# ROLE OF EXTRACELLULAR MATRIX CROSSLINKING ENZYME TISSUE TRANSGLUTAMINASE IN TRABECULAR MESHWORK HOMEOSTASIS AND REGULATING INTRAOCULAR PRESSURE

**DISSERTATION**

Presented to Graduate Council of the Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements for the Degree of

**DOCTOR OF PHILOSOPHY**

By

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Fort Worth, Texas

November 2017

**ROLE OF EXTRACELLULAR MATRIX CROSSLINKING ENZYME TISSUE TRANSGLUTAMINASE 2 IN TRABECULAR MESHWORK HOMEOSTASIS AND REGULATING INTRAOCULAR PRESSURE**

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## ABSTRACT

Increased intraocular pressure (IOP) is one of the major known risk factors for primary open angle glaucoma (POAG). The main cause of IOP elevation is obstruction to aqueous humor (AH) outflow at the trabecular meshwork (TM) in the eye. Cellularity and ECM turnover rates affect the normal physiology of the TM tissue. Various factors including transforming growth factor (TGFβ2) have been found responsible for a large number of glaucomatous changes. Increased expression and activity of tissue transglutaminase (TGM2), an enzyme induced by TGFβ2, has been seen in the glaucomatous TM. TGM2 can covalently crosslink ECM proteins including collagens, fibronectin and elastin. The purpose of this study was to investigate the role of TGM2 in ocular hypertension.

For this, we overexpressed TGM2 using adenovirus serotype 5 (Ad5), which has tropism for TM cells, in TM cells *in vitro* and in mouse eyes *in vivo*. *In vitro*, we validated overexpression of TGM2 using Ad5.TGM2 and also found increased crosslinking following overexpression. For our animal studies we used BALB/cJ and C57BL/6J mice. In our mouse models, following intravitreal injection of Ad5.TGM2, we saw a significant increase in IOP and decrease in AH outflow facility in TGM2 overexpressed eyes compared to contralateral eyes. Immunohistochemical staining showed that there was increased expression of TGM2 and

increased crosslinking in the TM region. There also appeared to be increased fibronectin at the TM region.

We followed this with a knockout (KO) study to determine whether TGM2 KO could affect IOP. For this we used TGM2 floxed mice. Following intravitreal injection of Ad5.Cre, we find a significant reduction in IOP. We also found that KO of TGM2 significantly reduced TGFβ2 induced ocular hypertension.

We further tested a small molecule TGM2 inhibitor ZM 449829. This inhibitor binds to TGM2 and inhibits crosslinking activity by locking it in an inactive state. In our findings, we observed that when treated with 5nM ZM 449829, at 48 hours it inhibited TGM2 crosslinking in 3 primary human glaucomatous cell strains. It also appears to reduce fibronectin deposition. We performed some preliminary tests of this drug *in vivo*. 5μM of the drug dissolved in PBS was administered as eye drops as once daily dosing. At 3 weeks, gross morphology of the eye and cornea looked normal. IOP measurements taken once a week till 3 weeks also did not show any aberrant changes.

Overall, our findings suggest that TGM2 plays a significant role in inducing ocular hypertension. This makes TGM2 a potential therapeutic target; therefore inhibition or amelioration of TGM2 crosslinking activity such as with potent inhibitors like ZM 449829 should be further studied as a novel therapeutic strategy for glaucoma.

## ACKNOWLEDGEMENTS

I would like to acknowledge each and every one of you who have been instrumental in my journey till here. I begin and end with thanking my mentor Dr. Clark. I find it hard to put it in words what would have been if not for his unwavering support, meticulous guidance and faith in me. Thank you for helping me hold my life together when so many things seemed to fall apart. You really are an extraordinary mentor and human being and I look forward to carrying your lessons and values in science and otherwise throughout my life. I want to sincerely thank my committee members Drs. Raghu Krishnamoorthy, Weiming Mao, Gulab Zode and Michael Allen. I have received support and understanding from each one of you during my tough times and I am grateful to all of you for that. Your feedback at every step of my graduate study has helped me develop my critical thinking and analysis. Drs. Cameron Millar for your help with animal work, Tara Tovar-Vidales for everything from cell culture to western blots and so many million other things, Yang Liu for your expert advice on intricate details of experiment planning and life, Iok-Hou Pang for all the statistical analysis I learnt, Dr. Colleen M. McDowell for your impeccable knowledge of mouse genetics and help in critical thinking, I have learnt so much from all of you.

Each and every one of my lab members past and present, thank you for all the times you all helped bounce ideas off, discuss science and all the non-science talk! This was a huge part of my

life and thank you to all of you for making this so memorable. Knowingly and unknowingly, I have learnt so much from each and every one of you.

To my family, my parents and my little brother, it does not get said enough that the amount of unconditional love I have received from you means everything to me. When I see you celebrate my victories more than myself, I feel all the efforts are worth it. Thank you for being so very understanding and bearing with my moods this whole time. I also would like to thank Pratik, I feel so lucky to have found my life partner in my best friend. Thank you for all the times you pushed me to keep going and stood by me during so many of my struggles.

A special thank you to all my teachers and mentors I have had in my life. I have been extremely lucky to have had teachers such as Mrs. Vimla, Dr. Nadig and so many others who have shaped me in a million ways.

To all my amazing friends all over the world, thank you for being there at all times of the day and night for me. You all have believed in me always, literally for decades! Thank you Roshni, Priya, Shri, Kayenat, Divya, Sampoorna, Diana, Araditha, Amita, Kirti, Arya, Shalini, Rini, Swagata, Priyanka, Girish, Bhavani, Shruti and so many others.

I dedicate this work to each and every one of you who have wished me well and helped me in this journey

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**LIST OF ABBREVIATIONS**

ECM – Extracellular matrix

FN- Fibronectin

TGF-β2- Transforming growth factor β2

TGM2 - Tissue transglutaminase

Ad5 - Adenovirus serotype 5

KO – Knockout

EGFR - Epidermal growth factor receptor

IGFIR - Insulin-like growth factor 1 receptor

MMP - Matrix metalloproteinases

LOX - Lysyl oxidase

IOP- Intraocular pressure

POAG – Primary open angle glaucoma

JCT – Juxtacanalicular region

SC – Schlemm's canal

GTM – Glaucomatous trabecular meshwork

BMP – Bone morphogenic protein

Ah – Aqueous humor

CLAN – Cross linked actin network

ADAM – Disintegrin-like and metalloproteinases

SD – Sheath derived

GGEL - N-ε (γ-glutamyl) lysine

CMV – Cytomegalovirus

MTM – Mouse trabecular meshwork

DMEM - Dulbecco's modified Eagle's medium

MOI – Multiplicity of infection

PFU – Plaque forming units

GFP – Green fluorescent protein

ANOVA – Analysis of variance

SEM – Standard error of mean

αSMA – Alpha smooth muscle actin

PBS – Phosphate buffer saline

PFA – Paraformaldehyde

DAPI - 4′,6-diamidino-2-phenylindole

ONH – Optic nerve head

RGC – Retinal ganglion cell

# CHAPTER I

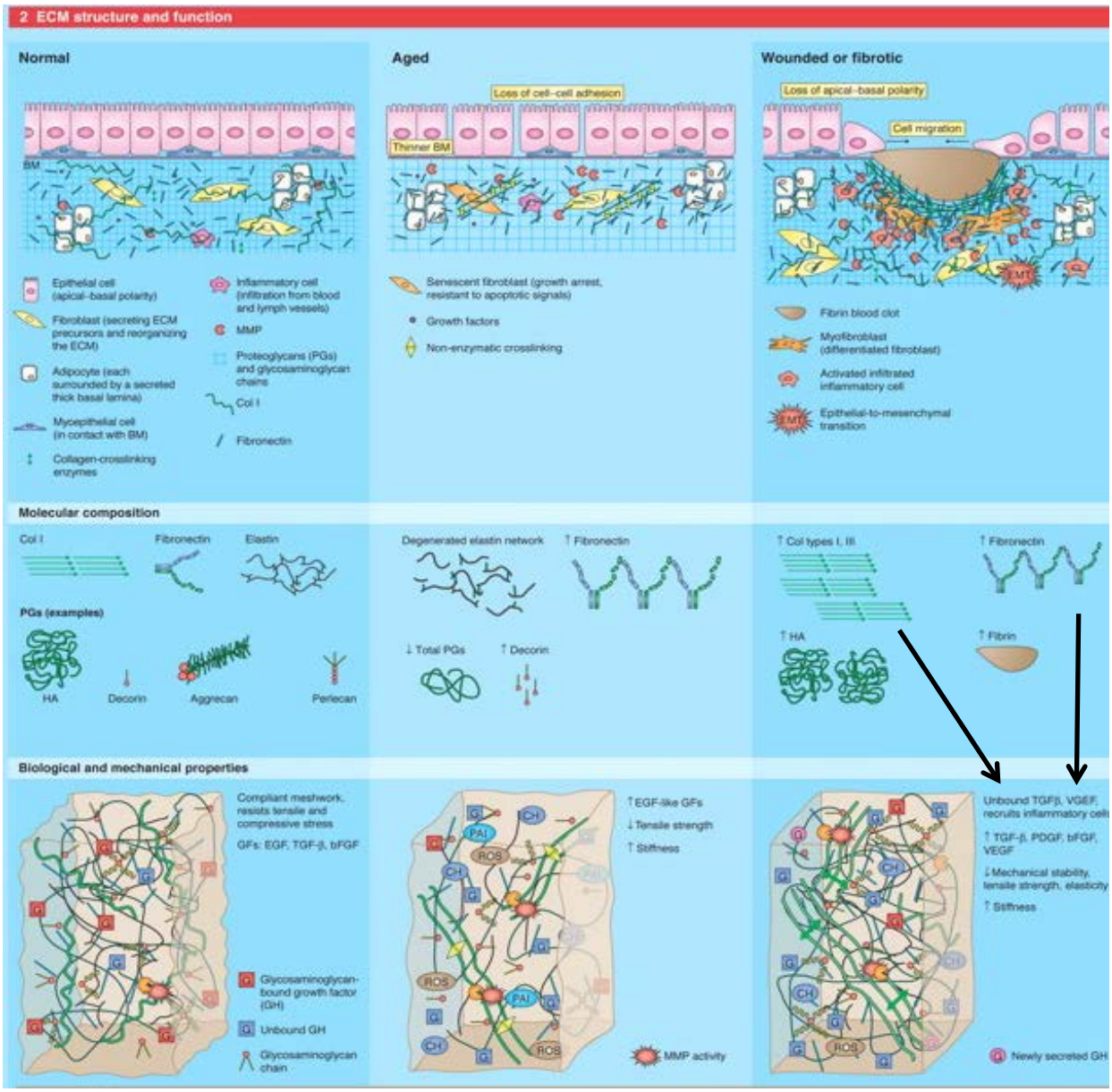
# INTRODUCTION

## Extracellular matrix, aging and fibrosis

The extracellular matrix (ECM) of a tissue provides structural and functional integrity and is one of the most important regulators of homeostasis (*1*) . The ECM is composed of water, proteins including fibrillar and non-fibrillar collagens, elastin, fibronectin (FN) and sugars including glycosaminogycans (GAG). Proteoglycans have a wide variety of functions that manifest in their hydration, binding and force-resistance properties (*2*). Collagen is the most abundant protein in the ECM, and its structure, organization and distribution plays a major role in ECM stability (*3*). Other ECM proteins such as elastin and fibronectin are closely associated with collagen. Elastin, which is formed from its precursor tropoelastin, provides recoil and flexibility to tissues that constantly undergo stretch. Elastin fibers are usually surrounded by glycoprotein microfibrils which are essential constituents of their structure (*4*). Fibronectin is a large dimeric protein and contains sites for binding of other ECM molecules and integrins (*5*). Understandably, along with the obvious function of the ECM to provide a structural scaffold to any tissue, it is also involved in various signaling pathways including binding and activation of epidermal growth factor receptor (EGFR), transforming growth factor β (TGFβ) and insulin-like growth factor 1 receptor (IGFIR) (*3*). Depending on the tissue, an appropriate rate of turnover of all ECM components maintains normal physiological functioning of the tissue.

As a tissue ages, intercellular signaling as well as cell to ECM interactions get dysregulated (*6*). Loss of junctional proteins such as cadherins, catenins and occludins causes functional deficits in signaling ultimately affecting tissue function. Fibroblasts in aged tissue also undergo senescence, which leads to upregulated levels of FN, matrix metalloproteinases (MMP's), cytokines and growth factors (*7*). With age, tissues exhibit gradual disorganization of their structure and decline of their particular functions.

Fibrosis differs from aging in that there is aberrant activation or deactivation of certain pathways leading to functional loss, which is much more pronounced than their age matched controls (Figure 1). One of the main players of fibrosis is transforming growth factor β2 (TGFβ2), and its role in up regulation of ECM proteins is well known (*8, 9*). Under stress or injury, fibroblasts rapidly activate latent TGFβ2, which in turn increases expression of ECM proteins fibronectin, collagen and elastin. It also induces expression of crosslinking enzymes lysyl oxidase (LOX) and tissue transglutaminase (TGM2) (*2*). In a fibrotic process, these crosslinking enzymes play a crucial role. As the synthesis of ECM proteins increases, these enzymes continue to irreversibly crosslink the proteins. In disease conditions, accelerated crosslinking stabilizes the ECM and changes tissue properties (*10*).




**Main players in glaucomatous fibrotic changes**

**FIGURE 1: The extracellular matrix at a glance.** Christian Frantz, Kathleen M. Stewart, Valerie M. Weaver. J Cell Sci 2010 123: 4195-4200

## Tissue transglutaminase (TGM2)

TGM2 belongs to a family of thiol and $Ca^{2+}$ dependent acyl transferases (*11*). It is a member of the transglutaminase family which are characterized by their similarity in the enzymatic reactions they catalyze (*12*). Other members of this family include the erythrocyte band 4.2, fXIIIA and

TGM1to TGM7 (*11*, *13*). Phylogenetic analysis of evolution of transglutaminase gene shows that at least 2 main branches probably arose from a common ancestral gene: the genes for erythrocyte band 4.2, TGM2, TGM3, TGM5, TGM6 and TGM7 and another for genes TGM1 and fXIII A and also for invertebrate TGMs and TGM4 (Figure 2). Although they have structural similarity, the mechanisms of transcriptional regulation are not homologous. This is evident from tissue-specific and developmental expression(*11*).

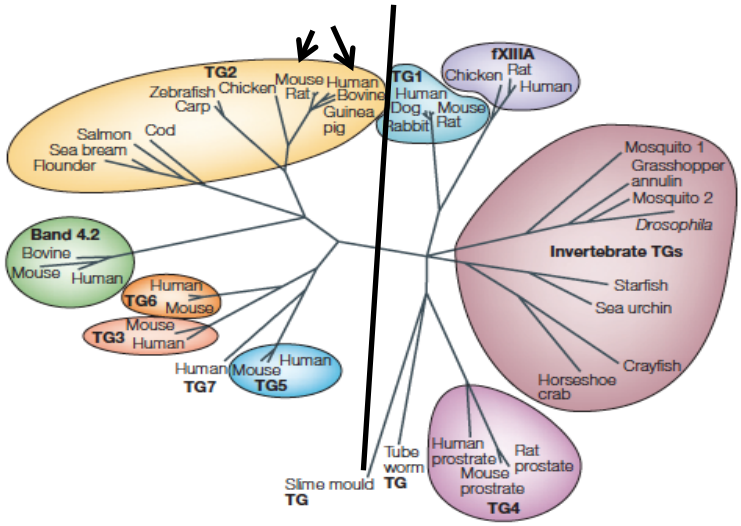



**FIGURE 2: Phylogenetic tree of papain-like transglutaminases**. L. Lorand, R. M. Graham, Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4, 140-156 (2003).

## Gene and protein

Human TGM2 is located on Chromosome 20 (20q12) (Accession numbers: Nucleotide NM_004613.2, Protein NP_004604.2; EC 2.3.2.13) (*12*). All the TGMases except fXIIIA and TG1 have 13 exons and 12 introns, fXIIIa and TGM1 have 15 exons and 14 introns and also have an extra exon encoding the 5' untranslated region. Exon 9 of TGM2, TGM3, TGM4 and band 4.2 is equivalent to exons 10 and 11 of fXIIIa and TGM1, separated by an intron (*14*). The splice variants of the gene are highly conserved.

The most ubiquitous isoform of the TGM family is TGM2 and it is 687 amino acids in length. It consists of 4 domains, an N-terminal β-sandwich domain, catalytic domain and two C-terminal β-barrel domains (Figure 3) (*15*, *16*). Crystal structures of TGM2 bound to GDP, ATP and irreversible inhibitors have been elucidated till date and they all show that TGM2 undergoes a large conformational change depending on whether it binds to GDP or irreversible inhibitors. GTP binding to TGM2 forms a closed structure and inactivates it while in the presence of $Ca^{2+}$ TGM2 gets activated and takes the open conformation (*15*).

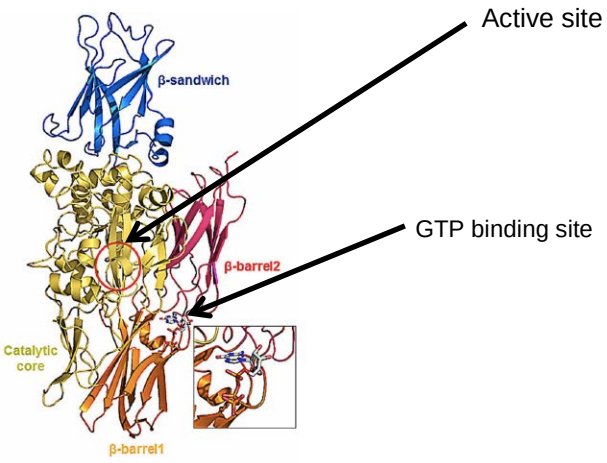



**FIGURE 3: Stoichiometry of TMG2 in solution**: T.-H. Jang *et al.*, Crystal Structure of Transglutaminase 2 with GTP Complex and Amino Acid Sequence Evidence of Evolution of GTP Binding Site. *PLOS ONE* **9**, e107005 (2014)

## Expression and localization

TGM2 is found in various cellular and extracellular compartments including the cytoplasm, nucleus, mitochondria and extracellular space (*11*, *17*). The cytoplasmic pool is the largest compared to distribution in other compartments. Intracellularly, in the closed conformation, TMG2 has some GTPase activity but this is not the most predominant function of TGM2. A variety of factors can trigger induction of TGM2 such as growth factors and chemokines which can trigger the release of $Ca^{2+}$ in the intracellular space and cause transamidating function

intracellularly (*17*). TGM2 also tends to become a plasma membrane associated complex as seen in case of EGF induction.

Extracellular TGM2 is found both near the cell surface in close association with the plasma membrane and in the ECM (*17*). It is known to covalently interact with fibronectin and integrins. It binds with soluble fibronectin and promote its deposition into the ECM. With the help of this interaction, TGM2 promotes cell-ECM adhesion, cell migration and assembly of fibronectin fibrillar matrices. Interestingly, TGM2 interacts with syndecan-4 where it interacts with integrins causing cell adhesion to fibronectin and in turn adhesion dependent development of focal adhesions, actomyosin contractions and stress fibers through RhoA (*18*).

**Unconventional secretion of TGM2**

TGM2 does not have a secretory signal sequence, is not localized in ER/Golgi complex and little is known about its secretion. In fibroblasts, TGM2 is externalized by recycling endosomes (*19*). In an unconventional method, the newly synthesized TGM2 is targeted to and delivered inside perinuclear recycling endosomes. However, whether TGM2 in these endosomes are exported by fusion and budding or any other method is still unknown. Reports indicate that the oxidative state is crucial for retention of TGM2 on the cell surface and its translocation to the ECM.

**Crosslinking mechanism**

TGM2 binds to its first substrate at a glutamine residue (Figure 4). This is called an ‘acceptor’. This is accompanied by release of ammonia or amine. The second substrate is called a ‘donor’. The donor binds to the acylenzyme intermediate, this is thought to be the rate limiting step. Two

main ways of protein crosslinking are – 1) De novo direct polymerization of proteins and 2) Enzymatic 'spotwelding' of proteins (*11*).

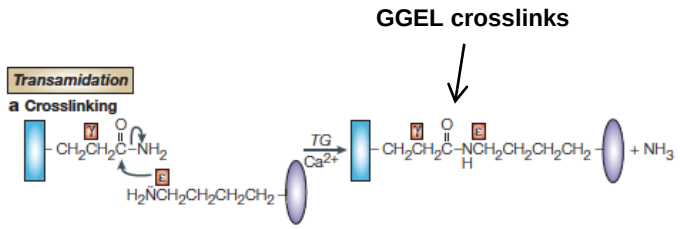



**FIGURE 4: Transamidation by transglutaminases.** L. Lorand, R. M. Graham, Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4, 140-156 (2003).

## TGM2 in fibrosis

Transglutaminase crosslinking activity is implicated in a variety of fibrotic diseases such as renal, hepatic and pulmonary fibrosis (*20*-*22*). Extracellularly, TGM2 binds the ECM proteins collagen, elastin and fibronectin and causes a fibrotic phenotype when turnover of ECM is dysregulated due to excessive crosslinking.

## Glaucoma and TGM2 in glaucoma

Glaucoma is an optic neuropathy characterized often by increased intraocular pressure (IOP), increased cup to disc ratio and a gradual loss of visual field as the disease progress. Of the different types of glaucoma, increased IOP is the highest risk factor for glaucoma (*23*). Increased resistance in aqueous outflow through the trabecular meshwork, a thin rim of tissue along 360$^{o}$ of the eye, is the main cause of increased IOP (*24*).

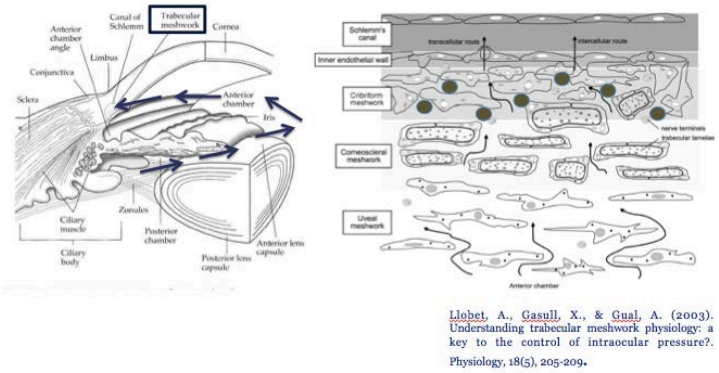



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**FIGURE 5: Aqueous humor outflow pathway and ultrastructure of TM**

The role of ECM deposition at the TM tissue has been studied since a long time to better understand ways to modulate outflow facility (Figure 5) (*25-30*). The sheath derived (SD) plaques seen at the inner region of the Schlemm's canal, corresponding to the juxtacanalicular region (JCT) region of the TM, were increased and arrangements of collagen fibrils were more irregular in glaucomatous eyes compared to age matched controls (*27*, *28*, *31*). This led to studying the role of enzymes including TGM2 that cause ECM crosslinking and thereby deposition.

Normally, the endogenous MMPs and disintegrin-like and metalloproteinases (ADAMs) are can break and turnover ECM in a balanced rate and maintain ECM homeostasis. However, in glaucoma, there seems to be an imbalance between ECM formation and degradation. The increased ECM deposition seen in glaucomatous TM may be attributed to irreversible covalent ECM protein crosslinking by the major crosslinking enzymes TGM2, LOX and lysyl-oxidase like enzymes (LOXL1 - 4). These enzymes crosslink major ECM proteins such as fibronectin, collagens and elastin. The reactions mediated by these enzymes lead to irreversible covalent bond formation, which increases the stability of the crosslinked ECM. There are no known endogenous enzymes that can break these bonds. Chronic covalent crosslinking decreases ECM

turnover and can consequently lead to biophysical changes in the TM, and over a prolonged period, this crosslinking can cause the TM tissue to gain a fibrotic phenotype. In POAG, there is significant thickening of elastic fiber sheaths in TM. For example, there is increased formation of 'sheath-derived' (SD) plaques in TM of POAG patients compared to age-related controls. This can cause increased TM stiffness thereby decreasing the ability of TM to facilitate AH outflow.

Previously, we have shown that glaucomatous TM cells and tissues have increased expression of TGM2 compared to age-matched controls. TGFβ2 induces TGM2 expression in TM cells utilizing the canonical Smad-signaling pathway. TGM2 is involved in calcium dependent covalent crosslinking of the ECM proteins collagen and FN through N-ε (γ-glutamyl) lysine (GGEL) linkages. Previous studies have shown that the TM of human eyes expressed TGM2 and increased expression was seen in glaucomatous eyes. We therefore wanted to see if overexpression of TGM2 in mouse eyes increased IOP through increased crosslinking activity at the TM region.

**Overall hypothesis**: We hypothesize that TGM2 plays a role in ECM crosslinking in the trabecular meshwork and thereby reduces aqueous humor outflow resistance and increases intraocular pressure.

We addressed our hypothesis under 3 specific aims.

**Specific aims:**

**Specific aim 1: To determine whether overexpression of TGM2 in mouse eyes will increase crosslinking in the TM region and lead to increased IOP and decreased AH outflow facility**

**Specific aim 2: To determine whether knockout of TGM2 can significantly reduce TGFβ2 induced ocular hypertension**

**Specific aim 3: To test a small molecule TGM2 inhibitor and determine its effect on crosslinking in glaucomatous TM cell strains**

**Significance:**

ECM deposition is a feature observed in patients with POAG. TGM2 over-expression causes dysregulation of ECM homeostasis and can lead to increased ECM deposition. Therefore this study helps in understanding a novel mechanism that could be responsible for increased IOP as seen in glaucoma. Our animal study also could be used as a model for ocular hypertension.

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# CHAPTER II

## Tissue Transglutaminase Elevates Intraocular Pressure in Mice

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ABSTRACT

Purpose: Tissue transglutaminase (TGM2) is elevated in glaucomatous trabecular meshwork (TM) tissues. We investigated whether increased expression of TGM2 increases extracellular matrix (ECM) crosslinking in the TM, thereby increasing aqueous humor (AH) outflow resistance and elevating intraocular pressure (IOP) in mouse eyes.

Methods: GTM3, primary human GTM 125-05 and cultured mouse TM (MTM) cells were transduced with Ad5.TGM2 (MOI-75) and fixed for immunocytochemistry (ICC). To test the effect on IOP in living eyes, Ad5.TGM2 was injected intravitreally into one eye of BALB/cJ (n=18) or C57BL/6J mice (n=9). The uninjected contralateral eye and Ad5.GFP served as controls. Daytime conscious IOPs were measured twice/week. Aqueous outflow facility (C) was measured by constant flow infusion on completion of IOP measurements. Immunohistochemistry (IHC) was performed on BALB/cJ mouse eyes to study TGM2 expression and activity.

Results: Treatment of cultured TM cells with Ad5.TGM2 increased immunostaining of GGEL crosslinks. Ad5.TGM2 injection significantly increased IOP in BALB/cJ (15.86 mmHg (injected) vs. 10.70 mmHg (control)) and in C57BL/6J (17.09 mmHg (injected) vs. 12.01 mmHg (control)) mice. Mean C in injected eyes of BALB/cJ (0.013 μL /min/mmHg) and C57BL/6J (0.012 μL /min/mmHg) was significantly lower than uninjected control eyes [BALB/cJ (0.021 μL/min/mmHg) and C57BL/6J (0.019 μL/min/mmHg)]. Ad5.TGM2 transduction of mouse eyes increased TGM2 expression in the TM region and increased GGEL crosslinks.

Conclusion: Increased expression of TGM2 in the TM increases GGEL crosslinking in the TM, increases aqueous outflow resistance and elevates IOP in mice. TGM2 may be at least partially responsible for ocular hypertension in POAG.

## INTRODUCTION

Primary Open Angle Glaucoma (POAG) is a multifactorial ocular disease characterized by optic neuropathy and is a leading cause of irreversible vision loss worldwide.[1, 2] One of the major risk factors associated with POAG is high intraocular pressure (IOP). Increased IOP is associated with changes in cellular and extracellular matrix (ECM) components of the trabecular meshwork (TM) that lead to increased aqueous humor (AH) outflow resistance and ocular hypertension.

Transforming Growth Factor – β2 (TGFβ2) is a profibrotic cytokine that is increased in the AH and TM of glaucomatous eyes compared to controls.[3-7] The ECM in TM tissues undergoes constant turnover, and TGFβ2 increases expression of ECM molecules such as collagen I, III, IV, and VI, elastin, fibronectin (FN), laminin and the crosslinking enzymes tissue transglutaminase (TGM2) and lysyl oxidase (LOX).[7-11] TGFβ2 plays a major role in ECM remodeling through secretion of ECM proteins along with Plasminogen Activator Inhibitor-1 (PAI-1), which inhibits matrix metalloproteinase (MMP) activity.[12] The bone morphogenic protein (BMP) antagonist gremlin is also elevated in glaucomatous trabecular meshwork (GTM) cells and causes ECM deposition by potentiating the fibrotic effects of TGFβ2.[13, 14] Since the major outflow pathway for AH is through the TM, modulation of ECM architecture can affect AH outflow through this tissue.[15] Understandably, glaucoma research has focused on studying pathways that can modify the TM structure and thereby affect its filtration function.

Existing pharmacological treatments for glaucoma provide symptomatic relief and are primarily targeted to lower IOP.[16] Surgical intervention by laser trabeculoplasty, trabeculectomy[17] or implantation of artificial drainage devices[18] is used when medications no longer adequately

control IOP. However, even with extensive research in this field, there presently is no cure for glaucoma. This is likely in part due to inadequate understanding of the complete pathophysiology of the disease in the TM and lack of treatments that directly inhibit glaucomatous damage to the TM. Active areas of research include genetic causes, for example myocilin mutations that are responsible for a subset of POAG.[19, 20] Cytoskeletal rearrangements leading to cross-linked actin network (CLAN) formation are found in glaucomatous TM cells and tissues and may be responsible for increased cell stiffness and biomechanical alterations in the TM contributing to outflow resistance.[21, 22]

One of the major causes for obstruction of outflow at the TM could be increased crosslinking and deposition of ECM proteins. There is increased production and deposition of ECM proteins such as collagen and FN in glaucoma.[23, 24] Normally, the endogenous MMPs and disintegrin-like and metalloproteinases (ADAMs) are capable of breaking down this ECM, thereby maintaining ECM homeostasis.[25] However, in glaucoma, there seems to be an imbalance between ECM formation and degradation. The increased ECM deposition seen in glaucomatous TM may be attributed to irreversible covalent ECM protein crosslinking by the major crosslinking enzymes TGM2, LOX and lysyl-oxidase like enzymes (LOXL1 - 4). These enzymes crosslink major ECM proteins such as fibronectin, collagens and elastin. The reactions mediated by these enzymes lead to irreversible covalent bond formation, which increases the stability of the crosslinked ECM.[26] There are no known endogenous enzymes that can break these bonds. Chronic covalent crosslinking decreases ECM turnover and can consequently lead to biophysical changes in the TM, and over a prolonged period, this crosslinking can cause the TM tissue to gain a fibrotic phenotype. In POAG, there is significant thickening of elastic fiber sheaths in TM. For example, there is increased formation of ‘sheath-derived’ (SD) plaques in TM of POAG patients compared to age-related controls.[27, 28] This can cause increased TM stiffness thereby decreasing the ability of TM to facilitate AH outflow.

Previously, we have shown that glaucomatous TM cells and tissues have increased expression of TGM2 compared to age-matched controls.[29] TGFβ2 induces TGM2 expression in TM cells

utilizing the canonical Smad-signaling pathway.[7] TGM2 is involved in calcium dependent covalent crosslinking of the ECM proteins collagen and FN through N-ε(γ-glutamyl) lysine (GGEL) linkages.[30] These crosslinked proteins are deposited in the TM and may reduce overall turnover of ECM proteins leading to a fibrotic phenotype. We therefore wanted to mimic the effects of TGM2 overexpression in the TM and study its effects on IOP and AH outflow resistance in mice.

## MATERIALS AND METHODS

### Ad5.TGM2 viral vectors and cell culture transduction

Adenovirus serotype 5 expressing human tissue transglutaminase 2 (Ad5.TGM2) using a cytomegalovirus (CMV) promoter was obtained from Vigene Biosciences (Ad5.TGM2 in PBS buffer) (Rockville, Maryland, USA) and from Vector Biolabs (Ad5.TGM2 in DMEM with 2% BSA & 2.5% Glycerol) (Malvern, Pennsylvania, USA). Basic Local Alignment Search Tool (BLAST) was performed for sequence homology in Uniprot, and human and mouse TGM2 protein homology is 84.1%. Ad5.Null (Vector Biolabs) and Ad5.GFP (Vigene Biosciences) were used as experimental controls in cell culture and mouse work, respectively.

A transformed human glaucomatous TM cell line (GTM3),[31] a mouse primary TM (MTM) cell strain[32] and a primary glaucomatous TM (GTM 125-05) cell strain (isolated from a 78 year old female donor eye obtained from Lions Eye Institute for Transplant and Research, Tampa, Florida) were cultured in Dulbecco's modified eagle's medium (DMEM, low glucose, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Fetal Bovine Serum (FBS, Atlas Biologicals, Collins, CO, USA), L-glutamine (GE Healthcare Life Sciences, Logan, UT, USA, 0.292 mg/mL) and penicillin (100 U/mL)-streptomycin (0.1 mg/mL) (Sigma-Aldrich Corp) as previously described.[7,9-11,13,14,24,29,33] Medium was changed every 2 to 3 days, and cells were grown to confluency in T-75 flasks. Approximately 50,000 GTM3, MTM and GTM125-05 cells

were plated onto coverslips in 24 well plates. GTM 125-05, GTM3 and MTM cells were treated with Ad5.Null (Vector Biolabs) and Ad5.TGM2 (Vector Biolabs) at an MOI of 75, and medium was changed after 24 hours. 48 hours after medium change, human TM cells were fixed for immunocytochemistry. MTM cells were grown for 5 days after medium change and then fixed for immunocytochemistry.

**Immunocytochemistry for TGM2, FN and ε-(γ-glutamyl) lysine (GGEL) bonds**

Two days (GTM3 and GTM125-05 cells) or five days (MTM cells) after transduction, cells were washed 3 times with PBS. Cells on coverslips were then fixed in 4% PFA for 30 minutes and washed with PBS 3 times. Cells were blocked in PBS superblock (Thermo Fisher Scientific, Waltham, MA, USA) for 2 hours, followed by incubation in primary antibodies TGM2 in GTM 125-05 cells, GTM3 and MTM cells (Rabbit monoclonal (EP2957) to Transglutaminase 2, Abcam (Cambridge, MA, USA), diluted 1:500), GGEL in GTM 125-05, GTM3 and MTM cells (ab424 abcam, diluted 1:50), and FN in GTM 125-05 cells (AB1945, Millipore, Temecula, California, diluted 1:500) in PBS Super block overnight at 4°C. Cells were then washed 3 times with PBS and incubated for 2 hours with the appropriate secondary antibodies (Goat anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA, USA) for TGM2, diluted 1:1000; Goat anti-Mouse IgM Cross Adsorbed Secondary Antibody, DyLight 594 (Thermo Fisher Scientific, Waltham, MA, USA) conjugate for GGEL, diluted 1:100; donkey anti-rabbit Alexa Fluor 488 for FN, diluted 1:1000) in PBS super block. Cells were washed with PBS 3 times followed by 3 quick washes with water (continuous washes without incubation) and mounted with ProLong Gold Antifade Mountant containing 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Slides were allowed to dry overnight and fluorescence imaging was done at the end of the process using a Keyence all-in-one fluorescence microscope (Itasca, IL).

Fluorescence intensity of TGM2, GGEL and FN were determined by ImageJ. To quantify, 2 locations in 3 coverslips per treatment group were imaged at low magnification (100X). Intensity was measured in ImageJ (version 1.50i), and nuclei in the same area were counted in Adobe Photoshop (Adobe Systems, San Jose, CA, USA). The fluorescence intensity was normalized to DAPI counted nuclei for all treatment groups in GTM 125-05 and MTM cells. In GTM3 cells,

DAPI count was challenging at low magnification. However, since equal number of cells were plated, fluorescence intensity of different treatment groups under 100X was compared.

**Decellularization**

GTM 125-05 cells plated separately onto coverslips in a 24 well plate were decellularized prior to fixing. [33, 34] Briefly, 3 days after transduction with Ad5.TGM2, cells were washed 3 times with PBS. Cells were incubated in 1 ml of 0.2% Triton X-100 in water at room temperature. Solubilized and detached cells were removed with 3 PBS washes. Subsequently, 1ml of 3% ammonium hydroxide in water was slowly added to the wells and incubated in room temperature for 5 minutes, incubated at -80$^{o}$ for 5 minutes followed by thawing in room temperature for 5 more minutes. Plates were checked under a phase contrast microscope to ensure decellularization and washed 3 times with PBS. Following decellularization, ECM was fixed with 4% PFA and blocking and staining for GGEL and DAPI was continued as described above.

**Mouse studies**

All experiments were conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and the UNTHSC Institutional Animal Care and Use Committee regulations. A total of thirty-five female BALB/cJ retired breeder mice (17 for first study and 18 for the repeat study) and nine female C57BL/6J mice aged 6 to 10 months obtained from The Jackson Laboratory (Bar Harbor, ME, USA) were used for our experiments. The animals were maintained on a 12-hour light/dark cycle (lights on at 0600 hrs). Food and water were available ad libitum.

**Adenoviral injections**

At Day 0, mice were anesthetized using inhalation anesthesia (Isoflurane 2.5%; $O_2$ (g) 0.8 L/min) prior to intravitreal injection with Ad5.TGM2 or Ad5.GFP. Eyes were pretreated with 1 to 2 drops of Alcaine® (Alcon Laboratories Inc., Fort Worth, TX, USA ; proparacaine hydrochloride (HCl) ophthalmic solution, 0.5%) prior to injections. Ad5.GFP (1x $10^6$ plaque forming units (pfu)) was injected intravitreally (left eye) in BALB/cJ retired breeder mice (n=5) for the pilot study and Ad5.CMV.TGM2 (1-50 × $10^6$ pfu) was injected intravitreally (left eye) in BALB/cJ retired breeder mice (n=6 for pilot study and n=18 for repeat) or C57BL/6J mice (n=9), using a 10 μL glass microsyringe (Hamilton Company, Reno, NV, USA) fitted with a ½" 33G needle with a 12° bevel to deliver 2 μL, as previously described.[35] The uninjected (right) eyes served as control. For our first study, we also used 6 naïve uninjected mice (female BALB/cJ retired breeders) to establish that our experimental conditions did not affect IOP. Eyes were observed at each IOP measurement with a direct ophthalmoscope (Welch-Allyn, Skaneateles Falls, NY, USA) for signs of inflammation of the anterior segment (including iridial hyperemia, corneal edema/opacity, discharge, synechia(e), or lenticular cataract). The pupillary light reflex was also observed. The presence or absence of lenticular opacity observed ophthalmoscopically in each eye was scored (Table 1).

**Conscious IOP measurements and aqueous humor outflow studies**

IOP measurements were taken using the Tonolab rebound tonometer (icare TONOVET, Icare Finland Oy, Vantaa, Finland) as previously described.[36] Mice were handled and acclimatized for approximately 2 weeks. Conscious baseline IOPs were measured the day prior to intravitreal injection (Day -1). Injection took place on day 0. Following injection, IOP was measured in conscious animals twice per week for up to 4 weeks. In order to circumvent issues associated with circadian variation in IOP, measurements were taken in the afternoon at the same time of day commencing between 1:30pm and 2:30pm.

Upon completion of IOP readings, six of the eighteen BALB/cJ mice and three of the nine C57BL/6J mice were used for aqueous humor outflow experiments. AH outflow facility was measured using a constant flow infusion methodology as previously published.[37-39] Briefly, animals were anesthetized with an intraperitoneal injection of an anesthetic cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg), given in a volume of 10 mL/kg (for induction). During the experiment, maintenance doses (approximately ¼× to ½× the initial dose) were given as required, and the animals were placed on an electrically heated pad to maintain body temperature at 37°C. The anterior chambers of the mouse eyes were cannulated with a 30G needle. After allowing up to 30 minutes for stabilization, pressure within the eye was determined at different flow rates (0.1 μL/min to 0.5 μL/min in 0.1 μL/min increments). Following conclusion of the experiment, anesthetized animals were removed from the perfusion apparatus and humanely sacrificed via exposure to 100% $CO_2$ (g) in a chamber until breathing stopped. A thoracotomy was then immediately performed to ensure death. Outflow facility (C) was computed from the reciprocal of the slope of the regression line drawn through the plot of pressure as ordinate and flow rate as abscissa.

**Immunohistochemistry for TGM2, alpha smooth muscle actin (αSMA), ε-(γ-glutamyl) lysine (GGEL), and (FN) in mouse TM**

At the end of the time course of IOP readings, mice were humanely sacrificed as above and eyes were enucleated, fixed in 4% PFA, and paraffin-embedded for immunohistochemistry (IHC). Five-micron sagittal sections were mounted on slides, and the slides were warmed and deparaffinized prior to staining (3 sections per eye were stained and imaged for all staining. Number of pairs of eyes used for each staining differed). Co-staining for αSMA and TGM2 was performed. Slides were stained for GGEL and co-stained for GGEL and FN.

Briefly, for αSMA and TGM2 co-staining, sections from 6 BALB/cJ mouse eyes (n=6 pairs) were used. Deparafffinized slides were placed in antigen retrieval buffer (citrate buffer, 0.01M, pH 6) and placed in a water bath at 60 °C for 2 hours for heat induced epitope retrieval followed by incubation in blocking buffer (10% goat serum/ 0.1% Triton-X in PBS super block) overnight at 4°C. Sections were then co-incubated with αSMA antibody (ab7817, Mouse monoclonal (1A4) to alpha smooth muscle actin, diluted 1:500] and with TGM2 primary antibody [Rabbit monoclonal (EP2957) to Transglutaminase 2, Abcam; diluted 1: 500]. Sections were then washed in PBS 3 times for 10 minutes each followed by washing with PBS-Tween-20 (0.05%) once and incubated for 10 minutes followed by 2 quick washes with water. Sections were then incubated in appropriate secondary antibodies (goat anti-mouse Alexa Fluor 594 for αSMA, diluted 1:1000 and goat anti-rabbit Alexa Fluor 488 for TGM2, diluted 1:1000) in PBS super block for 2 hours. Sections were then washed in PBS 3 times for 10 minutes each followed by wash with PBS-Tween-20 (0.05%) once and incubated for 10 minutes followed by 2 quick washes with water. Tissue sections were stained with DAPI to detect nuclei and allowed to dry overnight prior to imaging using Keyence all-in-one fluorescence microscope (Itasca, IL).

For GGEL staining (n=7 pairs of BALB/cJ mouse eyes), following deparaffinization, antigen retrieval was performed with 0.01M citrate buffer (pH 6) followed by blocking with mouse on mouse blocking solution (M.O.M. Kit, Basic, Vector Biolaboratories, Inc.) for 2 hours, followed by incubation with GGEL primary antibody (ab424; diluted 1:100) in PBS super block overnight at 4°C. Sections were incubated with an appropriate secondary antibody (Goat anti-Mouse IgM Cross-Adsorbed Secondary Antibody, DyLight 594 for GGEL, diluted 1:200) in PBS super block for 2 hours and washed with PBS + Tween 20 (0.05%) followed by PBS washes. Tissue sections were stained with DAPI to detect nuclei and allowed to dry overnight prior to imaging using the Keyence all-in-one fluorescence microscope (Itasca, IL).

For GGEL and FN co-staining (n=3 pairs of eyes, 1 BALB/cJ and 2 C57BL/6J), slides were deparaffinized and directly blocked with PBS super block overnight at 4°C. Slides were then co-incubated with primary antibody to FN (AB1945, rabbit anti-human FN, Millipore, Temecula,

California, diluted 1:500) and GGEL primary antibody (ab424; diluted 1:100) in PBS super block overnight at 4°C. Sections were then washed in PBS 3 times for 10 minutes each followed by incubation with PBS-Tween-20 (0.05%) for 10 minutes followed by 2 quick washes with water. Sections were incubated in appropriate secondary antibodies (goat anti-mouse IgM Cross-Adsorbed Secondary Antibody, DyLight 594 for GGEL, diluted 1:200 and goat anti-rabbit Alexa Fluor 488 for FN, diluted 1:1000) in PBS super block for 2 hours. Sections were then washed in PBS 3 times for 10 minutes each followed incubation with PBS-Tween-20 (0.05%) once and incubated for 10 minutes followed by 2 quick washes with water. Tissue sections were stained with DAPI to detect nuclei and allowed to dry overnight prior to imaging using Keyence all-in-one fluorescence microscope (Itasca, IL).

**Statistics**

All statistical tests were conducted using the software provided by GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) and SigmaPlot 12.5 (Systat Software, Inc, San Jose, CA, USA).. Multiple comparisons were conducted using analysis of variance (ANOVA) followed by Holm-Sidak *post-hoc* testing. For immunocytochemistry quantification of GTM 125-05 and MTM cells, fluorescence intensity normalized to cell number (DAPI), and the Ad5.Null treated group was compared to Ad5.TGM2 transduced group using paired Student's t-test (2-tailed). In GTM3 cells, total fluorescence intensity for Ad5.Null treated group was compared to Ad5.TGM2 transduced group using paired Student's t-test (2-tailed). For aqueous outflow facility, values for injected eyes were compared with their fellow uninjected contralateral control eyes using the paired Student's t-test (2-tailed). In all cases a P-value of $< 0.05$ was considered to be significant.

# RESULTS

## TGM2 crosslinking activity in GTM 125-05, GTM3, and MTM cells

Previously, we and others and reported that Adenovirus Serotype 5 efficiently transduces TM cells and expresses the transgene of interest. [40, 41] Prior to *in vivo* injections, we wanted to test and validate our viral TGM2 transgene in cultured TM cells.

To determine overexpression of TGM2, GTM 125-05, GTM3, and MTM cells were transduced with Ad5.TGM2 and with Ad5.Null as a treatment control. GTM 125-05 and GTM3 cells treated with Ad5.TGM2 (Figures 1B&D and 2B, F&H) showed a significant increase (Figure 1I &2I; p=0.0035 & p=0.01, n=6) in TGM2 expression compared to cells treated with Ad5.Null (Figures 1A&C and 2A, E&G). MTM cells treated with Ad5.TGM2 (Figures 3B, F &H) showed an apparent increase (Figure 3I; p=0.08, n=6) in TGM2 expression compared to cells treated with Ad5.Null (Figures 3A, E&G).

To validate crosslinking activity of TGM2, transduced GTM 125-05, GTM and MTM cells were fixed in 4% PFA and stained for GGEL bonds. The GGEL antibody detects covalent crosslinks mediated by TGM2 between glutamine and lysine side chains (GGEL) of ECM proteins. There appeared to be increased GGEL staining (Figures 1J, 2J & 3J; p=0.1, p=0.09 & p=0.1, n=6) in GTM 125-05, GTM3 and MTM cells treated with Ad5.TGM2 (Figures 1F&H; 2D, F&H; 3D,F & H) compared to cells treated with Ad5.Null respectively (Figures 1E&G; 2C,E & G ; 3C,E & G). This indicates that increased TGM2 crosslinking activity correlated with increased expression of TGM2 as seen in Ad5.TGM2 transduced cells compared to controls. There was no apparent increase in FN in Ad5.TGM2 transduced cells (Figure 1 F&H) compared to Ad5.Null treated cells (Figure 1 E&G) (Fig 1K, p=0.5, n=6). This is likely due to the early time point (48

hours), which was not sufficient time for accumulation of the crosslinked FN. There also appeared to be some co-localization between GGEL and FN.

To ensure that the antibody stains extracellular crosslinks, we decellularized cultured GTM 125-05 cells and stained the ECM with the GGEL antibody. The extracellular matrix derived from cells treated with Ad5.TGM2 showed higher crosslinking (n=3) (Figure 4B) compared to ECM from untreated cells (n=3) (Figure 4A). Coverslips were stained with DAPI to identify nuclei, and the lack of any visible nuclei ensured that all cells were removed (Figure 4C&D).

**Immunofluorescence of TGM2 transduced mouse eyes showed increased TGM2 expression and increased crosslinking following intravitreal injection of Ad5.TGM2**

After confirming expression and activity of our Ad5.TGM2 transducing vector in vitro, one eye of each mouse (BALB/cJ and C57BL/6) was injected with Ad5.TGM2 to evaluate *in vivo* expression and activity. Six BALB/cJ mice were used for TGM2 immunofluorescence. Images were assessed by 2 independent masked observers and each observer identified increased TGM2 staining at the iridocorneal angle including the TM in 5 out of 6 of the Ad5.TGM2 transduced eyes (Figure 5 B&D) compared to its uninjected control (Figure 5 A&C). Increased TGM2 expression was observed in the TM. Sections were co-stained with αSMA to identify TGM2 staining in the TM, and interestingly co-localization of TGM2 and αSMA was observed in the TM. Positive staining of TGM2 was also seen in ciliary body as previously reported[42, 43].

To determine whether increased TGM2 expression led to increased ECM crosslinking activity, we performed GGEL immunofluorescent staining of mouse eye sections. The primary antibody identified GGEL isopeptide bonds. Three independent masked observers assessed GGEL staining between Ad5.TGM2 transduced (Figure 6 B&D) and their contralateral uninjected eyes (Figure 6 A&C) in BALB/cJ. 2 out of 3 masked observers identified increased GGEL crosslink staining in the TM region in 7 out of 7 Ad5.TGM2 injected eyes compared to their contralateral

uninjected controls, and the remaining masked observer identified increased crosslink staining in 5 out of 7 Ad5.TGM2 injected eyes compared to their contralateral uninjected controls.

We also wanted to see if increased TGM2 expression and crosslinks affected FN expression. We tested for GGEL and FN co-expression in 2 C57BL/6J and 1 BALB/cJ mouse eyes. We found increased fibronectin staining in 2 out of the 3 Ad5.TGM2 transduced eyes (Figure 7 B&D) compared to their contralateral uninjected controls (Figure 7 A&C). However, we were unable to see colocalization because the optimal antigen retrieval and blocking methods for GGEL and FN staining varied (i.e. the method optimum for FN was unable to sufficiently stain GGEL at the TM).

**Intravitreal injection of Ad5.TGM2 increases IOP in mouse eyes**

To assess whether overexpression of TGM2 in the mouse TM could affect the IOP, we transduced living mouse eyes with Ad5.TGM2. We first did a pilot study with 17 BALB/cJ mice, 6 in the naïve group, 5 in the Ad5.GFP transduced group and 6 in the Ad5.TGM2 transduced group (Figure 8A). In all mice, intravitreal injections were performed in the left eyes only, while the right eyes were used as a contralateral uninjected control. Ad5.GFP transduction was assessed one week following injection by examination of each eye in a darkened room under ultraviolet illumination (Illumatool® Model LT-9900; Lightools Research, Encinatas, CA, USA) through a filter (excitation 470 nm, emission 515 nm) for fluorescence as an indication of presence of GFP in the anterior chamber. Green fluorescence was observed in the Ad5.GFP transduced eyes *in vivo* at 7 days, which confirmed Ad5.GFP transduction. Ad5.TGM2 transduced eyes showed significantly increased IOP on Day 9 that continued for 2 weeks. Differences between the left and their contralateral right eye (ΔOS-OD) show a significant difference in IOP ($p<0.0001$) in Ad5.TGM2 transduced eyes compared to Δ (OS-OD) of the naïve or Ad5.GFP group (Figure 8B&C). This experiment validated viral transduction as fluorescence was observed in Ad5.GFP transduced eyes. Ad5.GFP eyes did not show IOP elevation nor was IOP affected in the contralateral eyes. In addition, we have observed a similar

result in earlier studies.[39, 44] We therefore followed our next experiment using both BALB/cJ (n=18) and C57BL/6J (n=9) mice.

In BALB/cJ mice, IOPs were increased in the transduced eyes (Figure 9A) with significant increases on days 14, 19 and 22 and a maximum difference of 5.16 mmHg on day 19. The mean IOP of injected eyes on day 19 was 15.86 ± 1.06 (Mean ± SEM) while the mean IOP of uninjected eyes was 10.7 ± 0.48 (Mean ± SEM), which was the day with a maximum difference in IOPs ($p < 0.001$). Similarly, in C57BL/6J mice, injection of Ad5.CMV.TGM2 increased IOP from day 13 with maximum difference at day 17 with mean IOP of 17.09 ± 2.03 mmHg (injected) vs. 12.01 ± 0.47 mmHg (control) ($p<0.05$) (Figure 9B). There was little evidence of inflammation or altered IOP in any of the contralateral control eyes. In injected eyes, we saw none to mild lenticular opacity (score = 0-1), which has previously shown to not affect IOP.[40]

Statistical analysis was performed to ensure that there was no significant difference between eyes prior to injection. In BALB/cJ mice, baseline IOP readings were taken 2 days prior to injections (Figure 9A). Holm-Sidak's multiple comparisons test was performed and the adjusted P value for Day -2 (baseline) was greater than 0.9999. In C57BL/6J mice, baseline IOP readings were taken 1 day prior to injections. Holm-Sidak's multiple comparisons test was performed and the adjusted P value for day -1 (baseline) was 0.5461 (Figure 9B). For our studies, p values less than 0.05 were considered significant, therefore the difference between baseline IOP of both eyes were not significant.

The Baseline IOP dropped over the course of the study in the second BALB/cJ group, by day 8 (adjusted p value was 0.7710) onwards, and persisted until the end of the study day 22 (adjusted p value was 0.5091). A similar result was seen in the C57BL/6J group by day 9, although in this group, the difference was lost by day 20. We feel that these drops over time were modest (≤ 2 mmHg) and were likely a reflection of the animals' settling into the procedure of conscious IOP measurement, and becoming more comfortable with the procedure. These drops were also

considerably smaller than the rise in IOP seen in the Ad5.TGM2-injected groups of between 3 and 5.5 mmHg. However, we acknowledge that the drop in the vehicle-injected groups may have also reflected a masking effect on the rise in the Ad5.TGM2-injected groups

**Overexpression of TGM2 in mouse eyes decreases aqueous humor outflow facility**

Aqueous humor outflow facility (C) measurements were performed using a constant flow infusion method after final IOP readings. In BALB/cJ mice, the mean aqueous outflow facility of the TGM2 transduced eyes (0.013 ± 0.002 μL/min/mmHg) was significantly lower compared to the control eyes (0.021 ± 0.002 μL/min/mmHg) ($p=0.01$) (Figure 10A&B). In C57BL/6J mice, the mean aqueous outflow facility of TGM2 transduced eyes (0.012 ± 0.0018 μL/min/mmHg) was significantly lower compared to control eyes (0.019 ± 0.0036 μL/min/mmHg) the ($p<0.05$) (Figure 10C&D). Therefore, significantly increased outflow resistance was observed in Ad5.TGM2 transduced eyes in both strains.

## DISCUSSION

Our current study addresses the hypothesis that increased expression of TGM2 in the TM may lead to physiological changes in the TM causing increased outflow resistance and elevated IOP. We investigated the effects of using Adenovirus serotype 5 to overexpress TGM2 in the TM of mouse eyes. Overexpression of TGM2 protein and activity (i.e. increased GGEL crosslinks) was confirmed by immunohistochemistry. With an increase in TGM2 expression and activity, we observed a significant decrease in aqueous outflow facility and a significant increase in IOP in TGM2 overexpressing eyes (Figure 11).

Endogenous expression of TGM2 in TM tissues and cells has been previously reported.[29, 42, 43] Overexpression of TGM2 in the mouse TM mimicked the increased expression of TGM2 in the

TM of human glaucomatous eyes as reported previously.[29] Our immunocytochemistry studies were performed at 48 hours or 5 days (in MTM cells), where Ad5.TGM2 transduction significantly elevated TGM2 expression and also showed a trend for increased GGEL immunostaining. While increased TGM2 expression validates our adenovirus (Ad5.TGM2) and an increase in expression of TGM2 corresponds with an increase in its crosslinking activity, 48 hours or 5 days may not be sufficient to cause significantly increased crosslinking of FN, one of the main substrates for TGM2, since ECM turnover is a dynamic process. We believe that longer time points would eventually show significantly increased GGEL crosslinking and FN accumulation. ECM crosslinking through these irreversible covalent bonds between FN molecules and other ECM proteins would slow ECM degradation, lead to reduced turnover and greater ECM deposition.

Recently, Yang et al.[46] have reported that inhibition or induction of LOX, another ECM crosslinking enzyme, affected outflow resistance by modulating ECM crosslinking in perfused human and porcine anterior segments.[45] In the present study, we have determined that increased crosslinking by TGM2 alone can significantly elevate IOP in mouse eyes and can therefore be a potential therapeutic target. However, it will be interesting to see whether inhibition of TGM2 crosslinking activity can significantly reduce IOP or whether other crosslinking enzymes such as LOX and LOXL1-5 compensate it.

TGFβ2 is elevated in the AH and TM of POAG patients.[3-7] Excess TGFβ2 activates the profibrotic pathway. TGFβ2 increases production of ECM proteins along with ECM crosslinking enzymes (i.e. TGM2 and LOXs). A fibrotic phenotype is a result of dysregulation in the formation of ECM and/or its breakdown. TGM2, which is elevated in POAG eyes, is associated with other fibrotic diseases.[29] Classic actions of TGM2 include crosslinking ECM proteins causing increased tissue stiffness and loss of homeostasis.[46, 47] The presence of increased GGEL crosslinks indicates that increased expression of TGM2 in the TM is associated with increased crosslinking activity.[29] The present study suggests that an increase in crosslinking can decrease turnover of ECM in the TM thereby promoting ECM deposition that leads to increased outflow

resistance through the TM. While we believe that this is the most significant factor leading to ECM deposition and increased outflow resistance, TGM2 is also known to activate TGFβ2 from its latent form.[48] This pathway could therefore also be partially responsible for the downstream effects of TGFβ2 (including a TGFβ2-mediated increase ECM production). Fibronectin is an ECM substrate for TGM2, and increased expression of FN has been shown in the TM of POAG eyes[24]. Similarly, other ECM proteins can be crosslinked by TGM2, thereby contributing to ECM deposition. It would be interesting to further investigate which of the ECM proteins are crosslinked by TGM2 in the glaucomatous TM tissue. Intracellular TGM2 also has a variety of signaling functions,[49] which may affect IOP or AH outflow facility and will require further study.

A remaining intriguing question in POAG is, among all the pathways and genes activated by TGFβ2, which are capable of reducing aqueous outflow and significantly increasing IOP? In the past, ex vivo cultures perfused with MMPs and more recently wild-type mice injected intracamerally with AAV-MMP-3 decreased IOP, suggesting that the TM ECM regulates IOP.[15, 47, 50] In our present study, TGM2 overexpression decreased the AH outflow facility suggesting that ECM remodeling in the TM significantly increased IOP. From the modified Goldmann equation,[37,51] and assuming reported values for aqueous humor formation rate (Fin), uveoscleral outflow (Fu), and episcleral venous pressure (Pe), in BALB/cJ mice[51] the theoretical mean IOP of the injected eyes as calculated from the outflow facilities is 15.2 mmHg (the measured mean value by rebound tonometer the day before was 14.70 ± 1.01 mmHg), whereas the theoretical mean IOP of the uninjected eyes is 11.7 mmHg (the measured mean value the day before by rebound tonometer was 10.69 ± 0.32 mmHg). [The modified Goldmann equation states that IOP = [((Fin-Fu)/C) + Pe]. Thus, for naïve (uninjected) BALB/cJ mouse eyes (assuming that C = 0.021 microliters/min/mmHg): IOP = [((0.17 – 0.05)/0.021) + 6] = 11.7 mmHg (assuming Fu = 30% of Fin). For Ad5.TGM2-injected BALB/cJ mouse eye (assuming C has dropped to 0.013 microliters/min/mmHg): IOP = [(0.17 – 0.05/0.013) + 6] = 15.2 mmHg (assuming Fu is 30% of Fin). These computed IOP values are very close to those we actually measured in BALB/cJ mice. The aqueous humor dynamic parameters for BALB/cJ mice are from a previous publication[51]. Therefore, the TGM2 induced IOP elevation corresponded closely with the TGM2

mediated reduction of the outflow facility. For our outflow studies on C57BL/6J, we did not have a large enough "n" to obtain a better significance. In the future, it will be interesting to determine whether TGM2 leads to the dense fibrillar depositions that have been observed in POAG eyes.

In POAG, increased resistance to aqueous outflow elevates IOP, which likely contributes to the damage to the optic nerve. Consistently high IOP or IOP fluctuations in POAG could be the result of fibrotic changes in the TM.[52,53] In normal conditions, the IOP is constantly maintained. But in pathological conditions there is a dysregulation in the TM, which affects the tissue dynamics and leads to a fibrotic phenotype. During the initial stages of disease progression, the tissue probably has higher compensation capability leading to the fluctuations. As the disease progresses, the ECM deposition increases to an extent that cannot be compensated any further leading over time to a high IOP. Our model has features of increased ECM crosslinking, increased outflow resistance and elevated IOP, which phenocopies ocular hypertension in POAG. TGFβ2 has a number of effects on the TM, so in the future it will be interesting to determine whether TGFβ2-induced ocular hypertension is mediated at least partially by increased TGFβ2 induced TGM2 expression and activity. Consequently, inhibition of TGM2-mediated ECM crosslinking by could be a potential therapeutic approach. However, the challenge would be to determine if TGM2 inhibition alone would suffice or whether TGM2 inhibition in combination with other IOP lowering agents would be more effective as a cure for glaucoma.

Acknowledgements: The authors would like to sincerely acknowledge the technical assistance of Sherri Harris and Sandra Maansson. We also thank Dr. Iok-Hou Pang, Dr. Tara Tovar-Vidales and Dr. Colleen M. McDowell for freely sharing their knowledge and expertise.

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## Figures and figure legends

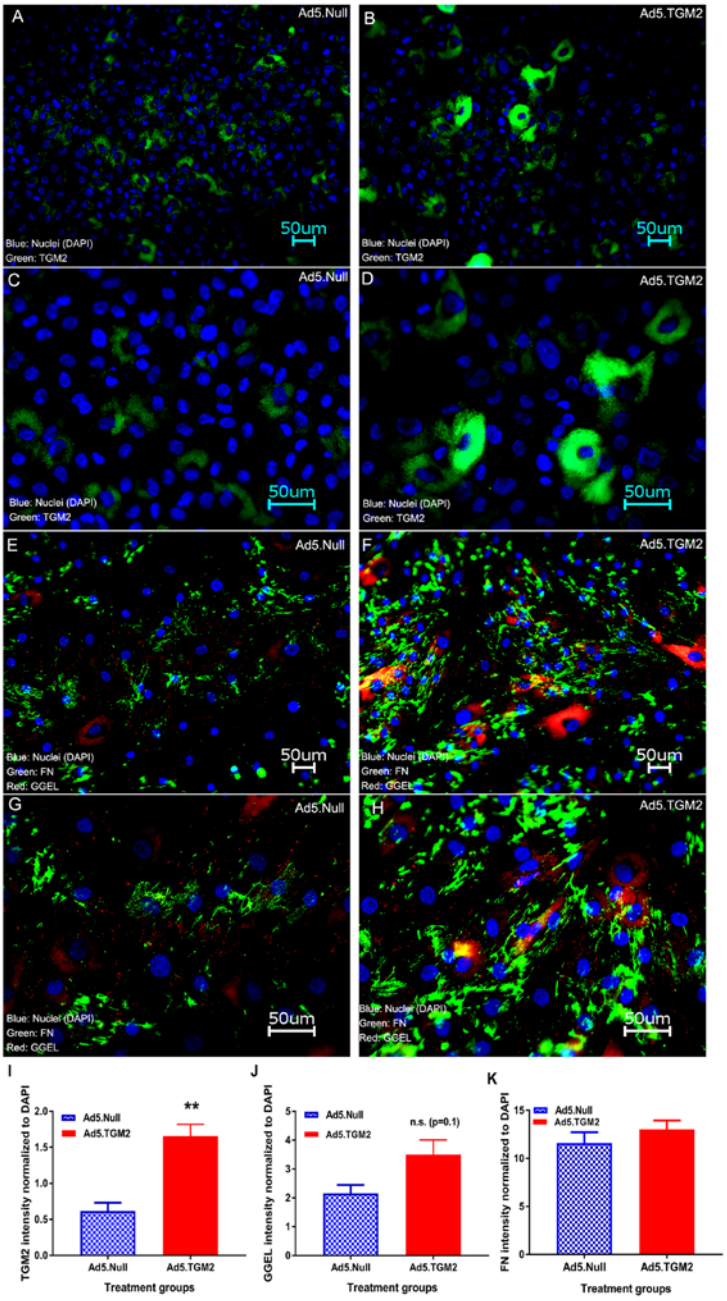

**Figure 1. Ad5.TGM2 transduction of cultured primary cell strain GTM 125-05 increases TGM2 expression, increases GGEL crosslinks and fibronectin expression.** Increased TGM2 staining is observed in Ad5.TGM2 transduced GTM 125-05 cells (B, D) compared to Ad5.Null treated control (A, C) (Quantification of TGM2 expression-I; p=0.0035, n=6). [A&B-100X; C&D-400X]. Increased GGEL crosslinks and fibronectin co-staining in GTM 125-05 cells transduced with Ad5.TGM2 (F&H) compared to Ad5.Null treated control (E&G) (Quantification of GGEL-J, p=0.1 (n.s.) and FN-K, p=0.5 (n.s.); n=6) [A&B; E&F-100X; C&D; G&F-400X]. Green: TGM2; Red: GGEL, Blue (DAPI): Nuclei

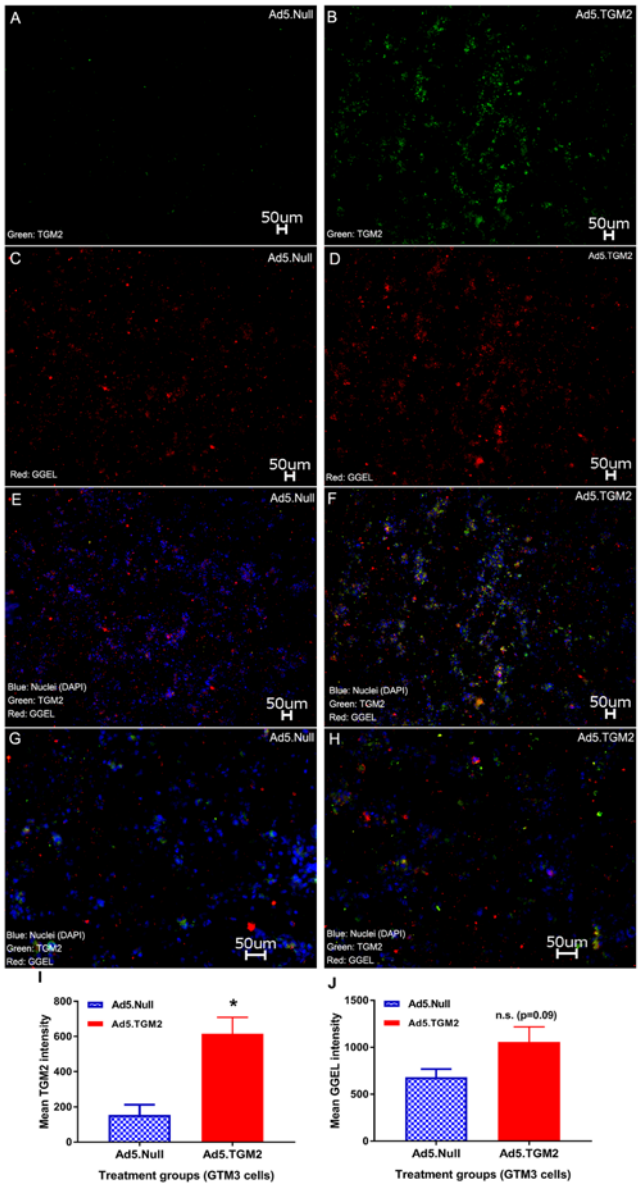


**Figure 2. GTM3 cells transduced with Ad5.TGM2 showed increased TGM2 expression and GGEL crosslinks.** Increased TGM2 staining is observed in Ad5.TGM2 transduced GTM3 cells (B, F&H) compared to Ad5.Null treated control (A, E&F) (Quantification of TGM2 expression-3I, p=0.01; n=6). Increased GGEL crosslinking in GTM3 cells transduced with Ad5.TGM2 (D, F&H) compared to untreated control (C, E&G) (Quantification of GGEL -3J, p=0.09 (n.s.), n=6). (A-F-100X, G-H-200X)

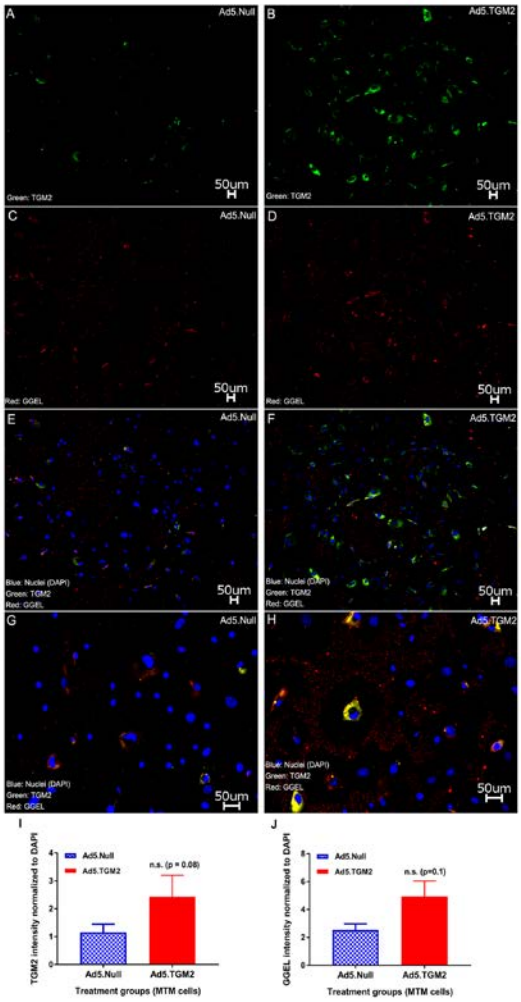


**Figure 3. MTM cells transduced with Ad5.TGM2 showed increased TGM2 expression and GGEL crosslinks.** Increased TGM2 staining is observed in Ad5.TGM2 transduced MTM cells (B, F&H) compared to Ad5.Null treated control (A, E&F) (Quantification of TGM2 expression- I;p=0.08 (n.s.); n=6). Increased GGEL crosslinking is observed in MTM cells transduced with Ad5.TGM2 (D,F&H) compared to untreated control (C,E&G) (Quantification of GGEL – J ;p=0.1(n.s.); n=6). (A-F-100X, G-H-200X)

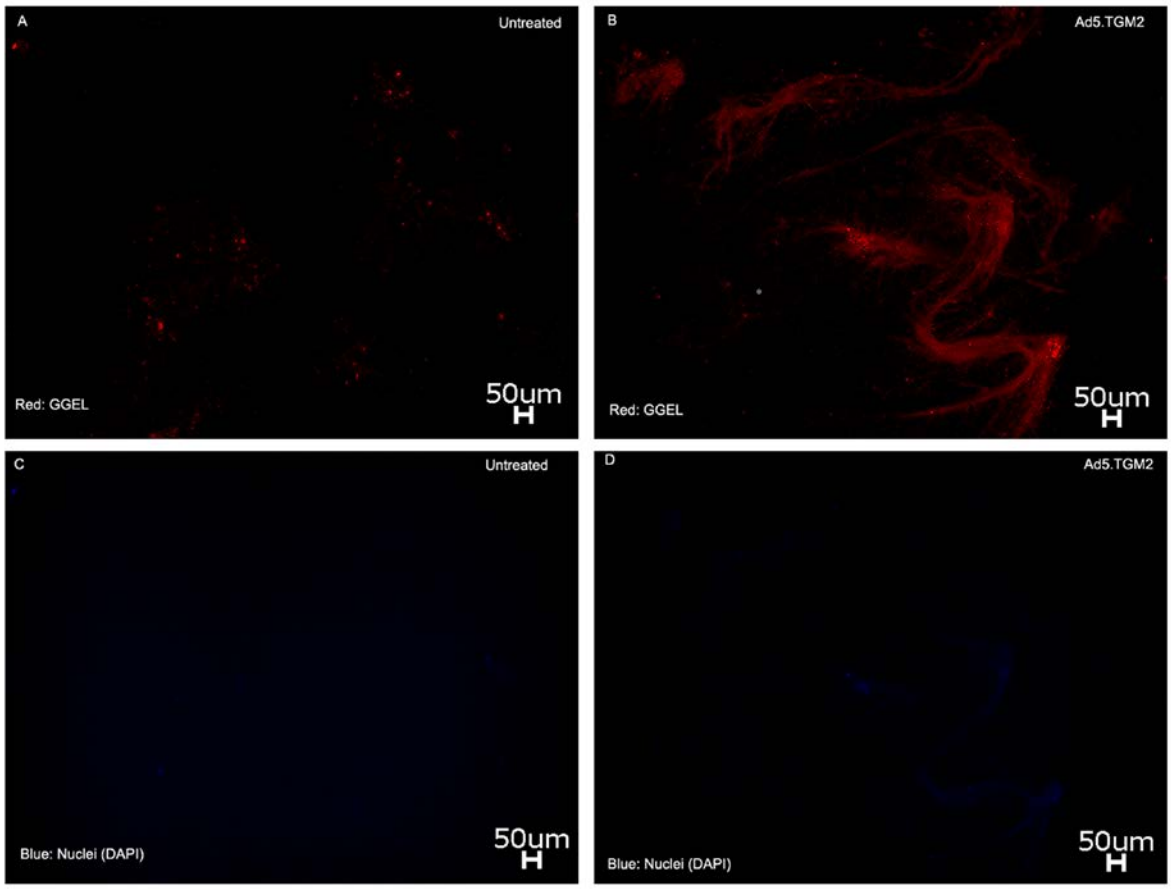


**Figure 4. GGEL staining after decellularization of GTM 125-05** (A-D). Increased GGEL crosslinks in the ECM of cells transduced with Ad5.TGM2 (B) compared to untreated control (A). DAPI staining showed no visible nuclei following decellularization (C&D) (n=3). Red: GGEL, Blue (DAPI): Nuclei

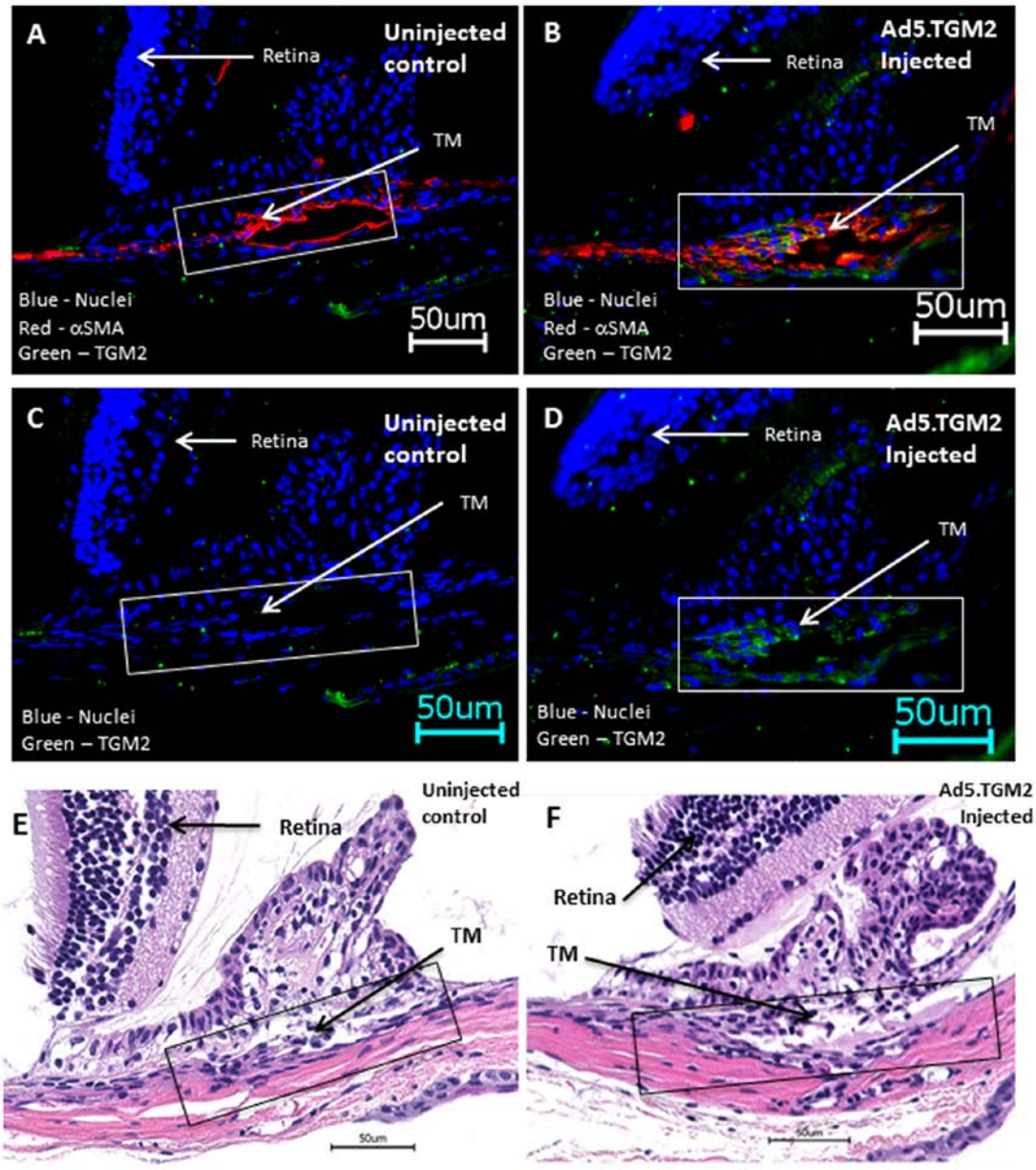
A
Uninjected control
Retina
TM
Blue - Nuclei
Red - αSMA
Green – TGM2
50um
B
Ad5.TGM2 Injected
Retina
TM
Blue - Nuclei
Red - αSMA
Green – TGM2
50um
C
Uninjected control
Retina
TM
Blue - Nuclei
Green – TGM2
50um
D
Ad5.TGM2 Injected
Retina
TM
Blue - Nuclei
Green – TGM2
50um
E
Uninjected control
Retina
TM
50um
F
Ad5.TGM2 Injected
Retina
TM
50um

**Figure 5. Effect of Ad5.TGM2 transduction of mouse eyes on expression of TGM2 in the TM region**. Ad5.TGM2 increased TGM2 expression in the iridocorneal angle including the TM of Ad5.TGM2 transduced eyes (B&D) compared to control eyes (A&C) determined by immunofluorescent staining (N=6 pairs of eyes). Co-staining with αSMA stained the TM, ciliary muscle and endothelium of Schlemm's canal (A&B) (Red: αSMA, Blue (DAPI): Nuclei). TGM2 staining only (C&D) without αSMA staining of the same sections as A&B respectively (Blue (DAPI): Nuclei, Green: TGM2). TM- Trabecular meshwork. White boxes highlight TM region. H&E images (E&F) of sections from the same eyes represented above (A-D). Black boxes highlight TM region.

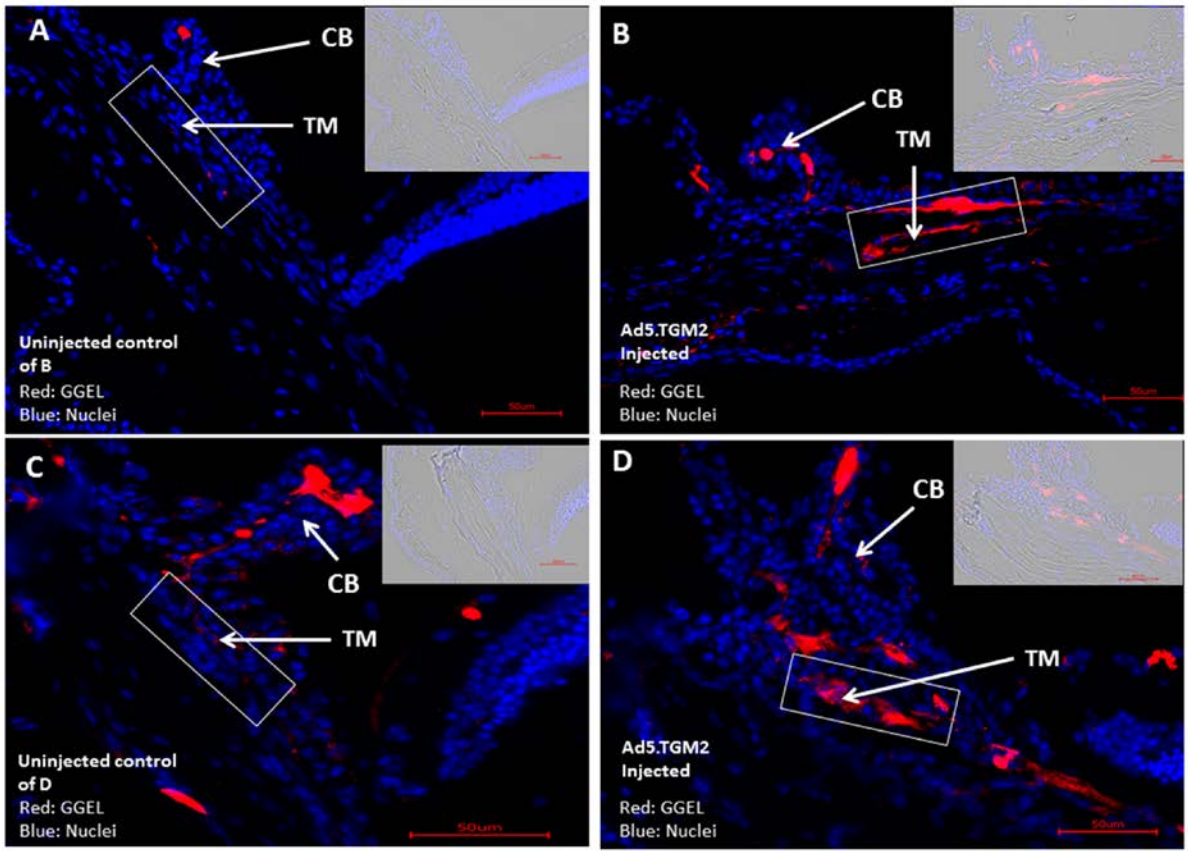


**Figure 6. Effect of Ad5.TGM2 transduction of mouse eyes on GGEL crosslinks in the TM region**. Ad5.TGM2 increased TGM2 crosslinking activity (GGEL) in the TM region of Ad5.TGM2 transduced mouse eyes (B&D) compared to respective contralateral controls (A&C) as determined by immunofluorescent staining (n=7). Representative images from 2 pairs of eyes are shown. CB - ciliary body; TM – trabecular meshwork. Scale 400 X. White boxes highlight TM region. Inserts are brightfield images of the same sections.

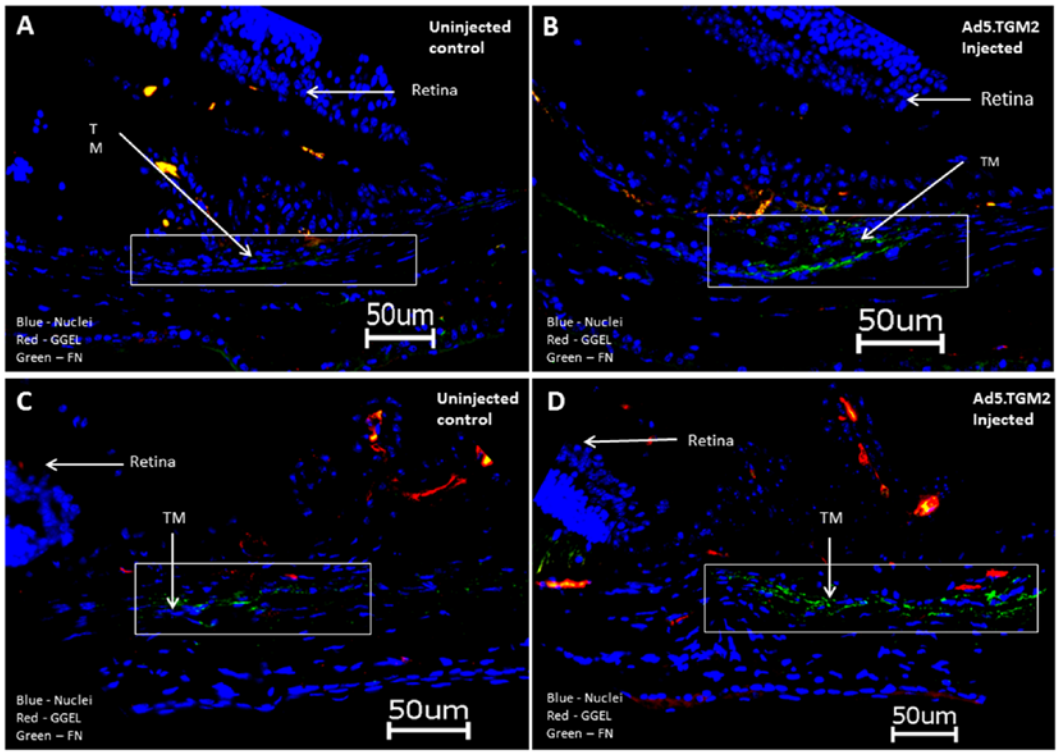


**Figure 7. Effect of Ad5.TGM2 transduction of mouse eyes on FN in the TM region.** Ad5.TGM2 transduction of mouse eyes shows increased FN staining (B&D) compared to uninjected controls (A&C respectively) as determined by immunofluorescent staining (n=3). Co-staining with GGEL (Red). Representative images from 2 pairs of eyes are shown (Blue (DAPI): Nuclei; Green: FN).

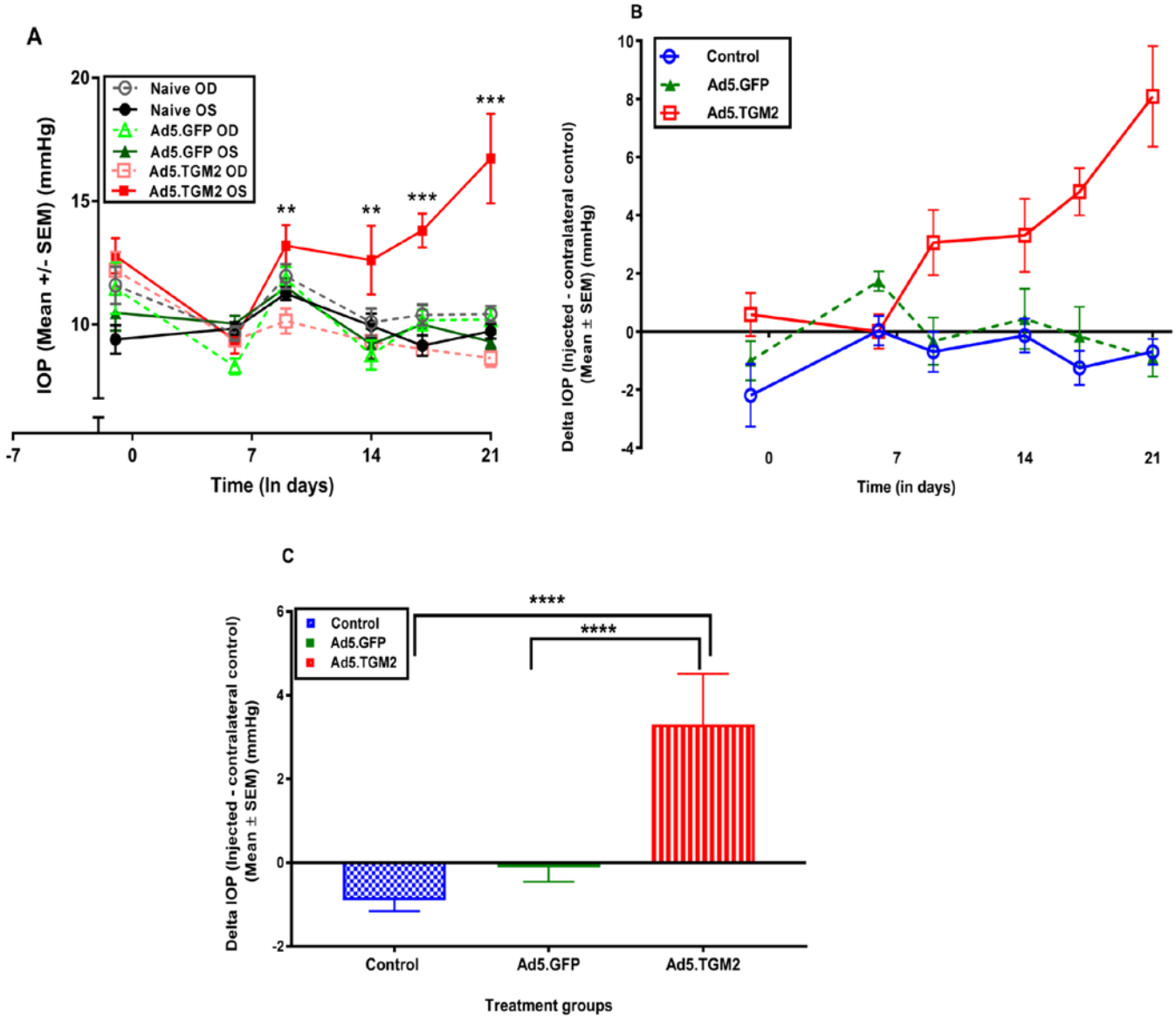
A
IOP (Mean +/- SEM) (mmHg)
Naive OD
Naive OS
Ad5.GFP OD
Ad5.GFP OS
Ad5.TGM2 OD
Ad5.TGM2 OS
**
**
***
***
Time (In days)
B
Delta IOP (Injected - contralateral control) (Mean ± SEM) (mmHg)
Control
Ad5.GFP
Ad5.TGM2
Time (in days)
C
Delta IOP (Injected - contralateral control) (Mean ± SEM) (mmHg)
Control
Ad5.GFP
Ad5.TGM2
****
****
Control
Ad5.GFP
Ad5.TGM2
Treatment groups

**Figure 8. Effect of Ad5.GFP and Ad5.TGM2 transduction on mouse IOP.** Ad5.TGM2 transduction of left eyes of BALB/cJ mice (OS) significantly increased IOP compared to uninjected right eyes (OD) (n=6). Ad5.GFP transduction in the left eye (OS) did not affect IOP of either eye (OD or OS) in the Ad5.GFP group (n=5). IOPs of naïve mice (n=6) were measured in same experimental conditions to ensure that environmental conditions did not affect IOP readings (A). **P = 0.01; ***P < 0.001; Significance between Injected TGM2 and non-injected groups was analyzed by 2-Factor ANOVA followed by Holm-Sidak post-hoc analysis. Difference between left (injected) eye and right (uninjected contralateral control) eye Δ(OS-OD) shows a significant difference in IOP (P<0.0001) in Ad5.TGM2 transduced eyes compared to Δ(OS-OD) of the naïve or Ad5.GFP group (p<0.0001; ****=p<0.0001) (B&C)

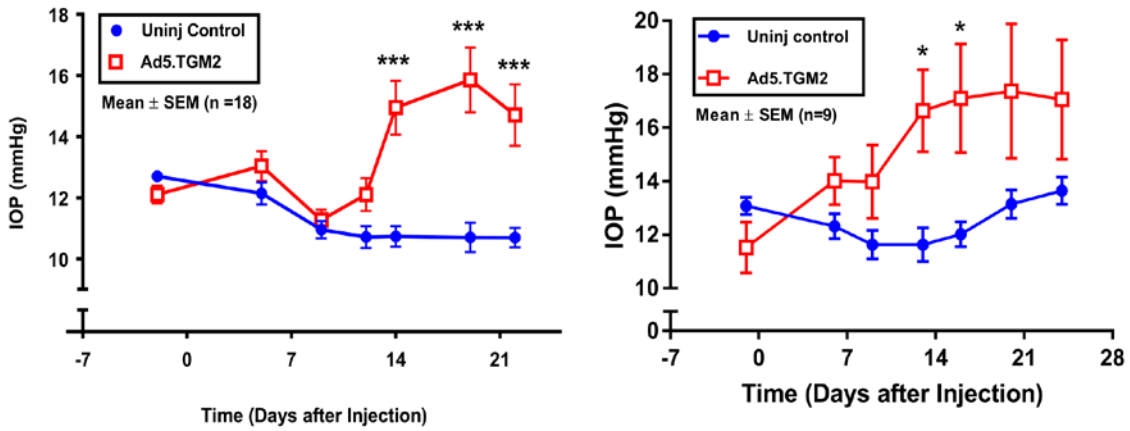


**Figure 9. Ad5.TGM2 elevates IOP in mouse eyes.** (A) In BALB/cJ mice (n=18), injection of Ad5.TGM2 significantly increased IOP from days 14 to 22, with the maximum difference elevation at day 19, (15.86 ± 1.06 mmHg in Ad5.TGM2 injected versus 10.7 ± 0.48 mmHg in uninjected controls (mean ± SEM, $p<0.0001$, ANOVA)). (B) In C57BL/6J mice (n=9), injection of Ad5.CMV.TGM2 increased IOP from day 13 with maximum difference at day 17 (17.09 ± 2.03 mmHg (injected) vs. 12.01 ± 0.47 mmHg (control) ($p<0.05$)). * $p < 0.05$, *** $p< 0.005$

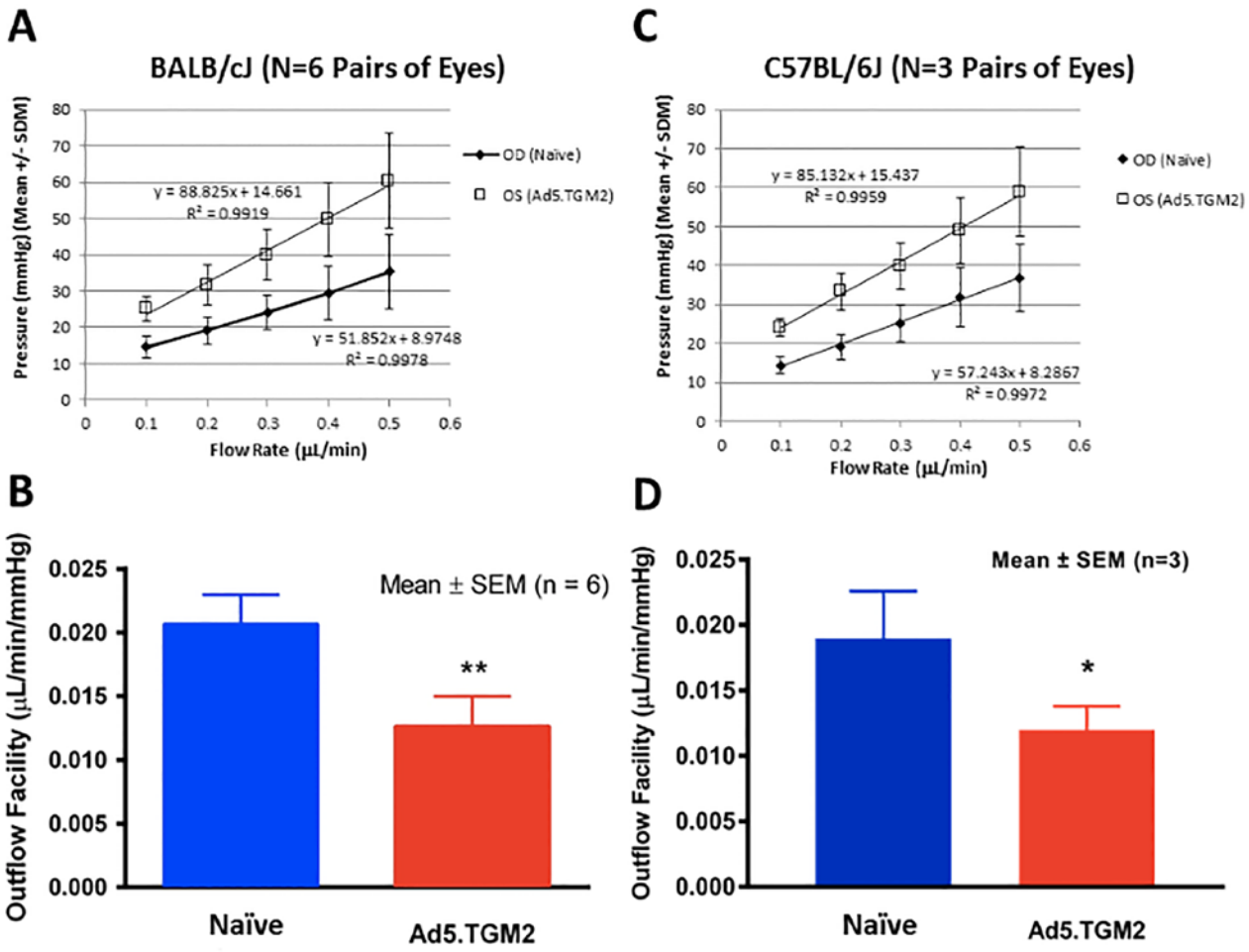
A
BALB/cJ (N=6 Pairs of Eyes)
Pressure (mmHg) (Mean +/- SDM)
Flow Rate (µL/min)
OD (Naïve)
OS (Ad5.TGM2)
y = 88.825x + 14.661
R² = 0.9919
y = 51.852x + 8.9748
R² = 0.9978
C
C57BL/6J (N=3 Pairs of Eyes)
y = 85.132x + 15.437
R² = 0.9959
y = 57.243x + 8.2867
R² = 0.9972
B
Outflow Facility (µL/min/mmHg)
Mean ± SEM (n = 6)
**
Naïve
Ad5.TGM2
D
Mean ± SEM (n=3)
*

**Figure 10. Ad5.TGM2 decreases aqueous outflow facility in Ad5.TGM2 transduced eyes.**

(A): BALB/cJ mouse pressure-flow rate curves in eyes of live animals (N = 6) (AC Perfusion). Error bars shows SDM. Over the flow rate range 0.1 to 0.5 μL/min, pressure in Ad5.TGM2 transduced eyes (pressure range of 24.94 ± 3.39 to 60.36 ± 12.95 mmHg) was significantly higher compared to uninjected controls (pressure range of 14.58 ± 3.12 to 35.31 ± 10.19 mmHg). SDM-Standard deviation of mean.

(B): Corresponding mean C was significantly lower in TGM2 transduced BALB/cJ eyes compared with uninjected control eyes (0.013 +/- 0.002 μl/min/mmHg vs. 0.021 +/- 0.002 μL/min/mmHg (mean +/- SEM)) ($p = 0.01$), and correlated closely with the associated increase in IOP.

(C): C57BL/6J mouse pressure-flow rate curve in eyes of live animals (N = 3) (AC Perfusion). Error bar shows SDM. Over the flow rate range 0.1 to 0.5 μL/min, pressure in Ad5.TGM2 transduced eyes (pressure range of 24.02 ± 2.27 to 58.79 ± 11.44 mmHg) was significantly higher compared to uninjected controls (pressure range of 14.43 ± 2.22 to 36.75 ± 8.61 mmHg). SDM-Standard deviation of mean.

(D): Corresponding mean C was significantly lower in TGM2 transduced C57BL/6J eyes compared with uninjected control eyes (0.012 +/- 0.0018 μL/min/mmHg vs. 0.019 +/- 0.0036 μL/min/mmHg (mean +/- SEM)) ($p < 0.05$), and correlated closely with the associated increase in IOP.

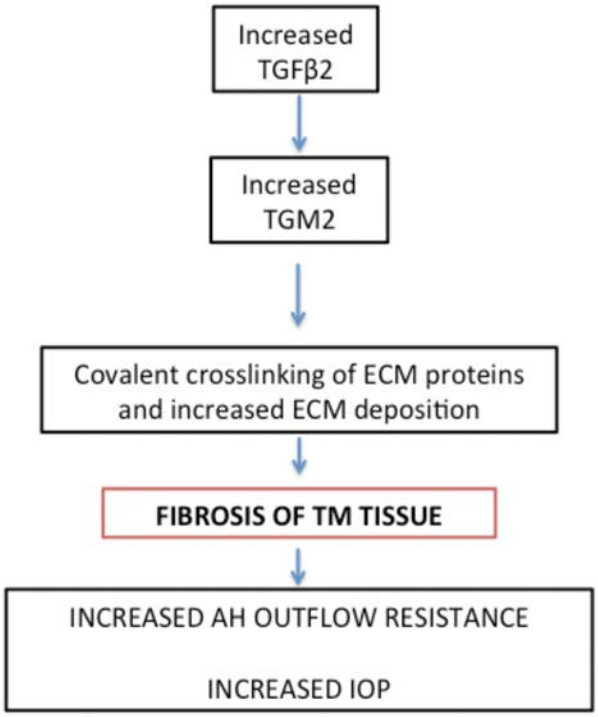


**Figure 11. Summary of effects of TGFβ2 and TGM2 on TM**

***Lenticular Opacity Scoring***

| LENTICULAR OPACITY SCORE | APPEARANCE OF LENS VIA DIRECT OPHTHALMOSCOPY |
|---|---|
| 0 | Normal appearance. |
| 0.5 | Barely visible small discrete opacity. |
| 1 | More readily visible small discrete opacity. **UNACCEPTABLE FOR STUDY.** |
| 2 | Larger opacity or several smaller opacities, but not involving entire lens. **UNACCEPTABLE FOR STUDY.** |
| 3 | Opacity of entire lens. **UNACCEPTABLE FOR STUDY.** |
| 4 | Rarely seen. Severe opacity of entire lens. Lens has dried, hardened, crusted appearance. **UNACCEPTABLE FOR STUDY.** |

**Table 1. Lenticular opacity score**

# CHAPTER III

## Knockout of tissue transglutaminase ameliorates TGFβ2 induced ocular hypertension: a novel therapeutic target for glaucoma?

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## Abstract

Glaucoma is a vision threatening optic neuropathy. It affects millions of people worldwide. There are various types of glaucoma such as primary open angle, normal tension and secondary closed angle glaucoma. Except in the normotensive cohort, increased IOP is the main risk factor for glaucoma. Consequently, studies into the mechanisms of causes of increased IOP show fibrotic changes in the trabecular meshwork that are different from age matched control. TGM2, an ECM crosslinking enzyme, is known to covalently crosslink ECM proteins and cause ECM deposition which, if occurs in excess in the TM, could cause increased IOP. We therefore wanted to study the effect of knockout of TGM2 on IOP in TGM2 floxed mice. From our results, we see that knockout of TGM2 can significantly decrease IOP by itself and also in the TGFβ2 induced ocular hypertensive model. This makes TGM2 a potential therapeutic target for glaucoma.

The hallmarks of aging such as genomic instability, epigenetic alterations, and reduction of proteolysis leading to loss of physiological structures and functions of a tissue are some of the characteristic features of aging (*34*). Pioneering work done by the Alvarado and Lütjen-Drecoll groups show a significant correlation between age and physiological changes in the trabecular meshwork (TM), including decreased cellularity and increased deposition of sheath derived (SD) plaque material (*31, 35*). The changes observed in the TM of glaucomatous eyes differ from that of age-matched including the aqueous humor (AH) outflow facility (*24, 28, 31, 36*). Increased

fibrillar depositions surrounding the central sheaths in the TM and the inner and outer wall of Schlemm's canal were observed in glaucomatous eyes compared to age-matched controls.

The distribution and balance between extracellular matrix (ECM) of the TM and its cellular components determine the path and rate of aqueous humor outflow through the TM into the Schlemm's canal (SC) (*24, 37*). Over the years, significant progress has been made in identifying and understanding various modifiable factors in the TM that can modulate intraocular pressure (IOP); examples include reducing ECM formation, decreasing ECM deposition or reducing TM cell stress (*29, 33, 38-44*). Recently, we have shown that overexpression of the ECM crosslinking enzyme tissue transglutaminase (TGM2) in mouse eyes increases IOP and decreases outflow facility by increasing ECM crosslinking in the TM region (*45*). TGM2 has been an enzyme of interest in primary open angle glaucoma (POAG) since increased ECM deposition was first observed in glaucomatous eyes, and TGM2 is well known for its irreversible ECM protein crosslinking activity which can lead to ECM deposition (*32, 33, 44*). This interest further grew when the predominant profibrotic cytokine TGFβ2 was found to induce TGM2 expression in TM cells (*44, 46-48*). In our current study, we show that partial knockout of TGM2 significantly reduced IOP in TGFβ2 induced ocular hypertensive mice.

In humans, the gene for TGM2 is located on chromosome 20 (20q11-12) and in the mouse on chromosome 2 (*14, 49*). The molecular weight of TGM2 ranges from 74-80 kD (*13*). Transglutaminases (TGMases) are a family of $Ca^{2+}$ dependent acyltransferases that mediate peptide bonds between intermolecular and intramolecular glutamine and lysine residues (*11, 49*). TGM2 also has G-protein signaling and GTPase activity (*13*). Extracellular protein crosslinking activity of TGM2 is well studied. In TGM2 knockout mice, TGM2 expression was ubiquitously inactivated and other TGMases did not compensate for this loss. The mice were viable with normal separation of digits and open eyelids (*13*). The floxed mice were therefore a good model for us to study targeted knockout in the TM.

We first wanted to confirm knockout of TGM2 in the TM of TGM2 floxed mice. For this, $TGM2^{t/t}$ floxed mice were obtained from The Jackson Laboratory (B6.129S1-*Tgm2*$^{tm1Rmgr}$/J) (*13, 50*). All experiments were conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and UNTHSC IACUC regulations. The breeder pair and first 2 generations of litters were genotyped by PCR to confirm the floxed mutant (~230bp amplicon) and the wild type C57BL/6J control (~184bp amplicon) and gave identical results to that listed on The Jackson Laboratories website (*51*)). Six mice aged 12 to 16 months were transduced with Ad5.CMV.Cre (Gene Transfer Core Facility, University of Iowa, Iowa City, IA) because Ad5 has tropism for trabecular meshwork tissue (*52*). 2 $x10^7$ pfu (in 2 µl) Ad5.CMV.Cre was injected intravitreally in the left eye, and the right eye was used as contralateral uninjected control. Mice were sacrificed according to our approved IACUC protocol, and eyes were collected at 2 time points (day 10, n=3 and day 26, n=3). The TM ring was isolated as previously published (*53*). Briefly, the sclera was cut along the equatorial plane just behind the limbus leaving minimal scleral tissue, and from the anterior half, the cornea was cut along the ciliary body keeping the TM ring intact. Care was taken to ensure that equal amount of tissue was collected from all eyes. RNA was isolated using the RNA isolation kit (RNeasy mini kit, Qiagen). Briefly, tissues were collected in centrifuge tubes (on dry ice), buffer was added, tissues were immediately homogenized, and RNA was isolated. 250 ng of RNA was used for cDNA synthesis, and qPCR was performed with GAPDH as the housekeeping control. Primers used include: TGM2 (F-5'-GGCAAGGTCTGAGAAAGCAC-3', R-5'CACGTAGACTGTTCCCAGCA-3'), GAPDH (F-5'-CCCAGCTTAGGTTCATCAGG-3', R-5'-TGGCAACAATCTCCACTTTG-3'). qPCR data analysis shows decreased TGM2 expression with fold change of 0.17 ± 0.05 (n=3) on day 10 and 0.52 ± 0.13 (n=3) on day 26 in Ad5.Cre transduced eyes compared to contralateral uninjected eye (Baseline at 1 represents TGM2 expression in the uninjected control eyes) (Figure 1A).

Following this we wanted to see whether knockout of TGM2 could modulate IOP. TGM2 floxed mice aged 2 to 8 months were used for all IOP studies. We performed conscious IOP measurements (*54*) on floxed mice. Baseline IOP readings were taken from 6 TGM2 floxed mice. The next day the left eyes were injected intravitreally with Ad5.CMV.Cre (2 X $10^7$ pfu in

2µl). Conscious IOP readings were recorded for 20 days. On Day 17, we see a significant decrease in IOP in Ad5.Cre transduced eyes (9.47 ± 0.8 (Mean ± SEM)) compared to contralateral control (13.89 ± 1.1 (Mean ± SEM)) (Figure 1B). To compare difference in IOP between transduced eyes and contralateral controls, statistical analysis was performed using paired t-test (two-tailed), p = 0.039. This moderate yet significant decrease in IOP indicated that TGM2 knockout probably modulated ECM crosslinking in the TM and thereby reduced resistance in the outflow facility.

TGFβ2 stimulates the synthesis of collagen, elastin, fibronectin, and the enzymes (LOXs and TGM2) that crosslink these ECM proteins (*30, 44, 46, 55, 56*). In glaucomatous eyes, the TM is exposed to excessive TGFβ2 leading to fibrotic changes and deterioration of TM function. TGM2 is implicated in numerous fibrotic conditions such as lung fibrosis, renal fibrosis, and atherosclerosis (*11, 12, 57*). We further wanted to test if targeted knockout of TGM2 in the TM could reduce TGFβ2-induced ocular hypertension in mice. We have previously published that Ad5.hTGFβ2$^{226/228}$ significantly increased IOP in mice (*54, 58*). To test our hypothesis that knockout of TGM2 can ameliorate TGFβ2 induced ocular hypertension, we used 2 groups of TGM2 floxed mice and measured IOP under isoflurane anesthesia. Ad5.hTGFβ2$^{226/228}$ (2 X $10^7$ pfu/2µl; n=8) and Ad5.CMV.Cre + Ad5.hTGFβ2$^{226/228}$ (2 X $10^7$ pfu/2µl; n=10) were injected intravitreally in the left eye one day after baseline IOP readings were measured. In all mice, the right eyes were used as uninjected controls, and IOP readings were taken for 21 days. Statistical analysis was performed using two-way ANOVA. We observed a significant IOP increase in the Ad5.hTGFβ2$^{226/228}$ transduced group, which was consistent with our previous publications (*52, 58*) (transduced vs. contralateral control (maximum difference): 18.45 mmHg ± 1.3 vs. 11.65 mmHg ± 1.01 (Mean ± SEM)). Interestingly, Ad5.CMV.Cre transduction in eyes treated with Ad5.hTGFβ2$^{226/228}$ showed a slight increase in IOP but was not significant (transduced vs. control (maximum difference): 15.2 mmHg ± 1.37 vs. 13.0 mmHg ± 0.78 (Mean ± SEM)) (Figure 2A). The increase in IOP in the Ad5.CMV.Cre + Ad5.hTGFβ2$^{226/228}$ can be attributed to the fact that multiple pathways are activated by TGFβ2 that lead to excessive ECM protein formation or inhibition of MMP's (*30, 46, 55, 56*). The analysis of difference of means of transduced eyes versus their contralateral eyes was analyzed using the Student's t-test (two-

tailed). Δ(OS-OD) of transduced groups showed significant difference (p=0.03) indicating that TGFβ2 induced ocular hypertension can be reduced by TGM2 knockout (Figure 2B). Trichrome staining of the anterior segment was performed to ensure open angle (Figure 2C).

TGM2 has been an interesting therapeutic target in a lot of fibrotic diseases. TM remodeling by TGFβ2 as seen in glaucomatous eyes are similar to fibrotic changes seen in other tissues characterized by increased ECM deposition, decreased cellularity, disorganized arrangement of ECM proteins such as collagen, and thickening of elastin fibrils. Current medications in glaucoma aim in symptomatic relief by reducing IOP (*59*). With current advancements in research, we should be able to target the cause of the disease. CRISPR/Cas9 mediated gene knockout is progressively explored as a future therapeutic option(*42*). Although complete knockout of TGM2 would not be physiologically favored, we can develop ways to moderate knockout so as to prevent pathological progress of the disease that would still retain physiological levels. A number of small molecules that inhibit TGM2 crosslinking activity have been studied(*60, 61*). With small molecule inhibitors, the key is to design drugs that can inhibit the extracellular crosslinking activity without affecting the intracellular signaling of TGM2 (*17*). In the future, TGM2 crosslinking inhibitors could be tested for therapeutic purposes to slow down or inhibit the progression of the glaucomatous damage to the TM either by itself or in combination with other glaucoma therapeutic drugs.

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## FIGURES AND FIGURE LEGENDS

**FIGURE 1**

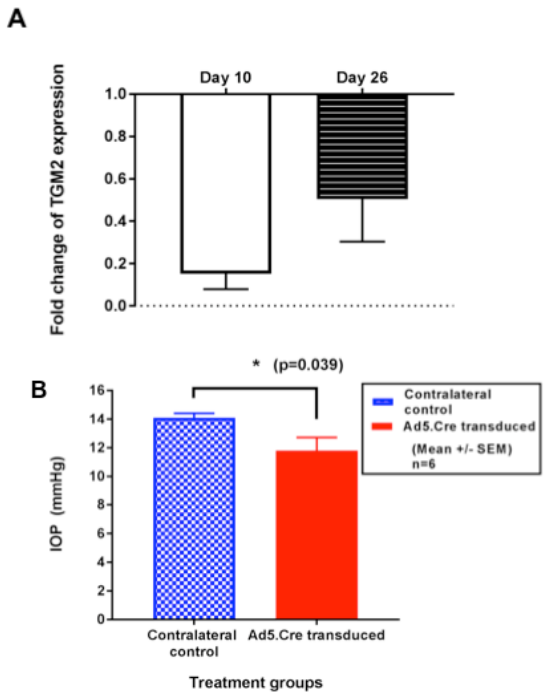


**Figure 1. Effect of TGM2 knockout on IOP.** All TGM2 floxed mice were transduced in one eye with Ad5.CMV.Cre (2 x$10^7$ pfu in 2 µl) and contralateral eyes were used as controls. Decreased TGM2 expression was seen in TGM2 KO mouse eyes compared to their contralateral control seen by a negative fold change of 0.17 ± 0.05 (n=3) on day 10 and 0.52 ± 0.13 (n=3) on day 26 as determined by qPCR (Figure 1A). Decreased IOP was seen in eyes transduced with Ad5.CMV.Cre compared to their contralateral control (Maximum IOP difference: 9.47 ± 0.8 versus 13.89 ± 1.1 (Mean ± SEM), transduced versus contralateral control), n=6, paired t-test, * - $p<0.05$ (Figure 1B).

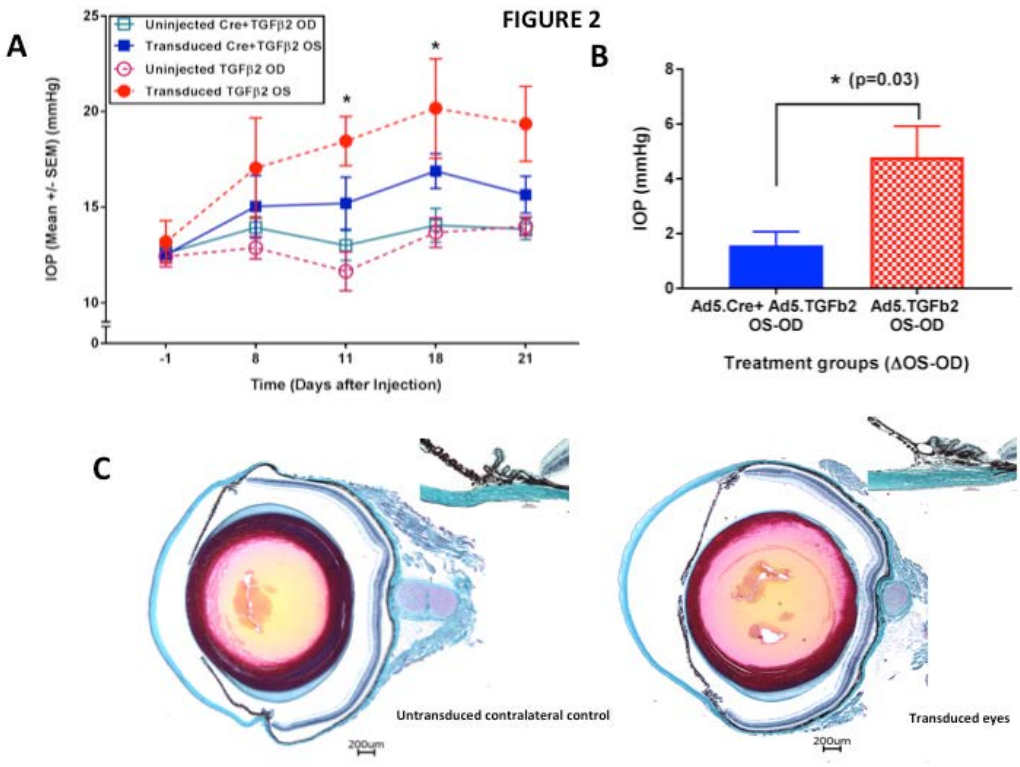


**Figure 2. Partial knockout of TGM2 significantly reduces IOP in TGFβ2-induced ocular hypertension model.** IOP values for Ad5.hTGFβ2$^{226/228}$ transduced group - (transduced vs. contralateral control (maximum difference): 18.45 mmHg ± 1.3 vs. 11.65 mmHg ± 1.01 (Mean ± SEM)) and Ad5.CMV.Cre + Ad5.hTGFβ2$^{226/228}$ transduced group - (transduced vs. control (maximum difference): 15.2 mmHg ± 1.37 vs. 13.0 mmHg ± 0.78 (Mean ± SEM)) shows that Ad5.hTGFβ2$^{226/228}$ caused significant IOP elevation and this IOP elevation was significantly alleviated by TGM2 knockout. Two-way ANOVA compared each treatment group to their contralateral control. * - Difference between transduced and contralateral eyes on the day (Figures 2A). Analysis of difference of means of transduced eyes versus their contralateral eyes was performed using the Student's t-test (two-tailed). Δ (OS-OD) of transduced groups showed significant difference (p=0.03) (Figure 2B). Representative image of trichrome staining of the anterior segment shows open angle of eyes with and without treatment (Figure 2C).

# CHAPTER IV

## Could intervention by an ECM crosslinking inhibitor halt glaucomatous fibrotic changes?

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**Abstract**

The main line of treatment of POAG still remains to be IOP lowering agents. Although there are some new line of drugs such as Rho kinase inhibitors in the pipeline, other drugs are being studied as mechanisms for IOP elevation are better understood. TGM2 is an ECM crosslinking enzyme that increases IOP when overexpressed in the mouse eyes and knockout showed significant reduction of IOP in the TGFβ2 model of ocular hypertension. We therefore wanted to test a small molecule inhibitor of TGM2 for inhibition of crosslinking activity. The drug ZM 449829 is found to be a potent inhibitor of TGM2 crosslinking activity. It also confers the advantage of not being active in a reducing environment such as intracellularly. This makes ZM 449829 a desirable small molecule as we could expect inhibition of TGM2 crosslinking activity extracellularly without affecting other intracellular and signaling functions of TGM2. In our study we show that ZM 449829 inhibits crosslinking in 3 primary human glaucomatous cell strains *in vitro*. Also, when given as eye drops it does not exhibit corneal toxicity or inadvertent IOP changes in our 21 day study.

## Introduction

Primary open angle glaucoma (POAG) is an optic neuropathy with worldwide prevalence of glaucoma estimated to reach about 80 million by the year 2020 and 111.8 million by the year 2040 and is the second leading cause of blindness, the first being cataract (*1, 2*). The Baltimore Eye Survey indicates that family history and age are risk factors for glaucoma and the African American population have a strong association with incidence of POAG (*3*). The ocular hypertension treatment study shows that males are at a higher risk and thinner central corneal measurement was also predictive of glaucoma (*4*). With the help of these extensive cross-sectional and longitudinal studies done to understand the progress of glaucoma, we now can intervene at early stages by monitoring intraocular pressure (IOP), cup/disc ratio and visual perimetry tests for visual field loss (*5*). In POAG, there is gradual loss of visual field ultimately leading to blindness. Increased IOP is a risk factor for most POAG cases (*4, 6-8*). Ocular hypertension increases the pressure within the eye, causing retinal ganglion cell (RGC) loss and damage to optic nerve head (ONH). RGC axons passing through the ONH get damaged due to bending of the lamina cribrosa and this can lead to blindness (*9*).

IOP lowering drugs are the main line of glaucoma medications (*10, 11*). Beta-blockers decrease aqueous humor formation and prostaglandin analogues increase aqueous humor (AH) outflow in the eye and these are the first line medications for POAG. If IOP does not decrease by 15-30% compared to baseline or the patient experiences adverse reactions, one of the drugs is changed with a drug from a class of second line of medications such as α-agonists and topical carbonic anhydrase inhibitors. Reducing IOP relieves ocular hypertension therefore reduces the extent and rate of optic nerve damage. Surgical approaches or laser therapies such as trabeculectomy, argon laser trabeculoplasty (ALT) and selective laser trabeculoplasty (SLT) are also performed when IOP lowering agents are not sufficient by themselves to control IOP (*12*). However in most cases the progress of the disease can only be slowed and there is yet no cure for glaucoma.

With advancements in the understanding of interplay of the cellular and extracellular components of the TM to maintain AH outflow, new therapeutic targets have been identified to lower IOP. Modulation of the Rho kinase and Endothelin-1 pathway may increase TM outflow by modulation of TM contractility. Intervention of transforming growth factor β2 (TGFβ2) and connective tissue growth factors (CTGF) pathways may remodel extracellular matrix (ECM) thus lowering IOP by increasing AH outflow through the conventional pathway. Other ways to decrease IOP could be through Angiotensin II and Serotonin modulation, which may increase uveoscleral outflow (*13*). Another exciting approach is gene therapy wherein targeted knockout of genes of interest (GOI) in the TM for example myocilin could rescue the glaucoma phenotype (*14*).

In POAG, ECM modulation has been a target in altering outflow as ECM depositions are observed at the juxtacanalicular region (JCT), which is the site of maximum resistance to outflow facility (*15-18*). Previously we have shown that overexpression of the ECM protein crosslinking enzyme tissue transglutaminase (TGM2) can elevate IOP in mice and TGM2 knockout using the CRE-LOX method can decrease TGFβ2 induced ocular hypertension (chapter 3) (*19*). We therefore wanted to test a small molecule crosslinking inhibitor as a potential therapeutic drug.

The role and pathways of TGM2 mediated fibrosis have been extensively studied in renal, pulmonary and hepatic fibrosis and also in cardiovascular diseases such as atherosclerosis (*20-24*). Recent findings in the role of TGM2 mediated protein aggregates in neurodegenerative diseases such as Huntington's disease and Alzheimer's disease have made TGM2 a very exciting therapeutic target (*25, 26*). Subsequently, various small molecules, for example ZM 449829, have been screened from the chemical libraries LOPAC and Prestwick following by *in vitro* experiments to determine potency and efficacy (*27, 28*).

The parent drug of ZM 449829 is ZM33923, which belongs to the class of naphthyl ketones and is highly potent; ZM 449829 can inhibit TGM2 in the nanomolar (nM) range (5 ± 5 nM). These compounds can also bind to the ATP binding site of a tyrosine kinase, Janus kinase 3 with reported $IC_{50}$ in the range of 4.4-7 μM (*27, 29*). We wanted to test if ZM 449829 can inhibit crosslinking in primary human glaucomatous cell strains *in vitro* and perform preliminary toxicity studies such as effect on the cornea when administered as eye drops and also determine whether it had any effect on IOP.

## Materials and methods

### Cell viability using CellTiter-Glo® 2.0 Assay

Two primary glaucomatous cell strains (GTM 125-05 and GTM 626-02) were plated on a 96 well flat bottom opaque plate. ZM 449829 was obtained from Tocris (Bio-Techne Corporation, Minneapolis, MN) and dissolved in Dimethyl sulfoxide (DMSO) to make 100mM stock. Cells were grown to confluency and treated with increasing concentrations of ZM 449829 (0.5nM to 1μM) in 100μl medium for 72 hours. DMSO was used as vehicle control. All treatments were performed in triplicates. After 72 hours, 100μl CellTiter-Glo (Promega Corporation, Madison, WI, USA) reagent was added, mixed in an orbital shaker for 2 minutes for contents to be released, incubated for 10 minutes in room temperature and luminescence was recorded using a Tecan M200 plate reader (Durham, North Carolina).

**Immunocytochemistry for ε- (γ-glutamyl) lysine (GGEL) bonds, fibronectin and phalloidin in primary glaucomatous cell strains**

Three primary human glaucomatous cell strains (GTM 125-05, GTM 60A and GTM 466-07)(*30*) were grown in Dulbecco's modified Eagle's medium (low glucose) (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Atlas Biologicals, Collins, CO, USA), penicillin (100 U/mL)-streptomycin (0.1 mg/mL) (Sigma-Aldrich Corp) and 1% L-glutamine (GE Healthcare Life Sciences, Logan, UT, USA, 0.292 mg/mL) as previously described (*30-32*). Cells were then plated on coverslips in 24 well plates and grown till confluency. Cells were treated with 5nM ZM 449829 and DMSO was used as vehicle control. All treatments were performed in triplicates. After 48 hours, medium was removed, cells were washed with PBS and fixed with 4% PFA for 30 minutes and washed with PBS 3 times. Cells were then blocked in PBS superblock (Thermo Fisher Scientific) for 2 hours, followed by incubation in primary antibody for GGEL (ab424 Abcam (Cambridge, MA, USA), diluted 1:100) in PBS Super block overnight at 4°C. Cells then were washed 3 times with PBS and incubated for 2 hours at room temperature with appropriate secondary antibody (Goat anti-Mouse IgM Cross Adsorbed Secondary Antibody, DyLight 594 conjugate (Thermo Fisher Scientific, Waltham, MA, USA) for GGEL, dilution 1:200) in PBS Super block. After 2 hours, cells were washed with PBS 3 times followed by 3 quick washes with water and mounted with ProLong Gold Antifade Mountant containing 4′, 6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Slides were allowed to dry overnight and imaged using a Keyence all-in-one fluorescence microscope (Itasca, IL).

GTM 125-05 cells were plated on a separate 24 well plate and grown to confluency as described above. Cells were treated with 5nM ZM449829, 5ng/ml TGFβ2 (R&D Systems, Minneapolis, MN) or co-treated with ZM449829 (5nM) + TGFβ2 (5ng/ml). All treatments were performed in triplicates. After 48 hours, cells were washed with PBS and fixed as described above. After blocking, cells were incubated with primary antibodies to fibronectin (AB1945, Millipore, Temecula, California, diluted 1:500 in PBS Superblock) or phalloidin (A12379; Alexa Fluor 488

Phalloidin (conjugated) diluted 1:2500 in PBS Superblock) overnight at 4°C. Cells incubated with primary antibody to fibronectin were then washed and incubated with secondary antibody to fibronectin (Donkey anti-rabbit Alexa Fluor 488, diluted 1:1000) for 2 hours at room temperature, washed with PBS 3 times followed by 2 quick washes with water and mounted using DAPI. Slides were allowed to dry overnight and imaged using a Keyence all-in-one fluorescence microscope (Itasca, IL).

**Western blotting for TGM2**

Conditioned medium was collected from GTM 125-05 cells plated with DMSO and ZM 449829 for immunocytochemistry; western immunoblotting was performed and probed for TGM2. Briefly, 40µl conditioned medium was added to 4x Laemmli Sample buffer (Bio-Rad, Hercules, CA) as loading dye (with beta-mercaptoethanol), boiled for 10 minutes, spinned down and loaded, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed and then transferred by electrophoresis on to a Immobilon-P PVDF membrane (EMD Millipore, Massachusetts, USA). Membranes were blocked using TBS Superblock (Thermo Fisher Scientific) for 2 hours followed by incubation in TGM2 primary antibody (TGase II Ab-3, Neomarkers; dilution 1: 2000, in TBS Super block) overnight at 4°C. Blots were then washed with Tris-buffered saline Tween (TBST; 20 mM Tris, 0.5M NaCl, and 0.05% Tween 20 (pH 7.4)) for 3 washes, 10 minutes each and incubated in secondary antibody (Goat anti-mouse IgG-HRP, Santa Cruz Biotechnology; dilution 1: 5000 in TBS Super block) for 2 hours at room temperature and then washed with TBST for 30 minutes. Clarity Western ECL Blotting substrate (Bio-Rad, Hercules, CA) was used to detect chemiluminescence and images were taken using the Bio-Rad ChemiDoc imaging system (Bio-Rad).

**Mouse studies**

Fifteen female BALB/cJ retired breeders were obtained from The Jackson laboratory (Bar Harbor, ME, USA). All experiments were conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and the UNTHSC Institutional Animal

Care and Use Committee regulations. Animals were maintained on a 12-hour light and dark cycle and provided food and water ad libitum.

Ten mice were given 5μM ZM 449829 eye drops (diluted in PBS) on both eyes once a day for 21 days. Five mice were given DMSO (1:2000, vehicle control) eye drops on both eyes. Prior to eye drop administration, baseline IOP measurements were taken under isoflurane using the Tonolab rebound tonometer as previously described (*33, 34*). IOP readings were taken once a week and eyes were observed under a slit-lamp at 21 days for effects of the drug on cornea.

## Results

### Cell viability with ZM449829

GTM 125-05 and GTM 626-02 cells were treated with increasing dose of the drug (0.5nM to 1 μM) and luminescence was recorded at 72 hours. The drug did not exhibit cell death up till 1μM (Figure 1). Therefore the 5nM dose used further for our 48 hour studies were safe.

### Crosslinking inhibition in 3 primary human glaucomatous cell strains

At 48 hours, all 3 glaucomatous cell strains showed decreased staining with GGEL antibody in cultures treated with ZM 449829 compared to DMSO treated cultures. This indicates inhibition of TGM2 crosslinking activity following treatment with ZM 449829 at 5nM (Figure 2 A-F).

**TGM2 expression after treatment with 5nM ZM449829**

Conditioned medium from GTM 125-05 cells treated with ZM 449829 and DMSO for 48 hours was probed for TGM2 expression by western immunoblotting. As expected, the expression of TGM2 does not change upon treatment with the inhibitor (Figure 3A). Coomassie blue staining of gel is shown as loading control since conditioned medium was used and protein concentration could not be quantified (Figure 3B).

**Effect of ZM 449829 and TGFβ2 on fibronectin and phalloidin in GTM 125-05**

GTM 125-05 cells treated with the inhibitor appears to have marked decrease in fibronectin and treatment with TGFβ2 shows increase in fibronectin compared to inhibitor as well as when compared with DMSO. Combination of ZM449829 (5nM) + TGFβ2 (5ng/ml) does not appear to show any definite change in fibronectin at 48 hours (Figure 4A-D).

GTM 125-05 cells treated with the inhibitor and stained with phalloidin mostly appear to be elongated compared to DMSO treated cells. Most cells in the TGFβ2 treated group appears to lose their typical elongated shape. Most cells treated with the combination of ZM449829 (5nM) + TGFβ2 (5ng/ml) do not seem as elongated as cells treated only with ZM 449829 neither do that look as dome shaped as cells treated only with TGFβ2 (Figure 4E-H).

**Effect of ZM449829 on mouse eyes**

BALB/cJ mouse eyes treated with DMSO or the inhibitor ZM 449829 did not show any changes on the corneal surface. No corneal edema or hyperemia was observed and overall gross morphology of eyes from both treatment groups appeared similar (Figure 5A-D). IOP readings

were taken once a week. As expected, there was no significant change in IOP in either treatment group (Figure 5E).

**Discussion**

ZM 449829 is found to be a potent inhibitor of TGM2 crosslinking (*27, 28*). It can bind to TGM2 and lock it in its inactive form thereby inhibiting its transamidating activity at doses as low as 5nM (*27, 29*). Because of the inability of this inhibitor to inhibit TGM2 under reducing conditions, it is less likely to be inhibitory to intracellular TGM2 because of reducing environment within the cell. We therefore believe that this drug inhibits extracellular crosslinking activity of TGM2. In this paper we show that *in vitro*, ZM 449829 decreases crosslinking in primary human GTM cells and possibly also affects fibronectin deposition in ECM and affects cell shape as evident from phalloidin staining. In our preliminary *in vivo* 21-day study we see that 5μM ZM 449829 eye drops do not cause any visible toxicity on corneal surface or affect IOP.

ZM 449829 at a higher dose is known to be a JAK3 inhibitor but chemical library screenings (LOPAC and Prestwick) and high throughput studies have showed that at $IC_{50}$ as low as 5 ± 5nM it can inhibit TGM2 without inhibiting the JAK-STAT pathway(*27, 29*). Transglutaminase has implications in a variety of fibrotic diseases as well as neurodegenerative conditions such as Huntington's disease and Alzheimer's disease. TGM2 is well studied (*21, 24-26*) and inhibitors of TGM2 activity are constantly been tested. The potency and selective extracellular activity of ZM 449829 makes it a promising molecule to be studied. In our study, the cell survival assay with 2 glaucomatous cell strains show that up to 1μM, the inhibitor does not show cell death at 72 hours (Figure 1). We thereafter performed our *in vitro* studies using 5nM of the drug and studied their effects at 48 hours.

Remarkable inhibition of crosslinking activity was seen at 5nM in all 3 cells strains (Figure 2A-F). The GGEL antibody stains specifically for ε- (γ-glutamyl) lysine bonds, which are formed due to TGM2 transamidating activity, and is a direct indication of TGM2 activity. Decreased staining in inhibitor treated cells compared to DMSO control probably indicates that once cells were treated with the inhibitor, further TGM2 crosslinking activity was inhibited while in the DMSO control group there was unopposed TGM2 crosslinking activity and thereby the apparent increased staining. Glaucomatous TM cells are also known to have increased TGM2 crosslinking activity compared to age matched controls (*35*), therefore inhibition of further crosslinking could probably help to regain homeostasis. Although literature suggests that doses as low as 5nM of ZM 449829 do not affect the JAK-STAT pathway, further studies could be done to test whether this inhibitor affects the JAK-STAT pathway in human glaucomatous TM cell strains at different concentrations.

While ZM 449829 binds to TGM2 and locks it in the inactive conformation, it is not known to affect expression levels of TGM2. Our western immunoblotting results show that the expression of secreted TGM2 in conditioned medium was not affected with the inhibitor (Figure 3). The advantage of solely being able to inhibit extracellular crosslinking activity of TGM2 without affecting expression of TGM2, as would happen in a knockout model, is that the other intracellular and signaling activities of TGM2 would probably not be affected (*36*).

Inhibition of crosslinking activity of TGM2 would indicate lesser ECM deposition as ECM turnover decreases if ECM proteins such as fibronectin, collagen, elastin and laminin get covalently crosslinked. Crosslinking by TGM2 is irreversible and there are no known endogenous mechanisms that can break these bonds (*36, 37*). Consistent with that, we notice that inhibition of TGM2 mediated crosslinking decreases fibronectin compared to DMSO control (Figure 4A,B). There is increased FN in TGFβ2 treated cells but in the inhibitor + TGFβ2 treated cells it is unclear whether there is any significant change in FN deposition at 48 hours (Figures 4C,D). When ECM proteins do not get covalently crosslinked, they can undergo normal turnover with the help of proteases such as matrix metalloproteases (MMP's) (*38*). Increased

fibronectin deposition has consistently been observed in POAG (*35, 39*). Implications of being able to reduce fibronectin deposition could mean decreased resistance to AH outflow at the TM which might alleviate thus caused ocular hypertension. In future higher doses of drugs or longer time points could be tested to determine if that would cause significant inhibition of fibronectin deposition with co-treatment of inhibitor with TGFβ2.

We also observed changes in the shape of cells in the inhibitor treated group. TM cells generally are elongated in shape but glaucomatous TM cells are known to have dome shaped actin fiber formations called cross linked actin networks (CLANs) (*40*). We observed that the inhibitor treated cells look more elongated compared to DMSO controls or TGFβ2 treated cells (Figure 4E-H). This could probably be due to less stress to cells in the inhibitor treated cells as there is less ECM deposited around the cells in the presence of the inhibitor. In future atomic force microscopy (AFM) studies could be done to test whether this inhibitor can affect ECM stiffness in glaucomatous TM cells (*41*).

After determining that this drug can inhibit crosslinking in primary human glaucomatous TM cell strains, we wanted to do some preliminary testing to see whether this drug had any effect on the cornea when administered as eye drops or if it affected the IOP. We used 5μM ZM 449829 as only a small amount crosses the cornea and reaches the anterior chamber. Slit lamp microscopy showed that there was no corneal damage or irritation or hyperemia in either group of mice treated with the eye drops (Figure 5A-D). The IOP over 3 weeks remained stable as expected (Figure 5E). No significant change was observed in either group. Since this drug inhibits crosslinking, which is a gradual process, an immediate decrease in IOP was not expected. But because of other known effects of this compound, we wanted to confirm that none of the other mechanisms cause inadvertent changes in IOP. In future, we could perform high performance liquid chromatography-mass spectrometry (HPLC-MS) studies to determine corneal penetration ability of the drug. An interesting study would be overexpression of TGM2 and testing whether this drug can inhibit TGM2 overexpression induced ocular hypertension (*19*). There are very few drugs in the pipeline for POAG that target the cause of the disease. This drug is therefore a

potential molecule that could be assessed for its safety and efficacy as a possible medication for POAG either by itself or in combination with other antiglaucoma medications.

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## FIGURES AND FIGURE LEGENDS

FIGURE 1

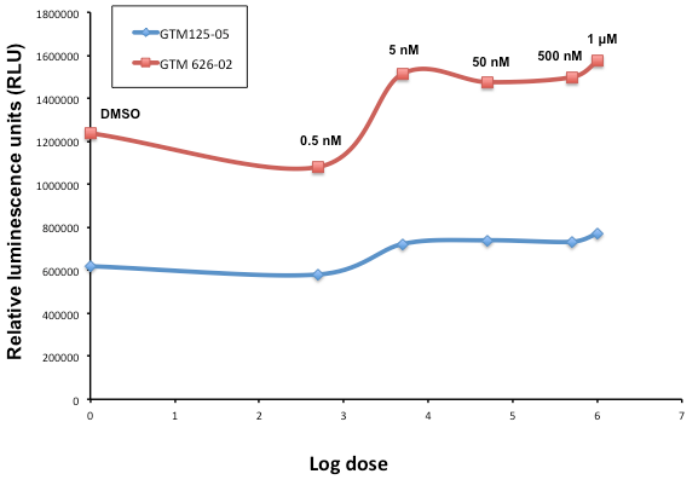


**Figure 1: Cell viability using CellTiter-Glo® 2.0 Assay.** GTM 125-05 and GTM 626-02 cells were treated with increasing dose of the drug (0.5nM to 1 µM) and luminescence was recorded at 72 hours. The drug did not exhibit cell death up till 1µM.

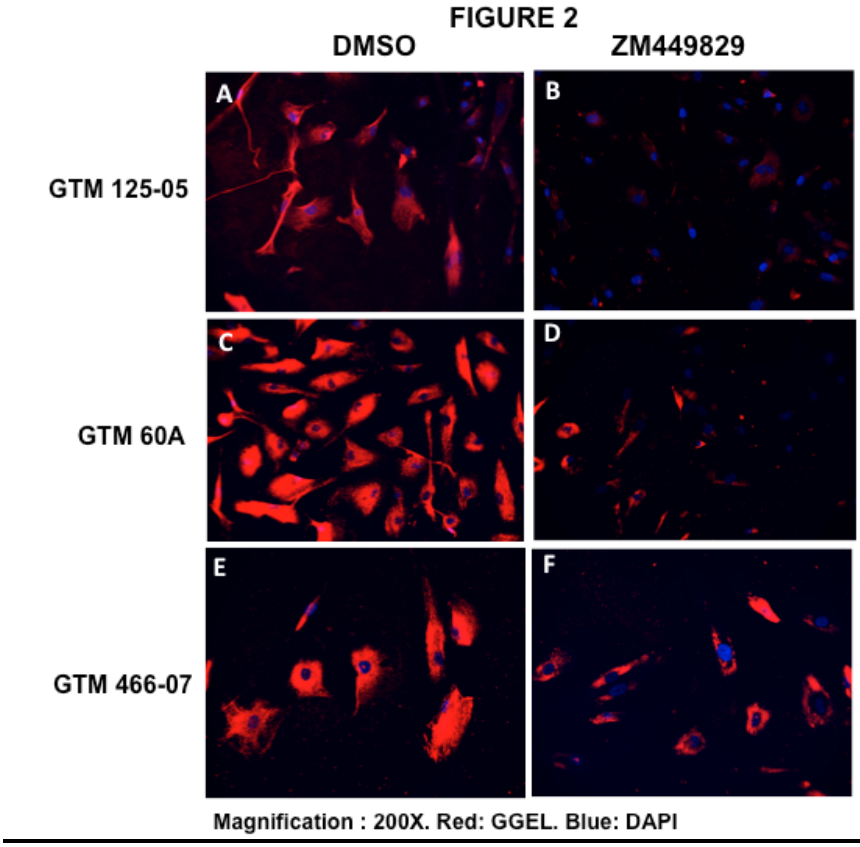


**Figure 2. Crosslinking inhibition in 3 human glaucomatous cell strains.** At 48 hours, all 3 glaucomatous cell strains showed decreased staining with GGEL antibody in cultures treated with ZM 449829 compared to DMSO treated cultures (Figures 2A-F).

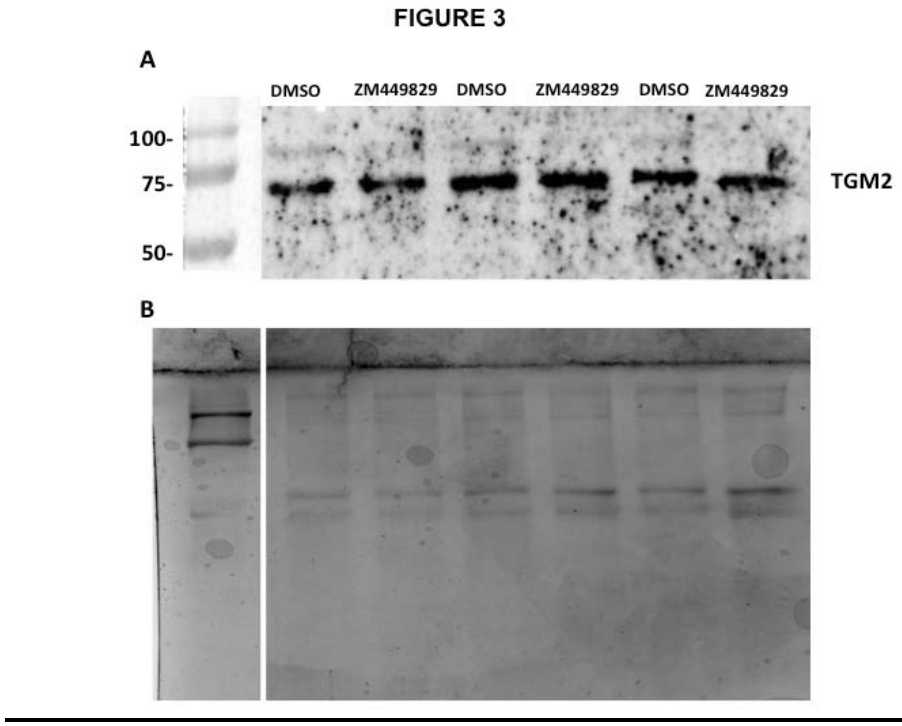


**Figure 3: Western immunoblotting for TGM2 expression after treatment with 5nM ZM449829.** Expression of TGM2 does not change upon treatment with the inhibitor (Figure 3A). Coomassie blue staining of gel is shown as loading control since conditioned medium was used and protein concentration could not be quantified (Figure 3B).

FIGURE 4

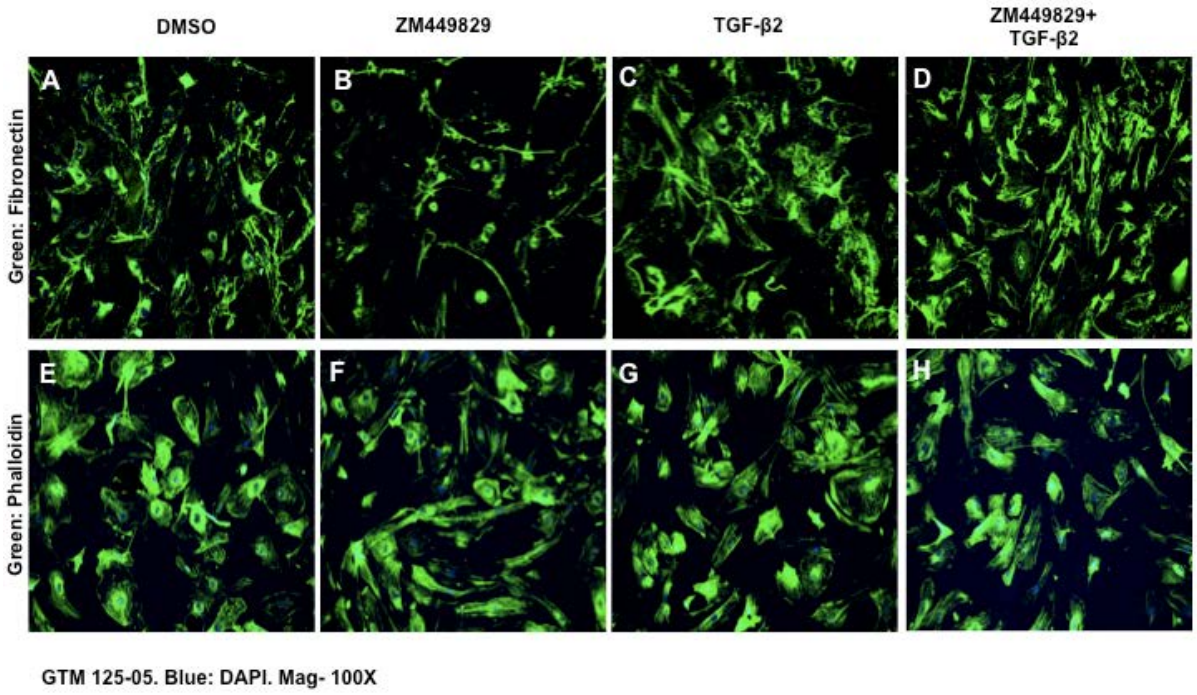


**Figure 4: Effect of ZM 449829 and TGFβ2 on fibronectin and phalloidin in GTM 125-05.** GTM 125-05 cells treated with the inhibitor appears to have marked decrease in fibronectin and treatment with TGFβ2 shows increase in fibronectin compared to drug as well as with DMSO. Combination of ZM449829 (5nM) + TGFβ2 (5ng/ml) does not appear to show any definite change in fibronectin at 48 hours (Figure 4A-D). GTM 125-05 cells treated with the inhibitor and stained with phalloidin mostly appear to be elongated compared to DMSO treated cells. Most cells in the TGFβ2 treated group appears to lose their typical elongated shape. Most cells treated with the combination of ZM449829 (5nM) + TGFβ2 (5ng/ml) do not seem as elongated as cells treated only with ZM 449829 neither do that look as dome shaped as cells treated only with TGFβ2 (Figure 4E-H).

## FIGURE 5

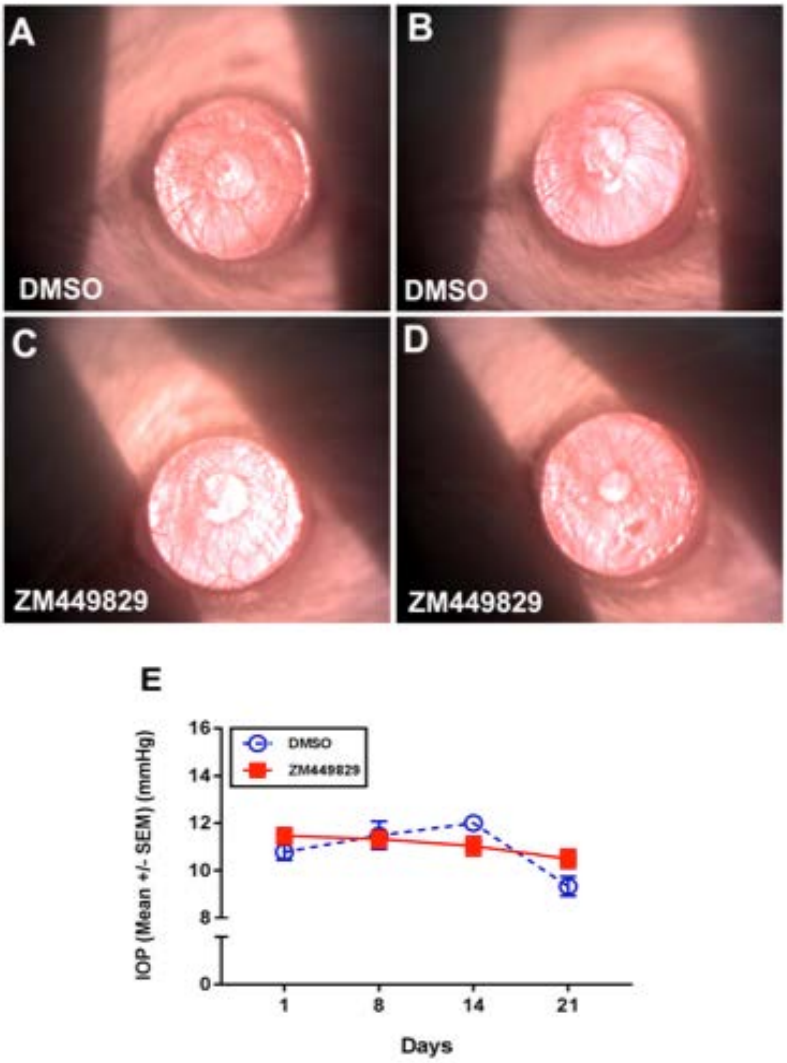


**Figure 5. Effect of ZM449829 on mouse eyes.** 5μM eye drops did not show corneal irritation, edema or hyperemia at 21 days (Figure 5A). IOP readings taken once a week did not show any inadvertent changes till 21 days (Figure 5B).

# Chapter V

## Conclusions and future directions

Our overall study demonstrates an important pathway in ocular hypertension. Increased TGFβ2 is seen in 50% POAG patients and TGFβ2 is known to induce TGM2 expression. TGFβ2 induces TGM2 expression in TM cells utilizing the canonical Smad-signaling pathway. TGM2 is involved in calcium dependent covalent crosslinking of the ECM proteins collagen and FN through N-ε (γ-glutamyl) lysine (GGEL) linkages. These crosslinked proteins are deposited in the TM and may reduce overall turnover of ECM proteins leading to a fibrotic phenotype. We therefore wanted to mimic the effects of TGM2 overexpression in the TM and study its effects on IOP and AH outflow resistance in mice.

In our study, we show that TGM2 significantly elevated IOP in 2 different strains of mice. The presence of increased GGEL crosslinks in the TM region in our immunohistochemical findings indicates that increased expression of TGM2 in the TM is associated with increased crosslinking activity. The present study suggests that an increase in crosslinking can decrease turnover of ECM in the TM thereby promoting ECM deposition that leads to increased outflow resistance through the TM. This corroborated by our finding that in BALB/cJ mice, the mean IOP of injected eyes on day 19 was 15.86 ± 1.06 (Mean ± SEM) while the mean IOP of uninjected eyes was 10.7 ± 0.48 (Mean ± SEM), which was the day with a maximum difference in IOPs ($p <$

0.001). Similarly, in C57BL/6J mice, injection of Ad5.CMV.TGM2 increased IOP from day 13 with maximum difference at day 17 with mean IOP of 17.09 ± 2.03 mmHg (injected) vs. 12.01 ± 0.47 mmHg (control) ($p<0.05$). This was in conjunction with the results from our outflow studies where we see increased outflow resistance in Ad5.TGM2 transduced eyes as opposed to their contralateral control. Immunohistochemical analysis of also shows increased crosslinking at the TM region. This data strongly correlates increased expression of TGM2 with ocular hypertension. We next want to test if inversely, knockout of TGM2 could reduce IOP.

In our TGM2 floxed mice, we see that partial knockout of TGM2 can reduce IOP in a TGFβ2 induced ocular hypertensive model. We observed a significant IOP increase in the Ad5.hTGFβ2$^{226/228}$ transduced group (transduced vs. contralateral control (maximum difference): 18.45 mmHg ± 1.3 vs. 11.65 mmHg ± 1.01 (Mean ± SEM)). Interestingly, Ad5.CMV.Cre transduction in eyes treated with Ad5.hTGFβ2$^{226/228}$ showed a slight increase in IOP that was not significant (transduced vs. control (maximum difference): 15.2 mmHg ± 1.37 vs. 13.0 mmHg ± 0.78 (Mean ± SEM)). The analysis of difference of means of transduced eyes versus their contralateral eyes was analyzed using the Student's t-test (two-tailed). Δ (OS-OD )of transduced groups showed significant difference ($p=0.03$) indicating that TGFβ2 induced ocular hypertension can be reduced by TGM2 knockout. The TGM2 knockout study along with the TGM2 overexpression study shows that TGM2 is an important player that could be responsible for IOP elevation in POAG.

Further, we tested a potent TGM2 inhibitor and observed decrease in crosslinking *in vitro* and observation of gross morphology of mouse eyes treated with 5μM of the drugs did not show corneal damage or aberrant IOP at 21 days. Our study identifies a target that could be responsible for changes in seen in POAG. In future, we could study to see whether TGM2 affects cell stiffness as seen in glaucomatous TM cells that show increased TM stiffness. Further small molecules such as ZM 449829 or similar drugs that can selectively inhibit TGM2 activity could be studied as a potential therapeutic treatment for glaucoma.

Overall, various mechanisms responsible for fibrotic changes in the TM are studied in POAG. Most of these changes are believed to be mediated by the profibrotic cytokine TGFβ2. Among other changes mediated by TGFβ2, overexpression of the crosslinking enzyme TGM2 is studied in various fibrotic conditions. ECM crosslinking by TGM2 is largely responsible for slowing ECM degradation and thereby turnover. This leads to imbalance in ECM structure and function. In the TM, implications of excessive ECM deposition includes increased resistance to AH outflow. As outflow through the TM is the major pathway, increased resistance in the TM, near the JCT, could lead to increased IOP.

Whether this is the sole mechanism in most cases of increased IOP or this mechanism adds to changes caused by other factors such as decrease in cellularity and cellular functions is yet to be studied. However, from various electron microscopy studies done earlier, ECM deposition seems to be one of the obvious changes seen in majority of patients with primary open angle glaucoma.

Therefore if increased crosslinking activity by TGM2 is responsible for this phenotype, it makes TGM2 an interesting target for intervention. If interference of the progression of the disease is possible, then inhibiting crosslinking would allow ECM turnover in a relatively normal rate. This would decrease overall ECM deposition in the outflow pathway. However whether that would be compensated by other mechanisms yet remains to be studied.

Multiple pathways are known to be responsible for glaucoma. The key to targeting the disease is through personalized approach. If we can determine whether one mechanism is predominant than others in every individual, we could treat each individual targeting the cause of disease progression in them. TGM2 knockout, among others, could lead to decreased ECM deposition and would be able to decrease overall outflow resistance and may therefore lead to rescue of the glaucomatous phenotype. A lot remains to be studied to determine whether this would be clinically feasible. However our in vivo models seem like a promising beginning for such studies.