiate smad3 phosphorylation, it did elicit a significant and very reproducible increase in TGF-β2, CTGF, and ppET-1 mRNA content (Figure 22B). The presence of exogenous TGF-β2 did not, however, alter TM cellular responses to elevated hydrostatic pressure (Figure 22B) confirming that canonical TGF-β2 signaling is not an early mediator in hydrostatic pressure mediated cellular responses.

**Elevated Hydrostatic Pressure Increases CTGF, TGF-β2, and ppET-1 mRNA Expression Independent of TRPV Mechanosensitive Ion Channels.**

The mechanosensitive ion channel TRPV 4 has been implicated in the regulation of AH outflow through the conventional pathway. In vitro, inhibition of TRPV4 prevents formation of actin stress fibers in TM cells induced by mechanical stress. Independent studies have also demonstrated the importance of TRPV1 in pressure-induced apoptosis in retinal ganglion cells. Activation of mechanosensitive ion channels, however, is heavily dependent upon shear forces that physically distort the plasma membrane and elicit a conformational change in these receptors. Given that hydrostatic pressure is an isotropic, non-deforming mode of physical stress, the role of TRP mechanosensitive ion channels in sensing and responding to elevated hydrostatic pressure remains unclear.
To determine if elevated hydrostatic pressure elicits changes in gene expression through activation of TRPV channels, transformed human TM cells were serum starved (x24), pretreated with vehicle or specific TRP channel inhibitors, and subsequently cultured at ambient or elevated hydrostatic pressure (+ 30mmHg, x6h), as indicated. As we have observed before, compared to ambient pressure controls, elevated hydrostatic pressure reproducibly increases the expression of TGF-β2, CTGF, and ppET-1 mRNA expression (Fig. 23). Pretreating (x30 min) TM cells with maximally effective concentrations of AMG-9810 (a selective TRPV1 antagonist) or HC-067047 (a TRPV4 antagonist) did not alter elevated hydrostatic pressure mediated changes in TGF-β2, CTGF, and ppET-1 mRNA expression (Fig. 23 A-C) Consistent with hydrostatic pressure being an isotropic, non-deforming mode of physical stress, these data strongly suggest that mechanosensitive TRP ion channels do not play a significant role in the mechanism by which transformed human TM cells respond to elevated hydrostatic pressures. The possibility that primary human TM cells invoke TRP channels in response to elevated hydrostatic pressure, however remote, remains to be evaluated.

**Elevated Hydrostatic Pressure Induced Cell Stress Independent of Oxidative Stress.**

It has been well demonstrated that aqueous humor from patients with POAG contains increased levels, compared with healthy controls, of superoxide dismutase and glutathione peroxidase as well as elevated levels of malondialdehyde (a marker of oxidative stress) in their TM.\(^{187, 188}\) More recently it was shown that TGF-β2 expression is regulated, at least in part, by ROS\(^{208}\)
Figure 23. Elevated hydrostatic pressure increases CTGF, TGF-β2, and ppET-1 mRNA expression independent of TRPV channel activation. Transformed (GTM3) human TM cells were pretreated x 30min with either vehicle (0.05% DMSO), TRPV 1 (AMG-9810), or TRPV 4 (HC-067047) channel antagonists, as indicated, and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, x6h). Relative mRNA content was quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes from a representative experiment, repeated once, and expressed as the mean ± SD (N=3). * p <0.05, * Student’s t-test relative to ambient pressure control.
Exposing retinal ganglion cells to high levels of elevated hydrostatic pressure, *in vitro*, induces mitochondrial dysfunction and enhances compensatory antioxidant expression. Similarly, previous findings observed here show increased cellular stress in TM cells exposed to elevated hydrostatic pressure (Figure 12). Collectively, these results suggest that elevated hydrostatic pressure mediated changes in TGF-β2, ET-1, and CTGF mRNA expression may result from increased cellular oxidative stress (Figure 15).

To determine whether oxidative stress plays a role in elevated hydrostatic pressure mediated events, transformed TM cells were serum starved (x24), pretreated (x24h) with vehicle or with the antioxidants trolox (a water-soluble analogue of α-tocopherol) or resveratrol (a stilbenoid polyphenol naturally enriched in the skin of grapes), and subsequently exposed to ambient or elevated hydrostatic pressure (+30mmHg) for up to 24h, as indicated. Pretreatment with antioxidants alone did not alter MTT reduction or increase LDH release (data not shown). Transformed TM cells pretreated with trolox and exposed to elevated hydrostatic pressure (1.5h-24h) exhibited changes in MTT reduction (Figure 24A), LDH secretion (Figure 24C), and TGF-β2, CTGF, and ppET-1 mRNA expression (Figure 24E) that were indistinguishable from ambient pressure controls (Figure 24A, C). By comparison, pretreatment with resveratrol modestly, yet significantly, protected TM cells against hydrostatic pressure-induced decreases in MTT reduction (Figure 24B) and increased LDH secretion (Figure 24D). Resveratrol pretreatment, however, failed to protect against elevated hydrostatic pressure mediated changes in TGF-β2, CTGF, and ppET-1 mRNA expression (Figure 21F).

Collectively, these findings argue against oxidative stress playing a major role in the
gene expression in human TM cells.

**Figure 24. Elevated hydrostatic pressure induces cell stress independent of oxidative stress.** Transformed (GTM3) TM cells were conditioned in serum free media (x 24h), pretreated (x30min) with either vehicle (0.1% DMSO) or antioxidants as indicated, and subsequently cultured at ambient or elevated hydrostatic pressure (+30 mmHg, x6h). Mitochondrial function and membrane permeability were quantified by (A, B) MTT res’tion and (C, D) LDH release, respectively. (E, F) Relative changes in CTGF, TGF-β2, and ppET-1 mRNA content were quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes from 1-2 and expressed as the mean ± SD (N=3-8). * p <0.05, One-way ANOVA with a Bonferroni’s post hoc analysis.
Physiological Consequences of Increased Perfusion Flow Rate on TM Tissue in situ.

It is well established that different models of mechanical stimuli elicit diverse cellular responses, including changes in gene expression.\textsuperscript{158,161} While our findings clearly show an experimental effect of elevated hydrostatic pressure on cultured human TM cells, the physiologic relevance of these \textit{in vitro} findings must be elucidated to fully understand the nature by which aberrantly elevated IOP impacts TM regulated outflow facility in patients with POAG. To address the physiological relevance of our findings, we examined the effect of increased fluid sheer stress on TM cellular responses \textit{in situ} using anterior segment perfusion. Porcine anterior segments were prepared as described in \textit{Methods and Materials} and allowed to stabilize at physiological perfusion flow rate of 4.5 µl/min. Following this stabilization period, anterior segments either continued to be perfused at physiologic flow rates (4.5 µl/min) or challenged by increasing the perfusion flow rate 2-fold (9 µl/min) for up to an additional 24h. At various times (1.5h, 6h, and 24h), the TM tissue was harvested from perfused anterior segments and changes in matricellular gene expression were analyzed. Increasing the perfusion flow rate from 4.5 ul/min to 9.0 ul/min produced a rapid, yet transient, increase in IOP that peaked within 1.5h, after which a new, slightly elevated (16 %), steady-state IOP was established (\textbf{Figure 25A}), demonstrating the compensatory compliance of the TM to enhanced IOP. Exposing TM tissue to increased perfusion flow rates did not appear to alter TM cellularity when compared to physiological perfusion flow rate controls (\textbf{Figure 25 E, F}). In contrast, time-dependent, differential changes in the content of CTGF and ppET-1 mRNA expressed by TM tissue, but not TGF-β2 mRNA, were observed in response to increased perfusion flow rates. These findings suggest that abrupt and possibly sustained
increases in IOP *in situ* elicits compensatory changes in the conventional outflow pathway that may initiate marked changes in TM matricellular and ECM gene expression.

**Figure 25. Physiological consequences of increased perfusion flow rate on TM tissue *in situ.*** Porcine anterior segments were allowed to stabilize for 24h, and subsequently perfused at physiological (4.5 µl/min) or increased (9 µl/min) perfusion flow rates for up to an additional 24h. (A) Relative changes in IOP in response to increased perfusion flow rates (dashed line = baseline). Data shown are normalized to baseline pressure (N=4-9). Porcine TM was removed at 1.5, 6, and 24 hours and relative changes in (B) CTGF, (C) TGF-β2 and (D) ppET-1 mRNA content were quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes and expressed as the mean ± SEM (N=4-9). * p <0.05, Student’s *t*-test relative to ambient pressure controls. Representative H&E photomicrographs of porcine TM infused at (E) physiological and (F) increased perfusion flow rates.
Glaucoma is a group of optic neuropathies characterized by optic nerve damage leading to irreversible blindness. The most prevalent form, POAG, is a slowly progressing disease that affects over 60 million worldwide, with this number expected to climb to 112 million by the year 2050.\textsuperscript{1,2} In the US alone, the socioeconomic cost of POAG exceeds 1.9 billion annually, and despite its overwhelming financial burden, treatment of POAG remains both refractive and palliative.\textsuperscript{3} The pathophysiology of POAG is not fully understood and consequently, therapeutic strategies that specifically target the underlying causes of this disease of have yet to be developed. Instead, current therapeutics are limited to nonspecific forms of intervention designed to lower elevated IOP, a major risk factor for the initiation and progression of this disease. This pathological elevation of IOP in patients with POAG, is believed to result from increased resistance to AH through the TM.\textsuperscript{10} Reported limitations of all FDA approved drugs for the treatment of POAG include decreased efficacy over time, limited bioavailability and the need for frequent dosing.\textsuperscript{211} It is also important to note that no approved therapeutic strategy targets the TM itself, instead working by various mechanisms to decrease AH production or increase uveoscleral outflow. Thus, the development of novel therapeutic
strategies that specifically target the both the TM and the pathogenic causes of POAG are necessary to advance patient care.

The lack of a therapeutic strategy that specifically targets the TM stems, in part, from an incomplete understanding of the mechanism by which resistance to AH outflow through the TM is elevated. Previous reports have linked this aberrant increased resistance to AH outflow to both alterations in the actin cytoskeleton and changes in the extracellular milieu of the TM.\textsuperscript{14, 83, 212, 213} In support of this, post mortem analysis of patient TM shows enhanced deposition of sheath-derived plaques as well as a 20-fold increase in TM stiffness.\textsuperscript{39, 51} Experimental evidence suggests a causal role for the profibrotic cytokine TGF-β2 in initiating both the formation of actin stress fibers and accumulation of ECM, thereby altering TM function and increasing outflow resistance.\textsuperscript{60, 63, 99, 214}

Whereas the role of TGF-β2 in elevating IOP has been fairly well studied, the effect of elevated pressure on the TM itself is less well understood. An increasing body of evidence has shown that the physical properties of a cell’s microenvironment are strong determinants of a cell’s behavior.\textsuperscript{215} The biophysical changes in the TM resulting from elevated IOP may be an important upstream factor in the progression of POAG. In this regard, TM cells cultured on stiffer substratum (thereby mimicking a glaucomatous ECM) respond by enhancing the expression myocilin, CTGF, fibronectin and SPARC, four genes implicated in the pathogenesis of POAG.\textsuperscript{216, 217} Interestingly, TGF-β2 has also been demonstrated to elicit increased expression of all four proteins, implying that mechanical stimuli may modulate TGF-β2 signaling in TM cells.\textsuperscript{218, 219} Chronic elevation
of IOP is known to exert three separate forms of mechanical stimuli upon the TM: increased physical stretch, enhanced fluid sheer stress, and elevated hydrostatic pressure. In comparison to physical stretch or fluid sheer stress, elevated hydrostatic pressure is the least often studied and consequently, least well understood mode of mechanical stress experienced by the TM. These, \textit{in vitro}, studies were designed to: (1) confirm TGF-β2 induced changes in gene expression in human TM cells, and (2) characterize TM cell responses to elevated hydrostatic pressure. A comprehensive understanding of the pathological mechanisms underlying POAG will reveal novel therapeutic targets and allow for the advancement of patient care.

\textbf{TGF-β2 Alters Gene Expression in TM Cells.}

\textit{In vitro} studies have established TGF-β2 as a key mediator of profibrotic signaling, inducing increased expression of matricellular and ECM components in the TM.\textsuperscript{47,76,77} Consistent with previous results, TGF-β2 markedly enhanced the expression of CTGF mRNA in both primary and transformed TM cells.\textsuperscript{126,220} CTGF is known to play an essential role in the regulation of tissue repair and fibrosis by integrating molecular cues and signals from the ECM to cell surface proteins such as integrins.\textsuperscript{221,222} Adenoviral overexpression of CTGF in the mouse eye induces a glaucomatous phenotype, including increased expression of fibronectin and smooth-muscle actin, elevated IOP, and optic nerve damage.\textsuperscript{120} \textit{In vitro}, CTGF can enhance the expression of ECM components and TM cell contractility independent of TGF-β2 signaling.\textsuperscript{121} To date, elevated CTGF content in POAG patient AH has not been reported, although AH samples
taken from patients prior to phacoemulsification may not necessarily detect local increases in CTGF secretion from the TM. Immunohistochemical analysis of patient TM, post mortem, may be a more appropriate method to detect local increases in CTGF content in patients with POAG. Collectively, these results suggest an important role for CTGF in the pathogenesis of POAG. It is unclear, however, whether CTGF is causative or simply an important downstream mediator of TGF-β2 dependent fibrosis in TM of POAG patients.

Not surprisingly, TGF-β2 treatment also elicited a concomitant and selective increase in mRNA content of numerous structural ECM protein subunits (Col1α1, Col1α2, Col4,) in both primary and transformed TM cells. Transformed (NTM5, GTM3) TM cell lines are widely used and generally exhibit characteristics similar to primary TM cells. Here, however, we observed a differential response to TGF-β2 in the regulation of FN mRNA content. Whenever possible, primary cells were used to confirm results originally demonstrated in transformed cell lines. In the event of a differential response between primary and transformed cells, the response exhibited by primary TM cells was considered more likely to mimic the true phenotype of the TM. Except for certain ECM components, no differences in TM cell responses were observed between cell lines.

TGF-β2 also markedly enhanced ppET-1 mRNA content in both primary and transformed human TM cells, though this response was much more robust in transformed cells. To date, constitutive expression of ET-1 from TM cells has not been observed. Previous findings from our lab have shown, however, that TGF-β2 induces a significant increase in ET-1 secretion from TM cells. Despite numerous studies, the functional
role of ET-1 in regulation of IOP remains unclear. Intravitreal injection of ET-1 into Dutch-belted rabbits elicits a transient increase in IOP.\textsuperscript{113} Similarly, we and others have shown that administration of ET-1 to perfused anterior segments transiently increases IOP.\textsuperscript{112} By comparison, ET-1 mediated contraction of the ciliary muscle leads to distension of the TM and a subsequent increase in outflow facility.\textsuperscript{223} In addition, ET-1 inhibits Na+/K+ ATPases in the nonpigmented ciliary epithelium, decreasing aqueous humor production.\textsuperscript{224} Collectively these results suggests that ET-1 plays a complex role in IOP homeostasis, with its functional effect depending upon the location of action.

Within the TM, ET-1 binds to the ET\textsubscript{A} receptor, stimulating RhoA GTPase activity and contraction of the actin cytoskeleton leading to increased resistance to AH outflow.\textsuperscript{100} Interestingly, exogenous TGF-β2 also decreases ET\textsubscript{A} receptor mRNA in both primary and transformed TM cells. ET\textsubscript{A} receptor expression is regulated by many factors, including ET-1 itself.\textsuperscript{225-227} TGF-β2 may be directly attenuating ET\textsubscript{A} mRNA content either by inhibiting transcription or decreasing ET\textsubscript{A} receptor mRNA stability. Alternatively, secreted ET-1 in response to TGF-β2, may act in an autocrine manner to attenuate ET\textsubscript{A} receptor mRNA. TGF-β2 mediated decreases in ET\textsubscript{A} receptor content may represent a homeostatic mechanism designed to lower IOP by preventing ET-1 mediated contraction of the actin cytoskeleton in TM cells.

The ET\textsubscript{B} receptor was not expressed to a measurable extent at the mRNA level in vehicle or TGF-β2 treated cells, consistent with previously published studies.\textsuperscript{108} This is most likely a result of the mRNA destabilizing motif “AUUUA” located in the 3’ noncoding region of ET\textsubscript{B} receptor mRNA, resulting in a shortened mRNA half-life.\textsuperscript{108} ET\textsubscript{B} receptor protein however, is expressed constitutively by TM cells although its role in
IOP homeostasis is currently not well understood. ET<sub>B</sub> receptor activity is typically thought to increase nitric oxide production leading to vasodilation, but also been shown to induce contraction in smooth muscle cells. Treatment of both bovine and human TM cells with the ET<sub>B</sub> receptor specific agonist IRL-1620 rapidly increases intracellular calcium while application of IRL-1620 to isolated bovine TM strips induces a small, but detectable, contraction. These results suggest that ET<sub>B</sub> receptor activation may induce contraction of the TM, increasing outflow resistance. In human TM cells, however, ET-1 activates endothelial nitric oxide synthase by binding to the ET<sub>B</sub> receptor to increase production of nitric oxide. While not conclusive, several reports suggest that nitric oxide donors may induce relaxation of the TM, decreasing outflow resistance. As such, the role of ET<sub>B</sub> receptor in the TM remains unclear.

TGF-β2 also markedly attenuated the expression of the B2 receptor in human TM cells. BK, a well characterized nonapeptide with potent vasodilatory properties, is known to lower IOP in various animal models through activation of the B2 receptor. Earlier studies found tissue kallikrein to be present in freshly isolated human TM and kinases I and II in human AH. Here we demonstrate constitutive expression of the B2, but not the B1 receptor, in both primary and transformed TM cells. TGF-β2 mediated decreases in B2 receptor content occur through activation of the canonical Smad3 dependent pathway, but independent of Rho GTPase signaling. Taken together, these findings suggest a role for the local production and action of BK in the TM under normal physiological conditions. It has been previously shown that chronic inhibition of endogenous BK activity leads to a significant increase in arterial BP. Thus, it is plausible that elevated content of TGF-β2 in the AH of POAG patients may elevate IOP,
in part, by attenuating constitutive B2 receptor expression and activation within the conventional outflow pathway.

The Therapeutic Potential of Targeting TGF-β2-mediated Signaling.

Results shown here conclusively demonstrate that TGF-β2 enhances the mRNA expression of specific ECM components, consistent with previous studies.\textsuperscript{214,230} We also highlighted a unique mechanism by TGF-β2 may elicit an aberrant increase in AH outflow resistance, through perturbation of the kallikrein-kinin system. TGF-β2 mediated rearrangements in the actin cytoskeleton are also thought to induce elevated IOP in patients with POAG.\textsuperscript{83,213} Collectively, these results suggest that directly targeting TGF-β2 may be an effective strategy to lower IOP in patients with POAG. While likely to decrease outflow resistance through the TM, directly targeting TGF-β2 could also elicit numerous side effects as TGF-β2 is a pleiotropic cytokine involved in a wide variety of cellular processes.\textsuperscript{231} As such, targeting events or functional outcomes that are both downstream of initial TGF-β2 mediated signaling, and involved in the pathogenesis of POAG represents a more viable therapeutic strategy.

Here we demonstrate that TGF-β2 markedly enhances mRNA content of CTGF and certain structural ECM components and previous studies have shown a direct causal link between ECM accumulation and increased outflow resistance.\textsuperscript{19,42,230} Anti-fibrotics, therefore, may represent a novel therapeutic strategy to attenuate TGF-β2 mediated accumulation of ECM in the TM. In this regard, the anti-fibrotic agents 5-fluorouracil and mitomycin C are commonly used in glaucoma filtration surgery to lower
postoperative IOP and prevent scarring. More recently, Pattabiraman et al. demonstrated that the anti-fibrotic pirfenidone effectively attenuates TGF-β2 mediated increases in Col1α1 content in cultured human TM cells. These previously published results as well as our findings presented here, strongly support the therapeutic potential of anti-fibrotics to prevent TGF-β2 mediated accumulation of ECM in the TM.

TGF-β2 also markedly enhanced ppET-1 mRNA content, and previous studies from our lab have demonstrated a concomitant increase in secreted ET-1 from human TM cells. ET-1, a potent vasoconstrictor, enhances formation of actin stress fibers and elevates IOP through activation of the small, monomeric, GTPase RhoA. Administration of inhibitors of the Rho effector molecule, Rho kinase (ROCK), inhibits the formation of F-actin reducing outflow resistance through the conventional pathway. Not surprisingly, several inhibitors of the Rho effector molecule, ROCK, are currently in either Phase II or Phase III clinical trials. While initial results of these trials seem promising, it is also important to note that a large number ROCK inhibiting compounds have already failed to pass clinical trials, typically due an inability lower IOP over long periods of time. In addition, ROCK inhibitors would be unlikely to protect against TGF-β2 mediated changes in B2 receptor content as this occurs selectively, through the canonical smad3-dependent pathway. Thus, while promising, the long term clinical efficacy of ROCK inhibitors remains uncertain.

BK itself represents a potential therapeutic strategy for patients with POAG due to its ocular hypotensive effects. Exogenous BK significantly increases outflow facility in both bovine and human anterior segment perfusion. Similarly, intravitreal injection
of BK into Dutch-Belted rabbits with naturally high IOP significantly decreases IOP\textsuperscript{130}, although this effect is transient. The recent discovery of the non-peptide BK analogue FR-190997 may represent a hypotensive agent with more favorable pharmacokinetic properties.\textsuperscript{29} Previous studies demonstrating the therapeutic efficacy of BK or FR-190997, however, did not account for the TGF-β2 mediated perturbation of the kallikrein-kinin system. It is plausible that elevated content of TGF-β2 present in POAG patient AH may reduce B2 receptor content in the TM, reducing the hypotensive effect of both BK and FR-190997.

While each of these therapeutics represents a novel and promising therapeutic strategy, a better understanding of the underlying mechanisms involved in the pathogenesis of POAG is necessary to truly advance patient care. To this end, we sought to elucidate the cellular response and mechanism of action of elevated hydrostatic pressure on TM cells, and how this unique mechanical stimulus may initiate a feed-forward loop that further elevates IOP.

**Elevated Hydrostatic Pressure Induces Cell Stress and Apoptosis in TM Cells.**

TM cells are known to sense and respond to fluctuations in IOP caused by blinking, eye movement, or ocular pulse. It has been well demonstrated that transient elevations in IOP elicit changes in ECM turnover and alterations in the actin cytoskeleton leading to decreased resistance to AH outflow.\textsuperscript{153, 154} The effect of prolonged mechanical stress on the TM as result of elevated IOP, however, is not as well understood. Neuronal PC12 cells exposed to extremely elevated hydrostatic pressures tend to exhibit
characteristic markers of oxidative stress and apoptosis.\textsuperscript{180} Similarly, we demonstrate that a sustained (x24h) exposure of cultured human TM cells to elevated hydrostatic pressure elicits mitochondrial dysfunction, as measured by the MTT assay. In addition, cleavage of caspase 3, a characteristic marker of apoptosis, was observed following even more prolonged exposure (~48h) to elevated hydrostatic pressure. Patients with POAG are known to exhibit decreased TM cellularity, when compared to healthy individuals, which is thought to contribute to elevated AH outflow resistance.\textsuperscript{234} These findings suggest that prolonged exposure to elevated hydrostatic may induce TM cell apoptosis in patients with POAG.

**Elevated Hydrostatic Pressure Alters the Actin Cytoskeleton in TM Cells.**

Prior to onset of apoptosis, TM cells are known to respond to mechanical stimuli by altering their cytoskeletal network and cell shape.\textsuperscript{198} Enhanced organization of the actin cytoskeleton of TM cells, especially in the JCT area, is known to increase outflow resistance through the conventional pathway leading to elevated IOP.\textsuperscript{235} When mechanical stretch is applied to TM cells, \textit{in vitro}, stress fibers form parallel to the direction of stretch.\textsuperscript{236} Actin stress fiber formation is correlated with TM tissue stiffness and enhanced resistance to aqueous humor outflow.\textsuperscript{237} Elevated hydrostatic pressure has also been shown to modulate cytoskeletal elements eliciting increased expression of heat-shock protein-27 in human astrocytes leading to stabilization of intermediate filaments networks.\textsuperscript{172} The effect of elevated hydrostatic pressure on the cytoskeletal network of TM cells, however, has not been previously reported.
Here we demonstrate a marked accumulation in F-actin in response to elevated hydrostatic pressure. The balance between the assembly of globular (G)-actin into F-actin, and subsequent disassembly back into G-actin, directly affects the activity and subcellular localization of actin binding proteins and certain transcription factors. Actin binding proteins have been shown to stimulate or inhibit steroid receptor hormones, indirectly regulating transcription. A shift in G/F actin ratio in TM cells following exposure to elevated hydrostatic pressure could indirectly regulate gene transcription by enhancing or inhibiting the activity of various actin binding proteins. Altered expression of nuclear actin was not observed in response to elevated hydrostatic pressure, but phalloidin cannot always detect nuclear actin. It seems unlikely that elevated hydrostatic pressure enhanced cytoplasmic F-actin content without a concomitant change in nuclear actin occurring. Nuclear actin has been demonstrated to play integral role in transcriptional regulation as it is (1) utilized by all three nuclear RNA polymerases, (2) may affect mRNA processing by associating with small nuclear ribonucleoproteins, (3) and is an essential component of chromatin-remodeling and histone acetyl transferase complexes. Thus it is plausible that hydrostatic pressure mediates changes in the actin content may play a more direct role in regulating gene expression by interacting directly with the machinery necessary for transcription.
Elevated Hydrostatic Pressure Elicits Selective Changes in Gene Expression in TM Cells.

Using a predesigned “POAG” nanoarray, we confirmed that elevated hydrostatic pressure induces marked changes in the gene expression of TM cells. Of the 16 genes measured, each of which has been previously indicated in the pathogenesis of POAG, only CDKN1A, VEGF-A, and CTGF mRNA content were significantly enhanced in response to elevated hydrostatic pressure. While this result clearly underrepresents the true number of genes affected, it does demonstrate, for the first time, that hydrostatic pressure elicits specific changes in gene expression.

CDKN1A is well known to inhibit cyclin dependent kinases, causing irreversible arrest of the cell cycle. A single nucleotide polymorphism (SNP) in this protein encoding a DNA-binding zinc finger domain is correlated with the prevalence of POAG in a specific Chinese population. This SNP prevents CDKN1A from binding to its target, sensitizing cells to apoptosis. While elevated hydrostatic pressure did elicit a small increase in CDKN1A mRNA content, it is unlikely to affect CDKN1A function. Pressure mediated increases in CDKN1A mRNA would have no effect on CDKN1A binding to its target, and thus would be unlikely to sensitize TM cells to apoptosis. As such, the physiological relevance of this finding remains uncertain.

Cultured TM cells also exhibited a transient increase in VEGF-A mRNA in response to elevated hydrostatic pressure. VEGF-A has been previously implicated in initiating and controlling the progression of a secondary form of glaucoma, neovascular glaucoma. Patients with this disease exhibit a 40-fold increase in VEGF-A content in
their AH, compared to healthy controls. This trend, however, is not reflected in POAG patient AH, and as such, the role of VEGF-A in POAG is less clear. In some patients with age-related macular degeneration, anti-VEGF therapy induces elevated IOP suggesting that VEGF-A may have hypotensive effects. Supporting this, administration of VEGF-A, \textit{ex vivo}, increases outflow facility in perfused porcine anterior segments. Recently, Micera \textit{et al.} examined differential protein expression in TM tissue isolated from medically managed POAG patients and healthy control samples. This study is somewhat unique in that most gene and proteomic studies are conducted on TM tissue acquired post mortem. In this study, TM was acquired from POAG patients undergoing trabeculectomies and 46 differentially expressed proteins were identified including VEGF-A. This implies that not only is VEGF-A constitutively expressed by the TM, \textit{in vivo}, but upregulated in patients with POAG. Interestingly, mechanical stretch has been shown to enhance VEGF-A secretion from TM cells. While the TM is known to express VEGF receptors, administration of exogenous VEGF-A to TM cells does not significantly alter transendothelial electrical resistance (TEER). In contrast, treatment of SC endothelial cells with VEGF-A markedly decreases TEER, enhancing cell permeability. Thus, elevated hydrostatic pressure mediated increases in VEGF-A from the TM may represent a compensatory mechanism that lowers outflow resistance by allowing AH to more easily cross the endothelial lining of SC.
Elevated Hydrostatic Pressure Elicits a Sustained Increase in CTGF mRNA content and Protein Secretion.

Elevated hydrostatic pressure markedly enhanced the synthesis and secretion of CTGF, despite this matricellular protein already being one of the most highly expressed genes in the TM. Constitutive expression of CTGF is necessary for proper maintenance of the actin cytoskeleton and ECM turnover. By comparison, enhanced expression of CTGF in the TM may be a primary protective response to oxidative stress. The TM is highly susceptible to oxidative damage due to diminished antioxidant defenses, and TGF-β2, a potent inducer of CTGF, is well known to regulate reactive oxygen species production. In experimental models of diabetic neuropathy, increased oxidative stress is typically paralleled by enhanced CTGF expression, suggesting a protective role for CTGF. In this regard, supplementation with CTGF prior to oxidative insult has been shown to be beneficial to TM cell viability, in vitro, while targeted knockdown of CTGF expression prior to a oxidative insult results in increased cell death. Collectively, these findings suggest that a rapid increase in CTGF expression represents an important protective mechanism designed to protect against oxidative or other forms of cell stress. Adenoviral overexpression of CTGF, however, promotes a glaucomatous phenotype including elevated IOP, alterations in the actin cytoskeleton, enhanced deposition of ECM components, and optic nerve damage. Therefore, a sustained increase in CTGF content as a result of elevated hydrostatic pressure, as demonstrated here, may ultimately lead to enhanced resistance to aqueous humor outflow through the TM.
Elevated Hydrostatic Pressure Transiently Increases TGF-β2 Content.

Elevated hydrostatic pressure also markedly increased the synthesis and secretion of TGF-β2 from TM cells in a time-dependent manner. It is well established that TGF-β2 content is elevated in the AH of patients with POAG.\textsuperscript{55,57-59} Post mortem analysis has localized TGF-β2 expression to multiple tissues in the anterior chamber including lens epithelial cells, conjunctival stroma, the ciliary body, and the TM itself.\textsuperscript{67,68} Previous studies from our lab clearly demonstrate constitutive synthesis and secretion of TGF-β2 from both primary and transformed TM cells suggesting that the source of elevated TGF-β2 in patient AH may be the TM itself.\textsuperscript{60} The mechanisms governing pressure mediated increases in TGF-β2 are unknown but most likely involve both increased Rho GTPase activity and histone acetylation. Inhibition of the small monomeric Rho GTPases, RhoA markedly attenuates constitutive secretion of TGF-β2 from TM cells.\textsuperscript{75} In contrast, TGF-β2 expression is enhanced by hyperacetylation of histones associated with the TGF-β2 promoter.\textsuperscript{80} Interestingly, fluid shear stress is known to modulate both HDAC and Rho GTPase activity in vascular endothelial cells.\textsuperscript{251,252} Elevated hydrostatic pressure may have a similar effect in TM cells, enhancing Rho GTPase activation and histone acetylation leading to increased TGF-β2 expression.

ET-1 Synthesis and Secretion is Enhanced in Response to Elevated Hydrostatic Pressure.

Similar to TGF-β2 and CTGF, ET-1 synthesis and secretion from the TM was markedly upregulated in a time-dependent manner in response to elevated hydrostatic
The local production of ET-1 would most likely induce a marked contraction of the actin cytoskeleton, enhancing AH outflow resistance. In this regard, elevated hydrostatic pressure did significantly alter F-actin expression as previously discussed. There was also a significant decrease in ET\(_A\) mRNA content in response to elevated hydrostatic pressure (x24h). Decreased ET\(_A\) receptor mRNA content may represent a homeostatic mechanism designed to lower IOP by decreasing ET-1/ET\(_A\) receptor mediated contraction of the actin cytoskeleton. While elevated hydrostatic pressure may be affecting ET\(_A\) receptor content directly, this effect observed may also be a result of secreted TGF-\(\beta\)2 or ET-1 acting in an autocrine manner.

**Elevated Hydrostatic Pressure Elicits Altered Expression of Proteases in Primary TM Cells.**

As both TGF-\(\beta\)2 and CTGF secretion was enhanced in response to elevated hydrostatic pressure, it is logical to assume that there may have been a similar response in ECM production. To this point, primary TM cells exposed to elevated hydrostatic did elicit a significant, yet transient increase in various structural ECM protein subunits in primary TM cells. Transformed TM cells, however, typically did not exhibit similar responses, and as such, the relevance of these findings is unclear. It is still plausible that elevated hydrostatic pressure may affect ECM protein content without significantly altering gene expression, by either inhibiting or promoting ECM degradation. Alternatively, elevated hydrostatic pressure may alter the transcription of genes relevant to ECM remodeling such as growth factors, proteases, and inhibitors of proteases.
Interestingly, exposure to elevated hydrostatic pressure markedly enhanced the expression of both MMP3 and PAI-1 mRNA from primary TM cells, both of which have been previously indicated in the pathogenesis of POAG. These results suggest that elevated hydrostatic pressure markedly affects the extracellular milieu of TM cells. By altering ECM turnover. Further studies are warranted to further elucidate the effect of elevated hydrostatic pressure on ECM proteins and aqueous humor outflow through the TM.

**TM Cells Respond to Elevated Hydrostatic Pressure by Increasing Bradykinin B1 Receptor mRNA Expression.**

Elevated hydrostatic pressure induced a marked, yet transient, increase in B2 receptor mRNA in primary TM cells. In contrast, B1 receptor mRNA expression in primary TM cells was enhanced in a sustained manner. We and others have shown that specifically in TM cells, the B1 receptor protein is not expressed to a measurable extent at the protein level and application of B1 receptor specific agonists fail to elicit a functional outcome. Expression of the B1 receptor in other tissues, however, is strongly upregulated following exposure to trauma, inflammation, or certain cytokines (tumor necrosis factor-α, IL-1β, and IL-8). The marked increase in B1 receptor mRNA observed implies that there may be a similar increase in B1 receptor protein. The B1 receptor is typically coupled to the same intracellular signaling cascades as B2 receptor. As such, the B1 receptor is known to activate protein kinase C, tyrosine kinase cascades, and the MAPK pathway, induce nuclear translocation of NF-κB, and
stimulate PI hydrolysis. Activation of B1 receptor, however, does tend to elicit a much
different pattern of intracellular signaling when compared to B2 receptor, mostly due to
differences in receptor internalization patterns. The B2 receptor is normally expressed in
a number of tissues, and present on the plasma membrane in the absence of its agonist,
BK. Following binding of BK, however, the B2 receptor undergoes rapid internalization
in clathrin-dependent manner. In contrast, binding of agonists to the B1 receptor
actually reduces the rate of receptor internalization from the plasma membrane. BK is
potent yet short lived hypotensive agent that acts by binding to the B2 receptor. If B1
receptor protein expression is in fact enhanced in the TM in response to elevated
hydrostatic pressure, B1 agonists may have a more prolonged hypotensive effect. Further
studies are warranted to characterize kallikrein-kinin system, specifically the B1 receptor,
under hypertensive conditions.

**TM Cells are Sensitive to Clinically Relevant Changes in Hydrostatic Pressure.**

Findings presented here clearly demonstrate that elevated hydrostatic pressure
induces cellular stress leading to apoptosis, enhances F-actin content, and selectively
alters gene expression in human TM cells, each of which may contribute to the aberrant
elevation of IOP in patients with POAG. When considering the relevance of these
findings, however, it is important to note the difference between physiological and
pathological levels of hydrostatic pressure. In these studies, +30 mmHg was chosen to
mimic a hydrostatic pressure load similar to that experienced by patients with POAG.
Application of increasing levels of hydrostatic pressure, however, has been previously
demonstrated to elicit a differential cellular response, somewhat mimicking a pharmacological dose response to a drug or ligand.\textsuperscript{203, 204} By comparison, we did not observe any significant differences in magnitude of TM cell responses to increasing hydrostatic pressures (+15-45 mmHg). It should be noted that the high pressure condition (+45 mmHg) was less than the hydrostatic pressure loads most commonly used in experiments in other ocular tissues (60-100 mmHg).\textsuperscript{174, 182} TM cells may exhibit differential responses when subjected to a larger range of hydrostatic pressures. By comparison, hydrostatic pressure loads as low as +15 mmHg are rarely used in an experimental setting, but would represent a marked increase \textit{in vivo}. A recent study found an average increase of only 8.8 mmHg in the IOP of patients with POAG when compared to healthy, age-matched controls.\textsuperscript{256} While caution should always be used in extrapolating results obtained \textit{in vitro} to \textit{in vivo} situations, these findings do indicate that TM cells may be remarkably sensitive to clinically relevant changes in hydrostatic pressure. Thus, the magnitude of increased IOP exhibited by patients with POAG may elicit a similar increase in the synthesis and secretion of matricellular proteins from TM cells as observed here.

The Role of the ET\textsubscript{b} Receptor in Elevated Hydrostatic Pressure Mediated Changes in Gene Transcription.

As both TGF-\textbeta 2 and ET-1 have been shown to induce CTGF expression,\textsuperscript{126, 220, 257} it is plausible that each may play a role in upregulating matricellular gene expression in response to elevated hydrostatic pressure. Pretreatment with specific antagonists to the
ET\textsubscript{A} receptor or TGF-\textbeta RII however, did not have a significant effect upon changes in gene transcription observed. In contrast, a specific antagonist to the ET\textsubscript{B} receptor markedly attenuated hydrostatic pressure mediated increases in both TGF-\textbeta 2 and ppET-1 mRNA. Although TM cells do not constitutively secrete ET-1, we have demonstrated that elevated hydrostatic pressure induces secretion of ET-1 within 3 hours of exposure. Collectively these results suggest that secreted ET-1 may bind to the ET\textsubscript{B} receptor in an autocrine manner, potentiating transcription of TGF-\textbeta 2 and ppET-1. While the role of the ET\textsubscript{B} receptor in IOP homeostasis remains unclear, recent evidence suggests that ET\textsubscript{B} receptor may be a key contributor to RGC cell death in patients with POAG.\textsuperscript{103} Post mortem analysis of glaucomatous tissue shows increased ET\textsubscript{B} receptor immunoreactivity in optic nerve head astrocytes.\textsuperscript{258} Similarly, ET\textsubscript{B} receptor expression is increased in areas of degeneration that colocalize with astrocytic markers in a non-human primate model of glaucoma.\textsuperscript{258} \textit{In vitro}, treatment of primary RGCs with ET-1 or ET-3, a selective ET\textsubscript{B} receptor agonist, induces apoptotic cell death to a similar extent.\textsuperscript{103} \textit{In vivo}, treatment with the combination ET\textsubscript{A}/ET\textsubscript{B} receptor inhibitor bosentan protects against axonal degeneration in the DBA/2J mouse model of glaucoma.\textsuperscript{259} In Morrison’s ocular hypertension model, ET\textsubscript{B} receptor expression in the nerve fiber and retinal ganglion cell layer is markedly upregulated prior to the onset of axonal degeneration.\textsuperscript{104} Furthermore, selective knockout of ET\textsubscript{B} receptor enhances RGC survival in this same model. Elevated IOP in patients with POAG, therefore, may enhance the expression and subsequent activation of ET\textsubscript{B} in the retina, contributing to RGC cell death in POAG. A similar increase in ET\textsubscript{B} receptor content or activation in the TM may contribute to hydrostatic pressure mediated apoptosis, ultimately leading to the decreased TM cellularity observed
in POAG patients. While elevated hydrostatic pressure markedly attenuated ET\textsubscript{A} receptor mRNA content, changes in ET\textsubscript{B} receptor mRNA content were not observed. Further studies are warranted to more fully characterize the effect of elevated IOP on ET\textsubscript{B} receptor expression and function in the conventional pathway.

**Elevated Hydrostatic Pressure Does not alter TGF-\(\beta\) Signaling in TM Cells.**

While it is clear that pressure mediated changes in gene expression in TM cells does not involve, at least initially, activation of T\(\beta\)RI, it still seemed plausible that elevated hydrostatic pressure may modulate TGF-\(\beta\)2-mediated signaling. Exposure of TM cells to elevated hydrostatic pressure, however, failed to induce smad3 phosphorylation. In addition, pretreatment with exogenous TGF-\(\beta\)2 had no observable effect upon pressure mediated changes in mRNA content. Collectively, these results suggest that elevated hydrostatic pressure mediated does not alter TGF-\(\beta\)2 signaling. Increased secretion of active TGF-\(\beta\)2 however, does not occur prior to 24h exposure to elevated hydrostatic pressure. As secreted ET-1 has been shown to acts in an autocrine manner to enhance ppET-1 and TGF-\(\beta\)2 mRNA transcription, it seems likely that secreted TGF-\(\beta\)2 may also elicit autocrine effects following a more prolonged exposure.
The Role of Reactive Oxygen Species in Cellular Responses to Elevated Hydrostatic Pressure.

Post mortem analysis of glaucomatous tissue suggests role of ROS in the pathogenesis of POAG as markers of oxidative stress including increased end-product lipid peroxidation and oxidative DNA damage in patients with POAG.\textsuperscript{184,185} Optic nerve head astrocytes exposed to elevated hydrostatic pressure display decreased mitochondrial fission, indicative of oxidative stress.\textsuperscript{179,182} More recently, it was shown that both CTGF and TGF-β2 expression are regulated, at least in part, by ROS.\textsuperscript{208,221} As such, it seemed plausible that the mitochondrial dysfunction and cell death, changes in F-actin content, and alterations in gene expression observed here may have resulted from increased ROS induced by elevated hydrostatic pressure.

To clarify the role of oxidative stress, TM cells were pretreated with two different antioxidants, and subsequently exposed to elevated hydrostatic pressure. Pretreatment with the antioxidant Trolox did not significantly alter pressure mediated changes in mitochondrial function, membrane permeability, or gene transcription. By comparison, resveratrol did have a slight, but significant protective effect, delaying changes mitochondrial function and membrane permeability. Elevated hydrostatic pressure mediated changes in gene expression, however, were not significantly altered by resveratrol. Taken together, these results suggest that oxidative stress does not play a significant role in the observed findings presented here. It is possible that the dose of antioxidants used here were simply insufficient to protect against the amount of oxidative damage induced by elevated hydrostatic pressure. Alternatively, prolonged exposure
(48h) to antioxidants themselves may act as a unique insult, sensitizing cells to oxidative stress. This is unlikely however, as resveratrol and Trolox by themselves did not significantly affect MTT formation or LDH release compared to ambient pressure controls. In addition to its antioxidant properties, resveratrol is also known to be anti-inflammatory and anti-proliferative due to its modulation of NF-κB, p53, bcl2, and the inhibitor of HDAC, sirtuin1. Thus resveratrol may be delaying deficits in mitochondrial function by a mechanism that is independent of oxidative stress.

**TM Cells Respond to Elevated Hydrostatic Pressure by a Mechanism Independent of TRPV Channel Activation.**

Previous studies conducted in other cell types have examined the downstream cellular effects of hydrostatic pressure, but little is known about the mechanism by which cells sense and respond to this stimulus. Ryskamp et al. recently identified the putative mechanosensitive ion channel TRPV4 as a key transducer of mechanical stretch in TM cells. In support of this, both selective TRPV4 agonists and substrate stretch enhance F-actin formation in primary TM cells. By comparison, topical administration of a TRPV4 antagonist simultaneously lowers IOP and protects against retinal damage in a mouse model of glaucoma. Alternatively, TRPV1 has been shown to be a key transducer of hydrostatic pressure in microglia and retinal ganglion cells, *in vitro*. The principal mechanism of TRPV channel activation involves changes in plasma membrane tension, either as a result of fluid sheer stress or cell swelling/shrinking, leading to a conformational change and subsequent influx of Ca²⁺ or other cations. As hydrostatic
pressure is an isotropic, nondeforming mode stress, it is unlikely to activate TRPV channels by inducing membrane deformation. However, recent evidence has demonstrated that additional diverse stimuli including phorbol esters, metabolites of arachidonic acid, acidity, and capsaicin can also activate these channels. Consequently while a role for TRPV1 and TRPV4 channels in TM cells in sensing and responding to elevated hydrostatic pressure does not seem likely, it could not be ruled out. Here, we demonstrate that specific TRPV1 and TRPV4 antagonists have no effect upon pressure mediated changes in gene transcription. This strongly supports the hypothesis that mechanosensation of hydrostatic pressure in TM cells is independent of TRPV 1 and 4 activation.

**Incorporating Hydrostatic Pressure into the Tensegrity Model of Mechanosensation.**

An alternative mechanism by which TM cells may sense elevated hydrostatic pressure involves a concept of cellular and tissue mechanotransduction known as the tensegrity model. This model views the ECM and cells within a tissue as one continuous network. The ECM is physically linked to the internal cytoskeletal lattice by transmembrane adhesion complexes, predominantly integrins, mechanically coupling the extracellular environment to the intracellular cytoskeletal scaffold. Within an individual cell, cytoskeletal elements (microtubules, intermediate filaments, and actin) simultaneously generate and resist mechanical forces determining cell shape while providing a framework for cytokinesis, intracellular communication, and the trafficking
of organelles, vesicles and proteins. When a traditional, deforming stimuli is applied to a
tissue, force is distributed across the continuous network of ECM fibers and subsequently
transmitted by transmembrane adhesion complexes to intracellular, load-bearing
cytoskeletal elements. This change in intracellular tension can alter the state or
polymerization of microtubules, intermediate filaments, and actin. Actin dynamics in
turn, have been shown to regulate the localization of some transcription factors and actin
binding proteins. For example, myocardin-related transcription factors (MRTFs) are
typically sequestered in the cytoplasm, associated with G-actin. Following mechanical
stimulation and actin polymerization, MRTF translocates to the nucleus and associates
with serum response factor to initiate transcription. Within the context of systemic
scleroderma, MRTF expression and nuclear translocation has been implicated in
maintaining a feedforward loop of increased contractility and matricellular protein
extpression leading to further mechanical stress and ECM modification. Within the TM,
the interaction between serum response factor and MRTF is critical to RhoA induced
expression of profibrotic genes. Here we demonstrated that elevated hydrostatic
pressure elicits a marked accumulation of intracellular F-actin. This shift in actin
dynamics most likely modulates the subcellular localization of transcription factors
including MRTF, leading to the enhanced expression of matricellular genes observed
here.
The Physiological Consequences of Increased Perfusion Flow Rate on the TM.

The TM experiences many different types of mechanical stress including fluid and *in vitro* modeling of each of these mechanical stimuli often results in unique and sometimes conflicting outcomes. This simultaneously enhances the difficulty of extrapolating *in vitro* cellular responses to mechanical stress to an *in vivo* situation, and increases the importance of comparing the effects of multiple mechanical modalities. To this end, we examined the effect of increased fluid sheer stress on matricellular gene expression in porcine TM cells. Increased perfusion flow rate elicited an immediate increase in pressure, as expected. After 1.5h, however, pressures began to decline before stabilizing 16% above baseline. TM cells initially respond to elevated IOP by altering ECM turnover in order to lower outflow resistance. This homeostatic response is clearly demonstrated by the decrease in pressure observed following 1.5h of increased perfusion rate. Similarly, Vittal *et al.* demonstrated that perfusion of human anterior segments at high pressures increases MMP2, lowering outflow resistance. In contrast to the effects of elevated hydrostatic pressure on TM cells, we did not observe any significant changes in TGF-β2 mRNA content in response to increased fluid shear stress in porcine anterior segments. There was also a statistically significant decrease in ppET-1 and CTGF mRNA. Attenuated expression of CTGF and ppET-1 represents yet another rapid, homeostatic response designed to lower outflow resistance similar to that previously demonstrated by Vittal *et al.* Evidence suggests, however, that increased deposition of ECM components and enhanced TM stiffness occur only after chronic over activation of this homeostatic response. It is plausible that a more prolonged exposure to high perfusion rates may elicit enhanced expression of matricellular proteins that more closely
mirrors the observed effects of elevated hydrostatic pressure on TM cells, *in vitro*. Furthermore, as fluid sheer stress and hydrostatic pressure are most likely detected by different mechanisms, it is logical each mechanical stimulus may elicit unique responses. To this point, it has recently been demonstrated that multimerization of the ECM protein cochlin and subsequent functional interaction with TREK-1 is involved in fluid shear stress dynamics in the TM.\textsuperscript{264} To date, however, no link has been demonstrated between elevated hydrostatic pressure and cochlin or TREK-1. Alternatively, perfusion of anterior segments at elevated pressures has been shown to induce differential regulation of collagens and matricellular proteins indicating increased fluid sheer stress may influence the expression of genes also regulated by hydrostatic pressure\textsuperscript{265, 266} Findings presented here clearly demonstrate that two types of mechanical stimuli experienced by the TM, fluid sheer stress and hydrostatic pressure, regulate CTGF and ET-1 expression in the TM.

**Summary and Conclusion.**

In this thesis, three unique mechanisms by which TGF-β2 elicits elevated IOP in patients with POAG are detailed. (1) TGF-β2 was found to markedly enhance the expression of matricellular and structural ECM genes, (2) TGF-β2 simultaneously induces increases in the expression of the potent vasoconstrictor ET-1, and (3) TGF-β2 was found to attenuate constitutive expression of Bradykinin B2 receptor (Figure 26). Each of these mechanisms represents a novel target for the therapeutic management of IOP in patients with POAG. Previously published studies have also shown TGF-β2
Increased TGF-β2 content in patient AH

Perturbation of the kallikrein-kinin system in the TM
• Attenuated B2 receptor expression

Altered TM gene expression
• CTGF
• ppET-1
• Col1α1, Col1α2, Col1α4

Increased resistance to AH outflow through the TM

Elevated IOP/elevated hydrostatic pressure

ET₁₆ channel activation

Cellular Stress
Altered actin cytoskeleton
Selective changes in gene expression
Enhanced matricellular protein release

Figure 26: Mechanism by which TGF-β2 elevates IOP and subsequent TM cellular responses.

A clear understanding of the underlying signaling events relevant to the pathogenesis of POAG is necessary to truly advance patient care. To better understand the role that elevated hydrostatic pressure may play in POAG, we characterized the response of TM cells to this unique mechanical stimulus. Elevated hydrostatic pressure induced cellular stress leading to apoptosis, alterations in the actin cytoskeleton, and selective and time-dependent changes in gene expression. Most notably TM cells exposed
to elevated hydrostatic pressure exhibited a transient increase in TGF-β2 and ET-1 content as well as a sustained increase in CTGF expression. The mRNA expression of several proteases (Table 5) involved in ECM remodeling was also enhanced, suggesting that elevated hydrostatic pressure may lead to ECM dysregulation. The mechanism by which TM cells sense and respond to hydrostatic pressure remains unknown but data presented here suggests that this mechanism is independent of TRPV channel activation, TGF-β2 signaling, or oxidative stress. By comparison, activation of ET_B receptor potentiates pressure mediated changes in TGF-β2 and ppET-1 content indicating this receptor may be involved.

Collectively, our findings suggest that elevated hydrostatic pressure may initiate a feed-forward loop by enhancing local secretion of matricellular proteins, enhancing F-actin content, altering the extracellular milieu, and decreasing TM cellularity ultimately leading to further enhanced resistance to AH outflow through the conventional pathway. The use of targeted therapeutics such as ROCK inhibitors, anti-fibrotics, or even ET_B receptor antagonists to interrupt this cycle may represent a novel viable strategy which to lower IOP in affected POAG patients
BIBLIOGRAPHY


123


The author, Jonathan Lautz, was born in Winfield, IL on April 7, 1988 to Anne and David Lautz. He attended Hope College in Holland, Michigan where he earned a Bachelor’s of Arts in Chemistry with a minor in Neuroscience in May, 2011. After graduation, Jonathan matriculated into the Neuroscience Graduate Program at Loyola University Chicago and began his graduate education under the mentorship of Dr. Evan B. Stubbs, Jr.

Jonathan’s dissertation focused on the role of transforming growth factor-β2 and hydrostatic pressure in the pathogenesis of primary open-angle glaucoma. During his studies, Jonathan applied for, and received two grants from the Illinois Society for the Prevention of Blindness. In his final year, Jonathan was a recipient of the Arthur J. Schmitt Fellowship in Leadership & Service. After completion of his graduate studies, Jonathan will pursue a one year Postdoctoral Fellowship in the lab of Dr. Stephen E.P. Smith.