The Role of Wnt/β-catenin Signaling in the Trabecular Meshwork Relating to Ocular Hypertensive Primary Open Angle Glaucoma

Ocular hypertension is the greatest causative risk factor of primary open angle glaucoma (POAG), the most prevalent subset of age-related glaucoma. Wnt signaling antagonist sFRP1 is increased in the trabecular meshwork (TM) of patients with POAG and induces ocular hypertension in human ex vivo eyes and in mice, which is resolved upon downstream Wnt/β-catenin signaling activation. The molecular mechanisms behind this remain unknown. β-catenin plays a role as an accessory protein to classical cadherin cytosolic domains, connecting these cell-cell adhesion proteins to the actin cytoskeleton. In other cell types, Wnt/β-catenin signaling crosstalks with the TGFβ/SMAD pathway, which is overactive in the POAG TM and is implicated in ocular hypertension. Our hypothesis is that **the Wnt/β-catenin signaling pathway maintains TM cell adhesion and intraocular pressure by stabilizing cadherins junctions on the TM cell membrane and by inhibiting the POAG-related TGFβ/SMAD pathway.** We used primary or transformed human non-glaucomatous TM (NTM) cells for all molecular and cell-based studies. NTM cells were treated with reporter viruses to study DNA binding element activity, recombinant protein to modulate Wnt/β-catenin or TGFβ/SMAD pathways, or siRNA to knockdown pathway mediators or cadherins. After treatment, NTM nucleic acid or protein was isolated or probed for Wnt/β-catenin or TGFβ signaling markers or cadherins. Some NTM cells were also plated for Real Time Cell Analysis (RTCA) cell impedance assays. Ad5.CMV recombinant adenoviruses encoding K-cadherin and/or

sFRP1 were injected into BALB/cJ mouse eyes. Conscious IOP was assessed for up to 35 days. We found that Wnt/β-catenin signaling cross-inhibits TGFβ signaling in a β-catenin and Smad4-dependent manner. This cross-inhibition resulted in a decreased K-cadherin and fibronectin expression. Wnt/β-catenin signaling also enhanced mRNA, protein, and membrane-bound levels of K-cadherin, the most highly expressed cadherin isoform in the TM. In vivo, K-cadherin reduced the ocular hypertensive effects of sFRP1. RTCA assays showed that Wnt/β-catenin signaling and K-cadherin are responsible for maintenance of TM cell adhesion. Wnt/β-catenin signaling is responsible for intraocular pressure maintenance through increased expression of K-cadherin-mediated TM cell adhesion and through inhibition of TGFβ/SMAD signaling.

THE ROLE OF WNT/β-CATENIN SIGNALING IN THE TRABECULAR MESHWORK RELATING TO OCULAR HYPERTENSIVE PRIMARY OPEN ANGLE GLAUCOMA

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Hannah C. Webber, B.S.

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CHAPTER V

CHAPTER I. INTRODUCTION

Author: Hannah C. Webber

North Texas Eye Research Institute

University of North Texas Health Science Center

Acronyms:

RGC – retinal ganglion cell

POAG – primary open angle glaucoma

ONH – optic nerve head

IOP – intraocular pressure

LGN – lateral geniculate nucleus

CSF – cerebrospinal fluid

CSFP – cerebrospinal fluid pressure

TLCPD – trans lamina cribrosa pressure differential

TM- trabecular meshwork

AH – aqueous humor

Glaucoma

Glaucoma is the most prevalent neurodegenerative disease in the world, with over 70 million people currently suffering from this blinding optic neuropathy.[1,2] Glaucoma is an incurable, age-related disease that is also a leading cause of irreversible blindness. Due to the ever-increasing human life span, Tham et al mathematically predicted a 74% increase in global cases of glaucoma between 2013 and 2040.[1] Glaucoma is a progressive optic neuropathy, which means that glaucoma patients will continue to lose their vision over time if a cure is not found, leading to very low quality of life for millions of elderly individuals.

Glaucoma is a group of diseases with several subtypes. However, all subtypes of glaucoma converge on a single qualifying characteristic – damage and death of retinal ganglion cells (RGCs) and their axons in the eye, leading to a loss of connection between the retina and the visual center of the brain, which causes blindness.[3-5] Understanding the pivotal role that RGCs play in transmitting messages from the retina to the brain is necessary when characterizing and understanding glaucoma. The eye itself is considered an extension of the brain – the eye and its neurons, including RGCs, are part of the central nervous system. This creates an issue that when studying optic neuropathies such as glaucoma, in contrast to peripheral nervous system axons that are able to regenerate, central nervous system neurons show no evidence of regeneration on their own. RGC cell nuclei are all located in the retina, with RGC axons projecting from these somas to form a thin axonal layer at the inner surface of the retina termed the retinal nerve fiber layer (RNFL). These axons all converge at the ONH, travel back through the optic nerve, and synapse with the lateral geniculate nucleus (LGN) of the brain. Neurons from the LGN then project to the visual cortex. The RNFL layer and the RGC layer form the 2nd and 3rd innermost

layers of the neural retina, respectively. The nuclei of retinal non-RGC cells are located in two layers: the inner nuclear layer and the outer nuclear layer. The inner nuclear layer includes the nuclei of bipolar, amacrine, and horizontal cells; the outer nuclear layer includes nuclei of photoreceptor cells. The outmost layer is the retinal pigmented epithelium layer that forms a blood-retinal barrier. The photoreceptor layer of the retina receives stimulation from photons and transmits that light energy into chemical energy, and the bipolar, amacrine, and horizontal cells of the inner nuclear layer reformat that chemical energy into a recognizable signal, and then the RGCs relay the summary of these signals to the brain. Without this vital connection between retina and brain, sight is lost.

In glaucoma, RGCs are the only affected cells of the neural retina. RGC axons exit the eye and turn 90 degrees from the RNFL into the optic nerve through a porous region termed the lamina cribrosa, which consists of collagen, elastin, laminin, astrocytes, and resident lamina cribrosa cells.[6,7] Since RGCs are non-myelinated at and turn sharply into the lamina cribrosa region, they are susceptible to intraocular pressure (IOP) changes. The progression of RGC damage in glaucoma can continue over many decades and therefore the vision loss that a patient is experiencing can be "unnoticeable" until it is severe, which gives glaucoma the nickname the "thief of sight". This is also because RGC axons and bodies that provide peripheral vision are lost first. Once glaucomatous RGC death begins, it cannot be stopped. When elevated IOP in glaucoma patients is controlled, RGC death and damage still progress, though at a significantly lower rate. Interestingly, normotensive glaucoma patients who receive IOP-lowering agent treatment also have significantly slower glaucoma progression than untreated normotensive

glaucoma patients[8], suggesting that RGC damage due to ocular hypertension can be slowed by lowering IOP. However, once RGC death initiates, it cannot be reversed.

Cerebrospinal fluid pressure (CSFP) at the back of the eye which results from CSFP inside the brain may affect the RGC axon in the lamina cribrosa region.[9] The myelinated optic nerve from the back of the eye to the brain is surrounded by the 3 layers of meninges including a space filled with cerebrospinal fluid (CSF). In contrast to IOP, which "pushes" the lamina cribrosa anteriorly, CSFP "pushes" the lamina cribrosa region posteriorly. IOP and CSFP therefore meet right at the lamina cribrosa, and create a trans-lamina cribrosa pressure differential (TLCPD), calculated as IOP - CSFP = TLCPD. In this scenario, if IOP is raised, and/or if CSFP is lowered, then the TLCPD is increased, leading to lamina cribrosa pathology and glaucomatous RGC axon damage. Several studies show that normotensive primary open angle glaucoma patients have lower CSFP[10], and other studies have shown that lower CSFP is independent of IOP.[11] However, research in this research area has technical difficulties: the CSFP behind the eye may not be equivalent to the pressure measured from a lumbar puncture. Lumbar puncture is an invasive procedure typically used to measure CSFP that is rarely used in research studies and has been replaced in some studies with a flawed calculation based on blood pressure. It has also been proposed that inherent fibrotic changes to the lamina cribrosa tissue is responsible for damage to the RGC axons housed there.[7] Further investigation is needed to test this hypothesis and to determine whether certain characteristics or parameters of the lamina cribrosa contribute to this optic nerve damage.

Regardless of the causative factor of glaucoma, once RGC axon damage and cell death begin, there is no currently available therapeutic to stop this process, even when the causative factor is removed after the initial insult. This leads to the apoptosis cascade hypothesis wherein once RGCs are injured, progressive cell death by apoptosis continues. Many insults have been associated with RGC damage and death in glaucoma and in glaucoma models. These insults include vascular insufficiency related to ocular hypertension-induced ischemia, excitotoxicity of RGC cells, the halting of axonal transport of mitochondria and neurotrophic factors, neuroinflammation, reactive gliosis mandated by astrocytes of the retina and ONH, increased oxidative stress, and deregulated autophagy.[3,12-15] Many groups are studying neuroprotective and neuroregenerative approaches to protect RGCs in the glaucoma eye. However, these goals cannot be achieved without first removing the most important glaucomatous insult - ocular hypertension.

Glaucomatous damage to the RGCs and retina can be assessed by multiple clinical tests, each with their own advantages and pitfalls. Humphrey visual field tests measure the peripheral vision which is lost first in glaucoma. The drawbacks to the Humphrey visual field test are 1) it is not sensitive since it only detects vision loss after significant loss of RGCs; and 2) it is a subjective test, which requires patient training and compliance. Fundoscopy acquires images of the structures at the back of the eye including the retina, macula, and the optic nerve head (ONH). RGC axons atrophy in glaucoma, leading to a loss of the optic nerve bundle represented by "cupping" at the ONH.[4] Hemorrhage at the ONH is also a risk factor for the progression of glaucoma that can be seen with fundoscopy. Fundoscope examination is very useful in screening ONH damage. Optical coherence tomography is used to produce high-quality cross-section

images of the ONH, distinguishing the 10 layers of the retina and the optic nerve head. OCT can be used to assess ONH cupping, RGC swelling and damage, RGC death, and thinning of the RNFL. However, OCT is not suitable for vasculature imaging. Therefore, these standard clinical tests must be combined for patient evaluation.

As described previously, glaucoma is a group of diseases with several subtypes - ocular hypertensive or normotensive referring to intraocular pressure (IOP); 'closed angle' or 'open angle' referring to the anterior angle between the iris root and cornea in the anterior chamber; primary or secondary referring to an unknown or known initial cause; and congenital glaucoma are caused by developmental abnormalities, which are frequent associated with genetic mutations.

Ocular hypertension is a condition defined as an IOP of 21mmHg or higher. These individuals are at a high risk of developing glaucoma because ocular hypertension is the leading causative risk factor of glaucoma. However, not all patients with ocular hypertension develop glaucoma, and there are individuals with a normal IOP who still develop glaucoma, termed normotensive glaucoma. There are multiple ways to measure intraocular pressure clinically, including non-contact tonometry that is typically used in a standard eye exam, and the more accurate Goldmann applanation tonometry, where a device is lightly pressed against a patients' cornea. Other options such as electronic tonometry or rebound tonometry are also available. The angle between the iris root and the end of the corneal endothelium must remain open so that aqueous humor, the clear liquid in the eye that provides nutrients to ocular tissues, can freely flow through this angle and out of the eye. If this angle becomes "closed", IOP will immediately and severely rise. This acute

spike in intraocular pressure can lead to glaucomatous damage to RGCs. Closed angle glaucoma accounts for an estimated 10% of glaucoma cases. If this anterior chamber angle remains open but glaucomatous damage is seen, this is termed open-angle glaucoma. The anterior chamber angle can be evaluated by using the gonioscope, a special optic device that enables ophthalmologists to visualize the structure of the angle . Typically, patients with closed-angle glaucoma will immediately have severe pain and headaches if there is acute angle closure crisis, and this condition will force those patients to seek medical assistance.

Primary open angle glaucoma (POAG) is less understood, and is currently the focus of glaucoma research. The "primary" open angle subtype refers to an 'unknown' or 'non-secondary' source of pathology. These primary open angle glaucoma patients can be ocular hypertensive or normotensive. Currently, ocular hypertensive POAG is thought to be due to pathology of the aqueous humor outflow pathway, causing IOP elevation which leads to glaucomatous damage to RGCs over time. However, some POAG patients do not have ocular hypertension. In these normotensive POAG patients, there is seemingly no source of pathology, yet glaucomatous damage to the RGCs ensues. More research must be done to find the insults that cause RGC death and blindness in these individuals.

The "secondary" subtype refers to glaucoma with a known source of pathology. Secondary glaucomas include steroid-induced glaucoma, pigmentary glaucoma, exfoliation glaucoma, and neovascular glaucoma. In these glaucomas, the primary diseases lead to blockage of aqueous humor drainage, causing ocular hypertension and leading to glaucoma. Pigmentary dispersion results from clogging of AH drainage by iris pigments; exfoliation glaucoma results from lens

and iris tissues rubbing together and sloughing off residue that clogs the drainage tissue; and neovascular glaucoma results from the formation of pathological blood vessels through the iris and into the drainage tissue. Steroid-induced ocular hypertension occurs in about 30-40% of the general population with steroid treatment for more than 4 weeks.[16,17] In these patients, steroids affect the drainage pathway of aqueous humor, decrease aqueous humor outflow, and induce ocular hypertension[18] which can result in glaucoma.

Our study focused on ocular hypertensive primary open angle glaucoma, a subtype that includes high IOP, an open angle, and no secondary causes of damage. In this subtype, inherent pathology to the aqueous humor outflow pathway has been pinpointed as the cause of ocular hypertension that leads to glaucoma, yet the exact molecular mechanisms driving this pathology remain unknown.[19]

Primary open angle glaucoma

Primary open angle glaucoma (POAG) is the most common subtype of glaucoma in the world with an estimated 57.5 million patients currently suffering from this disease as of 2015.[20] It is estimated that 6 million people will go blind from this disease in 2020[21], and since POAG is age-related, millions more will develop this disease and go blind each year that there is no cure. In fact, age is currently a risk factor for POAG, as the prevalence increases from 1% at age 40 to 7% by age 80.[1] POAG is a subtype of glaucoma that is currently defined by exclusion criteria. The "primary" refers to the fact that there is no obvious secondary cause of glaucoma such as steroid-induced glaucoma, and the "open angle" refers to the fact that these patients do not have angle closure at the iris root and corneal endothelium that is leading to pathology. Yet despite the

lack of a secondary cause or a closed angle, these patients still often have ocular hypertension, and always develop the RGC damage and death.

High intraocular pressure is the most important causative factor and risk factor of POAG. Other risk factors include anatomical features such as a thin cornea or myopia; ethnicity, particularly black and hispanic populations[22]; high blood pressure; family history especially when POAG is present amongst siblings[23]; and the presence of one of the 15 risk alleles found associated with POAG, although these associations are still considered mild and their functions are not fully understood.[19] There are also 3 genes associated with POAG in an autosomal dominant inheritance pattern – MYOC, OPTN, and WDR-36. It is now thought that mutations in the MYOC gene cause ocular hypertension and are found in 3-4% of all POAG cases including juvenile-onset open angle glaucoma (JOAG).[24,25] This suggests that although POAG phenotypes are similar, they may result from different disease mechanisms. In cases of ocular hypertensive POAG, it has been shown that damages to the conventional outflow pathway tissues cause ocular hypertension, which subsequently causes RGC damage.

Aqueous humor (AH) is a clear solution that is formed from the blood-aqueous barrier at the ciliary body processes. AH is secreted by the outer non-pigmented epithelium of the ciliary body into the posterior chamber, flows between lens and iris into the anterior chamber, and exits the eye through one of two pathways: the conventional pathway and the unconventional pathway. The production of AH by the ciliary body and drainage of AH through these pathways complement one another in a healthy eye, so that the presence of a consistent volume of AH helps maintain a physiological IOP.[26] The AH itself not only provides pressure maintenance in

the eye, but also supplies the anterior tissues of the eye with oxygen, anti-oxidants, nutrients, proteins, and immunoglobulins while maintaining the clarity necessary for light to enter the eye and reach the retina. The production rate of AH is relatively constant and not sensitive to pressure, so IOP is mostly maintained by the AH outflow pathways.[27] The conventional pathway is a pressure-dependent route through which the majority of AH flows out of the eye. Conventional outflow describes the pressure-dependent route of AH draining the eye through the 360 degree trabecular meshwork (TM), into the Schlemm's canal that lines the outside of the TM, through collector channels, aqueous veins and into the episcleral venous drainage. The unconventional pathway is the route a small amount of AH takes out of the eye through the iris root, the uveal meshwork, ciliary body muscle and into the suprachoroidal space and through the sclera. This pathway is not pressure-dependent. A blockage in the TM of the conventional AH drainage is implicated as the source of ocular hypertension in ocular hypertensive POAG.

Ocular hypertension is defined as an IOP of 21mmHg or higher. It is important to note that this is a reference value, as 21mmHg is simply two standard deviations plus the mean IOP of general population. Ocular hypertension can be diagnosed without any signs of glaucoma. Conversely, there are patients who never exhibit ocular hypertension, but do develop glaucoma. Interestingly, all glaucoma patients, regardless their IOP, who take IOP-lowering drugs show less disease progression,.[28] There are a variety of IOP-lowering drugs, and each of them has their own downsides. A common downside to every clinically available IOP-lowering drug in the US is that none of them target the TM pathology that reduces AH outflow and causes ocular hypertension. Prostaglandin analogues are IOP-lowering drugs that act by enhancing AH outflow through the unconventional outflow pathway. Multiple other drugs lower IOP by decreasing AH

formation such as beta blockers, alpha adrenergic agonists, and carbonic anhydrase inhibitors. Drugs such as cholinergic agonists can be used to contract the iris, pulling the TM open and allowing more AH outflow. However, these drugs can cause low vision and their IOP-lowering effects last only a few hours. In fact, all of these available IOP-lowering drugs have to be applied daily or multiple times per day, decrease in efficacy over time, and patients often have to use multiple IOP-lowering drugs, which results in low patient compliance. Ocular inserts that allow slow and sustained release of IOP-lowering drugs are currently being studied to address these issues. Other procedures such as laser trabeculoplasty which uses laser to "burn" the trabecular meshwork and trabeculectomy which involves surgical opening of the outflow pathways are also performed on glaucoma patients to lower IOP. However, these procedures have risks or are invasive and are usually used in glaucoma when ocular hypotensive drugs fail.[29]

The trabecular meshwork (TM)

The TM is a tissue of the conventional outflow pathway situated at the anterior angle of the eye near the iris root and the end of the corneal endothelium. It is a 360° dynamic tissue that offers resistance to AH outflow in a pressure-dependent and segmental manner.[30,31] The TM is made up of 3 layers of tissues, varying in porosity, cell density, and extracellular matrix (ECM).[32] From the innermost layer that first meets AH to the outermost layer that is situated against the schlemm's canal, the TM layers are the uveal meshwork, the corneoscleral meshwork, and the cribiform or juxtacanilicular meshwork. The pore size, through which AH passes, decreases from the uveal to the juxtacanilicular meshwork, and it is thought the juxtacanilicular layer provides the most resistance to AH. The uveal meshwork is made up of TM cells and connective tissue that extend to the ciliary body muscle. The corneoscleral meshwork contains TM cells on top of

a basement membrane that line lamellae beams made up of ECM. The juxtacanilicular meshwork is made up of a dense ECM and TM cells next to the inner wall of Schlemm's canal discontinuous basement membrane.[33] TM cells are endothelial-like cells, but do not form the same sheet-like membranes that are seen in tissues like the corneal endothelium. TM cells wrap around ECM beams with long, finger-like projections and make contact with other nearby TM cells via adherens junctions and gap junctions.[34]

The TM must respond to changes in AH in a multitude of ways to regulate IOP. TM cells are phagocytic, which helps them clear debris from the AH. They express alpha smooth muscle actin and can contract the actin cytoskeleton to reduce the space between cells and increase AH resistance, or relax the actin cytoskeleton to increase space and decrease resistance and lower IOP. The ECM of TM beams includes collagen I and IV, laminin, fibronectin, hyaluronic acid, elastin fibers, glycosaminoglycans, and proteoglycans.[35] In response to stretch or strain on TM cells due to an increase in AH or IOP, TM cells increase production and activity of matrix metalloproteinases to degrade ECM and increase AH outflow facility.[36,37] ECM expression and production also decrease with a rise in AH or IOP, perpetuating the TM's dynamic ability to regulate AH outflow by regulating ECM density.[38] It has also been suggested that the TM has autonomic and sensory innervation which may release different neurotransmitters to modulate TM and Schlemm's canal contraction and permeability.[39,40]

Many, if not all, of these facets that allow the TM to dynamically regulate AH outflow and IOP are damaged in the ocular hypertensive POAG TM tissue. The progression of ocular hypertensive POAG is associated with impaired homeostasis in the TM that no longer allows it

to be a dynamic regulator of AH outflow. In the ocular hypertensive POAG TM, cellular density is decreased, though the mechanism behind this cell loss remains unknown.[41,42] Glaucomatous TM cells show increased actin cytoskeletal rearrangement, forming cross-linked actin formations thought to halt them from properly responding to the AH and stabilizing IOP.[43] In the POAG TM tissue, ECM accumulation and thickening are also noted.[44] These changes result from a multitude of factors including overproduction of ECM such as elastin, collagen and fibronectin, increased crosslinking of ECM proteins, and decreased ECM degradation.[45-47] Studies have also shown increased stiffness in the POAG TM tissue and cells versus their non-glaucomatous counterparts.[48] Further studies highlighted the fact that when grown on thicker, stiffer substrates, TM stiffness increases, leading to a perpetuated tissue stiffness and phenotype that does not allow for dynamic AH outflow.[49] Essentially there is an overall fibrosis of the TM that is associated with decreased outflow and increased IOP, and some researchers have even suggested that an epithelial to mesenchymal transition is associated with TM pathology and may help drive this fibrosis.[50] Oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction have all also been associated with TM pathology during POAG.[51-54] Certain mutations in the MYOC gene contribute to 4% of the POAG cases including JOAG.[55] The MYOC gene encodes the protein product myocilin, though the exact function of wildtype myocilin is not fully elucidated. Myocilin knockout results in no phenotype changes in mice, suggesting that a gain-of-function of mutant myocilin leads to the POAG phenotype. Wild type myocilin may interact with the ECM and play a role in cell adhesion, while mutant myocilin accumulates in the cell, and causes ER stress.[52] These mutant myocilin-induced pathological changes are similar to those observed in the TM of ocular hypertensive POAG.[51,56-58]

Research has also shown that TM pathology is linked to changes in major cell signaling pathways such as the transforming growth factor β (TGFβ) pathway, bone morphogenetic protein (BMP) pathway, and the Wnt/β-catenin pathway.[59-61] The TGFβ signaling pathway has been shown to be overactive in the POAG TM, due to increased levels of TGFβ2 found in the AH and TM. The level of active and inactive TGFβ2 are doubled in 50% of POAG patients,.[62] In the non-glaucomatous eye, TGFβ2 is an important immune system regulator. However at pathological levels, TGFβ2 effects contribute to the POAG TM phenotype and increases IOP.[63,64] Increased levels of TGFβ2 affect TM cells by increasing contractility and CLANs formation[65], the production of ECM molecules such as fibronectin, and the production of ECM cross-linkers such as lysyl oxidases and transglutaminase-2.[47,66,67] TGFβ signaling includes many branches and cross-talks with other pathways in the TM such as BMP signaling and toll-like receptor (TLR) signaling.[68,69] BMP is responsible for negative regulation of TGFβ signaling, but in POAG eyes, the BMP antagonist gremlin blocks BMP's activities, leading to unrelenting TGFβ signaling and a furthering of the pathological phenotype. An increase in TLR4 activation by TGFβ2-induced fibronectin expression is found to in TM cells, suggesting a feed-forward mechanism by which TGFβ contributes to fibrosis.[68] Other than TGFβ2 and Gremlin, the Wnt/β-catenin signaling antagonist sFRP1 is also increased in the TM of ocular hypertensive glaucoma patients.[61] However, the relationship between Wnt/β-catenin and the other cell signaling pathways implicated in glaucoma as well as the molecular mechanisms through which sFRP1 leads to ocular hypertension remain unknown. Researchers should not only search for cause-and-effect mechanisms driving the initial pathology of POAG, but also look at converging downstream mechanisms and targets that can be manipulated to treat POAG.

Wnt signaling and the POAG TM

Wnt ligands such as Wnt3a bind to frizzled g-protein transmembrane receptors outside of the cell. Upon binding, Wnt signaling activation will disassemble the β-catenin-degradation complex consisting of Axin, APC, GSK-3, and CK1 by phosphorylation of these proteins. This disassembly will allow β-catenin to accumulate because it is no longer targeted for degradation by phosphorylation and ubiquitination by the degradation complex (Figure 1, left panel). The combination of Wnt ligand and receptor subtypes and the specific cell types determines whether Wnt/β-catenin signaling is activated or inhibited in each tissue. When β-catenin accumulates in the cytosol, it translocates into the nucleus and acts as a transcription factor, binding to the Wnt-associated TCF/LEF binding domain.[70] Inhibition of Wnt/β-catenin signaling by antagonists such as sFRP1 involves increased degradation of β-catenin in the cytosol and a halt of nuclear translocation and transcriptional activity (Figure 1, right panel).[71] The Wnt/β-catenin pathway is responsible for cell growth, development, cell adhesion, control of fibrosis, and other biological processes.[70] Wnt/β-catenin signaling is also often associated with cancers and diseases of aging, suggesting that the regulation of this pathway in each tissue must be tightly controlled.[40,72]

In 2008, Wang et al. found that sFRP1 is overexpressed in ocular hypertensive glaucomatous TM cells at the mRNA and protein levels.[61] They showed that sFRP1 treatment decreases outflow facility of perfusion-cultured human eyes and increases IOP in mice together with a decrease of β-catenin expression. Further studies have confirmed that Wnt-associated genes are expressed in the TM, particularly those associated with the Wnt/β-catenin signaling pathway, and that when activated in the TM by Wnt signaling agonists, β-catenin accumulated in the cytosol and translocated to the nucleus for TCF/LEF binding and enhanced activity.[73,74] Other studies

showed that Wnt signaling activation reduces stress fiber formation in the TM, suggesting a cytoskeletal component to Wnt signaling activation and that antagonism of Wnt signaling by sFRP1 could influence stress fiber formation in POAG.[75] However, our previous studies did not show sFRP1 or Wnt3- induced cytoskeletal reorganization.[74] Inhibition of Wnt signaling by sFRP1 also leads to an increase in cell stiffness, and activation of Wnt/β-catenin signaling inhibits TGFβ-induced matricellular protein expression, connecting Wnt signaling with the TM tissue pathology seen in POAG.[76]

The TGFβ/SMAD pathway has long been implicated in TM pathology during POAG because it is responsible for the over-production of ECM molecules and increased IOP.[59] In other cell types and diseases, the TGFβ/SMAD and Wnt/β-catenin pathways crosstalk with one another, but whether this crosstalk is positive or negative depends on the cell type. In adult bone marrow mesenchymal stem cells, Smad3 is protective of β-catenin, they aide one another's translocation into the nucleus, which is necessary for downstream TGFβ signaling.[77] In murine embryonic primary medial-edge epithelium cells, LEF1, a Wnt/β-catenin signaling transcription factor, associates with Smads to form an inhibitory complex to repress cadherin gene transcription and induce mesenchymal markers such as fibronectin.[78] In glomerular mesangial cells of the kidney, which are similar in structure and function to TM cells, Wnt/β-catenin signaling protects against TGFβ-induced fibrosis.[79] In TM cells, one study showed that indirect activation of Wnt/β-catenin signaling using lithium chloride (LiCl) decreased TGFβ-induced matricellular and ECM protein expression.[80] It is necessary to fully understand the interaction between Wnt/β-catenin and TGFβ signaling to develop drug targets to properly manipulate these pathways in a diseased TM.

It has also been shown that the accumulation of cytosolic β-catenin due to Wnt signaling activation can increase the β-catenin available for adherens junctions since β-catenin is an accessory protein that links classical cadherin protein cytosolic domains to the actin cytoskeleton.[81] Antagonism of Wnt/β-catenin signaling has also been shown to remove β-catenin from adherens junctions, destabilizing the junctional structure and negatively affecting cell-cell connections.[82,83] β-catenin's role as a necessary accessory protein to cadherin junctions is important for cellular homeostasis because classical cadherins are now known to serve many functions in the cell. The entire "adherens junction" are made up of cadherin proteins, cytosolic accessory proteins, and the entire structure is connected to the actin cytoskeleton. Cadherins themselves are calcium-dependent cell-cell adhesion molecular that are present as transmembrane proteins on the cell membrane of multicellular organisms.[84] Many isoforms of cadherin proteins exist in the human body.[85] Cadherins dimerize and form homophilic linkages with identical cadherin isoforms on adjacent cells in vivo, though cadherin isoforms can form heterophilic linkages in vitro.[86,87] Cadherins are pertinent to development as they pay a role in cell sorting, cell migration and motility, tissue patterning, and synaptogenesis.[88-92] The cytosolic domain of cadherin proteins is connected to the actin cytoskeleton via accessory proteins such as β-catenin.[93] With their extracellular and intracellular domains, cadherins effectively communicate with cells' actin cytoskeletal networks, allowing tissues to act in conjunction with one another. In tissues such as the epithelium or endothelium layer, these connections are very important to maintain/allow the dynamic regulation of the tissues and vessels they surround. Cadherin junctions also affect cell-ECM junctions such as focal adhesions, which comprise of transmembrane integrin proteins bound to ECM molecular in the extracellular space. Cadherin junctions and focal adhesion can regulate each other's expression and stability on the membrane

as one study showed that cadherin binding to cadherin-coated beads through β-catenin decreased focal adhesion binding.[94] Conversely, integrin binding to fibronectin-coated beads decreases cadherin and β-catenin expression, showing that these connections cooperate with each other in the cell depending on the extracellular enviroment. Cadherin engagement with other cadherins can also influence cell signaling by inducing nuclear translocation of certain transcription factors and influencing gene transcription.[95]

Because cadherins have a strong cytoskeletal link and regulate cell-cell as well as cell-ECM junctions, they are considered mechanosensors.[96] Since cells must respond to AH and pressure changes, the TM requires molecules that can properly detect mechanic signals so that the tissue can properly respond by altering contractility, ECM production and degradation, as well as cell adhesion. If these mechanosensors are decreased with age or disease, the TM cell homeostasis cannot be properly maintained. It is now known that different cadherin isoforms have different structures, different strength of adhesion, different roles in governing migration, and therefore they have different effects on cells. It is important to find out which cadherin isoforms are key to TM homeostasis and maintaining IOP.

Our study focused on the role of Wnt/ β-catenin signaling in the TM related to POAG pathology and ocular hypertension. To address this hypothesis, we carried out three specific aims:

1) Crosstalk between TGFβ and Wnt signaling pathways in the human TM. Using luciferase assays, we studied TGFβ/SMAD and Wnt/β-catenin signaling crosstalk in transformed or normal

human non-glaucomatous TM (NTM) cells. We also used siRNA knockdown to determine the role of TGFβ/SMAD and Wnt/β-catenin main mediators Smad4 and β-catenin in this crosstalk.

2) The role of Wnt/β-catenin signaling and K-cadherin in the regulation of IOP. We used qualitative polymerase chain reaction, western immunoblotting, and immunofluorescence to study β-catenin and cadherins in NTM cells. Mice eyes were injected with recombinant viruses containing sFRP1, K-cadherin, or both to study the effects on IOP.

3) The role of cadherins in TGFβ/Wnt signaling crosstalk and TM cell physiology. We performed western immunoblotting and immunofluorescence to study the effects of TGFβ/SMAD and Wnt/β-catenin cross-inhibition from SA1. We also performed cell impedance assays on NTM cells to study cell adhesion associated with Wnt/β-catenin signaling and K-cadherin.

Figures

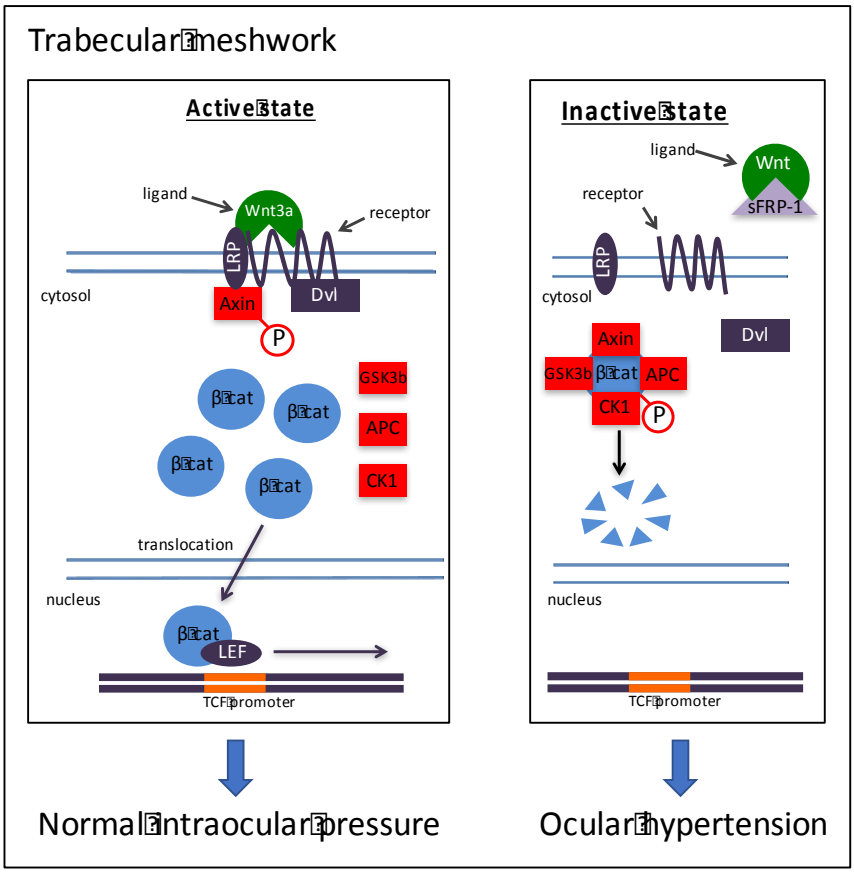

Figure 1. Wnt/β-catenin signaling in the trabecular meshwork normalizes intraocular pressure, while the addition of the POAG-associated Wnt/β-catenin antagonist sFRP1 causes ocular hypertension.

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CHAPTER II. CROSSTALK BETWEEN TGFβ AND WNT SIGNALING PATHWAYS IN THE HUMAN TRABECULAR MESHWORK

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Authors

Hannah C. Webber, Jaclyn Y. Bermudez, Anirudh Sethi, Abbot F. Clark, Weiming Mao

North Texas Eye Research Institute

University of North Texas Health Science Center

Acronyms:

AH - Aqueous humor

CLANs - Cross-linked actin networks

Co-Smad - common mediator Smad

Dkk1 - Dickkopf1

ECM - Extracellular matrix

GTM - Glaucomatous trabecular meshwork

IOP - Intraocular pressure

LRP5/6 - lipoprotein receptor-related protein 5/6

MOI - Multiplicity of infection

NTM - Non-glaucomatous trabecular meshwork

POAG - Primary open angle glaucoma

p-Smad - Phospho-Smad

RLU - Relative luciferase unit

R-Smad - receptor Smad

SBE - Smad binding element

SFRP1 - Secreted frizzled related protein-1

TCF/LEF - T-cell factor/lymphoid enhancer factor

TGFβ2 - Transforming growth factor beta-2

TGFβR - Transforming growth factor beta receptor

TM - Trabecular meshwork

Abstract

Primary Open Angle Glaucoma (POAG) is an irreversible, vision-threatening disease that affects millions worldwide. The principal risk factor of POAG is increased intraocular pressure (IOP) due to pathological changes in the trabecular meshwork (TM). The TGFβ signaling pathway activator TGFβ2 and the Wnt signaling pathway inhibitor secreted frizzled-related protein 1 (SFRP1) are elevated in the POAG TM. In this study, we determined whether there is a crosstalk between the TGFβ/Smad pathway and the canonical Wnt pathway using luciferase reporter assays. Lentiviral luciferase reporter vectors for studying the TGFβ/Smad pathway or the canonical Wnt pathway were transduced into primary human non-glaucomatous TM (NTM) cells. Cells were treated with or without a combination of 5μg/ml TGFβ2 and/or 100ng/ml Wnt3a recombinant proteins, and luciferase levels were measured using a plate reader. We found that TGFβ2 inhibited Wnt3a-induced canonical Wnt pathway activation, while Wnt3a inhibited TGFβ2-induced TGFβ/Smad pathway activation ($n=6$, $p<0.05$) in 3 NTM cell strains. We also found that knocking down of Smad4 or β-catenin using siRNA in HTM5 cells transfected with similar luciferase reporter plasmids abolished the inhibitory effect of TGFβ2 and/or Wnt3a on the other pathway ($n=6$). Our results suggest the existence of a cross-inhibition between the TGFβ/Smad and canonical Wnt pathways in the TM, and this cross-inhibition may be mediated by Smad4 and β-catenin.

Primary open angle glaucoma (POAG) is a leading cause of blindness worldwide characterized by progressive loss of retinal ganglion cells. The primary causative risk factor for the development and progression of POAG is elevated intraocular pressure (IOP) due to increased aqueous humor (AH) outflow resistance at the trabecular meshwork (TM) and the adjacent Schlemm's canal, where the majority of AH drains out of the eye [1-4]. At molecular level, excessive extracellular matrix (ECM) deposition and formation of cross-linked actin networks (CLANs) in the TM are associated with POAG [5-7]. Excessive ECM deposition "clogs" the TM outflow pathway while excessive CLAN formation in TM cells may increase the stiffness of TM cells, both of which have been recognized as contributing factors to increased outflow resistance and IOP [5,8].

Recently, studies have shown that abnormal levels of growth factors and associated cell signaling pathway activities can cause these glaucomatous changes in the TM [9,10]. Two important POAG-associated growth factors are transforming growth factor beta-2 (TGFβ2), an activator of the TGFβ pathway [11], and secreted frizzled-related protein 1 (sFRP1), an inhibitor of the Wnt signaling pathway [9].

TGFβ2 activates the Smad-dependent TGFβ (Smad/TGFβ) pathway by binding to the type II receptor (TGFβRII). This binding phosphorylates and activates the type I receptor (TGFβRI), which phosphorylates the intracellular receptor Smad (R-Smad) proteins Smad2 and/or Smad3. Phospho-Smad2/3 (p-Smad2/3) associates with the common mediator Smad (co-Smad) protein Smad4, and translocates into the nucleus. The complex binds to the Smad binding element (SBE) and changes gene expression. The TGFβ superfamily has long been implicated in several types of glaucoma [10,12-14]. Many studies showed that TGFβ2 is increased in the TM and AH of POAG patients [11,15,16]. TGFβ2 induces excessive ECM deposition of proteins such as fibronectin,

inhibitors of extracellular matrix degradation such as PAI-1, cross-linking proteins such as lysl oxidase (LOX), LOX-like enzymes, and transglutaminase-2 [16-19]. Besides ECM and related proteins, TGFβ2 also increases CLANs in bovine and human TM cells [6,7,20] and elevates IOP in human, porcine, and mouse eyes [10,21-23].

SFRP1 inhibits the Wnt signaling pathway by binding and sequestering Wnt ligands in the extracellular space, prohibiting their binding with the receptor [24,25]. When Wnt proteins are uninhibited, they can bind to their transmembrane receptor, Frizzled and the co-receptor, lipoprotein receptor-related protein 5/6 (LRP5/6). With the assistance of Disheveled, the cytosolic β-catenin degradation complex that consists of Axin2, APC, CK1 and GSK3β is disassembled via phosphorylation. Without this degradation complex, cytosolic β-catenin is no longer phosphorylated for proteasome degradation, and therefore can accumulate. Some cytosolic β-catenin will translocate into the nucleus, where they associate with the T-cell factors 1, 3, 4 (TCF1/3/4) or lymphoid enhancer-binding factor 1 (LEF-1), bind to the TCF/LEF binding element, and change gene expression. This is the β-catenin-dependent/canonical Wnt signaling pathway [26,27]. Our previous studies showed that there is a functional canonical Wnt pathway in the human TM (HTM) [28]. In the human glaucomatous TM (GTM), we found increased mRNA and protein levels of sFRP1 as well as decreased β-catenin, the latter of which is the key mediator of the canonical Wnt pathway as described previously [9,29]. We showed that the inhibition of the canonical Wnt signaling activity by SFRP1 or Dickkopf1 (Dkk1, an inhibitor that specifically inhibits the canonical Wnt pathway via the inhibition of LRP 5/6) elevates IOP in mouse eyes and perfusion cultured human eyes [9,28]. This IOP elevation can be blocked by co-treatment with a small molecule that activates the downstream canonical Wnt pathway [9]. Exactly how canonical Wnt signaling maintains IOP, however, is still under investigation.

Since both the TGFβ and Wnt pathways play important roles in the homeostasis of TM and regulation of IOP, it is very important to determine whether the two pathways crosstalk in the TM. Such crosstalk has been found in various non-TM cells, and more importantly, in fibrotic diseases. In renal fibrosis, the canonical Wnt pathway antagonizes the TGFβ/Smad pathway and protects the tissues from fibrotic damage [30]. Due to the fact that GTM alterations are very similar to those in fibrotic diseases (loss of resident functional cells and excessive ECM deposition), a similar crosstalk likely exists in the TM.

To determine whether a crosstalk exists in the TM, we used luciferase transcription reporter assays (also called luciferase assays) to measure whether the activation of one pathway by recombinant protein is able to affect the other pathway's activity. We transduced human primary non-glaucomatous TM (NTM) cells with lentiviral firefly luciferase reporter vectors containing the SBE for studying the TGFβ/Smad pathway (SBE virus, Qiagen, Valencia, CA) or vectors containing the TCF/LEF binding element for studying the canonical Wnt pathway (TCF/LEF virus, Qiagen). TM cells were also co-transduced with the lentiviral renilla luciferase reporter vector containing a minimal CMV (mCMV) promoter as an internal control (renilla control virus, Qiagen). Because the transcriptional activity of the minimal CMV promoter is not affected by any signaling pathways, the amount of renilla luciferase can be used to normalize firefly luciferase for the difference in cell numbers and transduction efficiency. On day 1, $3x10^4$ NTM cells were seeded into individual wells of 96 well white opaque plates in DMEM-low glucose medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin (Thermofisher, Waltham, MA). On day 2, when cells reached 50% confluence, SBE or TCF/LEF lentivirus together with renilla control virus were mixed in serum-free and antibiotics-free medium containing a transduction reagent (SureEntry, 1:4000, Qiagen) and

added to individual wells. The multiplicity of infection (MOI) was 75 for SBE or TCF/LEF lentivirus, and 50 for renilla control lentivirus. On day 3, medium was changed to serum-free medium. On day 4, cells were treated with the TGFβ pathway activator TGFβ2 (5ng/ml, R&D Systems, Minneapolis, MN) and/or the Wnt pathway activator Wnt3a (100ng/ml, R&D Systems) in serum-free medium. On day 5, firefly and renilla luciferase levels were measured using the DualGlo kit (Promega, Madison, MI) and a plate reader (Infinite M200, Tecan, San Jose, CA). Experiments were performed in replicates (n=6) and luciferase luminescent signals were read three times per well and averaged. Statistical analysis was performed using Prism Graphpad (GraphPad Software, La Jolla, CA) using one-way ANOVA. Multiple-comparison post-hoc tests were applied. Three primary NTM strains were studied and representative data were shown. The TM cell strains were previously characterized by a combination of TM cell markers including collagen IV, laminin, α-smooth muscle actin, as well as dexamethasone-induced myocilin expression and formation of cross-linked actin networks at Alcon (Fort Worth, TX) and were a kind gift. Cell strain information:
NTM340-07 male donor at age 80; NTM210-05 female donor with age unknown; NTM176-04 male donor at age 72.

The TGFβ or Wnt signaling pathway activity was expressed as relative luciferase units (RLU), which represent firefly luciferase levels normalized by renilla luciferase levels. We found that TGFβ2 and Wnt3a were able to activate their respective pathways (n=6, $p<0.05$) (Figure 1). However, co-treatment with TGFβ2 and Wnt3a significantly inhibited TGFβ2-induced TGFβ signaling activation as well as Wnt3a-induced Wnt signaling activation (n=6, $p<0.05$) (Figure 1). These data showed a cross-inhibition between these two pathways. In contrast, TGFβ2 or Wnt3a treatment alone had no effect on the basal activity of the other pathway except in NTM1022-05

cells in which Wnt3a inhibited basal TGFβ signaling (Figure 1C), suggesting that a concurrent activation of both pathways is required for this cross-inhibition.

Since both TGFβ and Wnt pathways have multiple subpathways (Smad-dependent and independent TGFβ pathways; canonical and non-canonical Wnt pathways), we used siRNA to knock down Smad4 or β-catenin, the key mediators of TGFβ/Smad pathway and canonical Wnt pathway, respectively, and performed similar luciferase assays. Our rationale is that without Smad4 or β-catenin, the non-Smad pathway or non-canonical Wnt pathway remains functional, respectively. However, the TGFβ/Smad or the canonical Wnt pathway will be disabled. This approach enabled us to dissect the subpathways involved in this cross-inhibition.

We performed luciferase assays using the transformed human NTM cell line HTM5 because it was technically challenging to conduct lentiviral transduction together with siRNA knockdown in primary TM cells. For these experiments, $3x10^4$ HTM5 cells were plated in 96 well opaque plates in Opti-MEM medium (Invitrogen, Grand Island, NY) without antibiotics but with 5% FBS and 1% glutamine. Right after cells were seeded, they were transfected with 50µl transfection mixture containing 100ng TCF/LEF or SBE luciferase reporter plasmids (Cignaling Reporter, Qiagen), 30 nM siRNA against β-catenin, Smad4 or non-targeting siRNA (OnTarget siRNA, GE Dharmacon, Lafayette, CO), and 0.6µl transfection reagent (Attractene, Qiagen). Different from lentiviral luciferase vectors, the luciferase plasmids are premixed with the TCF/LEF or SBE firefly luciferase reporter vector and mCMV renilla luciferase reporter vector according to manufacturer instructions. On day 2, medium was changed to serum-free Opti-MEM. On day 3, cells were treated with 100ng/ml Wnt3a, 5µg/ml TGFβ2, or both. On day 4, luciferase levels were measured as previously described. Experiments were performed in replicates (n=6), and data were analyzed as described previously.

Besides, to validate siRNA-mediated knockdown of target genes, we seeded $2x10^5$ HTM5 cells into a 12-well plate. Right after seeding, cells were transfected with a mixture (200 µl) with or without 30nM siRNA and/or 6.4 µl Attractene (Qiagen). Three day post-transfection, whole cell lysate was extracted using the M-PER Mammalian Extraction Reagent (Thermofisher) with 1:100 protease inhibitors. Proteins were separated using SDS-PAGE, transferred onto an Immobilon-P transfer membrane, blocked with 5% dry milk, and immunoblotted with anti-Smad4, anti-β-catenin (Cell Signaling, 1:500), and anti-GAPDH antibodies (Cell Signaling, 1:10,000) followed by HRP-linked anti-rabbit or anti-mouse IgG secondary antibody. Signal was developed using the Clarity Western ECL Blotting substrate (Bio-Rad, Hercules, CA). Images were taken using the FluroChem imaging system (Cell Biosciences, Santa Clara, CA) or the Bio-Rad ChemiDoc imaging system (Bio-Rad). Densitometry was performed and the level of the protein of interest was normalized to GAPDH. The level of non-targeting siRNA treated samples was set at 1. One-way ANOVA plus post-hoc tests were used to compare protein levels.

We found that HTM5 cells had similar responses to Wnt3a and/or TGFβ2 (Figure 2A and D) as in primary NTM cells (i.e. Wnt3a inhibited TGFβ2-induced TGFβ signaling and TGFβ2 inhibited Wnt3a-induced Wnt signaling). Knocking down Smad4 or β-catenin resulted in complete inhibition of TGFβ or Wnt pathway activation, respectively (Figures 2B and 2F), confirming the effectiveness of our siRNA in luciferase assays. β-catenin knockdown did not affect TGFβ2-induced TGFβ pathway activation (Figure 2C) and Smad4 knockdown did not affect Wnt3a-induced Wnt pathway activation (Figure 2E), demonstrating the specificity of our siRNA. With the knockdown of either Smad4 or β-catenin, TGFβ2 and Wnt3a co-treatment were unable to inhibit TGFβ2-induced TGFβ signaling (Figures 2B and 2C) or Wnt3a-induced Wnt signaling (Figures 2E and 2F), suggesting these pathway mediators are necessary for cross-

inhibition to occur. Our Western immunoblotting and densitometry results showed that there was about 70% decrease in Smad4 and β-catenin proteins ($p<0.01$, $n=3$), confirming the effectiveness of our siRNAs (Figure 2G and H).

In summary, our studies showed that there exists a cross-inhibition between the TGFβ/Smad pathway and the canonical Wnt pathway in the HTM. This inhibition requires both pathways' key mediators Smad4 and β-catenin.

Very few studies on such crosstalk in the TM have been reported. Villareal and colleagues showed that LiCl, a non-specific Wnt pathway activator, inhibits TGFβ2-induced expression of a subset of ECM and matricellular proteins [31]. However, the same study suggested that this inhibition is mediated by canonical Wnt signaling as well as miR-29b, and the latter may be due to the non-specific effect of LiCl. Also, whether there is a direct inhibition of the TGFβ signaling by the Wnt pathway or vice versa was not reported.

In non-TM cells and tissues, the crosstalk between the TGFβ and Wnt pathways has been well studied. However, it has different mechanisms and also shows various effects in different cells and tissues. Jian and colleagues found that in bone marrow-derived adult human mesenchymal stem cells, Smad3 and β-catenin form a complex to facilitate their translocation [32]. In chondrocytes, Zhang and colleagues reported that both Smad2 and Smad3 are required for the interaction with β-catenin, and they protect β-catenin from being degraded [33]. Nawshad and colleagues found that the p-Smad2-Smad4-LEF1 complex inhibits TGFβ3-induced E-Cadherin expression in mouse palate medial edge epithelial cells [34]. Nishita and colleagues showed that Smad4 is able to complex with β-catenin during Xenopus development [35]. In cancer cells, Smad4 and β-catenin can form a complex to regulate each other's activity [36,37]. The impaired crosstalk between the two pathways is also associated with cancer development and epithelial to

mesenchymal transition in metastasis [38,39]. Because the interaction between the two pathways is highly cell/tissue specific, elucidating the exact mechanism that mediates the cross-inhibition between Smad/TGFβ and canonical Wnt pathways in the TM is very important. Based on published studies and our findings that both Smad4 and β-catenin are required for this cross-inhibition, it is our hypothesis that the two transcription factors form a complex, and this complex represses both pathways' activity due to conformational changes and/or blockage of protein binding sites.

In this study, we used luciferase assays, a well-established approach to study pathway crosstalk. Although the luciferase assay offers specific and quantitative measurement of pathway activities, this artificial promoter-based reporter assay does not reflect the complexity of the cis-element involved in transcription including silencers, enhancers, and epigenetic factors that may fine regulate the crosstalk between TGFβ2 and Wnt pathways. Therefore, more research including ex vivo and in vivo studies is needed to elucidate this cross-inhibition in the TM.

In the healthy TM, Wnt signaling may function as a negative regulatory mechanism by which the effects of TGFβ2/overactivated TGFβ/Smad signaling on ECM, CLANs, and other cellular changes are alleviated. Since elevated TGFβ2 and SFRP1 are found in the AH and TM of many POAG patients, it is very likely that they co-exist in a number of POAG eyes. Under this situation, the inhibition of Wnt signaling by SFRP1 very likely disables the potential Wnt negative regulatory mechanism. Therefore, TGFβ2 and SFRP1 may contribute to POAG in a synergistic manner. Again, further studies are required to determine the molecular mechanism of this cross-inhibition and the exact molecules involved. This information may help us to identify novel therapeutic targets to provide new disease-modifying treatments for POAG.

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Figures

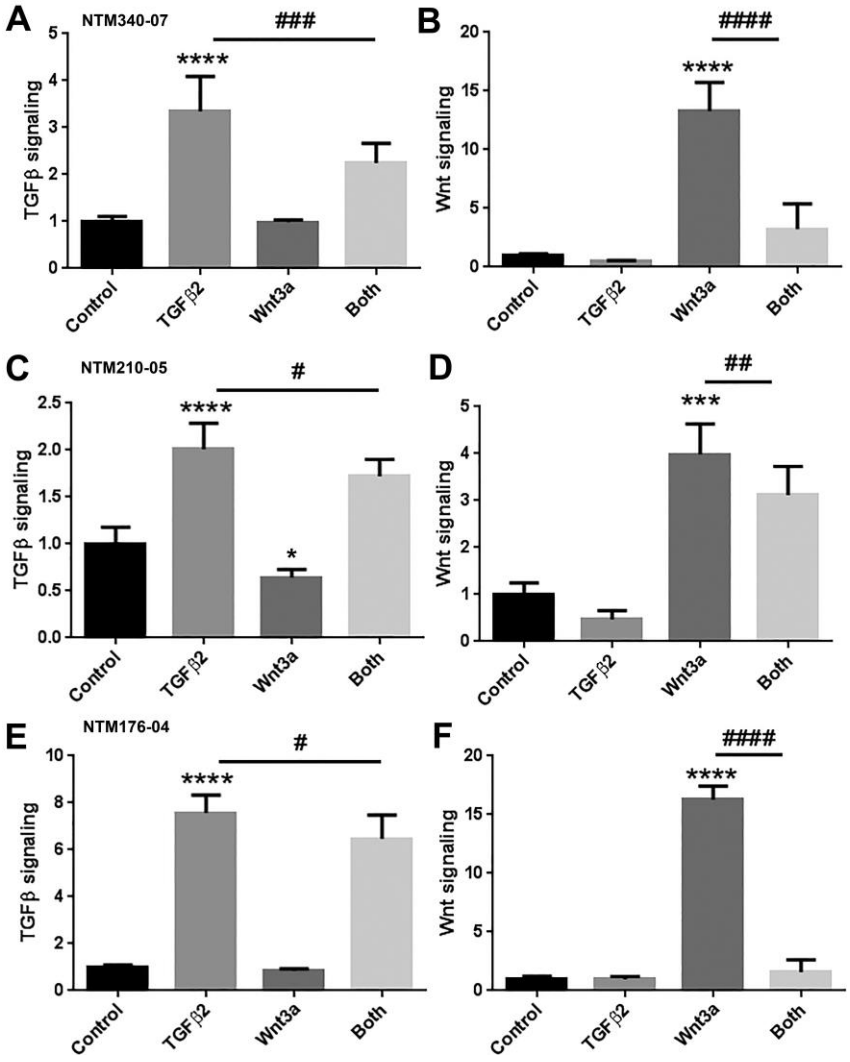

Figure 1. TGFβ and Wnt pathways inhibit each other's activity in primary NTM cells.

Primary NTM cells were transduced with the SBE (A, C and E) or TCF/LEF (B, D and F) lentiviral luciferase reporter vectors to study the TGF pathway (A, C, and E) and Wnt pathway (B, D, and F) activities, respectively. Cells were treated with or without 5ng/ml TGFβ2, 100ng/ml Wnt3a, or both (TGFβ2+Wnt3a) for 24 hours. Columns and bars: means and standard deviations of measured RLU, which represent individual pathway activity. *, ***, ****: $p<0.05$, 0.001, or 0.0001, compared to Control (A-F); #, ###: $p<0.05$ or 0.001, compared to TGFβ2 (A, C and E); ##, ####: $p<0.01$ or 0.0001 compared to Wnt3a (B, D, and F). N=6.

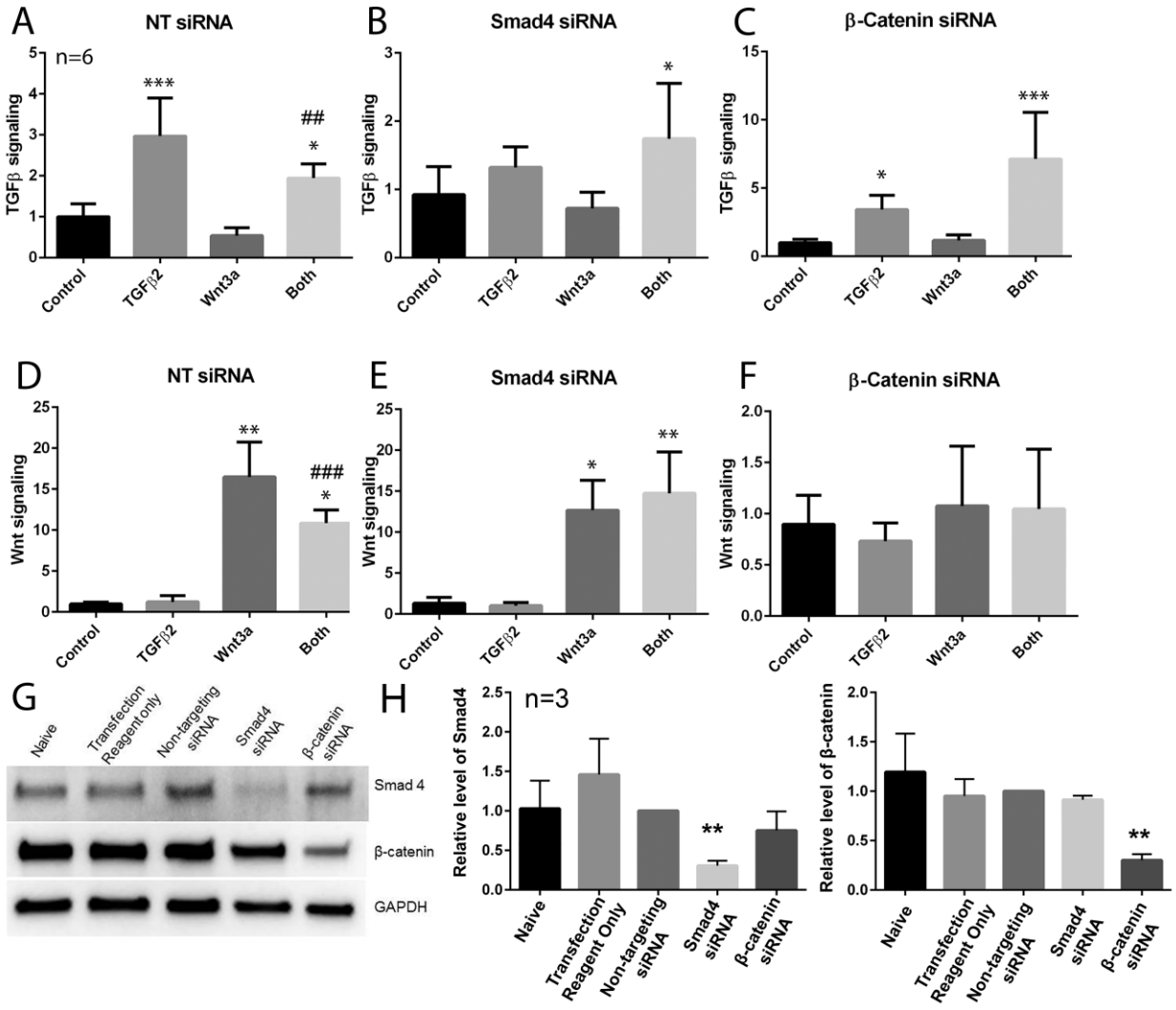

Figure 2. Smad4 and β-catenin are required for the cross-inhibition between TGFβ and Wnt pathways.

Smad4 (B and E) or β-catenin (C and F) was knocked down in HTM5 cells using siRNA. Non-targeting siRNA (NT) was used as a control (A). Cells were treated with or without 5ng/ml TGFβ2, 100ng/ml Wnt3a, or both (TGFβ2+Wnt3a) for 24 hours. TGFβ (A-C) and Wnt (D-F) pathway activities were measured using SBE or TCF/LEF plasmid-based luciferase assays, respectively. Columns and bars: means and standard deviations of measured RLU, which represent individual pathway activity. *, **, or ****: $p<0.05$, 0.01 or 0.0001, compared to

Control; ##: $p<0.001$, compared to TGFβ2 (A); ###: $p<0.001$ compared to Wnt3a (D); NS: not significant ($p>0.05$). N=6. Also, Western immunoblotting was used to confirm protein knockdown in siRNA transfected HTM cells (G). Densitometry and one-way ANOVA plus post-hoc tests were used to compare relative protein levels (H); columns and bars: means and standard deviations; **: $p<0.01$, compared to the non-targeting siRNA group; n=3.

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CHAPTER III. THE ROLE OF WNT/β-CATENIN SIGNALING AND K-CADHERIN IN THE REGULATION OF INTRAOCULAR PRESSURE

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Authors: Hannah C. Webber, Jaclyn Y. Bermudez, J. Cameron Millar, Weiming Mao, Abbot F. Clark

Institutions:

North Texas Eye Research Institute

University of North Texas Health Science Center

Abstract

Purpose: Primary open angle glaucoma (POAG) is the most prevalent form of glaucoma and is associated with pathological changes in the trabecular meshwork (TM) that likely lead to increased aqueous humor outflow resistance and increased intraocular pressure (IOP). Wnt/β-catenin signaling in the TM normalizes IOP, although the mechanism(s) behind this are unknown. We hypothesize that Wnt/β-catenin signaling regulates IOP via cadherin junctions through β-catenin.

Methods: Normal human TM (NTM) cells were treated with or without 100ng/ml Wnt3a, 1μg/ml sFRP1, or both for 4-48 hours. Cells were immunostained for β-catenin, total cadherin, or cadherin isoforms. Membrane proteins or whole cell lysates were isolated for western immunoblotting (WB) and probed for cadherin isoforms. RNA was extracted for cDNA synthesis and qPCR analysis of cadherin expression. Ad5.CMV recombinant adenoviruses encoding K-cadherin, and/or sFRP1 were injected into eyes of 4-6 month old female BALB/cJ mice (n=8-10). Conscious IOP was assessed for up to 35 days.

Results: Upon Wnt3a treatment, total cadherin expression increased and β-catenin accumulated at the TM cell membrane and on connections formed between TM cells. QPCR showed that Wnt3a significantly increased K-cadherin expression in NTM ($p<0.01$, n=3) and WB showed that Wnt3a increased K-cadherin in NTM, which was inhibited by the addition of sFRP1. Our in vivo study showed that K-cadherin significantly decreased sFRP1-induced ocular hypertension ($p<0.05$, n=6). Mouse anterior segment tissue also showed that K-cadherin alleviated sFRP1-induced β-catenin decrease.

Conclusions: Our results suggest that cadherins play a role in the regulation of TM homeostasis and IOP via the Wnt/β-catenin pathway.

Introduction

Glaucoma is an incurable, blinding disease characterized by optic nerve degeneration. Glaucoma is age-associated, and study from the 2005-2008 NHANES cohort shows that 2.91 million Americans over the age of 40 years suffer from some form of the condition[1]. As the average life span continues to rise, so too will the prevalence of this blinding progressive neuropathy. Primary open angle glaucoma (POAG) is the most common form of glaucoma, and a meta-analysis of 81 studies on POAG prevalence showed that in 2015, 57.5 million people were affected by POAG globally[2]. This number is projected to rise to 65.5 million by 2020[3], with some 11.1 million people expected to be bilaterally blinded[3] (150,000 in the USA alone[1]). POAG is a heterogeneous disease associated with multiple risk factors including ethnicity, family history, aging, and ocular hypertension (OHT)[4]. OHT is defined by an intraocular pressure (IOP) of 21mmHg or higher, and is the main causative and only clinically modifiable risk factor. In POAG with OHT, IOP increases when aqueous humor resistance through the trabecular meshwork (TM) increases. POAG is associated with inherent pathological changes to the TM that lead to this increased aqueous humor outflow resistance in patients with OHT. However, there are no currently available therapeutics that target TM pathology, and the causes of these pathological changes to the TM remain unknown. Comparisons between the healthy TM and the POAG TM have shown decreased TM cellularity, increased ECM in trabecular beams, increased TM cell stiffness, increased actin contractility, an increase in cross-linked actin networks (CLANs), and deregulation of TM cell signaling pathways including the transforming growth factor beta (TGFβ), bone morphogenic protein (BMP), and Wnt/β-catenin signaling pathways[5-8]. Increased expression of the growth factors transforming growth factor beta-2 (TGFβ2), Gremlin,

and secreted frizzled-related protein 1 (sFRP1) in the TM have been shown to lead to dysregulation of these homeostatic pathways[9-16].

sFRP1 is an antagonist of the Wnt signaling pathway that sequesters Wnt ligands outside of the cell, thereby blocking binding to Frizzled receptors.[17,18] When this happens, the intracellular "Wnt/β-catenin inhibitory complex" made up of GSK-3β, Axin, APC, and CK1 proteins tag the Wnt/β-catenin signaling mediator β-catenin for destruction through phosphorylation, ubiquitination, and proteasomal degradation. Therefore, β-catenin will be constantly degraded in the cell and will cease to translocate into the nucleus to regulate transcription of Wnt regulated genes via binding at the T-cell factor/lymphoid enhancer factor (TCF/LEF) promoter[19]. Expression of of Wnt antagonist sFRP1 is increased in the OHT glaucomatous TM compared to healthy TM[11]. When overexpressed, sFRP1 leads to increased IOP in perfusion-cultured human eyes and in mice eyes. Co-treatment with a Wnt path activator inhibits sFRP1-induced OHT in mouse eyes. Recent studies have shown that Wnt/β-catenin signaling genes are actively expressed in the TM, and that Wnt ligand Wnt3a activates the Wnt/β-catenin signaling pathway through β-catenin, but not the other non-canonical Wnt pathways[20,21]. Indirect activation of Wnt/β-catenin signaling in the TM through lithium chloride treatment has also been found to negatively regulate production of some extracellular matrix and matricellular proteins, as well as negatively regulate the TGFβ pathway and TGFβ-induced ECM production[22]. We have shown that the Wnt/β-catenin and TGFβ/SMAD pathways can cross-inhibit one another in non-glaucomatous primary TM (NTM) cells, and that this cross-inhibition relies on these pathways' main transcription factors β-catenin and Smad4[23]. However, the mechanisms by which Wnt/β-catenin signaling regulates IOP remain unclear.

β-catenin is the key transcription factor for Wnt/β-catenin signaling, but performs another function in the cell as an accessory protein to classical cadherins junctions. Cadherins are transmembrane proteins whose extracellular domains form calcium-dependent homophilic binding interactions to connect cells together[24]. The classical cytosolic domain of cadherins binds directly to β-catenin, among other accessory proteins such as α-catenin and p120-catenin[25]. Cadherins are connected to and stabilized by the actin cytoskeleton through these catenin accessory proteins, and in fact β-catenin is required for the cadherin-actin complex. Phosphorylation of β-catenin at different sites and the localization of β-catenin determine whether β-catenin remains bound to cadherins, and the lack of β-catenin-cadherin binding disrupts the cadherin-actin complex[26,27]. Since β-catenin plays major roles in both Wnt/β-catenin signaling and adherens junctions, it is likely that Wnt/β-catenin signaling regulates the anchoring of cadherins junctions to the actin cytoskeleton by regulating membrane associated β-catenin. Currently, the connection between Wnt/β-catenin signaling and cadherins junctions has not been explored in the TM.

Cadherins also play key roles in directing tissue patterning and forming neural connections during development, epithelial to mesenchymal transition (EMT), and serving as mechanosensors that relay mechanical perturbation from the cells' environment to the actin cytoskeleton to facilitate a tensile or migratory response[28-31]. Many isoforms of cadherin proteins exist in multiple species including humans, and their expression and distribution are highly tissue-specific. Different cadherin isoforms convey different physiological properties to cells. In the TM, Wecker and colleagues showed that in HTM cells, N-cadherin and OB-cadherin are up-regulated by treatment with TGFβ2 [32]. However, the expression other cadherin isoforms is still unclear in the HTM. In addition, the role of these cadherins in IOP regulation is unknown.

In this study, we determined the expression and localization of 4 cadherins in primary HTM cells and the regulation of these cadherins by Wnt/β-catenin signaling. We also studied the role of cadherins in sFRP1-induced OHT in mice.

Methods

Immunocytofluorescence (ICF)

NTM cells (NTM895-03: Female donor, age 63 years; NTM176-04: Male, age 72 years; NTM875-03: Male, age 77 years) were cultured on glass coverslips in DMEM-low glucose medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin (Thermofisher, Waltham, MA). When cells were confluent, culture medium was changed to serum-free medium, and the cells were cultured for an additional 24 hours. The cells were then treated with or without 100ng/ml recombinant mouse Wnt3a and/or 1μg/ml recombinant human sFRP1 for 24 hours (R&D Systems, Minneapolis, MN). At the end of treatment, cells were fixed with 4% paraformaldehyde at 4 °C, treated with 0.05% TritonX-100, blocked with Superblock (Thermo Scientific, Waltham, MA, USA) and immunostained with rabbit anti-β-catenin (1:250, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-K cadherin (1:250, Abcam, Cambridge, United Kingdom) or rabbit anti-Pan-cadherin antibody (1:250, Abcam). After incubation with a secondary donkey anti-rabbit antibody conjugated with Alexafluor 488 (1:500, Thermo Fisher Scientific), some cells were stained with with Phalloidin conjugated with Alexafluor 594 (1:1000, Life Technologies, Carlsbad, CA, USA) for 1 hour. After staining, glass coverslips were mounted on slides using the ProLong gold anti-fade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies). All images were captured using

a Nikon Eclipse Ti-U Epifluorescent microscope (Nikon, Minato, Tokyo, Japan) in combination with a Nuance imaging system (Nuance Communications, Burlington, MA, USA).

Quantitative Polymerase Chain Reaction (qPCR)

NTM cells were cultured in 12-well plastic dishes until they were confluent. They were serum-starved for 24 hours, then treated for an additional 24 hours with or without 100ng/ml Wnt3a and/or 1μg/ml sFRP1. Triplicates were used for each treatment. After treatment, cells were used for total RNA isolation using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed into cDNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA), and qPCR was performed using the SsoAdvance Universal SYBR Green Supermix (Bio-Rad) in a CFX96 thermocycler (Bio-Rad). The thermoprofile consisting of 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, followed by a temperature dissociation curve. GAPDH primers were used as an internal control. All the primers used were designed to span or flank exon-exon junctions. The primer (synthesized by Sigma Aldrich, St. Louis, MO, USA) sequences are as follows:

Axin2:

Forward: CAGATCCGAGAGGATGAAGAGA

Reverse: AGTATCGTCTGCGGGTCTTC

K-cadherin (CDH6):

Forward: GGCAGATCAGTTGATTCAGA

Reverse: GCCGTGTTGTCTTTGTTGTC

OB-cadherin (CDH11):

Forward: CAAAGTTTCCGCAGAGCGTA

Reverse: GCTTTATCACCCCCTCCTGT

N-cadherin (CDH2):

Forward: ATCCTGCTTATCCTTGTGCTG

Reverse: CCTGGTCTTCTTCTCCTCCA

GAPDH[33]:

Forward: GGTGAAGGTCGGAGTCAAC

Reverse: CCATGGGTGGAATCATATTG

Cadherin-19 (CDH19) A:

Forward: TGCAGGCTCTGGTCAGGTA

Reverse: GAAGACAGGTTCTTCTTGAAGGTT

Cadherin-19 (CDH19) B:

Forward: AGCACAAGCGTCTGTAACTCTG

Reverse: GGAAACAGGACGTCACTAACAA

Western immunoblotting (WB)

NTM cells were cultured in 60mm dishes until they were confluent. The cells were serum-starved for 24 hours, and treated with 100ng/ml Wnt3a and/or 1μg/ml sFRP1 for an additional 4, 24, or 48 hours. After treatment, the membrane protein fraction was isolated using the Eukaryotic Membrane Extraction Kit (Thermo Fisher Scientific), while the cytosolic and nuclear fractions were isolated using the NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Scientific). Total protein was isolated using the M-PER Mammalian Extraction Reagent (Thermo Fisher). Equal amounts of protein from each sample were separated using SDS-PAGE, transferred onto an Immobilon-P transfer membrane, blocked with 5% dry milk, and immunoblotted overnight at 4°C with rabbit anti-K-cadherin antibody (1:500, Abcam), rabbit anti-OB-cadherin antibody (1:500, Abcam), rabbit anti-β-catenin antibody (1:500, Cell Signaling), rabbit anti-GAPDH antibody (1:10,000, Cell Signaling), or mouse anti-Lamin A/C antibody (1:1000, Cell Signaling). The blots were then rinsed and incubated in secondary HRP-linked anti-rabbit or anti-mouse IgG antibody (1:5000, Cell Signaling Technology). Signals were developed using the Clarity Western ECL Blotting substrate (Bio-Rad). Images were taken using the Bio-Rad ChemiDoc imaging system (Bio-Rad).

Adenoviral vectors

Adenovirus serotype 5 (Ad5) vectors that overexpress the human K-cadherin and mCherry (Ad5.K-cadherin), mouse sFRP1 (Ad5.sFRP1), as well as a null vector (Ad5.Null) were obtained commercially from Vector Labs (Burlingame, CA, USA). Expression of each exogenous gene was driven by its own CMV promoter including mCherry in the Ad5.K-cadherin vector.

Viral transduction in mouse eyes

All mouse studies were conducted in compliance with the UNTHSC Institutional Animal Care and Use Committee and the ARVO Statement on the use of Animals in Ophthalmic and Vision Research. Female BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice aged between 4-6 months were used for intravitreal adenoviral injection. Prior to use, all animals' eyes were examined (hand-held ophthalmoscope, Welch-Allyn, Skaneateles Falls, NY, USA) to confirm that they presented a normal appearance. Immediately before viral injection, mice were anesthetized with a cocktail of ketamine/xylazine, administered intraperitoneally at 100mg/kg and 10mg/kg, respectively. A proportion of mice were instead anesthetized using inhalation anesthesia (isoflurane (2.0-2.5%), in combination with O_2 (0.8 L/min)). After anesthesia, equal numbers of infectrious units (IFU) were injected into the vitreous chamber of the left eye. $3x10^7$ infectious units in 1-5µl were slowly injected over a period of 1-2 minutes using a glass microsyringe (Hamilton Company, Reno, NV, USA) fitted with a 33G needle. The uninjected right eyes served as paired controls.

The treatment groups are as follows; each group comprised of 8-10 mice:

Ad5.K-cadherin ($1.5x10^7$ IFU) + Ad5.sFRP1 ($1.5x10^7$ IFU)

Ad5.K-cadherin ($1.5x10^7$ IFU) + Ad5.Null ($1.5x10^7$ IFU)

Ad5.sFRP1 ($1.5x10^7$ IFU) + Ad5.Null ($1.5x10^7$ IFU)

Ad5.Null ($1.5x10^7$ IFU)

Conscious mouse IOP measurement

Conscious mouse IOP was assessed using a TonoLab® rebound tonometer (Colonial Medical Supply, Franconia, NH, USA) in a masked manner. Baseline IOP was measured before viral injection, and IOP post viral injection was monitored on indicated days. At days 9 and 21, two mice from Ad5.K-cadherin, Ad5.sFRP1, and Ad5.K-cadherin + Ad5.sFRP1 groups were sacrificed for analyses.

Mouse issue analyses

Mice were sacrificed by exposure to 100% carbon dioxide at different time points for analysis. Immediately after sacrifice, eyes were enucleated and placed in ice-cold PBS.

The anterior segments of some eyes were dissected under a dissection microscope with the lens, ciliary body and iris removed. The anterior segment tissue was placed in the T-PER Tissue Protein Extraction Reagent (Thermo Scientific) with 1:100 protease inhibitors. Protein was extracted using the Tissuelyser (Qiagen). An equal amount of protein from each sample was separated using SDS-PAGE, transferred onto an Immobilon-P transfer membrane, blocked with 5% dry milk, and immunoblotted with anti-mCherry (1:500, Abcam), anti-β-catenin (1:500, Cell Signaling Technology), or anti-GAPDH antibody ((1:2,000, Cell Signaling Technology), overnight at 4°C, rinsed, then incubated in secondary HRP-linked anti-rabbit or anti-mouse IgG antibody (1:5000, Cell Signaling Technology). Signal was developed using the Clarity Western ECL Blotting substrate (Bio-Rad). Images were taken using the Bio-Rad ChemiDoc imaging system (Bio-Rad).

Other eyes were fixed with 4% paraformaldehyde at 4°C for 2 hours. These eyes were then washed with PBS, dehydrated, embedded in paraffin, sectioned, and mounted on glass slides. Some sections were subjected to hematoxylin & eosin (H & E) staining. The other sections were immunostained for sFRP1, mCherry, and β-catenin. Those sections were baked at 60°C for 30 mins, de-waxed, and rehydrated through a series of incubations with Xylene, 100% ethanol, 95% ethanol, 50% ethanol, and water. Tris-EDTA buffer (pH9.0) was used for antigen retrieval together with the 2100 Retriever (Electron Microscopy Sciences, Hatfield, PA). After antigen retrieval, the sections were treated with Triton X-100, blocked with Superblock Blocking Buffer (Thermo Scientific), and incubated with 1:100 rabbit anti-sFRP1 antibody (Novus Biological, Littleton, CO, USA), overnight at 4°C. The sections were then incubated with the donkey anti-rabbit secondary antibody conjugated with Alexafluor 488 (Thermo Scientific, 1:500) at room temperature for 2 hours, and mounted using the ProLong gold anti-fade reagent with DAPI (Life Technologies). Images were taken using the Nikon Eclipse Ti-U Epifluorescent microscope and the Nuance imaging system.

Results

Wnt signaling pathway regulated membrane-associated β-catenin and cadherins in TM cells

We first validated that the NTM cell strains and recombinant proteins were suitable to study Wnt signaling. The NTM cells were treated with or without 100ng/ml Wnt3a and/or 2μg/ml sFRP1 for 24 hours, and WB was performed using nuclear and cytosolic fractions (Figure 1A). We found that Wnt3a induced nuclear β-catenin translocation as well as cytosolic β-catenin accumulation, and this induction was blocked by co-treatment with sFRP1 (Figure 1A). Our

findings matched our published results[20], and showed that the cell strains and recombinant proteins were suitable for this study.

After validation tests, we then studied whether the Wnt signaling pathway regulated membrane-associated β-catenin in the TM. After treatment with or without 100ng/ml Wnt3a and/or 2μg/ml sFRP1 for 24 hours, NTM cells were used for ICF to study the distribution of β-catenin (Figure 1B). We found that Wnt3a induced accumulation of cytosolic β-catenin, translocation of nuclear β-catenin, as well as membrane associated β-catenin. Some membrane associated β-catenin was localized to filopodia-like connections that connected TM cells together (Figure 1B, white arrows).

Since β-catenin is part of the cadherin junctional complex, we then determined whether the Wnt pathway also regulates cadherins in the TM. NTM cells were treated with or without Wnt3a for 24 hours, and immunostained using an antibody (anti-Pan-cadherin antibody) that recognizes all types cadherins (total cadherins) (Figure 1C). We found that Wnt3a slightly increased total cadherin expression, and led to cadherin expression spread more throughout the cell as well as on the cell membrane when compared to control. This suggests that canonical Wnt signaling also uses β-catenin to stabilize cadherins on the membrane, which is associated with an increase in cadherin expression.

Wnt signaling pathway regulated the expression of K, OB, and N-cadherins in the TM

Since the cadherins family contains many isoforms, we first determined which cadherins are expressed in the TM. We searched the Iowa Ocular Tissue Database, and found that K-cadherin (CDH6), OB-cadherin (CDH11), cadherin19 (CDH19), and N-cadherin (CDH2) are the most abundant cadherins in the TM tissue[34]. To confirm the expression of these cadherins in the TM, we performed PCR using RNA isolated from NTM cells. K, OB, and N-cadherins were detected in the TM (Figure 2). In contrast, cadherin19 was not detected using two different set of primers (data not shown).

We then studied whether the expression of the 3 cadherins is regulated by the Wnt signaling pathway. We treated NTM cells with or without Wnt3a and/or sFRP1 for 24 hours, and compared the level of K, OB, and N-cadherins using qPCR. Axin2, whose expression is induced by the Wnt pathway, is frequently used as a marker for Wnt signaling activation. We found that K and OB-cadherins were significantly increased by Wnt3a treatment. Co-treatment with sFRP1 completely inhibited Wnt3a-induced K-cadherin expression, but not OB-cadherin. In contrast, Wnt3a did not change the expression of N-cadherin in TM cells. Interestingly, K-cadherin does not have the TCF/LEF binding element sequence within its promoter, so the increase of K-cadherin production may be a secondary effect of Wnt/β-catenin signaling activation.

Wnt3a increased membrane-associated K-cadherin expression in TM cells

Since the induction of K-cadherin by Wnt3a matched the Wnt signaling activity induced by Wnt3a (β-catenin translocation and Axin2 expression, as shown in Figures 1A and 2A), we further studied whether the distribution, especially membrane-associated distribution, of K-

cadherin is regulated by the Wnt/β-catenin signaling in the TM. We first treated NTM cells with or without Wnt3a together with or without sFRP1, and compared K-cadherin using ICF (Figure 3A). We found that that Wnt3a treatment increased the expression of K-cadherin in TM cells, and this increase was partially inhibited by co-treatment with sFRP1.

To compare membrane-associated K-cadherin quantitatively, we treated primary NTM cells with or without Wnt3a and/or sFRP1, and extracted the membrane protein fraction or whole cell lysate, for WB (Figure 3B). Membrane-associated K-cadherin increased 4 hours after Wnt3a treatment, but total K-cadherin did not, which suggest that Wnt signaling alters K-cadherin distribution at early stage before transcriptional regulation occurs. By 24 hours, total K-cadherin increased with Wnt3a treatment, as well as a more profound accumulation of β-catenin. This increase in membrane-associated and total K-cadherin as well as β-catenin was maintained for 48 hours. Similarly, all these inductions were inhibited by co-treatment with sFRP1. These data show that the Wnt/β-catenin signaling plays key role in regulating K-cadherin expression and localization in different intracellular compartments.

K-cadherin inhibited sFRP1-induced OHT

Since Wnt/β-catenin signaling regulates K-cadherin in the TM and inhibition of the Wnt/β-catenin induces OHT[11], we explored whether K-cadherin plays a role in maintaining IOP. We transduced mouse TM tissues by injecting Ad5 with or without the sFRP1 or K-cadherin expressing cassette, or a mixture of both vectors into the vitreous chamber of the left eye of 4-6 month old BALB/cJ mice. The right eye served as an injected naïve control. Our published studies have shown that Ad5 has tropism for the TM.[35] Conscious IOPs of both eyes were

recorded by using a rebound tonometer. Baseline IOP at day 0 and post-injection IOP at indicated days were plotted over time (Figure 4).

We found that Ad5.sFRP1 induced OHT ($p<0.01$ or 0.001 compared to Ad5.Null or contralateral control), which matched our published studies[11,20]. We also found that Ad.K-cadherin did not affect IOP ($p>0.05$ compared to controls). However, a co-transduction with Ad5.K-cadherin significantly inhibited sFRP1 induced OHT from day 6 to day 20 ($p<0.001$ compared to sFRP1 alone), and it completely inhibited OHT at day 35 ($p>0.05$ compared to controls).

During and at the end of the experiment, we collected mouse TM tissues for analyses (Figure 5). We first studied the morphology of the anterior segment using H&E staining to establish that there were no posterior, anterior, or peripheral anterior synechiae, mediated by inflammation and fibrin formation, which could lead to a partial or even complete closure of the chamber angle, leading to OHT. We did not find obvious signs of inflammation, inflammatory infiltrates, or synechiae, and the angle remained open (Figure 5A). Also, during the course of the experiments, routine ophthalmoscopic examinations performed each time IOP was assessed indicated that the eyes appeared normal (data not shown).

We then studied the expression of sFRP1 and K-cadherin in the TM tissues using immunofluorescence (IF) and WB. Our Ad5.K-cadherin viral vector contained an mCherry tag driven by a separate promoter. Since sFRP1 is a secreted protein and our Ad5.sFRP1 vector did not have a tag protein, we compared sFRP1 expression using IF instead of WB because the latter does not show secreted sFRP1 protein levels. We found that sFRP1 (endogenous and exogenous)

was expressed in multiple tissues including the cornea, sclera, iris, ciliary body, and retina. However, there was more sFRP1 in the TM in Ad5.sFRP1 transduced eyes (Figure 5B). We also compared the expression of K-cadherin (as shown by mCherry) and β-catenin in dissected anterior segment tissues using WB. We found that K-cadherin was expressed in both Ad5.K-cadherin and Ad5.sFRP1 + Ad5.K-cadherin anterior segments on day 9 post viral injection (Figure 5C).

Also, to confirm that overexpressed sFRP1 was functional, we probed anterior segment tissues for β-catenin using WB (Figure 5D). We found that total β-catenin was decreased in the anterior segments transduced with the Ad5.sFRP1 injected eyes, as compared to the fellow uninjected control eyes. Interestingly, co-transduction with K-cadherin seemed to at least partially block this inhibition of β-catenin.

Discussion

This study is a crucial step in understanding the molecular mechanisms involved in how the TM Wnt/β-catenin signaling pathway that regulates IOP. We found that K-cadherin is highly expressed in normal human TM at the mRNA and protein levels and that K-cadherin expression and mobilization to the TM membrane is governed by Wnt/β-catenin signaling. We also showed that K-cadherin overexpression can significantly reduce the ocular hypertensive effects of sFRP1 in vivo. The addition of Ad5.K-cadherin to Ad5.sFRP1 significantly decreased IOP when compared to Ad5.sFRP1 alone ($p<0.001$ from days 9 to 35). This suggests that the effects of K-cadherin can compensate for or reverse the effects of sFRP1 to normalize IOP, and/or that sFRP1 induces ocular hypertension by disrupting K-cadherin junctions. Interestingly, we found that K-

cadherin alone does not significantly alter IOP compared to control (Figure 4b). This suggests that addition of K-cadherin expression will not change outflow facility under normal conditions, but rather will allow the TM to sense and respond to extracellular changes, such as changes in ECM deposition or cell stiffness. We have also shown that upon Wnt signaling activation, some membrane associated β-catenin was localized to filopodia-like connections that connected TM cells together, which may define a novel structure by which TM cells maintain cell-cell connections.

K-cadherin, or fetal kidney cadherin, was so named for its high expression in the developing kidney[36]. K-cadherin is also expressed in the kidney well into adulthood, and in other tissues of the body such as the ventricles of the brain and central nervous system during adulthood and development [37-39]. Cadherins play many roles in the cell as homophilic cell-cell adhesion molecules, cell migration promoters or inhibitors, and as mechanosensors relaying external mechanical stimulation to the actin cytoskeleton. Different cadherin isoforms may confer upon adult cells and tissues different physiological properties and characteristics, due to protein structural differences (for example K-cadherin has only 35% protein homology to E-cadherin[40,41]). It is necessary to study the specific characteristics that K-cadherin confers upon the adult TM in order to understand its contribution to IOP regulation at the molecular level.

We show that active Wnt/β-catenin signaling and K-cadherin expression are important in the TM and in IOP regulation. Our findings are consistent with recent studies showing that active Wnt/β-catenin signaling combats the fibrotic processes in the TM, and that the POAG-associated Wnt antagonist sFRP1 promotes these fibrotic processes and increases IOP. We have also previously shown that Wnt/β-catenin signaling and Smad/TGFβ signaling cross-inhibit one another in the TM[23]. A crucial next step in TM research is to study how this POAG-associated

Wnt/β-catenin signaling and TGFβ signaling crosstalk affects TM cadherin expression, cadherin and TM cell function, and finally IOP regulation. Wecker et al. examined TGFβ2 and its effects on β-catenin and two cadherins expressed in the TM, OB-cadherin and N-cadherin. They found that TGFβ-2 and the subsequently activated TGFβ pathway can increase expression of β-catenin, N-, and OB-cadherin in the TM, and that TGFβ-2 can increase TM cell-cell adhesion[32]. However, they did not study β-catenin associated with Wnt/β-catenin signaling, nor did they study K-cadherin expression in the TM or the effects of cadherin expression on IOP. Our study shows that K-cadherin may be very important to TM physiology and may be governed by Wnt/β-catenin signaling. Based on our findings and on the Wecker 2013 study, we believe that TGFβ signaling promotes OB-cadherin expression and that Wnt/β-catenin signaling promotes K-cadherin expression, suggesting a potential mechanism by which these two signaling pathways may regulate one another by promoting opposing cadherins on the TM membrane, that may ultimately confer different physiological properties upon the TM tissue. In other cell types and in cancer cells, it has been shown that K-cadherin and OB-cadherin play opposing yet complementary roles in the cells in which they are expressed. The TM may be no exception[42-44]. Future studies should include analyses of expression of other highly-expressed cadherins in the TM such as OB-cadherin, to understand how K-cadherin becomes the dominantly expressed membrane-bound cadherin on the TM membrane in response to Wnt/β-catenin signaling.

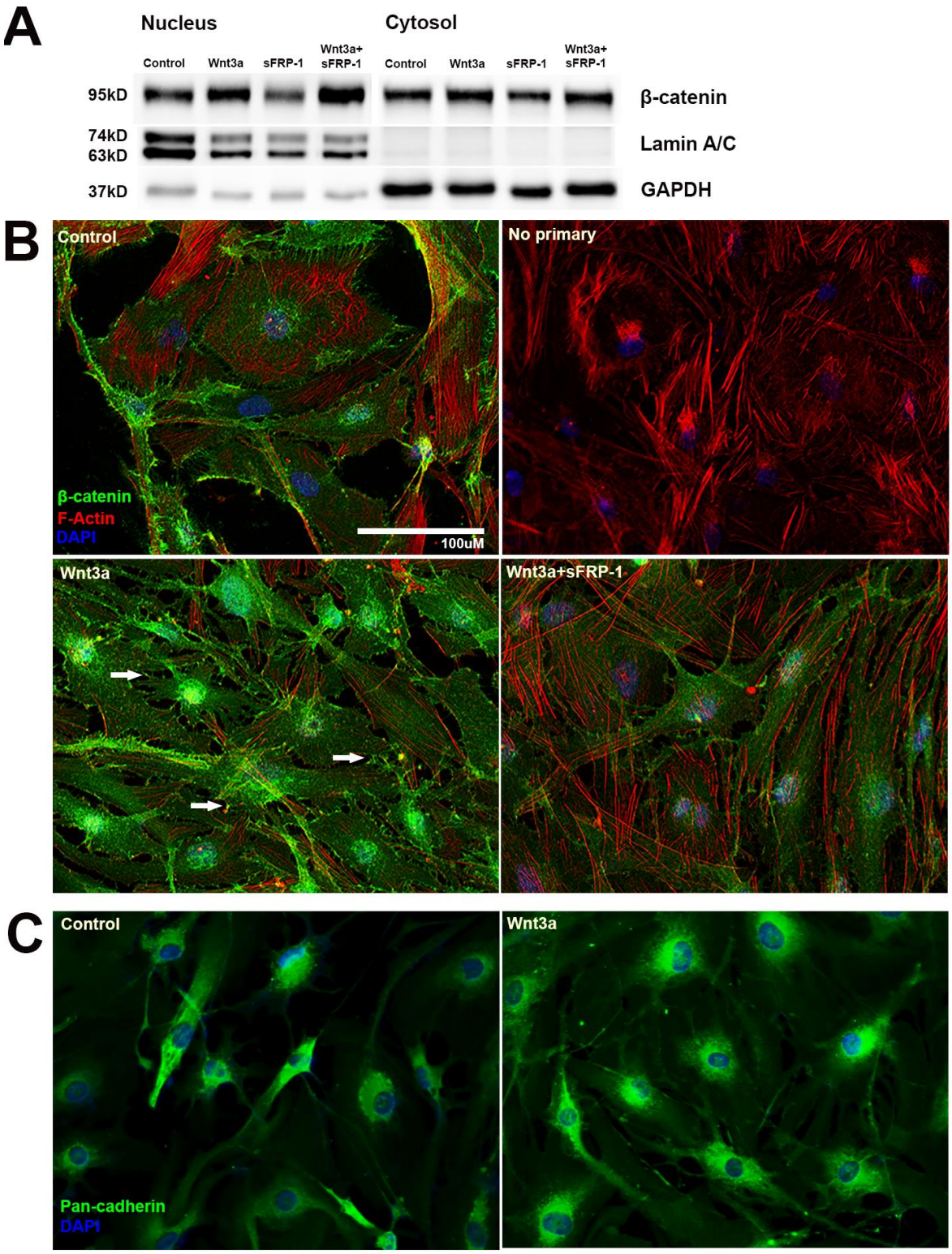
The mechanosensing properties of cadherins confer important cell processes such as contraction/relaxation of the actin network, and modulating cell to extracellular matrix connection through focal adhesions.[45] The actin cytoskeleton of the POAG TM has increased extracellular matrix deposition, increased contractility, and forms CLANs to increase IOP.[7,46] The pathologic antagonism of Wnt/β-catenin signaling that we observe in POAG decreases K-

cadherin expression and therefore may turn off the mechanosensing functions that K-cadherin may offer to TM cells and the control over the actin cytoskeleton and extracellular matrix. TM stiffness increases with antagonism of Wnt/β-catenin signaling[47], suggesting that the decrease in K-cadherin expression and function may increase TM cell stiffness and contribute to POAG TM pathology. Ultimately, TM mechanosensing of the extracellular environment and the ability to regulate the actin cytoskeletal network in response to extracellular cues may be harmed by Wnt/β-catenin antagonism. We need to determine how K-cadherin expression on the TM cell membrane may control the cell cytoskeletal response to restore a functional actin cytoskeleton during POAG.

Acknowledgements

NIH training grant T32 AG 020494 (HW and JB).

Figures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95kD
β-catenin
74kD
63kD
Lamin A/C
37kD
GAPDH
B
Control
β-catenin
F-Actin
DAPI
100uM
No primary
Wnt3a
Wnt3a+sFRP-1
C
Control
Pan-cadherin
DAPI
Wnt3a

Figure 1. Wnt signaling activation resulted in increased membrane-associated β-catenin and total cadherin in primary NTM cells.

Primary NTM cells were treated with or without Wnt3a and/or sFRP1 for 24 hours in serum-free medium. Cells were used for nuclear and cytosolic protein extraction followed by WB (A) or were used for ICF (B&C). (A) Lamin A/C was used as a nuclear protein loading control, and GAPDH was used as a cytosolic protein loading control. (B) ICF staining of β-catenin (green), F-actin (red), and nuclei (blue). F-actin and nuclei were stained using phalloidin-Alexa 594 and DAPI, respectively. White arrows indicate filopodia-like cell to cell connections lined with β-catenin. (C) ICF staining of total cadherins (green) and nuclei (blue).

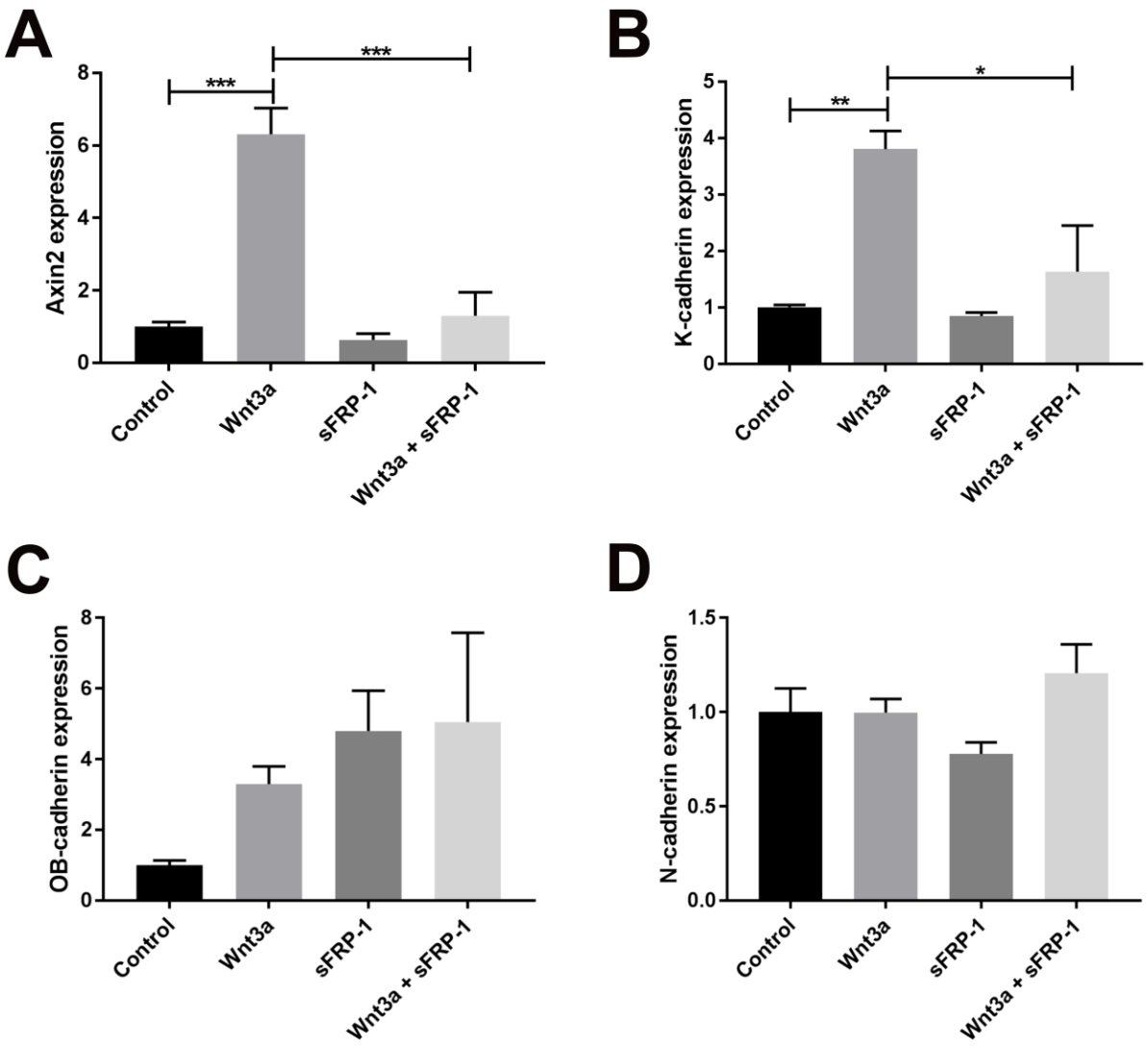

Figure 2. Wnt/β-catenin signaling activation induced the expression of K-cadherin at the mRNA level.

Primary NTM cells were treated with or without Wnt3a and/or sFRP1 for 24 hours, and RNA was extracted for reverse transcription and qPCR. Axin2 was used as a marker of the activation of Wnt/β-catenin signaling. Columns and error bars: means and standard error of mean (SEM). One-way ANOVA with the Dunnett post-hoc test was used for statistical analysis. N=3.*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

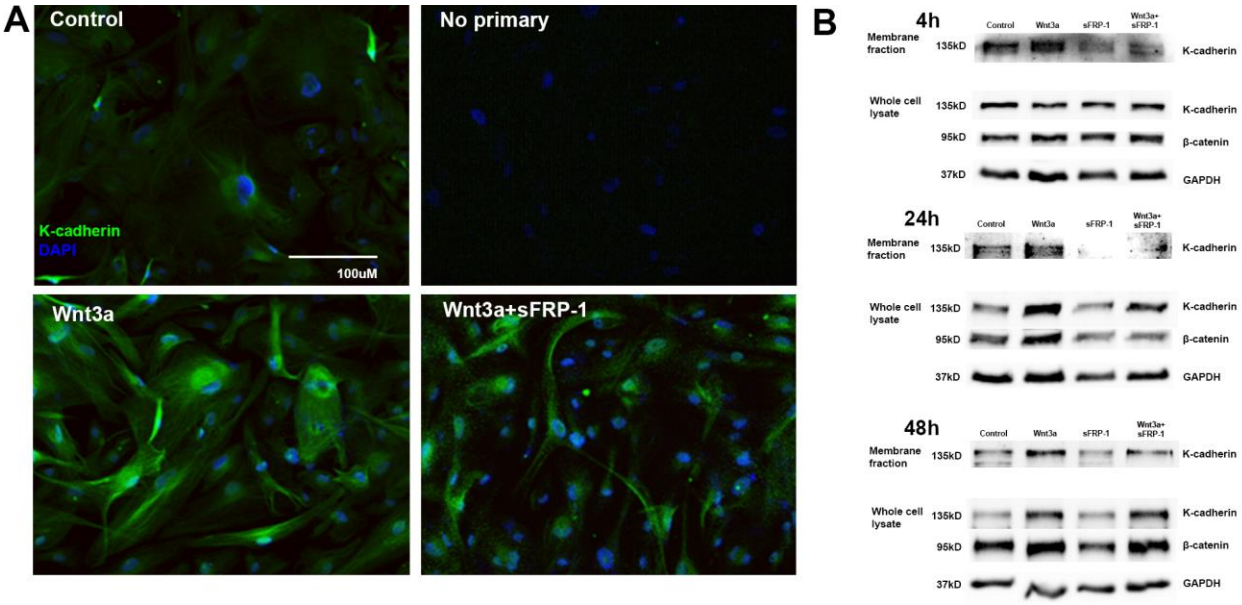

Figure 3. K-cadherin was regulated by Wnt/β-catenin signaling.

(A) Primary NTM cells were treated for 24 hours with or without Wnt3a and/or sFRP1. Cells were fixed stained using IFC for K-cadherin (green) and DAPI (blue). (B) Primary NTM cells were treated for 4, 24, or 48 hours with or without Wnt3a and/or sFRP1. Membrane protein fraction or whole cell lysate was isolated and probed for K-cadherin, β-catenin, and GAPDH.

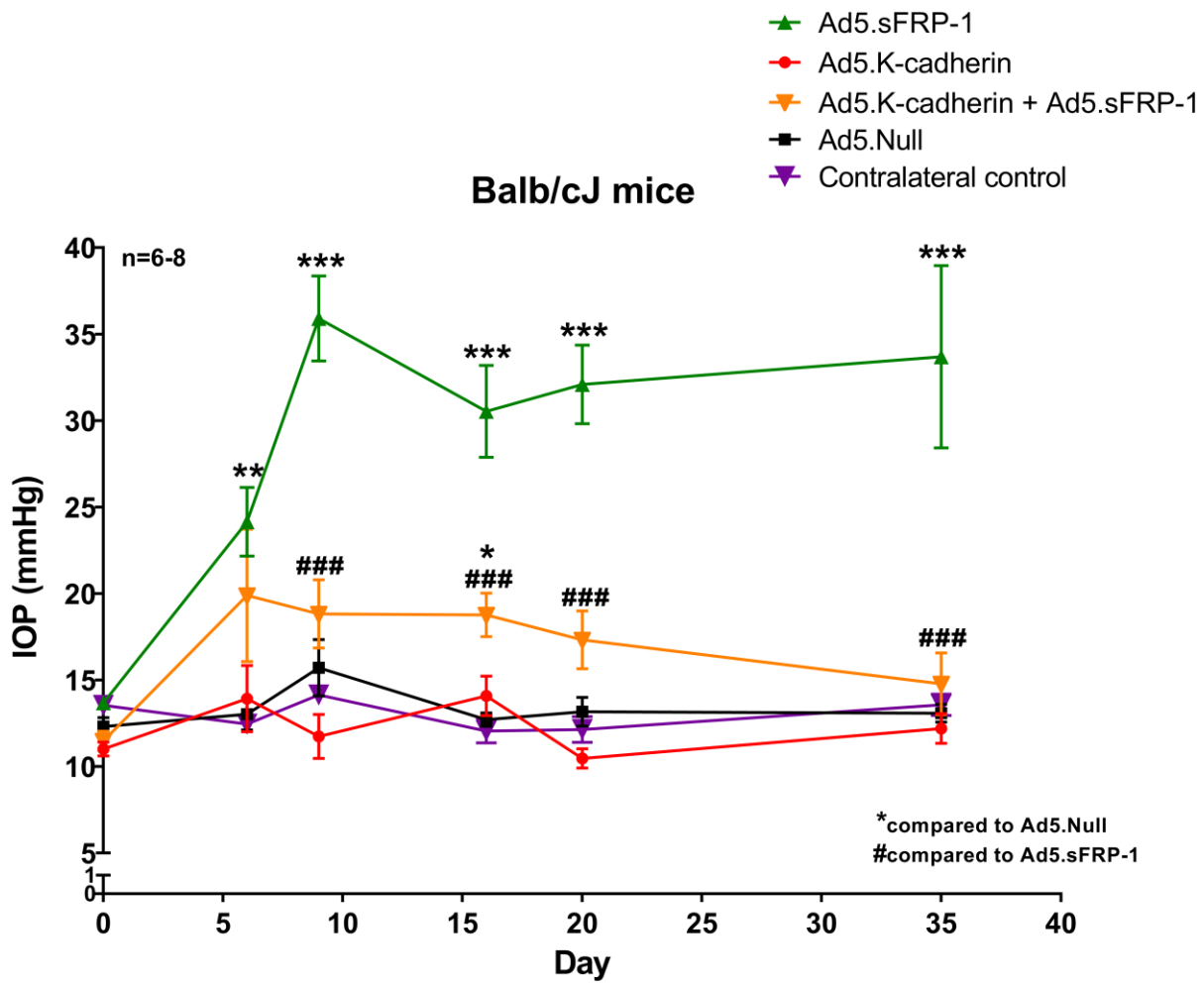

Figure 4. K-cadherin inhibited sFRP1-induced OHT in mouse eyes.

BALB/cJ mice were intravitreally injected with $3x10^7$ IFU Ad5 viral vectors encoding no foreign genes (Null), human sFRP1, murine K-cadherin, or a mix of two viral vectors (n=6-7 per group). IOP was measured before injection (day 0, baseline), as well as on days 6, 9, 16, 20, and 35 post-injection. The uninjected eyes were used as the contralateral control (naïve control). One-way ANOVA was performed for each time point with a Tukey post-hoc test. *p<0.05, **p<0.01, and ***p<0.001 as compared to Ad5.Null transduced eyes. ###p<0.001 as compared to Ad5.sFRP1 transduced eyes.

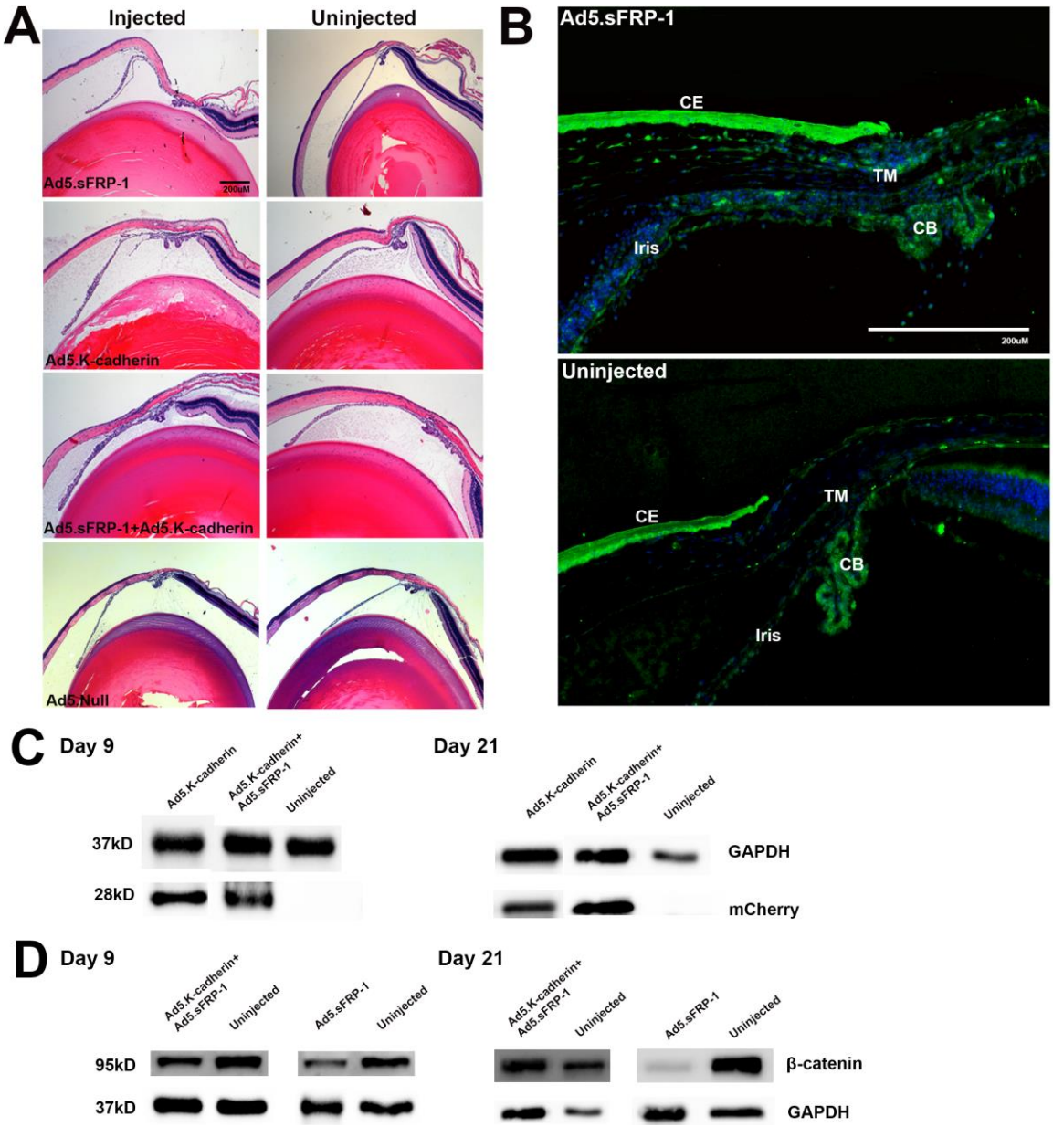

Figure 5. Morphological, histological, and biochemical analyses of the mouse eyes.

(A) On day 35 post-injection, paired injected eyes and uninjected contralateral control eyes from each group were enucleated, fixed, sectioned, and stained with H&E. (B) Twenty-one days post injection, mouse eyes transduced with or without Ad5.sFRP1 were immunostained for sFRP1 (green) and DAPI (blue). (C) and (D) On day 9 and 21 post-injection, mouse anterior segment tissues transduced with or without Ad5.sFRP1, Ad5.K-cadherin, or both were isolated for WB.

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CHAPTER IV. THE ROLE OF CADHERINS IN TGFβ/WNT SIGNALING CROSSTALK AND TRABECULAR MESHWORK CELL PHYSIOLOGY

Authors: Hannah C. Webber, Weiming Mao, Abbot F. Clark

Institutions:

North Texas Eye Research Institute

University of North Texas Health Science Center

Abstract

Purpose: We have shown cross-inhibition of the primary open angle glaucoma (POAG)-associated Wnt/β-catenin and SMAD/TGFβ signaling pathways in trabecular meshwork (TM) but the downstream effects of this crosstalk are unknown. Wnt/β-catenin signaling activation increases K-cadherin expression in TM cells and can reverse the ocular hypertensive effects of Wnt antagonist sFRP1. In this study, we report how Wnt/β-catenin and TGFβ signaling crosstalk affect the nuclear translocation of β-catenin and Smad4, and how this crosstalk affects the expression of K-cadherin and fibronectin. We also report how Wnt signaling and K- and OB-cadherins influence NTM cell impedance properties.

Methods: Confluent NTM cells were treated for 24 hours with or without 100ng/mL Wnt3a or 5ng/mL TGFβ2 and their and protein isolated for western immunoblotting or cells fixed for immunocytofluorescence. Samples were probed for β-catenin, Smad4, GAPDH, fibronectin (FN), K-, or OB-cadherin, or stained for FN or K-cadherin. Some NTM cells were plated on the Acea Real Time Cell Analysis (RTCA) iCelligence system. These cells were either grown to 80% confluence then transfected with 1nM K-cadherin, OB-cadherin, or non-targeting siRNA, or were grown to confluence then treated with or without 100ng/mL Wnt3a or 1ug sFRP1. Cellular impedance measurements were collected every hour for 72-96 hours. Maximum and minimum cell impedance values over this time period were analyzed using One-way ANOVA and reported as Mean+/- SEM.

Results: Co-treatment of Wnt3a and TGFβ2 in NTM cells decreased the expression of K-cadherin and fibronectin compared to Wnt3a or TGFβ2 alone, respectively. However, co-treatment still resulted in nuclear translocation of β-catenin and Smad4. Treatment of NTM cells with Wnt3a increased cell impedance and addition of sFRP1 decreased cell impedance. Addition

of sFRP1 alone to TM cells also decreased impedance. Transfection with anti-K-cadherin siRNA decreased NTM cellular impedance compared to non-targeting siRNA control. Anti-OB-cadherin siRNA decreased impedance as well, but to a lesser extent.

Conclusion: The TM Wnt/β-catenin signaling pathway inhibits Smad4 activity in the nucleus to regulate the TGFβ pathway and enhances cell adhesion through K-cadherin expression.

Introduction

Wnt/β-catenin signaling has been established as an important regulator of trabecular meshwork (TM) health and intraocular pressure (IOP) regulation.[1-4] The Wnt/β-catenin antagonist secreted frizzled-related protein 1 (sFRP1) is increased in the ocular hypertensive glaucomatous TM and can increase IOP in ex vivo and in vivo models.[2] We have shown that Wnt signaling increases K-cadherin expression on the TM membrane, and that Ad5.K-cadherin expression in the TM can rescue sFRP1-induced ocular hypertension in mice (See Chapter III). Cadherin proteins make up a large family of transmembrane proteins that form calcium-dependent, homophilic, dimerized linkages for cell-cell adhesion[5]. K-cadherin itself is an isoform highly expressed in the central nervous system, kidney, and the TM.[6] However, the level of cell-to-cell adhesion that Wnt/β-catenin signaling and K-cadherin offers to TM cells has not been studied.

TGFβ2 is a ligand of the TGFβ/SMAD signaling pathway that is overexpressed in the TM and aqueous humor of POAG patients.[7] Overactive TGFβ signaling increases fibronectin expression and extracellular matrix crosslinking to a pathological level in the TM, causing a decreased outflow facility and increased IOP due to backed up aqueous humor.[8-10] We have shown that direct Wnt/β-catenin signaling and TGFβ/SMAD signaling activation cross-inhibit one another in the human TM, and that this cross-inhibition requires the transcription factors β-catenin and Smad4[11], but the intracellular involvement of β-catenin and Smad4 in this cross-inhibition have not yet been shown, nor has the effect of this crosstalk on K-cadherin protein expression or fibronectin expression. It is important to understand to what extent these two POAG-related signaling pathways interact to properly treat the TM during POAG.

In this study, we report how Wnt/β-catenin and TGFβ signaling crosstalk affect the nuclear translocation of the intracellular mediators β-catenin and Smad4 in primary human non-glaucomatous trabecular meshwork (NTM) cells, and how this crosstalk affects the expression of Wnt signaling-associated K-cadherin and the TGFβ-associated ECM product fibronectin. We also report how Wnt signaling and cadherins that are expressed in the TM influence NTM cell impedance properties using the Acea Real Time Cell Analysis (RTCA) iCelligence system.

Methods

Cell culture

Primary human non-glaucomatous trabecular meshwork (NTM) cells from human donor eyes were used in each experiment. Cells were obtained from Alcon as a gift and grown up in low glucose Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum, 1% Glutamine, and 1% Penicillin Streptomycin. NTM cell strains used in these experiments are NTM 496-05, 82 years, female; 1022-02, 67 years, male.

Immunocytochemistry

NTM cells were cultured on top of glass coverslips until confluent in DMEM-low glucose medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin (Thermofisher, Waltham, MA). At confluence, medium was changed to serum-free medium for 24 hours. Cells were then treated for 24 hours with or without 100ng Wnt3a, 1ug sFRP1, 5ng/mL TGFβ2, or with nothing (control). At 24 hours, Cells were fixed in 4% paraformaldehyde, treated with 0.05% TritonX100, blocked with PBS blocking buffer, and stained with rabbit anti-fibronectin or anti-K cadherin antibody (1:500, Cell Signaling

Technology, Danvers, MA). NTM cells were then incubated with donkey anti-rabbit antibody tagged with fluorochrome 488 (Thermo Fisher Scientific, Waltham, MA). Glass coverslips were then mounted on ProLong gold anti-fade reagent with DAPI (Life technologies, Carlsbad, CA). All images were taken with a Nikon Eclipse Ti-U microscope under the same wavelength and exposure time on a set protocol in Nuance imaging software.

Western immunoblotting (WB)

After 24 hours of treatment with or without 100ng Wnt3a or 5ng/mL TGFβ2, NTM cell cytosolic and nuclear fractions were isolated using the NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Scientific) and NTM cell total protein was isolated using the M-PER Mammalian Extraction Reagent (Thermofisher) with 1:100 protease inhibitors. Equal micrograms of protein from each sample was separated using SDS-PAGE, electrophoretically transferred onto an Immobilon-P transfer membrane, blocked with 5% dry milk, and immunoblotted overnight at 4°C with rabbit anti-K-cadherin antibody (1:500, Abcam, Cambridge, United Kingdom), rabbit anti-OB-cadherin antibody (1:500, Abcam), rabbit anti-β-catenin antibody (1:500), rabbit anti-Smad4 antibody (1:500, Cell Signaling Technology) rabbit anti-GAPDH antibody (1:10,000), or mouse anti-Lamin A/C (1:1000) antibody (all Cell Signaling Technology). The immunoblots were then rinsed and incubated in secondary HRP-linked anti-rabbit or anti-mouse IgG antibody (1:5000, Cell Signaling Technology). Signals were developed using the Clarity Western ECL Blotting substrate (Bio-Rad, Hercules, CA). Images were taken using the Bio-Rad ChemiDoc imaging system (Bio-Rad).

Transfection

NTM cells allowed to grow to 80% confluence in Acea plates coated with copper electrodes. Transfection mixes were made so that each well was treated with 1nM K-cadherin, OB-cadherin, or non-targeting siRNA (OnTarget siRNA, GE Dharmacon, Lafayette, CO) along with 0.83uL Attractene (Qiagen transfection reagent) per well. Attractene and siRNA were incubated together in a transfection mix in OptiMEM alone for 20 minutes. NTM cell media was completely aspirated and 300 uL OptiMEM 5% FBS 1% glutamine was added to each well, and 200uL of transfection mix was added on top.

Cell impedance assays

Acea RTCA iCelligence system which was set to record "sweeps" or measurements of cellular impedance once every hour. NTM cells were plated in Acea plates coated with copper electrodes and their baseline impedance measured for 48 hours. Cells were then treated with or without 100ng Wnt3a or 1ug sFRP1 for 72 hours, or were treated with 1uM anti-K-cadherin siRNA, anti-OB-cadherin siRNA, or Non-targeting siRNA (control group) for 96 hours. The maximum impedance measurement taken for each treatment group over the last 72 hours of treatment divided by the baseline cell impedance was presented as "Maximum" for each treatment group. The minimum impedance measurement taken for each treatment group over the last 72 hours of treatment divided by the baseline cell impedance was presented as "Minimum" for each treatment group. Cell impedance measurements two hours before treatment was considered baseline for each experiment.

Results

TGFβ/Wnt signaling decreased fibronectin and K-cadherin expression, but still led to translocation of transcription factors Smad4 and β-catenin

We found that the cross-inhibition of Wnt/β-catenin and TGFβ/SMAD pathways in the TM leads to decreased K-cadherin and fibronectin expression (Figure 1). These findings are consistent with our previous study which found that co-treatment of Wnt3a and TGFβ2 leads to a decrease in TCF/LEF and TGFβ/SMAD DNA binding element activity. However, this cross-inhibition still involves nuclear translocation of the transcription factors β-catenin and Smad4 (Figure 1C). This suggests that β-catenin and Smad4 are not performing their transcriptional functions once inside the nucleus together.

Wnt/β-catenin signaling activation and K-cadherin maintain TM cell adhesion properties

To begin addressing how Wnt/β-catenin signaling activation and resulting increases in cadherin expression physiologically affect NTM cells, we performed cell impedance assays using the iCelligence system (Figure 3). These assays often are used to study cadherins and cell adhesion with the general principle that the more cadherins expressed, the greater cell adhesion and the greater cell impedance will result. We first activated or inhibited the Wnt/β-catenin signaling pathway in TM cells and measured cell impedance over a 72-hour period (Figure 2A). We found that Wnt/β-catenin activation significantly increased cell impedance while antagonism of Wnt/β-catenin by the POAG-associated protein sFRP1 significantly decreased cell impedance ($n=4$, $p<0.001$). Since we have shown in our past study that sFRP1 antagonizes Wnt/β-catenin signaling to decrease K-cadherin expression, we then tested whether K-cadherin knockdown by siRNA transfection would decrease cell impedance (Figure 2B). We also knocked down OB-

cadherin to see whether impedance-saving properties were specific to K-cadherin or whether OB-cadherin could also offer them to TM cells. We found that both anti-K-cadherin siRNA and anti-OB-cadherin siRNA significantly decreased cell impedance versus non-targeting control siRNA ($n=6$, $p<0.001$). However, K-cadherin knockdown decreased cell impedance greater than OB-cadherin knockdown, suggesting that the adhesion properties K-cadherin offers may be stronger than OB-cadherin.

Discussion

We found that activation of both the Wnt/β-catenin and TGFβ/SMAD pathways in the TM leads to decreased K-cadherin and fibronectin expression. These findings are consistent with our previous study which found that co-activation of these pathways by Wnt3a and TGFβ2 leads to a decrease in TCF/LEF and TGFβ/SMAD DNA binding element activity.[11] However, we have now shown that the transcription factors β-catenin and Smad4 show increased expression in the nucleus upon co-activation of Wnt β-catenin and TGFβ/SMAD pathways, suggesting that β-catenin and Smad4 are translocating into the nucleus but not functioning properly as transcription factors. In other cell types, Smad4 and β-catenin can interact to form an inhibitory complex.[12] This may also be the case in the TM.

We also found that Wnt signaling and K-cadherin maintain TM cell adhesion using RTCA cell impedance assays. We think that Wnt/β-catenin signaling regulates IOP in a two-fold manner: by stabilizing K-cadherin on the TM membrane, contributing to cell adhesion, and by negatively regulating the TGFβ/SMAD pathway, leading to decreased fibronectin expression and fibrotic TM effect. The specific relationship between cell-cell junctions and cell-ECM junctions through their shared attachment to the actin cytoskeleton remains to be investigated in the TM –

it would be interesting to see how K-cadherin-K-cadherin engagement affects fibronectin-integrin engagement in TM cells, and whether cadherin engagement helps the TM increase outflow facility.

In ocular hypertensive POAG, TGFβ signaling is over activated due to increased expression of the agonist TGFβ2, and Wnt/β-catenin signaling is inhibited due to increased expression of the antagonist sFRP1.[2,7] In this state, the TGFβ pathway is unregulated by the Wnt/β-catenin pathway, and produces ECM molecules such as fibronectin. At the same time, Wnt/β-catenin signaling isn't being stimulated to increase expression and stabilization of K-cadherin, a molecule that offers important physiological properties to the TM such as adhesion and IOP regulation. Though there has not been a study to confirm that the patients with an increase in sFRP1 also have an increased TGFβ2, we and many others have shown that these molecules can lead to the same POAG-like pathology in the TM and lead to increased IOP. K-cadherin is a cell adhesion molecule that may be important to TM homeostasis, and therefore we believe may be a viable option for treatment of POAG whether these pathways' pathologies are one in the same or whether they converge on the ocular hypertensive phenotype.

Restoring Wnt signaling in the TM tissue may not be a viable option for treatment of POAG, as aberrant Wnt signaling has long been associated with cancerous growths.[13] However, our data suggests that the restoration of K-cadherin expression in the TM may be a viable option for helping TM cells restore their adhesive properties and regulate IOP. How K-cadherin's regulation of cell adhesive properties leads to a healthy TM and normalized IOP remains unknown, and must be studied.

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Figures

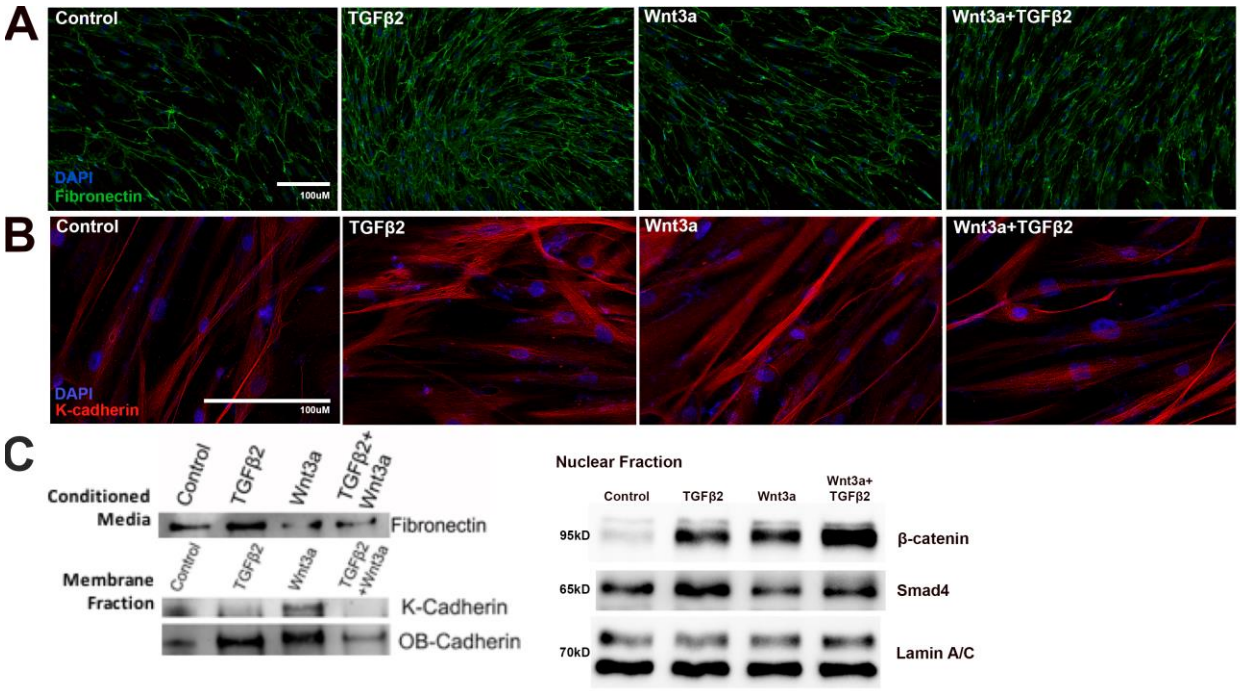

Figure 1. Co-activation of TGFβ and Wnt signaling decreased fibronectin and K-cadherin expression in primary NTM cells, but increased Smad4 and β-catenin nuclear translocation.

Primary NTM cells were treated with or without Wnt3a and/or TGFβ2 for 24 hours in serum-free medium and used for immunocytofluorescence (ICF). (A) ICF staining of fibronectin (green) and nuclei (blue). (B) ICF staining of total K-cadherin (red) and nuclei (blue). (C) Cells were also used for conditioned media, membrane, and nuclear protein extraction followed by western immunoblotting. Lamin A/C was used as a nuclear protein loading control.

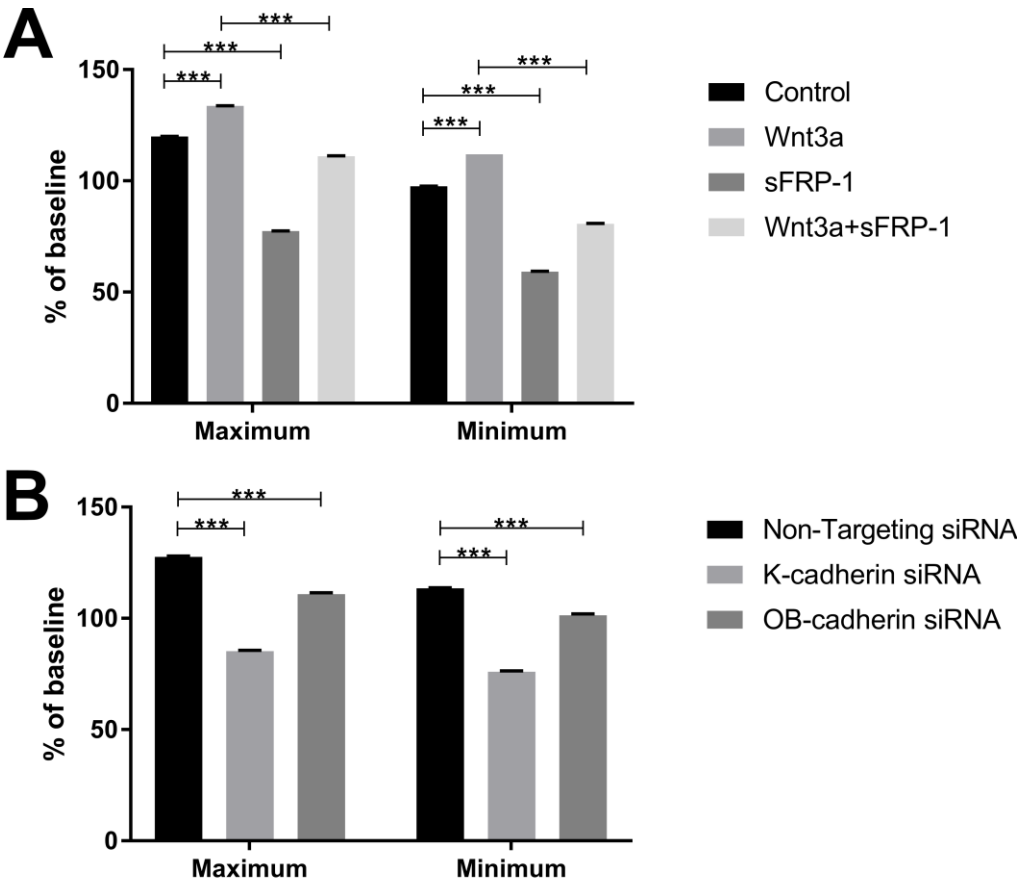

Figure 2. Wnt/β-catenin antagonism and knockdown of cadherins decreased NTM cell impedance.

Primary NTM cells were plated in Acea electrode-coated plates and their baseline cell impedance measured for 48 hours. (A) Cells were treated with or without Wnt3a and/or sFRP1 for and their impedance measured once per hour for 72 hours (n=4) or (B) cells were transfected with either non-targeting (NT) siRNA (n=4), anti-K-cadherin siRNA, or anti-OB cadherin siRNA (n=6) with impedance measurements once per hour for 96 hours. (A&B) Mean and SEM are reported. Maximum and minimum cell impedance values over the treatment period was normalized to baseline cell impedance, groups were compared using a one-way Anova with multiple comparisons. ***$p<0.001$.

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CHAPTER V. DISCUSSION

Author: Hannah C. Webber, Abe F. Clark, Weiming Mao

Acronyms:

POAG – primary open angle glaucoma

TM – trabecular meshwork

OHT – ocular hypertension

AH – aqueous humor

ECM – extracellular matrix

IOP – intraocular pressure

Future Directions

We have shown a cross-inhibition between the Wnt/β-catenin pathway and the TGFβ/SMAD pathway in the non-glaucomatous TM.[1] In POAG TM, Wnt signaling is inhibited by increased expression of secreted frizzled-related protein 1 (sFRP1).[2] Also, TGFβ signaling is over-activated by increased expression of transforming growth factor beta-2 (TGFβ2).[3] Therefore the TGFβ pathway has multiple layers of activation due to increased TGFβ2 and a lack of inhibition by the Wnt signaling pathway. However, it has yet to be determined whether OHT POAG patients who have increased sFRP1 expression in the TM also have increased TGFβ2 expression. If these proteins are elevated simultaneously in POAG patients, therapies that target both the Wnt/β-catenin and TGFβ/SMAD pathways must be developed. Understanding patient growth factor expression profiles is necessary in order to understand how Wnt/β-catenin and TGFβ/SMAD cross-inhibition fit into the molecular mechanisms driving TM pathology.

If increased sFRP1 in the TM is the initiator in the POAG process, then the loss of membrane associated K-cadherin may be the downstream mechanism that leads to ocular hypertension. However, if TGFβ2-induced over-activation of the TGFβ pathway is the initiator, Wnt/β-catenin signaling inhibition and K-cadherin loss may be a result of cross-inhibition by TGFβ signaling. This "chicken-and-egg" issue is difficult to study in a disease like POAG that progresses over many decades and only presents clinical damage after the disease process has advanced However, it is necessary to elucidate the time course of TM pathology in POAG in order to correctly treat the disease. A future study to resolve this issue could include an in vivo study, with two groups receiving either Ad5.TGFβ2 or Ad5.sFRP1 to induce TM pathology and ocular hypertension. Over a time course after injection, TM samples could be analyzed for mRNA

levels of TGFβ- and Wnt- related genes and proteins such as fibronectin, Smad4, K-cadherin, and β-catenin. AH samples could also be acquired in the time course and ELISA assays used to measure the levels of TGFβ- and Wnt- signaling activators and inhibitors in injected eyes vs. Ad5.null eyes and vs. contralateral control eyes. This study would give evidence to support that TGFβ2-induced TM pathology leads to a loss of Wnt/β-catenin signaling and a loss of TM homeostasis, or that sFRP1-induced TM pathology induces TGFβ-induced TM pathology.

We found that K-cadherin is responsible for maintaining cell adhesion and viral-mediated K-cadherin overexpression reduces the ocular hypertensive effects of sFRP1. However, we do not fully understand why Wnt/β-catenin preferentially induces the expression of this cadherin isoform, or why the TM expresses this specific isoform over other isoforms. The dynamics of the K-cadherin isoform are not fully understood either, though it has been shown in kidney cells and in development that K-cadherin and E-cadherin are differentially regulated in response to engagement with adjacent cells.[4,5] Studies have also shown that K- and OB-cadherin may play complementary roles in development and other cell types.[6-8] This may be the case in TM cells, where K-cadherin is preferentially regulated by Wnt/β-catenin signaling, as our data suggest, through β-catenin preferentially binding to the K-cadherin isoform or by inducing K-cadherin protein in a feed-forward manner. We also need to determine whether K-cadherin-mediated cell adhesion in the TM is able to withstand challenges such as increased intraocular pressure (IOP), and whether K-cadherin expression can help maintain AH outflow facility. If Wnt/β-catenin signaling is antagonized by increased sFRP1 expression, cadherins are disrupted, which leads to decreased cell adhesion. This could lead to loss of cell proximity, loss of cell-cell signaling, and therefore loss of TM cellularity or cell density over time. Cadherins also enhance cell control

over the actin cytoskeleton, and if these cadherin junctions are lost or disrupted, TM cells may lose their ability to act in syncytium as a tissue. To address the relationship between K-cadherin and TM cellularity, a study could be designed that includes a conditional knockout of the K-cadherin gene in the TM using the Cre-Lox system in vivo, measuring TM cell count and IOP over a time course. This could help us to further understand how K-cadherin affects cellularity, and how this cellularity loss affects IOP.

Cell-ECM junctions are also mediated through the actin cytoskeleton and when engaged, cell-ECM junctions can decrease expression of cadherins and their accessory proteins such as β-catenin. Conversely, formation of cell-cell cadherin junction may lead to a decrease in cell-ECM junctions.[9] Therefore, the loss of cadherins may strengthen the cell-ECM connections, leading to further cell-cell connection loss and increased ECM expression and fibrosis. Cadherins in the TM may enhance cellularity, cell signaling and regulation, control over the actin cytoskeleton, and regulation of cell-ECM connections. These are all facets of a healthy TM, all lost in the POAG TM.[10-13] It is important to continue to understand how cadherins may rescue or prevent TM pathology that leads to POAG.

Connections to current research

In the clinic, IOP measurements assess the risk of developing glaucoma due to ocular hypertension. Ocular hypertension used to be a diagnosable factor for glaucoma, but now retinal examination takes precedence since glaucoma is not dependent on "high" IOP. Ocular hypertension needs to be redefined as a biological marker in the context of individuals. Those with normotensive glaucoma may be ocular hypertensive based on their own personal baseline

and anatomy, even though their IOP is below 21mmHg. A study on the Korea National Health and Nutrition Survey (2008-2011) found that POAG patients' average IOP was in the "normal" range, yet was significantly higher than non-POAG controls.[14] A better way of defining ocular hypertension is a percentage increase in IOP - if IOP increases a certain percentage within a specified time frame, a patient can be diagnosed with ocular hypertension and watched for glaucomatous damage. Given that we don't truly know a relevant biological definition for ocular hypertension, is possible that the pathological effects of antagonism of Wnt signaling in the TM may apply to all POAG patients, and that the molecular mechanisms driving ocular hypertensive POAG may be similar to normotensive POAG. Until a biologically relevant ocular hypertension cutoff is defined, we will not be able to definitively differentiate POAG subtypes.

Genetic testing is also available and used for those with congenital glaucoma or with strong familial patterns of glaucoma. There are 15 alleles associated with age-related glaucoma, though none of these genes are cadherins or Wnt/β-catenin signaling mediators. It would be interesting to study whether the pathological cellular and tissue effects of these alleles included would interfere with the ability of Wnt/β-catenin signaling to enhance K-cadherin on the TM membrane, or to determine if the pathologies caused by these genes could be rescued by K-cadherin.

Actin and ECM modulator drugs are being studied in the TM to treat POAG pathology. Adenosine monophosphate-activated protein kinase (AMPK) suppressed the expression of ECM proteins and F-actin in the TM, and AMPK-null mice had higher IOP than controls. This suggests that proteins like AMPK that decrease expression of cytoskeleton or ECM proteins can

decrease IOP.[15] Rho-associated kinase (ROCK) inhibitors inhibit TGFβ2-induced outflow alterations in a 3D bioengineered TM. ROCKs induce stress fibers and focal adhesions, contributing to cell contractility and ECM binding. ROCK inhibitors have been shown to increase outflow through CD-constructed TM tissue and resolve CLANs, targeting TM pathology.[16,17] However, actin modulators such as ROCK inhibitors still did not treat the pathology of POAG TM, and they need to be applied daily in order to be effective. For a longer term solution to ocular hypertension, studies looking further upstream to reinstate the regulation of actin and actin contractility, including the manipulation of mechanosensors like cadherins, must be performed. Caveolins have also been shown to be mechanosensors in the TM, and the CAV1/2 allele is associated with POAG. The caveolin proteins could also be candidates for restoring mechanosensing properties in the TM.[18,19]

Recent studies attempted to replenish TM cells with induced pluripotent stem cells, and have shown that those TM-like cells have phagocytic activity.[20-22] Stem cell therapy would serve to restore function and cellularity in the POAG TM. Functional mechanosensors must be expressed in these cells such as K-cadherin or caveolins for this "regrown" TM to be properly functioning.

Steroid-induced glaucoma is a secondary, open-angle glaucoma that occurs in 30-40% of general population who take ocular steroids [23,24] The TM of steroid-induced glaucoma may benefit from K-cadherin's mechanosensing properties, since the TM of steroid-induced glaucoma patients has a similar phenotype to OHT POAG including increased ECM deposition and increased CLANs formation.[25-28]

Broad Implications

We showed that cadherins play a physiological role in TM cell adhesion and IOP regulation as well as that cadherins are heavily influenced by Wnt/β-catenin signaling, effectively linking TM physiology to cell biology. Neither cell signaling nor cell physiology can exist on its own, as cell and tissue response to mechanical stimulation is heavily coupled with cell signaling. In a tissue as dynamic as the TM, physiological effects such as mechanosensing of the extracellular environment need to be considered alongside cell signaling when searching for a cure to help the TM effectively regulate IOP.

We show a crosstalk between two pathways implicated in POAG pathology, the Wnt/β-catenin pathway and the TGFβ/SMAD pathway. Until now, these two pathways have only been studied separately in the TM save for a few published experiments, and these pathways have always been studied separately in human POAG patients. Our results suggest that these two pathways are closely tied in maintenance of TM health, and therefore must be studied together in the POAG TM. We are not the only group to show crosstalk between two POAG-associated pathways or pathologies. Therefore, we need to understand whether the road to TM pathology can converge on the same end pathology, whether TM scientists are describing different facets of a pathology that exist simultaneously within the same patients, or whether there are subsets of ocular hypertensive POAG that need to be treated separately based on TM pathology. The first step in understanding the complete molecular pathology of POAG is a proteomics study on a large group of ocular hypertensive POAG patient TM tissue, measuring the levels of sFRP1, TGFβ2, gremlin, and other proteins associated with TM pathology such as caveolin, fibronectin, transglutaminase-2 (TGM2), toll-like receptor 4 (TLR4), lysyl oxidase (LOX) proteins, K-

cadherin, myocilin etc. To develop suitable drugs or therapies for POAG, we must better understand how each of these POAG-associated pathways and proteins interact and characterize the molecular mechanisms driving POAG in the TM.

Figures

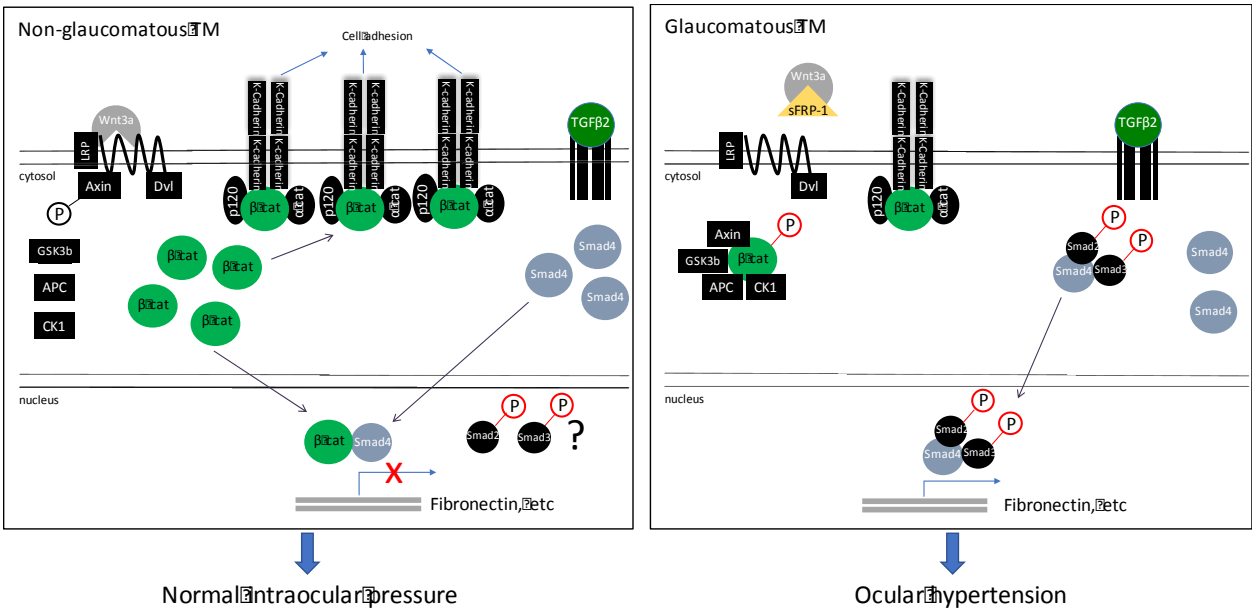

Figure 1. The proposed mechanism of the role of Wnt/β-catenin signaling in the maintenance of intraocular pressure.

In non-glaucomatous TM (left), Wnt/β-catenin signaling maintains K-cadherin total and membrane-bound expression, maintains cell adhesion, and inhibits the TGFβ/SMAD pathway induction of fibronectin. In the POAG TM (right) when the expression of Wnt/β-catenin signaling antagonist sFRP1is increased, K-cadherin total and membrane-bound expression and TM cell adhesion decrease, and uninhibited TGFβ/SMAD signaling will increase fibronectin expression.

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