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Glaucoma is a heterogeneous group of optic neuropathies that damage the optic nerve that leads to progressive visual loss and irreversible blindness in over 80 million people worldwide. Primary open angle glaucoma (POAG) is the most common form of glaucoma, and elevated intraocular pressure (IOP) is the major risk factor for the development and progression of this ocular disease. One of the potential mechanisms responsible for elevated IOP in POAG patients is the excessive accumulation of extracellular matrix (ECM) proteins within the trabecular meshwork (TM). Damage to the TM impairs the aqueous humor outflow and increases aqueous humor outflow resistance, and elevates IOP. Discovering potential new disease modifying targets to lower IOP is necessary to develop effective therapies to inhibit the progression of glaucoma. Here, we explored a novel molecular mechanism involved in the development of glaucomatous TM ECM damage. The effects of transforming growth factor beta 2 (TGFβ2) signaling pathways in the TM ECM have been extensively studied. TGFβ2 is a profibrotic signaling component in ocular hypertension development that increases ECM deposition in the TM.

As a member of the toll-like receptor family, toll-like receptor 4 (TLR4) was originally identified as the receptor for lipopolysaccharide (LPS). TLR4 can also be activated by endogenous ligands known as damaged associated molecular patterns (DAMPS), which are generated as a result of injury, cell damage, and ECM remodeling. DAMP-induced TLR4 activation has been

linked to fibrosis, ECM protein deposition, and augmented TGFβ2 signaling and downstream fibrotic responses. Recently, we identified TGFβ2-TLR4 signaling crosstalk that regulates the TM ECM, and mutation in *Tlr4* rescues TGFβ2-induced ocular hypertension in mice.

The goal of this work was to investigate the role of the endogenous TLR4 ligand fibronectin containing extra domain A isoform (FN-EDA), and the TLR4 downstream signaling molecule NFκB in TGFβ2-induced ocular hypertension in mice. Overall, we discovered that TLR4, FN-EDA and NFκB are necessary for TGFβ2-induced ocular hypertension and ECM deposition in mice. In addition, constitutively active EDA mice spontaneously develop ocular hypertension. These data provide new targets to lower IOP and inhibit glaucoma disease progression.

KEYWORDS: Ocular Hypertension · Nuclear factor kappa B (NFκB) · Damaged associated molecular patterns (DAMPs) · Toll-like Receptor 4 (TLR4) · Fibronectin Extra Domain A (FN-EDA) · Transforming growth factor beta 2 (TGFβ2)

THE REGULATION OF TRANSFORMING GROWTH FACTOR BETA-2-INDUCED OCULAR HYPERTENSION AND GLAUCOMATOUS ENVIRONMENT IN THE TRABECULAR MESHWORK

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CHAPTER I

INTRODUCTION TO THE STUDY

1.1 Anatomy and function of the human eye

The human eye is a highly specialized organ that is compartmentalized into anterior and posterior segments (Figure 1.1). The anterior chamber is bound anteriorly by the cornea and posteriorly by the anterior iris and lens surfaces.

The trabecular meshwork (TM) is a biomechanical sensitive tissue located at the junction of the cornea and iris. The TM contains cell-cell junctions of the endothelial-like TM cells. The TM cells have long finger-like projections which wrap around extracellular matrix (ECM) beams of fibronectin, laminin, glycoproteins, elastin fibers, proteoglycans, collagen I & IV, and hyaluronan. The TM modulates the aqueous humor (AH) outflow drainage by adjusting AH outflow resistance and in so doing maintains intraocular pressure (IOP) within the normal physiological range. IOP is the fluid pressure in the eye that is regulated by the rate of production and resistance to drainage of the AH [1, 2].

Adjacent to the TM is the Schlemm's canal (SC). SC is an endothelium-lined structure located at the inner space of the corneoscleral junction. SC function is to maintain AH homeostasis [3]. AH outflow resistance induced at inner wall of SC is predominantly due to the narrow extracellular spaces within the connective tissue [4] and the compact endothelial cell lining joined with numerous tight junctions between cells [5]. The inner wall of SC contains a basement membrane composed of a matrix of microfibril sheets. These properties allow the basement membrane to play a role in the modulation IOP [6].

The posterior chamber is located posteriorly from the anterior chamber and between the posterior surface of the iris, anterior lens, and zonule fibers. Projecting into the posterior chamber is the anterior portion of the ciliary muscle and the ciliary processes. The ciliary muscle controls the shape of the lens to which is attached by means of the zonular fibers. The ciliary processes are lined with a double-layered epithelium, consisting of a deeper layer of pigmented ciliary epithelial cells, and a surface layer of non-pigmented ciliary epithelial cells. The ciliary epithelium is involved in the production and active secretion of AH.

The posterior segment of the eye is adjacent to the anterior segment and comprises the vitreous cavity, retina, uvea (choroid), and optic nerve. The vitreous cavity is filed with a transparent viscoelastic gel called the vitreous humor. The retina is located posteriorly to the vitreous body. The retina is composed of a highly ordered sensory layer of nervous tissue consisting of an inner neurosensory component, consisting of nine layers, and a posterior retinal pigment epithelium. Within the retina are many neurons, interconnected by synapses. These include photoreceptors that transduce light from the external environment into neural impulses (action potentials), which are then passed via bipolar cells to the retinal ganglion cells (RGCs) and their axons in the retinal nerve fiber layer. These axons the run via the optic nerve to the lateral geniculate nucleus and the adjacent pulvinar located in the thalamus of the brain. There they synapse with optic radiations which course to the visual cortex in the occipital lobe for visual recognition. The unmyelinated RGC axons bundle together at the optic nerve head (ONH) to form the neuroretinal rim of the optic nerve, where RGC axons pass through the lamina cribosa and become myelinated as they pass into the optic nerve itself.

The lamina cribosa is a mechanosensitive tissue that is formed at the bottom of the optic cup through the ONH. The main functions of the lamina cribosa are as follows: (1) to allow RGCs and central retinal vein to exit the eye and the central retinal artery to enter the eye;
(2) to form a barrier between the intraocular space and extraocular space; (3) to behave as a mechanosensitive tissue that respond to IOP and retrobulbar cerebrospinal fluid (CSF) pressure in the retrobulbar part of the optic nerve [7]. The lamina cribrosa responds to the IOP and intracranial pressure (ICP) [8]. ICP is the pressure exerted by secreted cerebrospinal fluid inside the skull and on brain tissue. The difference between IOP and ICP across the lamina cribosa constitutes the translaminar pressure gradient (TPG). Exiting the lamina cribosa, the RGC axons become myelinated and form the inner part of the optic nerve.

The optic nerve is responsible for transmitting electrochemical signals to the brain and projects through the optic chiasm. In the optic chiasm the retinal nerve fibers from the nasal half of each retina cross to the opposite side of the brain, while the nerve fibers from the temporal half of the retina remain on the ipsilateral side of the brain. Exiting the optic chiasm, the optic tract is formed and project to the lateral geniculate nucleus and pulvinar in the thalamus. A small proportion also project to the pretectum and the superior colliculus. From the thalamus, second-order neurons residing in these areas project their axons via the nerve fibers of the optic radiation to transmit electrochemical signals to the primary visual cortex within the occipital lobe (V1) to be processed into images.

1.2 Aqueous humor production and drainage outflow pathways

Aqueous humor (AH) is a clear fluid that is continuously secreted into the posterior chamber at an average rate of approximately 2.5 μL/min by non-pigmented epithelial cells lining the ciliary processes. From the posterior chamber, AH flows between the iris and lens to pass through the pupil to enter the anterior chamber [2]. AH functions to nurture avascular tissues in the anterior segment, transport Vitamin C as an antioxidant agent, maintain the shape and internal alignments of the eye, remove pathogens and wastes, and maintain IOP. IOP is regulated by AH production rate from the ciliary processes and resistance to AH drainage by the conventional (or trabecular) outflow pathway. A proportion of A also egresses from the anterior chamber by the unconventional (or uveoscleral) outflow pathway [1, 9]. Within the normal physiological range of IOP, flow rate of AH across the conventional pathway is dependent on IOP, whereas flow rate of AH across the unconventional pathway is slightly altered by changes in IOP. The unconventional outflow pathway accounts for 10-35% of total AH drainage from the eye [10], and perhaps as much as 50% in very young monkeys [11]. AH passes through the root, then between individual bundles of ciliary muscle, into the suprachoroidal space, through the sclera, into the orbit, and then drains into the lymphatic system [12].

In contrast, the conventional outflow pathway is very much pressure-dependent and is responsible for approximately 65% to 90% of AH outflow[12]. The conventional outflow pathway provides the majority of the resistance to AH outflow [4]. The cytoplasmic vacuoles, which are traditionally called giant vacuoles, are transcellular pores that are pressure dependent outpouchings of inner wall endothelium of SC that bulge into the lumen of SC [13]. These giant vacuoles allow AH to egress the TM and enter inner wall endothelium [6] and then the lumen of SC [14] [13, 15]. AH egresses from the lumen of SC by scleral collector channels, anterior aqueous

veins, episcleral veins, ophthalmic veins of the venous system [16]. AH outflow is mainly mediated by changes within the extracellular matrix (ECM) surrounding TM cells, and changes within the TM cells themselves and inner wall of SC. These changes include cytoskeletal reorganization and increased cell stiffness. In turn, these modifications modulate contractility and tension of the TM and SC inner wall endothelial cells [17].

1.3 Trabecular meshwork

The TM incorporates a series of beams and sheets of ECM composed of a variety of ECM proteins that are lined with TM cells [18]. The primary function of the TM is to regulate AH outflow from the anterior chamber by generating resistance, which in turn regulates IOP. In response to elevated IOP, the TM and inner wall of SC undergoes ECM remodeling until an acceptable physiological IOP range [19] and a normal AH outflow resistance have been reached [20] [21] [22]. The TM is divided into three regions called the uveal meshwork, the corneoscleral meshwork, and cribriform meshwork (also known as the juxtacanalicular apparatus (JXT)) [23] (Figure 1.2). The uveal meshwork is the outermost layer composed of TM cells and connective tissue of the TM. The connective tissue is attached to the corneal tissue and ciliary muscle. During TM contraction, the uveal meshwork favor AH passage through the large intertrabecular spaces. The corneoscleral meshwork is composed of lined collagen and elastic tissue beams wrapped in basement membrane material located underneath the TM cells. The phagocytotic properties of the TM cells within the uveal meshwork and corneoscleral meshwork [24] leads to the removal of debris from the AH so that this material does not clog the outflow pathway. The cribriform meshwork is the inner-most TM layer that is adjacent to inner endothelial wall of SC. These latter two areas are thought to be responsible for majority of AH outflow resistance [3, 25]. The cribriform meshwork is composed of

TM cells that have the smallest pore sizes [26] and lowest porosity [18, 25] [3] compared to the cribriform meshwork and uveal meshwork.

Overall, the TM structure is dependent on the expression and distribution of a variety of ECM composition of the TM. The TM ECM turnover involves the degradation, biosynthetic replacement, and organization of ECM remodeling components to effectively respond to dynamic IOP changes in order to maintain the homeostatic state. A disruption in the TM's function and recovery can lead to the development of glaucoma, which is a chronic neurodegenerative disease.

1.4 Pathology of glaucoma

Glaucomas are a collection of chronic and multifactorial neurodegenerative diseases leading to optic neuropathy and irreversible vision loss [27, 28]. The clinical characteristics of glaucoma include: (1) progressive thinning of the retinal nerve fiber layer; (2) progressive loss/death of RGC axons and their somas by apoptosis [27]; (3) cupping and excavation of ONH or optic disc and progressive increase in the cup: disk ratio [29]; (4) a thinning of the neuroretinal rim; (5) visual field loss [30]; and in many cases (6) elevated IOP [31].

Currently over 80 million people suffer from glaucoma and this number is predicted to continue rising pass 112 million by 2040 [32]. In healthy patients, ECM turnover is adjusted to shift balance toward higher or lower AH outflow resistance. This homeostatic adjustment appears to begin with selective proteinase secretion and activation, targeted ECM cleavage and fragment uptake, and the replacement of ECM components. Contrary to healthy patients, in glaucomatous eyes there is an abnormal ECM turnover that impairs the balance between deposition and degradation of TM ECM, and damage to inner wall of endothelium SC. This results in obstruction of the AH outflow system, which increases the resistance to egress of AH.

In TM cells, secreted protein acidic and rich cysteine (SPARC) is a extracellular matrix protein that is an essential component in the transforming growth factor beta 2 (TGFβ2) effect on IOP [33] [34]. TGFβ2 is a cytokine responsible for maintaining tissue integrity and homeostasis. It is well established that TGFβ2 induces ECM protein production in the TM and significantly decreases cell viability of both GTM3 cells and primary human TM cells [35]. Thrombospondin-1 (TSP1) and Thrombospondin-2 (TSP2) are matricellular proteins involved in profibrotic remodeling and are upregulated by TGFβ2 [36]. As a component of the ECM, TSP1 is induced by mechanical forces on TM cells associated with glaucoma [37]. TSP1 is involved in profibrotic remodeling [36]. TSP2 is involved in pathogenesis of primary open angle glaucoma and contributes to increasing fibronectin deposition within the ECM in response to TGFβ2 [38].

Decreased TM cellularity in the glaucomatous TM [39]results in increased AH outflow resistance, or reduced AH outflow facility, remodeling of the TM cytoskeleton [40] [41], and increased tissue stiffness. The elevation of tissue stiffness is caused by the increase expression of actin stress fibers, [42] [43], reorganization of cross-linked actin networks (CLANS) in the TM cells and tissue [44], accumulation of aberrant amounts and types of ECM proteins, decrease ECM breakdown, and increase expression of intra-trabecular compression and trabecular beam thickening [45] [46]. Anatomical changes in endothelium wall of SC in glaucomatous patients include smaller circumference, area, diameter, and pore density than normal patients [47] [48] [49].

1.5 Glaucomatous optic neuropathy

Glaucomatous optic neuropathy is caused by damage to the optic nerve head (ONH). Glaucomatous damage leads to large cup to rim ratio [50], RGC death, RGC axon damage and degeneration, ONH remodeling, increased ECM deposition in ONH [51, 52], actin cytoskeleton

reorganization, and retinal nerve fiber layer atrophy [53] (Figure 1.4). The initial site of ocular hypertension-induced damage in glaucoma is at the ONH [54]. Prolonged IOP elevation leads to anatomical modifications within the ONH which includes collagen IV and fibronectin deposition. Ocular hypertension induced damage causes progressive neurodegeneration of the RGC axons as they pass through the ONH [55]. The glaucomatous ONH develops into a large and progressively increasing cup to rim ratio, ONH remodeling, ECM deposition, and increase TGFβ2 levels [52]. The occipital cortex and lateral geniculate nucleus may show clinical characteristics in the brain that are targets of RGC neurons also degenerate in glaucoma [56].

Surrounding the RGC axon bundle are astrocytes and lamina cribrosa cells [57, 58]. In humans, the mechanical stimulus of IOP-induced stress and strain in the lamina cribosa may cause shearing pressure in the laminar plates, leading to inhibition of axoplasmic transport in the RGC axons, induce significant fragmentation in the lamina cribosa, and cause deprivation of neurotropic factors for RGC growth and survival. This in turn will initiate the ONH astrocytes and lamina cribosa cells to send signals to induce chronic lamina cribosa ECM remodeling in an attempt to return to a homeostatic environment. During glaucoma-induced injury, activated microglial cells redistribute within the retina, ONH, optic nerve, and optic nerve tract to overexpress inflammatory molecules, leading to the progression of glaucomatous damage [59] and RGC death [60].

The pathogenesis of glaucoma is not fully understood, and each type of glaucoma is different.

1.6 Overview of types of glaucoma

Two major categories of glaucoma are angle-closure and open-angle glaucoma. Angle closure glaucoma is characterized as the iris bulges forward to narrow or block the drainage angle formed at the cornea and the iris and prevents AH from exiting the eye. This leads to ocular

hypertension development. Two forms of angle closure glaucoma are acute angle closure glaucoma and childhood developmental glaucoma. Acute angle closure glaucoma occurs when the flow of AH is blocked and pressure inside the eye rapidly increases very quickly [61]. Childhood developmental glaucoma is rare and occurs in babies within their first years of life and in young children. This disease is caused by improper development of the eye's drainage system (anterior segment dysgenesis) that leads to IOP elevation. Open angle glaucoma (OAG) is characterized as the eye angle between the iris and cornea is sufficiently open and the TM is clinically visible using a goniolens. Normal tension glaucoma is a subset of primary open angle glaucoma (POAG) in which optic nerve damage occurs without eye pressure exceeding the normal range.

Secondary glaucoma is blocking of AH outflow from debris or small molecules leading to elevated IOP. Two common types of secondary glaucoma are: (1) pigmentary glaucoma, which results from pigment granules from the iris flaking off and blocking the drainage system, thereby increasing IOP and (2) exfoliation glaucoma, which abnormal accumulation of proteins in the drainage system and other structures of the eye blocking the drainage system, thereby increasing IOP. POAG is the most common form of glaucoma [62]. In POAG patients, the imparied AH outflow drainage through the TM and inner wall of SC is caused by an increase of TM ECM deposition decrease of ECM degradation, reduced TM cell density, and abnormal TM cell function. As a result, AH outflow resistance increases leading to prolonged IOP elevation. Elevated IOP causes neurodegeneration of RGCs and their axons. Without proper treatments to reduce IOP levels, RGC atrophy will continue and lead to irreversible visual impairment and vision loss. Of all the risk factors for POAG, the only risk factor that can be modified via by surgery or drug therapy is elevated IOP.

1.7 Additional POAG risk factors

Risks factors for developing POAG in healthy subjects include advancing age, individuals of African or Mexican descent, reduced corneal thickness, family history, and glaucomatous *MYOC* mutations. In older individuals, increasing age leads to TM cells working less efficiently and causes disruption of the AH outflow pathway. Furthermore, POAG patients with increasing age have faster visual field changes [63] [50]. Individuals of African and Mexican decent disproportionally have a higher probability of developing POAG compared to Caucasians [64] (Figure 1.3). Individuals with thinner cornea thickness are also more likely to develop POAG [50]. Lastly, a positive family history of glaucoma increases one's risk by 3-to 4 fold [65] [66]. Known mutations in MYOC and other genes are known to cause glaucoma.

MYOC is a secreted glycoprotein that encodes myocilin and regulates IOP. MYOC is highly expressed in the TM and its normal function is unknown. *MYOC* mutations are responsible for the inheritance of autosomal dominant juvenile glaucoma and adult-onset POAG [67]. Glaucomatous *MYOC* mutations occur in a small percentage of POAG patients [68], causes non-secretion, and gain-of-function phenotype and ER stress which leads to compromised TM function and IOP elevation [69]. Working knowledge of understanding progressive risk factors for glaucoma and identifying growth factors that modulate these risk factors is essential in actively treating patients at risk for glaucoma progression.

1.8 Current treatments for glaucoma

There is a pressing need for developing novel therapies to reduce elevated IOP because elevated IOP correlates with axonal loss in the ONH, which is due to impaired axoplasmic flow leading to RGC loss [70] [71] and damage to the optic nerve at the lamina cribrosa region [72].

Currently, the only effective treatment for glaucoma is to lower IOP, either pharmacologically or via laser or surgical techniques to create an artificial AH outflow channel, or by surgical implantation of a drainage device. Pharmacological approaches are in general the first line of treatment. Progression to laser or surgical techniques usually come later after pharmacological approaches have ceased to yield sufficient IOP lowering efficacy. Most common and effective IOP-lowering drug therapies that slow the progression of glaucomatous damage and prevent RGC damage/death include: (1) relaxing the TM to increase conventional outflow pathway using a rho kinase inhibitors [73] [74]; (2) decreasing AH production rate using beta-blockers, alpha-2 agonists, or carbonic anhydrase inhibitors (CAIs); and (3) enhancement of AH flow by the unconventional outflow pathway using tF-class prostaglandins (e.g. latanoprost) [75] [76, 77]. Unfortunately, these drug therapies have shortcomings including: (1) not being uniformly effective; (2) progressively losing efficacy over a period of years; and (3) even when given in combinations, the efficacy can gradually be lost[78]. Recent studies suggest that identifying and understanding pathogenetic signaling pathways in the glaucomatous TM can facilitate developing novel drugs that target such pathways to lower elevated IOP and inhibit the progression of the disease.

1.9 Transforming growth factor β overview

TGFβ is a multifunctional collection of cytokines that maintains both tissue integrity and homeostasis. TGFβ signaling prevents fibrosis in normal tissue by mediating pathological increase of ECM protein secretion and deposition [79]. TGFβ is synthesized in the ciliary epithelium, lens epithelium, and the TM as part of an inactive large latent complex (LLC) [80] [81]. In the LLC, TGFβ isoforms noncovalently binds to the latent TGFβ binding protein and the latency associated protein. Upon secretion of the LLC, the complex covalently binds to ECM and serves as a local

TGFβ isoform reservoir. Liberated active TGFβ binds to TGFβ receptor II causing TGFβ receptor II to dimerize and phosphorylate TGFβ receptor I.

TGFβ signals through either the SMAD or non-SMAD signaling pathways. The SMAD signaling pathway of TGFβ leads to the recruitment and phosphorylation of the SMAD2/SMAD3 complex. The p-SMAD2/3 complex dissociates from the TGFβ receptors and dimerizes to signal transducer Smad4. The pSMAD2/3-Smad4 complex translocate into the nucleus to bind to TGFβ response elements on DNA that target gene expression. During non-SMAD TGFβ signaling, MAP kinases ERK, JNK, and p38 MAP kinases are activated and translocate into the nucleus to regulate gene expression (Figure 1.5) [82] [83] [84] [85].

Studies suggest that transforming growth factor-β (TGFβ) is involved in the pathogenesis of certain forms of glaucoma [86] and may influence the major risk factor for the development and progression of POAG. Secreted by TM cells [79], TGFβ is involved in cell development, wound healing, proliferation, migration, apoptosis, and differentiation [79]. One essential role of TGFβ in the TM is to regulate ECM deposition in human TM cells by mediating ECM deposition and suppressing TM cell proliferation [79].

TGFβ isoforms in the mammalian eye are TGFβ1, TGFβ3, and TGFβ2. These TGFβ isoforms share 60-80% homology in amino acid composition and have different roles [87] [88]. TGFβ1 triggers wound healing [89], released in large quantities from platelets, and regulate various cell activities such as cell growth, proliferation, differentiation, motility, and apoptosis [90]. Secreted in tissue and muscles, TGFβ3 is involved in the formation of blood vessels, regulation of bone growth, wound healing, and regulation of the immune system.

TGFβ2 is the most abundant TGFβ isoform [91] that activates both canonical and noncanonical signaling pathways in the anterior and posterior segments of the eye. Significantly elevated TGFβ2 levels have been identified in the anterior chamber of glaucomatous eyes. Dysregulated TGFβ2 expression can promote TGFβ2 SMAD signaling to act as a glaucomatous insult. In the TM, these insults are as follows: (1) dysregulate fibrotic ECM gene expression that promotes elevated synthesis of ECM proteins [92, 93] [4] and alter ECM in human TM cells [94]; (2) disrupt healthy tissue remodeling leading to tissue dysfunction [95] [96]; (3) elevate crosslinking enzyme levels such as transglutaminase (TGM2) [97] and Lysyl oxidase (LOXs) [94]; (4) reorganize TM actin cytoskeleton into cross-linked actin networks (CLANs) [98] [99] [44] ; (5) inhibit TM cell proliferation [100]; (6) increase expression of both plasminogen activator inhibitor (PAI-1) [101] [102] and tissue inhibitors of metalloproteinases (TIMPs) expression [103]; and (7) prevent matrix metalloproteinases activation [104].

Studies have also shown TGFβ2-treated cultured human TM cells [105] [35] and TGFβ2-treated perfused anterior eye organ culture model [106] [107] [93] increases ECM protein synthesis . In addition, TGFβ2 treatment increases outflow resistance in organ culture experiments [106] [93]. Understanding the crosstalk that TGFβ2 pathways have with other signaling pathways in the pathogenesis of glaucoma will provide novel ideas for developing new and effective therapeutic agents to target these pathways and inhibit the progression of glaucoma.

1.10 TGFβ2 crosstalk with other key signaling pathways in a glaucomatous environment

1.10.1 Crosstalk between TGFβ2-Wnt signaling

Canonical Wnt signaling is a signal transduction pathway that inhibits the activity of TGFβ in primary non-glaucomatous TM cells. TGFβ2 increases β-catenin expression in human

TM cells [108]. β-catenin is a key transcription factor for Wnt/ β-catenin signaling and an accessory protein for classical cadherin junctions expressed in human TM cells [108]. In conjunction with Smad4, β-catenin is required for the cross-inhibition between TGFβ and Wnt signaling pathways in HTM cells to regulate TM homeostasis [109].

1.10.2 Crosstalk between TGFβ2-BMP7 signaling

Bone morphogenetic proteins (BMPs) are a collection of signaling molecules belonging to TGFβ superfamily of proteins and play a critical role in inducing bone and cartilage formation [110]. Previous studies have demonstrated that BMPs alter signaling pathways of TGFβ2 [111]. Previous studies show that treatment of cultured TM cells with TGFβ2 caused a substantial increase in fibronectin, collagens IV and collagen VI expression [112]. Whereas the co-treatment of cultured TM cells with TGFβ2 and BMP7 reduced TGFβ2-effects [112]. Furthermore, TGFβ2-treated human TM cells upregulated mRNA and protein expression of connective tissue growth factor (CTGF) and these effects were blocked by BMP7-TGFβ2 co-treatment [112]. The antagonizing effects of BMP7 on TGFβ2-induced ECM protein expression of CTGF can be block by knockdown of Smad7 (siRNA Smad7) [113]. Smad7 is a TGFβ signaling inhibitor that binds to TGFβ type I receptor intracellularly, thus preventing Smad2/3 to associate with TGFβ receptor and mediating the antagonizing effects of BMP7 on TGFβ2 signaling. Overall, the physiological role of TGF-β2 and BMP7 signaling in the TM may serve a critical role for mediating the TM ECM function and structure.

1.10.3 Crosstalk between TGFβ2-gremlin-BMP4

Gremlin is a secreted BMP antagonist that binds to BMP-4 and suppresses TGFβ2 induced TM ECM protein expression [92, 114] and signals through the Smad3-dependent pathway [115]. Studies show that the expression Gremlin is elevated in cell lysates and cultured medium of glaucomatous human TM cell lines, perfusate medium of cultured human anterior segments, human AH [92]. Studies have shown that Gremlin-treated perfusion cultured human eye anterior segments [92] and overexpression of Gremlin in mice [115] significantly elevates IOP and increases ECM protein expression. These findings suggest that elevated Gremlin levels in glaucomatous TM in mice and humans may be responsible for elevated IOP associated with glaucoma. The crosstalk between Gremlin and TGFβ2 results in a pathogenetic fibrotic response in the TM. TGFβ2 elevates Gremlin expression and Gremlin elevates TGFβ2 expression while inhibiting TGFβ2 activity. Gremlin may signal through other unknown signaling pathways that are independent from TGFβ2 to mediate BMP4 activity in the TM.

1.10.4 Crosstalk between TGFβ2-glucorticoids

Glucocorticoids (GCs) are a collection of natural (cortisol) or synthetic (dexamethasone (DEX)) ligands that that bind the glucocorticoid receptor to maintain normal metabolism, homeostasis, and immune regulation. Studies have shown that DEX increases TGFβ2 levels in AH and TM of mouse model of DEX-induced ocular hypertension [116]. In addition, DEX activates TGFβ2 SMAD signaling pathway in primary human TM cells [116]. This led to GC-induced TM ECM accumulation and elevated IOP. To confirm TGFβ2 signaling was associated within glucocorticoid signaling pathways, DEX-induced ocular hypertension was blocked SMAD3 deficient mice [116]. In addition, DEX induces extracellular deposition in the TM as well as CLAN

formation in TM cells and tissue associated with steroid-induced ocular hypertension [117] [118]. Sustained use of GC in humans leads to ocular hypertension development and other open angle glaucoma phenotypes [119]. The crosstalk between TGFβ2 signaling and GC may play an important role in the pathophysiology of POAG and manipulations of these interactions can provide a novel intervention for both POAG and GC-induced glaucoma.

1.10.5 Crosstalk Between TGFβ2-TLR4

Toll-like receptor 4 (TLR4) is a member of the Toll Like Receptor (TLR) family [120]. TLR4 activation by damage associated molecular patterns (DAMPs) can initiate a fibrotic response [121] and augment TGFβ signaling as shown in *in vitro* and *in vivo* studies [122] [123] [35]. *In vitro* studies have shown that inhibition of TLR4 signaling blocks TGFβ2-induced ECM production in primary human TM cells and that mutated TLR4 blocks TGFβ2-induced ocular hypertension in mice [35]. The proposed pathway of TGFβ2-TLR4 signaling crosstalk leads to elevated TGFβ2 signaling, ECM deposition, progressive fibrotic response in the TM, and ocular hypertension (Figure 1.7). Overall, the crosstalk between TGFβ2-TLR4 provides insight into a novel molecular mechanism involved glaucomatous TM damage.

TLR4 signaling is a regulator of bone morphogenetic protein (BMP) and activin membrane bound inhibitor (BAMBI) expression. BAMBI is a transmembrane glycoprotein that inhibits BMP, activin, and TGFβ signaling [13] [114] and is involved in the production and regulation of TM ECM [124]. Upon TLR4-DAMP signaling, MyDyD88-NFκB activation downregulates BAMBI and enhances TGFβ signaling which increases ECM production [125]. Recently, we discovered that TGFβ2 regulates BAMBI expression and increased ECM production in TM cells. Increased AH outflow resistance and ocular hypertension are induced by the selective knockdown of *Bambi* in

mice. Knockdown of *Bambi* also induces ECM production of fibronectin and collagen-1 in mouse TM cells [124]. Overall, the pathway of TLR4 signaling is linked with BAMBI expression.

1.11 Toll-like Receptor 4 signaling pathway

TLR4 is a type I transmembrane protein consisting of an extracellular domain, a single transmembrane domain (TMD), and an intracellular Toll-interleukin 1 receptor (TIR) domain that are required for downstream signal transduction [126]. TLR4 signaling is activated by exogenous ligands such as lipopolysaccharide (LPS) or endogenous damage associated molecular patterns (DAMPs) [121]. DAMPs are danger molecules released from damaged and/or dying cells and tissues. Several DAMPs that activate TLR4 signaling as a result of cell damage, ECM remodeling, or oxidative stress [127] [121] are low molecular weight hyaluronic acid (LMWHA) [128], Tenascin C [127], and fibronectin isoforms that contain extra domain A (FN-EDA) [129]. TLR4 activation initiates the fibrotic process via recognizing DAMPs that are derived cellular motifs from tissue known as endogenous ligands [121]. The extracellular domain of TLR4 recognizes and becomes activated by DAMPs and initiates DAMP-TLR4 complex signal transduction through TLR4's intracellular adaptor molecule TIR-domain by either the myeloid differentiation factor 88 (MyD88) independent or dependent pathways.

In the TLR4-MyD88 dependent pathway, MyD88 recruits IL-1 associated receptor kinases (IRAK1, IRAK2, IRAK4, TRAFK6) to activate the TAB2, TAB3, TAK1 complex. This complex phosphorylates the stimulus-responsive IκB kinase complex (IKK), triggers proteasome-mediated degradation of IkB [130] [131] [132] [133], thereby freeing of the cytosolic nuclear factor kappa B (NFκB). Free NFκB translocate into the nucleus and mediates fibrotic responses by altering gene expression [134] [123] [135] [136] (Figure 1.6). Dysregulated DAMP-activated TLR4 signaling promotes

unresolved renal and hepatic fibrosis and augments TGFβ2 signaling [123]; [136]; [137]. Recently, TLR4 signaling has been shown to be associated with a novel molecular mechanism that contributes to ocular hypertension [35] [124] (Figure 1.7). TLR4 is expressed in human TM cells [35] and TM tissue. TLR4 expression is higher in the TM of POAG human donor eyes compared to healthy eyes [107]. TGFβ2-induced ocular hypertension in perfusion cultured human anterior segments was blocked by concomitant treatment with TLR4 inhibitor TAK-242 [138].
TLR4 antagonist, TAK-242, binds to the intracellular TLR4 domain to disrupt TLR4 interaction with adaptor molecules, thereby inhibiting TLR4 signal transduction [139].

TLR4 signaling is an important regulator of TGFβ2-induced ocular hypertension and ECM protein increase. TLR4 mutant mice (C3H/HeJ) have a spontaneous missense mutation in the *Tlr4* gene (P712H) in the cytoplasmic portion of TLR4 [140] that inhibits TLR4 downstream signaling transduction. As a result, TGFβ2-induced ocular hypertension and increased ECM protein expression in the TM is blocked in *Tlr4* mutant mice [35]. These findings provide evidence that TLR4 signaling pathway is an effective target to inhibit IOP elevation and reduce profibrotic mediators in TM in this model. These data demonstrate that TLR4 signaling can enhance TGFβ signaling forming a TGFβ2-TLR4 crosstalk. The TLR4 downstream signaling molecule nuclear factor kappa B (NFκB) has the potential to mediate fibrotic response in the TM leading to the development of ocular hypertension.

1.12 Nuclear Factor kappa B canonical signaling pathway

Nuclear Factor kappa B (NFκB) is a downstream signaling molecule in different pathways, including the TLR4 signaling pathway [131]. As a transcription factor that is activated by a number of signaling receptors., NFκB is highly regulated and controls DNA transcription [141], cytokine and ECM protein production, cell survival [131] [131] [130] [129] [129] [129], proliferation, and differentiation [142]. Within the cytosol, latent NFκB protein subunits are p50, p65, and IκB [143] [131].

After TLR4 is activated by DAMPs, TLR4 signaling transduction is initiated, and the stimulus-responsive IκB kinase complex (IKK) phosphorylates the IκB subunit (p-IκB) which is responsible for retaining inactive NFκB in the cytosol. The p-IκB triggers proteasome-mediated degradation of IκB [130] [132] [133] to free and thereby activate the NFκB complex. Following activation, the NFκB complex binds to importin at its nuclear localization sequence and translocate into the nucleus to bind to its DNA response elements [130]. Bound to DNA, NFκB targets gene expression of IκB and ECM proteins [133]. The mechanisms and consequences of NFκB activation leading to a fibrotic response in the TM are not well understood. Previous studies have shown that MyD88-NFκB dependent pathway signaling downregulates BMP and activin membrane-bound inhibitor (BAMBI) expression in other tissues [122] [136] [135].

BAMBI is a transmembrane glycoprotein that inhibits BMP, activin, and TGFβ signaling [144] and maintains IOP homeostasis [124]. BMPs are a family of growth factors involved in regulation of ECM that suppresses TGFβ2-induced ECM deposition [92, 112]. In hepatic satellite cells of the liver, the p50 subunit of NFκB represses BAMBI mRNA expression, thus enhancing TGFβ signaling and fibrogenesis [145]. Thus, NFκB signaling may play a critical role in regulating TM homeostasis, ECM protein expression in the TM, and IOP levels.

1.13 Fibronectin

. Fibronectin (FN) is a large dimeric multidomain glycoprotein and a major TM ECM protein [146] [147]. FN is responsible for mediating cellular interactions with other ECM material and is involved with different cellular functions such as cell adhesion, migration, growth and differentiation [148] [149, 150]. FN is located within the sheath material surrounding the elastin tendons connected to elastin fibers in the cribriform meshwork, and inner wall of Schlemm's canal to help contribute to TM/SC contraction and relaxation, scattered throughout basement membrane of inner wall of Schlemm's canal, and in the core of the trabecular beams [147].

In POAG patients, FN plays a role in developing a fibrotic response that leads to excessive deposition of connective tissue which includes increased deposition of a wide variety of ECM proteins that impairs the structure and function of the AH, SC, TM and ONH [151] [152] [146] [153] [154]. It is possible that the cause of increase FN expression in POAG patients compared to normal patients is due to elevated levels of TGFβ2 in the TM and AH [81] [97]. Numerous studies demonstrate TGFβ2 elevates FN expression in cultured TM cells *in vitro* and *in vivo* [155] [104]. Treatment of TGFβ2 on cultured TM cells and *in vivo* mouse studies elevates FN expression [35, 124]. Studies show that the action of TGFβ2 on FN is largely mediated through connective tissue growth factor (CTGF). CTGF is a matricellular protein and a TGFβ2 target gene with high constitutive expression in the TM [156].

FN messenger RNA (mRNA) can undergoes alternative splicing isoforms [157] associated with fibrosis. FN is found either as a soluble dimer in plasma (pFN) or as insoluble fibrils in ECM tissues known as cellular FN (cFN). The differences between the two is pFN is secreted by hepatocytes directly in the bloodstream for blood clotting and would healing, and cFN is

synthesized by many cell types including fibroblasts and endothelial cells. cFN is a mixture of FN isoforms including alternatively spliced extra domain A (EDA) or extra domain B (EDB).

FN-EDA is a splice variant of FN that is age-related and can change during development and pathological processes [151]. Previous studies have shown that cFN-EDA acts as a DAMP and promotes pulmonary fibrosis [158] and liver fibrosis [159]. Recently studies have shown FN-EDA activates TLR4 and augments TGFβ signaling [35] and stimulates NFκB activation via TLR4 [160]. In POAG patients, elevated FN expression in the TM is correlated with increased FN-EDA expression [155]. *In vitro* studies show that cFN-EDA treated human TM cells induced intracellular FN expression to the same degree as TGFβ2 [35]. More studies are needed to fully understand the role of FN and FN-EDA in glaucomatous eyes. FN plays an important role in the development of fibrosis; therefore, it is critical for future studies to focus on discovering animal models that develop excessive FN and FN-EDA expression in eye regions that are targeted by POAG.

1.14 Animal models of POAG

Glaucoma research and drug development are hindered by a limited selection of preclinical models that recapitulate human disease. Developing animal models that mimic human glaucoma disease facilitates in the discovery of important glaucoma-associated changes in the outflow pathways, improves our understanding of AH outflow physiology, and is useful tool for discovering targets for new therapeutic drugs. Animal models used to study the progression of glaucoma include: (1) Rhesus monkeys with hypertensive POAG [161]; (2) Kayo Santiago macaques which are a valuable model for human normotensive and hypertensive POAG [161]; (3) POAG in Beagle dogs [162] [163]; and (4) primary congenital glaucoma in cats [164].

Disadvantages of using monkeys, dogs and cats as animal models of glaucoma are that they are costly and have limited availability. Alternatively, rodents have been increasingly used as glaucoma models because of the following advantages: (1) ocular anterior segments that are anatomically and physiologically similar to the human eye; (2) well-developed trabecular meshwork which connects to inner wall of SC by a network of elastic fibers, (3) true SC; (4) conventional outflow pathway function in mice that is generally similar to those in human eyes; (5) pharmacologic responses to many compounds are similar to human eye; (6) transgenic mouse strains expressing genes associated with POAG are easy to generate; and (7) rodents can be transfected with genes using viral expression vectors. Generally, the combination of these advantages with continued advancements in cell biology and genetic tools make the mouse an excellent system for discovering molecular pathways that control glaucoma pathophysiology.

DBA/2J mice develop a secondary form of glaucoma called pigmentary glaucoma. This type of secondary glaucoma is due to pigment dispersing iris disease that leads to progressive IOP elevation and RGC loss [165] [166]. In DBA2/J mice eyes, ocular hypertension development is age-related and asynchronous. DBA/2J mice have been used extensively to study the pathogenesis of ocular hypertension induced RGC death associated with glaucoma [167]. Unfortunately, there are limitations with the DBA/2J mouse glaucoma model due to a variability of onset, variable damage between the paired eyes, and late onset of ocular hypertension [168]. Research in DBA/2J mice studies in human glaucoma have provided new mechanisms and ideas on the development of ocular hypertension induced RGC loss. However, understanding the anatomical and physiological modifications due to glaucoma within the anterior segment is limited for DBA/2J mice.

Transgenic mice are very useful to implicate a specific gene or pathogenic pathway in any area of the eye, including the TM and optic nerve. These mouse models allow one to identify as

many mutations as possible for the purpose of understanding genotype-phenotype relationships that are essential to understanding the disease [169]. MYOC gene encodes the glycoprotein myocilin. Myocilin has an unknown function and is secreted into the AH from the TM of the eye. MYOC mutations causes myocilin accumulation in the TM cells and develop glaucomatous phenotype. For example, the *Y437H* mutation in human *MYOC* in the Tg-*MYOC(Y437H)* transgenic mouse develops elevated IOP, RGC death, and RGC axonal degeneration [170]. Mutant myocilin accumulates in the endoplasmic reticulum (ER) of the TM and induces ER stress [69]. Other inducible mutant human *MYOC* mice strains have been identified as having ocular hypertension [171] and optic nerve damage [172]. Other studies that express mutant mouse myocilin have much milder glaucoma phenotypes [173] [174].

A new transgenic mouse model for glaucoma is the connective tissue growth factor (βb1-CTGF) mouse. In this animal model, the lens-specific overexpression of profibrotic connective tissue growth factor (CTGF), which is transcriptionally activated by TGFβ, leads to ECM changes in the cytoskeleton of the TM followed by elevated IOP and progressive RGC axon loss in the optic nerves and increase IOP [175] [156]. Furthermore, the (βb1-CTGF) transgenic mouse could serve as a promising model to better understand the pathobiological mechanisms in POAG. Overall, transgenic mice represent a valuable tool to study the genetics, function, and treatment of glaucomatous myocilin mutations.

Utilizing viral vector-based models of POAG in rodents have been promising in research. A common viral vector used to target TM cells is adenovirus 5 (Ad5). Adenoviruses are linear, non-integrative, non-enveloped, double-stranded DNA viruses that can be intravitreally injected into the eyes of mice. Advantages of Ad5 are as follows: (1) tropism for TM with no non-specific effects on AH hydrodynamics [176]; (2) a large cloning capacity of 36kB; (3) rapid and high

efficiency of gene delivery to both non-dividing and dividing TM cells; (4) high success rate of transducing TM cells in mice [177] [35] [124]; and (5) use of a relatively low titer [35] [124] [178]. On the other hand, disadvantages of using Ad5 are as follows: (1) a strong immunogenicity leading to mild-moderate anterior segment inflammation (also depending on transgene) and (2) expression is transient in the TM due to inflammation or immune response in young mice. Adenovirus vectors are a powerful gene targeting tool that can be used to infect specific types of cells efficiently both *in vitro* and *in vivo.* Not only is the type of viral vector used important, but the proper route of administration to target TM is essential.

Two experimental approaches to inject a viral vector to transduce foreign genes that target the TM cells of rodent eyes is to perform intravitreal or intracameral injections into rodent eyes with a viral vector. Intravitreal injections are more favorable than intracameral injections because intravitreal injections yield a better response in rodents because the vitreous serves as a slow-releasing depot for intravitreally injected virus; whereas, the conventional outflow pathway can wash out a high proportion of the intracameral virus injection in the rodent eye [178]. This leads an insufficient amount of the virus left for productive TM cell transduction.

A rodent model used in glaucoma research is the gene knockout mouse model. This model disrupts or inactivates one or more targeted genes of interest to conduct *in vivo* studies of gene function and biological mechanisms. An experimental technique used to conduct conditional knockout strategies is the CRE/LOX method in which one can combine floxed mice with Ad5.Cre injection. One example is intravitreally injecting Ad5.Cre in B6.129S1-Bambi^{tm1Jian}/J mice to knockdown *Bambi* in the TM. BAMBI normally functions to inhibit TGFβ2 signaling.

In this study, knockdown of *Bambi* led to ocular hypertension, increased ECM expression including FN and collagen-1 proteins, and reduced outflow facility [124]. Another example is

intravitreally injecting Ad5.Cre in TGM2$^{t/t}$ floxed mice (B6.129S1-*Tgm2*^{tm1Rmgr}/J) to partially knockout tissue transglutaminase (TGM2) in the TM. TGM2 is an ECM crosslinking enzyme that covalently crosslinks ECM proteins and causes excessive ECM protein deposition in TM, thereby increasing IOP. Partial knockout of TGM2 in the TM reduced TGFβ2-induced ocular hypertension and reversed TGFβ2 mediated decreased AH outflow facility [179]. In summary, gene knockout mouse models are useful to understand the role of a specific gene or pathogenic pathway by comparing the knockout organism to a wildtype with a similar genetic background.

1.15 Experimental models of POAG

Several experimental approaches have been developed and utilized to create an induced ocular hypertension models including: (1) Adenovirus 5 (Ad5) transduction of *MYOC* mutants into mouse eyes, which closely reflected the human genotype / phenotype correlation seen in humans with MYOC glaucoma [180]; (2) Ad5 transduction of a bioactivated form of TGFβ2 (Ad5.hTGFβ2$^{C226/228S}$) into mouse eyes [180]; (3) episcleral vein injection of hypertonic saline to sclerose the AH outflow pathway in rats [181] [182]; (4) microbead injection into the anterior segment of rats and mice [183]; and (5) glucocorticoid-induced ocular hypertension [52, 184].

Of the methods listed, intravitreally injecting Ad5.TGFβ2 to transduce this foreign gene for target cell expression is the technique we used to validate the pathogenic signaling pathways of glaucoma in rodent eyes. Ad5.TGFβ2 (Ad5.hTGFβ2$^{C226/228S}$) is a bioactivated form of TGFβ2 that reduces AH outflow and elevates IOP in the mouse animal model. Ad5.hTGFβ2$^{C226/228S}$ is a mutated human TGFβ2 coding sequences at positions 226 and 228 in the latency-associated peptide (LAP) from cystine to serine [180]. Mutant human TGFβ2 overcomes the hurdle of requiring proteolytic removal of LAP from the newly synthesized latent TGFβ2 in rodent eyes.

1.16 Conclusion

Overall, POAG is a sight-threatening disease with complicated pathophysiology that is far from being completely understood. Continued development of mouse models of POAG and in-depth study of its pathology will contribute to identifying molecular mechanisms responsible for the glaucomatous environment within the TM. Moreover, this information can be used to develop novel drug therapies to elucidate the progression of POAG and return IOP to a homeostatic range that will prevent RGC neurodegeneration and irreversible vision loss.

1.17 Specific Aims

Background

Glaucoma is a heterogeneous group of optic neuropathies that damage the optic nerve and lead to progressive visual loss and irreversible blindness in over 80 million people worldwide. Primary open angle glaucoma (POAG) is the most common form of glaucoma, and elevated intraocular pressure (IOP) is the major risk factor. One of the potential mechanisms responsible for elevated IOP in POAG patients is the excessive accumulation of extracellular matrix (ECM) proteins within the trabecular meshwork (TM). The ECM composition of the TM plays a major role in regulating aqueous humor outflow and IOP. The effects of transforming growth factor beta (TGFβ) signaling pathways on the TM ECM have been extensively studied. TGFβ2 is a signaling component in ocular hypertension development that causes ECM protein accumulation in the TM cells and elevates fibrosis in TM.

As a member of the toll-like receptor family, toll-like receptor 4 (TLR4) was originally identified as the receptor for lipopolysaccharide (LPS). TLR4 can also be activated by endogenous ligands known as damaged associated molecular patterns (DAMPs), which are generated in situ as

a result of injury, cell damage, and ECM remodeling. DAMP-induced TLR4 activation has been linked to fibrosis, ECM protein deposition, augmented TGFβ2 signaling, and downstream fibrotic responses. Recently, we identified TGFβ2-TLR4 signaling crosstalk regulates changes in the TM ECM and mutation in *Tlr4* prevents TGFβ2-induced ocular hypertension in mice.

Here, we investigate the role of an endogenous TLR4 ligand, fibronectin containing extra domain A isoform (FN-EDA), and a downstream signaling molecule of TLR4, NFκB, in TGFβ2-induced ocular hypertension in mice.

Hypothesis

Endogenous pathological TLR4 ligand FN-EDA activates TLR4 and augments TGFβ2 signaling sensitivity, leading to glaucomatous TM damage and elevated IOP. Mutated NFκB will inhibit TGFβ2-induced ocular hypertension and glaucomatous TM damage

SAI. Determine whether the endogenous TLR4 ligand FN-EDA is necessary for TGFβ2-induced ocular hypertension and glaucomatous TM damage in mice.

Determine whether Ad5.TGFβ2 can induce ocular hypertension in C57BL/6J (B6) mice and experimental mice B6.EDA$^{-/-}$, B6.EDA$^{-/-}$/TLR$^{-/-}$, B6.EDA$^{+/+}$/ TLR4$^{-/-}$, B6.TLR4$^{-/-}$, B6.EDA$^{+/+}$mice by intravitreally injecting 2.0 μL Ad5.TGFβ2 (2.5 x 10^7 pfu) into one eye, while the contralateral uninjected eye is used as a negative control.

Expectations

We anticipate Ad5.TGFβ2 will induce ocular hypertension in both experimental cohorts of C57BL/6J, B6.EDA$^{+/+}$/ TLR4$^{-/-}$ and B6.EDA$^{+/+}$ mice. The *Tlr4* - *EDA* double knockout mutant mice and *EDA* knockout mutant mice may rescue ocular hypertension phenotype and attenuate

morphological changes within the ECM of the TM. Ad5.TGFβ2 may induce ocular hypertension in B6.EDA$^{+/+}$/ TLR4$^{-/-}$ mice due to FN-EDA binding to other receptors that may induce a fibrotic response. For protein analysis using IHC, we expect total fibronectin and FN-EDA isoform protein expression to increase in B6.EDA$^{+/+}$ mice and Ad5.TGFβ2 injected C57BL/6J and B6.EDA $^{+/+}$ mouse strains. We expect intravitreal injections of Ad5.null (2.5 x 10^7 pfu) will have no effect on any of the mouse strains.

SAII. Determine whether NFκB is necessary for TGFβ2-induced ocular hypertension and glaucomatous TM damage in mice.

Determine whether Ad5.TGFβ2 can induce ocular hypertension in C57BL/6J mice and experimental mice B6.Cg-Nfκb1^{tm1Bal}/J mice which lack the p50 subunit of NFκB. We will intravitreally inject Ad5.TGFβ2 into one eye, and the contralateral uninjected eye will be used as a negative control.

Expectations

We anticipate Ad5.TGFβ2 will induce ocular hypertension in C57BL/6J. Mutation in NFκB will prevent TGFβ2-induced ocular hypertension and attenuate morphological changes within the ECM of the TM. We expect total fibronectin and FN-EDA isoform protein expression to only increase in the C57BL/6J mouse strain. We expect intravitreal injections of Ad5.null will have no effect in any of the mouse stains.

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CHAPTER I. LIST OF FIGURES

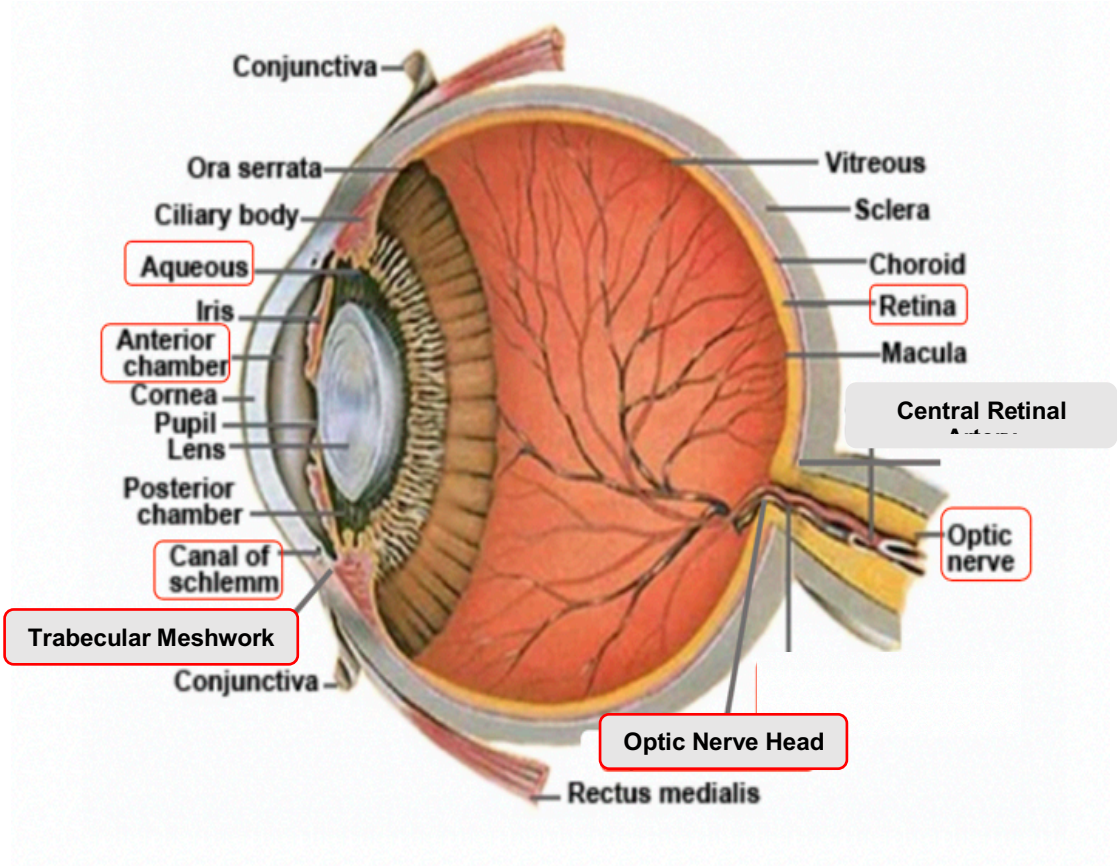

Figure 1.1 Anatomy of the Human Eye: Cross-section view of the human eye. Red boxes represent areas associated with ocular hypertension development and glaucomatous optic neuropathy and neurodegeneration http://www.mastereyeassociates.com/eye-anatomy.

Modified by Amanda Roberts 2020.

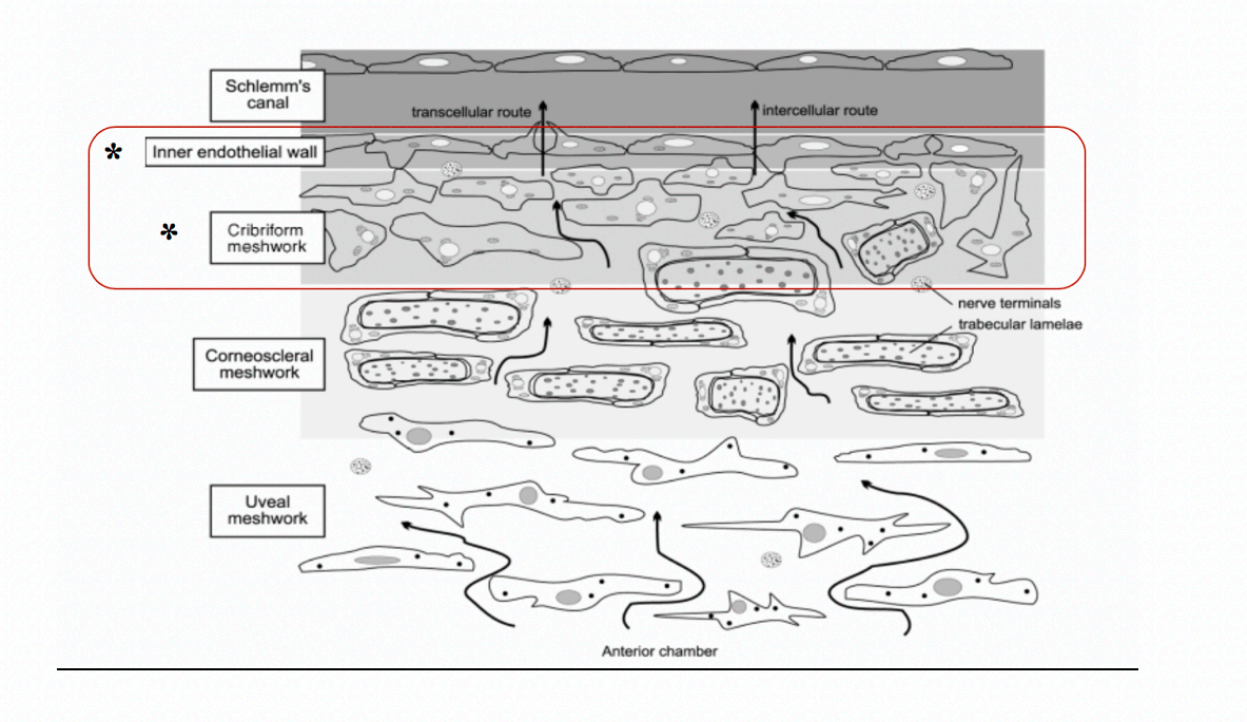

Figure 1.2 Aqueous humor outflow through the trabecular meshwork:

AH flows through the uveal meshwork, corneoscleral meshwork, and cribriform meshwork. Both the cribriform meshwork and inner endothelial wall are thought to be the sites with the highest resistance against AH outflow resistance [22]. *Red box represents the areas of highest aqueous humor outflow resistance. Modified by Amanda Roberts 2020.

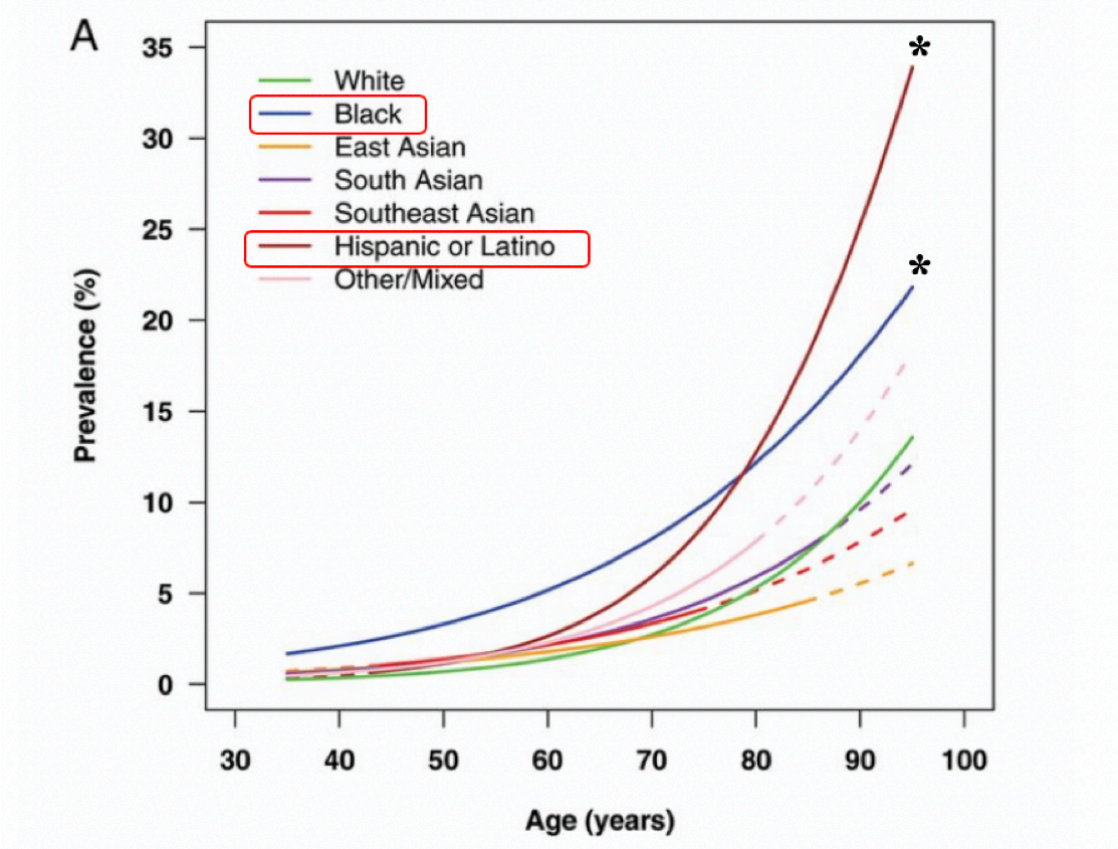

Figure 1.3 Estimated prevalence (%) of POAG with age for both men and women by ethnicity: Black populations (Blue line) have the highest absolute levels of POAG prevalence at each age before 80 years. Hispanic/Latino populations. (Red line) have the highest levels of POAG prevalence after 80 years [62]. Red box represent the two groups with the highest POAG risk. Modified by Amanda Roberts 2020.

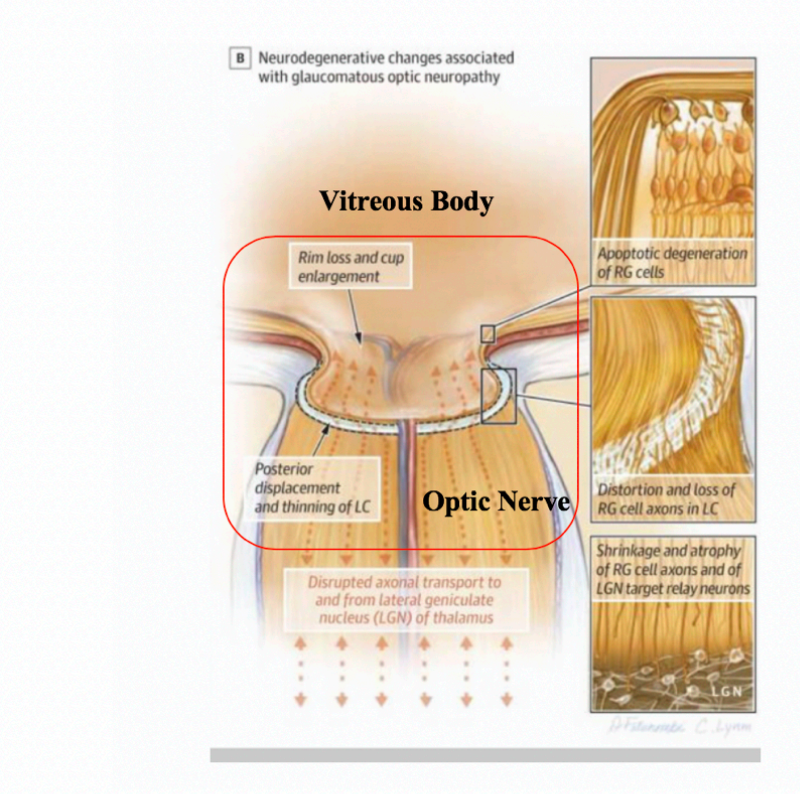

Figure 1.4 Schematic illustration of Neurodegenerative changes of glaucomatous optic neuropathy: Glaucomatous optic neuropathy is characterized by optic disc tissue and lamina cribosa damage, loss of optic rim neural tissue (unmyelinated RGC axons) causing optic cup enlargement, and degeneration of RGC axons Red box represents the regions of the most impact from glaucomatous optic neuropathy.

. http://medsphere.wordpress.com/2016/11/20/poag/)

Modified by Amanda Roberts 2020.

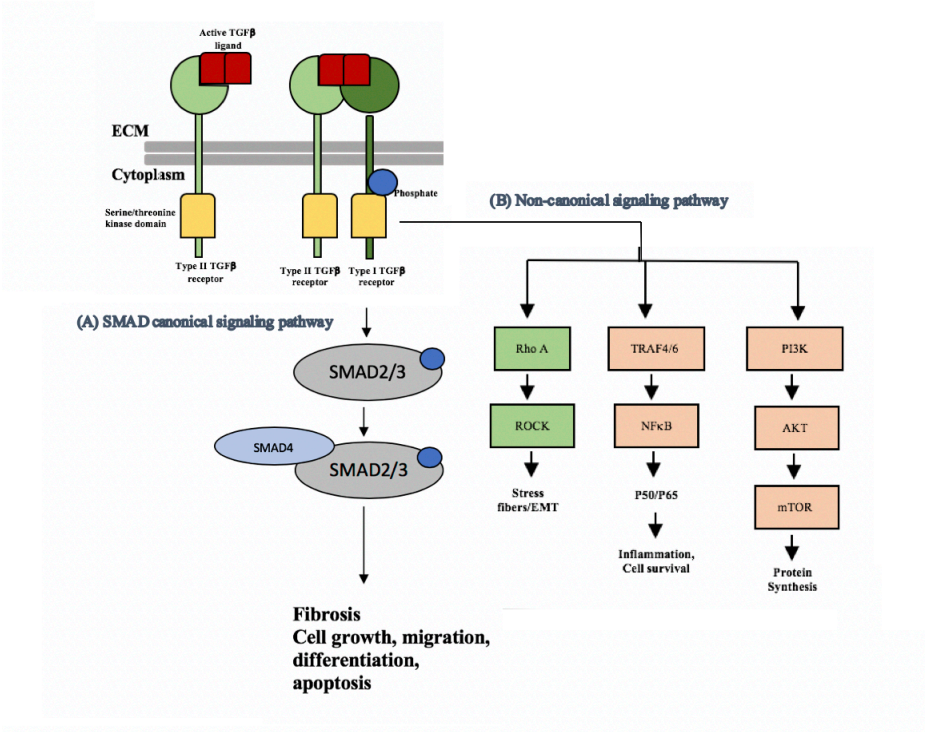

Figure 1.5 TGFβ2 canonical and non-canonical signaling pathways: (A) In the SMAD/Canonical signaling pathway, activated type I receptors phosphorylate Smads 2 & 3, forming a complex with Smad4. Smad 2/3/4 complex translocate into the nucleus to interact with distinct transcription factors to turn on and off the transcription of TGFβ responsive genes to regulate cell activity. (B) In the Non-Canonical Signaling pathway of TGFβ2 signaling, non-SMAD kinase pathways are activated and regulate of TGFβ-mediated functions.

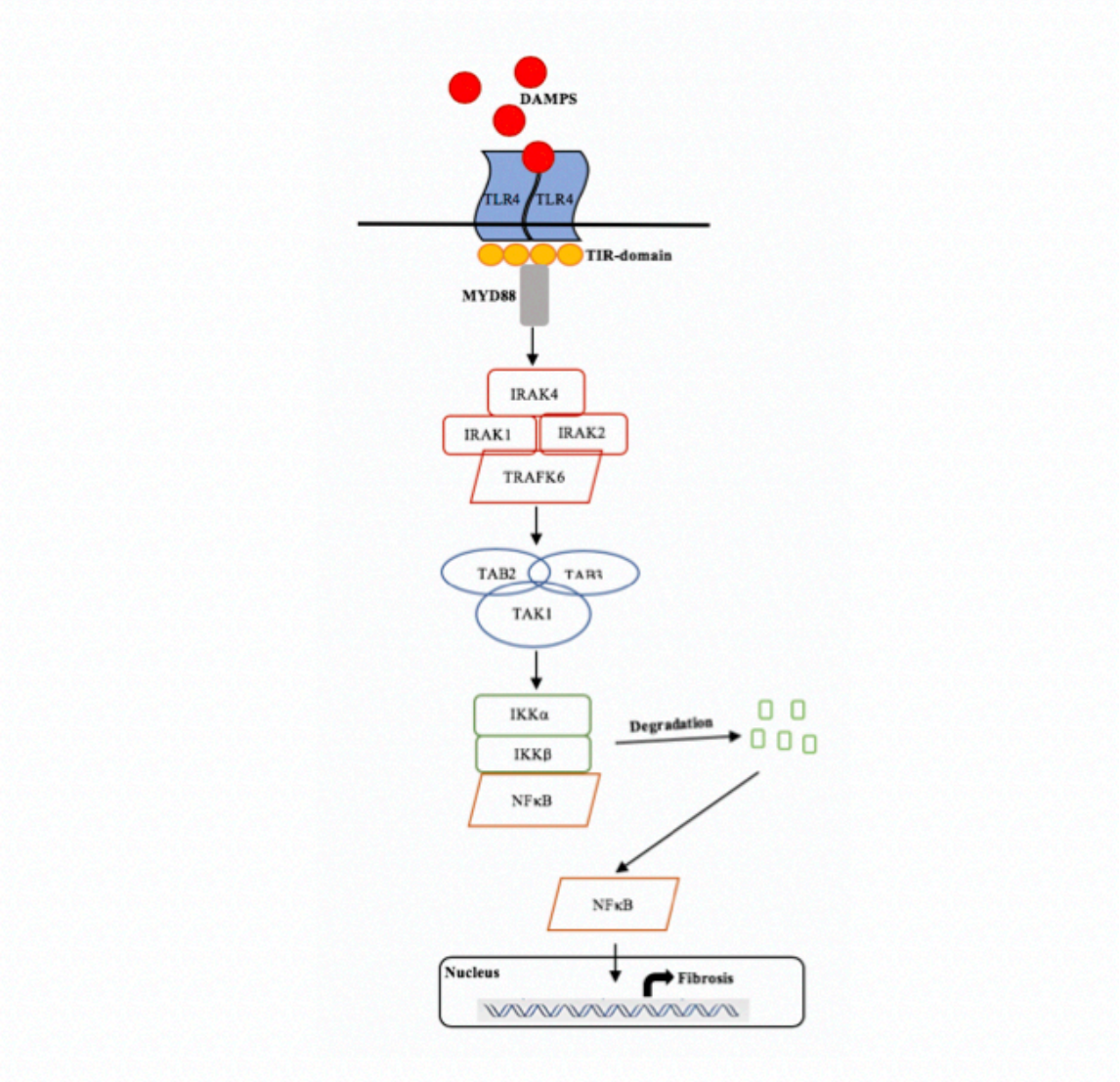

Figure 1.6 TLR4 intercellular signaling pathway: The TLR4 signaling pathway can be activated by DAMPs through activation of the MyD88 independent pathway. The recruitment of IL-1 associated receptor kinases leads to phosphorylation of IκB kinase complex to trigger IκB degradation, freeing of NFκB to translocate into the nucleus and induce a fibrotic response.

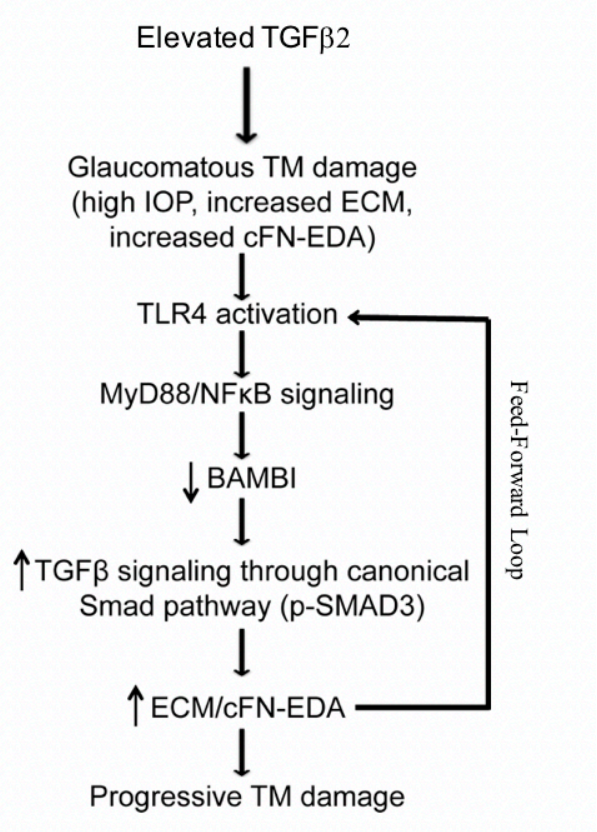

Figure 1.7 Scheme diagram of the proposed pathway of TGFβ2-TLR4 signaling crosstalk in the glaucomatous trabecular meshwork: Elevated TGFβ2 leads to glaucomatous Tm damage which will induce the production of DAMPs such as FN-EDA**.** FN-EDA activates TLR4 and the MyD88-NFκB signaling pathway. This leads to down regulation of BAMBI expression and elevation of TGFβ2 signaling through the canonical SMAD pathway. In turn, elevated ECM deposition and cFN-EDA expression will prolong the progressive TM damage and cFN-EDA will activate TLR4 signaling and continue the cycle of progressive fibrosis and TM damage [(4)]. Modified by Amanda Roberts 2020

CHAPTER II

NUCLEAR FACTOR-KAPPA BETA SIGNALING IS REQUIRED FOR TRANSFORMING GROWTH FACTOR BETA-2 INDUCED OCULAR HYPERTENSION

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Nuclear factor-kappa beta signaling is required for transforming growth factor Beta-2 induced ocular hypertension

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ABSTRACT

A major risk for the development of primary open-angle glaucoma (POAG) is elevated intraocular pressure (IOP). Elevated IOP is caused by increased outflow resistance due in part to excessive extracellular matrix (ECM) deposition in the trabecular meshwork (TM). The role of transforming growth factor beta 2 (TGFβ2) in inducing ECM production is well understood. Recent studies suggest that toll-like receptor 4 (TLR4) plays an important role in fibrogenesis. We have previously described a crosstalk between TGFβ2 and TLR4 in the development of ocular hypertension and glaucomatous TM damage. Nuclear factor-kappa beta (NF-κB) is critical for TLR4 signaling. To determine the transactivation of NF-κB, TM cells were stimulated with cellular fibronectin containing the EDA isoform (cFN-EDA), TGFβ2, or lipopolysaccharide (LPS) in combination with a selective TLR4 inhibitor. cFN-EDA, TGFβ2, and LPS all induced transactivation of NF-κB and inhibition of TLR4 blocked the effect of each treatment paradigm. To evaluate the role of NF-κB in IOP regulation, we utilized our inducible mouse model of ocular hypertension by injection of Ad5.TGFβ2 in mice harboring a mutation in NF-κB and wild-type controls. IOP was measured over time and eyes accessed by immunohistochemistry for the ECM protein FN and the specific FN-EDA isoform. Ad5.TGFβ2 induced ocular hypertension and expression of FN and FN-EDA in wild-type mice, but mutation in NF-κB blocked the effect. These data suggest that NF-κB is necessary for TGFβ2-induced ECM production and ocular hypertension and the transactivation of NF-κB is dependent on both TGFβ2 and TLR4.

The glaucomas are a heterogeneous group of optic neuropathies and intraocular pressure (IOP) is known to be a significant causative risk factor (Quigley, 1993). Aqueous humor (AH) production and drainage from the eye determines and regulates the IOP. The trabecular meshwork (TM) is an important tissue in AH drainage and functions to impart a normal resistance to AH outflow. The ECM composition of the TM plays a major role in the regulation of IOP and it is well known that there are significant changes to the ECM composition of the TM in glaucoma which is associated with increased AH outflow resistance and increased IOP (Lutjen-Drecoll, 1999; Rohen and Witmer, 1972). The regulation of the ECM architecture is significantly influenced by the transforming growth factor beta 2 (TGFβ2) signaling pathway. TGFβ2 levels are significantly elevated in the AH of POAG patients (Inatani et al., 2001; Ochiai and Ochiai, 2002; Ozcan et al., 2004; Tripathi et al., 1994). TGFβ2 treatment of primary and transformed TM cells alters the ECM composition and organization (Fleenor et al., 2006; Fuchshofer et al., 2007; Hernandez et al., 2017; Sethi et al., 2011; Tovar-Vidales et al., 2011; Welge-Lussen et al., 1999; Wordinger et al., 2007). Importantly, we recently demonstrated that over-expression of a bioactivated form of TGFβ2 in mouse eyes causes ocular hypertension, which is dependent on toll-like receptor 4 (TLR4) expression (Hernandez et al., 2017; Shepard et al., 2010).

TLR4 is a member of the toll-like receptor family and was originally identified as the receptor for lipopolysaccharide (LPS) (Poltorak et al., 1998). After LPS binds to TLR4, the LPS-TLR4 complex initiates signal transduction through intracellular adaptor molecules such as myeloid differentiation factor 88 (MyD88), which leads to recruitment of IL-1 receptor–associated kinases and activation of nuclear factor-kappa beta (NF-κB) (Kawai and Akira, 2010). TLR4 can also be activated by endogenous ligands, known as damage-associated molecular patterns (DAMPs) (Miyake, 2007; Piccinini and Midwood, 2010). One such DAMP, known as fibronectin extra domain A (FN-EDA), is an isoform of FN which is upregulated as a response to tissue injury, repair, or remodeling, and during disease states (Ffrench-Constant et al., 1989;

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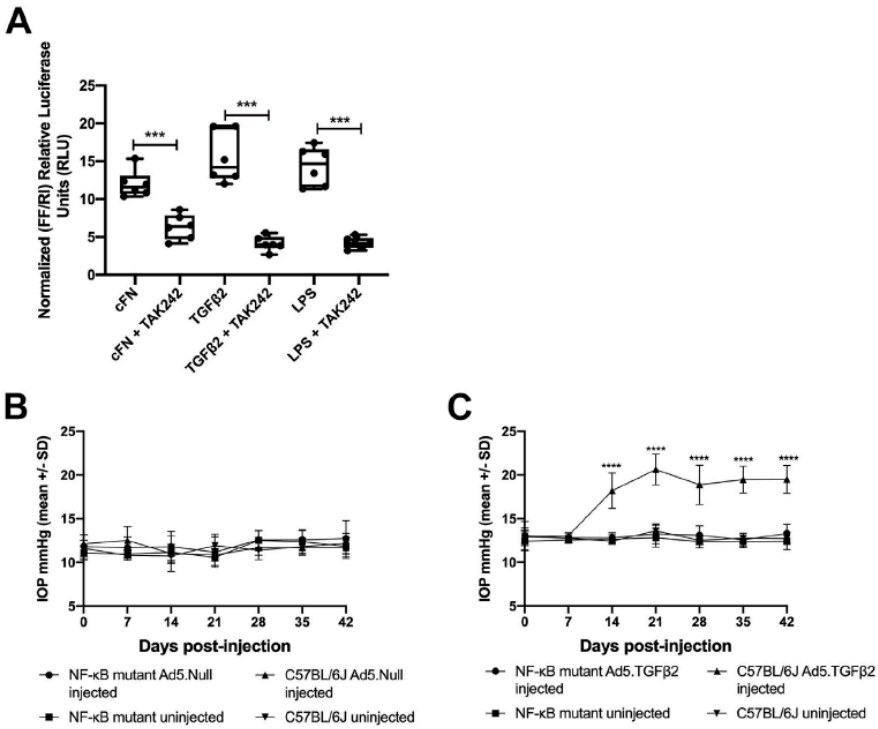

Fig. 1. NF-κB response to TLR4 and TGFβ2 signaling. (A) Immortalized glaucomatous TM cells (GTM3) cells were transfected with 100 ng of NF-κB dual-luciferase reporter (Qiagen) using Lipofectamine 3000 transfection reagent (Invitrogen). Cells were pre-treated with selective TLR4 inhibitor (TAK-242, 15 μM) for 60 min, followed by TGFβ2 (5 ng/mL), TLR4 ligand (cFN-EDA 10 μg/mL) or LPS as a positive control (100 ng/mL) for 24 h. Luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega) and the Infinite M200 (Tecan). Transfection efficiency was normalized to Renilla luciferase activity. Data are represented as normalized relative light units. ***$p < 0.001$, $n = 6$ independent replicates/treatment. (B, C) NF-κB is required for Ad5.TGFβ2-induced ocular hypertension. (B) Ad5.Null had no effect on IOP in C57BL/6 J mice ($n = 5$) or NF-κB mutant mice ($n = 9$). (C) Ad5.TGFβ2 induced elevated IOP starting at 14 days post-injection until day 42 post-injection in wildtype C57BL/6 J mice ($n = 10$). No difference in IOP was detected at any timepoint in NF-κB mutant mice ($n = 10$). Significance was calculated by one-way ANOVA followed by Tukey post hoc analysis at each time point and p values reported are comparison of injected eye with the corresponding uninjected control eye for each treatment, ****$p < 0.0001$.

Hino et al., 1995; Kuhn et al., 1989; Muro et al., 2003). In addition, activation of TLR4 by FN-EDA has been shown to be NF-κB dependent (Kelsh-Lasher et al., 2017; Okamura et al., 2001). DAMP activated TLR4 signaling has been linked to fibrosis and the regulation and production of ECM proteins in hepatic fibrosis, renal fibrosis, lesional skin and lung in scleroderma patients, and most importantly here to TM damage and ocular hypertension (Bhattacharyya et al., 2013; Campbell et al., 2011; Hernandez et al., 2017; Pulskens et al., 2010; Seki et al., 2007). We and others have shown that DAMPs can activate TLR4 and augment TGFβ signaling and downstream fibrotic responses (Bhattacharyya et al., 2013; Guo and Friedman, 2010; Hernandez et al., 2017; Yang and Seki, 2012).

We previously demonstrated a TGFβ2–TLR4 signaling crosstalk that contributes to the development of ocular hypertension (Hernandez et al., 2017, 2018). Our *in-vitro* data show that blocking TLR4 signaling prevents the production of ECM proteins in primary TM cells in culture (Hernandez et al., 2017). In addition, a spontaneous mutation in *Tlr4* inhibited the production of ECM proteins and prevented TGFβ2-induced ocular hypertension in mice (Hernandez et al., 2017). We also demonstrated that the DAMP, FN-EDA, enhances the TGFβ2-induced response in primary TM cells, and inhibition of TLR4 blocks this effect (Hernandez et al., 2017). The exact molecular interaction between DAMPs and TLR4 has yet to be fully elucidated. However, it is known that the DAMP, HMGB1, binds TLR4 and signals through adaptor molecules via the Toll/IL-1 receptor-domain to MyD88, IRAK, TRAF and ultimately to NF-κB (Yang et al., 2010). Similar to HMGB-1, FN-EDA has been shown to activate TLR4 leading to NF-κB activation and induction of downstream responses (Kelsh-Lasher et al., 2017; Okamura et al., 2001). Interestingly, TLR4 activation also downregulates the TGFβ pseudoreceptor BMP and the activin membrane-bound inhibitor (BAMBI). BAMBI normally functions to inhibit TGFβ signaling (Bhattacharyya et al., 2013; Seki et al., 2007; Yan et al., 2009). BAMBI downregulation by TLR4 is regulated by the NF-κB-dependent pathway (Guo and Friedman, 2010; Seki et al., 2007; Yang and Seki, 2012). We have also shown that knockdown of BAMBI induces ocular hypertension in mice (Hernandez et al., 2018). Together, these data demonstrate that the TLR4 signaling pathway is an important regulator of IOP homeostasis. Since TLR4 signaling ultimately leads to NF-κB activation, we hypothesized that NF-κB would be important in the development of ocular hypertension.

NF-κB resides in the cytoplasm in its inactive form and is composed by the heteromeric complex of p50, p65, and the inhibitor of the nuclear factor kappa B family of proteins (IκB alpha) (Fraczek et al., 2013; Phan et al., 2015; Strnad and Burke, 2007). Activation of TLR4 leads to proteasomal degradation of I Kappa Beta (IκB), release and exposure of the nuclear localization signal of RelA-p50 (NF-κB), and translocation to the nucleus. To test the hypothesis that NF-κB plays a role in TGFβ2-TLR4 crosstalk in the TM, transactivation studies were carried out on

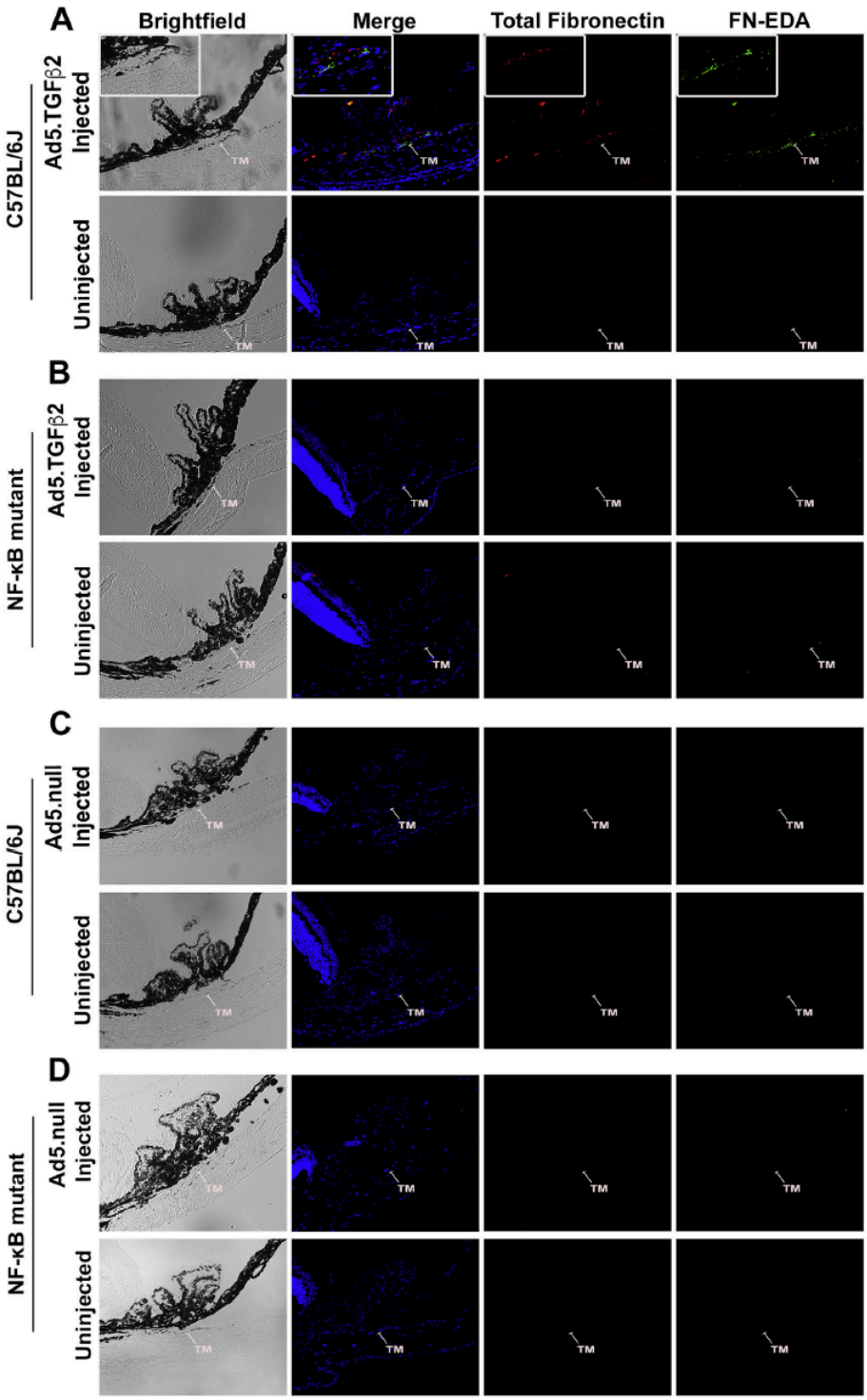

Fig. 2. NF-κB is required for Ad5.TGFβ2-induced fibronectin production. (A) Ad5.TGFβ2 induced expression of both FN and FN-EDA in the TM at 42-days post-injection in C57BL/6 J mice (n = 10), inset shows TM region or each image. (B). Ad5.TGFβ2 had no effect on FN or FN-EDA expression in NF-κB mutant mice (n = 10). (C, D) Ad5.null had no effect on FN or FN-EDA expression in C57BL/6 J (n = 5) mice or NF-κB mutant mice (n = 9).

transformed TM cells (GTM3). The transformed GTM3 cell line has previously been described (Pang et al., 1994). Since primary TM cells are difficult to transfect (Stamer and Clark, 2017), GTM3 cells were used in this study. Cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA) and supplemented with penicillin (100 units/mL), streptomycin (0.1 mg/mL), and L-glutamine (0.292 mg/mL) (Thermo Fisher Scientific, Rockford, IL, USA). Briefly, GTM3 were plated at a density of 1×10^5 per mL and transfected with the NF-κB luciferase reporter vector constitutively expressing *Renilla* luciferase vector or negative control vector (Quigen, Germantown, MD, USA) and the Dual-Glo Luciferase Assay System (Promega, Madison, Wisconsin, USA). Cells underwent transfection with serum free media overnight according to the manufacturer's instructions. Cells were treated with TAK-242 (CLI-095; InvivoGen, San Diego, CA, USA) at 15 μM for 60 min, followed by treatment with cFN-EDA (F2518; Sigma-Aldrich Corp., St. Louis, MO, USA) or LPS (100 ng/mL) (Sigma-Aldrich Corp., St. Louis, MO, USA). Interestingly, cFN-EDA, TGFβ2, and LPS all increased the activity of NF-κB, and pretreatment with the TLR4 inhibitor, TAK-242, blocked the effect of each individual treatment (Fig. 1A). No signal was detected from the negative control vector (data not shown). These data suggest that cFN-EDA, TGFβ2, and LPS activate NF-κB, NF-κB transactivation is mediated by the TLR4 pathway, and TGFβ2 signaling likely leads to the expression of TLR4 ligands which initiate downstream transactivation of NF-κB.

To further test the role of NF-κB in TGFβ2-induced ocular hypertension, we employed our established mouse model of ocular hypertension using Adenovirus 5 (Ad5) viral vector expressing human $TGFβ^{c226s/c228s}$ (hereafter referred to as Ad5.TGFβ2) (University of Iowa Vector Core, Iowa City, IA, USA) (Hernandez et al., 2017; Shepard et al., 2010). All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee Guidelines and Regulations. C57BL/6J and B6.Cg-$NFκB1^{TM1Bal}$/J (referred to as NF-κB mutant mice hereafter) mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were 5–8 months old at the start of the experiment. Ad5.TGFβ2 was used to overexpress TGFβ2 as previously described (Hernandez et al., 2017; Shepard et al., 2010). Adenovirus 5.Null vector (Vector Biolabs, Malvern, PA, USA) was used as a negative control (Fig. 1B). Briefly, 2 μL of 2.5×10^7 plaque-forming units (pfu) was intravitreally injected into one eye, and the contralateral eyes were used as negative controls. To evaluate the role of NF-κB on the development of ocular hypertension, intraocular measurements were performed using a rebound tonometer on isoflurane anesthetized mice as previously described (Hernandez et al., 2018). As expected, we observed that C57BL/6 J wild-type mice develop ocular hypertension starting at 14 days post-injection and remain significantly elevated throughout the 42-day time course, ($n = 10$, $p < 0.0001$ days 14–42) (Fig. 1C). However, the NF-κB mutant mice ($n = 10$) did not develop ocular hypertension at any time point after injection with Ad5.TGFβ2 (Fig. 1C). Ad5.Null had no effect in either mouse strain ($n = 5$ C57BL/6 J, $n = 9$ NF-κB mutant mice) (Fig. 1B). These data suggest that NF-κB is an important regulator of TGFβ2-induced ocular hypertension.

We further analyzed the TM of Ad5.TGFβ2 injected and Ad5.null injected C57BL6/J mice and NF-κB mutant mice at the end of the 42-day time course. We have previously demonstrated that TGFβ2 induces ECM changes to the TM both *in vivo* and *in vitro* (Hernandez et al., 2017, 2018; McDowell et al., 2013, 2015). Here, we investigated whether Ad5.TGFβ2 is able to induce the expression of total FN and FN-EDA isoform in the TM of both wildtype and NF-κB mutant mice by immunohistochemistry (Fig. 2A and B). Eyes were enucleated and fixed in 4% paraformaldehyde for 24 h, processed and embedded in paraffin. Five micrometer sections were cut, and sections were transferred to glass slides. Paraffin sections were dewaxed 2 times in xylene, 100% ethanol, and 95% ethanol for 2 min each. Slides were then soaked in PBS for 5 min. Sections were labeled with primary rabbit-anti–cFN-EDA antibody (1:100, NBP1-91258; Novus Biologicals) and mouse-anti-FN antibody (1:100, clone IST-4; Sigma-Aldrich), followed by secondary antibodies Alexa Fluor–labeled 488 donkey anti-rabbit Ig (1:500, Life Technologies) and Alexa Fluor-labeled 594 donkey anti-mouse Ig (1:500, Life Technologies). The mouse–anti-FN antibody recognizes epitopes on the N-terminus of FN, which are expressed by all FN isoforms. As expected, Ad5.TGFβ2 was able to induce the expression of FN-EDA isoform and total FN in C57BL/6 J mice (Fig. 2A). Similar to the IOP phenotype, Ad5.TGFβ2 did not induce the expression of either total FN or FN-EDA isoform in the NF-κB mutant mice (Fig. 2B). Ad5.null had no effect on FN or FN-EDA expression in C57BL/6 J mice or NF-κB mutant mice (Fig. 2C and D). These data suggest that NF-κB activation may be the critical step involved in generating the TGFβ2-TLR4 induced ECM changes in the TM.

In the present study, we provide a novel role for NF-κB in the development of ocular hypertension. We have previously described a TGFβ2–TLR4 crosstalk mechanism that plays an important role in the development of ocular hypertension. Here, we show that NF-κB is necessary for TGFβ2-induced ECM production and ocular hypertension. Further, the transactivation of NF-κB is dependent on both TGFβ2 and TLR4. The molecular mechanisms by which NF-κB upregulates the ECM is not fully understood, but our data suggests it is important for development of ocular hypertension and ECM changes in the TM. These data describe a novel molecular pathway that could be used to identify innovative therapeutic targets to lower IOP and prevent glaucomatous TM damage.

Declaration of competing interest

H. Hernandez, None; **A.L. Roberts**, None; **C.M. McDowell**, None. All authors have approved this final manuscript as being true in representation of the experimental findings.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2020.107920.

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CHAPTER III

FIBRONECTIN EXTRA DOMAIN A (FN-EDA) ELEVATES INTRAOCULAR PRESSURE THROUGH TOLL-LIKE RECEPTOR 4 SIGNALING

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Fibronectin extra domain A (FN-EDA) elevates intraocular pressure through Toll-like receptor 4 signaling

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Elevated intraocular pressure (IOP) is a major risk factor for the development and progression of primary open angle glaucoma and is due to trabecular meshwork (TM) damage, which leads to impaired aqueous humor outflow. Here, we explore a novel molecular mechanism involved in glaucomatous TM damage. We investigated the role of an endogenous Toll-like receptor 4 (TLR4) ligand, fibronectin-EDA (FN-EDA), in TGFβ2-induced ocular hypertension in mice. We utilized transgenic mouse strains that either constitutively express only FN containing the EDA isoform or contain an EDA-null allele and express only FN lacking EDA, with or without a mutation in *Tlr4*, in our inducible mouse model of ocular hypertension by injection of Ad5.TGFβ2. IOP was measured over time and eyes accessed by immunohistochemistry for total FN and FN-EDA expression. Constitutively active EDA caused elevated IOP starting at 14 weeks of age. Ad5.TGFβ2 induced ocular hypertension in wildtype C57BL/6J mice and further amplified the IOP in constitutively active EDA mice. TLR4 null and EDA null mice blocked Ad5.TGFβ-induced ocular hypertension. Total FN and FN-EDA isoform expression increased in response to Ad5.TGFβ2. These data suggest that both TLR4 and FN-EDA contribute to TGFβ2 induced ocular hypertension.

Glaucoma is a heterogeneous group of optic neuropathies with progressive degeneration of the optic nerve leading to vision loss and irreversible blindness[1]. The prevalence of individuals diagnosed with glaucoma worldwide by 2020 is predicted to be over 80 million and by 2040 over 112 million[2]. Glaucoma is characterized by cupping of the optic disc, death of retinal ganglion cells, and optic nerve degeneration[1]. Primary open angle glaucoma (POAG) is the most common form of glaucoma[3] and elevated intraocular pressure (IOP) is the most significant causative risk factor for glaucoma[4]. IOP is the fluid pressure inside the eye that is regulated by the production of aqueous humor in the ciliary body and drainage of aqueous humor by the trabecular meshwork (TM) and uveoscleral outflow[5,6]. As a result of the aqueous humor primarily passing through the conventional TM outflow pathway, the TM is the major regulator of IOP. The TM is a biomechano-sensitive tissue located at the junction of the iris and cornea. The TM is composed of a series of fenestrated beams and sheets of extracellular matrix (ECM) covered with endothelial-like TM cells[7,8]. Of the three regions of the TM (uveal, corneoscleral, and cribiform), the cribiform region and the inner wall of the Schlemm's canal, is the major site of aqueous humor outflow resistance[7]. Overall, the ECM composition of the TM is important in regulating aqueous humor outflow and forming a fluid flow pathway for proper aqueous humor drainage[9,10]. It is also known that POAG patients have an increased accumulation of ECM proteins within the TM[11,12], increased ECM production leads to an increase in aqueous humor (AH) outflow resistance[13], decreases AH outflow facility[14–18], and causes ocular hypertension[13,15,19,20].

Although the pathology of the disease is well studied, many of the current drug therapies used to lower elevated IOP focus on suppressing the aqueous humor formation and enhancing uveoscleral outflow; however, these particular therapies do not target the molecular pathology of the disease at the TM. Many of these therapies are also not uniformly effective, can progressively lose efficacy, and only slow vision loss progression[21]. Recently, new therapies have begun to target the TM and the underlying pathology such as the Rho kinase/norepinephrine transporter inhibitor netarsudil[22]. However, there is a still a need to identify additional novel molecular

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mechanisms responsible for glaucomatous damage in which a drug therapy can target the pathology of the disease to lower elevated IOP and prevent further TM damage.

The transforming growth factor beta 2 (TGFβ2) signaling pathway has been well studied in the TM and it is known to be elevated in the aqueous humor and TM tissue of glaucomatous eyes[23–26]. TGFβ2 has also been shown to mediate fibrosis development and ECM deposition within the TM, and induce ocular hypertension in both mice and in *ex vivo* perfusion organ culture systems[13,15,20,27,28]. We and others have also shown that TGFβ2 signals through both the canonical SMAD pathway, alters the ECM in human TM cells[29–35], is essential for TGFβ2-induced ocular hypertension in mice[36], and has the ability to crosstalk with TLR4 signaling[13,15].

TGFβ2 signaling increases the production of ECM proteins, including fibronectin (FN). We and others have identified FN, a dimeric multidomain ECM glycoprotein, to be elevated in glaucomatous TM tissues and aqueous humor[15,18,28,37]. FN functions as a regulator of cellular processes, directs and maintains tissue organization and ECM composition, directs ECM-ECM and ECM-cell interactions, and regulates activity of growth factors and proteins associated with ECM remodeling. The multi-domain dimer is composed of type I, type II, and type III domains with over 20 alternatively spliced isoforms. FN is composed of either cellular FN (cFN) or plasma FN (pFN) isoforms. cFN has multiple isoforms generated by alternative processing of a single primary transcript at 3 domains: extra domain A (EDA), extra domain B (EDB), and the type III homologies connecting segment[38]. During embryonic development, the fibronectin EDA (FN-EDA) isoform is abundant[39]; however, in adults the presence of FN-EDA is minimal and primarily functions as a structural scaffold and signaling molecule that regulates cell adhesion, proliferation, and migration[40]. In addition, the expression of FN-EDA is upregulated as a response to tissue injury, repair, or remodeling[41], and during disease states such as epithelial fibrosis[42], wound healing[43], and rheumatoid arthritis[44]. Importantly, FN-EDA isoform is elevated in glaucomatous trabecular meshwork tissue compared to normal trabecular meshwork tissue[12] and amplifies the response of TGFβ2 in primary TM cells in culture[15].

Recently, we discovered that FN-EDA enhances the TGFβ2-induced ECM response in primary TM cells, and this effect can be blocked by inhibition of toll-like receptor 4 (TLR4) signaling[15]. TLR4 is a member of the TLR family of proteins. Historically, TLR4 was first identified as the receptor for lipopolysaccharide (LPS). It is now known that TLR4 can also be activated by damage associated molecular patterns (DAMPs) as a result of tissue damage, cell injury, or ECM remodeling in other diseases[45–47]. FN-EDA is a known DAMP and activator of TLR4[48]. Our data suggests that TGFβ2 signaling increases ECM production, including production of FN-EDA, leading to activation of TLR4, and increased IOP. Activation of TLR4 downregulates the TGFβ2 antagonist, BMP and activin membrane bound inhibitor (BAMBI), leading to uninhibited TGFβ2 signaling, and a continuation of a pathogenic feed forward loop[45]. These data suggest a TGFβ2-TLR4 signaling crosstalk in the development of glaucomatous TM damage. Here, we identify the importance of FN-EDA in the development of ocular hypertension using transgenic mice that either constitutively express the EDA isoform or contain an EDA null copy, with or without knockdown of *Tlr4*.

Results

Anterior segment anatomy of transgenic mice. Previously, Chauhan and colleagues generated and characterized several EDA and TLR4 transgenic mouse strains used here: B6.EDA$^{-/-}$ (EDA null), B6.TL4$^{-/-}$ (TLR4 null), B6.EDA$^{+/+}$ (constitutively active EDA), B6.EDA$^{-/-}$/TLR4$^{-/-}$, and B6.EDA$^{+/+}$/TLR4$^{-/-}$[49,50]. To determine whether there are any gross anatomical changes to the eye of these transgenic mice, we performed a clinical slit-lamp exam comparing each strain to C57BL/6J controls. Both frontal and lateral images were taken of the exterior eye globe at 15, 30, and 60 days post-natal (Supplemental Fig. 1). Irises of all mouse strains at each age group appear densely pigmented with a complex morphology, as previously described for wildtype C57BL/6J mice[50]. The pupils are round, corneas clear, and no obvious clinically observed anterior segment morphometric abnormalities. In addition, histological sections and H&E analysis was performed on the same mice at each time point (Supplemental Fig. 2). The iridocorneal angles are open and TM morphology appears normal for all strains and ages analyzed. Our data suggests that there are no anatomical differences in the anterior segment of the mouse eyes between strains and there no obvious developmental morphometric abnormalities in B6.EDA$^{-/-}$, B6.TL4$^{-/-}$, B6.EDA$^{+/+}$, B6.EDA$^{-/-}$ /TLR4$^{-/-}$, or B6.EDA$^{+/+}$/TLR4$^{-/-}$ mice. We also characterized the TM of each mouse strain at 60 days of age (Fig. 1). All mouse strains showed a defined TM and Schlemm's canal by H&E staining. The TM of all mouse strains stained positive for alpha-SMA, a known protein highly expressed in TM cells, and collagen-1, a known ECM marker in the TM. These data suggest that the TM in all the mouse strains analyzed is present and anatomically normal.

Constitutively active FN-EDA causes elevated IOP in mice. Clinical and histological analysis of the transgenic EDA and TLR4 strains demonstrated normal TM development. However, when comparing baseline IOP readings from each mouse strain (C57BL/6J, B6.EDA$^{-/-}$, B6.EDA$^{-/-}$ /TLR4$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$, B6.TL4$^{-/-}$, and B6.EDA$^{+/+}$) at 5 months of age, IOP in B6.EDA$^{+/+}$ is significantly elevated compared to each of the other strains, including B6.EDA$^{+/+}$/TLR4$^{-/-}$ (Fig. 2A). These data suggest that EDA can cause elevated IOP and it is TLR4 dependent. To further analyze the effect of EDA on IOP, we measured IOP in C57BL/6J and B6.EDA$^{+/+}$ mice starting at age 8 weeks through 32 weeks of age (Fig. 2B). Significant IOP elevation began at 14 weeks of age in B6.EDA$^{+/+}$ mice compared to C57BL/6J controls and continued through 32 weeks of age. As expected, C57BL/6J mice maintained a normal IOP throughout the 32 weeks as previously reported[15]. These data demonstrate that although the TM develops normally in EDA$^{+/+}$ mice, and EDA$^{+/+}$ mice at 8 weeks of age have a normal IOP, constitutively active EDA causes elevated IOP to develop over time.

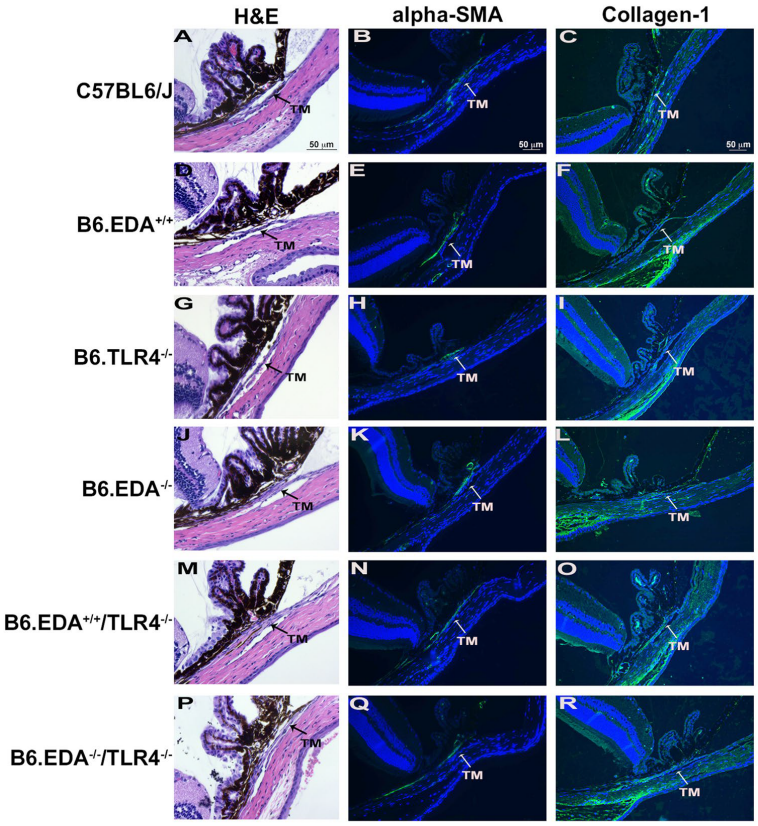

Figure 1. Histological exam of trabecular meshwork of EDA and TLR4 transgenic mice. (**A,D,G,J,M,P**) H&E staining of histological sections from C57BL/6J, B6.EDA$^{+/+}$ B6.TL4$^{-/-}$, B6.EDA$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$, and B6.EDA$^{-/-}$ /TLR4$^{-/-}$ mice. (**B, E, H, K, N, Q**) Alpha-SMA labeling of the TM in C57BL/6J, B6.EDA$^{+/+}$ B6.TL4$^{-/-}$, B6.EDA$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$, and B6.EDA$^{-/-}$ /TLR4$^{-/-}$ mice. (**C,F,I,L,O,R**) Collagen-1 labeling of the TM in C57BL/6J, B6.EDA$^{+/+}$ B6.TL4$^{-/-}$, B6.EDA$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$, and B6.EDA$^{-/-}$ /TLR4$^{-/-}$ mice. All animals were 60 days old at time of analysis, n = 5 mice/strain. All images taken at 200x magnification.

FN-EDA and TLR4 are necessary for TGFβ2 induced ocular hypertension. To test the crosstalk between TGFβ2 and TLR4, we utilized our established mouse model of ocular hypertension using Ad5.TGFβ2 virus, which contains a bioactivated form of TGFβ2[15,19,36,51]. Ad5.TGFβ2 was injected intravitreally into one eye of each animal and the contralateral uninjected eye was used as a negative control. In order to determine whether FN-EDA and TLR4 are necessary for Ad5.TGFβ2-induced ocular hypertension phenotype, we tested wildtype C57BL/6J ($n = 17$), B6.TLR4$^{-/-}$ mice (n = 8), B6.EDA$^{+/+}$ mice (8), B6.EDA$^{-/-}$ (n = 18), B6.EDA$^{-/-}$ /TLR4$^{-/-}$ (n = 23) and B6.EDA$^{+/+}$/ TLR4$^{-/-}$ (n = 16) mice in our model system (Fig. 3A). As expected, C57BL/6J mice eyes injected with Ad5.TGFβ2 developed ocular hypertension compared to their contralateral uninjected eye[15,51]. Ad5.TGFβ2 also significantly elevated IOP in B6.EDA$^{+/+}$ mice compared to their contralateral uninjected eye from days 7-42 post-injection. In addition, the IOP in Ad5.TGFβ2 injected eyes from B6.EDA$^{+/+}$ mice are significantly elevated compared to Ad5.TGFβ2 injected eyes in wildtype C57BL/6J mice from days 0-21 post-injection, demonstrating an enhanced effect in these mice. Similar to the data in Fig. 2, uninjected B6.EDA$^{+/+}$ eyes have significant IOP elevation compared to uninjected C57BL/6J eyes. Previously, we reported that C3H/HeJ mice harboring a spontaneous mutation in *Tlr4* are also resistant to TGFβ2-induced ocular hypertension[15]. Here, we recapitulate this data on the C57BL/6 J (B6) genetic background in B6.TLR4$^{-/-}$ mice which had no significant IOP changes in response to Ad5.TGFβ2 injection, and in B6.EDA$^{+/+}$/TLR4$^{-/-}$ mice as mutation in *Tlr4* blocked EDA and TGFβ2-induced IOP elevation. Mutation in EDA$^{-/-}$ also blocked TGFβ2-induced ocular hypertension compared with C57BL/6J controls and uninjected control eyes. In addition, similar to our previous reports Ad5. Null virus has no effect on IOP in any mouse strain (Fig. 3B)[15]. These data suggest that FN-EDA and TLR4 are both necessary for TGFβ2-induced ocular hypertension.

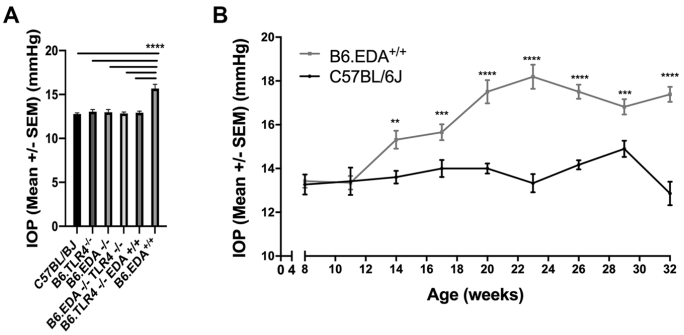

Figure 2. Constitutively active FN-EDA causes elevated IOP in mice: **(A)** IOP at 5 months of age in C57BL/6J (n = 17), B6.TL4$^{-/-}$ (n = 8), B6.EDA$^{-/-}$ (n = 18), B6.EDA$^{-/-}$ /TLR4$^{-/-}$ (n = 23), B6.EDA$^{+/+}$/TLR4$^{-/-}$ (n = 16), and B6.EDA$^{+/+}$ (n = 8) mice. B6.EDA$^{+/+}$ mice had significantly elevated IOP compared to each of the other strains. Statistical significance determined by one-way ANOVA followed by Tukey analysis. **(B)** IOP from 8 weeks of age to 32 weeks of age in C57BL/6J and B6.EDA$^{+/+}$ mice. There was no significant difference in IOP between C57BL/6J and B6.EDA$^{+/+}$ mice at 8–12 weeks of age. At 14 weeks of age, ocular hypertension developed in B6.EDA$^{+/+}$ mice and remained elevated through 32 weeks of age compared to C57BL/6J mice. Statistical significance determined by Student's t-test at each time point. At least n = 6–20 eyes (3–10 mice) were measured at each time point. ****P < 0.0001, ***P < 0.001, **P < 0.05.

TGFβ2 increases FN and FN-EDA expression in the trabecular meshwork. Next, we explored the effect of TGFβ2 on FN and FN-EDA expression in the TM of each of the mouse strains (Fig. 4, Supplemental Figs. 3 and 4). Ad5.TGFβ2 significantly increased both FN levels and FN-EDA levels in the TM of C57BL/6J mice (Fig. 4A,D,M,N). B6.EDA$^{+/+}$ mice also expressed the FN-EDA isoform in the TM of uninjected naïve eyes, and TGFβ2 further increased the expression of FN and EDA (Fig. 4B,E,O,P). TGFβ2 had no effect on FN or FN-EDA expression in the TM of B6.TLR4$^{-/-}$ mice, demonstrating again that TLR4 is necessary for TGFβ2-induced TM damage (Fig. 4C,F). Trace amounts of FN and FN-EDA were evident in the TM of B6.EDA$^{+/+}$/ TLR4$^{-/-}$ mice (Fig. 4G,J), but there was no difference between TGFβ2-injected and control eyes. Correlating with the IOP data, TGFβ2 also had no effect on the expression of FN or FN-EDA in the TM of Ad5.TGFβ2-injected and uninjected eyes of B6.EDA$^{-/-}$, B6.EDA$^{-/-}$ /TLR4$^{-/-}$, and B6.TL4$^{-/-}$ mice (Fig. 4C,F,H,K,I,L).

Discussion

Here we identified FN-EDA as a necessary molecule in pathogenic TGFβ2-TLR4 signaling cross-talk in the development of ocular hypertension in mice. Previously we showed that FN containing the EDA isoform can increase ECM production and enhance the effects of TGFβ2 in primary human TM cells in culture, and this effect could be blocked by inhibition of TLR4[15]. We also demonstrated that mutation in *Tlr4* blocked TGFβ2-induced ocular hypertension in mice[15]. We further expanded on this hypothesis here and demonstrated that exclusion of the EDA exon in B6.EDA$^{-/-}$ mice blocked TGFβ2 induced ocular hypertension and constitutive inclusion of the EDA exon in B6.EDA +/+ caused ocular hypertension and further exacerbated the effect of TGFβ2. Importantly, the effect of EDA$^{+/+}$ on ocular hypertension was dependent on TLR4. These data highlight a novel role of EDA in ocular hypertension and provides a new therapeutic target to lower IOP that is relevant to the pathology of glaucomatous TM damage.

TLR4 is a member of the TLR family of the innate immune system. Recent evidence suggests that endogenous ligands of cell compartments and matrix can activate TLR4, a phenomenon that can occur as a result of tissue damage or extracellular matrix remodeling[45–47]. These ligands, also known as damage associated molecular patterns (DAMPs), have the potential to cause a cellular response. Endogenous DAMPs include FN-EDA, HMGB-1, and low molecular weight hyaluronic acid amongst others. Here we recapitulate our earlier report that TLR4 is necessary for TGFβ2-induced ocular hypertension[15], and further demonstrate FN-EDA is necessary and dependent on TLR4 for TGFβ2-induced ocular hypertension.

TLR4 is a relevant pathway to study in the context of glaucoma. Several polymorphisms have been identified in *TLR4* in human SNP studies of glaucoma patients. Shibuya el al identified multiple SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, and rs7037117) in the *TLR4* gene associated with the risk of normal tension glaucoma (NTG)[52]. In addition, Takano el al identified in Japanese individuals with POAG, NTG, and exfoliation glaucoma (XFG), the allele frequency of rs2149356 was the most significant. Further, the SNPs at rs10759930, rs1927914, rs1927911, and rs2149356 were all significantly higher in the glaucoma groups compared to the control group[53]. Navarro-Partida el al evaluated SNPs Asp299Gly (rs4986790 A/G) and Thr399Ile (rs4986791 C/T) in Mexican patients with POAG compared to controls and found that the *TLR4* coding SNPs Asp299Gly and Thr399Ile was significantly higher in the POAG patients, suggesting that there is a genetic susceptibility alleles for POAG in the Mexican population[54]. However, these results may be population specific as another report of a NTG Korean population demonstrated no statistical significance difference between the NTG patients and controls for

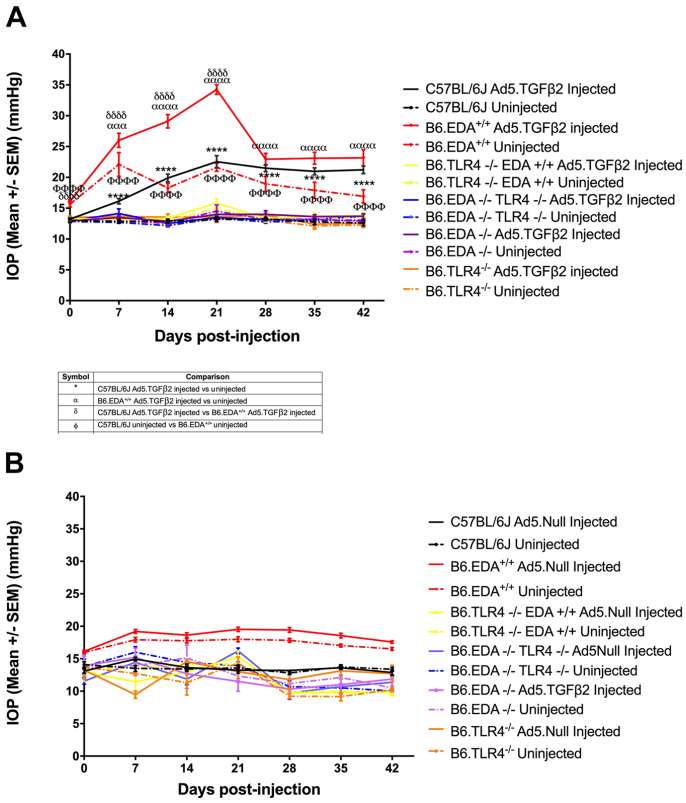

Figure 3. FN-EDA and TLR4 are necessary for TGFβ2-induced ocular hypertension: Ad5.TGFβ2 significantly elevated IOP in C57BL/6J mice (16.1 +/− 0.4 mmHg) and B6.EDA$^{+/+}$ mice (26.0 +/− 1.1 mmHg) compared to their respective uninjected contralateral eyes (C57BL/6J: 12.8 +/− 0.3 mmHg; B6.EDA$^{+/+}$: 22.1 +/− 1.9 mmHg) starting at 7-days post injection and remained significant through 42 days post-injection (C57BL/6J injected: 22.2 +/− 0.6 mmHg, C57BL/6J uninjected: 12.6 +/− 0.5 mmHg; B6.EDA$^{+/+}$ injected: 23.2 +/− 1.3 mmHg, B6.EDA$^{+/+}$ uninjected 16.9 +/− 1.1 mmHg). Ad5.TGFβ2 elevated IOP in B6.EDA$^{+/+}$ mice significantly above the IOP in Ad5.TGFβ2 injected C57BL/6J mice through 21 days post-injection (C57BL/6J injected: 22.5 +/− 1.0 mmHg; B6.EDA$^{+/+}$ injected: 34.3 +/− 0.8 mmHg). C57BL/6J uninjected eyes compared to B6.EDA$^{+/+}$ uninjected eyes were significantly different throughout the time course. Ad5.TGFβ2 had no effect on B6.TLR4$^{-/-}$, B6.EDA$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$, or B6.EDA$^{-/-}$ /TLR4$^{-/-}$ mice at any time point. Average IOP at day 42 for B6.TLR4$^{-/-}$ 12.6 +/− 0.3 mmHg injected, 12.3 +/− 0.4 mmHg uninjected; B6.EDA$^{-/-}$ 13.7 +/− 0.4 mmHg injected, 12.9 +/− 0.2 mmHg uninjected; B6.EDA$^{+/+}$/TLR4$^{-/-}$ 13.8 +/− 0.5 mmHg injected, 13.5 +/− 0.3 mmHg uninjected; B6.EDA$^{-/-}$/TLR4$^{-/-}$ 13.7 +/− 0.3 mmHg injected, 13.1 +/− 0.2 mmHg uninjected. C57BL/6J ($n = 17$), B6.EDA$^{-/-}$ (n = 18), B6.EDA$^{+/+}$ mice (n = 8), B6.TLR4$^{-/-}$ mice (n = 8), B6.EDA$^{-/-}$ / TLR4$^{-/-}$ (n = 21–23) and B6.EDA$^{+/+}$/ TLR4$^{-/-}$ (n = 16). Statistical significance determined by one-way ANOVA followed by Tukey analysis at each time point, *, α, δ, ϕ, ω all indicate significance of at least $P < 0.001$. All values represent mean +/− SEM. **(B)** Ad5.Null had no effect on IOP in C57BL/6J, B6.EDA$^{+/+}$ B6.TL4$^{-/-}$, B6.EDA$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$, or B6.EDA$^{-/-}$ /TLR4$^{-/-}$ mice compared to the contralateral uninjected eye at any time point. N = 3-5 mice/strain.

SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953) in the *TLR4* gene[55]. And, Abu-Amero and colleagues evaluated the SNP at s4986790 in the *TLR4* gene of Saudi POAG patients, and found no statistical difference compared to controls[56]. Importantly, the role of TLR4 in fibrogenesis has also been identified and confirmed by specific SNP alleles in *TLR4* being associated with a delayed progression of fibrosis in liver disease and conferring an overall protective effect[57,58].

Fibronectin is an extracellular glycoprotein that is elevated in the aqueous humor and glaucomatous TM tissues[18,37,38]. Fibronectin provides structural support, signaling and regulates growth factors involved in ECM remodeling. Interestingly, fibronectin can bind to itself, other ECM molecules, growth factors, and receptors.

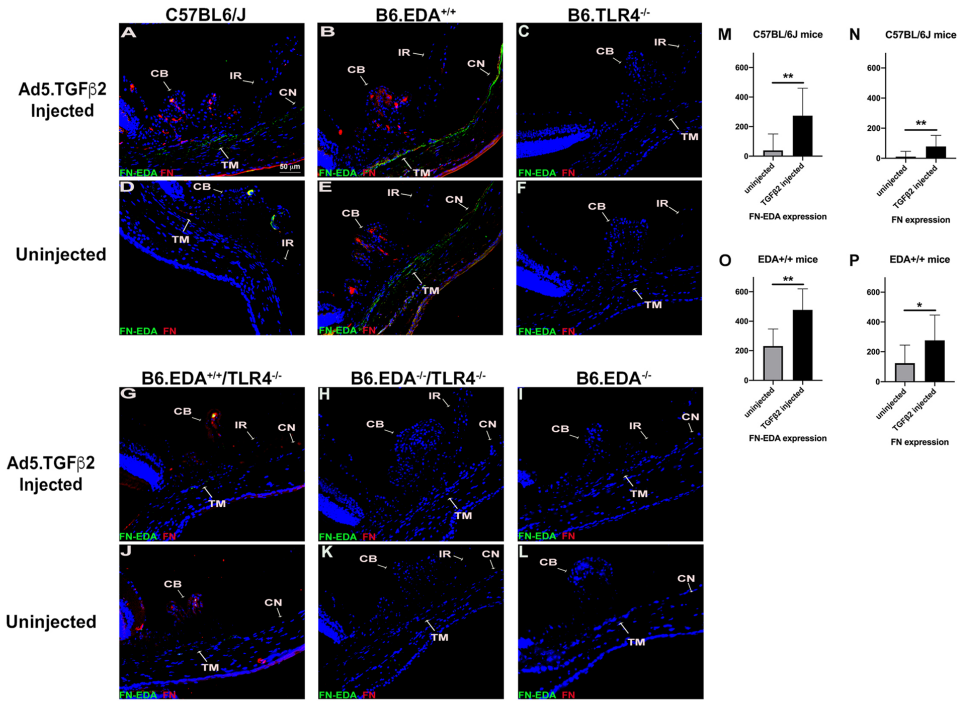

Figure 4. TGFβ2 induces FN and FN-EDA expression in C57BL/6J and EDA + / + mice. All analyses were performed 6-7 weeks post-injection of Ad5.TGFβ2. **(A,D)** Ad5.TGFβ2 increased both FN and FN-EDA expression in the TM of C57BL/6J mice. **(B,E)** Ad5.TGFβ2 increased both FN and FN-EDA expression in the TM of B6.EDA$^{+/+}$ mice. **(C,F)** Ad5.TGFβ2 had no effect on FN or FN-EDA expression in the TM of B6.TLR4$^{-/-}$ mice. **(G,J)** Trace amounts of FN-EDA and FN was detected in B6.EDA$^{+/+}$/TLR4$^{-/-}$ mice, no difference was observed between Ad5.TGFβ2 injected and uninjected eyes. **(H,I,K,L)** No detectable FN-EDA expression was observed in B6.EDA$^{-/-}$ or B6.EDA$^{-/-}$ /TLR4$^{-/-}$ mice, and no difference was observed between Ad5.TGFβ2 injected and uninjected eyes. **(M,N)** FN and FN-EDA expression quantified by ImageJ analysis for mean intensity/area in the TM of C57BL/6J mice. **(O,P)** FN and FN-EDA expression quantified by ImageJ analysis for mean intensity/area in the TM of B6.EDA$^{+/+}$ mice. C57BL/6J mice (n = 11), B6.EDA$^{+/+}$ mice (n = 6), B6.TLR4$^{-/-}$ mice (n = 8), B6.EDA$^{-/-}$ (n = 18), B6.EDA$^{-/-}$ /TLR4$^{-/-}$ (n = 22) and B6.EDA$^{+/+}$/ TLR4$^{-/-}$ (n = 16). Statistical significance determined by Student's paired t-test *P < 0.05.

Cellular FN containing the EDA domain has been shown to play important roles in tissue damage[44,59–61] and fibrogenesis[41,62–65]. Medina et al. showed that human normal TM and glaucomatous TM cells and tissues express the cFN isoforms (EDA and EDB) and that the EDA isoform is elevated in the glaucomatous TM tissue[66]. EDA expression is also induced by TGFβ2 and dexamethasone in primary TM cultures[12,67]. Here we demonstrate elevated expression of EDA in the mouse TM in response to TGFβ2.

Functionally, it is known that EDA acts as an endogenous ligand for toll-like receptor 4 (TLR4)[48]. The activation of TLR4 is also dependent upon the expression of MD-2 and other TLR4 accessory proteins[48,68]. Recently, α4β1 integrin was identified to function as a TLR4-coreceptor to initiate an EDA-dependent response[69]. In addition, increased EDA levels led to further production of the EDA isoform in dermal fibroblasts[69]. The mechanism in which FN-EDA activates TLR4 in TM cells remains to be identified, but these data support our hypothesis of a progressive feed-forward mechanism of pathogenic TLR4 signaling involving the fibronectin EDA isoform.

EDA null and constitutively active EDA mice with and without mutation of *Tlr4* were previously developed and characterized[41,49]. These transgenic mice provide a means to study the function of the EDA isoform and its dependence on Tlr4 in a controlled strain specific manner using our inducible model of ocular hypertension. Interestingly the TM of B6.EDA$^{-/-}$ mice appears to develop normally as shown by gross clinical and histological analysis, and these mice are completely resistant to TGFβ2 induced ocular hypertension. B6.EDA$^{-/-}$ mice also express very little FN in the TM. Previously, it has been shown that depletion of the EDA-domain can lead to a 40% decrease in the remaining FN levels[70]. However, this phenomenon appears to be tissue specific as it is also known that B6.EDA$^{-/-}$ mice have normal levels of FN in the heart and brain[41]. These data highlight that the EDA

isoform of FN is necessary for TGFβ2-induced ocular hypertension. Our data also demonstrates that TGFβ2 overexpression in the TM induces expression of both FN as well as expression of the EDA isoform in wildtype C57BL/6J mice and constitutively active EDA$^{+/+}$ mice as measured 7-weeks after TGFβ2 injections, complimenting the increase in IOP in these mice. It is important to note that the anti–cFN-EDA antibody (NBP1-91258; Novus Biologicals) used here is made against the C-terminal region of the Fibronectin protein (within residues 2250–2300). Mouse-anti-FN antibody (clone IST-4; Sigma-Aldrich) recognizes an epitope located within the 5th type III repeat of human plasma fibronectin, which is common to all fibronectin forms. However, the exposure and accessibility of the 5th type III repeat can be affected by unfolding of the FN protein, FN-FN interactions during fibril formation, and placement or interaction within the ECM[71–73]. Therefore, the binding of this antibody may be affected in certain tissues and disease states and the presence or absence of the EDA domain. This hypothesis is supported by previous studies where both antibodies were used in human TM tissues, and a similar phenotype was noted as we see here, where EDA did not always co-localize with the total FN antibody[12]. Therefore, it is likely that we are underestimating the amount of total FN produced in our experiments.

We also demonstrated that when EDA is constitutively expressed the mice develop high IOP by 14 weeks of age and persists for at least 32 weeks of age. Therefore, constitutively active EDA mice represent a novel mouse model of ocular hypertension. These mice are an excellent resource for the field of ocular hypertension research and TM pathology as they develop ocular hypertension by an early age, maintain open irideocorneal angles, and produce a homogenous response between animals. IOP was also amplified in the TGFβ2 injected eyes of B6.EDA$^{+/+}$ mice compared to the TGFβ2 injected eyes of C57BL6/J mice for 21 days post-injection. Starting at 28 days post-injection the IOP in the TGFβ2 injected eyes of B6.EDA$^{+/+}$ mice lowered to that of the C57BL/6J injected eyes. However, the IOP in the TGFβ2 injected eyes of B6.EDA$^{+/+}$ mice remained significantly elevated compared to their uninjected control eye from day 14 until the end of the time course. These data suggest that there may be an additional compensatory mechanism that is regulating the IOP with the overexpression of both EDA and TGFβ2. We have previously shown a decrease in IOP at around 21 days after Ad5.TGFβ2 injection in other mouse strains[36] which is thought to be due to genetic background differences. Therefore, it could be that the constitutive expression of EDA not only causes elevated IOP and an initial amplification of TGFβ2 responses, but it may also induce a compensatory pathway that can partially reduce the effects. Further analysis of EDA$^{+/+}$ mice and downstream signaling pathways are needed to fully address this phenomenon. A more detailed analysis of the TM over time will help elucidate the exact molecular and pathological changes occurring in the TM that result in the ocular hypertension phenotype. In addition, further characterization will determine whether this TM damage leads to additional glaucomatous phenotypes in the retina ganglion cells and optic nerve of these animals.

In conclusion, we have demonstrated that both Tlr4 and FN-EDA are necessary for TGFβ2-induced ocular hypertension. These data provide a model system to study glaucomatous TM damage and develop novel therapeutic strategies.

Materials and Methods

Animals and Adenovirus Injection. All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of North Texas Health Science Center (UNTHSC; Fort Worth, TX, USA) and the University of Wisconsin-Madison (Madison, WI) Institutional Animal Care and Use Committee (IACUC) Guidelines and Regulations. The generation of FN-EDA$^{-/-}$, FN-EDA$^{+/+}$, FN-EDA$^{+/+}$/TLR4$^{-/-}$, and FN-EDA$^{-/-}$/ TLR4$^{-/-}$ mice has previously been described[41]. B6.EDA$^{+/+}$ mice were generated to contain spliced sites at both splicing junctions of the EDA exon and therefore constitutively express only FN containing EDA. B6.EDA$^{-/-}$ mice contain an EDA-null allele of the EDA exon and express only FN lacking EDA. All animals were housed in the UNTHSC vivarium or the UW-Madison vivarium. Adenovirus 5 (Ad5) viral vector expressing human TGFβ$^{c226s/c228s}$ (referred to throughout as Ad5.TGFβ2) (University of Iowa, Iowa City, IA, USA) was used to overexpress TGFβ2 as previously described[34–36]. Ad5.Null virus (Vector Biolabs, Malvern, PA, USA) was used as a negative control. Briefly, 2μL of 2.5×10^7 plaque-forming units (pfu) was intravitreally injected into one eye, and the uninjected contralateral eyes were used as negative controls as previously described[13,15].

Intraocular Pressure Measurements. Intraocular pressure was measured as previously described[13]. Briefly, IOP was measured on isoflurane anesthetized mice using the Tonolab tonometer (Colonial Medical Supply, Franconia, NH, USA). All IOP measurements were performed during the same time period of the light-on phase. Statistical significance was determined by one-way ANOVA followed by Tukey analysis at each time point, comparing the injected eyes and contralateral uninjected eyes between each strain. All mice were at least 5 months old for adenoviral injection experiments.

Immunohistochemistry of Mouse Eyes. After completion of the IOP time course after Ad5.TGFβ2 injection, mouse eyes were enucleated and fixed in 4% PFA overnight. Eyes were embedded in paraffin, cut into 5-μm sections, and transferred to glass slides. Slides were heated for 2 hours on a heated plate. Deparaffinization was performed by washing with xylene, 100% ethanol, and 95% ethanol, twice for 2 minutes each. Slides were soaked in distilled water and antigen retrieval (citrate buffer) was performed in 65 °C water bath for 30 minutes. Tissues were cooled off to room temperature and washed with 1X PBS three times. Tissues were stained with hematoxylin and eosin or blocked using Superblock Blocking Buffer in PBS (Thermo Fisher Scientific) for 60 minutes and further processed for immunohistochemistry as follows. For TM morphology analysis, primary rabbit-anti-smooth muscle actin (1:00, ab5694, Abcam) and rabbit-anti-collagen-1 (1:100, ab34710, Abcam) were used followed by secondary antibody Alexa Fluor-labeled Donkey-anti Rabbit Ig (ThermoFisher), 1:500 dilution. For FN analysis, primary rabbit-anti–cFN-EDA antibody (1:100, NBP1-91258; Novus Biologicals) and mouse-anti-FN antibody (1:100, clone IST-4; Sigma-Aldrich) were used followed by secondary antibodies Alexa Fluor–labeled anti-rabbit

Ig (Life Technologies) 1:500 dilution and Alexa Fluor-labeled donkey anti-mouse Ig (Life Technologies) 1:500. The mouse–anti-FN antibody recognizes epitopes on the N-terminus of FN, which are expressed by all FN isoforms. Slides were mounted with Prolong Gold mounting medium containing DAPI (Invitrogen-Molecular Probes). Images were taken using Keyence microscope BZ-X710 (Itasca, IL) or Zeiss Axio Imager Z2 microscope. All images were taken at ×200 magnification. Mean florescence intensity/area was measured in a masked manner for each image using ImageJ analysis. Statistical significance was determined by Student's paired t-test for each animal comparing the TGFβ2-injected eye to the contralateral uninjected eye.

Slit-lamp examination. Anterior segment phenotypes were assessed with a slit-lamp (SL-D7; Topcon, Tokyo, Japan) and photo documented with a digital camera (D100; Nikon, Tokyo, Japan). Images were taken with identical camera settings and prepared with identical image software processing.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions
A.L.R. and C.M.M. designed the study and wrote the manuscript. A.L.R., C.M.M., T.A.M., T.E.P., S.M.C., S.L.H. conducted the experiments and the data analysis. A.K.C. generated the transgenic mice and reviewed and edited the manuscript.

Competing interests
The authors declare no competing interests.

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Fibronectin extra domain A (FN-EDA) elevates intraocular pressure through Toll-like receptor 4 signaling

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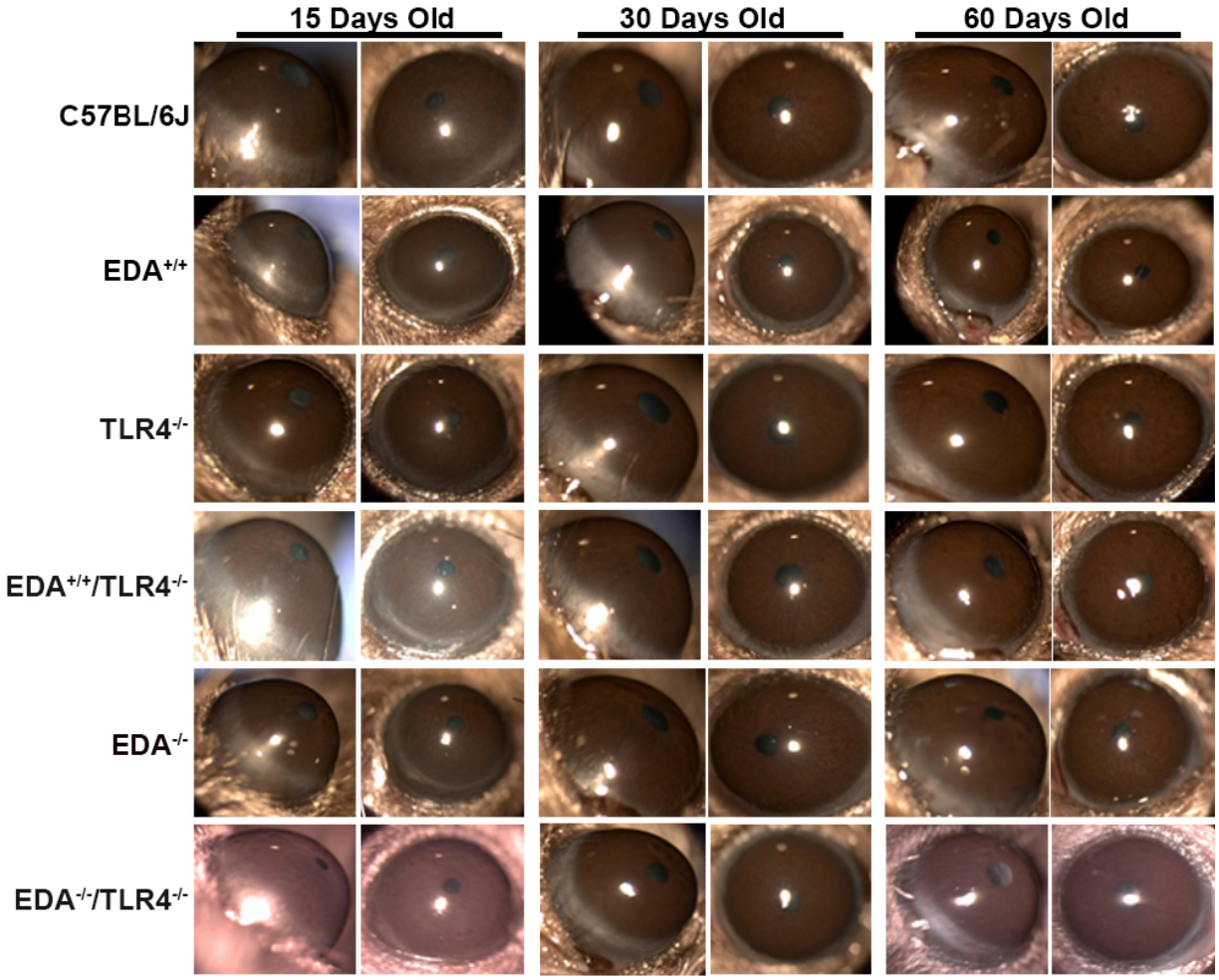
Supplemental Figure 1. Clinical exam of anterior chambers of EDA and TLR4 transgenic mice. Clinical slit-lamp images of frontal and lateral views of anterior chamber in C57BL/6J, B6.EDA$^{-/-}$, B6.TL4$^{-/-}$, B6.EDA$^{+/+}$, B6.EDA$^{-/-}$ /TLR4$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$ mice. No significant gross anatomical changes were identified 15, 30, and 60 days post-natal in any of the mouse strains.

Supplemental Figure 2. Histological exam of anterior chambers of EDA and TLR4 transgenic mice. H&E staining of histological sections from C57BL/6J, B6.EDA$^{-/-}$, B6.TL4$^{-/-}$, B6.EDA$^{+/+}$, B6.EDA$^{-/-}$ /TLR4$^{-/-}$, B6.EDA$^{+/+}$/TLR4$^{-/-}$ mice. No significant gross anatomical changes were identified 15, 30, and 60 days post-natal in any of the mouse strains. All images taken at 200x magnification.

Supplemental Figure 3. TGFβ2 induces FN and FN-EDA expression in C57BL/6J and EDA+/+ mice. (A-F) Ad5.TGFβ2 increased both total FN and FN-EDA expression in the TM of C57BL/6J mice. **(G-L)** Ad5.TGFβ2 increased total FN expression in the TM of B6.EDA$^{+/+}$ mice. **(M-R)** Ad5.TGFβ2 had no effect on FN or FN-EDA expression in the TM of B6.TLR4$^{-/-}$ mice. C57BL/6J mice (n=11), B6.EDA$^{+/+}$ mice (n=6), and B6.TLR4$^{-/-}$ mice (n=8).

Supplemental Figure 4. B6.EDA$^{-/-}$ and B6.TLR4$^{-/-}$ mice are resistant to TGFβ2-induced ECM changes in the TM. (A-F) Trace amounts of FN-EDA and FN was detected in B6.EDA$^{+/+}$/TLR4-/- mice, no difference was served between Ad5.TGFβ2 injected and uninjected eyes. **(G-R)** No detectable FN-EDA expression was observed in B6.EDA$^{-/-}$ or B6.EDA$^{-/-}$ /TLR4$^{-/-}$ mice, and no difference was observed between Ad5.TGFβ2 injected and uninjected eyes. B6.EDA$^{-/-}$ (n=18), B6.EDA$^{-/-}$ /TLR4$^{-/-}$ (n=22) and B6.EDA$^{+/+}$/ TLR4$^{-/-}$ (n=16).

Supplemental Figure 1



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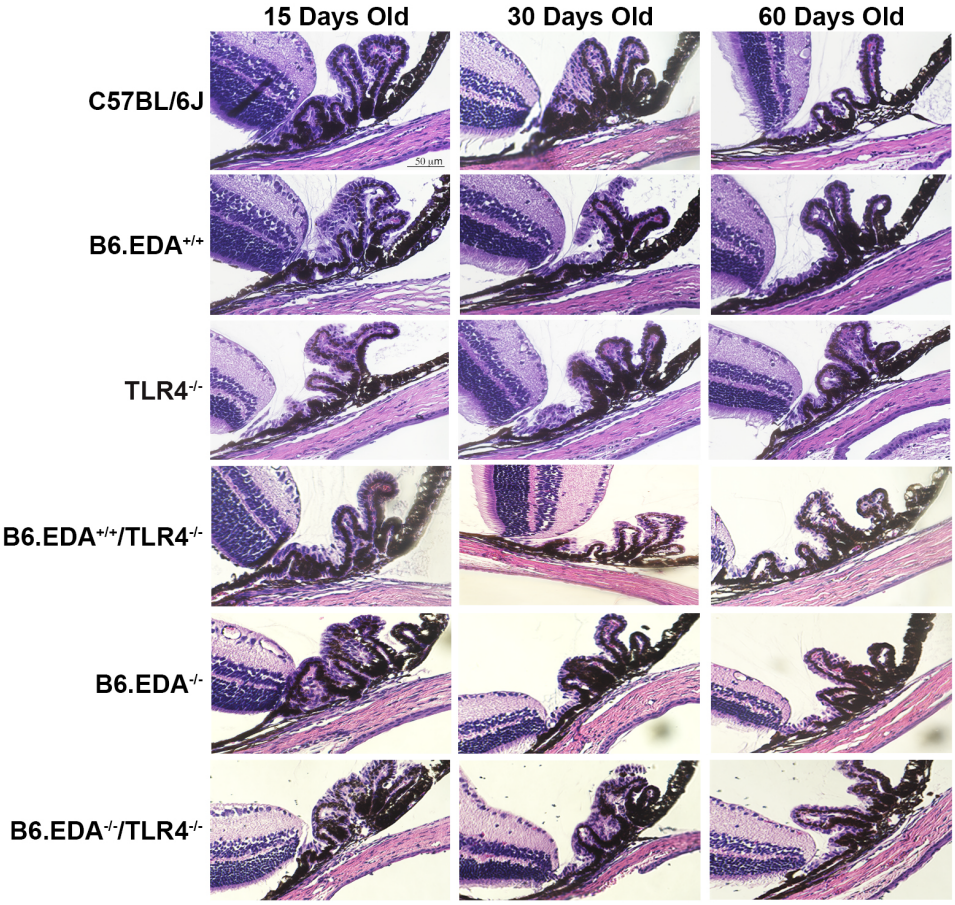
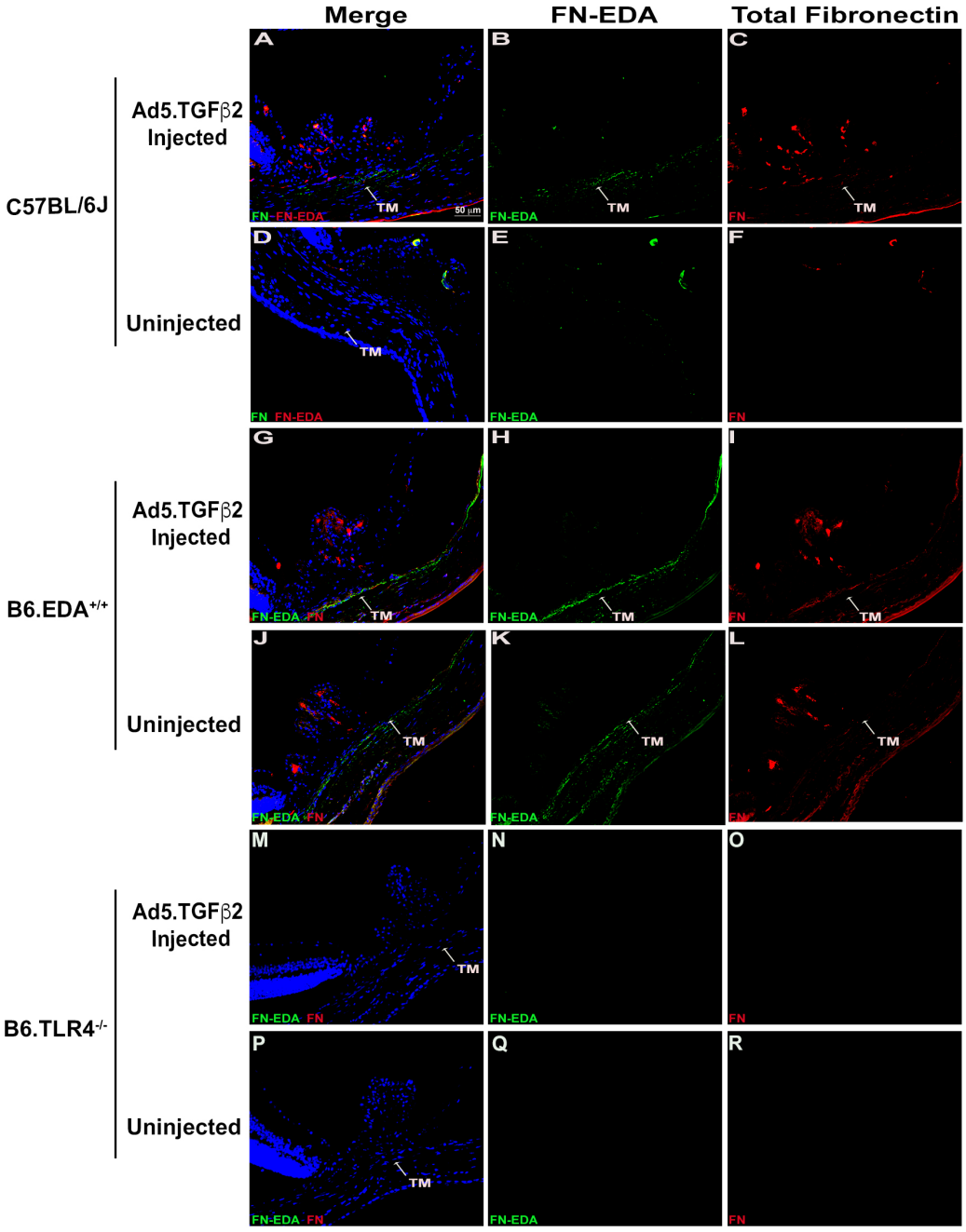
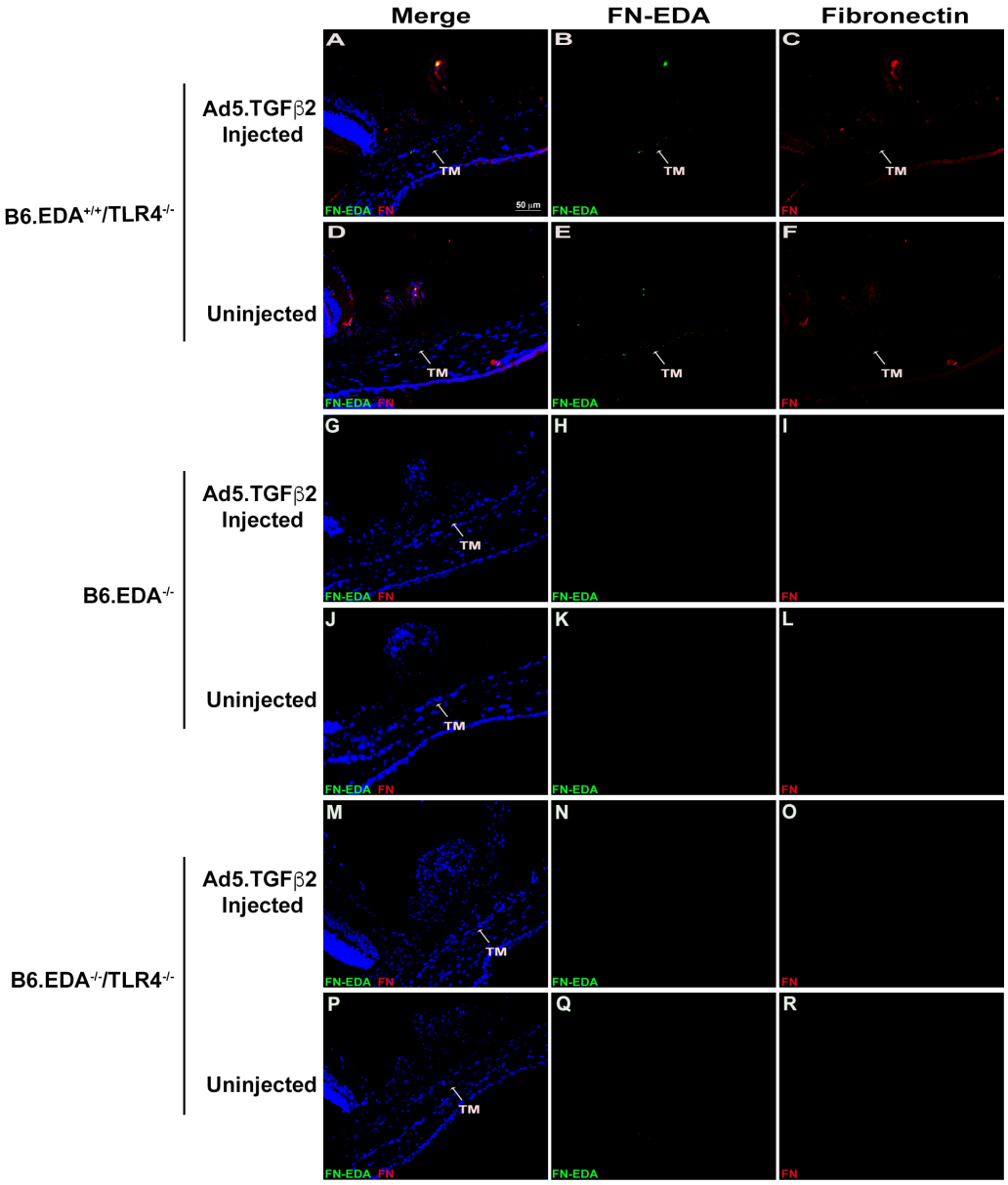


Supplemental Figure 3



Supplemental Figure 4



CHAPTER IV

DISCUSSION

4.1 Role of fibrosis and TGFβ2 in glaucomatous damage to TM

Primary open angle glaucoma (POAG) continues to be the most common form of glaucoma within the United States and is the leading cause of irreversible blindness. The major risk factor of POAG is elevated intraocular pressure (IOP), which results from abnormal aqueous humor (AH) outflow drainage through the trabecular meshwork (TM) and prolong elevated AH outflow resistance and ocular hypertension development.

The fibrotic response in the trabecular meshwork (TM) of primary open angle glaucoma (POAG) includes excessive synthesis and persistent deposition of extracellular matrix (ECM) molecules [1]. This leads to dysfunctional TM ECM, modification of cellular pathways, improper aqueous humor (AH) outflow drainage [2, 3], ongoing elevation of aqueous humor (AH) outflow resistance [4, 5] and ocular hypertension development [4, 6-8]. Transforming growth factor beta 2 (TGFβ2) is a profibrotic cytokine that regulates ECM synthesis and breakdown [9, 10].

Dysregulated TGFβ2 signaling through the canonical SMAD pathway alters TM ECM reorganization in normal human TM cells. In POAG patients, TGFβ2 levels are elevated in the aqueous humor and trabecular meshwork (TM) tissue of glaucomatous eyes [11-13]. Studies show that dysregulated TGFβ2 signaling through the canonical SMAD pathway alters TM ECM reorganization in normal human TM cells [14] [15] [16]. Mice [17] and ex vivo perfusion organ culture systems [18] [8, 19] treated with exogenous TGFβ2 induces ocular hypertension. In addition, TGFβ2 perfused in the human anterior segment organ culture model contributes to the reduction outflow

facility [20]. These studies and many others indicate that TGFβ2 is involved in the pathogenesis of glaucoma.

Previous studies show that the crosstalk between TGFβ2 and other molecules such as toll-like receptor 4 (TLR4) are involved in the fibrotic response in primary human TM cells and human TM tissue [4, 6, 8]. TLR4 is a member of the TLR family. TLR4 signaling plays a role in fibrogenesis [21] [22] [6] when activated by the endogenous ligands called damage associated molecular patterns (DAMPs). The expression of DAMPs are upregulated in response to tissue repair, injury, wound healing, or excessive ECM remodeling [23, 24]. Interestingly TLR4 expression is significantly elevated in glaucomatous human TM sections compared to normal human TM sections[8]. To test if TGFβ2 and TLR4 signaling pathways are involved in ocular hypertension development in mice, we utilized C3H/HeJ mice which have a spontaneous mutation in *Tlr4* which disrupts TLR4 signaling. We discovered that C3H/HeJ mice are resistant to TGFβ2-induced ocular hypertension and ECM synthesis [6]. Furthermore, TLR4 inhibition decreases TGFβ2-induced fibronectin and collagen IV expression in paired anterior segments in human perfusion organ culture system [8]. Overall, these studies suggest that the TGFβ2-TLR4 crosstalk is an potential pathway that could be used as a target to lower IOP and further understand the pathogenies of glaucoma.

4.2 FN-EDA elevates IOP through TLR4 signaling

Fibronectin extra domain A (FN-EDA) is a DAMP that activates TLR4 signaling and plays an important roles in tissue damage [25] [26] [27, 28] [29] and fibrogenesis [30, 31] [32] [33] [34]. FN-EDA is abundant in glaucomatous human TM tissue [35]. Our previous studies demonstrated a significant increase in ECM protein expression in TGFβ2-treated primary human TM cells and co-treatment with cFN-EDA enhanced those effects. In addition, inhibiting TLR4 signaling in culture primary

TM cells prevented TGFβ2-induced ECM protein production [6]. These data supports the idea that TLR4 has a contributing role in ECM regulation and fibrosis within TM cells [4, 6].

In our current study, we assess the age in which spontaneous ocular hypertension developed in B6.FN-EDA$^{+/+}$ (constitutively expressing the FN EDA isoform) mice eyes. Interestingly, B6.EDA$^{+/+}$ mice spontaneously developed ocular hypertension starting at 14 weeks of age and continued through 32 weeks of age. Our results suggest that constitutively active EDA mice develop elevated IOP over time. These data offer an opportunity to study the function of the EDA isoform and its dependence on TLR4 using our inducible TGFβ2 model of ocular hypertension.

In our current studies we demonstrate the importance of FN-EDA in ocular hypertension development. In the presence of Ad5.TGFβ2, ocular hypertension was further amplified in B6.FN-EDA$^{+/+}$ mice compared to TGFβ2 injected eyes of C57BL/6J mice for 21 days post-injection. However, at 28 days post-injection, the IOP of B6.FN-EDA$^{+/+}$ mice dropped to levels similar to the TGFβ2 injected eyes of C57BL/6J mice while remaining at a significant higher level than the uninjected contralateral B6.FN-EDA$^{+/+}$ mice eyes. Although we have previously seen a decrease in IOP around 21 days post-injection of Ad5.TGFβ2 in other mouse strains [17], we have not explored why this occurs. It is possible that this occurs because Smad7, a competitive inhibitor of receptor-activated Smad2/3 phosphorylation, is regulating TGFβ2 signal transduction, thus establishing a negative feedback loop [36]. Future studies on Smad7 levels in the TM are needed to support this idea.

Furthermore, the continuous expression of FN-EDA may cause the initial amplification of IOP levels in B6.EDA$^{+/+}$ mouse eyes injected with Ad5.TGFβ2 and spontaneous development of ocular hypertension in uninjected B6.FN-EDA$^{+/+}$ mouse eyes. Decreased IOP after 28 days post-

injection may be caused by other pathways intervening to protect the integrity and function of the eye. Further analysis of B6.EDA$^{+/+}$ mice are needed to identify additional signaling pathways responsible for these effects. We have previously shown that inhibition of TLR4 signaling in cultured primary human TM cells prevents TGFβ2-induced ECM protein production of fibronectin [6]. The EDA and TLR4 null mice were resistant to TGFβ2 induced ocular hypertension (Figure 4.1). These data show that TLR4 and EDA are necessary for TGFβ2 induced ocular hypertension and FN-EDA elevates IOP through TLR4 signaling. We also showed B6.EDA$^{+/+}$/TLR4$^{-/-}$ mice are resistant to TGFβ2 ocular hypertension, which shows that TLR4 must be present for EDA to induce the ocular hypertension.

Constitutively active EDA mice represent a novel animal model for ocular hypertension research. There may be an additional mechanisms that regulates IOP when both EDA and TGFβ2 are overexpressed. Further analysis of EDA+/+ mice and downstream signaling pathways are needed to fully understand these results. Both TLR4 and FN-EDA are necessary for TGFβ2-induced ocular hypertension and therapeutically targeting FN-EDA and/or TLR4 can reduce IOP to help delay or prevent the progression of glaucomatous damage to the TM.

4.3 TGFβ2 induces FN and FN-EDA expression in C57BL/6J and EDA$^{+/+}$ mice

TGFβ2 is a key cytokine involved in glaucoma pathology and induces elevated levels of TM ECM protein expression and deposition. This cytokine alters the ECM composition and induce crosslinking within the TM [15, 35]. As a result, TGFβ2 can affect TM tissue microenvironment and induce abnormalities in the function of TM cells. Increased levels of TGFβ2 in aqueous humor of human glaucomatous eyes may play a role in the pathogenesis of POAG [11]. Studies show that the elevation of fibronectin expression disrupts the drainage of AH outflow through the TM [4, 37].

Fibronectin is a multifunctional glycoprotein in the ECM that provides structural support and mediates cellular interactions with ECM material and cellular properties [38] [39]. Recent studies show that total FN [35] [8] and FN isoform EDA [35] protein expression is elevated in human glaucomatous TM tissue compared to human normal TM [35]. We recently have shown exogenous TGFβ2 induces a fibrotic response in primary human TM cells by increasing fibronectin expression and co-treatment with cFN-EDA further enhances these effects [6]. Several studies have shown that exogenous TGFβ2 induced elevated FN expression in mouse animal models [4] [6].

Here we present evidence of a critical role of FN-EDA in TM progressive fibrosis in mice and demonstrate the importance of FN-EDA in ECM regulation and fibrosis in the TM. For the first time in history we have shown that constitutively active EDA mice have elevated FN expression within the trabecular meshwork. These findings complement our previous discoveries that fibronectin protein expression is elevated in the TM of human glaucomatous eyes [35], and cFN-EDA enhances TGFβ2 effects of elevating fibronectin expression in primary HTM cells[6]. Compared to the uninjected B6.EDA$^{+/+}$ eyes the contralateral Ad5.TGFβ2 injected B6.EDA$^{+/+}$ eyes had a significantly higher FN-EDA and total FN protein expression in the TM. These results support our idea that exogenous TGFβ2 induces ECM expression in the TM. These results confirmed a new role of FN-EDA which is to regulate ECM protein expression and fibrosis within the TM. The EDA and TLR4 null mice were resistant to TGFβ2 induced ECM protein expression (Figure 4.1). These data show that TLR4 and EDA are necessary for TGFβ2 induced ECM protein expression and the absence of TLR4 and/or EDA could protect TM from fibrotic response in the absence and presence of exogenous TGFβ2. Overall, we demonstrated elevated expression of FN and FN-EDA in the mouse TM in response to TGFβ2 and in constitutively active FN-EDA mice.

Our results suggest that FN-EDA and TLR4 play a critical role in the development of fibrosis in the TM.

4.4 NFκB signaling is required for TGFβ2-induced ocular hypertension and TGFβ2-induced ECM production in the TM

Nuclear Factor kappa B (NFκB) is a key transcriptional factor family that is involved in cell proliferations, fibrosis, and immune response [40]. Here we provide evidence that NFκB plays a critical role in ocular hypertension development and TGFβ2-induced ECM production in the TM. Furthermore, we demonstrated that transactivation of NFκB is dependent on TGFβ2 and TLR4 signaling. Previous studies have shown that activation of TLR4 signaling leads to NFκB activation and induction of downstream responses [41] [42]. Here we tested our hypothesis to determine if TLR4 and NFκB signaling are important in ocular hypertension development. Our results from transfecting transformed TM cells (GTM3) with NFκB luciferase reporter vector suggested that cFN-EDA and TGFβ2, activate NFκB signaling.

Furthermore, TLR4 signaling pathway mediates NFκB transactivation. We also utilized mice lacking the NFκB p50 subunit to disrupt NFκB signaling. The p50 subunit is necessary for NFκB to translocate into the nucleus and to induce gene transcription. Our results show that NFκB is necessary for TGFβ2-induced ocular hypertension and TGFβ2-induced FN production in the TM. Other ECM proteins such as collagen-1 should be assessed in the TM for future studies.

We have studied the role of BAMBI in the regulation of IOP. Our previous studies provide evidence demonstrating that overexpression of BAMBI is involved in inducing ocular hypertension and ECM protein expression in the TM of mice, one of the major phenotypes of glaucoma [4]. However, it is unknown if these effects are due to NFκB signaling in the TM that

leads to fibrotic response in mice and ocular hypertension development. Overall, we demonstrate that TGFβ2-TLR4 crosstalk is important in the development of a glaucomatous TM. In addition, this crosstalk could be used to identify an innovative therapeutic target to decrease elevated IOP and inhibit the progression of glaucomatous TM damage.

4.5 Relevance of results in perspective with previous literature

Identifying the role of TGFβ2 and TLR4 in pathogenic fibrosis in the TM is the main point of our studies. Previously we utilized *in vitro* and *in vivo* systems to determine whether the BMP and TGFβ2 antagonist BAMBI is important in the development of ocular hypertension and fibrosis in the TM [4]. Bone morphogenetic proteins (BMPs) are a family of growth factors that regulate ECM and prevents TGFβ2 from inducing ECM deposition [43]. A transmembrane glycoprotein that acts as a negative regulator of the TGF-β signaling pathway by interacting with BMP receptors and antagonizing BMP signaling is the pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) [44].

Recently, we have shown that the knockdown of Bambi alters ECM protein expression in cultured cells and mouse TM, reduces outflow facility, and causes ocular hypertension [4]. These data provide evidence to identify BAMBI as an important regulator of TM ECM and ocular hypertension. The activation by DAMP-TLR4 signaling increases NFκB signaling, down-regulates BAMBI activity and enhances TGFβ signaling in the TM. As a result, ECM deposition and protein expression increases. Future studies are needed to determine if the TLR4-mediated BAMBI downregulation of BAMBI promoter activity is regulated through its interaction with p50 subunit of NFκB.

We previously reported that BAMBI mRNA and protein expression is decreased in human TM cells [46]. In addition, BAMBI expression is downregulated by TLR4-NFκB signaling pathway [47]. BAMBI also antagonizes TGFβ signaling by interacting with TGFβ receptors[48]. In mouse animal studies we have shown that conditional knockdown of *Bambi* induces AH outflow resistance and ocular hypertension [4]. Our results in the current study that NFκB is necessary for TGFβ2-induced ocular hypertension and ECM production in the TM [49] correlates with our previous work that TLR4 activity regulates NFκB signaling and BAMBI expression. Together, the data suggest that TLR4 and NFκB are important regulators of BAMBI expression in the TGFβ2-TLR4 signaling crosstalk For instance, in the anterior segments of human perfusion organ culture system, we demonstrated that preventing TLR4 signaling decreases TGFβ2-induced fibronectin and collagen 4 expression [8]. Overall the studies support the idea that TLR4 is necessary for TGFβ2 to induce ocular hypertension and ECM protein expression.

The pathogenesis of progressive tissue fibrosis in the TM and other areas of the eye in glaucoma patients are not incompletely understood. Here we present evidence of a critical role of FN-EDA in inducing ocular hypertension and ECM protein accumulation in the TM of mice. We found that in the absence of FN-EDA or TLR4, mice fail to develop glaucomatous TM and ocular hypertension in the presence of Ad5.TGFβ2. Previous findings show that TGFβ2 regulates fibronectin pre-mRNA alternative splicing pattern in trabecular cells and significantly increases EDA+ isoforms [52]. It is critical for future studies to analysis the optic nerve head and endothelial wall of Schlemm's canal in B6.EDA$^{+/+}$ mice. This will help identify other mechanisms that may be involved in regulating the IOP pathological changes in B6.EDA$^{+/+}$ mice.

Fibrotic response in the eye also occurs in the posterior segment of POAG patients' eyes.. Within the posterior segment of the eye of POAG patients, the ECM of the optic nerve head (ONH)

undergoes fibrogenesis that may lead to retinal ganglion cell loss. In POAG patients, TGFβ2 levels are elevated in ONH. This leads to ECM remodeling and ECM deposition in the ONH [53]. Currently it is unknown whether there is increased FN-EDA expression in the ONH of B6.EDA$^{+/+}$ mouse eyes or if these mice develop glaucomatous ONH remodeling that causes retinal ganglion cell axon damage and degeneration. Future studies are needed to assess the ONH ECM and retinal ganglion cells functionality and structure in B6.EDA$^{+/+}$ mice.

4.6 Animal models for POAG for discovering new pathogenic pathways

Discovering new pathogenic pathways for TGFβ2 and ocular hypertension can facilitate in creating more effective disease modifying therapies and slow retinal ganglion cell loss and vision loss progression. Current glaucoma drug therapies reduce elevated IOP. The latest glaucoma drug therapy is Rho kinase/norepinephrine transporter inhibitor. This drug therapy increases the aqueous humor outflow through the TM and decreases aqueous humor production and episcleral venous pressure [54]. Focusing on TGFβ2 signaling pathways are essential because studies have shown that TGFβ2 is elevated in the AH [13] of POAG patients. Previous *in vivo* studies have shown that treating mice with exogenous TGFβ2 induced ocular hypertension [4, 6, 55]. The crosstalk between TLR4 and TGFβ2 is a promising target for breaking the cycle of progressive fibrosis in human TM cells treated with exogenous TGFβ2 [4, 6]. In our current studies we have shown that NFκB, TLR4, and FN-EDA contribute to and are necessary for TGFβ2-induced ocular hypertension.

Studying and identifying novel molecular mechanisms that are responsible for glaucomatous development can lead to discovering novel animal models that recapitulate human glaucoma disease. Animal mouse models are valuable tools to study glaucoma-related

characteristics. Aging DBA/2J mice are a model that develops progressive eye abnormalities which closely mimic human hereditary glaucoma [56]. However, this mouse strain has several limitations. For instance, DBA/2J mice have variability of onset, variable damage between the paired eyes, and late onset of OHT [56]. Although variable in disease onset and severity between individual mice, the model is well-characterized and the outcomes are very predictable as a whole.

For instance, the angiopoietin-1 (*Angpt1)* conditional knockout mice are a genetic model of ocular hypertension, exhibit reduced retinal ganglion cell density and optic nerve dysfunction, and defects in SC development and function [57]. Another mouse model used to study the molecular and physiological progression of secondary congenital glaucoma disease is the spontaneous mutation of the *Sh3pxd2b* gene in the B6.*Sh3pxd2b*^{nee} mutant animal model. The glaucoma-like characteristics that this model develops are ocular hypertension, decreased retinal ganglion cell density, and retinal ganglion cell axon degeneration [58]. Overall these animal models are effective tools to study new pathogenic pathways for ocular hypertension and other glaucoma characteristics.

4.7 Outstanding issues

Our work here showed TLR4-TGFβ2 signaling crosstalk in the TM, but there is a possibility this pathway is also regulating fibrotic changes in the ONH leading to glaucomatous changes in the retina and optic nerve. Studies have shown increased TGFβ2 expression in glaucomatous human ONH tissues and cells [53]. However, we need to conduct further analysis to determine if the TLR4-TGFβ2 signaling crosstalk is responsible for a glaucomatous environment within the ONH of the TGFβ2 injected eyes of the B6.EDA$^{+/+}$ mice. In our present study we

demonstrated that B6.EDA$^{+/+}$ mice spontaneous developed ocular hypertension and elevated ECM protein expression in the TM. In addition to the TM phenotype, there is likely also damage to the retina and ONH. Addressing these outstanding issues will help identify the potential role of TLR4 signaling in the fibrotic damage to the ONH and if there is TGFβ2-TLR4 crosstalk in the optic nerve head.

4.8 Future direction

Future experiments to further assess glaucoma related modifications within B6.EDA$^{+/+}$ mice are to analyze and identify any functional and structural changes to the optic nerve, RGCs and their axons. In our current studies we have identified that B6.EDA$^{+/+}$ mice is an excellent ocular hypertension animal model to study POAG. In addition, various studies have shown changes in the glaucomatous optic nerve damage have a direct relationship with elevated IOP [59, 60] [61]. Furthermore, monkeys with laser-induced high IOP levels demonstrated similar optic neuropathy development that was clinically and histologically indistinguishable from human POAG, including severe degenerative changes and blockage of RGC axoplasmic transport [62].

We will determine whether TM damage and ocular hypertension development in B6.EDA$^{+/+}$ mice eyes lead to additional glaucomatous phenotypes such as retina ganglion cell (RGC) neurodegeneration and optic neurotherapy. To determine the loss of retinal nerve fiber layer, decreased retinal ganglion cell complex thickness, and abnormal anatomical changes to the retina, we will use non-invasive optical coherence tomography (OCT) imaging in live control C57BL/6J and ocular hypertensive B6.EDA$^{+/+}$ mice. We expect the retinal layers of B6.EDA$^{+/+}$ mice to be thinner compared to the control mice. Next, we will examine the in vivo functional activity loss of retinal ganglion cells (RGCs) by measuring the pattern electroretinogram

(PERG) amplitudes and latencies in B6.EDA$^{+/+}$ and C57B/6J mice. We also will examine whether spontaneous ocular hypertension development in B6.EDA$^{+/+}$ mice lead to RGC loss by whole-mount retina immunostaining with RBPMS, a RGC specific marker, and quantification of immunostaining with Image J software. We expect a high percentage of RGC loss in B6.EDA$^{+/+}$ mice compared to C57BL/6J mice. Furthermore, to examine gross morphological differences in retinal layers, we will conduct H&E staining of B6.EDA$^{+/+}$ and C57BL/6J mouse retinal cross-sections. We expect cell loss and thinning in the RGC layer in B6.EDA$^{+/+}$ mice. These data could indicate that spontaneous ocular hypertension development in B6.EDA$^{+/+}$ mice leads to both structural and functional RGC loss.

Glaucomatous neurodegeneration can be associated with prolonged ocular hypertension, axonal transport defects, optic nerve demyelination, and activation of astrocytes and immune cell infiltration in the optic nerve head. Therefore, we will examine whether spontaneous ocular hypertension development in B6.EDA$^{+/+}$ mice cause optic nerve degeneration. To examine the optic nerve degeneration, the myelin around RGC axons is stained with *p*-phenylenediamine (PPD). These darkly stained axons represent damaged or dying axons. Semi-automated counting tool image analysis software will be used to quantify three areas of the optic nerve, which will be classified as normal, moderately damaged, or severely damaged. We expect the B6.EDA$^{+/+}$ mice to demonstrate darkly stained degenerating axons and a reduction in healthy RGC axons compared to control C57BL/6J mice in both the proximal and distal regions. Using transmission electron microscopy (TEM), we expect to observe other signs of optic neuropathy such as unmyelinated axons, RGC axonal swelling, and residual empty vacuoles in B6.EDA$^{+/+}$ mice eyes compared to control C57BL/6J mice eyes.

Ocular hypertension can lead to glaucomatous neurodegeneration and anterograde axonal transport defects in the optic nerve head of monkey eyes [58] and mice eyes [59]. To determine if the presence of axonal damage and whether anterograde transport mechanism is impaired the optic nerves of B6.EDA$^{+/+}$ mice eyes, we will intravitreally inject fluorescently tagged cholera toxin B (CTB) dye and trace the CTB dye through the entire optic nerve into the lateral geniculus nucleus and the superior colliculus within the brain. We expect a partial blockage of axonal transport at the optic nerve head with minimum detection of CTB throughout the entire optic nerve in B6.EDA$^{+/+}$ mice eyes compared to normal axonal transport of CTB throughout the entire optic nerve in control C57BL/6J mice eyes. Furthermore, we expect to detect no CTB transport in the superior colliculus of B6.EDA$^{+/+}$ mice compared to normal detection of CTB transport in the superior colliculus of control C57BL/6J mice. These data indicate that glaucomatous neurodegeneration in B6.EDA$^{+/+}$ mice affects the degree to which the RGC axons have functional loss of axonal transport.

Next, we will determine whether TGFβ2 leads to RGC neurodegeneration and optic neurotherapy in B6.EDA$^{+/+}$ mice intravitreally injected with bioactivated Ad5.TGFβ2 compared to non-injected B6.EDA$^{+/+}$ mice. We will perform the same experimental methods as we previously mentioned with the non-injected B6.EDA$^{+/+}$ mice. We expect B6.EDA$^{+/+}$ mice injected with Ad5.TGFβ2 to have more significant RGC loss and thinning of RGC layer, RGC axon degeneration, significantly reduced antegrade RGC axon transport, and less function and activity of RGCs.

The ONH consists of connective tissue beams that supports the opening in which retinal ganglion cells exit the eye [64] and is the initial site of ocular hypertension-induced damage [65].To identify the molecular and pathological changes occurring in the ONH ECM of B6.EDA$^{+/+}$ mice,

we would examine if the ONH had increase ECM protein expression of fibronectin, FN-EDA, and collagen-1. Studies in the human ONH have identified quiescent microglia in normal ONHs and activated microglia in the glaucomatous nerve [66]. We would assess the activation of microglia cells in ONH of B6.EDA$^{+/+}$ mice and compare those results C57BL/6J mice using allograft inflammation factor 1 (AIF-1). If the microglia cells are active in B6.EDA$^{+/+}$ mice, then we expect to see clusters of microglia cells. We could also identify the characteristics of activated astrocytes based on their migration, and shape modification, and access the neurotrophins such as brain derived neurotropic factors that are secreted to promote RGC survival.

4.9 Conclusion

In summary, our data conclude that FN-EDA, TLR4, and NFκB are all necessary for TGFβ2-induced ocular hypertension and ECM production in the TM. Discovering that B6.EDA$^{+/+}$ mice have the potential to become the next glaucoma animal model could help identify novel molecular mechanisms responsible for the development of glaucomatous environment within the TM. Figure 4.2 summarizes the mechanism and results in our studies. Regulating persistent TLR4 signaling by targeting endogenous ligand FN-EDA accumulation or the NFκB signaling pathway represents a potential novel strategy for breaking the cycle of progressive fibrosis in the glaucomatous trabecular meshwork.

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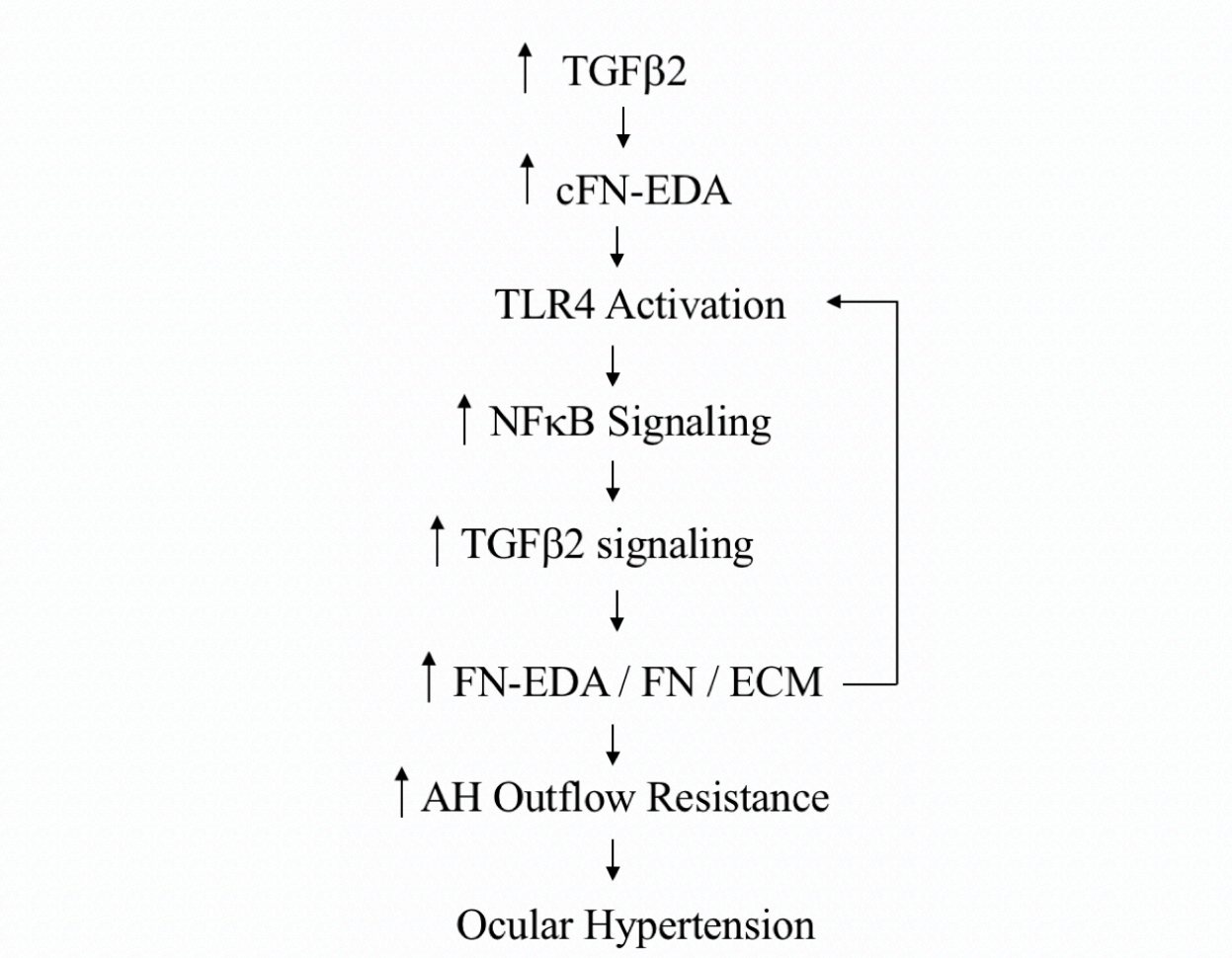

Figure 4.1 Outline of results: Elevated TGFβ2 increase FN-EDA expression the TM to activate TLR4 signaling transduction. Within TLR4 signaling NFκB is activated and elevates TGFβ2 expression and signaling. Excessive TGFβ2 activation further increases FN-EDA and other ECM protein expression. FN-EDA stimulates the feed-forward mechanism of pathogenic TLR4 signaling. ECM synthesis and deposition block aqueous humor outflow, resulting in elevated aqueous humor outflow resistance and ocular hypertension.

Mouse Strain	FN expression in Ad5.TGFβ2 Injected eye	FN-EDA expression in Ad5.TGFβ2 injected eye	FN expression Non-injected contralateral eye	FN-EDA expression Non-injected contralateral eye	IOP Level in Ad5.TGFβ2 injected eye	IOP Level in Non-injected contralateral eye
C57BL/6J (B6)	↑↑	↑↑	-	-	↑↑	-
B6.EDA$^{+/+}$	↑↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑↑	↑↑
B6.EDA$^{+/+}$/TLR4$^{-/-}$	↑	↑	-	↑	-	-
B6.TLR4$^{-/-}$	-	-	-	-	-	-
B6.EDA$^{-/-}$	-	-	-	-	-	-
B6.EDA$^{-/-}$/TLR4$^{-/-}$	-	-	-	-	-	-

Table 4.1 Summary of IOP and protein expression within the trabecular meshwork of each moue strain: Mouse strains included: constitutive expression of the FN-EDA isoform (B6.EDA$^{+/+}$), constitutive expression of the EDA isoform with knockout of Tlr4 (B6.EDA$^{+/+}$ / TLR4$^{-/-}$), EDA null (B6.EDA$^{-/-}$), with a knockout of Tlr4 (B6.TLR4$^{-/-}$), or both a EDA null with a knockout of Tlr4 (B6.EDA$^{-/-}$ / TLR4$^{-/-}$). All analyses were performed 6-7 weeks post-injection of Ad5.TGFβ2. (-) represents normal levels, one arrow presents elevated level, 2 arrows represent significant elevation, 3 arrows represent highly significant elevation, 4 arrows represent very highly significant elevation.