The Role of Cochlin in the Trabecular Meshwork

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THE ROLE OF COCHLIN IN THE TRABECULAR MESHWORK

By

Teresia Angelica Carreon

A DISSERTATION

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Doctor of Philosophy

THE ROLE OF COCHLIN IN THE TRABECULAR
MESHWORK

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Glaucoma affects over 70 million people worldwide and can result in irreversible progressive vision loss if left untreated. Primary open angle glaucoma is the most common form of the disease. This disease is characterized by an uncontrolled increase in intraocular pressure (IOP) and this characteristic remains the only modifiable risk factor. The trabecular meshwork (TM) is a filter-like structure responsible for aqueous humor filtration into Schlemm’s canal (SC) and into the episcleral veins (EV). It is believed that obstruction in the TM leads to impeded aqueous humor outflow resulting in an increase in IOP. It has previously been demonstrated that the extracellular matrix protein cochlin forms deposits in the TM of glaucomatous individuals compared to controls and may contribute to impeded aqueous humor outflow. Our group has shown how TM cells detect shear stress via the interaction of cochlin with the cell surface bound, stretch-activated channel TREK-1. Uncontrolled increase in resistance, a mechanism that typically contributes to the segmental outflow pattern in a regulated manner in the eye, is also thought to be a contributing factor to the increase in IOP. In this study, we characterize segmental outflow in the DBA/2J and DBA/2J-Gpnmb+/SjJ mouse model and further investigate the role the extracellular matrix protein cochlin plays in mechanotransduction of TM cells for IOP regulation.
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LIST OF ABBREVIATIONS

AA: arachidonic acid

ACAID: anterior chamber associated immune deviation

AH: aqueous humor

BM: basement membrane

CAI: carbonic anhydrase inhibitor

CB: ciliary body

ECM: extracellular matrix

EPV: episcleral venous pressure

EV: episcleral veins

FCH: factor C homology domain

GAG: glycosaminoglycan

IOP: intraocular pressure

JCT: juxtacanalicular tissue

MMP: matrix metalloproteinase

NTM: normal trabecular meshwork cells

OCT: optical coherence tomography

OHTS: Ocular Hypertension Treatment Study
PERG: pattern electroretinogram

POAG: primary open angle glaucoma

PVDF: polyvinylidene fluoride

RGC: retinal ganglion cell

SC: Schlemm’s canal

TF: transcription factor

TM: trabecular meshwork

TREK-1: TWIK-related K+ 1 channel

Tyrp1b: b allele of tyrosine related protein

VF: visual field

vWFA: von Willebrand factor A-like domain

VWF: von Willebrand factor
Chapter 1: The dynamics of aqueous humor outflow

Glaucoma is a disease that can result in irreversible progressive vision loss when left untreated. This disease affects over 70 million people worldwide with primary open angle glaucoma (POAG) being the most common form (Quigley, 1996, 2011; Quigley and Broman, 2006). Intraocular pressure (IOP) remains the only modifiable risk factor in glaucoma (Morrison, 2003). Uncontrolled increases in IOP lead to death of the retinal ganglion cells (RGCs) resulting in progressive peripheral vision loss. This increase in IOP results from altered aqueous humor (AH) production and outflow in the anterior chamber of the eye.

The ciliary processes actively secrete AH, which bathes the eye providing nutrients to the anterior structures and removing waste material. The outflow of the AH follows two distinct outflow pathways that we will cover in more detail in the following sections. Overall, maintenance of AH outflow and IOP is a major component in maintaining the overall homeostasis of the eye.

The maintenance of IOP in the anterior segment results from a number of components acting synergistically with one another. Although work has focused on elucidating the mechanism of AH outflow and resistance as a whole, there is a fundamental lack of knowledge regarding the underlying molecular mechanisms responsible for IOP maintenance. The goal of this thesis is to investigate the molecular mechanisms underlying the extracellular matrix (ECM) protein cochlin in glaucoma pathology as well as further understand segmental outflow by
utilizing DBA/2J and DBA/2J-Gpnmb+/SjJ mice. Knowledge into this area will open the door for research into the role cochlin plays in glaucoma pathology.

1.1 The aqueous humor outflow pathway

AH is a key component in maintaining overall homeostasis of the eye. As previously mentioned, AH is responsible for providing anterior segment structures with nutrients as well as removing waste material from the eye. It also plays a major role in maintenance of IOP, which ultimately results in eye shape and the eye’s refractive properties. The AH outflow pathway is comprised of various meshwork tissues and vessels responsible for providing a route for AH which, is actively secreted by the ciliary processes, to flow from the anterior chamber into the surrounding episcleral veins (EV). AH follows two distinct outflow pathways, the conventional pathway and the unconventional (uveoscleral) pathway (Figure 1.1A & B).

1.1.1 Conventional outflow pathway

The conventional pathway was first proposed in the 1870’s (Leber, 1873; Schwalbe, 1870) and eventually demonstrated in the 1900’s (Ascher, 1942; Seidel, 1921). In the conventional pathway, AH filters out through the trabecular meshwork (TM) into Schlemm’s Canal (SC). The structures in this pathway work synergistically to regulate outflow. Fluid flow through this pathway is dependent on a pressure gradient specifically between pressure inside (IOP) and pressure
outside the eye (episcleral venous pressure [EVP]) (Stamer, 2012). This difference allows the AH to move through the various components in this pathway. This route allows the return of AH to the venous system as it enters the distal outflow system following this pathway. This pathway is responsible for ~85-97% of AH outflow in the anterior segment (Stamer, 2012). Although it makes up a majority of the AH outflow pathway, the lack of drugs targeting this pathway for glaucoma treatment leaves this as an ideal avenue for research. In order to understand this outflow pathway as a whole is important to understand how each individual structure contributes to it.

1.1.1.1 *Trabecular meshwork (TM)*

The TM is one of the main structures in the conventional outflow pathway. It is primarily a filter-like structure that acts by filtering out cell debris, reactive oxygen species, and pigment from the AH. The cells of the TM are pseudoendothelial cells enveloped by connective tissue giving the TM a sponge-like composition. The TM is divided into distinct regions: the uveal meshwork, the corneoscleral meshwork, and the juxtacanalicular tissue (JCT). These regions are organized in a way in which the JCT region abuts the inner wall of the SC (Figure 1.1C). The area in which the JCT region and SC interact is the major site of resistance in the outflow pathway (Stamer and Acott, 2012). Resistance found in glaucomatous eyes localizes specifically in the JCT region although the contribution of TM cells, the ECM, or the inner wall of the SC to this resistance remains to be elucidated (Grant, 1963).
Figure 1.1 Schematic representation of outflow and areas of resistance in the anterior segment. A) The ciliary body (CB) actively secretes aqueous humor in the anterior chamber and follows two separate outflow pathways. The conventional pathway consists of aqueous humor passing through the trabecular meshwork (TM) into Schlemm’s canal (SC). Alternatively, the unconventional pathway completely bypasses the TM and the SC. Adapted from (Carreon et al., 2016). B) The major components of the conventional outflow pathways are the TM and the SC. C) The TM exhibits a filter-like conformation with cells organized in layers. The aqueous humor flows through the outer layers of the TM into the juxtacanalicular (JCT) region, into the inner wall (IW) of SC, and drains into the collector channels (CC). The aqueous humor eventually exits into the aqueous veins (AV). The red arrows indicate the major areas of resistance highlighting the pattern in which it increases from the outer TM into the JCT region and into the IW of SC. Figures B and C were adapted from (Carreon et al., 2017a).
1.1.1.2 Schlemm’s Canal (SC)

The SC is one of the first vessels that the AH encounters in the conventional outflow pathway. It is made up of endothelial cells and maintains a flattened tube structure. Its location within the eye is in the ocular wall of the limbus (region that connects the cornea to the sclera). Together, the SC endothelial cells and basement membrane (BM) provide a barrier for AH drainage. Recently, characterization of this unique vessel has shown that it expresses both blood and lymph vessel markers (Kizhatil et al., 2014). Specifically, the SC expresses the lymphatic marker Prox-1 but cannot be considered a true lymphatic vessel as it lacks expression of lymphatic vessel marker endothelial hyaluronan receptor (LYVE-1) (Truong et al., 2014; van der Merwe and Kidson, 2014). The inner wall of the SC is a component in outflow resistance although the exact contribution remains unknown. Although it is a key contributor to AH outflow regulation, this is not its only role in the eye.

The SC is also a key component in anterior chamber associate immune deviation (ACAID) in which antigens stimulate immune cells in the eye resulting in their exit through the SC into the systemic circulation. Once in circulation, these immune cells work to induce systemic suppression of specific immune responses to the detected antigen (Kizhatil et al., 2014). The SC provides a major contribution to the overall homeostasis of the eye.
1.1.1.3 Collector channels and aqueous veins

The outflow system beyond the TM is known as the distal outflow system and is comprised of the collector channels as well as the aqueous veins. Collector channel entrances are located near the SC external wall allowing for flow of AH from the TM to the SC into the collector channels. Following this path, the AH eventually ends up flowing into the aqueous veins from the collector channels. This entire flow regimen is dependent on IOP and venous pressure resulting in the pulsatile flow of AH.

Pulsatile flow refers to the coordinated responses elicited by tissue pathways responsible for regulating AH flow (Ascher, 1961; Johnstone et al., 2011). The ocular pulse arising in the SC is caused by changes in the choroidal vascular volume (oscillation between diastole and systole) referred to as the choroidal piston (Phillips et al., 1992). The change in the ocular pulse triggers pulsatile TM motion outward towards the SC reducing the total volume of AH in the SC lumen and an increase in SC pressure resulting in the movement of AH out of the SC (Johnstone et al., 2011; M. Johnstone, 2010). All of the tissues in this flow regime work synergistically to regulate the flow of AH and as a result the increase or decrease of IOP.

The collector channels are key components in the regulation of IOP and AH outflow. The structure of these collector channels as well as the number of collector channels present result in areas of high flow or low flow and have an effect on AH outflow as well as overall IOP. Recently, it has been suggested that active flow regions contain more collector channels than inactive flow regions
(Cha et al., 2016). Clinically, the reduction or lack of collector channels across 360° regions has been repeatedly observed in glaucomatous eyes (Grover et al., 2014; Grover et al., 2015; Johnstone and Grant, 1973). Collector channels lead to drainage of AH into the aqueous or episcleral veins. The characterization of collector channels at the molecular level remains to be understood but would provide more in depth knowledge into the outflow pathway as a whole.

1.1.2 Unconventional (uveoscleral) outflow pathway

The unconventional pathway refers to the flow of AH through the uvea, the ciliary body (CB) and muscle, into the choroid and sclera. It is known as the unconventional pathway and can amount to up to ~50% of AH outflow depending on the age as well as the health of the eye (Toris et al., 1999). This pathway was first identified in tracer studies (Fine, 1964). Further work in the 1960’s in monkeys by Anders Bill investigated the functionality of the “unconventional pathway” making it distinguishable from the conventional pathway (Bill, 1962, 1965, 1966a, b, c). Debate continues in regard to the final destination of AH in this pathway. It has been postulated that fluid flows through the sclera and episclera into the orbit where it is absorbed by the orbital vasculature (uveoscleral) (Bill, 1966a, 1975; Bill and Phillips, 1971; Gabelt and Kaufman, 1989). Alternatively, it is also postulated that the AH is absorbed osmotically by the choroid and enters into the vortex veins (uveovortex) (Barany, 1967; Pederson et al., 1977; Sherman et al., 1978). Finally, in a third pathway, it is thought that drainage of AH occurs into the lymphatic vessels of the CB
(uveolymphatic) (Yucel et al., 2009). Although the exact flow remains to be fully understood, overall, all three of these pathways likely contribute to the unconventional outflow pathway.

1.2 Segmental outflow in the aqueous humor outflow pathway

Outflow channels in the eye are not in a uniform pattern; instead, outflow is segmental (Figure 1.2). Regions of high flow and low flow contribute to this segmental pattern. This flow pattern identified as segmental pigmentation was observed in the TM of human eyes (Johnson, 1989). This segmental variability was further validated in normal and glaucomatous eyes with tracer experiments in human as well as monkey eyes (Buller and Johnson, 1994; de Kater et al., 1989; Epstein and Rohen, 1991; Vranka et al., 2015). The molecular mechanisms underlying segmental outflow remains to be elucidated but research has identified potential components contributing to this outflow pattern.

1.2.1 Major contributors to segmental outflow

The segmental outflow pattern present in the eye results from a variety of contributing factors. Outflow resistance, funneling of AH, and the presence of pores are the major contributors. Although it is not known which of these mechanisms contribute the most, each one plays a unique role to result in this outflow pattern. In order to understand the segmental outflow pattern, it is important to understand how each underlying mechanism works.
Figure 1.2 Segmental outflow en face representation in the anterior chamber. Q-tracker 655 injected into the anterior segment of a perfused human whole globe demonstrates the areas of high flow and low flow that make up this specific outflow pattern.

1.2.1.1 Outflow resistance

Resistance in the eye accumulates at the SC as well as the JCT region of the TM. The TM and SC inner wall cells appear to work synergistically to regulate resistance. IOP itself is a result of outflow resistance generation in these areas (Bahler et al., 2004a; Bahler et al., 2004b; Grant, 1963; Johnson et al., 1992; Maepea and Bill, 1992). The SC inner wall and JCT region regulate
resistance via a hydrodynamic mechanism known as “funneling” (Overby et al., 2009). In glaucoma, elevated IOP may result from increased resistance in the JCT region of the TM (Johnson et al., 2002; Johnson et al., 1992; Stamer, 2012; Stamer and Acott, 2012). The contribution of the TM and SC inner wall to outflow resistance remains to be fully understood. Each of these structures alone do not create enough resistance to produce elevated IOP.

1.2.1.2 Funneling in resistance

Funneling refers to the mechanism that takes place between the JCT region of the TM and the SC inner wall. The inner wall of the SC contains pore sites in which the AH that passes through the JCT must funnel into. This funneling increases resistance as the area of the JCT that actively filters AH is reduced. The fact that the TM cells in the JCT region tether to the inner wall of the SC create an environment ideal for this mechanism to occur resulting in an increase in resistance. The resistance decreases once these two structures are separated (Stamer and Acott, 2012). The spacing between the TM cells in the JCT region and the SC inner wall as well as the pore density in the SC inner wall affect overall resistance (Allingham et al., 1992; Ethier et al., 1986; Johnson et al., 1992; Johnstone and Grant, 1973; Overby et al., 2009). These observations were validated in perfusion tracer studies in living monkey eyes (Sabanay et al., 2000; Sabanay et al., 2004) and enucleated bovine eyes (Lu et al., 2008). In glaucomatous eyes, a lower pore density as well as impaired pore formation has been demonstrated to result in higher resistance (Braakman et al., 2015;
Johnson et al., 2002; Stamer and Acott, 2012). Variations in funneling throughout the JCT and SC inner wall regions are postulated to contribute to segmental flow (Battista et al., 2008; Lu et al., 2008). This alludes to the resistance from funneling as a characteristic of segmental outflow.

1.2.2 Extracellular matrix (ECM) changes as major components in segmental outflow

ECM components such as proteins with glycosaminoglycans (GAGs) as side chains (versican and hylaruanan) provide a majority of outflow resistance (Acott and Kelley, 2008; Keller et al., 2009a; Keller et al., 2012). This outflow resistance, as previously mentioned, contributes to segmental outflow. The ECM is constantly undergoing modifications from a variety of different proteins and proteases responsible for ECM turnover as well as stabilization (Keller et al., 2009a; Keller et al., 2009b; Keller et al., 2008). Changes in these proteins and proteases seem to influence overall segmental outflow.

Specific proteinases known as matrix metalloproteinases (MMPs) are major components in ECM maintenance as they function in ECM degradation. These proteases are first produced as inactive forms known as zymogens and are later converted into the active form of the specific enzyme via substrate activation. The gene expression of ECM proteases MMP-1 and MMP-10, and other ECM specific proteins such as cochlin, matrix gla protein, and type V
collagen, exhibit altered gene expression patterns in glaucoma (Vranka et al., 2015).

1.3 Elevated intraocular pressure (IOP) in glaucoma pathology

As previously mentioned, the IOP refers to the balance of AH entering and exiting the eye. IOP remains the only modifiable risk factor in glaucoma, even in those forms of glaucoma not associated with an increase in IOP (Anderson, 2011). The movement of aqueous humor throughout the anterior chamber of the eye is dependent on a specific pressure difference between inside pressure (IOP) and outside pressure (episcleral venous pressure [EVP]). Various components work in synergy to maintain the homeostasis of this pressure difference and overall IOP.

1.3.1 Areas contributing to glaucoma pathology

AH outflow dysregulation contributes to glaucoma pathology. The uncontrolled increase in IOP can lead to irreversible vision loss if left untreated. The area that primarily contributes to glaucoma pathology is the conventional outflow pathway. As mentioned in the previous section (Section 1.1.1), this pathway is comprised of the TM and Schlemm’s canal. Accumulation of resistance is thought to contribute to glaucoma pathology.
The TM and its filter-like structure is one of the main components associated with impeded aqueous humor outflow. There have been a variety of observations made in the last few years to corroborate TM dysfunction in primary POAG. Patients with POAG exhibiting high IOP have an increase in conventional outflow resistance, which is reduced after surgical removal of the TM (Grant, 1958, 1963). Directed laser activation of TM cells has shown to temporarily reduce overall outflow resistance and lower IOP in POAG eyes (Bradley et al., 2001; Latina et al., 1998). Clinical features in corticosteroid-induced glaucoma is parallel to the clinical features observed in POAG (Armaly, 1965; Becker, 1965). In vitro, corticosteroids increase TM cell stiffness as well as alter the cytoskeletal architecture potentially contributing to this increase in stiffness (Raghunathan et al., 2015; Zhuo et al., 2010). Additionally, mutations in myocilin, a specific gene identified in the TM, cause an increase in outflow resistance, high IOP, and ultimately POAG (Stone et al., 1997; Wilkinson et al., 2003). These observations together, lay a strong foundation for the implication of the TM in glaucoma.

The TM’s relationship with Schlemm’s canal in the conventional outflow pathway make it an area of interest in impeded aqueous humor outflow. The JCT region of the TM is the area in which the inner wall of SC and the TM interact. This area is where resistance is greatest in the conventional outflow pathway, as previously described (Figure 1.1C) (Bahler et al., 2004a; Bahler et al., 2004b; Grant, 1963; Johnson et al., 1992; Maepea and Bill, 1992). The generation of resistance is dependent on these two areas working synergistically with one another. The increase in resistance observed in glaucomatous eyes is linked to
the JCT region as well (Grant, 1963). The outflow facility in the JCT region occurs as a result of the presence of pores on SC inner wall and the mechanism referred to as “funneling”, described in previous sections (Section 1.2.1.2) (Johnson et al., 1992; Overby et al., 2009). The interaction of the TM and SC inner wall is referred to as the JCT region and is the area primarily implicated in increased resistance resulting in an increase in IOP. This overall mechanism contributes to glaucoma pathology.

1.3.2 Diurnal fluctuations in intraocular pressure

Diurnal fluctuations refer to circadian IOP fluctuations that occur throughout the day. This results in the cells of the TM experiencing a range of fluid shear throughout the day (Sit et al., 2008). Interestingly, fluid flow rates in normal and glaucomatous eyes are comparable throughout the daytime but nighttime flow rates are significantly higher in glaucomatous individuals (Larsson et al., 1995). The differences in diurnal fluctuations influence glaucoma pathology.

Variation in IOP that cannot be detected in the clinic is detrimental to progressive damage in glaucoma. Although tonometry measurements may record an IOP within normal range, peaks in IOP throughout the day, specifically at night, result in cumulative deterioration of the visual field (Alpar, 1983; Katavisto, 1964; M. Radnot, 1979; Zeimer et al., 1991). Further assessment of IOP using home tonometry (Alpar, 1983; Epstein, 1986; Jensen and Maumenee,
1973; Posner, 1965; Zeimer et al., 1983; Zeimer et al., 1986) or in-patient diurnal tonometry (M. Radnot, 1979) has demonstrated the presence of undetected peaks and IOP fluctuations that had not been previously identified (Katavisto, 1964). These uncontrolled peaks in diurnal fluctuations in patients previously exhibiting normal IOP in the clinic have been identified as a significant risk factor in glaucoma pathology. The measure of diurnal fluctuations in IOP is crucial for assessing and predicting glaucoma pathology (Asrani et al., 2000). Overall, the uncontrolled variation in daily diurnal fluctuations is a major risk factor in glaucoma progression.

1.3.4 Current treatment options

Glaucoma treatment drugs were first introduced into glaucoma therapy in 1875. The overall idea of glaucoma treatment is focused on maintaining patient quality of life by preserving visual function at an affordable cost. According to the European Glaucoma Society (EGS), treatment for glaucoma should be considered under the following conditions: 1) the patient is at risk of developing functional impairment that will result in deterioration of visual function – related to quality of life, 2) the patient with a glaucomatous visual field (VF), specifically progressive disease, 3) the patient with significant changes within the optic nerve head as well as the retinal nerve fiber layer, a characteristic of glaucoma (Society, 2014). Treatment is selected based on risk factors (IOP, age, central corneal thickness, vertical cup/disk ratio, and standard deviation pattern in VF) (European Glaucoma Prevention Study et al., 2007; Gordon et al., 2002),
progressive VF loss, and cost effectiveness (Kalouda et al., 2017). Currently, there are a series of first line drugs, prostaglandin analogues, beta-blockers, carbonic anhydrase inhibitors, and alpha agonists (Figure 1.3), followed by second line drugs known as parasympathomimetics (miotics).

Prostaglandin analogues (latanoprost, travoprost, bimatoprost, tafluprost) were first available for use in glaucoma treatment in the 1990s. This specific class of drug has replaced beta-blockers as the first-line of treatment. These drugs work by increasing uveoscleral outflow by inducing collagen degradation by activating tissue – remodeling enzymes and transcription factors. The overall efficacy in reducing IOP is 25 – 35% (Kalouda et al., 2017; Villumsen and Alm, 1992). Prostaglandin analogues are observed to have mild side effects, a low non-responder rate, and require only a once daily administration making them a widely favorable glaucoma treatment option (Gandolfi and Cimino, 2003; Williams, 2002).

Beta (β)-blockers (timolol, levobunolol, metipranolol, carteolol, befunolol, betaxolol) act by decreasing aqueous humor production specifically by competing with adrenergics for β-adrenoreceptors. The overall mechanism results from antagonism of the endogenous adrenergic responses. Timolol is known as the most effective anit-glaucoma drug aside from the prostaglandin analogues. The overall efficacy in lowering IOP is 20 – 25% and non-responder rate is around 20% (Kalouda et al., 2017; Silverstone et al., 1991). β-blockers have very few side effects but are systemically absorbed, which may alternatively lead to further complications. This drug class also has minimal nocturnal efficacy and a
standard dosing of twice daily (Lama, 2002; Ormiston and Salpeter, 2003; Schuman, 2000).

Carbonic anhydrase inhibitors [CAIs] (acetazolamide, methazolamide, dichlorphenamide, dorzolamide, brinzolamide) act by inhibiting active AH secretion. Carbonic anhydrase is an enzyme essential in aqueous humor production within the ciliary processes and inhibition of this process results in the reduction of IOP. CAIs are available as systemic drugs and topical drugs depending on the patient and the length of treatment. The systemic CAIs have a 30 – 40% efficiency in IOP reduction while the topical CAIs have a 20% efficiency in IOP reduction (Kalouda et al., 2017). Severe side effects are associated with hypersensitivity to this class of drugs. The systemic side effects present with the CAIs do not make them ideal for long-term use (Fraunfelder and Bagby, 2000).

Alpha agonists (apraclonidine, brimonidine, clonidine) are adrenergic agonists that work to reduce IOP by constricting the ciliary vasculature. This causes a decrease in AH production and alternatively there is an increase in uveoscleral outflow. This specific class of drugs are selective $\alpha_2$–adrenergic agonists. The effectiveness in reducing IOP ranges from 18 – 35% depending on the specific drug as they differ in selectivity of specific adrenergic receptors (Kalouda et al., 2017).

Finally, parasympathomimetics [Miotics] (pilocarpine, carbachol, echothiophate, demecarium) act by causing contraction of the ciliary muscle and scleral spur. This in turn results in the opening of the TM and Schlemm’s canal allowing for an increase in aqueous humor outflow to reduce IOP. The efficacy of
lowering IOP for this class of drugs is 20 – 25%. The severe side effects associated with miotics limit their utilization in glaucoma treatment (Gupta et al., 2008; Kalouda et al., 2017).

Glaucoma treatment options have improved throughout the years with surgical intervention being the last resort. It is important to point at that most times a single anti-glaucoma medication is not efficient enough to reach the target IOP in order to inhibit glaucoma progression. According to the Ocular Hypertension Treatment Study (OHTS), an estimated 39.7% of patients required more than a single anti-glaucoma medication to reach their target IOP (Kalouda et al., 2017; Kass et al., 2002). It is crucial to keep in mind side effects of each drug as well as patient history in order to prevent severe issues from the combination therapy. Recently, fixed combination therapy has been utilized resulting in a reduction in the number of bottles and drops used. This allows the patient to have a treatment dose of one drop of combined medication as opposed to two separate drops of different medications (Cox et al., 2008; Higginbotham, 2010; Higginbotham et al., 2002; Society, 2014). Further investigation into treatment options may assist in uncovering treatment avenues with greater efficacy in a larger group of patients to improve overall quality of life.

1.3.5 Future directions

Current anti-glaucoma medications have targets in the uveoscleral outflow pathway to increase AH outflow or reduce AH production (Figure 1.3). Although
the conventional outflow has been implicated to comprise a majority of AH outflow, it has been elucidated that the unconventional pathway can make up ~50% of outflow depending on the age and health of the individual (Toris et al., 1999). Currently, the drugs that target the conventional outflow pathway are lacking compared to the amount present that target the unconventional pathway. The fact that this outflow pathway contains many compensatory and backup mechanisms makes it difficult to find a specific drug target. Current treatment does not lower IOP efficiently enough to target IOP and in a majority of cases requires combination therapy. Future work into further understanding the molecular mechanisms underlying the conventional outflow pathway will allow for the investigation of new therapeutic targets. Gaining understanding of the TM extracellular matrix proteins/lipids, TM cell mechanotransduction, vasculature, and further characterizing animal models for investigation will provide new insights into new therapeutic approaches (Brubaker, 2003; Overby et al., 2009; Stamer, 2012).
Figure 1.3 Representative diagram of location of action of current drug therapies. Currently available drug therapies affect the unconventional outflow pathway resulting in an increase in aqueous humor outflow or a decrease in aqueous humor production. Adapted from (Carreon et al., 2016).

1.4 Specific aims of project

The pattern of outflow in the anterior chamber has been extensively studied yet the underlying molecular mechanisms remain to be elucidated. The mechanism by which TM cells are able to detect changes in IOP and adapt to these changes remains to be fully understood. As a result, the characterization of the DBA/2J and DBA/2J-Gpnmb+/SjJ mice is an important area in order to utilize these mice as a tool in glaucoma research. The primary goal of this thesis is to characterize these specific mouse models in an effort to better understand the underlying molecular mechanisms of IOP regulation as well as to understand the role the ECM protein cochlin plays in glaucoma pathology. This project proceeds with the following specific aims:

Specific Aim 1: Characterize high/low flow differences between DBA/2J and DBA/2J-Gpnmb+/SjJ mice

Specific Aim 2: Determine how the interaction of cochlin and mechanotransducing channel TREK-1 in trabecular meshwork cells influences the regulation of intraocular pressure

In the following sections, we describe the plan to address each of these specific aims in this thesis.
1.4.1 Specific aim 1

The segmental outflow in the anterior chamber results from the accumulation of resistance as previously described. Various tracer studies have demonstrated this specific flow pattern, which is comprised of high flow and low flow areas. The greatest limitation of these studies is that these tracers introduce an external component and as a result, there lacks ultimate certainty in the findings in comparison to natural in vivo outflow patterns. In order to understand the molecular make-up and mechanisms underlying segmental outflow, it is important to draw parallels between outflow in human eyes and model systems such as the murine model. The understanding of segmental outflow in DBA/2J and DBA/2J-Gpnmb+/SjJ mice will provide an avenue for further investigation into the molecular mechanisms surrounding outflow resistance as well as open the doors for research into future therapeutic avenues.

In Chapter 2, we utilize Q-tracker 655 beads in order to characterize segmental outflow in DBA/2J and DBA/2J-Gpnmb+/SjJ mice. We further characterize molecular differences between high flow and low flow areas using vascular markers in these mice. Taken together we draw parallels between human outflow and the DBA/2J mouse model as well as the DBA/2J-Gpnmb+/SjJ mouse model for further investigation into outflow resistance.
1.4.2 Specific aim 2

The change of ECM proteins in the eye is a major contributor to segmental outflow regulation and overall resistance. The expression levels and distribution differs from protein to protein. The ECM protein cochlin was identified to be uniquely present in glaucomatous TM compared to normal and followed a segmental distribution similar to the segmental outflow pattern (Bhattacharya et al., 2005a; Bhattacharya et al., 2005b). It has previously been shown that TM cells detect changes in aqueous humor fluid shear stress via the interaction of cochlin with the stretch-activated channel TREK-1 on the cell surface (Goel et al., 2011). Whether this interaction is a component of IOP regulation remains to be understood.

In chapter 3, we investigate how the interaction of cochlin and mechanosensitive channel TREK-1 in TM cells influences IOP regulation. We utilize biochemical techniques and functional assays to elucidate the molecular mechanisms of the cochlin/TREK-1 interaction. We demonstrate the importance of the interaction of cochlin and TREK-1 in maintaining overall IOP homeostasis.
Chapter 2: Characterization of high/low flow differences between DBA/2J and DBA/2J-Gpnmb+/SjJ mice

The following work encompassing all of chapter 2 has been published in *Experimental Eye Research* (Carreon et al., 2016) and *Progress in Retinal and Eye Research* (Carreon et al., 2017a). Segmental outflow has been identified and studied using tracers in human eye perfusion models, monkey eyes (Bill, 1965, 1975, 1977; Bill and Hellsing, 1965; Cha et al., 2016; de Kater et al., 1989; Epstein and Rohen, 1991; Vranka et al., 2015), and the murine model (Swaminathan et al., 2013). The fact that these tracers bring an external component to these studies results in limitations that do not allow for a complete comparison to mechanisms that occur in natural in vivo outflow patterns. The ability to draw parallels between DBA/2J and DBA/2J-Gpnmb+/SjJ mice outflow patterns and human outflow patterns will add an additional tool into the scientific arsenal to further investigate the mechanisms surrounding outflow resistance.

2.1 Purpose/Rationale

2.1.1 DBA/2J and DBA/2J-Gpnmb+/SjJ mice models

The DBA/2J mouse model is one of the most well characterized models that exhibit chronic age-related glaucoma phenotypes (John et al., 1997; John et al., 1998). This mouse is homozygous for mutations in two separate genes, the b allele of tyrosine related protein (Tyrp1b) and the transmembrane glycoprotein Gpnmb. These mice typically exhibit pathology in the anterior chamber with
pigment dispersion and iris stromal atrophy (Howell et al., 2007). In general, iris disease takes place at 6-8 months of age and shortly after an increase in IOP is exhibited (Libby et al., 2005). At the onset of high IOP at around 8 months of age, studies have shown that damage to the optic nerve is evident with axonal loss and optic nerve gliosis (Libby et al., 2005; Schlamp et al., 2006). Retinal degeneration in these mice is evident at around 10-12 months of age (Schlamp et al., 2006). Recently, we have identified a cohort of DBA/2J mice (5.3% of total mice) in which pigment dispersion is negligible and the angle remains open yet the IOP is elevated. These results were confirmed with a double Gonio lens microscope, optical coherence tomography (OCT), and slit lamp analyses. Furthermore, endpoint Fontana-Masson staining confirmed the lack of pigmentary dispersion within this cohort (Wang et al., 2015). This subset of mice are ideal candidates for a model of glaucoma pathology.

The DBA/2J-Gpnmb+/SjJ mouse model is a genetic match to the DBA/2J mouse model. The DBA/2J-Gpnmb+/SjJ mice are homozygous for the wild-type allele of Gpnmb on a DBA/2J genetic background. These mice do not exhibit a spontaneous increase in IOP between 4 months – 23 months of age and only have a mild form of iris disease resulting from the Tyrp1b mutation. Interestingly, these mice also lack damage to the optic nerve, which is commonly seen with DBA/2J mice at ~12 months of age (Howell et al., 2007). The lack of pathology in the DBA/2J-Gpnmb+/SjJ mice yet genetic similarity to the DBA/2J mice make them an ideal experimental control for studies involving DBA/2J mice.
The most prominent advantage in using the DBA/2J mouse is the ease of manipulation of certain genes and molecular pathways in glaucoma pathology. It is important to point out that these mice do exhibit variation in the development of the disease resulting in the need for careful selection to occur for experimental studies (Schlamp et al., 2006). Taken together the use of these mice strains in tandem can result in strong experimental results. The fact that the DBA/2J-Gpnmb+/SjJ mouse is a genetic matched control to the DBA/2J mouse adds a control element to studies that strengthens the evidence. The aim of this study is to characterize the segmental outflow in these mice to add more components to their use as a mouse model to investigate glaucoma pathology.

2.2 Methods

The University of Miami IACUC approved all study protocols accordingly. All methods were carried out in accordance with the approved guidelines. DBA/2J and DBA/2J-Gpnmb+/SjJ inbred mice were obtained from Jackson Laboratory (Bar Harbor, ME). Colonies were established at the Animal Facility of the University of Miami, Miller School of Medicine (Miami, FL) in a controlled environment. The animals were kept in rooms with 12 h light (6 AM – 6 PM)/dark (6 PM – 6 AM) cycles and were fed ad libitum.
2.2.1 Q-tracker 655 to track high flow/low flow areas

DBA/2J and DBA/2J-Gpnmb+/SjJ mice of varying ages (3, 6, 8 months) were anesthetized using intraperitoneal injection of ketamine (100 mg/kg) and xylazine (9 mg/kg). Following anesthesia, mice were given an intracameral injection of Q-tracker 655 fluorescent microspheres (Invitrogen) diluted 1:1000 in PBS. The mice were then placed on a heating pad and allowed to incubate with microspheres for ~2 hours. Following incubation, mice were euthanized with carbon dioxide adhering to animal procedures approved by the IACUC. Eyes were harvested and whole globes were imaged en face using a Leica DM 6000 B confocal microscope (Swaminathan et al., 2013).

2.2.2 Assessing vessel markers

The whole globes that were imaged were then treated with a sucrose gradient at 15%, 20%, and 30% sucrose for 1 hour each treatment. This was then followed by immersion into 1% paraformaldehyde for 24 hours for fixation. The eyes were then placed into OCT freezing medium (Sakura Finetek) and flash frozen. Tissue was sectioned and prepared for immunohistochemistry. Sections were blocked with 1XPBS + 0.2% bovine serum albumin (BSA) (EMD Chemicals) for 30 minutes and incubated overnight with primary antibodies for specific vessel markers (LYVE-1, CD31, podoplanin) at a 1:200 dilution. Tissue was washed 3 times with 1XPBS + 0.2% bovine serum albumin (BSA) at 10 minutes each and incubated for 1 hour with secondary antibodies at a 1:1000 dilution. The tissue
was washed again 3 times at 10 minutes each with 1XPBS + 0.2% bovine serum albumin (BSA). Imaging of the sections was done using a Leica DM 6000 B confocal microscope.

2.3 Results

2.3.1 Differences in outflow pathways (3, 6, 8 months DBA/2J and DBA/2J-Gpnmb+/SjJ mice)

The subset of DBA/2J mice previously described to lack pigmentary dispersion but exhibit elevated IOP have been termed as “pure ocular hypertensive” mice. This cohort accounts for 5.3 percent of total mice with 18 – 24 percent of hypertensive mice between 8 – 9 months of age and exhibit an open angle as well (Carreon et al., 2016; Wang et al., 2015). An advantage in using these mice is the availability of a genetic matched control in the DBA/2J-Gpnmb+/SjJ mice. The DBA/2J-Gpnmb+/SjJ mice lack the spontaneous increase in IOP and the characteristic neuropathy seen in DBA/2J mice (Howell et al., 2007). Previously, it has been shown through pattern electroretinogram (PERG) analyses (Porciatti et al., 2010) and lipid profile analyses (Wang et al., 2015) that there are marked functional as well as compositional differences between these mice. We aim to further characterize differences in segmental outflow in the pure ocular hypertensive DBA/2J and DBA/2J-Gpnmb+/SjJ mouse model.

We have identified differences in the distribution of high flow and low flow areas in 8 month old (IOP ≥ 18 mm of Hg) DBA/2J mice when compared to age
matched DBA/2J-Gpnmb+/SjJ mice (Figure 2.1). There was an evident reduction in high flow regions in 8 month old DBA/2J mice up to about 11-12 months of age, which is expected as IOP increase is observed at this time. In the DBA/2J-Gpnmb+/SjJ mice, outflow regions were more uniform with respect to flow distribution in mice aged 3 – 12 months of age. High flow regions seem to stay consistent in 3 month, 6 month, and 8 month DBA/2J-Gpnmb+/SjJ mice whereas in DBA/2J mice low flow regions dominate even in the 3 month old mice (Figure 2.1A). These differences demonstrate the importance of these mice in segmental outflow investigation (Carreon et al., 2016). Characterization of these mice involved an assessment of vessel markers in high flow and low flow regions.
Figure 2.1 Representative en face images of segmental outflow patterns in DBA/2J and DBA/2J-Gpnmb+/SjJ mice. A) Q-tracker 655 injected into the anterior chambers of 3, 6, 8 months old DBA/2J mice and their genetic counterpart DBA/2J-Gpnmb+/SjJ mice. High flow and low flow areas are visualized to depict the segmental outflow pattern in the anterior chamber \( n = 30 \) for each strain \( (n = 15 \) male and \( n = 15 \) female)]. A significant difference in distribution of high flow and low flow regions is observed in these strains. The noticeable lack of high flow regions in the DBA/2J mice corroborates with the blockage of fluid flow as being segmental consistent with observations in human glaucomatous eyes.

2.3.2 Differences in vessel markers (3, 6, 8 months DBA/2J and DBA/2J-Gpnmb+/SjJ mice)

The flow pattern in the eye is understood to follow a segmental pattern yet the molecular mechanisms underlying this flow pattern are not well understood. Certain proteins take on different expression patterns in the eye, some correlating with the segmental outflow pattern (Vranka et al., 2015). Differences in high flow and low flow regions have been demonstrated in perfused human eyes. Only recently have these regions been further characterized by studying gene expression differences. Specific collagen genes \( (\text{COL1A1, COL4A2, COL6A1, COL6A2, and COL16A1}) \) and some matrix metalloproteinases \( (\text{MMP1, 2, 3, and 12}) \) have been shown to follow an enriched pattern in high flow regions in perfused human eyes (Vranka et al., 2015). In order to further elucidate the underlying molecular mechanisms contributing to segmental outflow it is essential to further understand differences in DBA/2J and DBA/2J-Gpnmb+/SjJ mice.

The lymphatic system is a unique network in comparison to blood vessels (Witte et al., 2006). In the eye, there are conflicting results for the potential
contribution of lymphatics to aqueous humor clearance (Bradbury and Cole, 1980; Cole and Monro, 1976; Gausas et al., 1999; Gruntzig et al., 1977; Gruntzig et al., 1979; Klaushofer and Pavelka, 1975; Werner et al., 1987). Previously, the lack of lymphatic markers provided an obstacle in the investigation of this system (Yucel et al., 2009). Contrary to the lymphatic system, the vasculature in the eye has been studied rather extensively. The maintenance of the vasculature contributes to the homeostasis of the basement membrane (BM) in key structures in the eye, such as the TM (Carreon et al., 2017a). Vascular growth is dependent on growth cues from the BM (Warren and Iruela-Arispe, 2014) as well as the composition of the BM (provisional BM or assembled BM) (E.R. Clark, 1938). In order to characterize the high flow and low flow areas in DBA/2J and DBA/2J-Gpnmb+/-SjJ mice, lymphatic as well as vascular markers were used.

The markers utilized for this study were lymphatic endothelial hyaluronan receptor -1 (LYVE-1), podoplanin, and CD31 (Carreon et al., 2017a). LYVE-1 and podoplanin are both used extensively as lymphatic markers (Banerji et al., 1999; Herwig et al., 2014; van der Merwe and Kidson, 2014; Yucel et al., 2009) while CD31 is used as a vascular marker (Kizhatil et al., 2014; van Setten et al., 2016). Prior to our study, the limbal vasculature and aqueous vessels were studied in the murine model (C57BL/6J mice) (van der Merwe and Kidson, 2014). LYVE-1 was absent from the endothelial cells lining Schlemm’s canal in mice but was present in the lymphatic vessels in the limbus (van der Merwe and Kidson, 2014). Podoplanin is expressed in the conjunctiva (Cursiefen et al., 2002; Fogt et al., 2004; Regina et al., 2007), corneal epithelium as well as the endothelium
(Cursiefen et al., 2002), TM, and the iris of human adult eyes (Birke et al., 2010; Hamanaka et al., 2011; Watanabe et al., 2010).

To further investigate the high flow and low flow areas in the DBA/2J and DBA/2J-Gpnmb+/SjJ mice, immunohistochemistry was performed probing for LYVE-1, podplanin, and CD31 (Figure 2.2). High flow areas in the hypertensive DBA/2J mice expressed high levels of LYVE-1 and CD31 (Figure 2.2B). Age matched DBA/2J-Gpnmb+/SjJ mice showed the same expression pattern for these markers (data not shown). Low flow areas exhibited a markedly lower expression of LYVE-1 and CD31. Podoplanin exhibited low expression in both the high flow and low flow areas (Figure 2.2B). This data demonstrates the differences in lymphatic and vessel marker distribution in the distal outflow regions in relation to high flow/low flow areas specifically in DBA/2J and DBA/2J-Gpnmb+/SjJ mice.

Figure 2.2 Characterization of high flow and low flow areas in DBA/2J mice. A) Visualization of regions of high flow and low flow in a DBA/2J hypertensive mouse using Q-tracker 655 with trabecular meshwork (TM) and Schlemm’s canal (SC) as indicated demonstrates a non-uniform outflow pattern. B) High flow areas
in this DBA/2J hypertensive mouse demonstrate increased levels of LYVE-1 (green) with DAPI (blue) to stain the nuclei and CD31 (cyan) in contrast to low flow areas. Podoplanin (magenta) expression remains consistent between high flow and low flow areas. *Adapted from (Carreon et al., 2017a).*

**2.4 Discussion**

Segmental outflow can be described as a dynamic mechanism resulting from changes in the ECM, BM, and overall resistance in the outflow pathway. The identification and characterization of segmental outflow areas in the murine model (Figure 2.1 & 2.2), specifically in glaucoma models (DBA/2J and DBA/2J-Gpnmb+/SjJ mice), allows for further investigation into impeded aqueous humor outflow. Future research will provide insight into the influence potential segmental blockage may have on impeded outflow in glaucoma.

The investigation of various lymphatic and vascular markers within these areas (Figure 2.2B) provides insight into the molecular make-up of the high flow and low flow areas in the DBA/2J and DBA/2J-Gpnmb+/SjJ mouse model system. Taken together with previous investigations characterizing the limbal angioarchitecture (van der Merwe and Kidson, 2014; Yucel et al., 2009), this data corroborates the use of this specific model for investigation of segmental outflow. This investigation further supports the use of the DBA/2J and DBA/2J-Gpnmb+/SjJ mice as model systems for glaucoma and the investigation of the molecular mechanisms underlying segmental outflow.
Chapter 3: The interaction of cochlin and mechanotransducing channel

**TREK-1 in trabecular meshwork cells influences the regulation of intraocular pressure**

The following work encompassing all of chapter 3 has been published in *Scientific Reports* (*Carreon et al., 2017c*). The regulation of intraocular pressure depends on the contribution from a number of factors. These same components may also result in the segmental outflow pattern observed in the eye. Although a number of proteins such as versican, SPARC, and myocilin have been investigated, there lack studies into the role of the ECM protein cochlin in fluid flow regulation in the eye.

### 3.1 Purpose/Rationale

The ECM protein cochlin, as previously mentioned, was found to be uniquely present in abundance in the TM of glaucomatous individuals. Cochlin deposit formation was identified in the glaucomatous samples as well (*Bhattacharya et al., 2005c*). Further investigation has demonstrated the ability of cochlin to assist in IOP regulation in DBA/2J-Gpnmb+/SjJ mice (*Goel et al., 2012*). Previous work has also suggested syn-expression of cochlin and the stretch activated channel TREK-1 in human TM cells (*Goel et al., 2011*). Taken together, the interaction of cochlin and TREK-1 is an area of interest in the regulation of fluid flow mechanisms in the eye.
3.1 Cochlin as a mechanosensor in the eye

Cochlin was first identified in the cochlear and vestibular labyrinths of the ear. Mutations in the COCH (coagulation factor C homology) gene, which encodes cochlin, has been linked to the development of the hearing loss and vestibular disorder DFNA9 (Robertson et al., 1994; Robertson et al., 1998; Robertson et al., 1997). Further mutations have been implicated in some cases of Menière disease (Fransen et al., 1999) as well as presbyacusis (age related hearing loss) (de Kok et al., 1999).

The mature form of cochlin consists of a signaling peptide, an N-terminal factor C homology domain (FCH), and two von Willebrand factor A-like domains (vWFA1 and vWFA2) (Figure 3.1). Mutations that cause the diseases previously described are primarily present on the FCH domain. In bacteria, these mutations result in misfolding and aggregation of expressed FCH domains (Liepinsh et al., 2001). Human cochlin’s protein structure is highly conserved with 94% amino acid sequence identity to mouse protein and 79% identity to chicken protein. This secreted protein has three glycosylated isoforms with masses of ~40, ~46, and ~60 kDa (Ikezono et al., 2004; Robertson et al., 2003; Robertson et al., 2001). The isoforms with the lower mass (~40 and ~46 kDa) contain the vWFA-like domains but lack the amino-terminal FCH domain, specifically the area in which mutations occur (Robertson et al., 2003). Cochlin is thought to contribute to the architecture of the cochlea by binding components of the ECM (Robertson et al., 1998; Robertson et al., 1997). Interestingly, cochlin expression increases in the
TM of POAG patients with age followed by an increase in cochlin deposits around Schlemm's canal (Bhattacharya et al., 2005c).

Figure 3.1 Representative image of cochlin domains. Cochlin is comprised of a signaling peptide, an FCH domain, and two von Willebrand Factor A-like (vWFA) domains. The vWFA domains may contribute to the ability of cochlin to act as solution-phase mechanosensor in the trabecular meshwork.

3.1.1.1 Intraocular pressure regulation

The presence of cochlin in glaucomatous TM (Bhattacharya et al., 2005c) as well as in DBA/2J mouse TM (Bhattacharya et al., 2005a) begs the question; does cochlin play a role in IOP regulation? Because of the varying changes in shear stress to maintain IOP, cells exhibit a mechanism that allows them to detect and accommodate themselves to these changes. The properties that cochlin exhibits as a result of the vWFA-like domains, which will be discussed in detail in the next section, may allow it to perform mechanosensing mechanisms in the TM.

The ability of cochlin to regulate intraocular pressure has been demonstrated in the murine model. Overexpression of cochlin in DBA/2J-Gpnmb+/SjJ mice elicited a rise in IOP, which peaked between 8-30 days and appeared to be concomitant with cochlin expression (Goel et al., 2012). A protein
of a similar size, human serum albumin (HSA) did not elicit an increase in IOP demonstrating that cochlin’s effect is more than just a result of its size. Furthermore, silencing of cochlin using shRNA in 8-month-old DBA/2J mice, a strain that exhibits an increase in IOP at this age, showed a decrease in IOP for up to two months following injection (Goel et al., 2012). Taken together these results demonstrate cochlin’s ability to influence IOP regulation although we still lack full understanding of the underlying molecular mechanisms.

3.1.1.2 Von Willebrand Factor A (vWFA) – like domains and cochlin

*multimerization under shear stress*

The two vWFA-like domains present in cochlin, are thought to play a key role in the protein’s mechanosensing ability. These domains exhibit characteristics similar to von Willebrand factor (VWF) in the hemostatic system. In the blood and lymphatic systems, VWF is a key component in hemostasis and thrombus formation. VWF is a highly adhesive, multimeric glycoprotein found in plasma and produced in endothelial cells as well as megakaryocytes. There are varieties of VWF sizes ranging from low to ultra-large molecular weights (Budde et al., 2006; Goodeve, 2010). Each of these multimers has different hemostatic function based on their weights. Detection of changes in shear stress in their environment results in cleavage of these multimers by proteases such as ADAMTS13 (Levy et al., 2001; Siedlecki et al., 1996). Following cleavage, these multimers go on to perform a variety of hemostatic functions. Aggregation of VWF occurs because of induced hydrodynamic forces in solution and may act as
an influencing factor on cell adhesion rates in circulation (Shankaran et al., 2003). VWF has the ability to act as a mechanosensor to adapt to changes in shear stress. The vWFA-like domains present on cochlin allows us to draw parallels to its function and the function of VWF in the hemostatic system.

The vWFA-like domains likely contribute to cochlin deposit formation in the TM because of an increase in hydrostatic forces. This characteristic is shared with VWF in the hemostatic system. Previously, it has been shown that application of shear stress to purified recombinant cochlin induced aggregation. This aggregation is likely comprised of disulfide bond formation and interactions with the surrounding ECM. In glaucomatous TM, cochlin may remain for a prolonged period of time allowing it to interact through its vWFA-like domains to cause aggregation and further interaction with fibrillar collagens. This interaction with fibrillar collagens may result in collagen degradation accounting for the increase in cochlin and decrease in collagen levels through aging (Marchant et al., 1996; Pareti et al., 1987). Further corroborating this, cochlin itself demonstrates an age – dependent (40 – 85 years old) increase in its presence in glaucomatous TM although it is produced constitutively in normal TM (Bhattacharya et al., 2005c). Cochlin exhibits the necessary components to act as a mechanosensor in the TM and play a role in glaucoma pathology. It is important to understand that this mechanosensor does not work alone but needs the help of a cell-surface mechanotransducer to deliver signals to the TM cells.
3.1.2 **TREK-1 as a cell-surface mechanotransducer**

TWIK-related K+ 1 channel (TREK-1) is a mechanosensitive stretch activated potassium channel (Chemin et al., 2005) and the most extensively studied K_{2P} channel. This specific class of channel is important in setting the resting potential of a variety of different cell types (Patel and Honore, 2001). Interestingly, TREK-1 has unique gating properties allowing it to be activated by mechanical stimulation, intracellular acidosis, and warm temperature (Honore et al., 2002; Kang et al., 2005; Lesage and Lazdunski, 2000; Maingret et al., 2000; Patel et al., 1998). TREK-1 activation is known to alter the actin cytoskeleton architecture of the cell and is actively involved in the formation of actin-rich membrane protrusions (Chemin et al., 2005; Lauritzen et al., 2005). Interestingly, TREK-1 mRNA is expressed at the mRNA level in the TM. Further validation demonstrated the presence of TREK-1 at the protein level in the TM (Tran et al., 2014). The presence of TREK-1 in the TM and the characteristics of its function make it an ideal candidate as a mechanotransducer on the TM cell surface.

3.1.3 **Proposed model for mechanotransduction of TM cells in response to changes in shear stress**

The characteristics exhibited by cochlin as well as TREK-1 allude to a potential interaction between the two in the transduction of mechanosensing. This transduction may in turn lead to the remodeling of the TM cell cytoskeleton and overall cellular architecture to accommodate for changes in shear stress.
TREK-1 and cochlin co-localization has been demonstrated previously in TM cells (Goel et al., 2011). Although cochlin regulation has been demonstrated to influence IOP and excess cochlin expression has been linked with an increase in IOP (Goel et al., 2012), the direct interaction with TREK-1 for mechanotransduction has not been fully shown. We hypothesize a model in which cochlin acts as a soluble mechanosensor. After changes in shear stress cochlin multimerizes and interacts with TREK-1 on the TM cell surface resulting in changes in TM cell architecture to accommodate the changes in shear stress. The following evidence demonstrates the necessary presence of both cochlin and TREK-1 in IOP regulation as well as the difference in electrophysiological properties between monomeric and multimeric cochlin (Carreon et al., 2017c).

3.2 Methods

The University of Miami IACUC approved all animal study protocols. All methods were carried out in accordance with the approved guidelines. DBA/2J and DBA/2J-Gpnmb+/SjJ inbred mice were obtained from Jackson Laboratory (Bar Harbor, ME). Colonies were established at the Animal Facility of the University of Miami, Miller School of Medicine (Miami, FL) in a controlled environment. The animals were kept in rooms with 12 h light/dark cycles and were fed ad libitum.
3.2.1 Cochlin and TREK-1 manipulation in DBA/2J-Gpnmb+/SjJ mice

A cochlin transgene bearing lentivirus was constructed in HEK293T cells (ATCC). The cochlin gene (COCH) used to produce the cochlin expression vector was human (NM_004086). The cochlin expression clone (GeneCopoeia Inc.) was packaged into a lentivirus vector using the Lenti-Pac-FIV expression packaging kit provided by the manufacturer. The protocol provided a yield of $10^7$ infectious units/mL of the recombinant lentivirus.

TREK-1 downregulation was done using a TREK-1 shRNA virus made in HEK293T cells using the Trans-Lentiviral™ GIPZ Packaging System (Open Biosystem,) following the protocol provided by the manufacturer. This procedure typically yielded a viral stock of $10^8$ infectious units/mL. The shRNA was a set of 5 clones and transfection efficiency was determined to be 70%. This efficiency was validated using equal amounts of protein in an SDS-PAGE followed by TREK-1 expression detection via western blot. The membrane was stripped and re-probed for GAPDH to confirm equal loading.

DBA/2J-Gpnmb+/SjJ mice were given an intracameral injection targeting the TM with the cochlin overexpression vector alone or together with the TREK-1 shRNA vector. Mice IOP was measured prior to injection and throughout the course of the study with a hand held tonometer, TonoLab (Colonial Medical Supply). Prior to IOP measurement or intracameral injections, mice were anaesthetized with an intraperitoneal injection (0.1 µL) of ketamine (100 mg/kg) and xylazine (9 mg/kg). Failure to respond to touch indicated adequate anesthesia.
3.2.2 Fluorescein transport assay and rat-tail collagen expansion assay

The fluorescein transport assay was performed in an Ussing-type chamber (World Precision Instruments Inc.). Fluorescein flow (fluorescein dye) was measured across polyvinylidene fluoride (PVDF) membranes (Pall Life Sciences) layered with transfected TM cells. The TM cells were cultured on the PVDF membranes following incubation of PVDF membranes with rat-tail collagen (BD Biosciences) to facilitate cell adherence. The cells were allowed to form a confluent monolayer over a 16-24h period following the addition of multiple cell layers to achieve a confluent tri-layer of cells. The cells were transfected with the DNA of interest (TREK-1+cochlin, TREK-1+RPE65, TREK-1 only, transfection agent (Lipofectamine 2000, Invitrogen, Inc.), or non-transfected control). Following 24-36h post-transfection the membranes were placed in the Ussing-type chamber between the hemi-chambers. The entire apparatus was connected to a single-channel peristaltic pump (Watson-Marlow) with 1XPBS as the bathing medium. A set volume of fluorescein dye (1:100 dilution of 1 mg/mL) was added to one hemi-chamber. After 5 minutes, an equal volume was aspirated from the opposite side of the membrane at the opposing hemi-chamber opening and the fluorescein concentration was measured using a spectrophotometer.

The rat-tail collagen expansion assay was performed following a published procedure (Ilagan et al., 2010). The hydrogel solution was prepared in a serum-free environment with the following reagents: 10XMEM (Invitrogen), sodium bicarbonate (Sigma-Aldrich), L-glutamine (Sigma-Aldrich), and HEPES buffer (Invitrogen). Separate Eppendorf tubes were prepared with the hydrogel solution
for each transfection type. The transfection complexes were prepared in individual tubes containing a mixture of the transfection agent (Lipofectamine 2000, Invitrogen) and the desired DNA vectors with a transfection agent ratio (w/v) of 0.4µg/µL. TM cells were removed at >90% confluence from 25 cm² cell culture flasks (Becton Dickinson) using trypsin. Cells were centrifuged for 5 minutes at 800 rpm (77xG), supernatant was removed, and the cell pellet was re-suspended in serum-free 1XDMEM culture media (Corning). The cell suspension was then aliquoted into each respective transfection-complex tube. The reaction was incubated for 45 minutes followed by termination via the addition of cell culture media (1XDMEM+10%FBS). The suspension was gently mixed in the prepared hydrogel solution and the rat-tail collagen to initiate gel polymerization. Using a 1 mL single use syringe (Henk-Sass-Wolf), the suspension was injected into a borosilicate glass capillary tube with an inner diameter of 0.75 mm (WPI) to approximately half of its volume. Capillary tubes were placed on a black background with a millimeter scale and a digital picture was taken following the hydrogel injection. The capillary tubes were incubated in a moisture chamber to prevent dehydration in a cell culture incubator at 37°C and 5% CO₂. Pictures were taken every 24-48h and lengths were measured using NIH ImageJ (v.1.43u) software. Statistical analysis was done using Microsoft Excel 2007 (Microsoft Corp.).
3.2.3 Immunocytochemistry to visualize changes in the actin cytoskeleton

Normal human TM cells (NTM) were cultured in 12 well plates (Corning) on circular microscope cover slides (VWR International) with serum-free cell culture media (1XDMEM, Corning). Cells were incubated for 24h in an incubator at 37°C and 5% CO₂. Following the 24h incubation, monomeric or multimeric cochlin was added at a concentration of 10µg per well to specific wells. Multimeric cochlin was produced using a 30-gauge syringe and repetitive passage of monomeric cochlin through the syringe 15-20 times before addition to the wells. Following 24h incubation, cells were washed 3 times with 1XPBS. Cells were then fixed with 1% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes. Following fixation, cells were washed 3 times with 1XPBS and blocking solution (1XPBS+0.2% bovine serum albumin (BSA) (EMD Chemicals)) was added to the cells for 30 minutes. The primary antibody for cochlin (hCochlin#3 Aves Labs Inc.) was added at a 1:200 dilution and incubated overnight at 4°C. After the overnight incubation, cells were washed 3 times for 10 minutes each with block solution and placed into the corresponding secondary antibody (Donkey anti-chicken FITC, Abcam) at a 1:1000 solution. Cells were incubated for 1h at room temperature. After incubation, cells were washed 3 times for 10 minutes each with block solution. Following the washes, cells were incubated with 100nM rhodamine phalloidin (Cytoskeleton) for 30 minutes to stain the actin cytoskeleton. Cells were then washed 3 times for 10 minutes each with 1XPBS. The coverslips were mounted on glass microscope slides (VWR International) and stained with DAPI Vectashield (Vector Laboratories). The
slides were imaged using a Leica DM 6000 B confocal microscope (Leica, Inc.). The intensity of fluorescence was measured as a relative arbitrary unit under the same settings and conditions for each sample using ImageJ software. The cellular conformation was confirmed via individual counting performed by five different individuals blinded to cell treatment. The overall averages were taken from these calculations and presented as an arbitrary measurement of cells/area.

3.2.4 Immunocytochemistry on arachidonic acid (AA) treated TM cells

Normal human TM cells (NTM) were cultured in 12 well plates as described in the previous section (3.2.3). Specific wells of confluent cells were treated with 20µM AA diluted in serum-free DMEM media. Before AA treatment, cells were incubated with 5µM of indomethacin, a cyclooxygenase inhibitor, for 30 minutes in order to prevent AA metabolism. Following treatment of both compounds, cells were washed 3 times with 1XPBS and fixed with 1% paraformaldehyde for 15 minutes. Cells were then washed 3 times with 1XPBS and placed into block solution (as described in section 3.2.3) for 30 minutes. The primary antibody for TREK-1 (Santa Cruz Biotechnology, Inc.) was added at a 1:200 dilution and incubated overnight at 4°C. Cells were washed 3 times at 10 minutes each with block solution and placed into the corresponding secondary antibody (Goat anti-mouse Alexa Fluor 488, Invitrogen Molecular Probes) at a 1:1000 dilution for 1h at room temperature. Following the secondary antibody incubation, cells were washed 3 times for 10 minutes each with block solution. Cells were then incubated with 100nM rhodamine phalloidin for 30 minutes to
stain the actin cytoskeleton. Following staining, cells were washed 3 times for 10 minutes each with 1XPBS. The coverslips were placed on microscope slides, stained with DAPI Vectashield, and imaged using a Leica DM 6000 B confocal microscope. Intensity of fluorescence was measured as a relative arbitrary unit under the same settings and conditions for each sample using ImageJ software.

3.2.5 Functional assays: whole-cell patch clamp

All the whole-cell patch clamp experiments were performed in collaboration with Aida Castellanos and Xavier Gasull at the University of Barcelona, Barcelona, Spain. HEK293T cells and normal human TM cells (NTM) were cultured on 12-mm dishes at 37°C and 5% CO₂ in cell culture media (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine). Cells were transiently transfected with pIRES2-EGFP vector alone (control) or with pEZ-Lv205-hTREK-1 (GeneCopoeia, Inc.) using the FuGene transfection reagent (Roche), according to the manufacturer instructions. Patch-clamp experiments were performed on the cells 24-48h after transfection. Specifically for the TM cells, the K2 Transfection System from Biontex Laboratories GmbH (Planegg/Martinsried) was used according to manufacturer instructions.

The electrophysiological recordings were performed using a patch-clamp amplifier (Axopatch 200B, Molecular Devices). The patch electrodes were fabricated in a Flaming/Brown micropipette puller P-97 (Sutter instruments). The electrodes maintained a resistance between 2-4MΩ when filled with intracellular
solution. The intracellular solution consisted of the following (in mM): 135 KCl, 2.1 CaCl₂, 2.5 MgCl₂, 5 EGTA, 2.5 ATP, 10 HEPES at pH 7.3. The bath solution consisted of the following (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES at pH 7.4. The overall osmolality of the isotonic solution was adjusted with sorbitol to ≈300 mOsm/Kg. For the C-type gate experiments performed in high K⁺ solution, the following was used (in mM): 15 NaCl, 135 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES at pH 7.4.

The membrane currents were recorded using the whole-cell patch clamp configuration, filtered at 2kHz, digitized at 10 kHz, and acquired with pClamp 10 software. The data was analyzed with Clampfit 10 (Molecular Devices) and Prism 4 (GraphPad Software, Inc.). The series resistance was kept below 15MΩ and compensated at 70-80%. All recordings were performed at room temperature and the shear stress stimulation in transfected cell lines was achieved by increasing the overall bath perfusion rate from 0 or 0.1 mL/min (baseline) to 1 mL/min (shear stress). Cells in the whole cell configuration were held at -60 mV and 1s voltage ramps from -100 to +50 mV were used to record the TREK-1 mediated current. For all patch clamp experiments, monomeric cochlin (Mo) was diluted in recording solution in the absence of Ca²⁺. Multimeric cochlin was produced by passing the monomeric cochlin through a 30-gauge syringe as previously described (section 3.2.3) 15-20 times before adding to the well (Mu,shear) or by adding Ca²⁺ to the solution where cochlin was prepared (Mu,Ca²⁺). Arachidonic acid with a final concentration of 20µM was added to the bath.
3.3 Results

3.3.1 Cochlin and TREK-1 modulate intraocular pressure

The overexpression of cochlin in DBA/2J-Gpnmb+/SjJ mice elicits an increase in IOP (Goel et al., 2012). These mice are a genetically matched strain to the DBA/2J mouse but do not develop elevated IOP or glaucomatous optic nerve damage (Howell et al., 2007), as mentioned in the previous sections. We silenced TREK-1 in DBA/2J-Gpnmb+/SjJ mouse TM with the use of shRNA (Figure 3.2). The TREK-1shRNA reduced TREK-1 expression by ~70% (Figure 3.2B) compared to controls. In the mice, silencing of TREK-1 prevented the previously observed cochlin-induced increase in IOP compared to control animals injected with the cochlin overexpression vector alone (n=6; p<0.016). This provides evidence that both cochlin and TREK-1 are needed for IOP regulation. The silencing of TREK-1 without cochlin overexpression maintained a lower IOP as observed with cochlin overexpression and TREK-1shRNA in combination (n=6; p<0.016) (Figure 3.2A). The influence these two components have on IOP regulation in the DBA/2J-Gpnmb+/SjJ mice, led us to investigate the spatial changes that may occur in the ECM of the TM cells because of their interaction.
Figure 3.2 TREK-1 silencing alters the increase in IOP observed with cochlin overexpression. **A)** Cochlin expression lentivirus (▲), cochlin expressing lentivirus with TREK-1 shRNA (■), or TREK-1 shRNA alone (♦) were injected into the TM of DBA/2J-Gpnmb+/SjJ mice. An analyses of variance (ANOVA) confirmed a statistically significant difference amongst the three cohorts. Cochlin alone produced an increase in IOP statistically different from that observed with cochlin+TREK-1 shRNA (n = 6; *p<0.016) and TREK-1 shRNA only (n = 6; *p<0.016) treated groups according to Scheffe’s post hoc test. **B)** Representative western blot validating the effectiveness of the TREK-1 shRNA using control or TREK-1 shRNA treated mice (DBA/2J-Gpnmb+/SjJ) TM as indicated. Stripping was performed and the membrane was probed with a second antibody for GAPDH in order to validate equal protein loading. Adapted from (Carreon et al., 2017c).
3.3.2 Cochlin and TREK-1 act synergistically to modulate trabecular meshwork cells

Cochlin in glaucomatous TM is thought to be in its multimeric form as a result of shear stress or oxidative stress. This increase in cochlin expression seems to have an effect on TREK-1 expression. Interestingly, multimerized cochlin results in an increase in TREK-1 expression compared to monomeric cochlin in western blot analyses (Figure 3.3A). Further validation using a co-immunoprecipitation demonstrated that similar amounts of cochlin exposed to shear stress are associated with greater amounts of TREK-1 in human TM samples (Figure 3.3B). We established the influence that multimeric cochlin has on TREK-1 expression alluding to an interaction between the two molecules. We next sought to investigate the influence these two molecules had on TM cells.

The use of two techniques, the fluorescein dye transport assay and rat-tail collagen expansion assay allowed for the investigation of the influence the cochlin-TREK-1 interaction had on cellular organization within the TM cells (Figure 3.3C & D). Fluorescein dye transport was measured across a trilayer of TM cells cultured on a PVDF membrane (Figure 3.3C) in an Ussing-type chamber. Cells were transfected with TREK-1+cochlin (monomeric), TREK-1+Retinal Pigment Epithelium-Specific Protein 65 kDa (RPE65), TREK-1 alone, TREK-1shRNA, or were non-transfected controls. The RPE65 in this experiment served as a control since it is similar in molecular size to cochlin. The filter alone allowed for a large amount of dye to pass through while the addition of cells reduced the passage significantly. The TM cells transfected with TREK-1+cochlin
showed a significant increase in dye transport compared to the other cohorts of transfected cells (TREK-1+Retinal Pigment Epithelium-Specific Protein 65 kDa (RPE65), TREK-1 alone, TREK-1shRNA; n=10; *p<0.001) or non-transfected cells (n=10; *p<0.001). The higher magnitude of transport seen in cells transfected with TREK-1+cochlin (compared to the TREK-1shRNA+cochlin) demonstrates the influence the interaction of cochlin and TREK-1 has on changes in cell shape. This observation implies that both cochlin and TREK-1 are essential to alter the TM cell shape towards homeostatic regulation of fluid flow.

The rat-tail collagen gel expansion assay was performed to validate the changes in cell shape resulting from the TREK-1 and cochlin interaction (Figure 3.3D). TREK-1+cochlin transfected cells demonstrated a significant increase in gel expansion compared to cells only (n=10; *p<0.001). The TREK-1+RPE65 transfected cells and the TREK-1 only transfected cells exhibited minimal expansion when compared to TREK-1+cochlin transfected cells. Interestingly, the cells with TREK-1 downregulation transfected with TREK-1shRNA demonstrated contraction rather than expansion. This contraction was significant when compared to the cell only control (n=10; *p<0.001) or with TREK-1+cochlin (n=10; p<0.001). This data further supports the role the cochlin and TREK-1 interaction has on the modulation of TM cells. Following these findings, we focused on investigating the influence the cochlin and TREK-1 interaction had specifically on the TM cell cytoskeleton.
Figure 3.3 Cochlin and TREK-1 interaction changes fluid flow dynamics in trabecular meshwork cells. **A)** Western blot analysis demonstrates the increase in TREK-1 expression in the presence of cochlin multimerized under shear stress. Upper panel: cochlin with or without shear stress as indicated separated on a non-reducing but denaturing gel. Middle panel/lower panel: same experimental conditions as top panel but separated on a reducing and denaturing gel followed by probing for cochlin or TREK-1 as indicated. **B)** Co-immunoprecipitation performed to demonstrate the interaction of cochlin and TREK-1 in normal human TM as well as glaucomatous human TM samples (presumed in the presence of shear stress) followed by western blot analysis. **C)** Fluorescein dye transport across several layers of cells cultured on PVDF membrane within an Ussing-type chamber (as depicted in the top figure) was measured as fluorescence intensity. Cells were in the presence of TREK-1+cochlin, TREK-1+RPE65, TREK-1 only, TREK-1 shRNA+cochlin as indicated. Student’s t-test confirmed significant difference in transport between TREK-1+cochlin and all other transfected cohorts (n = 10; *p<0.001). TREK-1+cochlin also demonstrated a significant difference in transport compared to non-transfected controls (n = 10; *p<0.001). **D)** Rat tail collagen assay was performed by culturing TM cells transfected with TREK-1+cochlin, TREK-1+RPE65, TREK-1 only, TREK-1 shRNA+cochlin, or untransfected (hydrogel+cells) as indicated in rat tail collagen and injecting it into separate capillary tubes. Collagen expansion or retraction was measured and recorded. TREK-1+cochlin, TREK-1+RPE65, and TREK-1 only exhibited...
significant expansion compared to cells only (hydrogel+cells) by pairwise t-test (n = 10 per group; *p<0.001). Contraction was observed in the TREK-1 shRNA+cochlin cohort, which was statistically significant compared to cells only (n = 10 per group; *p<0.001). Adapted from (Carreon et al., 2017c).

3.3.3 Cochlin and TREK-1 influence trabecular meshwork cell architecture

The changes in overall TM cell architecture were visualized by performing immunofluorescence microscopy probing for actin on human TM cells (Figure 3.4). All cells in these experiments were treated with exogenous cochlin at physiological concentrations (10 µM). The untreated TM cells (Figure 3.4A) and the monomeric cochlin treated TM cells (Figure 3.4B) maintained a visibly organized cytoskeleton architecture whereas the multimeric cochlin treated TM cells demonstrated an increase in actin expression along with visible clustering of actin fibers (Figure 3.4C). This specific clustering is limited in the untreated cells and nowhere near the intensity seen in the multimeric cochlin treated cells.

Relative quantification of cochlin and actin in both the treated and untreated cells has been presented in Figure 3.4D-F using fluorescence/area. Additionally, a difference in cells shape is presented between all three cohorts (untreated, mono- and multi-meric cochlin treated). Untreated cells and monomeric treated cells (Figure 3.4A & B) maintained a uniformed flattened, circular shape compared to multimeric treated cells (Figure 3.4C), which exhibited a spindle-like conformation. Further validation through quantification demonstrated the changes in cell shape resulting from treatment with the different forms of cochlin (Figure 3.4G-J). These results demonstrate the changes in cellular architecture resulting from the interaction of multimeric cochlin with TREK-1.
Figure 3.4 Cochlin’s molecular state influences changes in TM cellular architecture. **A-C** Immunocytochemical images representative of human normal TM cells probed with primary antibody for cochlin (green) and actin (red) after 24-hour treatment with exogenous monomeric or multimeric cochlin as indicated. White areas highlight the regions with actin clustering. **D-F** Fluorescence intensity/area was measured for untreated, monomeric, or multimeric cochlin treated cells. This was measured as an arbitrary unit using ImageJ software under the same settings and conditions. **G-J** Cell number (cells/area) based on cellular morphology (circle-like or spindle-like) of untreated, monomeric, or multimeric treated cells was quantified as a relative arbitrary unit of cells/area. *Adapted from (Carreon et al., 2017c).*

Further experiments were conducted in order to investigate the effects of TREK-1 channel activation on the actin cytoskeleton of TM cells (Figure 3.5). Cellular lipids such as arachidonic acid (AA) activate TREK-1 channels (Lee and Goldberg, 2011; Patel and Honore, 2001; Pattabiraman et al., 2012). AA was utilized as a TREK-1 channel activator and changes in the actin cytoskeleton of
TM cells were observed (Figure 3.5B). TM cells were treated with 20µM of AA and the actin cytoskeleton was analyzed using immunofluorescence microscopy. TREK-1 expression remained at a normal state in untreated cells (Figure 3.5A) along with a normal actin distribution. The AA treated cells (Figure 3.5B) demonstrated a robust increase in TREK-1 expression as well as actin expression. Quantification using fluorescence/area as a relative arbitrary unit was performed as described in the previous experiments (Figure 3.5C). These observations demonstrate the influence TREK-1 activation has on the TM cell actin cytoskeleton. Inhibition of phospholipase A2, an AA hydrolyzing enzyme, causes a decrease in the actin cytoskeleton of porcine TM cells (Pattabiraman et al., 2012), corroborating the data presented here. The overall changes in actin cellular architecture is different from what was observed with exogenous multimeric cochlin treatment (Figure 3.4C). The results presented here taken together with previous observations (Goel et al., 2011; Goel et al., 2012), suggest the effect multimerized cochlin has on cellular architecture mediated by interaction with TREK-1. This demonstrates the way in which a high shear stress environment may lead to production of excess multimeric cochlin resulting in vast cytoskeletal changes in TM cells.
Figure 3.5 Effects of TREK-1 activation induced by arachidonic acid (AA) on actin cytoskeleton of TM cells. A & B) Immunofluorescence images representative of human normal TM cells probed with primary antibody for TREK-1 (green) and rhodamine phalloidin for actin (red) following no treatment or AA treatment. C) Fluorescence intensity/area was measured for TM cells under no treatment or AA treatment. This was measured as a relative arbitrary unit using ImageJ software under the same settings and conditions. Adapted from (Carreon et al., 2017c).

3.3.4 Multimerized cochlin causes changes in TREK-1 current

To further assess the cochlin and TREK-1 interaction, functional studies were performed to demonstrate the effects of cochlin on TREK-1 channel current (Figure 3.6). Human TREK-1 was transiently expressed in HEK293 cells and channel activity was analyzed using whole-cell patch clamping. The cells specifically expressing TREK-1 were voltage-clamped at -60mV and the following voltage ramps (refer to methods) were used to record the channel current. The vehicle alone, monomeric cochlin or multimeric cochlin were added to the bath (Figure 3.6). Vehicle alone did not produce significant effects (-1.6%±1.3%; n=11). Interestingly, the presence of multimerized cochlin at physiological
concentrations (10µM) reduced TREK-1 basal channel activity. Similar effects were seen when multimerized cochlin was produced by addition of Ca\textsuperscript{2+} (-33.4±4.1%; n=8; Figure 3.6A, B, &D) or by a shear stress protocol using a 30-gauge syringe (-30.0±5.1%; n=8; Figure 3.6D). Fluid shear stress was induced by increasing the bath perfusion rate and this increase resulted in an increase in TREK-1 channel activity (Figure 3.6B) as previously reported (Anishkin et al., 2014; Bittner et al., 2014; Brohawn et al., 2014; Nayak et al., 2009). There were no differences seen from shear stress in the vehicle group and cochlin groups (Figure 3.6D). In the presence of monomeric cochlin, a small but significant increase in TREK-1 activity was observed compared to the vehicle only (+3.8±1.4%; n=9; Figure 3.6C & D).

As previously mentioned, TREK-1 is activated by a variety of factors. Specifically, external protons, heat, and pressure act on TREK-1 via a common gate that exhibits C-type gate characteristics (Bagriantsev et al., 2011). The external area of the transmembrane segment M4 is a major component in the TREK-1 gating apparatus and regardless of activators or inhibitors of the channel, this mechanism is a conserved feature in different K\textsubscript{2}P channels. Interestingly, a mutation in this specific M4 segment (W275S) results in a gain of function TREK-1 channel exhibiting reduced sensitivity to extracellular protons or temperature, an effect mimicked by using a high extracellular potassium concentration (Bagriantsev et al., 2011). To assess the inhibitory effect of multimeric cochlin treatment on TREK-1 and determine whether this is mediated by the C-type gate, recordings were carried out in high K\textsuperscript{+} extracellular solution.
There was no significant effect observed in the high K+ solution (+2.5±3.2%; n=8; Figure 3.6D) in contrast to the inhibitory effect of multimeric cochlin, suggesting the inhibition elicited by cochlin may be acting through a C-type gate mechanism. In support of these observations, diminished sensitivity to shear stress was observed in high K+ solution (+10.4±3.9%; n=8; Figure 3.6D). These results suggest a direct interaction between cochlin and TREK-1 yet it remains unknown if these effects are inhibitory/excitatory and are directly related to changes in cell shape. TREK-1 effects on cell shape are independent of the channel's ion transport capability (Lauritzen et al., 2005).

Figure 3.6 TREK-1 recordings in HEK293 cells using whole-cell patch clamp. A) Multimerized cochlin (10 nM) was applied to the bath of a HEK293 cell expressing hTREK-1. A 2 hour Ca2+ treatment was used to multimerize cochlin. TREK-1 current was activated with 1 s voltage ramps from -100 to +50 mV every
5 s (as in B). Values were measured at +45 mV in each ramp and plotted versus time. A significant current reduction was observed in the presence of multimerized cochlin (Mu). Conversely, shear stress (increase in bath perfusion rate) caused an increase in current. B) Left: TREK-1 current resulting from a voltage ramp in basal conditions and after the addition of multimerized cochlin. Right: TREK-1 channels and increase in whole cell current resulting from increase in bath perfusion rates. C) Using the same protocol described in A, the effect of monomeric (Mo) cochlin was measured. D) Vehicle (n = 11), monomeric cochlin (Mo; 10 nM; n = 8), cochlin multimerized by Ca\textsuperscript{2+} addition for 2 hours (Mu; 10 nM; n = 8), and cochlin multimerized by passage through a 26-gauge needle 20 times [SS: shear stress] (n = 8) were the conditions used to quantify the effects of cochlin on TREK-1 current. The high K\textsuperscript{+} solution refers to the TREK-1 recordings performed in high extracellular K\textsuperscript{+} concentration (135 mM). In this condition, vehicle, multimeric cochlin (Mu; 10 nM) and shear stress stimuli were applied (n = 8). *p<0.05; ***p<0.01, Student’s t-test vs. vehicle. Adapted from (Carreon et al., 2017c).

Further whole-cell patch clamp was carried out in a human TM cell line transiently transfected with TREK-1. The TREK-1 current was recorded at a voltage ramp of -100 to +50 mV and challenged with multimeric cochlin following the previous procedure. Interestingly, the multimeric cochlin treatment induced a statistically significant decrease in TREK-1 current (36.8±11.2%; n=5; p<0.05 vs. baseline; Figure 3.7A & B). This observed effect was opposite of the well-known shear stress effects resulting from fluid flow or arachidonic acid (20µM) activation both of which increased overall TREK-1 current (72.1±21.9% & 148.7±50.1% respectively; n=5 each; Figure 3.7B).
Figure 3.7 TREK-1 recordings in TM cells using whole-cell patch clamp. A) Voltage ramp from -100 to +50 mV in basal conditions was used to elicit TREK-1 current in a human TM cell expressing hTREK-1. This was performed in basal conditions and after adding multimerized cochlin (10 nM) to the bath. B) TREK-1 current was measured in the transiently transfected TM cells after stimulation with multimerized cochlin (Mu; 10 nM; n = 5), shear stress (n = 5), and arachidonic acid (n = 5; 20 µM). *p<0.05, Student’s t-test vs. basal current. Adapted from (Carreon et al., 2017c).

3.4 Discussion

The identification of cochlin as a mechanosensing molecule in the solution phase or the extracellular matrix of the TM through the interaction of the cell-surface mechanotransducer TREK-1 is a novel finding. Aqueous humor outflow dysregulation is influenced by spatial changes in the ECM and remodeling of the cytoskeleton (Larsson et al., 1995; Morrison et al., 2005). In the TM, TREK-1 performs mechanotransduction with the aid of cochlin, a unique characteristic not observed in other systems. Cochlin helps to facilitate TREK-1’s mechanotransducing properties in the low fluid flow regime of the TM. Interestingly, TREK-1 function varies from location to location as is evident from work performed in alveolar epithelial cells. TREK-1 deficiency in this cell type
correlates with a decrease in actin stress fibers and TREK-1 overexpression correlates with an increase in stress fibers (Schwingshackl et al., 2015). The results presented here demonstrate a difference in effect in TM cells after multimeric cochlin treatment possibly resulting from difference in cell type as well as location.

The importance of cochlin in IOP regulation has been established through cochlin downregulation using shRNA (Goel et al., 2011, 2012). The TREK-1 downregulation performed in the above experiments may result in a decrease in the sensitivity of TM cells to detect mechanical stimulus necessary for the collagen expansion (Figure 3.3D) and corroborates the observations of decreased fluorescein transport (Figure 3.3C). These results further support the change in cell shape and modulation that occurs from the cochlin/TREK-1 interaction. This change in shape in the TM cells influences overall aqueous humor dynamics because of the TM tissue architecture (Goel et al., 2012). The overall changes elicited from the TM cells after multimeric cochlin treatment is consistent with our previous observations (Goel et al., 2011; Goel et al., 2012). These changes consisted of the cells taking on an elongated and spindle-like conformation (Figure 3.4C & J).

The model we have proposed in which the binding of multimerized cochlin to TREK-1 changes cell shape and motility is supported by the experiments we have carried out (Figure 3.8). Cytoskeletal changes mediated by TREK-1 appears to be independent of TREK-1 channel activity (Lauritzen et al., 2005). Cell relaxation may occur after TREK-1 activation resulting in increased outflow
in the normal state, a mechanism similar to the high-conductance calcium dependent K+ channel (BKCa) (Dismuke and Ellis, 2009; Soto et al., 2004). This hypothesis is supported by the small but significant increase observed in TREK-1 current after monomeric cochlin treatment (Figure 3.6C). Monomeric cochlin may interact with TREK-1 in the normal physiological environment resulting in a small increase in TREK-1 current exhibiting a positive effect on outflow and an overall reduction in IOP. In the diseased environment, as supported by the data presented here, multimerized cochlin binds to TREK-1 reducing TREK-1 current, which may in turn decrease outflow resulting from structural changes in the TM cells. This effect is induced specifically by the multimerized cochlin and TREK-1 interaction. The increase in IOP in the pathological state may occur because of impedance in aqueous humor outflow caused by multimerized cochlin's inhibition of TREK-1 current. Decline in TREK-1 expression has been demonstrated to promote cells to enter a contractile state such as in the uterus during pregnancy (Monaghan et al., 2011). These observations in other systems may serve as parallels for TREK-1 function in the TM in the presence of multimeric cochlin. For the first time we demonstrate the influence the cochlin and TREK-1 interaction may have in glaucoma pathology. Further investigation may render this interaction or each molecule separately as a future target for therapy or prevention.
Figure 3.8 Proposed mechanosensing model through cochlin and TREK-1 interaction. The cells of the inner wall (IW) of Schlemm’s canal (SC) and the trabecular meshwork (TM) specifically in the juxtacanalicular (JCT) region influence resistance. The TM cells may secrete monomeric cochlin, which is quickly degraded by proteases in the ECM. Under influence of shear stress, cochlin begins to multimerize and interacts with TREK-1 causing reorganization of the cytoskeleton. Adapted from (Carreon et al., 2017a).
Chapter 4: Concluding remarks

Glaucoma is a worldwide health concern that continues to worsen as the population ages. Identification of new therapeutic avenues requires an understanding of the molecular mechanism underlying this disease. By specifically understanding the fluid flow regime in the eye, future therapeutic avenues can be identified and further investigated.

Overall, further characterization of segmental outflow in the DBA/2J and DBA/2J-Gpnmb+/SjJ mouse models was performed. The DBA/2J mice exhibit more low flow areas even at younger ages compared to the DBA/2J-Gpnmb+/SjJ mice (Figure 2.1 & 2.2). The hypertensive 8 month old DBA/2J mice do in fact demonstrate a lack of high flow areas compared to 8 month old DBA/2J-Gpnmb+/SjJ mice (Carreon et al., 2016). The difference in vascular markers between the high flow and low flow areas (Carreon et al., 2017a) (Figure 2.2B) also demonstrates the molecular differences underlying the segmental outflow in these mice. Characterization of outflow in these mice further strengthens their use as models to investigate impeded aqueous humor outflow in glaucoma pathology.

The ECM protein cochlin influences IOP regulation in the trabecular meshwork through its interaction with the cell-surface mechanotransducer TREK-1. The interaction between cochlin and TREK-1 elicits changes in TM cell architecture (Figure 3.3C & D). Cochlin in its multimeric form also results in significant changes in the actin cytoskeleton of TM cells (Figure 3.4C, F, & J). Multimeric cochlin also causes a decrease in TREK-1 current that may influence
aqueous humor outflow (Carreon et al., 2017b) (Figure 3.6 & 3.7). An increase in TREK-1 current, elicited by interaction with monomeric cochlin, may cause the TM cell to relax resulting in increased outflow similar to what is observed with the high-conductance calcium dependent K+ channel (BKCa) (Dismuke and Ellis, 2009; Soto et al., 2004). However, the interaction with multimerized cochlin results in an inhibition of TREK-1 current contributing to disruption of the aqueous humor outflow. The data presented here suggests that cochlin and TREK-1 together (Figure 3.8) are involved in glaucoma pathology.

Taken together, this work presents a novel interaction between cochlin and TREK-1 (Figure 3.8) that has the potential as a therapeutic avenue. Further characterization of DBA/2J and DBA/2J-Gpnmb+/SjJ mice further established the use of these mice for outflow research. Future work will allow for the manipulation of the cochlin and TREK-1 interaction in these models to elucidate the molecular mechanisms influencing IOP regulation.

4.1 Future directions

The establishment of the interaction of cochlin and TREK-1 as a component in IOP regulation provides the opportunity for further investigation. Future work focuses on investigating the transcription of cochlin in normal versus glaucomatous states utilizing the DBA/2J and DBA/2J-Gpnmb+/SjJ mice characterized in specific aim 1. Transcription factors (TFs) are responsible for regulating protein expression induced by specific stress and stretch changes in the TM (Malone and Hernandez, 2007; Vittal et al., 2005). Previously, bioinformatics analyses identified a specific group of TFs that bind to the cochlin
promoter region. Biochemical analyses further demonstrated a difference in
these TFs between normal and glaucomatous TM samples (Picciani et al., 2009).
The specific group of TFs has not been studied in the DBA/2J and DBA/2J-
Gpnmb+/SjJ mice. The study of these TFs in these mice will provide a reservoir
for potential manipulation of these TFs to induce a reduction in cochlin
transcription in the glaucomatous state.

The utilization of electrophoretic mobility shift assay (EMSA) on the
nucleic protein of varying ages and conditions of DBA/2J and DBA/2J-
Gpnmb+/SjJ mice allow for the investigation of the expression as well as activity
of these TFs in the TM (Figure 3.9A). Preliminary results in normotensive (IOP
≤15 mm of Hg) versus hypertensive (IOP ≥18mm of Hg) DBA/2J mice has
demonstrated a difference in the TF Barx2 with an increase in hypertensive
versus normotensive mice (Figure 3.9B & C). Barx2 is related to the *Drosophila*
Bar family genes. This TF is expressed in a variety of epithelial tissues that utilize
branching morphogenesis (lacrimal gland, kidney, lung, mammary gland) (Tsau
et al., 2011). Further investigation will be carried out in varying ages of DBA/2J
and DBA/2J-Gpnmb+/SjJ mice for Barx2 and the remaining identified TFs.

The transcriptional regulation of cochlin in the TM has not been
investigated extensively. Bioinformatic analyses have identified potential TF
binding sites on the cochlin promoter region covering a span of 5000 bp from the
translational start site (Picciani et al., 2009). Promoter regions for specific cochlin
homologues, bone morphogenetic protein 2 and -4 (Helvering et al., 2000) as
well as the von Willebrand factor (Guan et al., 1999) are within 2000 bp from the
start site. Interestingly, elevated protein (Brn3a, Brn3b, Nrf2, Barx2, and FoxQ1) expression levels as well as protein-DNA complexes have identified in glaucomatous versus normal TM (Picciani et al., 2009). A decrease in FOXC1 was observed in glaucomatous versus normal TM. The investigation of these identified TFs in DBA/2J and DBA/2J –Gpnmb+/SjJ mice will allow for more in depth investigation into the upregulation of cochlin in the glaucomatous (Figure 3.9A).

![Figure 3.9](image)

**Figure 3.9** Electrophoretic mobility shift assay (EMSA) for Barx2 in normotensive versus hypertensive DBA/2J mice. A) Summary of study design investigating events leading up to and following increase in IOP in
normotensive (≤15 mmHg) versus hypertensive (≥18 mmHg) DBA/2J mice. B) Preliminary EMSA data of normotensive versus hypertensive DBA/2J mice TM nucleic protein probing for transcription factor Barx2. C) Quantification using densitometry of EMSA probed Barx2 shift.
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