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Glaucomatous MYOC mutations activate the IL-1/NF-κB inflammatory stress response and the glaucoma marker SELE in trabecular meshwork cells

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Purpose: Activation of the IL-1/NF-κB inflammatory stress pathway and induction of SELE expression in the trabecular meshwork (TBM) is a marker for high-tension glaucomas of diverse etiology. Pathway activation stimulates aqueous outflow and protects against oxidative stress, but may be damaging in the long-term. MYOC mutations have been causally linked to high-tension forms of primary open angle glaucoma (POAG). This study investigated a possible link between MYOC mutations and activation of the IL-1/NF-κB pathway and expression of SELE.
Methods: We constructed MYOC expression vectors with mutations at sites that cause POAG. Mutations (Q368X, Y437H, A427T) were selected to represent proteins with differing POAG-causing potency (Q368X > Y437H > A427T) and intracellular retention behavior (Q368X and Y437H retained, A427T released). The constructs were made in two different kinds of vectors; one a plasmid designed for transient transfection (pCMV6), and one a doxycycline-inducible lentiviral vector (pSLIK) for stable cell transduction. The immortalized human trabecular meshwork line TM-1 was used for all expression studies. Expression of IL1A mRNA was determined by reverse transcription (RT)–PCR, as well as a set of five other genes associated with signaling pathways linked to glaucoma: IL1B and IL6 (NF-κB pathway), TGFB2 and ACTA2 (TGF-β pathway) and FOXO1 (E2F1 apoptotic pathway). An ELISA was used to quantify IL1A protein released into culture media. To quantify intracellular NF-κB activity, we transiently transfected stably transduced cell lines with a luciferase expression vector under control of the IL8 promoter (containing an NF-κB response element).
Results: Transiently expressed wild-type MYOC was released into cell culture media, whereas mutant MYOCs Q368X and Y437H remained within cells. Both mutant MYOCs activated the IL-1/ NF-κB pathway, significantly stimulating expression of IL1A and IL1B. However Y437H, which causes a severe glaucoma phenotype, was less effective than Q368X, which causes a moderate glaucoma phenotype. In addition, the retained mutants stimulated expression of stress response genes ACTA2 and FOXO1. Unexpectedly, wild-type MYOC significantly *decreased* expression of IL6 and TGFB2, to approximately half of the control levels, and expression of IL1B and ACTA2 was also slightly decreased. Induction of MYOC mutants Q368X and Y437H in stably transduced cell lines significantly stimulated the level of IL1A protein released into culture media. Once again however, the effect of the severe MYOC mutant Y437H was less than the effect of the moderate MYOC mutant Q368X. In contrast, induced expression of the intracellularly retained mutant MYOC A427T or wild-type MYOC did not change the amount of IL1A protein in culture media. Induction of Y437H MYOC plus IL1A treatment increased NF-κB activity by 25% over IL1A alone. In contrast, induction of Q368X or A427T plus IL1A treatment did not significantly affect NF-κB activity over IL1A alone. However, wild-type MYOC expression inhibited IL1A-stimulated NF-κB activity. We also observed that endogenous MYOC expression was induced by IL1A in TM-1 cells and primary TBM cell cultures. SELE was co-expressed with MYOC in the primary cell lines.
Conclusions: These results indicate that POAG-causing MYOC mutants activate the IL-1/NF-κB pathway, with activation levels correlated with intracellular retention of the protein, but not POAG-causing potency. Unexpectedly, it was also discovered that wild-type MYOC inhibits activation of the IL-1/NF-κB pathway, and that activation of the IL-1/NF-κB pathway stimulates expression of MYOC. This is the first evidence that glaucoma-causing MYOC mutants can activate the inflammatory response and that wild-type MYOC has anti-inflammatory activity.

Glaucoma is the 3rd most prevalent cause of visual impairment and blindness among white Americans, and the leading cause among black Americans [1,2]. All forms of glaucoma have in common optic nerve degeneration characterized by typical visual field defects. Elevated intraocular pressure (IOP) is the major risk factor, and lowering IOP is the only proven treatment [3]. Many patients remain refractory to existing IOP-lowering drugs and eventually may

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become blind. Additional mechanistic information is needed to identify new targets for disease intervention.

Elevated IOP, also known as ocular hypertension, results from impaired drainage of aqueous humor through the TBM and Schlemm's canal [3]. The defect that causes primary open angle glaucoma (POAG) is at the cell and tissue level, and is influenced by genetic risk factors, the process of aging and environmental or physiologic stress [4-13]. Tissue changes include loss of TBM cells, collapse of trabecular beams, and accumulation of extracellular material [5,14,15]. Our team identified expression of the inflammatory marker endothelial leukocyte adhesion molecule-1 (ELAM-1), also known as E-selectin (SELE), as a defining feature of the diseased phenotype of the TBM in both open and closed angle forms of high-tension glaucoma of a variety of etiologies [16]. We further determined that the IL-1/NF-κB inflammatory stress response activates SELE expression, and we demonstrated the cytoprotective role of this response.

Interleukin-1 (IL-1) is a cytokine that had previously been demonstrated to lower the intraocular pressure (IOP) in rat, rabbit, and human models [17,18]. This may occur through stimulation of matrix metalloproteinase (MMP) expression [18,19], or by directly increasing paracellular permeability across Schlemm's canal [20]. However, we proposed that the IL1/NF-kappaB stress response would lead to the pathological hallmarks of glaucomatous trabecular meshwork if chronically activated. These findings and conclusions have been replicated and extended by other laboratories [7,8,11,21-25]. Chronic, low-grade inflammation has been suggested as an underlying mechanism linking major age-related diseases such as atherosclerosis, arthritis, osteoporosis, and cardiovascular diseases [26-28]. Inhibition of the proinflammatory mediators associated with aging and environmental stress delays the progression of several age-related pathologies [26,29]. The observed activation of a chronic inflammatory stress response in the TBM deserves special attention, as it could lead to new therapeutic approaches.

MYOC, encoding the glycoprotein called myocilin, was the first gene linked to familial forms of primary open angle glaucoma (POAG) [30]. Up to 70 different disease-causing MYOC mutations have been described, and it has been estimated that "myocilin glaucoma" accounts for 2%–4% of all cases of POAG [31-34]. MYOC has three exons and encodes a predicted 504 amino acid polypeptide containing two major homology domains: an N-terminal myosin-like domain and a C-terminal olfactomedin-like domain [34]. The majority of MYOC disease-causing variations are clustered in the olfactomedin homology domain of the third exon. Very recently, the crystal structure of the olfactomedin domain of myocilin was solved [35]. This reveals it to be a new addition to the small family of five-bladed beta-propellers, known for their ability to act as hubs for protein–protein interactions. Interacting partners identified thus far include Flotilin-1, a membrane-bound protein involved in vesicular trafficking [36], and optimedin, an olfactomedin-related extracellular protein [37]. The implications of these interactions for function remain unclear [38]. Haploinsufficiency has been excluded as the primary mechanism for POAG [39] and genetically increasing or decreasing wild-type MYOC expression also does not induce POAG [40,41]. Therefore, a gain-of-function disease mechanism has been hypothesized [42-44].

Although wild-type MYOC lacks the signal peptide sequence for routing into the classical secretion pathway, the protein is predominantly localized extracellular. One possibility is that export occurs on the surface of exosomes [45]. Most mutant MYOC forms are misfolded and aggregate in the endoplasmic reticulum, activating the unfolded protein response, a stress pathway [46,47]. Significantly, this represents a gain-of-function. It should be recognized however, that some glaucoma-causing mutations do not cause intracellular retention of MYOC protein. To account for this, an alternative mechanism has been suggested that relies on abnormal interactions of the secreted mutants with the extracellular matrix and/or cell/surface elements [48].

Recently, interest in the role of low-grade inflammation in glaucoma has been re-emerging [49]. In this study, we investigated a possible mechanistic connection between myocilin glaucoma and activation of the IL-1/NF-κB stress pathway. We hypothesized that mutant MYOC proteins would activate the IL-1/NF-κB pathway in proportion to the aggressiveness of their disease phenotype. To investigate this idea, we selected MYOC mutants that produce proteins with a range of POAG-causing potency (Q368X > Y437H > A427T) and intracellular retention behavior (Q368X and Y437H retained, A427T released).

METHODS

Cells: Throughout the text, HUGO nomenclature is used for genes and their products. Cells of the immortalized human TBM line, TM-1 [50], were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Atlanta Biologicals, Inc., Norcross, GA), 2 mM L-glutamine, 2.5 ug/ml amphotericin B, and 25 ug/ml gentamicin. Primary human TBM cells were prepared from corneal rims discarded at the time of surgical transplantation of donor corneas, as described [51,52]. Briefly, TBM tissue was dissected from the corneal rim under a dissecting microscope and transferred to

Table 1. Oligonucleotides used in this study

Site-Specific Mutagenesis		
MYOC mutation	**Direction**	**Oligonucleotide sequence (3′)**
Y437H	Sense	ATCATCTGTGGCACCTTGCACACCGTCAGCAGCTACACCT
	Antisense	AGGTGTAGCTGCTGACGGTGTGCAAGGTGCCACAGATGAT
Q368X	Sense	CCTGGAGCTGGCTACCACGGATAGTTCCCGTATTCTTGGG
	Antisense	CCCAAGAATACGGGAACTATCCGTGGTAGCCAGCTCCAGG
A427T	Sense	ATCCGTAAGCAGTCAGTCACCAATGCCTTCATCATCTGTG
	Antisense	CACAGATGATGAAGGCATTGGTGACTGACTGCTTACGGAT
RT–PCR		
Gene	**Direction**	**Oligonucleotide sequence (5’-3′)**
IL1A	Sense	ATCAGTACCTCACGGCTGCT
	Antisense	TGGGTATCCCAGGCATCTCC
IL1B	Sense	CTGAAAGCTCTCCACCTCCA
	Antisense	TCATCTTTCAACACGCAGGA
IL6	Sense	CACACAGACAGCCACTCACC
	Antisense	TTTCACCAGGCAAGTCTCCT
TGFB2	Sense	CGCCAAGGAGGTTTACAAAA
	Antisense	TGCTGAGACGTCAAATCGAA
FOXO1	Sense	AAGAGCGTGCCCTACTTCAA
	Antisense	CACCCTCTGGATTGAGCATC
ACTA2	Sense	ATGCCTCTGGACGTACAACTG
	Antisense	CGGCAGTAGTCACGAAGGAAT

wells of a 48 well plate containing DMEM with 10% FBS. A coverslip was placed over the tissue and the primary cells were allowed to grow out onto the bottom of the well over a period of several weeks. Once a confluent cell layer was obtained, cells were removed from the plate using trypsin and reseeded into the wells of a 6 well plate. After expansion, the identity of the cells was confirmed by morphology, by expression of the TBM cell marker AQP1, and by induction of the TBM cell marker MYOC after treatment with dexamethasone for 10 days. Primary TBM cells were used for experiments up to passage 8.

Expression constructs and transient expression: Wild-type MYOC cDNA cloned in the plasmid expression vector pCMV6 was purchased from Origene (Rockville, MD). In this vector, the inserted gene is placed under control of the strong, constitutive cytomegalovirus promoter. Mutations were created at sites that cause POAG (Q368X, A427T and Y437H) using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The oligonucleotides used for mutagenesis (Table 1) were designed according to the manufacturer’s protocol and purchased from Integrated DNA Technologies (Coralville, IA). The nucleotide changes were confirmed by sequencing (GENEWIZ, Newbury Park, CA), from both the 5′ and 3′ sides of the target DNAs.

For expression, TM-1 cells were transfected with MYOC expression constructs (1 µg) using Lipofectamine® LTX with Plus™ Reagent (Life Technologies, Carlsbad, CA), as per supplier’s instructions. Post-transfection, the cells were kept in complete medium overnight before collection of cells for further analysis.

Lentivirus constructs and stably transduced cell lines: Lentiviral expression vectors were created, viral particles were produced, and cell infection was performed as described [53]. Briefly, full-length wild-type MYOC and mutant MYOC cDNAs were amplified by PCR using high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA) and inserted into pre-lentiviral vector pENTT_mcs downstream of the Tetracycline Response Element (TRE). Then the target genes linked to the TRE were transferred into the lentiviral vector pSLIK-hygro by in vitro recombination (Invitrogen, Carlsbad, CA). For packaging, the lentiviral expression plasmids were co-transfected into HEK293T cells along with helper plasmids pMD.G1 and pCMVR8.91. Culture media containing viral particles were harvested after 48–72

h and used to infect immortalized TM-1 cells in the presence of 8 ug/ml Polybrene (Millipore Corp., Bedford, MA). Cell cultures were infected at a low multiplicity of infection (MOI) to ensure <30% infection frequency. Cells stably transduced with lentiviral expression vectors were selected by treating the cultures with 150 µg/ml Hygromycin (Invitrogen, Carlsbad, CA) for 3 weeks.

For induction of wild-type and mutant MYOCs, cells of stably transduced lines were seeded in 6 well plates at 200,000 cells per well. To induce the transgene, cells were then treated with doxycycline (100–1000 ug/ml) for up to 7 days. A stable cell line transduced with empty pSLIK vector was created to serve as a negative control.

Reverse transcription (RT)- PCR: Custom oligonucleotides primers (Table 1) were purchased from Integrated DNA Technologies (Coralville, IA). Total RNA was isolated from transfected TM-1 cell using TRIzol® reagent (Life Technologies). Real-time RT–PCR analysis was performed using the iScript one-step RT–PCR kit with SYBR Green (Bio-Rad, Hercules, CA) on an ABI PRISM 7900 HT sequence detection system (Life Technologies) according to the manufacturer's instructions. The cycling parameters were set at 95 °C for 10 s (denature), 58 °C for 10 s (annealing) and 72 °C for 30 s (extension) and 40 cycles were performed. Relative quantification was calculated as $2^{-\Delta\Delta Ct}$ by the comparative Ct method.

Western blotting: Cell-conditioned culture media were harvested and clarified by centrifugation. Cells were scraped from the 6-well plate in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) suspended in 100 µl of radioimmunoprecipitation assay buffer (RIPA buffer: Tris 50 mM, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) and protease inhibitors cocktail (Roche, Pleasanton, CA), then lysates were clarified by centrifugation. Proteins in the supernatants were separated by SDS–PAGE and transferred to PVDF membranes.

Antibodies to MYOC were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and to SELE (CD62E) and ACTB from Abcam (Cambridge, UK). The PVDF membranes were incubated with one of these primary antibodies with dilution of 1:2000 (mouse monoclonal MYOC antibody), 1:1000 (rabbit polyclonal SELE antibody) and 1:10,000 (mouse monoclonal ACTB antibody) overnight at 4 °C with gentle shaking, following the manufacturer's instructions. The membranes were then incubated with secondary antibody–horseradish peroxidase conjugates from Santa Cruz Biotechnology (Santa Cruz, CA) with dilution of 1:10,000 for 1 h. Specific signals were developed using the ECL kit components 1 and 2 (GE Healthcare UK Limited, Buckinghamshire, UK) for 1 min. Antibody binding to proteins on the membranes was visualized by chemiluminescence exposure of photographic film (LAS-4000; Fujifilm, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA): Wild-type and mutant MYOC inducible cell lines were plated in 6-well plates at 200,000 cells per well in medium with 10% serum to promote cell proliferation. When cells in each well attained confluence, the medium was changed to serum-free and cells were left untreated or treated with doxycycline. Subsequently, media were changed every 2 days. Cell conditioned media were saved at 4 days and 6 days for quantification of cytokines by ELISA. Samples of conditioned media were concentrated by adding trichloroacetic acid to 10% of the total volume. Proteins from 1 ml of each media sample were dissolved in 50 ul of PBS for ELISA (Cayman Chemical Company, Ann Arbor, MI). The protocol and calculation were performed according to manufacturer's manual. At 6 days, cell lysates were prepared western blotting.

Luciferase assay: A luciferase expression construct driven by the IL8 promoter (pIL8/luc) was a kind gift from Dr. Ebrahim Zandi (University of Southern California). Doxycycline-inducible MYOC cell lines were plated in 6-well plates at 70% confluence and co-transfected with pIL8/luc at 2 ug/well and a control pRL-TK renilla expression construct (Promega, Madison, WI) at 1.25 ug/well using Lipofectamin LTX with Plus Reagent (Life Technologies). After transfection, cells were incubated in complete media overnight, during which time they reached confluence. Then cells were transferred to serum-free medium and treated with IL1 alpha, with or without doxycycline. After 72 h, the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to passively process (without scraping) the cells according to manufacturer's manual, and then both renilla and luciferase activity was read using a VICTOR3V plate reader (PerkinElmer, Shelton, CT).

Statistical analysis: Results are expressed as the mean ± standard error of the mean (S.E.) The significance of differences in mean values between untreated and treated samples was determined by the Student *t* test after determining that the data fit a normal distribution. $p \leq 0.05$ was considered statistically significant.

RESULTS

Transient expression of MYOC mutants: We hypothesized that the misfolding of mutant myocilin would create stress and activate the NF-kappaB pathway, resulting in production of inflammatory cytokines and expression of SELE. To investigate this idea, we created wild-type and mutant MYOC expression constructs. We chose two MYOC mutations

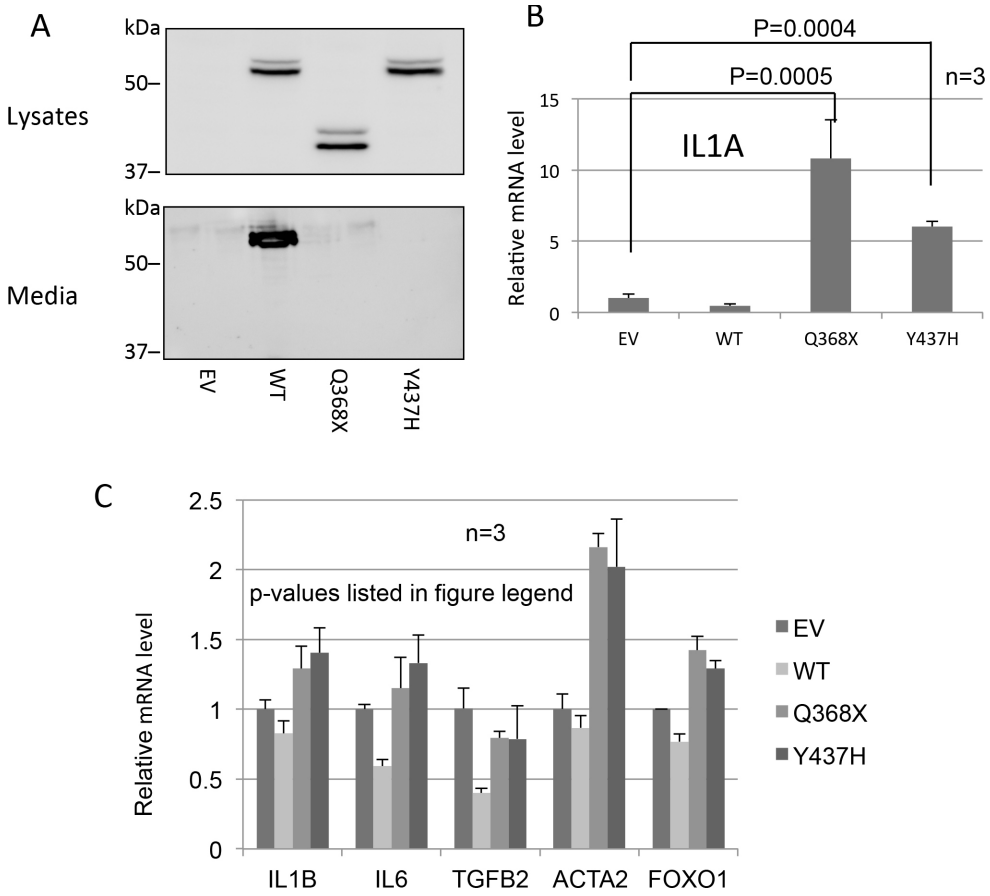

Figure 1. Effects of MYOC Mutations on Expression of IL1A and other Genes Associated with Glaucoma in Transient Transfections Cultures of TM-1 cells were transiently transfected with pCMV6 expression constructs containing wild-type (WT) or mutant (Q368X, Y437H) MYOC genes. A set of cells was also transfected with empty vector (EV) control for comparison. After 24 h incubation, cell lysates were collected for western blotting or for extraction of RNA and RT–PCR analysis. **A**: Lanes of an SDS-polyacrylamide gel were equally loaded with aliquots of each sample (20 ug) and proteins were separated by electrophoresis. Western blotting was performed with anti-MYOC antibody. The electrophoretic mobility of molecular weight size standards is indicated. **B**: Equal aliquots of total RNA were analyzed by qRT-PCR for IL1A mRNA, and normalized to ACTB, which served as an internal control. Results obtained using MYOC expression constructs were compared to EV controls. **C**: Equal aliquots of total RNA described in (B) were also analyzed by real-time reverse transcription-polymerase chain reaction (RT–PCR) for IL1B, IL6, TGFB2, ACTA2 and FOXO1 mRNAs, and normalized to ACTB, which served as an internal control. Results obtained using MYOC expression constructs were compared to EV controls. Mutant MYOC overexpression increased mRNA levels for IL1B (Q368X*), ACTA2 (Q368X** and Y437H*) and FOXO1 (Q368X** and Y437H**); wild-type MYOC overexpression decreased mRNA levels for IL1B* and IL6*, TGFB2* and FOXO1**; *p<0.05; **p<0.001; n=3.

located in the olfactomedin-like domain of exon 3 that cause different glaucoma phenotypes [54] correlated with their differing solubility and cell retention characteristics [48]. Y437H is associated with severe juvenile POAG, exhibiting a mean maximum IOP of 43.7 mmHg, a mean age at diagnosis of 19.9 years and age of youngest diagnosis as 4 years. Q368X is associated with juvenile and adult-onset POAG and has more moderate characteristics, with a mean maximum IOP of 21.1 mmHg, a mean age at diagnosis of 37.8 years and age of youngest diagnosis as 15.1 years. The protein products of both mutants are insoluble. When expressed in cultured cells, the proteins produced by both mutants are retained within the cell.

Figure 1 shows representative results of transient transfection experiment in which cell lysates and conditioned cell culture medium were collected for analysis after 72 h in cells of the immortalized cell line TM-1 cells. Wild-type MYOC protein was found entirely in cell culture medium, while mutant proteins produced by MYOCs Q368X and Y437H were expressed and retained entirely in the cell, as revealed by western blot analysis (Figure 1A). RT–PCR analysis revealed that the level of IL1A mRNA was significantly increased in cells transfected with these two mutant constructs as compared to cells transfected with vector alone (Figure 1B). This result is consistent with our original hypothesis. However, the Y437H MYOC mutant, that causes a severe glaucoma phenotype, was less effective than the Q368X MYOC mutant that causes a moderate glaucoma phenotype. Thus IL1A was increased more than 10 fold in cells transfected with MYOC Q368X and was increased by sixfold in in cells transfected with MYOC Y437H. Interestingly, IL1A mRNA was decreased in cells transfected with wild-type MYOC, however this decrease did not attain statistical significance.

For comparison, we also examined the effects of transient transfection with the MYOC constructs on expression of a set of five other stress response genes associated with glaucoma: IL1B and IL6 (NF-κB pathway), TGFB2 and ACTA2 (TGF-β pathway), and FOXO1 (oxidative stress; Figure 1C). Again consistent with our hypothesis, as well as with the finding for IL1A, cells transfected with mutant MYOCs significantly increased expression of all five cellular stress response genes. In this case, the effect of the two different MYOC mutations was not significantly different. Intriguingly, cells transfected with wild-type MYOC significantly decreased expression of IL1B, IL6, TGFB2 and FOXO1. This finding provided an initial suggestion that wild-type MYOC overexpression might affect cellular stress response pathways, the including NF-κB pathway.

Inducible expression of MYOC mutants in stable cell lines: Several groups have shown that transient expression of mutant MYOC forms causes cellular toxicity [47,55-57] and leads to cell death with long exposure [56]. This was also observed in our experiments (data not shown). Since this could be an independent factor in the observed changes in gene expression, we sought an approach that would reduce such toxicity by allowing us to control the level of mutant MYOC expression, and the length of time expressed. For this, we chose to create stably transduced TM-1 cell lines harboring wild-type or mutant MYOCs cloned into a lentivirus vector, placing expression under control of a doxycycline inducible promoter. We created such lentiviral vectors expressing wild-type MYOC and MYOCs Y437H and Q368X, the two mutants discussed above. For further comparison, we also created lentiviral vectors expressing MYOC A427T, a mutant that produces a mild, very late adult-onset POAG phenotype [58], and is and a mild variant from a protein stability/aggregation standpoint [59]. The mean maximum IOP for this mutant is 22 mmHg, the mean age at diagnosis is 61 years, and the age of youngest diagnosis is 46 years. Importantly, the protein product is soluble and does not accumulate intracellularly.

In several different experiments, cells were incubated with various doses (100–1000 ng/ml) of doxycycline in serum-free media and cell morphology was observed. Cells treated in this way appeared completely unaffected for up to 7 days. These results are consistent with another report in which stable, inducible cell lines were employed [60].

Figure 2 shows representative results examining expression of wild-type and mutant MYOCs in the stably transduced cell lines. Expression of the transgenes was induced by doxycycline in a dose-dependent manner, and the protein products were easily detectable by western blotting. As already demonstrated in transient transfection experiments described above, mutant proteins produced by MYOCs Q368X and Y437H were found almost entirely in cell lysates, while wild-type MYOC was found entirely in the cell culture media. In addition, and as predicted, the mutant protein produced by MYOC A427T was found almost entirely in the cell culture medium.

Having established a model of mutant MYOC expression without apparent toxicity, we then re-examined the question of MYOC effects on IL1A expression. In this case, we elected to measure IL1A protein released into cell culture media by ELISA. Figure 3 shows representative results. Results were similar to those obtained in transient expression experiments and were consistent with our hypothesis. Thus, doxycycline induction (1 ug/ml) of cell lines harboring the MYOC mutants Q368X and Y437H increased the amount of IL1A protein released into culture media by ~25-fold ($p<0.0007$) and 10 fold ($p<0.002$), respectively. Once again however, the effect of the severe MYOC mutation Y437H was less than the effect of the moderate MYOC mutation Q368X. On the other hand, induction of cell lines harboring the mutant MYOC A427T or wild-type MYOC transgenes did not discernably change the amount of IL1A protein released into the culture media.

We next asked whether induction of the mutant MYOC transgenes would also increase NF-kappaB activity. A representative experiment is shown in Figure 4. The four MYOC-inducible cell lines were transfected with a luciferase reporter construct driven by the NF-kappaB-inducible transcriptional promoter for IL8 (IL8/luc) to enable quantification of NF-kappaB activity. Cells were then treated with doxycycline at 100 or 500 ng/ml to induce the MYOC transgenes, one set of cells being left untreated as a control. Parallel cultures were also treated with IL1A protein (10 ng/ml) as a positive control. After 72 h, luciferase activity was measured.

As expected, addition of IL1A stimulated NF-kappaB activity in all four lines in which transgenes were not induced with doxycycline. Induced expression (at both 100 and 500 ng/ml) of MYOC Y437H significantly increased NF-kappaB activity. Doxycycline induction of MYOC Y437H in cells co-treated with IL1A also significantly increased NF-kappaB activity by 25% over IL1A alone ($p<0.05$). Induced expression of MYOC Q368X caused the highest elevation of NF-kappaB activity, as was observed for IL1A expression. However the results did not attain statistical significance, likely because the error bars were large.

The mild glaucoma-causing mutant A427T did not have a discernable effect on NF-kappaB activity. Intriguingly, induction of wild-type MYOC expression inhibited IL-1alpha-stimulated NF-kappaB activity by 31%, and this was significant ($p<0.04$). This is consistent with the initial

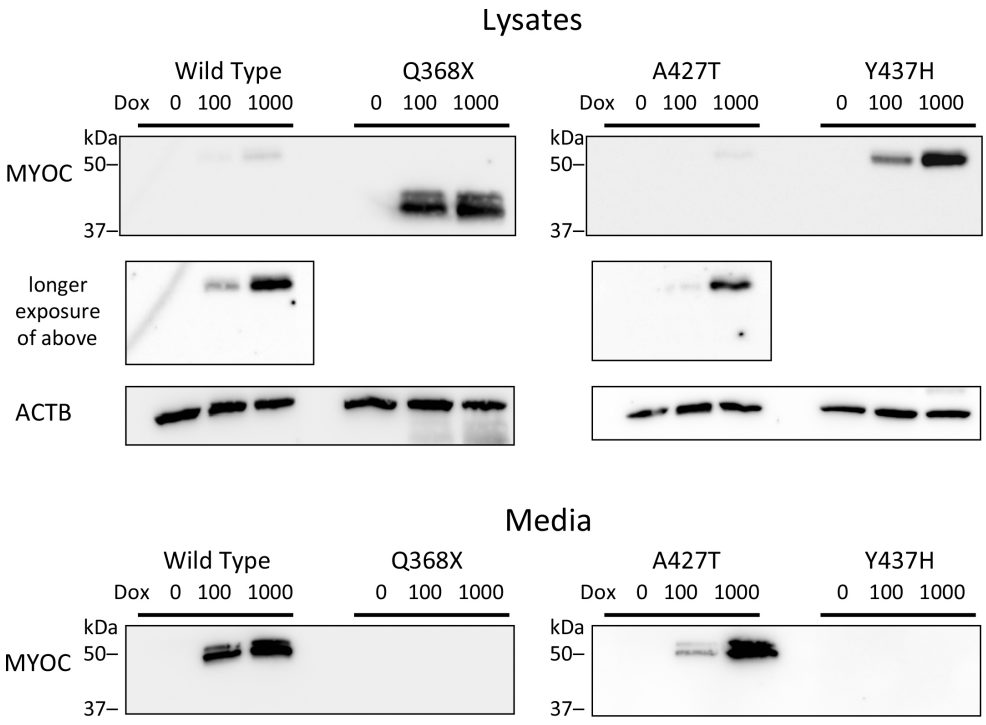

Figure 2. Expression of Wild Type and Mutant MYOCs by Stably Transduced Cell Lines MYOC expression was induced in TM-1 cell lines stably transduced with wild-type or mutant MYOC lentivirus expression constructs across a range of doxycycline concentrations (0, 100, and 1000 ng/ml). After 4 days time, conditioned media and cell lysates were collected for assay. Lanes of an SDS-polyacrylamide gel were equally loaded with aliquots of each sample (20 ul of cell culture media or 20 ug cell lysates) and proteins were separated by electrophoresis. Western blotting was performed with MYOC antibody to visualize MYOC proteins. Blots were re-probed with antibodies against ACTB as a control for equal loading of gel lanes. The electrophoretic mobility of molecular weight size standards is indicated.

suggestion provided by results of the transient transfection experiments, noted above (Figure 1).

Figure 5 shows an observation we made as part of another study using our stable cells lines harboring the MYOC expression vectors. Unexpectedly, we observed that endogenous MYOC expression is induced by IL1A in the TM-1 cell line harboring control lentivirus (Figure 5A). Co-treatment with IL1RA, a receptor antagonist, revealed that this induction occurred through the IL-1 receptor. Reprobing of the western blot revealed that SELE was co-expressed with MYOC under these conditions. At the time we made this observation it was novel, thus we followed up on its validity using two different primary TBM cell lines. We found that treatment with IL1A induced MYOC expression in both cell lines (Figure 5B). Additionally, MYOC was induced by treatment with dexamethasone, confirming an observation first made by Polansky's laboratory [61,62]. SELE expression was also induced by IL1 in these primary cell lines, as we have previously reported [16], but was not induced by dexamethasone. This is consistent with the known anti-inflammatory effects of dexamethasone [63].

DISCUSSION

Some years ago, we reported that activation of the IL-1/NF-kappaB inflammatory stress response is a defining feature of both open and closed angle forms of high-tension glaucoma of a variety of etiologies [16]. In this study, we now provide evidence to link an additional high-tension form of open angle glaucoma to this pathway: myocilin glaucoma. Our findings support our hypothesis that IL-1/NF-kappaB inflammatory stress response represents a common mechanism operative in the pathophysiology of high-tension glaucomas.

MYOC is the first gene in which mutations were identified as causative for familial forms of glaucoma [30]. The gene is expressed in several tissues of the body, but to our knowledge, mutations cause disease only in the eye [64]. Despite intensive investigation, the biologic function of MYOC has been elusive. MYOC may play a role in cell-extracellular matrix interactions [65,66], cell migration [67] and mitochondrial function [68]. MYOC is part of the dystrophin-associated protein complex and thus may play a role as a regulator of muscle hypertrophy pathways [69]. An interesting recent study has provided evidence that MYOC may serve as an extracellular chaperone [70]. It was also recently suggested that the MYOC functions as a plasma

membrane scaffold protein involved in receptor-mediated endocytosis [71].

As mentioned in the Introduction to this paper, most mutant MYOC forms are misfolded and aggregate in the endoplasmic reticulum, activating the unfolded protein response, a stress pathway [46,47]. Specific MYOC mutations may lead to different amounts of misfolding, with varying degrees of recognition by the ubiquitin degradation pathway. Wild-type MYOC appears to contain a cryptic peroxisomal targeting signal type 1 (PTS1) and glaucoma-associated mutations were shown to activate the targeting sequence [72]. It is suggested that mutant MYOC interaction with the PTS1 receptor may mean poorer clearance from the endoplasmic reticulum and greater TBM cell dysfunction, culminating in a higher IOP phenotype [72].

In this study, we investigated a possible mechanistic connection between mutant MYOC proteins with a range of disease phenotype and myocilin glaucoma and activation of the IL-1/NF-κB stress pathway. By mapping familial glaucoma-associated lesions onto the structure of the olfactomedin domain recently resolved by crystallography [35], three regions sensitive to aggregation have been identified. The largest number of disease-causing variants is found within the core beta-sheet belt of the propeller, particularly those variants that would be most destabilized and least tolerated. The Y437H mutation associated with severe juvenile POAG is located here. Substitutions in the molecular clasp region of the core appear better tolerated. Such variants affect the tethering of the strands and/or alignment of the disulfide bond in this region. Q368X is less severe, is located here. Several additional substitutions cluster near the Ca^{2+} coordination sphere, including the mild glaucoma-causing mutation MYOC A427T. The substitution likely weakens Ca^{2+} coordination.

We found that the two intracellularly retained mutants we tested, MYOCs Y437H and Q368X, activated the IL-1/NF-kappaB pathway. Using both the transient transfection and stably transduced cells and two different IL1 expression

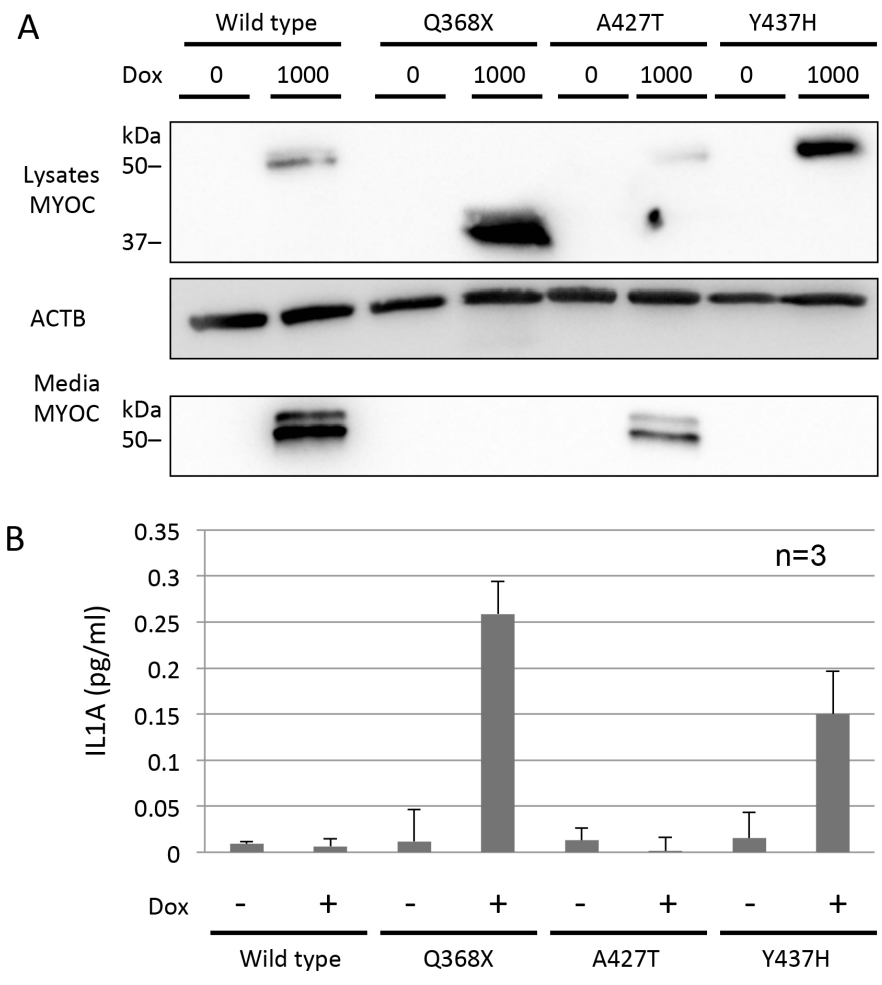

Figure 3. Effects of MYOC Mutations on Expression of IL1A in Stably Transduced Cell Lines MYOC expression was induced in TM-1 cell lines stably transduced with wild-type or mutant MYOC lentivirus expression constructs at 1000 ng/ml doxycycline. A parallel set of cells was left uninduced for comparison. After 6 days, conditioned media and cell lysates were collected for assay. **A**: Lanes of an SDS-polyacrylamide gel were equally loaded with aliquots of each sample (20 ul of cell culture media or 20 ug cell lysates) and proteins were separated by electrophoresis. Western blotting was performed with anti-MYOC antibody to quantify MYOC protein. Lysate blots were re-probed with ACTB as a control for equal gel loading. The electrophoretic mobility of molecular weight size standards is indicated. **B**: ELISA was used to quantify IL1A protein in equal volume samples of conditioned media.

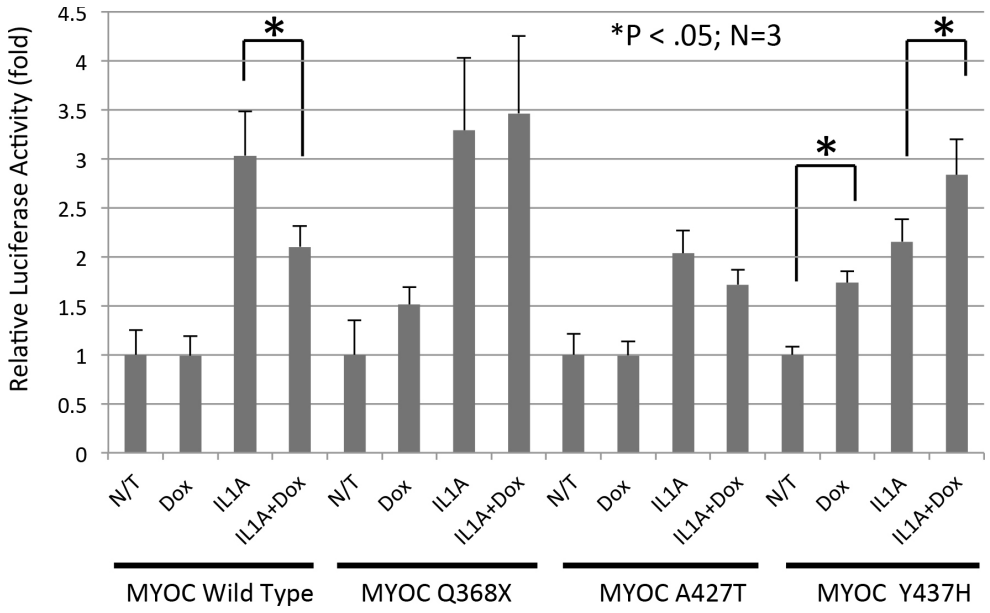

Figure 4. Effects of MYOC Mutations on NF-kappaB Activity in Stably Transduced Cell Lines TBM-1 cell lines stably transduced with wild-type or mutant MYOC lentivirus expression constructs were transfected with the IL8-luc expression construct or the renilla expression construct control. MYOC expression was then induced in at 1000 ng/ml doxycycline. A set of cultures was treated with IL1A and a parallel was left untreated for comparison. *p<0.05 n=3.

assays, we found that the moderate glaucoma-causing mutant had the greatest effect. This could be due to differences in transfection efficiencies or copy number differences, however, the western blots seemed to indicate that expression was equivalent. The reason for this observation will be followed up in future studies. Only the most severe glaucoma-causing mutant Y437H had a detectable effect on the NF-kappaB activation assay. In this case, however, error bars were large for the moderate glaucoma-causing mutant Q368X, which may have precluded the detection of true significance.

As also noted in the Introduction to this paper, some mutations that do not cause intracellular retention of MYOC protein can still cause glaucoma. To account for this, an alternative mechanism has been suggested that relies on abnormal interactions of the secreted mutants with the ECM and/or cell/surface elements [48]. In this study, we could not detect any effect of the secreted mutant A427T on activation of the IL-1/NF-kappaB pathway. However, it is important to note that this MYOC mutant has a much lower glaucoma-causing effect than the retained MYOC mutants we examined. If its ability

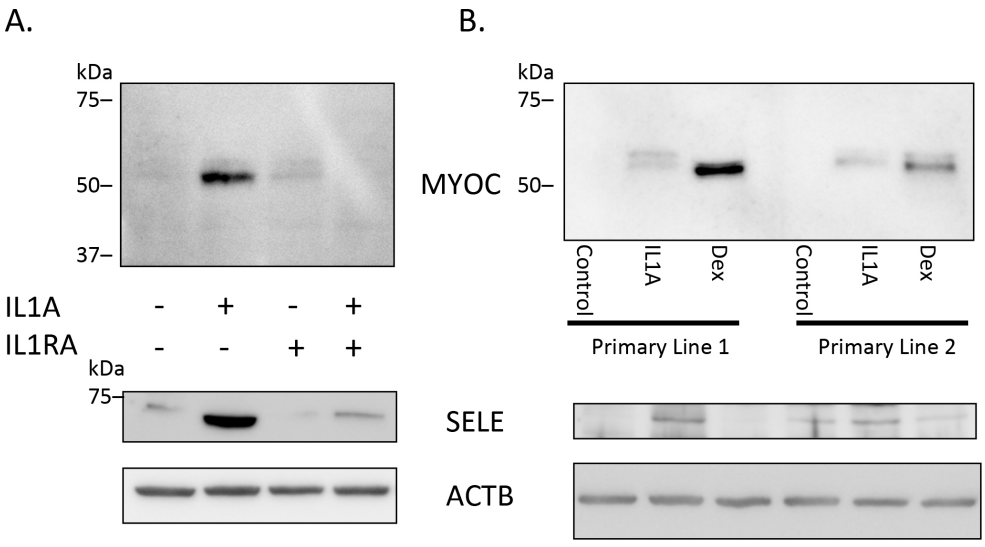

Figure 5. Effects of IL1A on Expression of Endogenous MYOC and SELE (**A**) western blot of lysates from cultures of TM-1 cells stably transduced with empty lentivirus vector. Cultures were treated for 72 h with IL1A (10 ng/ml), IL1RA (100 ng/ml), or both IL1A and IL1RA. Blots were probed for MYOC and SELE. ACTB was used as a control to demonstrate equal loading of gel lanes. **B**: Western blot of lysates from two primary trabecular meshwork cell lines derived from separate individuals (primary line 1 and primary line 2). Cells were plated and cultures were allowed to grow to confluence. They were then treated with IL1A (10 ng/mL) or dexamethasone (100 nM) for 10 days.

to activate the IL-1/NF-kappaB pathway is similarly low, then this effect may be beneath the level of detection of our tests.

Prior to our submission of this manuscript, a publication appeared reporting that inflammatory cytokine IL1A stimulates endogenous MYOC expression in the porcine system [70]. Independently, we found that IL1A stimulates endogenous MYOC expression in the human system. In addition, we extend this finding by our discovery that wild-type MYOC overexpression inhibits activation of NF-kappaB. Thus wild-type MYOC would participate in a negative feedback loop to inhibit activation of the IL-1/NF-kappaB inflammatory stress pathway.

We put this negative feedback loop together with the positive feedback loop activated by intracellularly retained mutant MYOC, as schematically diagramed in Figure 6. In individuals heterozygous for intracellularly retained mutant MYOC, activation of the IL-1/NFkappaB pathway by mutant MYOC should stimulate expression of both mutant and wild-type MYOC alleles; the mutant MYOC would further stimulate the pathway, but wild-type MYOC might at least partially counter this effect. However, in individuals homozygous for intracellularly retained mutant MYOC, only the positive feedback loop would be active.

MYOC was discovered in studies to understand the mechanisms of steroid-induced ocular hypertension and glaucoma identified as a protein highly induced by treatment of primary TBM cell cultures with glucocorticoids [61,62]. It has since been shown that MYOC expression can also be induced in TBM cells by TGFB1 [73], OPTN [74], and mechanical stretch [73]. The dexamethasone-stimulated increase in MYOC expression was recently shown to be dependent on activation of NFATC1 by calcineurin [75]. NF-kappaB and NFAT pathways can crosstalk [76], suggesting this as a candidate mechanism for stimulation of MYOC expression by IL1A. MYOC modulates Wnt signaling by interacting with components of this signaling pathway [77,78]. In addition, it was recently shown that MYOC binds and activates the plasma membrane receptor ErbB2/ErbB3 in the sciatic nerve [79]. The mechanisms whereby MYOC inhibits activity of NF-kappaB remain to be determined.

Living tissues are exposed to many different stress conditions. Short-term stress typically stimulates protective responses that help maintain tissue homeostasis. However, chronic exposure can lead to changes that contribute to the progression of many age-related degenerative conditions. In our previous study, we provided evidence that activation of the IL-1/NF-kappaB inflammatory stress response in glaucomatous TBM cells is protective in the short run, but amplification of the response as a result of prolonged

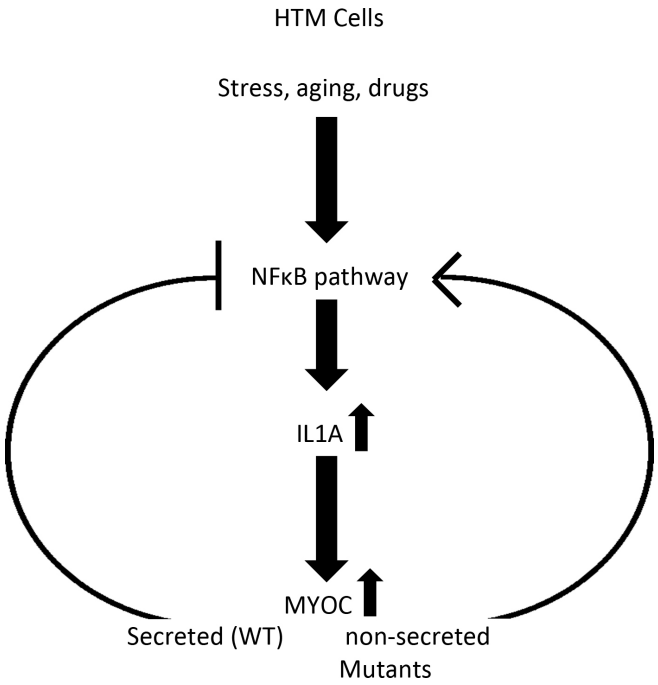

Figure 6. Schematic Representation A model is depicted illustrating the possible positive (retained MYOC mutants) and negative (WT MYOC) feedback loops controlling the IL1A/NFkappaB pathway in TBM cells.

stress would lead to pathological tissue changes resulting in ocular hypertension [16]. Here we identify another possible mechanism for amplifying the IL-1/NF-kappaB inflammatory stress response in a familial form of glaucoma. These studies are quite timely considering recent renewed interest in the role of low-grade inflammation in glaucoma [49]. They provide additional support for the use of anti-inflammatory agents in the treatment of high-tension glaucomas.

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REFERENCES

1. Quigley HA. Number of people with glaucoma worldwide. Br J Ophthalmol 1996; 80:389-93. [PMID: 8695555].
2. Congdon N, O'Colmain B, Klaver CC, Klein R, Munoz B, Friedman DS, Kempen J, Taylor HR, Mitchell P. Eye Diseases Prevalence Research G: Causes and prevalence of visual impairment among adults in the United States. Arch Ophthalmol 2004; 122:477-85. [PMID: 15078664].
3. Fautsch MP, Johnson DH. Aqueous humor outflow: what do we know? Where will it lead us? Invest Ophthalmol Vis Sci 2006; 47:4181-7. [PMID: 17003404].
4. Alvarado J, Murphy C, Polansky J, Juster R. Age-related changes in trabecular meshwork cellularity. Invest Ophthalmol Vis Sci 1981; 21:714-27. [PMID: 7298275].
5. Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. Ophthalmology 1984; 91:564-79. [PMID: 6462622].
6. Chen JZ, Kadlubar FF. A new clue to glaucoma pathogenesis. Am J Med 2003; 114:697-8. [PMID: 12798460].
7. Li G, Luna C, Liton PB, Navarro I, Epstein DL, Gonzalez P. Sustained stress response after oxidative stress in trabecular meshwork cells. Mol Vis 2007; 13:2282-8. [PMID: 18199969].
8. Liton PB, Luna C, Challa P, Epstein DL, Gonzalez P. Genome-wide expression profile of human trabecular meshwork cultured cells, nonglaucomatous and primary open angle glaucoma tissue. Mol Vis 2006; 12:774-90. [PMID: 16862071].
9. Izzotti A, Bagnis A, Sacca SC. The role of oxidative stress in glaucoma. Mutat Res 2006; 612:105-14. [PMID: 16413223].
10. Saccà SC, Izzotti A, Rossi P, Traverso C. Glaucomatous outflow pathway and oxidative stress. Exp Eye Res 2007; 84:389-99. [PMID: 17196589].
11. Liton PB, Gonzalez P. Stress response of the trabecular meshwork. J Glaucoma 2008; 17:378-85. [PMID: 18703948].
12. Liton PB, Gonzalez P, Epstein DL. The role of proteolytic cellular systems in trabecular meshwork homeostasis. Exp Eye Res 2009; 88:724-8. [PMID: 19046967].
13. Izzotti A, Sacca SC, Longobardi M, Cartiglia C. Mitochondrial damage in the trabecular meshwork of patients with glaucoma. Arch Ophthalmol 2010; 128:724-30. [PMID: 20547950].
14. Epstein DL, Allingham RR, Schuman JS. Chandler and Grant's Glaucoma. 4th ed. Baltimore, Maryland: Williams and Wilkins; 1996.
15. Lütjen-Drecoll E, Shimizu T, Rohrbach M, Rohen JW. Quantitative analysis of 'plaque material' in the inner- and outer wall of Schlemm's canal in normal- and glaucomatous eyes. Exp Eye Res 1986; 42:443-55. [PMID: 3720863].
16. Wang N, Chintala SK, Fini ME, Schuman JS. Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye defines the glaucoma disease phenotype. Nat Med 2001; 7:304-9. [PMID: 11231628].
17. Kee C, Seo K. The effect of interleukin-1alpha on outflow facility in rat eyes. J Glaucoma 1997; 6:246-9. [PMID: 9264304].
18. Bradley JM, Vranka J, Colvis CM, Conger DM, Alexander JP, Fisk AS, Samples JR, Acott TS. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. Invest Ophthalmol Vis Sci 1998; 39:2649-58. [PMID: 9856774].
19. Samples JR, Alexander JP, Acott TS. Regulation of the levels of human trabecular matrix metalloproteinases and inhibitor by interleukin-1 and dexamethasone. Invest Ophthalmol Vis Sci 1993; 34:3386-95. [PMID: 8225873].
20. Alvarado JA, Alvarado RG, Yeh RF, Franse-Carman L, Marcellino GR, Brownstein MJ. A new insight into the cellular regulation of aqueous outflow: how trabecular meshwork endothelial cells drive a mechanism that regulates the permeability of Schlemm's canal endothelial cells. Br J Ophthalmol 2005; 89:1500-5. [PMID: 16234461].
21. Wang NL, Chen JC, Fan ZG, Wu HP, Lin H, Qiao LY. The effect of interleukin-1alpha on intraocular pressure in rabbit eyes and its focal side effects. Zhonghua Yan Ke Za Zhi 2005; 41:647-51. [PMID: 16080902].
22. Diskin S, Kumar J, Cao Z, Schuman JS, Gilmartin T, Head SR, Panjwani N. Detection of differentially expressed glycogenes in trabecular meshwork of eyes with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2006; 47:1491-9. [PMID: 16565384].
23. Suarez T, Vecino E. Expression of endothelial leukocyte adhesion molecule 1 in the aqueous outflow pathway of porcine eyes with induced glaucoma. Mol Vis 2006; 12:1467-72. [PMID: 17167401].
24. Wang NL, Chen JC, Fan ZG, Wu HP, Lin H, Qiao LY. The effect of interleukin-1alpha on intraocular pressure in rabbit eyes and its focal side effects. Zhonghua Yan Ke Za Zhi 2005; 41:647-51. [PMID: 16080902].
25. Birke MT, Birke K, Lutjen-Drecoll E, Schlotzer-Schrehardt U, Hammer CM. Cytokine-dependent ELAM-1 induction and concomitant intraocular pressure regulation in porcine anterior eye perfusion culture. Invest Ophthalmol Vis Sci 2011; 52:468-75. [PMID: 20861478].
26. Chung HY, Kim HJ, Kim KW, Choi JS, Yu BP. Molecular inflammation hypothesis of aging based on the anti-aging mechanism of calorie restriction. Microsc Res Tech 2002; 59:264-72. [PMID: 12424787].
27. Chung HY, Sung B, Jung KJ, Zou Y, Yu BP. The molecular inflammatory process in aging. Antioxid Redox Signal 2006; 8:572-81. [PMID: 16677101].
28. Chung JH, Eun HC. Angiogenesis in skin aging and photoaging. J Dermatol 2007; 34:593-600. [PMID: 17727362].
29. Sung B, Park S, Yu BP, Chung HY. Modulation of PPAR in aging, inflammation, and calorie restriction. J Gerontol A Biol Sci Med Sci 2004; 59:997-1006. [PMID: 15528772].

30. Stone EM, Fingert JH, Alward WL, Nguyen TD, Polansky JR, Sunden SL, Nishimura D, Clark AF, Nystuen A, Nichols BE, Mackey DA, Ritch R, Kalenak JW, Craven ER, Sheffield VC. Identification of a gene that causes primary open angle glaucoma. Science 1997; 275:668-70. [PMID: 9005853].

31. Alward WL, Fingert JH, Coote MA, Johnson AT, Lerner SF, Junqua D, Durcan FJ, McCartney PJ, Mackey DA, Sheffield VC, Stone EM. Clinical features associated with mutations in the chromosome 1 open-angle glaucoma gene (GLC1A). N Engl J Med 1998; 338:1022-7. [PMID: 9535666].

32. Fingert JH, Heon E, Liebmann JM, Yamamoto T, Craig JE, Rait J, Kawase K, Hoh ST, Buys YM, Dickinson J, Hockey RR, Williams-Lyn D, Trope G, Kitazawa Y, Ritch R, Mackey DA, Alward WL, Sheffield VC, Stone EM. Analysis of myocilin mutations in 1703 glaucoma patients from five different populations. Hum Mol Genet 1999; 8:899-905. [PMID: 10196380].

33. Gong G, Kosoko-Lasaki O, Haynatzki GR, Wilson MR. Genetic dissection of myocilin glaucoma. Hum Mol Genet 2004; 13:Spec No 1R91-102. [PMID: 14764620].

34. Tamm ER. Myocilin and glaucoma: facts and ideas. Prog Retin Eye Res 2002; 21:395-428. [PMID: 12150989].

35. Donegan RK, Hill SE, Freeman DM, Nguyen E, Orwig SD, Turnage KC, Lieberman RL. Structural basis for misfolding in myocilin-associated glaucoma. Hum Mol Genet 2015; 24:2111-24. [PMID: 25524706].

36. Joe MK, Sohn S, Choi YR, Park H, Kee C. Identification of flotillin-1 as a protein interacting with myocilin: implications for the pathogenesis of primary open-angle glaucoma. Biochem Biophys Res Commun 2005; 336:1201-6. [PMID: 16198165].

37. Torrado M, Trivedi R, Zinovieva R, Karavanova I, Tomarev SI. Optimedin: a novel olfactomedin-related protein that interacts with myocilin. Hum Mol Genet 2002; 11:1291-301. [PMID: 12019210].

38. Resch ZT, Fautsch MP. Glaucoma-associated myocilin: a better understanding but much more to learn. Exp Eye Res 2009; 88:704-12. [PMID: 18804106].

39. Fingert JH, Stone EM, Sheffield VC, Alward WL. Myocilin glaucoma. Surv Ophthalmol 2002; 47:547-61. [PMID: 12504739].

40. Gould DB, Miceli-Libby L, Savinova OV, Torrado M, Tomarev SI, Smith RS, John SW. Genetically increasing Myoc expression supports a necessary pathologic role of abnormal proteins in glaucoma. Mol Cell Biol 2004; 24:9019-25. [PMID: 15456875].

41. Pang CP, Leung YF, Fan B, Baum L, Tong WC, Lee WS, Chua JK, Fan DS, Liu Y, Lam DS. TIGR/MYOC gene sequence alterations in individuals with and without primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2002; 43:3231-5. [PMID: 12356829].

42. Wiggs JL, Vollrath D. Molecular and clinical evaluation of a patient hemizygous for TIGR/MYOC. Arch Ophthalmol 2001; 119:1674-8. [PMID: 11709019].

43. Lam DS, Leung YF, Chua JK, Baum L, Fan DS, Choy KW, Pang CP. Truncations in the TIGR gene in individuals with and without primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2000; 41:1386-91. [PMID: 10798654].

44. Kim BS, Savinova OV, Reedy MV, Martin J, Lun Y, Gan L, Smith RS, Tomarev SI, John SW, Johnson RL. Targeted Disruption of the Myocilin Gene (Myoc) Suggests that Human Glaucoma-Causing Mutations Are Gain of Function. Mol Cell Biol 2001; 21:7707-13. [PMID: 11604506].

45. Hardy KM, Hoffman EA, Gonzalez P, McKay BS, Stamer WD. Extracellular trafficking of myocilin in human trabecular meshwork cells. J Biol Chem 2005; 280:28917-26. [PMID: 15944158].

46. Kwon YH, Fingert JH, Kuehn MH, Alward WL. Primary open-angle glaucoma. N Engl J Med 2009; 360:1113-24. [PMID: 19279343].

47. Joe MK, Sohn S, Hur W, Moon Y, Choi YR, Kee C. Accumulation of mutant myocilins in ER leads to ER stress and potential cytotoxicity in human trabecular meshwork cells. Biochem Biophys Res Commun 2003; 312:592-600. [PMID: 14680806].

48. Gobeil S, Letartre L, Raymond V. Functional analysis of the glaucoma-causing TIGR/myocilin protein: integrity of N-terminal coiled-coil regions and olfactomedin homology domain is essential for extracellular adhesion and secretion. Exp Eye Res 2006; 82:1017-29. [PMID: 16466712].

49. Vohra R, Tsai JC, Kolko M. The role of inflammation in the pathogenesis of glaucoma. Surv Ophthalmol 2013; 58:311-20. [PMID: 23768921].

50. Filla MS, Liu X, Nguyen TD, Polansky JR, Brandt CR, Kaufman PL, Peters DM. In vitro localization of TIGR/MYOC in trabecular meshwork extracellular matrix and binding to fibronectin. Invest Ophthalmol Vis Sci 2002; 43:151-61. [PMID: 11773026].

51. Alvarado JA, Wood I, Polansky JR. Human trabecular cells. II. Growth pattern and ultrastructural characteristics. Invest Ophthalmol Vis Sci 1982; 23:464-78. [PMID: 7118506].

52. Polansky JR, Weinreb RN, Baxter JD, Alvarado J. Human trabecular cells. I. Establishment in tissue culture and growth characteristics. Invest Ophthalmol Vis Sci 1979; 18:1043-9. [PMID: 383640].

53. Shin KJ, Wall EA, Zavzavadjian JR, Santat LA, Liu J, Hwang JI, Rebres R, Roach T, Seaman W, Simon MI, Fraser ID. A single lentiviral vector platform for microRNA-based conditional RNA interference and coordinated transgene expression. Proc Natl Acad Sci USA 2006; 103:13759-64. [PMID: 16945906].

54. Hewitt AW, Mackey DA, Craig JE. Myocilin allele-specific glaucoma phenotype database. Hum Mutat 2008; 29:207-11. [PMID: 17966125].

55. Sohn S, Hur W, Joe MK, Kim JH, Lee ZW, Ha KS, Kee C. Expression of wild-type and truncated myocilins in trabecular meshwork cells: their subcellular localizations and

cytotoxicities. Invest Ophthalmol Vis Sci 2002; 43:3680-5. [PMID: 12454036].

56. Liu Y, Vollrath D. Reversal of mutant myocilin non-secretion and cell killing: implications for glaucoma. Hum Mol Genet 2004; 13:1193-204. [PMID: 15069026].

57. Zillig M, Wurm A, Grehn FJ, Russell P, Tamm ER. Overexpression and properties of wild-type and Tyr437His mutated myocilin in the eyes of transgenic mice. Invest Ophthalmol Vis Sci 2005; 46:223-34. [PMID: 15623777].

58. Faucher M, Anctil JL, Rodrigue MA, Duchesne A, Bergeron D, Blondeau P, Cote G, Dubois S, Bergeron J, Arseneault R, Morissette J, Raymond V, Quebec Glaucoma N. Founder TIGR/myocilin mutations for glaucoma in the Quebec population. Hum Mol Genet 2002; 11:2077-90. [PMID: 12189160].

59. Burns JN, Turnage KC, Walker CA, Lieberman RL. The stability of myocilin olfactomedin domain variants provides new insight into glaucoma as a protein misfolding disorder. Biochemistry 2011; 50:5824-33. [PMID: 21612213].

60. Joe MK, Tomarev SI. Expression of myocilin mutants sensitizes cells to oxidative stress-induced apoptosis: implication for glaucoma pathogenesis. Am J Pathol 2010; 176:2880-90. [PMID: 20382707].

61. Nguyen TD, Chen P, Huang WD, Chen H, Johnson D, Polansky JR. Gene structure and properties of TIGR, an olfactomedin-related glycoprotein cloned from glucocorticoid-induced trabecular meshwork cells. J Biol Chem 1998; 273:6341-50. [PMID: 9497363].

62. Polansky JR, Fauss DJ, Chen P, Chen H, Lütjen-Drecoll E, Johnson D, Kurtz RM, Ma ZD, Bloom E, Nguyen TD. Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. Ophthalmologica 1997; 211:126-39. [PMID: 9176893].

63. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids–new mechanisms for old drugs. N Engl J Med 2005; 353:1711-23. [PMID: 16236742].

64. Borrás T. The effects of myocilin expression on functionally relevant trabecular meshwork genes: a mini-review. J Ocul Pharmacol Ther 2014; 30:202-12. [PMID: 24564495].

65. Goldwich A, Scholz M, Tamm ER. Myocilin promotes substrate adhesion, spreading and formation of focal contacts in podocytes and mesangial cells. Histochem Cell Biol 2009; 131:167-80. [PMID: 18855004].

66. Peters DM, Herbert K, Biddick B, Peterson JA. Myocilin binding to Hep II domain of fibronectin inhibits cell spreading and incorporation of paxillin into focal adhesions. Exp Cell Res 2005; 303:218-28. [PMID: 15652337].

67. Kwon HS, Tomarev SI. Myocilin, a glaucoma-associated protein, promotes cell migration through activation of integrin-focal adhesion kinase-serine/threonine kinase signaling pathway. J Cell Physiol 2011; 226:3392-402. [PMID: 21656515].

68. Sakai H, Shen X, Koga T, Park BC, Noskina Y, Tibudan M, Yue BY. Mitochondrial association of myocilin, product of a glaucoma gene, in human trabecular meshwork cells. J Cell Physiol 2007; 213:775-84. [PMID: 17516541].

69. Joe MK, Kee C, Tomarev SI. Myocilin interacts with syntrophins and is member of dystrophin-associated protein complex. J Biol Chem 2012; 287:13216-27. [PMID: 22371502].

70. Anderssohn AM, Cox K, O'Malley K, Dees S, Hosseini M, Boren L, Wagner A, Bradley JM, Kelley MJ, Acott TS. Molecular chaperone function for myocilin. Invest Ophthalmol Vis Sci 2011; 52:7548-55. [PMID: 21873671].

71. McKay BS, Congrove NR, Johnson AA, Dismuke WM, Bowen TJ, Stamer WD. A role for myocilin in receptor-mediated endocytosis. PLoS ONE 2013; 8:e82301-[PMID: 24367514].

72. Shepard AR, Jacobson N, Millar JC, Pang IH, Steely HT, Searby CC, Sheffield VC, Stone EM, Clark AF. Glaucoma-causing myocilin mutants require the Peroxisomal targeting signal-1 receptor (PTS1R) to elevate intraocular pressure. Hum Mol Genet 2007; 16:609-17. [PMID: 17317787].

73. Tamm ER, Russell P, Epstein DL, Johnson DH, Piatigorsky J. Modulation of myocilin/TIGR expression in human trabecular meshwork. Invest Ophthalmol Vis Sci 1999; 40:2577-82. [PMID: 10509652].

74. Park BC, Tibudan M, Samaraweera M, Shen X, Yue BY. Interaction between two glaucoma genes, optineurin and myocilin. Genes Cells 2007; 12:969-79. [PMID: 17663725].

75. Faralli JA, Clark RW, Filla MS, Peters DM. NFATc1 activity regulates the expression of myocilin induced by dexamethasone. Exp Eye Res 2015; 130:9-16. [PMID: 25450062].

76. Cai T, Li X, Ding J, Luo W, Li J, Huang C. A cross-talk between NFAT and NF-kappaB pathways is crucial for nickel-induced COX-2 expression in Beas-2B cells. Curr Cancer Drug Targets 2011; 11:548-59. [PMID: 21486220].

77. Kwon HS, Lee HS, Ji Y, Rubin JS, Tomarev SI. Myocilin is a modulator of Wnt signaling. Mol Cell Biol 2009; 29:2139-54. [PMID: 19188438].

78. Shen X, Ying H, Yue BY. Wnt activation by wild type and mutant myocilin in cultured human trabecular meshwork cells. PLoS ONE 2012; 7:e44902-[PMID: 23028669].

79. Kwon HS, Johnson TV, Joe MK, Abu-Asab M, Zhang J, Chan CC, Tomarev SI. Myocilin mediates myelination in the peripheral nervous system through ErbB2/3 signaling. J Biol Chem 2013; 288:26357-71. [PMID: 23897819].

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