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Age and sex affect TGFβ2-induced ocular hypertension in C57BL/6J mice

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Abstract

Glaucoma is a leading cause of blindness worldwide. The loss of vision in glaucoma patients is due to optic nerve damage. The most important risk factor of glaucoma is elevated intraocular pressure (IOP) which is due to glaucomatous changes in the trabecular meshwork. Animal models, especially mouse models for ocular hypertension (OHT), are important for studying glaucoma. Published studies showed that $2.5X10^7$ PFU adenoviral vectors expressing the biologically active form of human TGFβ2 elevate IOP in female C57BL/6J mice when they are intravitreally delivered. In this study, we found that $2.5X10^7$ PFU adenoviral TGFβ2 vector did not elevate IOP in 3- or 5-month old male C57BL/6J mice. In contrast, $5X10^7$ PFU of the same viral vectors elevated IOP in both 3- and 5-month old male C57BL/6J mice. Also, 5-month old mice showed earlier OHT and higher IOP compared to 3-month old mice. In summary, our data showed that age and sex play roles in adenoviral vector-mediated TGFβ2-induced OHT in C57BL/6J mice.

Keywords

Glaucoma; intraocular pressure; TGFβ2; mouse model; ocular hypertension

Glaucoma is one of the leading causes of irreversible blindness in the world (Resnikoff et al., 2004). The primary risk factor of glaucoma is increased intraocular pressure (IOP) (Investigators, 2000), which is due to elevated aqueous humor outflow resistance at the trabecular meshwork (TM). Glaucomatous TM changes include loss of TM cells (Alvarado et al., 1984; Kuehn et al., 2021), compromised TM cell functionality, formation of cross-linked actin networks (CLANs) (Clark et al., 1994; Hoare et al., 2009), and excessive deposition of extracellular matrix (ECM) proteins (Dan et al., 2005; Lütjen-Drecoll et al.,

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1989; Medina-Ortiz et al., 2013; Vranka et al., 2015). All these changes make the TM tissue stiffer and elevate outflow resistance (Raghunathan et al., 2015a; Raghunathan et al., 2015b; Wang et al., 2018; Wang et al., 2017).

Growth factors are important signaling molecules for TM homeostasis, and changes in growth factor levels have been identified in glaucomatous eyes (Mao et al., 2016). Among these factors, TGFβ2 has been well studied (Wordinger et al., 2014). Clinically, elevated TGFβ2 has been well documented in the glaucomatous aqueous humor (Inatani et al., 2001; Tripathi et al., 1994) and TM tissues (Tovar-Vidales et al., 2011). Experimentally, excessive amount of TGFβ2 induces ECM production and crosslinking (Fleenor et al., 2006; Sethi et al., 2011; Tovar-Vidales et al., 2008; Zhao et al., 2004), CLAN formation (Montecchi-Palmer et al., 2017), ocular hypertension (OHT) in perfusion cultured human eyes (Gottanka et al., 2004), as well as OHT in in vivo mouse eyes (Shepard et al., 2010).

The TGFβ2 protein is secreted in the latent form (Wordinger et al., 2014). Upon enzymatic cleavage, the active form of TGFβ2 binds and activates its receptors TGFβ receptor type I (TGFβRI) and TGFβ receptor type II (TGFβRII) which phosphorylate receptor Smad proteins including Smad2 and/or Smad3. Phosphorylated Smad2/3 then binds to Smad4 and translocate into the nucleus, where they bind to the Smad binding elements and regulate target genes.

Shepard et al. first reported that intravitreal injection of adenoviruses expressing human mutant TGFβ2$^{C226S;\ C228S}$ (a constitutively active form of TGFβ2) increases IOP in 2–5 month old Balb/cJ mouse eyes (sex unspecified) (Shepard et al., 2010). Therefore, it seems that both young and old Balb/cJ mice (sex unclear) respond well to mutant TGFβ2. In contrast, the role of sex in TGFβ2-induced OHT has not been specifically studied in C57BL/6J mice (Hernandez et al., 2020; McDowell et al., 2013; Roberts et al., 2020). Since sex is becoming an important consideration in biomedical research, we determined if sex also plays a role in the adenoviral vector-medicated TGFβ2-induced OHT mouse model.

Male C57BL/6J mice were procured from Jackson Laboratories (Bar Harbor, ME). We chose the C57BL/6J strain since this genetic background is frequently used in transgenic or knockout mouse studies. Mice were housed at the Laboratory Animal Resource Center at Indiana University School of Medicine, and all the procedures were approved by the Institutional Animal Care and Use Committee. The mice were housed under controlled temperatures and humidity with 8am light off and 8pm light on (reversed light cycle). Food and water were provided ad libitum. After acclimation for at least two weeks, the mice were anaesthetized using 2.5% isoflurane and baseline IOP was measured using a stabilized rebound tonometer (iCare Tonolab, Ayritie, Vantaa, Finland) to minimize tonometer movement. IOP was measured at around 2pm throughout the study. Each mouse eye was measured for 5 readings and the readings were averaged. After baseline IOP establishment, one of the mouse eyes was intravitreally injected with 2.5×10^7 PFU of Ad5-CMV-human TGFβ2$^{C226S;C228S}$ (referred to as “Ad5-CMV-TGFβ2” in the following text) while the fellow eye was injected with the same amount of Ad5-CMV-GFP. Some mouse eyes were injected with 5×10^7 PFU Ad5-CMV-TGFβ2 and their fellow eyes with 5×10^7 PFU Ad5-CMV-GFP. All adenoviruses and their titers were provided by Vector

Biolabs (Malvern, PA). Intravitreal injection was performed as described before. Briefly, the mice were anesthetized using 2–3% isoflurane together with proparacaine (Valent Pharmaceuticals, Bridgewater, NJ) eye drops. Two microliters of viruses were loaded into a 10ul Hamilton syringe with a 1-inch long 33 gauge needle protected by a short 28 gauge needle. The 28 gauge needle prevents the shaft of the 33 gauge needle from bending. The mouse eye was held using a forceps and the 33 gauge needle was inserted at the equator avoiding damaging any blood vessels or the lens. Adenovirus was slowly injected over 1–2 minutes. All procedures were performed under a dissection microscope. IOP measurement was resumed the next week after injection. To avoid bias, all mouse studies were conducted in a masked manner.

To determine if male C57BL/6J mice respond to Ad5-CMV-TGFβ2, we injected 2.5×10^7 PFU Ad5-CMV-TGFβ2 into one eye and 2.5×10^7 PFU Ad5-CMV-GFP (as a control) into the fellow eye of both 3-month old (n=10) and 5-month old male mice (n=10). IOP was monitored weekly for 11 weeks. In contrast to published results from female mice (Hernandez et al., 2020), we did not observe a significant IOP elevation in Ad5-CMV-TGFβ2 injected male mice (Student's paired t-test for comparisons between eyes "*": $P>0.05$ at all time points except at weeks 1 and 5 as well as weeks 1 and 2, respectively; one-way ANOVA for comparisons at a certain timepoint to baseline IOP "#": $P> 0.05$ except at week 1 for TGFβ2) (Figure 1A and 1B).

To determine if male C57BL/6J mice might need high viral doses to develop OHT, we injected another fourteen 3-month old and ten 5-month old male mice with $5X10^7$ PFU Ad5-CMV-TGFβ2 or $5X10^7$ Ad5-CMV-GFP viruses in a similar approach. We observed a significant IOP elevation in Ad5-CMV-TGFβ2 injected eyes in both age groups (Figure 2A and 2B) (Student's paired t-test for comparisons between eyes "*": $P<0.05$ from weeks 4 or 1; one-way ANOVA for comparisons at a certain timepoint to baseline IOP "#": $P<0.05$ from weeks 4 or 6). Also, we observed earlier (4 weeks vs. 1 week between GFP and TGFβ2 eyes) and higher IOP elevation (24.17 mmHg vs. 40.44 mmHg at week 9) in older mice compared to younger mice, respectively (Figure 2A and 2B).

To determine the level of exogenous TGFβ2 (human mutant TGFβ2C226S; C228S) in the aqueous humor, anterior chamber paracentesis was conducted in some mouse eyes using a 30 gauge needle and the aqueous humor was collected using the 25 μl glass capillary tube (Drummond Scientific Company, Broomall, PA) in anesthetized mice. Five aqueous humor samples of the same group were pooled for ELSIA and the assay was performed using the Human TGFβ2 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

To determine the level of exogenous TGFβ2 in the TM, the mouse eyes were enucleated after euthanasia. For some eyes, the TM region (a ring of tissue containing the TM since the TM is too small to be dissected accurately) was dissected under a dissection microscope for RNA extraction using the Qiagen RNeasy kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Alfred Nobel Drive Hercules, CA) using 200ng of total RNA

according to manufacturer's instructions. qPCR was conducted using the iTaq universal SYBR green kit in the CFX-96 thermocycler (Bio-Rad Laboratories). Primer sequences:

GAPDH (Martens et al., 2003):	Forward: 5'-GGTGAAGGTCGGAGTCA-AC-3′
	Reverse: 5′-CCATGGGTGGAATCATATTG-3′
TGFβ2 (Rayana et al., 2021):	Forward: 5′-AGAGTGCCTGAACAACGGATT-3′
	Reverse: 5′-CCATTCGCCTTCTGCTCTT-3′

The other eyes were used for immunofluorescence analysis without dissection. After 4% paraformaldehyde fixation, paraffin embedment, sectioning, and antigen retrieval using Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) and the 2100 Antigen retriever (Electron Microscopy Sciences, Hatfield, PA), the tissue sections were used for immunostaining. The Mouse on Mouse (M.O.M) immunodetection kit (Vector Laboratories, Inc, Burlingame, CA; Cat # BMK-2202) was used according to manufacturer's instructions. Briefly, the sections were exposed to M.O.M Mouse IgG blocking reagent for 1 hour. Then, the sections were probed with the primary mouse-anti-TGFβ2 (1:100, catalog# ab36495, Abcam, Cambridge, MA) or mouse-anti-Fibronectin-IST9 (1:250, catalog# ab6328, Abcam) antibody and then probed with the secondary Avidin conjugated antibody and the tertiary Alexa Fluor conjugated streptavidin (Thermofisher Scientific, Waltham, MA). A no primary antibody control was also included. At least 3 eyes from each viral/age group were studied. Images were taken using Zeiss LSM 700 Confocal microscopy (Zeiss, White Plains, NY). Some sections were deparaffined for hematoxylin and eosin staining.

We found that injection with either 2.5×10^7 PFU or 5×10^7 PFU, regardless of age groups, induced high levels of TGFβ2 expression in the TM region (Figures 1C and 2C, respectively) as well as the aqueous humor (Figures 1D and 2D, respectively).

We did not observe obvious angle closure in TGFβ2/GFP treatment groups or 3/5 month age groups (H&E staining, data not shown). We found elevated TGFβ2 expression in Ad5-CMV-TGFβ2 injected eyes, especially in the TM region, compared to Ad5-CMV-GFP injected control eyes regardless of viral dose or age (Figure 1E and 1F; Figure 2E and 2F), which matched ELISA and qPCR data described previously. Densitometry analysis of TGFβ2 fluorescence intensity showed increased TGFβ2 expression in the TM region (N=3; Student's t-test with all P values<0.05):

Three-month old, 2.5×10^7 PFU: 68.9 (mean optic density) ±15.7 (standard deviation) vs. 184.0±16.0; 2.7 fold increase

Five-month old, 2.5×10^7 PFU: 49.4±16.9 vs. 142.3±7.5; 2.9 fold increase

Three-month old, 5×10^7 PFU: 48.4±13.3 vs. 134.5±13.0; 2.8 fold increase

Five-month old, 5×10^7 PFU: 67.8±2.0 vs. 179.0±15.8; 2.6 fold increase

Similarly, we found that the expression of the fibronectin isoform EDA was also elevated in all Ad5-CMV-TGFβ2 injected eyes (Figure 1G and 1H; Figure 2G and 2H) (N=3; Student's t-test with all P values<0.05).

Three-month old, 2.5×10^7 PFU: 81.4 (mean optic density) ±22.3 (standard deviation) vs. 155.7±31.7; 1.9 fold increase

Five-month old, 2.5×10^7 PFU: 84.3±28.9 vs. 169.3±25.9; 2.0 fold increase

Three-month old, 5×10^7 PFU: 49.2±20.1 vs. 150.0±4.6; 3.0 fold increase

Five-month old, 5×10^7 PFU: 57.2±38.6 vs. 178.5±25.7; 3.1 fold increase

EDA has been shown to play a role in glaucomatous ECM changes (Filla et al., 2017; Hernandez et al., 2020; Medina-Ortiz et al., 2013).

Our data showed that sex plays a key role in the induction of OHT in C57BL/6J mice using the Ad5-CMV-TGFβ2 viral vector. Unlike female C57BL/6J mice in which OHT can be induced at the age of 3 months (data not shown) and 5 months using $2.5X10^7$ PFU Ad5-CMV-TGFβ2, male C57BL/6J mice require twice the number of viruses to develop OHT. Since we used the TGFβ2 model, one would expect that there would be more TGFβ2 and EDA in the high viral dose (5×10^7 PFU) group/OHT group. Surprisingly, we did not observe such changes (Figure 1C, 1D; Figure 2C, 2D; Figure 1E vs. 1F; and Figure 2E vs. 2F). We believed that this difference is unlikely to be due to viral quality since we successfully induced OHT in female C57BL/6J mouse eyes using 2.5×10^7 PFU viral particles produced by the same vendor (Miller et al., 2020).

We think that sex hormones might contribute to this sex-based difference in IOP response. These hormones regulate a variety of biological processes. Although we studied the fibronectin isoform EDA, there are many other proteins that are targets of TGFβ signaling which might have been affected by sex hormones. Eye size could be another contributing factor. Male mice are larger than female mice and therefore their eyes are likely larger and may contain more TM cells. Therefore, male eyes may require more viral particles to reach a similar moiety to infection ratio as that in female eyes.

Also, male C57BL/6J mice may have more "reserve" in their uveoscleral outflow compared to female mice. When the same amount of TGFβ2 was expressed in male and female eyes which resulted in similar damage in the TM, male mouse eyes might accommodate better due to their potentially high capacity in uveoscleral outflow reserve and therefore showed no OHT. A comprehensive measurement of mouse outflow facility, uveoscleral outflow, and episcleral vein pressure will be helpful to answer this question.

Another possibility is the differential effect of isoflurane on male vs. female mouse IOP. If male mice require longer induction time, this prolonged isoflurane exposure may further lower their IOP which may have masked their OHT. However, no such studies have been conducted specifically addressing this issue. Ding and colleagues reported that isoflurane-based general anesthesia lowers IOP especially elevated IOP in C57BL/6J mouse eyes, and this reduction can continue up to 30min (Ding et al., 2011). However, the authors did not

describe the sex of the mice that they used. Tsuchiya and colleagues showed similar results using a mixture of about 5-month old male and female C57BL/6J mice, and reported that this reduction is the greatest in the initial 3 minutes (Tsuchiya et al., 2021). A specific comparison between sex was not conducted in that study (Tsuchiya et al., 2021).

In addition to sex, we found that age should be considered in this mouse model. We found that older C57BL/6J male mice responded better than younger mice to TGFβ2. Therefore, we recommend using older mice if a relatively higher IOP is required for the study. Other studies also showed that older mice are easier or more suitable for OHT induction (Faralli et al., 2020; McDowell et al., 2012; Pang and Clark, 2020). In contrast, if a moderate IOP elevation is needed, then using younger mice might be a better choice.

Although we successfully induced OHT in both young and old C57BL/6J male mouse eyes using the high viral dose, we did not observe higher TGFβ2 levels (qPCR and ELISA for TM region tissues and AH samples, respectively) when comparing old mice receiving the high viral dose (5×10^7 PFU) and showing OHT to old mice receiving the low viral dose (2.5×10^7 PFU) and showing no OHT. Theoretically, higher viral doses should have induced more transgene expression. We believe that it might be due to

1. Excessive cell death induced by TGFβ2.

   It is known that high viral doses, including adenoviruses, are toxic to cells. Therefore, the mouse eyes receiving 5×10^7 PFU Ad5-CMV-TGFβ2 were likely to have more TM cell loss. The loss of TM cells would lead to A) relatively low TGFβ2 expression since there were not enough cells to produce TGFβ2; and B) OHT since TM cells are required to maintain normal outflow facility.

2. Endoplasmic reticulum (ER) stress.

   Due to the high viral dose of Ad5-CMV-TGFβ2, the transduced TM cells might express more TGFβ2 which could cause severe ER stress. Studies have also shown that ER stress in the TM contributes to OHT (Kasetti et al., 2017; Zode et al., 2014). In contrast, GFP is a relatively non-toxic protein. The control eyes receiving Ad5-CMV-GFP were likely to avoid severe ER stress and therefore did not show IOP elevation.

3. Inflammation.

   Adenoviruses cause ocular inflammation (Millar et al., 2008). The mouse eyes receiving more adenoviruses were likely to have more severe inflammation which could contribute to OHT. Although the control eyes received the same numbers of Ad5-CMV-GFP viral particles and they should have similar levels of inflammation, they did not show OHT. We predict that the inflammation in Ad5-CMV-TGFβ2 injected eyes might work synergistically with the overexpression of TGFβ2 to induce OHT.

In summary, both sex and age should be considered when developing the TGFβ2-induced OHT mouse model, at least in the C57BL/6J strain. Further research is needed to determine if such differences also exist in the other mouse strains.

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Abbreviations

Ad5	adenovirus serum type 5
CMV	cytomegalovirus
CLANs	cross-linked actin networks
EDTA	ethylene diamine tetra acetic acid
GFP	green fluorescent protein
IOP	intraocular pressure
mM	milli molar
OHT	ocular hypertension
PBS	phosphate buffered saline
PFU	plaque forming unit
TM	trabecular meshwork
ECM	extracellular matrix
TGFβRI	TGFβ receptor type I
TGFβRII	TGFβ receptor type II

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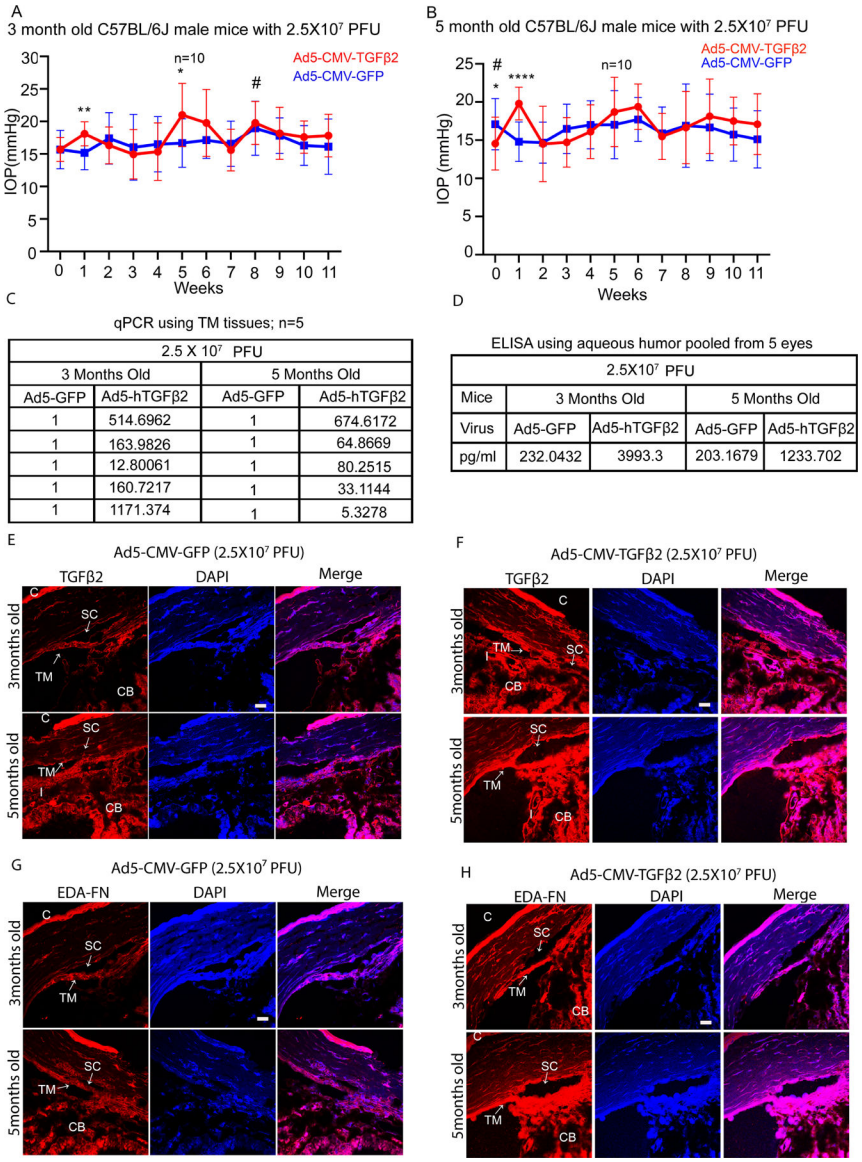
Highlights

- $2.5X10^7$ PFU Ad5-CMV-TGFβ2 did not induce significant intraocular pressure in 3 or 5-month old C57BL/6J male mice.
- $5X10^7$ PFU Ad5-CMV-TGFβ2 significantly elevated intraocular pressure in both 3 and 5-month old C57BL/6J male mice
- Older mice respond better to Ad5-CMV-TGFβ2



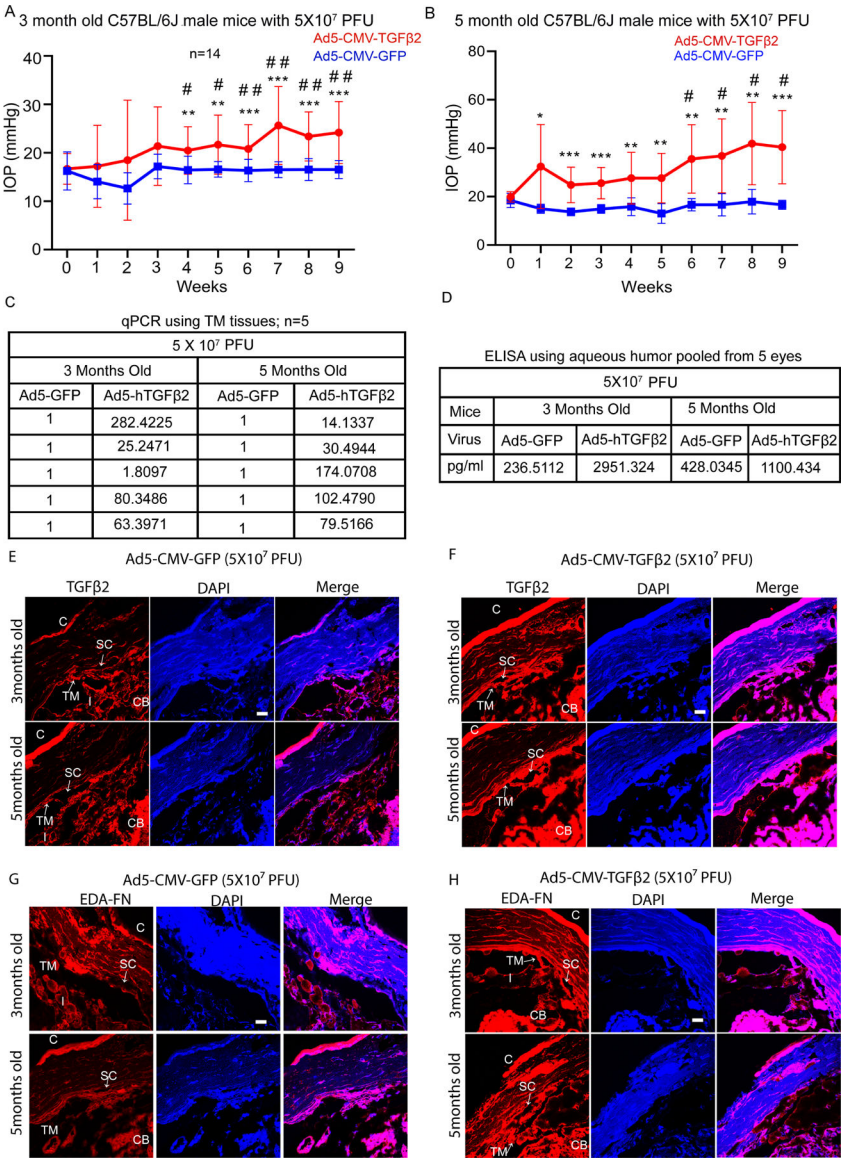
2.5 X 10⁷ PFU			
3 Months Old		5 Months Old	
Ad5-GFP	Ad5-hTGFβ2	Ad5-GFP	Ad5-hTGFβ2
1	514.6962	1	674.6172
1	163.9826	1	64.8669
1	12.80061	1	80.2515
1	160.7217	1	33.1144
1	1171.374	1	5.3278

2.5X10⁷ PFU				
Mice	3 Months Old		5 Months Old	
Virus	Ad5-GFP	Ad5-hTGFβ2	Ad5-GFP	Ad5-hTGFβ2
pg/ml	232.0432	3993.3	203.1679	1233.702

Figure 1:

C57BL/6J male mice injected with 2.5X10^7 PFU Ad5-CMV-TGFβ2 did not develop OHT. Three-month old (A) and five-month old (B) male C57BL/6J mice were intravitreally injected with Ad5-CMV-TGFβ2 (red dots and lines) to one of the eyes and the fellow eye received Ad5-CMV-GFP as a control (blue dots and lines). Student's paired t-test was used to compare IOP between paired eyes at each time points, and "*" was used to show significance. *: P<0.05; **: P<0.01, ****: P<0.0001. Also, one-way ANOVA was used to compare IOP at each time point with the baseline IOP, and "#" was used to show significance of TGFβ2 eyes. #: P<0.05. Error bars: standard deviations. (C): TGFβ2 expression levels in mouse TM tissues determined by qPCR. Five pairs of eyes were used for each age group and the level of TGFβ2 in Ad5-GFP injected eyes were set at 1. (D): TGFβ2 expression levels in the mouse aqueous humor determined by ELISA (the same eyes as in C). Five aqueous humor samples from the sample group were pooled. (E) and (F):

immunostaining of TGFβ2 in 3-month and 5-month old mouse eyes, respectively. (G) and (H): immunostaining of EDA fibronectin (EDA-FN) in 3-month and 5-month old mouse eyes, respectively. C: cornea; CB: ciliary body; I: iris; SC: Schlemm's canal; TM: trabecular meshwork. Scale bars: 20μM.



5 X 10⁷ PFU			
3 Months Old		5 Months Old	
Ad5-GFP	Ad5-hTGFβ2	Ad5-GFP	Ad5-hTGFβ2
1	282.4225	1	14.1337
1	25.2471	1	30.4944
1	1.8097	1	174.0708
1	80.3486	1	102.4790
1	63.3971	1	79.5166

5X10⁷ PFU				
Mice	3 Months Old		5 Months Old	
Virus	Ad5-GFP	Ad5-hTGFβ2	Ad5-GFP	Ad5-hTGFβ2
pg/ml	236.5112	2951.324	428.0345	1100.434

Figure 2:

C57BL/6J male mice injected with 5X10^7 PFU Ad5-CMV-TGFβ2 developed OHT. Three-month old (A) and five-month old (B) male C57BL/6J mice were intravitreally injected with Ad5-CMV-TGFβ2 (red dots and lines) to one of the eyes and the fellow eye received Ad5-CMV-GFP as a control (blue dots and lines). Student's paired t-test was used to compare IOP between paired eyes at each time points, and "*" was used to show significance. **: P<0.01, ***: P<0.001. Also, one-way ANOVA was used to compare IOP at each time point with the baseline IOP, and "#" was used to show significance of TGFβ2 eyes. #: P<0.05. ##: P<0.01. Error bars: standard deviations. (C): TGFβ2 expression levels in mouse TM tissues determined by qPCR. Five pairs of eyes were used for each age group and the level of TGFβ2 in Ad5-GFP injected eyes were set at 1. (D): TGFβ2 expression levels in the mouse aqueous humor determined by ELISA (the same eyes as in C). Five aqueous humor samples from the sample group were pooled. (E) and (F): immunostaining of

TGFβ2 in 3-month and 5-month old mouse eyes, respectively. (G) and (H): immunostaining of EDA fibronectin (EDA-FN) in 3-month and 5-month old mouse eyes, respectively. C: cornea; CB: ciliary body; I: iris; SC: Schlemm's canal; TM: trabecular meshwork. Scale bars: 20μM.