ABSTRACT

Stevenson, Cooper S., The Effects of Ad5.CMV.hTGFβ2$^{C226/228S}$ on AHD in Mice. Master of Science (Medical Sciences Research Track), April 16, 2018, 37 pp.; 8 figures, bibliography.

Introduction: Elevated intraocular pressure (IOP) is a key risk factor for the development of primary open-angle glaucoma (POAG), a leading cause of blindness in people over the age of 40 years. Transforming growth factor beta-2 is a cytokine known to contribute to the pathogenesis of POAG due to its deleterious effects on aqueous humor outflow via the conventional, or trabecular, outflow pathway in the eye. However, its effects on the rate of aqueous outflow (Fu) via the unconventional or uveoscleral outflow pathway, rate of aqueous humor production (Fin), and episcleral venous pressure (Pe) are unknown. Further, effects of euthanasia and enucleation in our hands on TGFβ2-mediated effects on Fu are also unknown. The goal of the present study was to quantify the impact of over-expression of TGFβ2 on aqueous humor dynamics (AHD) in the mouse eye, with special emphasis on Fu, Fin, and Pe in the mouse eye. **Methods:** To simulate TGFβ2 over-expression, left (OS) eyes were injected intravitreally (IVT) with a mutant form of TGFβ2 (Ad5.CMV.hTGFβ2$^{C226/228S}$, 2×10^7pfu in 2μL), while right (OD) eyes were injected IVT with a null virus (Ad5.CMV.null, same titer and volume). Following 14 days, after which time mean IOP (determined tonometrically in conscious mice) had become elevated in TGFß2-injected eyes (84.29% increase in IOP, $P < 0.001$), Fu was determined directly by cannulating the anterior aqueous chamber (AC) and perfusing it with fluorescein isothiocyanate-dextran (1×10^{-9} M), followed by dissection of the retina/choroid/iris-ciliary body/scleral shell, homogenization, and measurement of each sample's fluorescence, and then inference of flow rate using a standard curve. Those perfusion were performed in living eyes, also in eyes in situ in the animal immediately following euthanasia, and enucleated eyes perfused in vivo either (i) exposed to air, or (ii)

submerged in PBS. In a further group of experiments in living animals aqueous humor outflow conductance (C) (also known as aqueous humor outflow facility), and Pe were measured, and then Fin and Fu were calculated using a constant flow infusion method. Further, we sought to determine whether IOP elevation would lead to a reduction in RGC numbers in the retina, so retinal flat mounts from both treated and untreated eyes from 5 of our animals were prepared and RGC counts were made.

Results:

Direct assessment of Fu: For eyes perfused in-vivo, Fu was reduced in OS (0.0048 ± 0.0017 µL/min) compared to OD (0.0987 ± 0.0126 µL/min, $P = 0.025$). For eyes perfused in euthanatized mice, Fu was reduced in OS (0.0215 ± 0.0101 µL/min) compared to OD (0.1543 ± 0.0241 µL/min, $P = 0.010$). For eyes perfused ex-vivo while submerged in PBS, there was no difference in Fu between OS (0.0222 ± 0.0065 µL/min) and OD (0.0137 ± 0.0078 µL/min, $P = 0.175$). For eyes perfused ex-vivo while exposed to air, Fu was reduced in OS (0.0702 ± 0.0087 µL/min) compared to OD (0.1377 ± 0.0106 µL/min, $P = 0.008$).

Assessment of AHD using constant flow infusion: Fin showed a trend towards a reduction in the eyes in which TGFβ2 was over-expressed, but this effect did not reach statistical significance. There was a significant increase in Pe in eyes in which TGFβ2 was expressed (8.6 ± 0.7 mmHg in OS to 6.4 ± 0.2 mmHg in OD, $P = 0.015$). **Discussion:** Given these results, the present study further quantifies the effect of TGFβ2 in POAG, providing more insight into its mechanism of action in this disease.

THE EFFECTS OF Ad5.CMV.hTGFβ2$^{C226/228S}$ ON AHD IN MICE

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PRACTICUM REPORT

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

by

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Fort Worth, Texas

April 16, 2021

ACKNOWLEDGEMENTS

I would like to thank Dr. Cameron Millar for his willingness to mentor me over the duration of this project. His guidance, technical expertise, and passion for teaching have shaped my growth in the lab this past year, even more so considering the obstacles that both 2020 and 2021 have brought to us at UNTHSC. I have certainly gained a deeper appreciation for the scientific process as a whole, and how it can better inform scientists and clinicians in their pursuit to ease human suffering; I look forward to taking these experiences forward in my pursuit of a career in medicine. I would also like to thank my advisory committee, Dr. Dorota Stankowska and Dr. Tara Tovar-Vidales for their support throughout my project. I also want to thank Dr. Bindu Kodati for her help with some of the last, but most technically challenging, aspects of my project.

I also thank my girlfriend, Brooke, for her support throughout my academic and professional pursuits in these last two years at UNTHSC. I also owe a great amount of thanks to my family for their unending support of me as I chase my own goals, as well as inspiring and encouraging my pursuit of a career in medicine.

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Background

Glaucoma is a disease characterized by progressive damage to the optic nerve head and optic nerve, and retinal ganglion cell (RGC) somas and their axons,[16] and remains one of the

leading causes of irreversible blindness worldwide.[1, 6, 8] In primary open-angle glaucoma (POAG), by far the most common subset of the glaucomas, there is an increased resistance to aqueous humor outflow through the cells of the trabecular meshwork (TM).[5] While its pathophysiology is not fully understood, a major risk factor associated with the development of progressive loss of vision in POAG is elevated intraocular pressure (IOP).[1, 6, 8, 16] A reasonable approximation for IOP can be expressed mathematically in the modified Goldmann equation:

$$IOP = [(\frac{Fin-Fu}{C}) + Pe]$$

Where Fin = aqueous humor formation rate, Fu = uveoscleral outflow rate, C = aqueous humor outflow conductance, or the facility (the reciprocal of aqueous humor outflow resistance), and Pe = episcleral venous pressure. Increased intraocular pressure (IOP) will develop as a result of increased resistance to aqueous humor outflow through either the TM, or decreased Fu, or increased Fin, or increased Pe, or via some combination of two or more of these components.[6, 8-9]

Elevated levels of transforming growth factor-β2 (TGFβ2) in the aqueous humor of POAG patients has been identified as a contributor to the pathophysiology of elevated IOP in both human and rodent subjects,[2, 16] primarily due to its effects on the trabecular meshwork (TM)[2-4, 16, 21] and subsequent impediment of trabecular outflow by increasing resistance to aqueous humor flow through the TM. Elevated expression of TGFβ2 in human TM cell cultures has also been shown to summarily increase secretion of extracellular matrix (ECM) proteins such as PAI-1 and fibronectin. Increased deposition of ECM has also been seen in the TM in human POAG eyes, suggesting that TGFβ2 plays a role in the pathogenesis of POAG.[3] Injection of $TGF\beta2^{C226/228S}$ (an activated form of TGFβ2) has been demonstrated to elevate IOP and impede aqueous outflow facility in rodents. In the mouse, IOP has been shown to be significantly elevated 4 days after injection and remained elevated for 11 subsequent days.[2] However, the

effects of TGFβ2 on other principal aspects of aqueous humor hydrodynamics (specifically, aqueous humor formation rate (Fin), uveoscleral outflow (Fu), and episcleral venous pressure (Pe)) in animal models have to date not been demonstrated.

In the ECM, TGFβ2-related signaling results in proliferation of proteins such as perlecan, elastin, collagens α1 and α2, type 1 plasminogen activator inhibitor (PAI-1), and fibronectin, among others.[2, 3, 4, 16, 21] Increased deposition of such proteins in the TM leads to increased resistance to aqueous humor outflow,[16, 21] but the presence of these or similar effects on uveoscleral outflow are unknown. These effects on ECM protein deposition have been established in cultured human TM cells, pointing to the viability of modelling POAG using TGFβ2 in vivo. Fleenor et al.[3] were able to show an increase in fibronectin and PAI-1 secretion in cultured human TM cells. Moreover, they were able to demonstrate an increase in IOP in an ex vivo model of human donor ocular tissues injected with TGFβ2. The perfusates from the TGFβ2-treated tissues also displayed time-dependent increases in fibronectin an PAI-1, which was consistent with the results observed in cultured human TM cells.[3] Shepard et al. demonstrated that POAG can be modeled successfully in the rodent using Ad5.CMV.hTGFβ2$^{C226/228S}$, rather than wild-type human or mouse TGFβ2. Specifically, point mutations were introduced into human TGFβ2, changing cysteine residues at positions 226 and 228 into serine residues. In Chinese hamster ovary (CHO) and GTM3 cells transfected with p.hTGFβ2^{WT} and p.hTGFβ2$^{C226/228S}$, active TGFβ2 was produced in higher quantities by p.hTGFβ2$^{C226/228S}$ transfected cells. Specifically, active TGFβ2 comprised 25% of total TGFβ2 in p.hTGFβ2$^{C226/228S}$ transfected CHO cells compared to 8% in p.hTGFβ2^{WT} transfected cells; in GTM3 cells, 31% of total active TGFβ2 was found in Ad.TGFβ2$^{C226/228S}$ transduced cells, compared to 13% in to Ad.TGFβ2^{WT} transduced cells. Moreover, QRT-PCR analyses of

downstream TGFβ2 effectors such as connective tissue growth factor (CTGF) and PAI-1 showed significantly increased expression following induction by Ad.hTGFβ2$^{C226/228S}$.[2] This research initially characterized the ability of TGFβ2$^{C226/228S}$ to induce the proliferation of CTGF, PAI-1, the EDA splice variant of fibronectin (FN-EDA), and collagen 1A1 (COL1A1) in cultured TM cells, then demonstrated a significant increase in IOP in both BALB/cJ mice and Wistar rats.[2] However, this past work has focused on modeling POAG via an increase in IOP by decreasing trabecular outflow facility alone. The effects of intravitreal injection of Ad5.CMV.hTGFβ2$^{C226/228S}$ on the other aspects of aqueous humor dynamics, including Fin, Fu, and Pe have not yet been demonstrated. Thus, the focus of this research is to investigate whether there is also a response by the suprachoroidal space and sclera to increased levels of TGFβ2. We hypothesize that uveoscleral outflow through the suprachoroidal space and sclera will be modified following increased expression of TGFβ2 and will not be affected by euthanasia. Boussommier-Calleja et al. demonstrated that uveoscleral or pressure-independent outflow is not significantly different from zero in enucleated mouse eyes bathed in isotonic saline.[31-32] This group did also demonstrate non-zero pressure-independent flow in mouse eyes exposed to air, though they hypothesized this discrepancy was due to evaporation from the surface of the eye itself.[33-35] IOP elevation following intravitreal injection of Ad5.CMV.hTGFβ2$^{C226/228S}$ is known to result primarily from a decrease in aqueous outflow conductance (C) through deposition of ECM proteins in the tissues comprising the conventional or pressure-dependent outflow pathway, though its effects on Fin and Pe are unknown.[2]

Practicum Research Report

Specific Aims

The current research seeks to describe the effects of over-expression of TGFβ2 on Fin, Fu, and Pe in living mice. We also seek to corroborate earlier findings relating to C and IOP. We also seek to study the effects of over-expression of TGFβ2 on Fu in eyes perfused ex-vivo. Finally, we aim to describe the effects of TGFβ2-mediated increases in IOP on retinal ganglion cell (RGC) numbers.

Background

Aqueous humor is produced and secreted by the non-pigmented epithelium of the ciliary body, also known as the pars plicata, into the posterior chamber of the eye. From here, aqueous humor travels anteriorly, around the iris, and into the anterior chamber of the eye.[37] (Figure 1) Aqueous humor then drains through two outflow pathways; the first is via the trabecular meshwork, then to Schlemm's canal, and finally entering venous circulation in the episcleral veins.[7, 8] This outflow pathway is also known as the pressure-dependent pathway; in the living eye, aqueous outflow rate through the TM increases approximately linearly with IOP over the range of IOP from 5 mmHg to 40 mmHg.[9] The mechanisms of this relationship are due to a variety of molecular factors released in response to mechanical or chemical signaling in the TM, including gases, lipids, cytokines, and nucleotides.[10] Outflow via the TM has been thoroughly studied and is far more readily observable; indeed, there are a number of pharmacological interventions and procedures that can be used to alleviate increased resistance to aqueous

outflow across the TM in POAG, such as administration of Rho-kinase inhibitors (ROCK inhibitors).[10] Muscarinic agonists such as pilocarpine bind M3 receptors on the ciliary muscle, which pulls the TM open, facilitating aqueous egress. An artificial outflow channel can be created by scleral flap trabeculectomy, and laser trabeculoplasty can be used to alter the inherent outflow resistance of the TM.[6, 11, 12] Artificial drainage shunts can also be implanted surgically. Generally, POAG is first managed utilizing pharmacological intervention by one or more drugs that have different mechanisms of action by which they reduce IOP. With time though, the patient will likely become increasingly refractory to pharmacological intervention, and laser or surgical procedures will be indicated in order to effectively control IOP. However, laser and especially surgical interventions inherently carry a greater degree of risk, thus making pharmacological intervention a more desirable (and non-invasive) treatment option, at least in the initial stages of POAG.

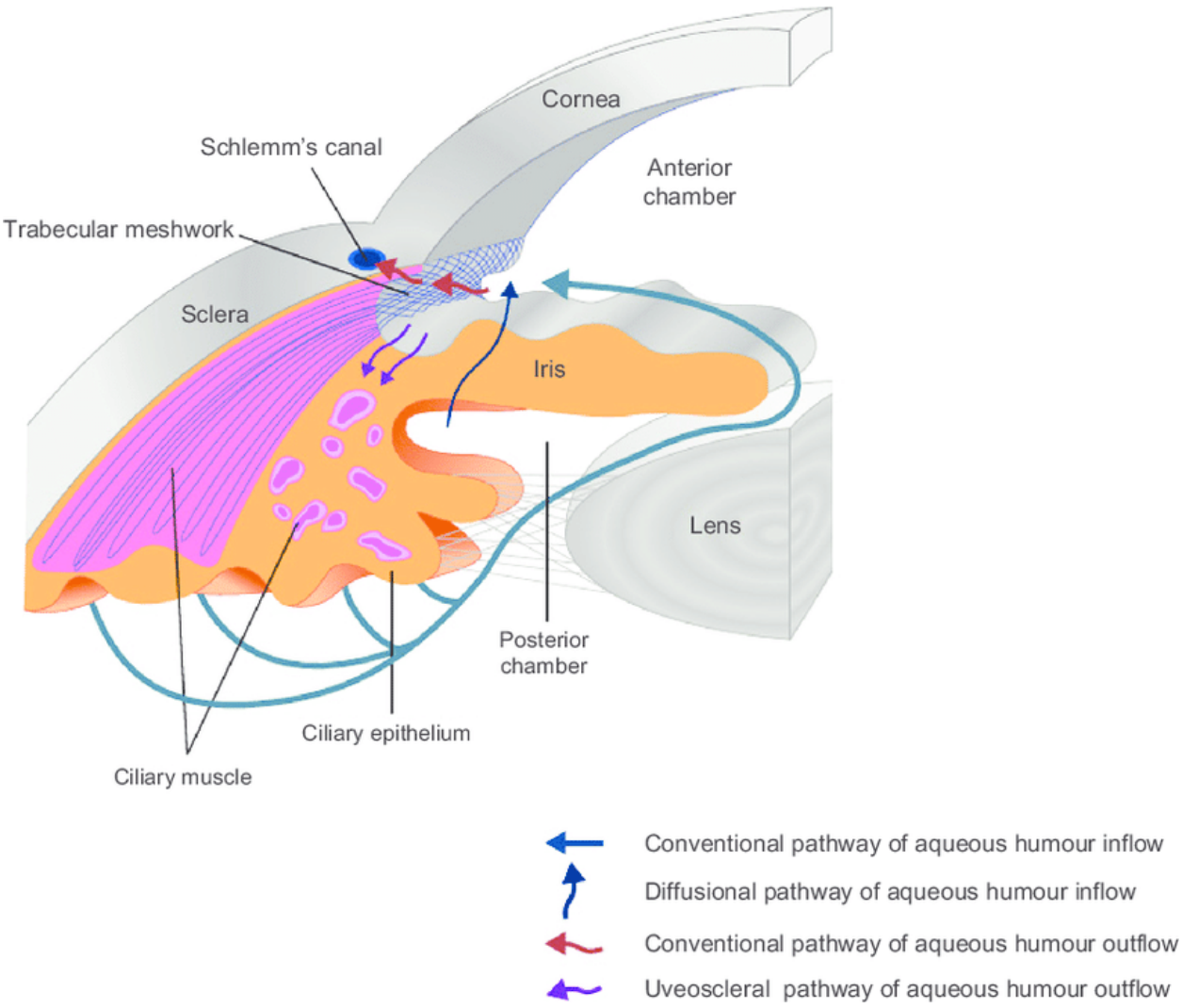


Figure 1: Diagrammatic representation of aqueous humor production and outflow in the eye. Source: Crawley L, Zamir SM, Cordeiro MF, Guo L. Clinical options for the reduction of elevated intraocular pressure. Ophthalmol Eye Dis. 2012;4:43-64. Published 2012 Apr 30. doi:10.4137/OED.S4909

Although the majority of aqueous humor flows through the trabecular meshwork, there is also another pathway for aqueous outflow from the anterior chamber: the uveoscleral, or unconventional pathway. In this pathway, aqueous flows directly through the spaces between the bundles of the ciliary muscle, and from there into the space between the sclera and the choroid, referred to as the suprachoroidal space. From here, aqueous drains through fenestrations in the sclera and into the orbit, where it reaches lymphatic vessels. A fraction of this aqueous may also

be drained by choroidal blood vessels; this fraction is considered to flow by the uveovortex route, as choroidal venous drainage occurs via the vortex veins and then the ophthalmic vein.[5, 14-15] Unlike the trabecular pathway, the uveoscleral pathway has been demonstrated to be influenced by a very minimal degree by changes in IOP (within physiological limits); simply, it has a small facility and is normally considered to be pressure-independent.[30] There are several reasons for this: the sclera provides little resistance to aqueous egress, and the vessels of the choroid can readily drain any aqueous collected there.[14] Moreover, given the contractile nature of the ciliary muscle, changes in its structure provide a source of resistance to aqueous outflow. As the ciliary muscle contracts, the space between the muscle bundles decreases, thus providing increased resistance to aqueous outflow.[14] Given these factors, the pressure gradient present in the uveoscleral pathway is established by the pressure in the anterior chamber and the pressures in the suprachoroidal space and supraciliary space. Uveoscleral outflow, while much more difficult to assess, has in recent years been shown to contribute far more to total aqueous outflow than once thought.[14]

Early research on human eyes showed that uveoscleral outflow only contributed to about 4-14%[15] of total aqueous outflow in the control group, though these eyes were all from individuals at least 35 years of age or older.[15] More recent studies have demonstrated a far greater contribution of uveoscleral outflow to total aqueous outflow, from 54% of total outflow in younger individuals to even as much as 46% in older individuals;[14, 22] as expected, uveoscleral outflow is even further reduced in glaucomatous eyes.[14] Thus, with regards to the aqueous outflow pathways in humans, uveoscleral outflow is a far greater contributor to total aqueous outflow facility than once thought, and is a promising target for pharmacological and surgical intervention.

As evidence mounted for the uveoscleral pathway as a viable means of treating glaucomatous conditions, pharmacological interventions soon became widely available. One of the most common pharmacological treatments for elevated IOP and POAG is the use of prostaglandin analogues,[6-7, 17] namely latanoprost.[17] Through its agonistic actions at the prostaglandin F receptor, latanoprost increases the permeability of the sclera to aqueous, thus enhancing the egress of aqueous out of the anterior chamber.[6, 17] Latanoprost also promotes ciliary muscle relaxation, and progressive dissolution of ciliary muscle matrix collagen types I and III via increased expression of MMPs. Alpha-2 adrenergic agonists like brimonidine and apraclonidine function similarly by increasing uveoscleral outflow (in addition to decreasing the rate of aqueous humor production by the ciliary body),[6, 18-19] while in rabbits has been shown to simultaneously decrease the rate of production of aqueous humor. Their effect on outflow resistance likely occurs through α-adrenergic stimulation of prostaglandin release,[18-19] which would further reduce ciliary muscle tone and increase uveoscleral outflow.

While surgical intervention in glaucoma has traditionally focused on increasing trabecular outflow, recent progress has demonstrated the uveoscleral pathway is a viable surgical target as well.[12, 27, 28] These interventions largely involve the implantation of shunts in either the supraciliary or suprachoroidal spaces. Devices such as the CyPass Micro-Stent (Alcon, Fort Worth, Texas, USA) are implanted into the supraciliary space and divert aqueous outflow from the anterior chamber into the suprachoroidal space.[28, 29] The SOLX gold shunt (SOLX Ltd., Waltham, Massachusetts, USA), iStent Supra (Glaukos Corporation, Laguna Hills, California, USA), Aquashunt (OPKO Health Inc., Miami, Florida, USA), and STARflo (iSTAR Medical, Isnes, Belgium) are all inserted into the suprachoroidal space and function in a similar manner, redirecting aqueous from the anterior chamber and into the suprachoroidal space.[27, 28] However,

these devices have yet to be approved by the FDA and are thus only available in an investigational setting.

Recent studies have implicated TGFβ2 in the pathogenesis of increased IOP and POAG.[2-4, 20] Through its action on TGFβ1 receptors, TGFβ2 increases secretion of PAI-1 and fibronectin in TM cells, which may contribute to its effects on aqueous outflow resistance.[21] Considering these effects in TM cells, there is potential for TGFβ2 having similar effects in the cells of the uvea, ciliary muscle, ciliary body, choroid, and sclera, the tissues in which uveoscleral outflow occurs. However, such effects have not been elucidated in the uveoscleral pathway in existing literature.

Uveoscleral outflow has typically been determined indirectly through direct measurement some of the aqueous humor hydrodynamic parameters in the eye as described in the modified Goldmann equation. In a typical experiment, IOP can be conveniently assessed using tonometry. Fin can be measured by fluorophotometry, and Pe by episcleral venamanometry, or artificial modification of IOP until blood is observed refluxing into the episcleral veins and Schlemm's canal. Fu can thus be derived via rearranging the modified Goldmann equation, such that:

$$Fu = Fin - C \times (IOP - Pe)$$

Fu has also been directly measured via the use of radiolabeled albumin or fluorescent tracers, though these methods require periodic blood draws or enucleation of the eye followed by sectioning and histology, respectively.[25-26] In recent years, techniques such as constant-flow infusion have allowed for the simultaneous measurement or assessment of such parameters in rodent models.[2, 9, 22-23] This has the obvious benefit of bypassing the need to derive certain parameters from a control group of animals or from population means given a specific strain.[9]

Murine eyes have also been shown in recent years to be a viable proxy for human or non-human primate eyes in the laboratory setting, both from structural and functional considerations.[24] Given the biological similarities between the anterior segments of the mouse eye and the human, the mouse serves as an even more attractive model for POAG given its short lifespan, ease with which to house and feed, and more than 20,000 strains available.[23] Given the invasive nature of direct measurement of aqueous humor dynamics, sacrifice of the animal is often necessitated, further demonstrating the utility of the mouse as a viable model for POAG and aqueous humor dynamics studies.[22]

Another concern with the measurement of aqueous humor dynamics is the conditions in which measurements are taken. Previous studies have found that uveoscleral outflow facility remains unchanged following euthanasia within the same eye.[9] This observation further confirms the long-standing hypothesis that uveoscleral outflow is a pressure-independent pathway;[14] given the direct assessment of uveoscleral outflow in the present study, this data is of utmost importance. Furthermore, the lack of change in uveoscleral outflow facility following euthanasia and subsequent loss of blood flow suggests that the uveovortex component of unconventional aqueous outflow is not an important contributor to total aqueous outflow.[14]

Research Design and Methodology

Subjects

For assessment of Fu, 20 female BALB/cJ mice between the ages of 4-5 months were used. For assessment of Fin and Pe, 20 female BALB/cJ mice of the same age were used. For retinal flat mounts and RGC counting to assess possible RGC loss, eyes from 5 mice which displayed significantly increased IOPs in this second cohort were used (n = 10). Mice were

purchased from Jackson Laboratories, Bar Harbor, ME, USA. Animals were given food and water ad libitum, and were kept on 12 hour dark/12 hour light conditions, with lights on at 6:00 a.m. All experimental procedures were conducted in accordance with and adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the University of North Texas Health Science Center (UNTHSC; Fort Worth, TX, USA) Institutional Animal Care and Use Committee (IACUC) regulations and guidelines.

Measurement of IOP

On day 0, IOP was measured in conscious animals using a TonoLab rebound tonometer (Colonial Medical Supply, Franconia, NH, USA). IOP measurements were performed on each eye and served as the baseline IOP measurement. Repeat IOP measurements were taken on day 7, as well as day 10 or day 14 to confirm IOP elevation.

Adenovirus Injections

On day 1, animals were placed under a surgical plane of anesthesia using 2.5% isoflurane administered in an anesthesia chamber. Upon full achievement of a suitable plane of anesthesia, each animal was removed from the chamber and placed in a mask to ensure maintenance of anesthesia. The left eye (OS) was given 1 to 2 drops of 0.5% proparacaine HCl as a topical local corneal anesthetic, then given a single intravitreal injection containing Ad5.CMV.hTGFβ2$^{C226/228S}$ suspended in 2 µL solution of PBS. The injections were administered using a Hamilton (Reno, NV) glass microsyringe using a 30-gauge needle. Each injection was administered over a period of 5 seconds. The needle was then be left in place for an additional 30 seconds and then carefully withdrawn.

Perfusion of Eyes for Assessment of Fu

After development of elevated IOP in the injected (OS) eyes, 5 animals were anesthetized using a mixture of ketamine 100 mg/kg (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine 10 mg/kg (Vetus; Burns Veterinary Supply) administered intraperitoneally. Following our previously described methodology,[9] the anterior chambers of each eye were cannulated and perfused with fluorescein isothiocyanate dextran (FITC-dextran) (2.0×10^{-3} M to 3.3×10^{-3} M; 10,000 ng/μL; Sigma Chemical Co., St. Louis, MO) at a flow rate of 0.5 μL/min for 10 minutes. The same procedure was repeated in 5 animals immediately following euthanasia induced by anesthetic overdose administered intraperitoneally. We also enucleated eyes from 10 animals (euthanized by inhalation of 100% CO_2). We submerged 5 of these eyes in 10% PBS and perfused them with FITC-dextran at the same flow rate for the same period of time. In the final 5 of these eyes, we kept them unsubmerged (in direct contact with air) and perfused them with FITC-dextran at the same flow rate for the same period of time. All uninjected (OD) eyes were treated as internal controls and perfused in the same manner as the contralateral (OS) eyes within each group.

Estimation of Fu

Immediately following perfusion, each eye was enucleated and dissected to isolate the retina/choroid/iris-ciliary body/scleral shell, cornea/TM, lens, and vitreous. The retina/choroid/iris-ciliary body/scleral shell were placed in a 1.5 mL tube with 150 μL PBS and homogenized. Each homogenate was centrifuged at 3000g for 5 minutes. 100 μL of each supernatant was decanted from each tube and placed in microcuvette. Fluorescence was read for each tube (excitation 460 nm, emission 515-575 nm) (QuantiFluor-P Handheld Fluorimeter, Promega Corporation, Madison, WI). Fu for each eye was calculated as:

$$Fu = [Conc. FITC - dex\ (\mu g/1000\mu L)\ \times\ 150\mu L/1000]/(10\mu g\ x\ 10\text{min})$$

For a total perfusion time of 10 minutes at an initial concentration of FITC-dextran of 10,000 ng/µL, or 10 µg/µL. Concentration of FITC-dextran in each tube was determined from a standard curve, as well as a PBS blank. All fluorescence values (standard curve and samples) were blank-corrected.

Determination of Aqueous Humor Dynamics by Constant Flow Infusion

An additional cohort of 6 female BALB/cJ mice between the ages of 4-5 months were injected with Ad5.CMV.hTGFβ2$^{C226/228S}$ as per the above protocol. This cohort was used to determine aqueous humor dynamics using our previously published methodology of constant flow infusion.[9, 22-23] Following induction of a surgical plane of anesthesia, pre-cannulation IOP was determined using a TonoLab rebound tonometer (Colonial Medical Supply, Franconia, NH, USA).

Pe was determined using a method previously described by Aihara et al.[36] Following cannulation, the 3-way stopcock was adjusted such that the perfusion line was open to the manometer, and the manometer was adjusted such that the pressure in the eye equals the pre-cannulation IOP. Once the IOP of each eye stabilized for a period of 5 minutes, the anterior chamber of the eye was observed using a dissection microscope under 10X to 30X magnification. The manometer was lowered by 1.36 cm such that the pressure in each eye decreased by 1 mmHg; waiting 1 minute after lowering the manometer, then viewing the anterior chamber again through the microscope. This was repeated until episcleral veins, aqueous veins, scleral collector channels, and Schlemm's canal were visibly distended with blood. The pressure at which this was observed was considered to be a good estimate for Pe. The eye was returned to its pre-cannulation IOP, then the manometer was switched off to the perfusion line.

C was determined mathematically following the conclusion of the constant flow infusion experiment. Mean pressure (mmHg) at each flow rate was recorded as dependent upon each flow rate (µL/min). The slope of this graph was determined using simple linear regression; C (µL/min/mmHg) was calculated as the reciprocal of this slope. Furthermore, C was determined under both live and euthanatized (dead) conditions.

To calculate Fu, the mean Fu values at each flow rate were determined using the following equation:

$$Fu = Flow\ rate = (mean\ pressure\ at\ the\ flow\ rate - C_{dead})$$

In this calculation, Fin and Pe are equal to zero following euthanasia.

Finally, Fin was calculated using the following equation:

$$Fin = |C_{live}\ (IOP - Pe)| + Fu$$

Retinal Flat Mounting

Eyes from 5 mice with elevated IOPs were enucleated and fixed in 4% PFA for 2 hours, then washed three times in PBS. The anterior segments were removed, then the retinas were removed and placed into PBS until processed. For pre-treatment, retinas were washed 2-3 times in PBS with 0.3% Triton X-100 (TX) for a total of 1 hour at room temperature with gentle agitation. Retinas were then treated with a blocking solution of 10% goat serum, 0.3% TX, and PBS for 1-2 hours at room temperature with gentle agitation. Next, a primary antibody (rabbit anti-RBPMS 1:200, GeneTex, Cat# GTX 118619) stain was applied, in a solution of 10% goat serum, 0.3% TX, and PBS at 4° C for 3 nights with gentle agitation. Following incubation, retinas were washed with PBS for 3-4 hours with gentle agitation, changing PBS several times. Next, a secondary antibody consisting of AlexaFluor488 goat anti rabbit in PBS with 0.1% TX was applied and incubated overnight in dark at 4° C with gentle agitation. Retinas were then

rinsed over 1-2 hours in PBS, changing PBS several times. Finally, retinas were mounted on slides with the RGC side up, making 2-4 small cuts towards the nerve head to flatten the retina. Extra PBS was removed, and a coverslip was placed with a mounting medium (Vectashield Mounting Medium with DAPI, Vector Laboratories, H-1200). 10g of weight was placed on top of the coverslip to further flatten the retina, keeping it for 15-20 minutes.

Statistical Analysis

Data are presented as mean ± 95% CI. A paired Student's *t*-test is used to compare Fu, Fin, Pe, and RGC counts between OS and OD in each animal. One-way ANOVA is used to compare measurements taken in all 4 experimental conditions of direct assessment of Fu, followed by a suitable post hoc test. $p<0.05$ is considered as significant. Two-way ANOVA is also used to compare IOPs between OS and OD over time in conscious animals.

Results

Direct Assessment of Fu

For eyes perfused in-vivo, Fu was reduced in OS (0.0048 ± 0.0017 μL/min) compared to OD (0.0987 ± 0.0126 μL/min, $P = 0.025$). For eyes perfused in euthanatized mice, Fu was reduced in OS (0.0215 ± 0.0101 μL/min) compared to OD (0.1543 ± 0.0241 μL/min, $P = 0.010$). For eyes perfused ex-vivo while submerged in PBS, there was no difference in Fu between OS (0.0222 ± 0.0065 μL/min) and OD (0.0137 ± 0.0078 μL/min, $P = 0.175$). For eyes perfused ex-vivo while exposed to air, Fu was reduced in OS (0.0702 ± 0.0087 μL/min) compared to OD (0.1377 ± 0.0106 μL/min, $P = 0.008$). (Figure 2)

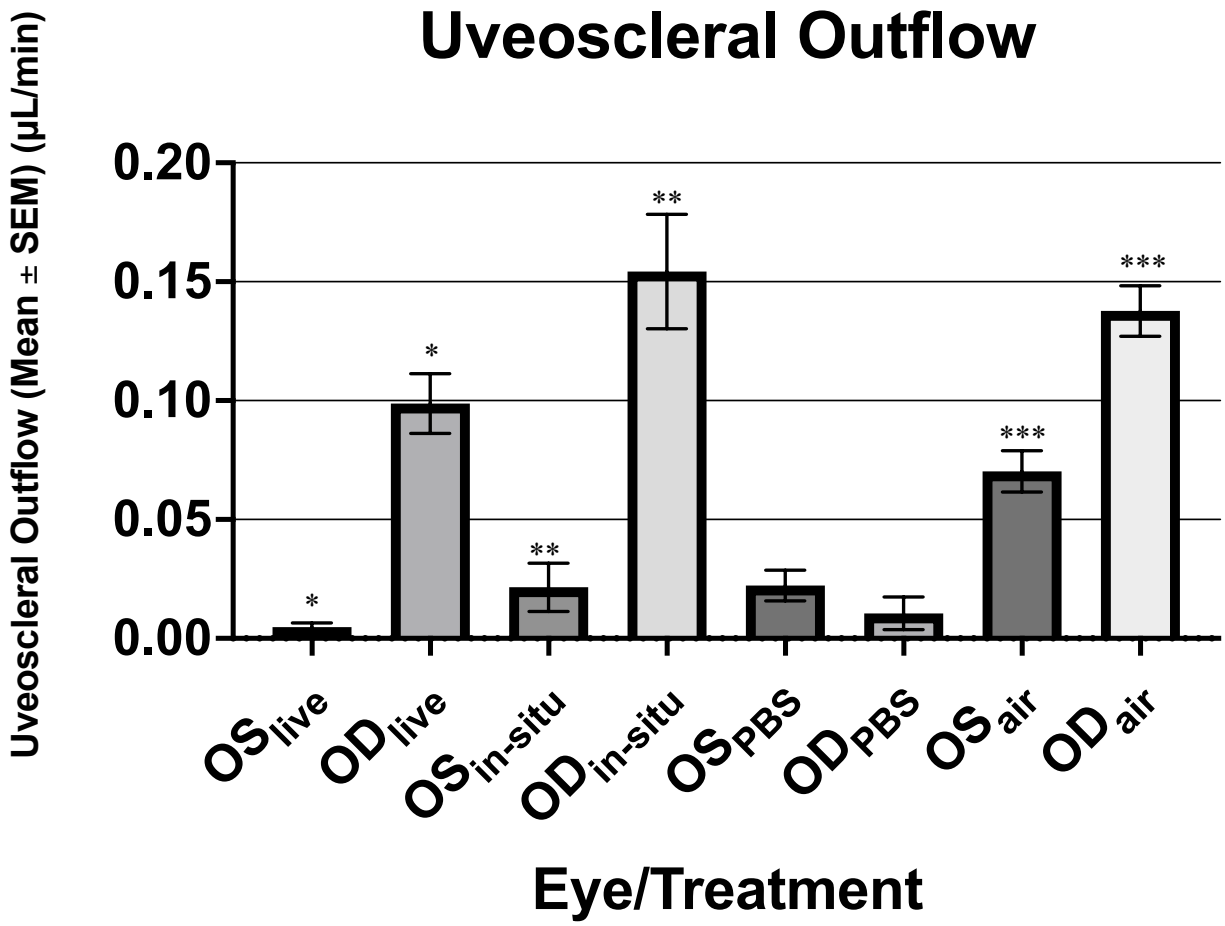


Figure 2: Comparison of Fu in treated (OS) eyes vs. untreated (OD) eyes in live mice, mice euthanized by anesthetic overdose, enucleated eyes bathed in PBS, and enucleated eyes exposed to air. There was a significant difference between OS_{live} and OD_{live} ($P = 0.025$), $OS_{in\text{-}situ}$ and $OD_{in\text{-}situ}$ ($P = 0.010$), and OS_{air} and OD_{air} ($P = 0.008$). There was no significant difference between OS_{PBS} and OD_{PBS} ($P = 0.175$). Bars equal mean ± SEM ($n = 5$ eyes per group).

Assessment of IOP

In our cohort of mice used for direct measurement of Fu, there was a significant difference in conscious IOP measured at day 7 between OS (22.33 ± 1.667 mmHg) and OD (13.63 ± 0.6424 mmHg; $P < 0.001$) and at day 14 between OS (26.30 ± 0.9773 mmHg) and OD (14.38 ± 0.3850 mmHg, $P < 0.001$). (Figure 3)

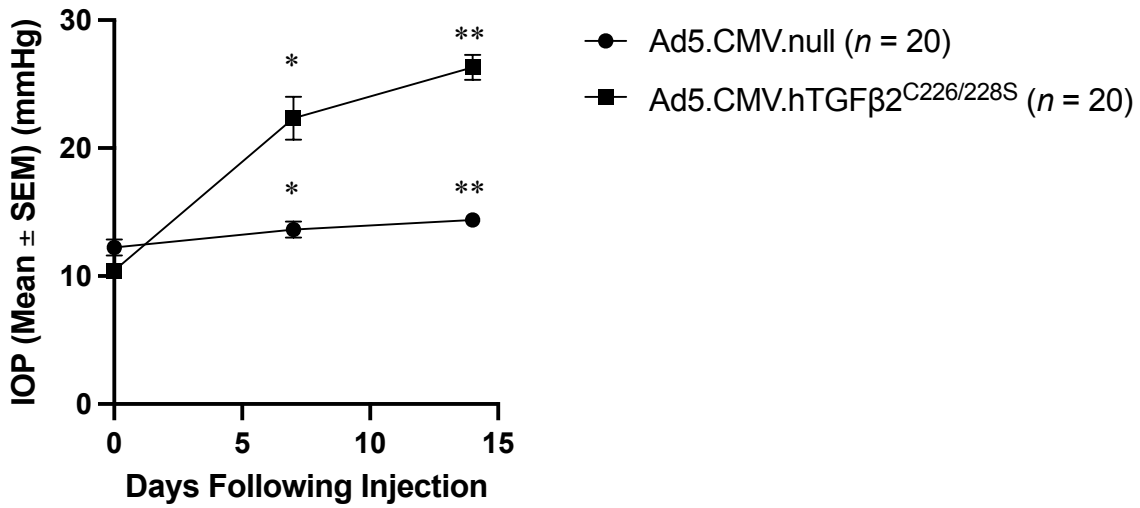


Figure 3: Assessment of IOP at 0, 7, and 14 days following intravitreal injection of Ad5.CMV.hTGFβ2$^{C226/228S}$ (OS) vs. Ad5.CMV.null (OD). Results are presented as mean ± SEM. $P < 0.001$ compared with Ad5.CMV.null group by two-way ANOVA.

In our cohort of mice utilized for assessment of AHD, there was a significant difference in conscious IOP measured at day 7 between OS (13.43 ± 0.8255 mmHg) and OD (10.53 ± 0.5290 mmHg, $P = 0.041$) and at day 10 between OS (21.98 ± 1.846 mmHg) and OD (12.80 ± 1.087 mmHg, $P = 0.002$). (Figure 4) However, following anesthesia, although there was a trend towards an increase in OS as compared with OD, there was no significant difference in anesthetized IOP (measured at day 13 and 14) between OS (13.21 ± 2.688 mmHg) and OD (9.718 ± 1.285 mmHg, $P = 0.535$). This was found to be because following anesthesia, the elevated IOP in 2 of the animals had unexpectedly collapsed to a very low level.

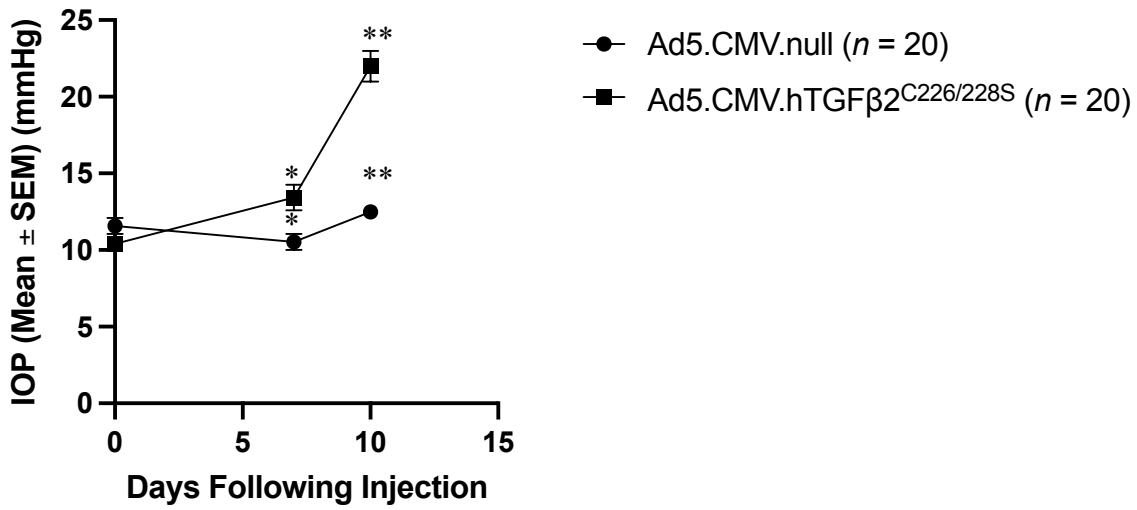


Figure 4: Assessment of IOP in mice used for AHD studies. Measurements were taken at 0, 7, and 10 days following intravitreal injection of Ad5.CMV.hTGFβ2$^{C226/228S}$ (OS)vs. Ad5.CMV.null (OD). Results are presented as mean ± SEM. $P < 0.001$ compared with Ad5.CMV.null group by two-way ANOVA.

Indirect Assessment of Fu

In our cohort of mice used for assessment of AHD, increased expression of TGFβ2 resulted in a significant difference in Fu between OS (0.1556 ± 0.0483 µL/min) and OD (0.0316 ± 0.0317 µL/min; $P = 0.044$) when indirectly measured via calculation using values recorded during constant flow infusion. Further, there was a trend towards a considerably higher value for Fu in those eyes in which IOP had collapsed.

Assessment of C

Increased expression of TGFβ2$^{C226/228S}$ resulted in a significant difference in C_{live} between OS (0.0237 ± 0.0065 µL/min/mmHg) and OD (0.0468 ± 0.0119 µL/min/mmHg, $P = 0.046$). (Figure 5)

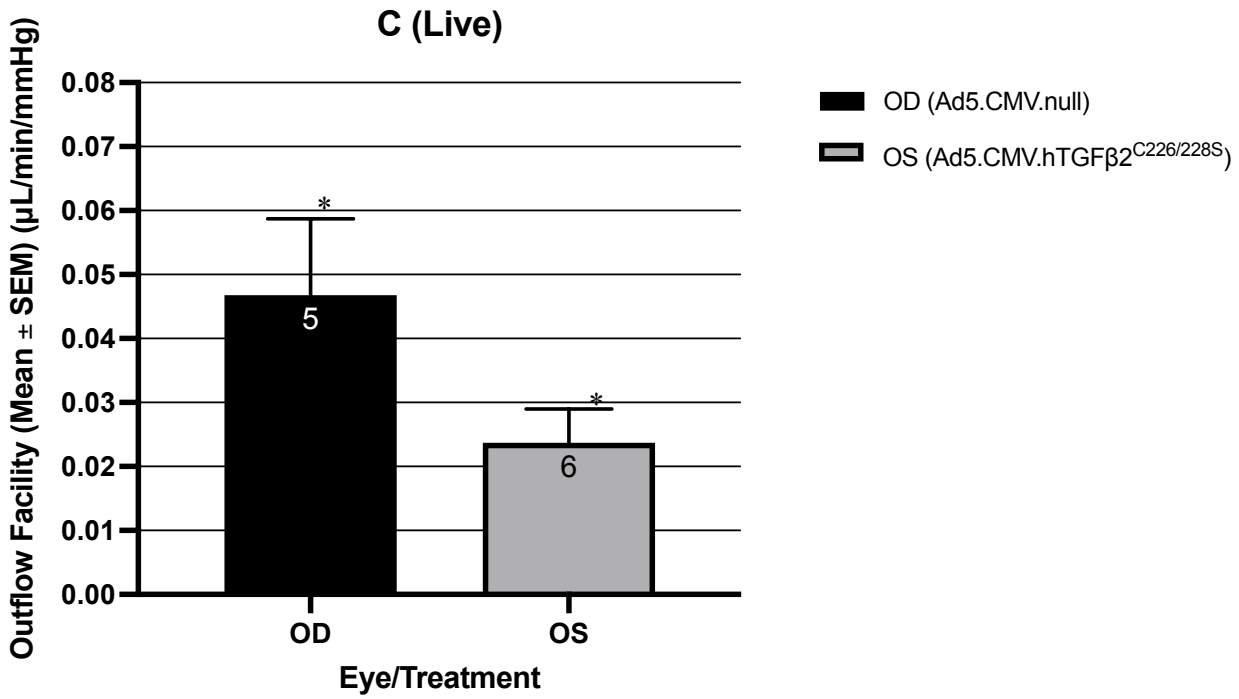


Figure 5: Assessment of C_{live} in 5 mice used for AHD studies. There was a significant difference between OS and OD ($P = 0.046$) as indicated by an unpaired Student's t-test, assuming equal population variances.

Assessment of Fin

Increased expression of TGFβ2$^{C226/228S}$ resulted in no significant difference in Fin between OS (0.2006 ± 0.0377 µL/min) and OD (0.2646 ± 0.1324 µL/min, $P = 0.673$) (Figure 6). However, there was a downward trend when comparing OS and OD.

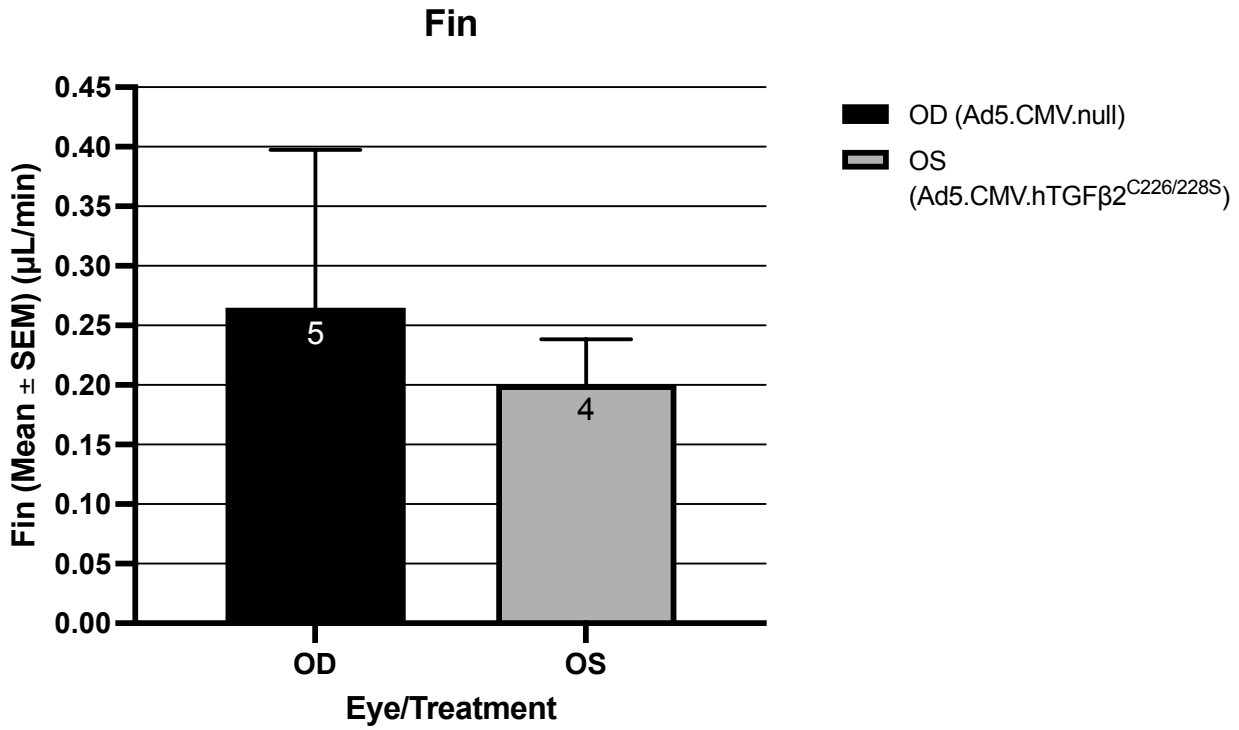


Figure 6: Comparison of Fin in treated (OS) eyes vs. untreated (OD) eyes. There was no significant difference between OS and OD ($P = 0.673$).

Assessment of Pe

In the present study, increased expression of TGFβ2$^{C226/228S}$ resulted in a significant difference in Pe between treated and control eyes (8.6 ± 0.7 mmHg in OS to 6.4 ± 0.2 mmHg in OD, $P = 0.015$). (Figure 7)

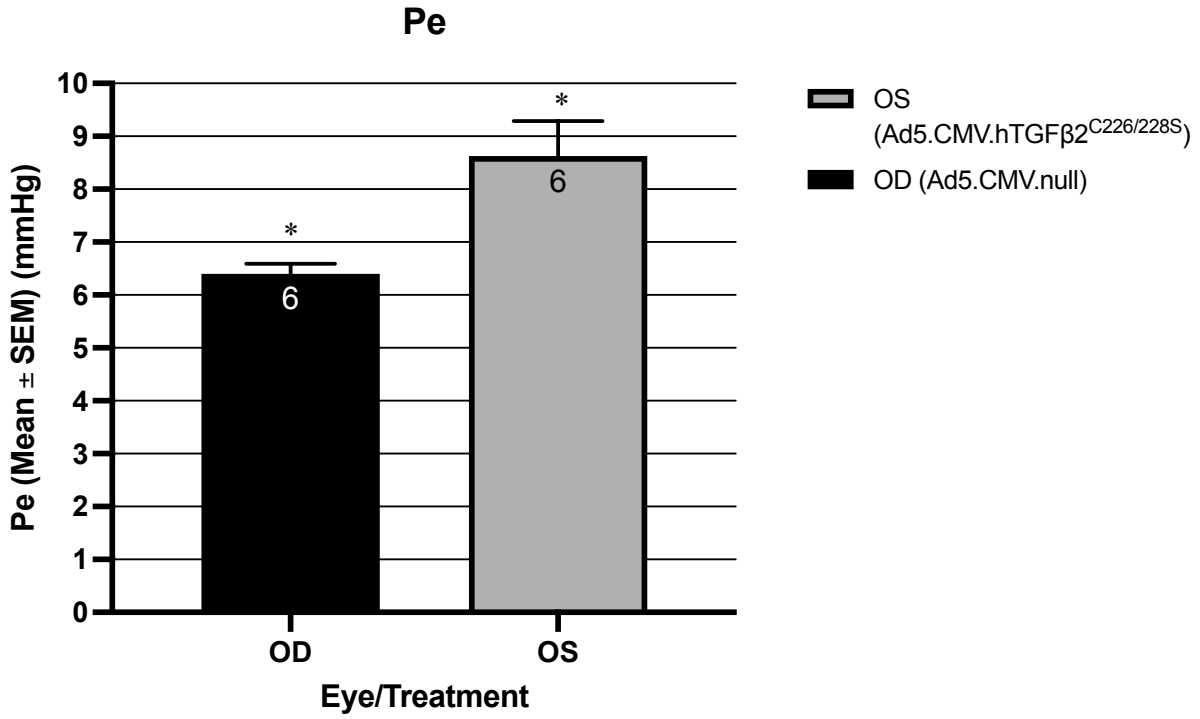
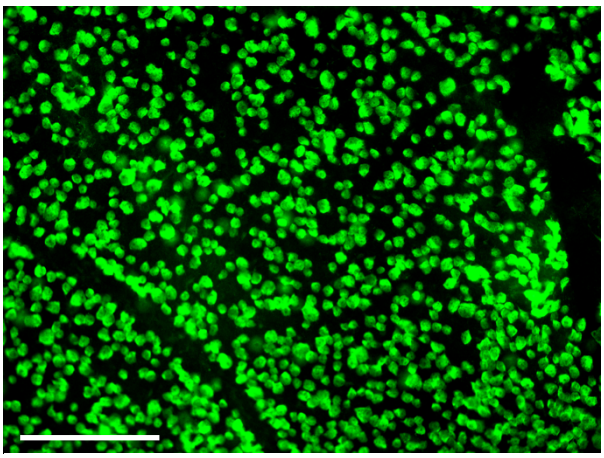


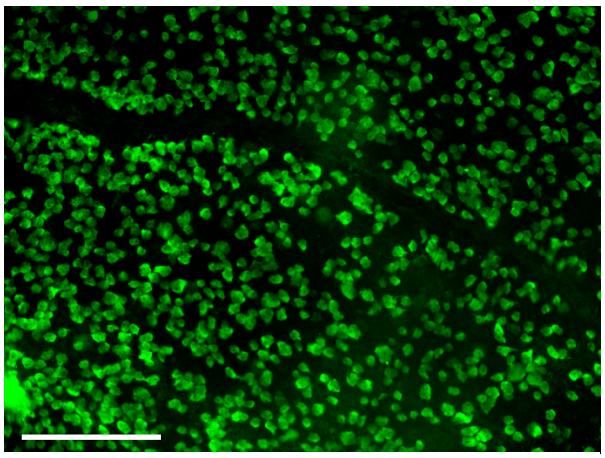
Figure 7: Comparison of Pe in treated (OS) eyes vs. untreated (OD) eyes. There was a significant difference between OS and OD ($P = 0.015$).

RGC Counting

In our treated (OS) eyes, there were an average of 1434.07 RGCs per field view at 20x magnification, compared to an average of 1300.78 RGCs per field view at 20x magnification in our untreated (OD) eyes, representing a 10.2% reduction in RGC numbers from OS to OD. However, this result was not statistically significant ($P = 0.264$). (Figure 8)



OD (untreated); AlexaFluor488 stain



OS (treated); AlexaFluor488 stain

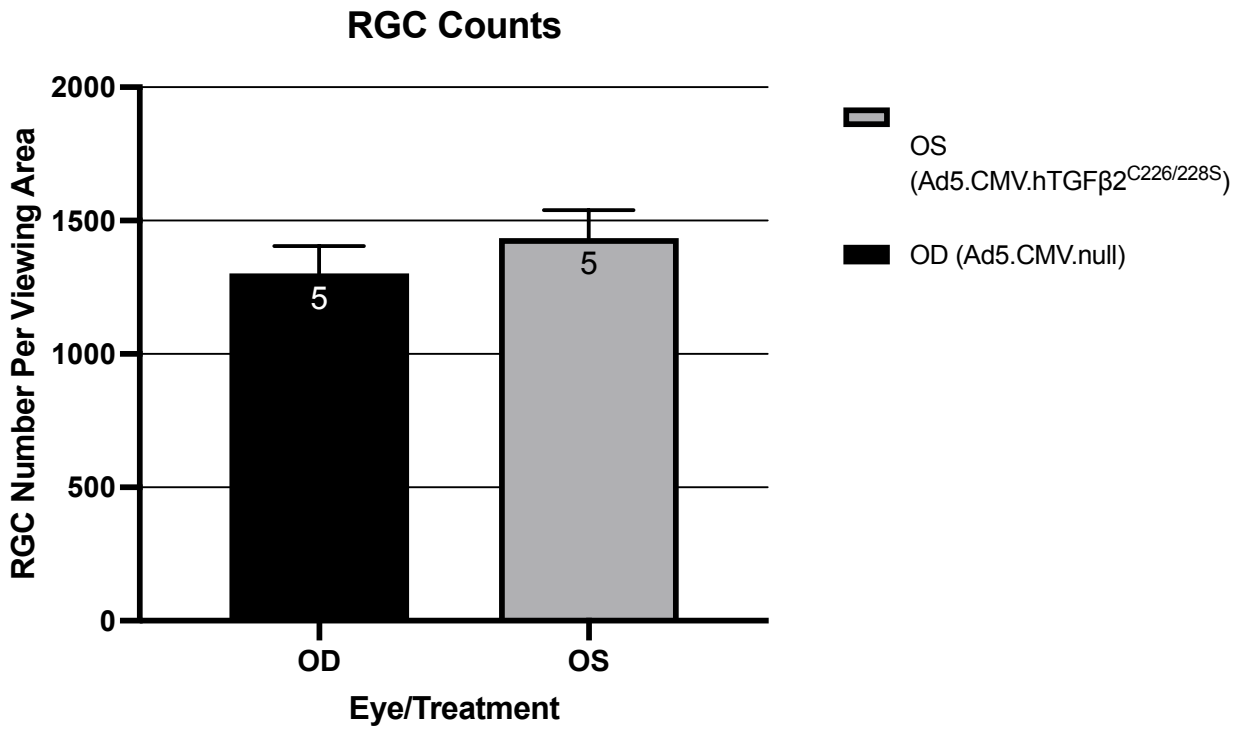


Figure 8: RGCs were stained with rabbit RBPMS antibody and secondary antibody of AlexaFluor488 goat anti-rabbit. Cell bodies were stained with DAPI. Top row, left: untreated (OD) eye. Top row, right: treated (OS) eye. There was no significant difference between OS and OD ($P = 0.264$). Scale bar represents 150 µm.

Discussion

In the present study, we have demonstrated that over-expression of hTGFβ2$^{C226/228S}$ results in significant decreases in Fu in the living mouse, in the euthanized mouse, and in

enucleated eyes exposed to air, but not in enucleated eyes bathed in PBS. Similar results were seen by Boussommier-Calleja et al. in regards to enucleated eyes exposed to air vs. eyes bathed in PBS, leading this group to conclude that Fu requires evaporation from the ocular surface, and that insufficient hydration of the ocular surface artificially increases Fu.[31] However, we also demonstrate a significant decrease in Fu in both the living and euthanized mouse, both of which represent experimental conditions that more accurately approximate the anatomical and physiological environment of the eye. These decreases in Fu were seen alongside the expected elevation in conscious IOPs after transient TGFβ2 increases.[2] The mechanism by which this TGFβ2-mediated decrease in Fu is unknown, but its effects in the sclera, suprachoroidal space, uvea, ciliary muscle, and ciliary body are likely similar to those seen in anterior segment tissues from mouse eyes as well as cultured GTM cells.[2] Moreover, there is data suggesting that in POAG, the sclera becomes more rigid and stiffer, i.e. less compliant.[39, 40] This has a more direct effect on the biomechanical environment of the optic nerve head, but also may have a degree of influence on Fu, in that it may cause target tissues to further impede aqueous egress. Given that TGFβ2 is known to promote secretion of various ECM proteins and in particular collagens 1α1 and 1α2, a collagenous tissue such as the sclera could indeed be an area in which TGFβ2 exerts its effects.

In our second cohort of 5 mice used for further AHD studies following expression of hTGFβ2$^{C226/228S}$, anesthetized IOPs were surprisingly not significantly different between treated and non-treated eyes. This was because, although IOP remained elevated in a subset of the animals at the time of perfusion (several days following the final conscious IOP measurement), in two of the animals IOP dropped to approximately 6 mmHg. This was likely due to a localized inflammatory response to the viral vector; expression and presentation of adenovirus proteins by

histocompatibility proteins is thought to be a mechanism by which there is an immune response to adenovirus-infected cells.[41, 42] In effect, this very well could have led to endogenous prostaglandin release and a large increase in Fu.

There was no significant difference between treated and untreated eyes when comparing calculated means for Fin and Pe, which could suggest that TGFβ2 has no effect on Fin in the mouse; however, there was a downward trend. But, given that Fin in particular is calculated from anesthetized IOP values as well as C_{live} values, an error in the determination of either of these two values could lead to an inaccurate assessment of Fin. Indeed, a 10% error in measurement of C could lead to a 15% error in the calculation of Fin, as well as up to a 36% error in the calculation of Fu.[22, 23]

Given the time constraints and external factors that our project faced, there were a limited number of animals that could be utilized. Moreover, potential sources for error during the constant flow infusion experiments such as needle blockage and leakage of aqueous from the cannulated eyes forced certain animals to be excluded from data analysis. Together, these two considerations likely reduced the statistical power of the presented results. In order to overcome this shortcoming, it will be necessary to simply utilize more animals as a continuation of these aforementioned experiments. Furthermore, further refinement of manual technique in cannulating the anterior chamber of the eye will reduce the chance of aqueous leakage and thus yield more accurate results with regards to direct measurement of C and Pe.

Finally, while we saw a 10.2% increase in RGC numbers in our untreated (OD) eyes, this was not a statistically significant result. This is likely due to the relatively short amount of time between significant IOP elevation and procurement of eyes for flat mounting. Had these eyes had longer exposure to TGFβ2-mediated IOP elevation (on the scale of weeks to months), there

would have likely been a reduction in RGC numbers in our treated eyes.[38] Moreover, the effects of TGFβ2-mediated IOP elevation on RGC numbers would better be addressed by utilizing transgenic mice engineered to chronically over-express TGFβ2, rather than by introducing a transient increase in TGFβ2 as in this study. This would have the benefit of avoiding an immune response to a transient increase in TGFβ2, which would then reduce IOP to normal levels.[2]

Conclusion

We assessed the effects of over-expression of hTGFβ2$^{C226/228S}$ on AHD in the young BALB/cJ mouse. There was a significant increase in IOP, a significant reduction in C, a significant increase in Pe, and a significant reduction in Fu when measured directly. This latter result for Fu was not mirrored as expected when determining this parameter indirectly from constant flow infusion data, but was due to an unexpected pressure drop in members of that cohort in animals, we suspect due to localized inflammation in response to the viral vector. These findings especially help to quantify the effects of TGFβ2 on Fu in the mouse eye, a crucial component of aqueous humor egress and contributor to variations in IOP in POAG.

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