Expressional and functional involvement of gap junctions in aqueous humor outflow into the ocular trabecular meshwork of the anterior chamber

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Purpose: The ocular trabecular meshwork (TM) responsible for aqueous humor (AH) drainage is crucial for regulating intraocular pressure (IOP) of the eye. An IOP elevation that causes distended TM is involved in the pathogenesis of glaucoma, suggesting intercellular connections important for the TM pathophysiology. The goal of this study was to examine whether gap junction proteins between endothelial cells in the TM are expressional and functional.

Methods: The expression levels of the gap junction channels in normal human TM cells were determined with real-time PCR and western blot assays. Immunohistochemistry (IHC) staining was performed to examine the localization of gap junction proteins in normal human TM cells and tissues. IOP and the outflow of AH were measured after intercameral injection of gap junction blockers in C57/BL6 mice.

Results: Gap junction proteins GJA1, GJA8, GJB6, and GJC1 were robustly expressed in human TM cells from three individuals. Among the four gap junction channels, GJA1 and GJA8 exhibited the most abundance in the TM. The IHC analysis further confirmed that these proteins were expressed on the membrane between adjacent cells. In the human TM tissues, GJA1, GJA8, GJB6, and GJC1 were also observed along the trabecular beams. Inhibition of gap junctions with intracameral injection of blockers resulted in a statistically significant increase in aqueous humor outflow resistance and IOP elevation in mice.

Conclusions: The GJA1 and GJA8 gap junction proteins, in particular, are robustly expressed in human TM cells and tissues. Pharmacological inhibition of gap junction channels causes an increased resistance of AH outflow and an elevation of IOP in mice. The present findings suggest the functional role of gap junction channels for regulation of AH outflow in the TM, and activation of gap junctions might represent a therapeutic strategy for treatment of glaucoma.

Glaucoma is a common blinding disease that results in an irreversible degeneration of the optic nerve and vision loss [1]. The major risk factor for most glaucoma is an elevation of intraocular pressure (IOP) predominantly caused by decreased removal of aqueous humor (AH) [2,3]. In the AH drainage system, the trabecular meshwork (TM) is the major tissue responsible for outflow of AH from the anterior chamber of the eye [2,4], and dysfunctional TM is involved in elevation of IOP [5,6]. The TM tissue comprises the trabecular beams which are covered with TM cells [7]. Direct cell-to-cell contact between adjacent TM cells indicates that gap junctions are critical for intercellular communication and maintenance of IOP [8-10].

Gap junction channels are mechanosensitive, and important for regulation of various physiologic processes through the exchange of molecules and ions [11]. The gap junction proteins consist of a family of connexin proteins that are four transmembrane segments (TMSs) with cytoplasmic N- and C- termini [12]. Among the 21 identified human gap junction proteins [13], GJA1 and GJA8 have been shown to be relevant to the pathogenesis of eye diseases. For instance, mutations in GJA1 (Gene ID: 2697; OMIM: 121014) can cause oculodentodigital dysplasia (ODDD) which is an autosomal dominant disease with numerous ocular abnormalities, including glaucomatous phenotypes [14-17]. In glucocorticoid-induced glaucoma, dexamethasone (DEX) can disrupt GJA1-based intercellular communication in the TM cells [18], and the candidate gap junction gene GJA8 (Gene ID: 2703; OMIM: 600897) is associated with cataract [19,20]. However, whether gap junction proteins are functionally expressed in human TM remains largely unknown. Therefore, it is important to examine the expression of gap junction proteins in the TM, and explore the role of gap junctions in regulation of IOP.

In this study, we found that four gap junction genes and proteins, including GJA1, GJA8, GJB6, and GJC1, are robustly expressed in human normal TM cells and tissues. These gap junction channels are also expressed along the trabecular beams. Blockage of the gap junctions in the TM
statistically significantly increases the resistance of AH outflow, and causes an elevation of IOP in mice. The present findings demonstrate the expressional and functional involvement of gap junction channels in regulation of AH outflow and IOP in the TM.

METHODS

Materials: Normal human TM cells were obtained from three healthy human donors that were enucleated within 6 h after death. The cells from two of the donors, named donor 1 and donor 2, were obtained from Lion Eye Bank of United States (Iowa, IA), and the third cell sample was from a Chinese donor (donor 3) from Beijing Tongren Hospital Eye Bank (Beijing). Two human TM tissue samples obtained from Lion Eye Bank of United States were designated as donor 4 and donor 5. Human TM tissues from donor 4 and donor 5 were fixed in 4% paraformaldehyde (Thermo, Waltham, MA) and embedded in optimum cutting temperature (OCT) compound (Sakura, Tokyo, Japan). According to our previous descriptions [21], the normal TM cells were cultured in biopsies and media (MEM-alpha [Gibco, Grand Island, New York], 10% fetal bovine serum [Hyclone, Logan, UT], and Primocin; Invivogen, San Diego, CA) at 37 °C in an incubator with 5% CO₂. The TM cells at passage 5 to 8 were used in this study. Eight-micrometer-thick sections were used for the immunohistochemistry study. The collection protocols for human TM tissues were approved by the Eye Bank Association of America and the Institutional Review Boards of Beijing Tongren Hospital.

Two-month-old male C57/BL6 mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) were used for measurement of IOP and aqueous humor outflow facility. Mice were kept under standard housing conditions with a 12 h:12 h light-dark cycle, controlled humidity of 45–55%, and room temperature of 22–24 °C. All experimental procedures conformed to institutional guidelines for laboratory animal care and use, and were approved by Qingdao University Medical Center.

Real-time PCR (RT–PCR): Total RNAs were extracted from three different normal TM cell samples using TRIzol reagent (Ambion, Austin, TX) and quantified with a NanoPhotometer (Implen, Germany). CDNAs were generated from mRNA through random primed reverse transcription reaction (Promega, Madison, WI). The samples were amplified in triplicate using the SYBR Green system (Bio-Rad, Hercules, CA). The primers for amplification of 21 human gap junction channel genes were designed using Beacon designer V7.9 (Table 1). GAPDH (Gene ID: 2597 OMIM: 138400) was used as a reference gene. PCR reaction was run at 95 °C for 15 min, followed by 50 cycles at 95 °C for 10 s, at 50 °C for 30 s and at 72 °C for 30 s.

Immunohistochemical staining: Human TM cells were grown in poly-D-lysine coated coverslips, and fixed with 4% paraformaldehyde at room temperature for 20 min. The coverslips and the sections from donor 4 and 5 were rinsed in Dulbecco’s PBS (1X; 145 mM NaCl, 8.1 mM Na₂HPO₄·12H₂O, 1.9 mM NaH₂PO₄·2H₂O, PH7.2-7.4; DPBS) for 5 min, and incubated in the blocking solution (DPBS with 1% bovine serum albumin, BSA) for 1 h before further incubation with primary antibodies which were diluted in the blocking solution (1:100) overnight. After rinsing with DPBS, the coverslips and sections were incubated with the corresponding secondary antibodies that were diluted in the DPBS (1:200) for 2 h. The nuclei of the TM cells and the tissues were stained with 4′,6-diamidino-2-phenylindole (DAPI; Santa Cruz, Dallas, TX) at room temperature for 30 min. The coverslips and the sections were mounted using Neutral Balsam (Solarbio, Beijing, China), and imaged with a confocal microscope (Nikon, Tokyo, Japan). The primary antibodies used in this study were rabbit polyclonal LAMA4 antibody (Abcam, Cambridge, MA), rabbit polyclonal TIMP3 antibody (Abcam), rabbit polyclonal GJA1 antibody (Abcam), mouse monoclonal GJC1 antibody (Abcam), rabbit polyclonal GJA8 antibody (Abcam), and mouse monoclonal GJB6 antibody (NOVUS, Littleton, CO). The secondary antibodies were Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG; Invitrogen, Carlsbad, CA) and Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen).

Dexamethasone treatment: For detection of trabecular meshwork–inducible glucocorticoid response, the expression of myocilin protein encoded by the human MYOC gene (Gene ID: 4653 OMIM: 602432) was examined in human TM cells in the presence of 100 nM DEX (Sigma) for 3 days [21], and 0.1% dimethyl sulfoxide (DMSO) was used as vehicle control. Fresh culture media were changed daily. Cell lysates were collected, and myocilin protein expression in TM cells was detected using western blotting.

Western blotting: Proteins from TM cells of three normal individuals were extracted using RIPA (Thermo), and quantified using the BCA Protein Assay Reagent Kit (Thermo). Total proteins (20 µg) were loaded on 5% sodium dodecyl sulfate (SDS)-acrylamide gel and separated with 10% SDS-acrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene fluoride membrane using Mini Trans-Blot Cell (Bio-Rad) under the condition of 150 mA for 1.5 h. The membrane was incubated in blocking buffer (Tris-buffered saline, 5% nonfat dry milk, and 0.1% Tween-20) at room temperature for 1 h before further incubation with primary
antibodies against GJA1 (Abcam), GJC1 (Abcam), GJA8 (Abcam), GJB6 (NOVUS), and myocilin (Abcam) at 4 °C overnight. After rinsing with Tris-Buffered Saline-Tween-20 (TBST) three times, the membrane was incubated with corresponding secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Immunoreactive bands were visualized under chemiluminescent detection with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) in a ChemiDoc XRS+ imaging system (Bio-Rad). Band intensity was determined with Image Lab software (BioRad), and the expression of the gap junction proteins was normalized to the amount of GAPDH. Each data point represents the results of three independent experiments.

Intracameral injection: Mice were sedated with 8% chloral hydrate (0.125 ml/20 kg), and kept on a heating blanket (37 °C) until recovery from the surgery. Gap junction blocker carbenoxolone (CBX; Sigma) or flufenamic acid (FFA; Abcam) at 100 μM was delivered into the anterior chamber of the eye using Hamilton syringes (Hamilton, Reno, NV) [22, 23]. Mice receiving an equal amount of PBS 1X were used as vehicle control.

IOP measurement: The mice were anesthetized with 2.5% isoflurane. After exposure to isoflurane for 5 min, IOP was measured using a rebound tonometer (TonoLab, Helsinki, Finland) as previously described [24]. All measurements were taken between 10 AM and 12 PM.

Trabecular outflow facility measurement: As previously described [24], mice were anesthetized with 8% chloral hydrate (0.125 ml/20 kg), and then cannulated with a 33-gauge needle (Becton Dickenson). About 0.9% saline was pumped into the eye through a 150 μl Hamilton syringe mounted on a computer-controlled syringe pump. The pressure in the system was monitored using a flow-through pressure transducer (Icumedical, San Clemente, CA). Flow rates (microliters per minute) for sustainment of 15, 25, and 35 mmHg were recorded using HemoLab software (Stauss Scientific, Iowa City, IO), and the outflow facility (μl/min/mmHg) was calculated.

Statistical analysis: Data were expressed as the mean ± standard error of the mean (SEM). One-way ANOVA and a t test were used for the statistical analyses among the samples in

| Gene   | Forward primer         | Reverse primer         |
|--------|------------------------|------------------------|
| GJA1   | TGTGGACATGCACCTTGAAGC  | TGTGGCCAGGGATCTCTTTT  |
| GJA3   | ATCATCTTCAAGACGCTTGT  | GTAGATCCACAGATGTGTA   |
| GJA4   | GTGACGACCAATCAGATTTC  | CCGACACGTAAATGACAT    |
| GJA5   | GAAGAAAGATCAGACGAT    | GGACCCATATTATTGCTGA   |
| GJA8   | TCATGTGTCTGTGCTGCTTCT| TGGCTTTCTGGAAGAGAGGAG|
| GJA9   | AGAGTTTGGAATGCGTGGAGG| TAGCGGCTCTAATGAAATC   |
| GJA10  | CGAGTTATGTCGCAATTCAGG| CAAATGAGGAATGCTCAGAG |
| GJB1   | GTGGGGTGATGAGAAATCTT  | TTCTCTATGTGCTGCTGCT  |
| GJB2   | TACACACAAGCAGCATTCTTCT| CAGACACTGCAATCATGAC   |
| GJB3   | CAAGTACTCAGACGCCTTCG| GTCAGCAGATGAGCTCAGAG |
| GJB4   | GTCCCTGTACGAACCTTGA   | CCGGAGATGTAACATGCTCA  |
| GJB5   | CTGGCCTTCTCTTGCTTCTC| TGTCAACAGGATAGCTGCA   |
| GJB6   | CCTGGGTTGGAAAATGTTGG | AACACACTTTCAGCAGAGG  |
| GJB7   | GGGGCTTATGTACGGTATTAT| CGTCTCTACGTTGTTGTGG  |
| GJC1   | GGAAGAAACGGAGAGGAGGCA| CACCTCAACACGGTCTTGG  |
| GJC2   | TCAACACATGGGCTCCTCA  | GTCTCTCTCTCTTCTCTCT  |
| GJC3   | GCCCTCTAGAAAGACATTT  | AGCTTTCTCTTCTGCTGCT  |
| GJD2   | ACACCTGCACCAATATCA   | GCCGGGACATAACATTCC    |
| GJD3   | AGCAACACACAGGAGGAGTC| TCACACATGAGAGCAGAAA  |
| GJD4   | TTCAACGTCTTATGCTCAG  | GAGAGAGAGGCTGATGATG   |
| GJE1   | ATCTTCTCCTCGGGTGTCTA| AAAGACCTCAGGAAACCGAG |
| GAPDH  | GGGTCCACGCTTACGCTTAC| CATTCTCGCCTTTCCTGCTG |
this study. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Characterization of human TM cells: Human normal TM cells were isolated from three human individual donors and cultured in biopsy medium with a spindle-like morphology (Figure 1A). Because isolated TM cells after eight passages begin to display numerous senescence properties [4], we used the TM cells from passages 5 to 8 in this study, after their viability was confirmed. Immunohistochemistry staining revealed that the isolated TM cells from three individual donors exhibited robust expression of biomarkers such as laminin alpha 4 (LAMA4) and tissue inhibitor of metalloproteinase 3 (TIMP3) proteins (Figure 1B). We also examined the expression of another biomarker myocilin in the TM cells after DEX treatment (Figure 1C). Western analysis revealed that the expression level of the myocilin proteins in the TM cells was statistically significantly increased after DEX treatment (Figure 1D). These morphological and functional observations demonstrate that the human primary TM cells used in this study feature endogenous TM cells.

Robust expression of gap junction genes in human TM cells: For detection of gene expression in normal TM cells, transcriptional levels of 21 human gap junction genes in the TM cells were screened and amplified with quantitative detection of the Ct (cycle threshold) using real-time PCR. The Ct levels are inversely proportional to the amount of the target genes in the samples. As shown in Figure 2A, the mean cycle threshold Ct values of five gap junction channel genes, $GJA1$, $GJA8$, $GJB6$ (Gene ID: 10804; OMIM: 604418), $GJC1$ (Gene ID: 10052; OMIM: 608655) and $GJD4$ (Gene ID: 219770; OMIM: 611922) were 19, 26, 25, 25, and 27, respectively, indicating their abundant gene expression, compared with the Ct value for the $GAPDH$ control. Based on the Ct value of $GAPDH$, the mRNA expression levels of $GJA1$, $GJA8$, $GJB6$, $GJC1$, and $GJD4$ were normalized. As shown in Figure 2B, the expression of the $GJA1$ gene, encoding the gap junction alpha-1 protein (also known as connexin 43, Cx43), from three donors was statistically significantly higher than that

![Figure 1](image_url)

Figure 1. Morphological and functional characterization of normal human TM cells from donors. A: A spindle-like morphology is shown in the isolated trabecular meshwork (TM) cells from donor 1 through donor 3. B: Positive staining of biomarkers laminin alpha 4 (LAMA4; red) and tissue inhibitor of metalloproteinase 3 (TIMP3; red) for TM cells at passage 5. Cell nuclei were labeled in 4′,6-diamidino-2-phenylindole (DAPI, blue). Bar = 50 µm. C: Immunoreactive bands of myocilin proteins were visualized. GAPDH was used as the reference protein. D: The intensity of the visualized bands in the control and dexamethasone (DEX)-treated cells from each donor. Data were quantified from three independent experiments (n=3). * p≤0.05, compared with control.
of the other gap junction channel genes in the human TM cells. The expression of the \textit{GJA8} (encoding for the gap junction alpha-8 protein) and \textit{GJC1} (encoding for the gap junction gamma-1 protein) genes was also relatively higher (Figure 2B–D). In donor 3, the expression of the \textit{GJB6} gene (encoding for the gap junction beta-6 protein, also known as connexin 30) was higher than that of the \textit{GJA8}, \textit{GJC1}, and \textit{GJD4} genes. To confirm the gene expression, the \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} proteins were further examined using western blot assay. Immunoreactive bands for the \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} proteins were visualized in the TM cells from all three donors (Figure 3A). As shown in Figure 3B–D, quantification analysis of band intensity with densitometry indicated that the expression of the \textit{GJA8} and \textit{GJA1} proteins was statistically significantly higher than that of \textit{GJB6} and \textit{GJC1}, suggesting their important roles in maintaining the integrity of the TM structure and function in vitro.

**Cellular localization of \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, \textit{and \textit{GJC1}} in the TM cells and tissues:** To further determine the localization of the \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} proteins in the TM cells, immunohistochemistry staining was performed in fixed TM cells. As shown in Figure 4, \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} from three donors could be detected on the membranes of the TM cells, especially the cell membranes between adjoining cells. The \textit{GJA1} protein was the most abundant on the membrane among the gap junction proteins expressed in the TM cells. The \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} proteins were expressed at a lower level than \textit{GJA1}. To further examine the localization of gap junction channels, we also performed immunohistochemistry staining of the \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} proteins in human TM tissues. As shown in Figure 5, the \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} proteins from donor 4 were robustly stained in the iridocorneal angle, iris, and ciliary body within the anterior segment. As seen in the high magnification images in the upright panels, \textit{GJA1}, \textit{GJA8}, \textit{GJB6}, and \textit{GJC1} were strongly stained along the trabecular beams in all three layers of the TM tissues. \textit{GJA1} showed the highest expression in the TM tissue, compared to the three
other gap junction proteins. To further confirm these results, tissue from another human donor eye (donor 5) was collected, and stained immunohistochemically for localization of gap junction proteins. As shown in Fig. S1, similar localization of GJA1, GJA8, GJB6, and GJC1 in the TM tissue was observed. These results demonstrate that GJA1 is the major gap junction protein likely responsible for cellular communications in vivo.

Increased resistance of aqueous humor outflow by pharmacological inhibition of gap junctions in the TM: To explore the functional role of gap junctions in regulation of AH outflow, two gap junction channel blockers, CBX and FFA, were individually injected into the anterior chamber of the C57/BL6 mice. As shown in Figure 6A, the outflow facility after FFA treatment was statistically significantly decreased with a value of 0.0055 μl/min/mmHg, compared to the vehicle control (0.0087 μl/min/mmHg, p=0.013). A trend of decreased outflow facility was also observed in the mice treated with CBX, although there was no statistical difference between the PBS group and the CBX group.

We also measured the IOP daily after the injection of the gap junction blockers. In the vehicle control group of mice, an average IOP with a value of 15.77±1.770 mmHg (n=28) was obtained (Figure 6B). In contrast, a single intracameral injection of gap junction blocker CBX or FFA at 100 µM resulted in a statistically significant elevation in the IOP with values of 17.91±4.480 mmHg (n=15, p=0.03) and 17.75±4.510 mmHg (n=19, p=0.04), respectively (Figure 6B). These results suggest that gap junctions in the TM may play an important role in regulation of AH outflow and subsequent IOP.

DISCUSSION
The TM tissue with resident cells along the trabecular beams in the iridocorneal angle is crucial in regulating AH drainage [3]. Previous studies have suggested that intercellular communication maintained by gap junctions between adjacent TM cells is crucial in regulation of AH outflow [10]. Mutations in GJA1 have been reported to cause ODDD with the open angle glaucomatous phenotype [25,26]. Mice harboring G60S in GJA1 have been shown to be pathologically relevant to glaucoma [27]. Previous published data indicated that disruption of the GJA1-based intercellular junction is relevant to the
TM functions in glucocorticoid-induced glaucoma [18]. The goal of the present study was to directly explore the roles of gap junctions in regulation of AH outflow. The functional tests in this study were performed after the existence of gap junction proteins in the TM tissue was confirmed.

In this study, we showed that four gap junction channel proteins (GJA1, GJA8, GJB6, and GJC1) are expressed in human TM cells and tissues, from several human eye donors. Although this is a small number of samples, and a larger collection of human eye donors is needed, the present investigation showed that among the gap junctions, GJA1 is robustly expressed at either the transcriptional or translational level, suggesting an important role in the TM. It has been reported that different gap junction subunits display different selectivity for small molecules in intercellular communication [28], and GJA1 can regulate the transfer of glucose,
glutamate, glutathione, adenosine, and ADP more efficiently than other gap junction proteins [29]. This robust expression of the GJA1 gap junction subunit in the TM supports the notion that glucose, glutamate, glutathione, adenosine, and ADP might be the necessary components for maintaining the role of the TM in regulating AH outflow [30] and IOP [31].

In the eye, the increased AH production or reduced AH outflow can induce an elevation of IOP [1,3]. The present data demonstrate that intracameral injection of gap junction blockers can cause a statistically significant elevation of IOP. Loss of connexin 43 has been determined to result in a chronic reduction of IOP [32-34]. In the present study, the acute elevation in the IOP by the gap junction blockers likely resulted from the increased resistance of AH outflow. To confirm this, the resistance of AH drainage was evaluated through measuring the outflow facility in the blocker-treated and control mice. When increased IOP was observed in the treated mice, decreased outflow facility was also detected. These observations indicate the important roles of gap junction channels in the TM for maintenance of regular AH outflow in the eye, and pharmacological regulation of gap

Figure 5. Immunohistochemistry staining of the GJA1, GJA8, GJB6, and GJC1 proteins in human anterior segment tissues. GJA1 (red), GJA8 (red), GJB6 (green), and GJC1 (green) are localized in the iris, ciliary body, and the trabecular meshwork with immunohistochemistry (IHC) staining. In the high-magnification images (upright panels), positive staining is shown along the trabecular beams. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI; blue). Bar = 50 µm.

Figure 6. Decrease in AH outflow facility and an IOP elevation in mice treated with CBX or FFA. A: Outflow facilities were measured in mice at 5 days after injection. Compared to the average value of the outflow facility in vehicle control, decreased outflow facility was observed after carbenoxolone (CBX) or flufenamic acid (FFA) treatment. * p≤0.05. B: Intraocular pressure (IOP) was measured every day after intracameral injection of CBX or FFA. Compared to the IOP in the control mice (n=28), the IOP was increased statistically significantly after treatment with either CBX (n=15) or FFA (n=19).
junction function may represent a potential therapy for treatment of glaucoma.

Regulation of the TM function is likely involved in gap junction-based transfers of small cytoplasmic molecules between adjacent TM cells. These events can trigger complicated downstream effects, including TM cell growth [10,35,36] and cytoskeleton change [18], which are important for regulating AH outflow. Intercellular communications through hemichannels formed by gap junction proteins have been shown to play important roles in regulating the cell physiology through intracellular signaling. For example, the GJA3-formed hemichannel has been shown to be an important regulator for ATP release in oocytes [11]. In human TM cells, the connexin-formed hemichannel has also been demonstrated to be actively involved in ATP release [8,37], which is important for MMP2 release, and reduction in the outflow resistance of the TM [8,38]. The immunohistochemical analysis and functional tests in the present study suggested that increased resistance of AH drainage in the treated mice might also be caused by the insufficient ATP release through the hemichannels in the TM cells.

It is of interest that inhibition of gap junctions by the blockers FFA and CBX can statistically significantly decrease AH outflow and increase IOP. FFA displays stronger effects on increasing outflow resistance than CBX. FFA and CBX have been reported to be common blockers for inhibiting gap junction and hemichannel activity [37]. FFA has been reported to be a potent inhibitor for blocking gap junction and hemichannel activity, but with minimal effects on the pannexin channel [39,40]. Nonetheless, CBX exhibits a substantially greater affinity for pannexin channel inhibition than connexin hemichannel inhibition [40]. FFA and CBX are commonly used gap junction antagonists, although they can affect other targets [41]. FFA is widely used for non-selective cation, chloride, potassium, calcium, and sodium channels. Among these channels, TRPV4 is a well-studied ligand-gated ion channel that is involved in regulation of IOP, and inhibition of TRPV4 can enhance the outflow facility and lower IOP in a mouse glaucoma model [42]. CBX has been reported to block calcium channels, p2×7 receptors, and NMDA-evoked currents [43], and the calcium channel blocker decreases IOP [44]. These observations suggest that other ion channel targets in the TM are also likely involved in the regulation of AH drainage.

In summary, robust expression of the GJA1, GJA8, GJB6, and GJC1 gap junction proteins was observed on the membranes between adjacent cells in normal human TM cells. Among the four gap junctions, GJA1 and GJA8 were the most abundant expressed along the trabecular beams in human TM tissues. Pharmacological inhibition of gap junctions statistically significantly decreased the outflow facility, and induced an elevation of intraocular pressure in mice. The present results highlight the therapeutic potential of enhancing gap junction function for treatment of glaucoma, through lowering of intraocular pressure in the eye.

APPENDIX 1. IMMUNOHISTOCHEMISTRY STAINING OF GJA1, GJA8, GJB6 AND GJC1 PROTEINS IN HUMAN ANTERIOR SEGMENT TISSUE FROM DONOR 5.

Gap junction proteins GJA1 (red), GJA8 (red), GJB6 (green) and GJC1 (green) are localized in trabecular meshwork in IHC staining. Positive staining is shown along the trabecular beams. Cell nuclei were stained with DAPI (blue). AC: anterior chamber, SC: schlemm's canal. Bar=50 µm. To access the data, click or select the words "Appendix 1."

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