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Histochemical Analysis of Glaucoma Caused by a Myocilin Mutation in a Human Donor Eye

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Purpose: Mutations in myocilin (*MYOC*) may cause either juvenile-onset open-angle glaucoma (JOAG) or adult-onset primary open-angle glaucoma. Myocilin encodes a glycoprotein that normally is secreted from trabecular meshwork cells that regulate intraocular pressure. Prior in vitro, transgenic rodent, and organ culture experiments have suggested that abnormal accumulation of MYOC protein within trabecular meshwork cells is a key step in glaucoma pathophysiology. We investigated the pathogenesis of *MYOC* glaucoma by examining a donor eye from a patient with JOAG caused by a Tyr437His *MYOC* mutation.

Design: Case-control immunohistochemical study of a donor eye from a patient with JOAG caused by a Tyr437His *MYOC* mutation and age-matched control donor eyes.

Participants: An eye from a 59-year-old man with JOAG caused by a Tyr437His *MYOC* mutation and eyes from 5 donors (51−66 years of age) with no known ocular disease were examined.

Methods: Frozen fixed sections of the iridocorneal angle were prepared from the donor eyes of the *MYOC* glaucoma patient and control eyes. We used antibodies directed against *MYOC*, collagen IV, and GRP78 (BiP) as well as wheat germ agglutinin and concanavalin A lectins to localize MYOC protein in the trabecular meshwork.

Main Outcome Measures: Qualitative comparison of MYOC protein labeling and localization in the trabecular meshwork of donor eyes from a glaucoma patient with a *MYOC* mutation and from control participants.

Results: Using immunohistochemistry, we detected more abundant MYOC protein within the trabecular meshwork of the *MYOC* glaucoma patient's eye than in control eyes. We further localized MYOC protein within the trabecular meshwork cells of the *MYOC* glaucoma patient's eye by colabeling with the endoplasmic reticulum (ER) marker GRP78 (BiP). Little to no MYOC was identified within the trabecular meshwork cells of control eyes. Minimal extracellular MYOC was detected in both *MYOC* glaucoma eyes and control eyes.

Conclusions: This histopathologic analysis of a rare donor eye from a glaucoma patient with a *MYOC* mutation supports our model of *MYOC*-associated glaucoma, in which *MYOC* mutations cause abnormal intracellular retention of MYOC within the ER of trabecular meshwork cells as a key step toward development of glaucoma. *Ophthalmology Glaucoma 2018;1:132-138 © 2018 by the American Academy of Ophthalmology*

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Glaucoma is the leading cause of irreversible blindness worldwide[1,2] and is a disease of the optic nerve and retinal ganglion cells. Optic nerve degeneration resulting from glaucoma has a characteristic appearance on clinical examination, cupping of the optic disc, and is associated with stereotypical patterns of vision loss. Higher intraocular pressure is associated with higher risk for glaucoma, but glaucoma can occur at any intraocular pressure. The most common type of glaucoma is primary open-angle glaucoma, which by definition is diagnosed after 40 years of age. Juvenile-onset open-angle glaucoma (JOAG) is diagnosed between 3 and 40 years of age and is notable for markedly high intraocular pressure, autosomal dominant inheritance, and strong family history.[3]

Primary open-angle glaucoma has a strong genetic basis. Some cases are caused by the combined action of many genetic risk factors. More than 20 such risk factors for glaucoma have been discovered.[4] Other cases show autosomal dominant inheritance patterns and are caused primarily by mutations in single genes.[4−7] Three of these glaucoma-causing genes have been identified: myocilin (*MYOC*),[5] optineurin (*OPTN*),[6] and TANK binding kinase 1 (*TBK1*).[7] Myocilin was the first glaucoma-causing gene to be identified, and mutations in this gene are the most common known genetic cause of glaucoma. Myocilin mutations are responsible for 2% to 4% of primary open-angle glaucoma cases worldwide[5,8−10] and 8% to 63% of JOAG cases.[11−14] More than 100 different *MYOC* mutations have been reported to date (www.myocilin.com). The vast majority of glaucoma-causing mutations in *MYOC* are located within exon 3, the olfactomedin-like domain, and are predicted to cause disease through dominant negative mechanisms.[3] Some genotype−phenotype relationships have been recognized. Many *MYOC* mutations cause JOAG (Pro370Leu and Tyr437His), whereas others are associated with adult-onset primary open-angle glaucoma (Gln368Stop).[8,9]

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Myocilin encodes a secreted glycoprotein of unknown function. Although *MYOC* mRNA expression is almost ubiquitous throughout the body and eye,[15,16] MYOC protein production has been reported primarily within the trabecular meshwork and other ocular tissues.[17–19] Studies of cultured trabecular meshwork cells, anterior segment eye culture, transgenic mice, and human aqueous humor from glaucoma patients have shown that wild-type MYOC is secreted into aqueous humor or culture media, whereas mutant MYOC protein is retained within cells.[10,20–23] Joe et al[20] demonstrated that accumulation of intracellular mutant MYOC protein in the endoplasmic reticulum (ER) causes ER stress, which has been replicated in transgenic mice.[21] These data have suggested a model of *MYOC*-associated glaucoma in which disease-causing mutations prevent secretion and cause mutant MYOC to accumulate in trabecular meshwork cells, which stimulates ER stress and ultimately leads to loss of trabecular meshwork cell function and glaucoma.

Although studies in cultured cells and animal models provide powerful and controlled insights into pathophysiologic features, analyses of human eyes harboring rare disease-causing mutations can offer a unique and robust understanding of how disease affects the production and localization of the mutant protein in the most relevant anatomic and phylogenetic context. In the current study, we investigated the localization of mutant MYOC protein in the first available human donor eye from a patient with glaucoma caused by a Tyr437His *MYOC* mutation. Immunohistochemical analyses of this human donor eye were consistent with our hypothesis that retention of mutant MYOC protein within trabecular meshwork cells occurs in *MYOC*-associated glaucoma.

Methods

Human Donor Eyes

A patient with JOAG caused by a Tyr437His *MYOC* mutation is a member of a large pedigree that has been described in previous reports.[5,9,10] He provided written informed consent for participating in this research project, which was conducted with the approval of the University of Iowa's institutional review board for human subjects research. At 59 years of age, the patient's right eye was painful and blind. His eye was removed (enucleated) and was made available for research. Control eyes were obtained as whole globes from human donors from the Iowa Lions Eye Bank (Iowa City, Iowa). Full consent for research was obtained from the donor's next of kin in all cases, and all experiments were performed in accordance with the tenets of the Declaration of Helsinki.

After enucleation, the patient's right eye immediately was fixed in 10% neutral-buffered formalin. Another eye from an age-matched donor (66 years of age) who had no known eye disease was placed in 10% formalin 3.5 hours after death and used as a control. After 96 hours in 10% formalin, the eyes were transferred to 10 mM of phosphate-buffered saline (PBS). We had access to additional control eyes that had been collected previously. These human donor eyes (51–59 years of age) with no known ocular disease had been fixed within 6 to 8 hours after death and were used as a second set of controls. One of these control patients (control 3) had been given a 3-day course of oral steroid 2 weeks before death and a 3-day intravenous course of steroids 1 week before death. The anterior segments of these additional eyes were fixed in 4% paraformaldehyde in 10 mM of PBS (pH 7.4) for 2 hours.

Anterior segments from all eyes were cryoprotected by passing through a sucrose gradient before being embedded in 20% sucrose in Optimal Cutting Temperature compound (Ted Pella, Redding, CA).[22] Sections of 7-µm thickness were collected from each sample using a Microm H505E cryostat (Waldorf, Germany) and mounted on Superfrost plus slides (Ted Pella).

Immunohistochemistry Analysis

Immunofluorescence procedures were performed as described previously.[23] Briefly, sections were blocked for 15 minutes using a PBS solution with 1 mg/ml bovine serum albumin. Sections then were incubated in the primary antibody solution for 1 hour, followed by rinsing 3 times with PBS and incubation in the appropriate Alexa 488-, Alexa 546-, and Alexa 633-conjugated secondary antibodies (Invitrogen, Eugene, OR) at a concentration of 10 µg/mL in PBS for 30 minutes. Sections were counterstained with 4′-6-diamidino-2-phenylindole, washed 3 times for 5 minutes in PBS, and coverslipped with Aquamount (Thermo Fisher Scientific, Waltham, MA). All PBS solutions contained 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Antibodies used for immunohistochemistry included anti-MYOC (HPA027364; Sigma-Aldrich, Darmstadt, Germany) used at a concentration of 0.5 µg/ml, anti-collagen IV (M3F7; Development Studies Hybridoma Bank, University of Iowa, Iowa City, IA) used at a concentration of 0.25 µg/ml, anti-CD45 (555480; BD Biosciences, San Jose, CA) used at a concentration of 2.5 µg/ml, and anti-GRP78 (14-9768-80; Thermo Fisher Scientific, Waltham, MA) used at a concentration of 10 µg/ml. Biotinylated wheat germ agglutinin (B-1025; Vector Laboratories, Burlingame, CA) and biotinylated concanavalin A (B-1005; Vector Laboratories) were used at a concentration of 100 µg/ml, followed by incubation in Dylight 488 Streptavidin (SA-5488; Vector Laboratories) for 30 minutes at a concentration of 10 µg/ml. Sections were viewed on an Olympus BX41fluorescence microscope (Shinjuku, Tokyo, Japan) with a SPOT RT camera (Sterling Heights, MI) and on a Leica SPS TCE confocal microscope (Wetzlar, Germany). Photographs were captured using identical exposure, laser intensity, and gain settings for all sections being compared. Hematoxylin–eosin staining was performed under the following conditions: hematoxylin (1 minute), rinse in double-distilled water, acid alcohol (3 seconds), rinse in water, 80% ethanol (1 minute), eosin (2 seconds), 95% ethanol (30 seconds), 100% ethanol (30 seconds), and xylenes (twice for 30 seconds each time). MM 24 mounting media (Leica, Wetzlar, Germany) was used to apply coverslips to slides stained with hematoxylin–eosin.

Optic Nerve Tissue Preparation

A segment of the optic nerve posterior to the eye was fixed in a 1/2K buffer overnight, dehydrated in acetone, and embedded in Spurrs resin, and 0.5-µm sections were acquired using a Leica EM UC5 Ultramicrotome (Wetzlar, Germany) with a Histo Diamond Knife (Diatome, Hatfield, PA). Sections were stained with a 1% paraphenylenediamine in a 1:1 isopropanol:methanol solution for 1 hour, rinsed twice with 1:1 isopropanol:methanol for 5 minutes, and finally rinsed with xylenes for 3 minutes. Slides were coverslipped with 1:1 xylenes:Permount (Thermo Fisher Scientific, Waltham, MA). Sections were viewed on an Olympus BX41fluorescence microscope (Shinjuku, Tokyo, Japan) with a SPOT RT camera (Sterling Heights, MI).

Results

In 1997, we identified a *MYOC* mutation (Tyr437His) in a very large pedigree that included more than 27 family members with

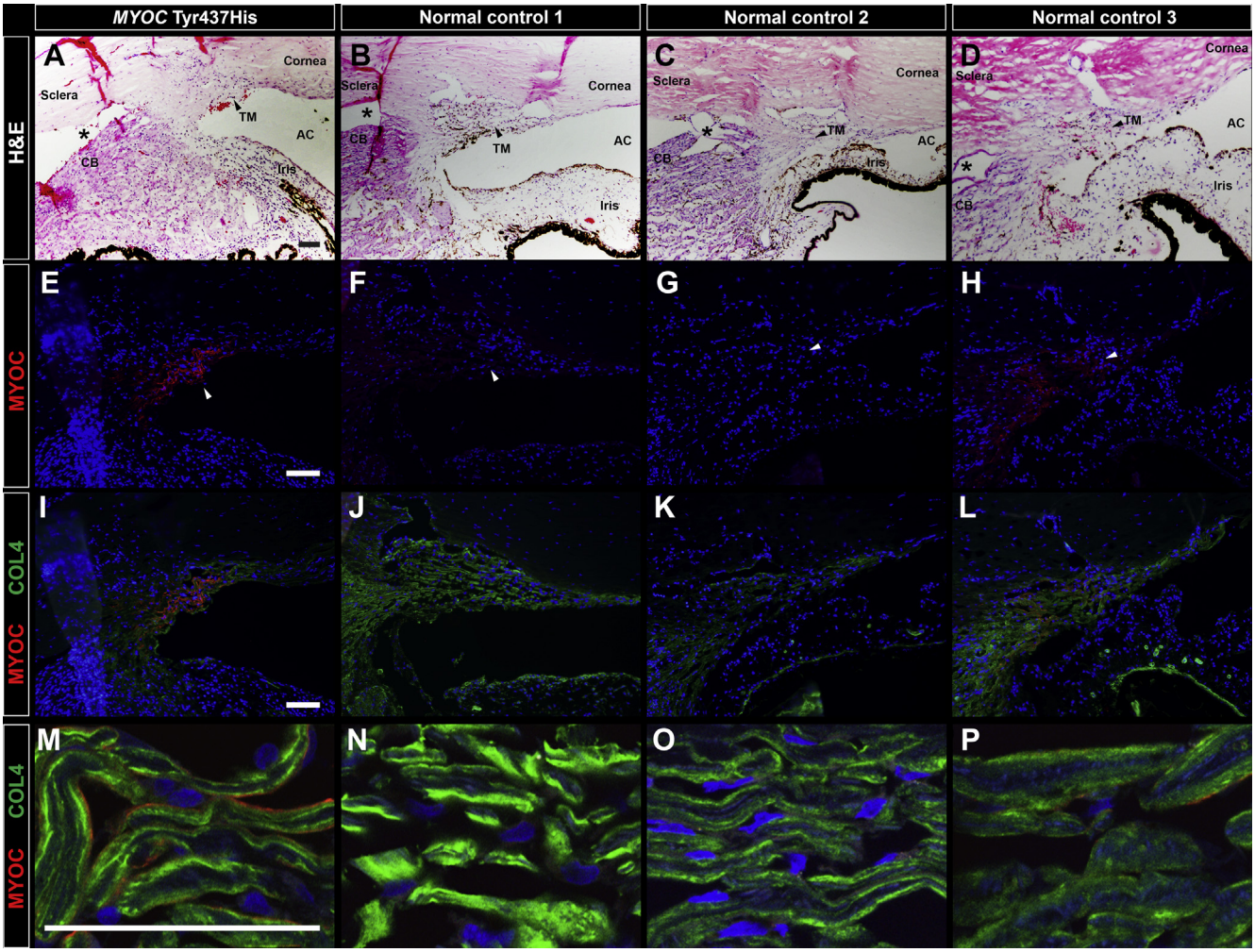

Figure 1. Immunohistochemical detection of myocilin (MYOC) protein in the trabecular meshwork of an eye with a MYOC Tyr437His mutation. Brightfield microscopic images show hematoxylin–eosin staining of the iridocorneal angle in (**A**) an eye with a MYOC Tyr437His mutation and (**B**–**D**) 3 normal control eyes. **E**, Intense MYOC immunoreactivity (red) was observed in the trabecular meshwork of the eye with a MYOC Tyr437His mutation. **F**, No MYOC immunoreactivity was observed in normal control 1, an age-matched donor with no known eye disease whose eye was fixed identically in 10% formalin to match fixation of the MYOC Tyr437His eye. Four additional normal control eyes from donors with no known ocular disease were fixed previously in 4% paraformaldehyde and were available for use as an additional set of controls. No MYOC immunoreactivity was observed in 3 of the 4 additional control eyes, as shown with (**G**) a representative image from normal control 2. **H**, One of the 4 additional control eyes, normal control 3, exhibited minimal positive MYOC labeling. **I**–**P**, The MYOC Tyr437His eye and all of the normal control eyes showed collagen IV immunoreactivity (green) of the trabecular meshwork. **M**–**P**, At higher magnification, collagen IV is seen deposited along the beams of the trabecular meshwork, with minimal to no colocalization with MYOC in (**M**) the MYOC Tyr437His eye or (**P**) in normal control 3. Asterisks indicate artifactitious tissue separation during processing. Black and white arrows point to the trabecular meshwork. Cell nuclei are labeled with 4′-6-diamidino-2-phenylindole (blue). AC = anterior chamber; CB = ciliary body; TM = trabecular meshwork. Scale bars = 100 μm.

juvenile-onset glaucoma.[5,24] One of the members of this pedigree donated his right eye for research at 59 years of age in 2017.

Clinical Course of Glaucoma Patient with *MYOC* Mutation Tyr437His

The patient showed typical features of JOAG, including early age at diagnosis, markedly high intraocular pressure, dominant inheritance of glaucoma, and a strong family history. He was diagnosed with JOAG at 16 years of age, and his highest recorded intraocular pressures were more than 60 mmHg in each eye. The patient and his family members were found to have a heterozygous glaucoma-causing *MYOC* mutation, Tyr437His.

Surgery (trabeculectomy) was required to control the patient's intraocular pressure adequately (left eye at 29 years of age and right eye at 30 years of age). The patient demonstrated good control of intraocular pressure for more than 20 years after surgery with use of topical glaucoma medications. At 53 years of age, however, he experienced a series of additional problems in his right eye that began with an ocular infection (endophthalmitis) that was treated with surgery (vitrectomy and lensectomy) and antibiotics. One month later, he sustained a retinal detachment in his right eye that was repaired with another surgery (vitrectomy and scleral buckle). After these surgeries, corneal defects developed, and the patient's intraocular pressure became more elevated in the right eye. At 54 years of age, the patient underwent another glaucoma surgery on the

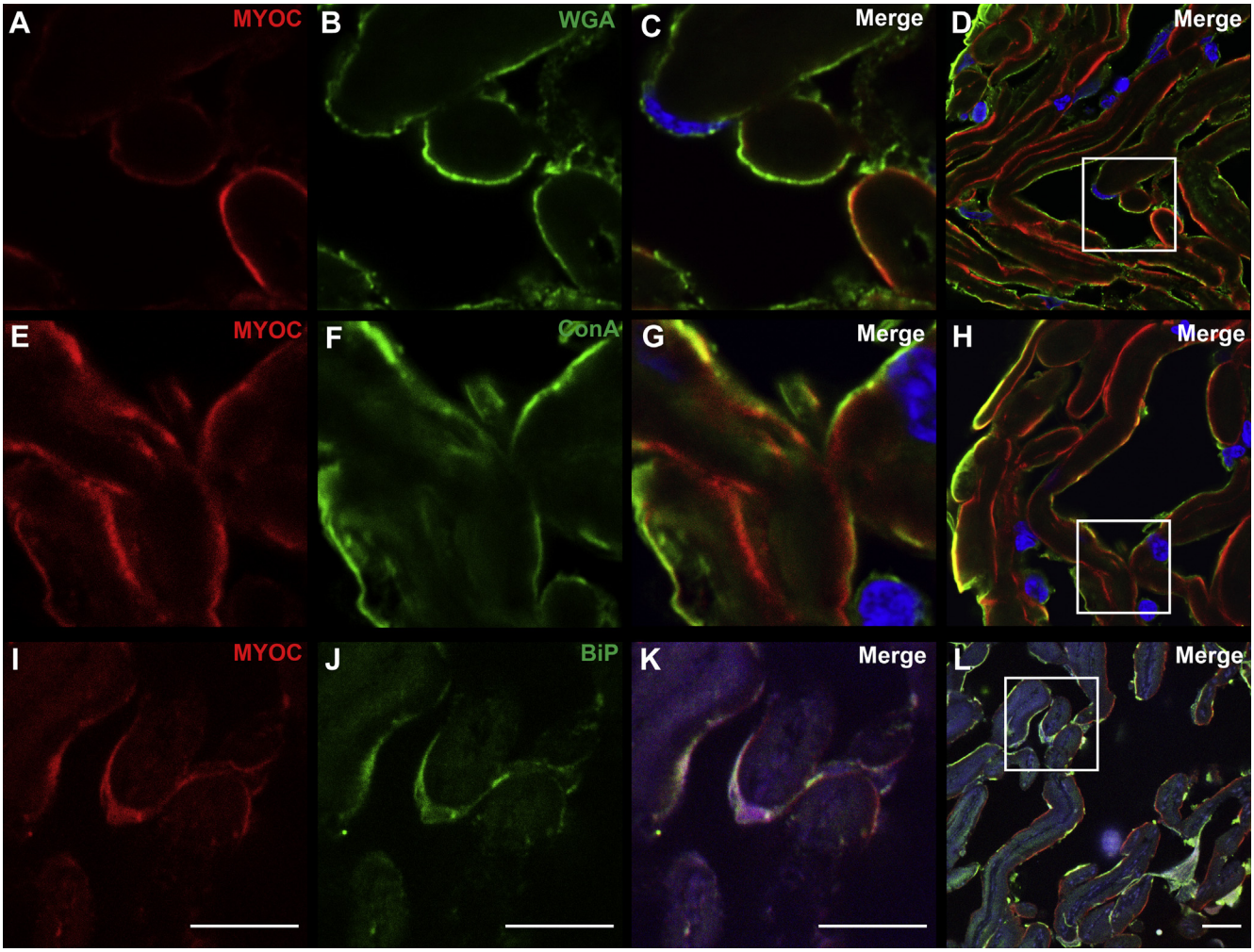

Figure 2. Confocal microscopy of myocilin (MYOC) protein within trabecular meshwork cells of an eye with a MYOC Tyr437His mutation. Confocal microscopy shows MYOC immunoreactivity in the trabecular meshwork of the eye with a MYOC Tyr437His mutation that colocalizes with 2 markers for trabecular meshwork cells, (**A**–**D**) wheat germ agglutinin (WGA) lectin and (**E**–**H**) concanavalin (ConA) lectin. Colabeling suggests localization of MYOC within trabecular meshwork cells. **I**–**L**, Myocilin immunoreactivity also colocalizes with GRP78 (BiP), a marker of endoplasmic reticulum stress. Cell nuclei are labeled with 4′-6-diamidino-2-phenylindole (blue). Scale bars = 10 μm.

right eye. A Baerveldt drainage device was placed to improve control of the intraocular pressure. Along with the drainage tube, a corneal transplant and an anterior chamber intraocular lens implant also were placed in an attempt to maximize and rehabilitate his vision. Despite these efforts, the vision in his right eye was limited to hand movements. At 59 years of age, the patient returned to the clinic with bare light perception and pain in his right eye. He was diagnosed with another intraocular infection (endophthalmitis) and was treated with antibiotics and topical steroid. The infection was treated successfully; however, the patient experienced persistent pain with bare light perception vision. Seventeen days after the onset of the infection, his right eye was removed for pain control. The eye underwent histopathologic examination and research analysis.

Immunohistochemistry of the Trabecular Meshwork from the *MYOC* Glaucoma Patient

The *MYOC* glaucoma patient's eye and that of an age-matched normal control eye were fixed identically in formalin, dissected, and analyzed for the presence of MYOC protein using various immunocytochemical approaches. Attention was focused on the trabecular meshwork, the primary site for fluid drainage from the eye, which is located at the juncture of the cornea and iris (the iridocorneal angle). We detected intense labeling of MYOC protein in the trabecular meshwork of the patient's eye with the Tyr437His mutation (Fig 1E, I, M), whereas we detected no MYOC in the normal control eye (Fig 1F, J, N). We confirmed that more MYOC was present in the trabecular meshwork of a *MYOC* glaucoma patient than in control eyes by examining more control eyes. Much less MYOC protein was detected in the trabecular meshwork of an additional 4 eyes from control participants (representative images shown in Figs 1G, H, K, L, O and P). Specificity of the MYOC antibody was confirmed by preincubation of the antibody with a ×10 excess of recombinant MYOC protein (H00004653-P01; Novus Biologicals, San Diego, CA; Fig S1, available at www.ophthalmologyglaucoma.org).

We further localized MYOC within the trabecular meshwork of the *MYOC* glaucoma patient's eye by colabeling with an

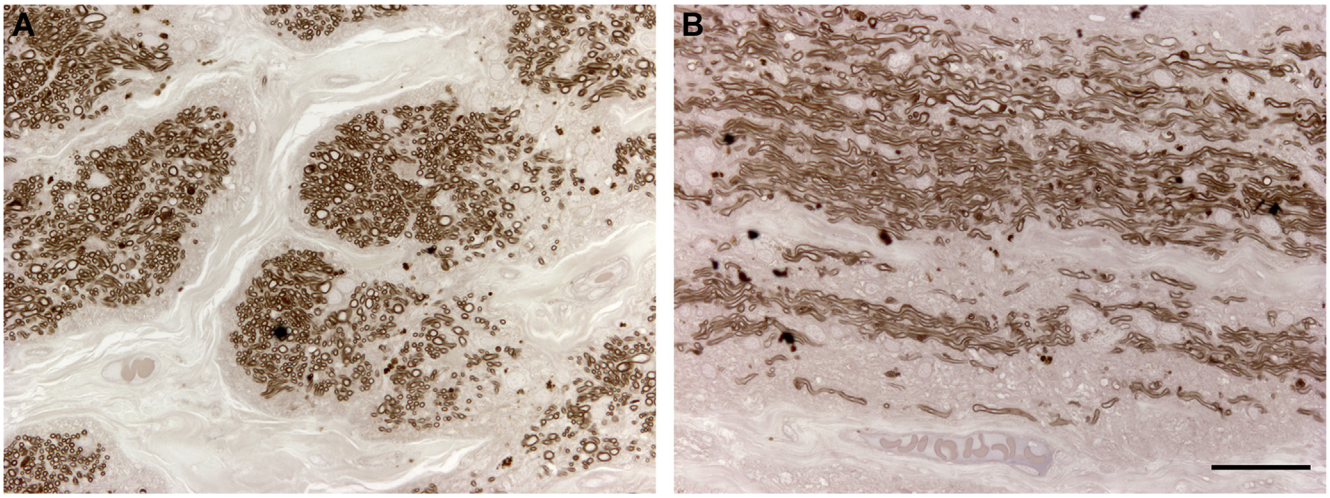

Figure 3. Photomicrographs showing histologic features of the optic nerve of an eye with a myocilin (MYOC) Tyr437His mutation. The myelin sheaths of optic nerve axons were stained with paraphenylenediamine. Extensive loss of axons caused by severe glaucoma is seen in (**A**) cross-section and (**B**) sagittal section of the patient's optic nerve. Scale bar = 100 μm.

antibody directed against collagen IV (COL4A). Collagen IV has been localized previously in the trabecular meshwork to the basal lamina between the trabecular meshwork cells and the trabecular beams, which largely are composed of collagen I, collagen III, and elastin.[25] The anti-COL4A antibody labeled the surface of the trabecular beams in a pattern consistent with the basal lamina (Fig 1I–P). Colabeling of MYOC and COL4A showed virtually no overlap, suggesting that MYOC is localized within the trabecular meshwork cells that line the surface of the trabecular beams.

We investigated MYOC localization within trabecular meshwork cells using 2 lectins, wheat germ agglutinin and concanavalin A, that have been shown previously to label trabecular meshwork cells.[26–28] We observed extensive colabeling of MYOC with wheat germ agglutinin along the surface of the collagen beams in a pattern consistent with localization of MYOC with trabecular meshwork cells (Fig 2A–D). Labeling with concanavalin A produced the same pattern of intracellular localization of MYOC (Fig 2E–H). Given prior reports demonstrating accumulation of mutant MYOC in the ER,[21] we further studied MYOC localization in trabecular meshwork cells using antibodies against the ER marker GRP78 (BiP). We detected colabeling of GRP78 and MYOC in trabecular meshwork cells of the *MYOC* glaucoma patient's eye (Fig 2I–L).

Finally, given the history of recent ocular infection, we tested for the presence of leukocytes in the trabecular meshwork by labeling with an anti-CD45 antibody. Although leukocytes were notable in the anterior chamber of this donor eye compared with controls, we observed no colabeling of MYOC and CD45 (Fig S2, available at www.ophthalmologyglaucoma.org), further indicating that MYOC is localized within trabecular meshwork cells rather than in infiltrating leukocytes.

Optic Nerve Histologic Analysis

We investigated the effects of glaucoma caused by the Tyr437His *MYOC* mutation on the *MYOC* glaucoma patient's optic nerve. Sections of the optic nerve were stained with paraphenylenediamine to visualize myelin sheaths surrounding axons (Fig 3). We detected a profound loss of axons in both cross-sections and longitudinal sections of the patient's optic nerve consistent with severe glaucoma.

Discussion

Human donor eyes from glaucoma patients with disease-causing *MYOC* mutations are extremely rare. Here we report a histologic study of a rare donor eye from a glaucoma patient with a *MYOC* mutation. Before histologic study, the patient's eye sustained trauma and inflammation from multiple surgeries, intraocular infection, and prior retinal detachment. Each of these events may have caused anatomic or functional changes in the trabecular meshwork of the patient's eye that may have influenced MYOC production, localization, or both. Moreover, MYOC production is known to be stimulated by steroids,[17] and the patient received topical steroid therapy for 17 days before removal of his eye. Steroid induction of MYOC production may have contributed to the increased amount of MYOC detected in the patient's eye. Given his complex clinical history with potential artifactual influences on MYOC protein production and localization, caution must be exercised in interpreting the results. Similarly, one control eye showed a small amount of MYOC in its trabecular meshwork cells and came from an individual who received systemic steroids before death (control 3), which may have altered MYOC expression in this eye. Despite these limitations, we believe that our studies of a rare human donor eye with a glaucoma-causing *MYOC* mutation have provided valuable insights into the pathogenesis of *MYOC*-associated glaucoma.

Previous studies of *MYOC* in human eyes have been limited to donor eyes (not known to carry a *MYOC* mutation)[18] and to surgical samples of aqueous humor and trabeculectomy specimens.[29,30] Here we present a pathologic analysis of

whole tissue from a rare human donor eye with glaucoma caused by a *MYOC* mutation. Our study of this eye provides compelling support for our model of *MYOC*-associated glaucoma that was based primarily on studies of transgenic animals and cell lines. Transfection or viral transduction of cultured human trabecular meshwork cells with *MYOC* vectors previously showed that wild-type MYOC protein is secreted, whereas mutant MYOC protein is retained within cells[29] and stimulates ER stress.[20] The same pattern of nonsecretion or retention of mutant MYOC within the trabecular meshwork cells also has been observed in transgenic Tg-*MYOC*$^{Tyr437His/+}$ mice[21] and in mice whose eyes were transduced with adenovirus carrying a mutant *MYOC* gene.[31] It has been hypothesized that intracellular retention of abnormal MYOC may be toxic to trabecular meshwork cells, may stimulate ER stress, and ultimately may lead to trabecular meshwork cell death. Such loss of trabecular meshwork cells and intraocular pressure regulation may be an important early step in glaucoma related to *MYOC* mutations. Herein, we showed that a naturally occurring *MYOC* mutation in a patient's genome caused intracellular retention of MYOC protein in the ER of trabecular meshwork cells of a human eye. Mislocalization and accumulation of MYOC within trabecular meshwork cells was a key step in the pathophysiologic features of glaucoma in our patient with glaucoma caused by a *MYOC* mutation. Moreover, colocalization of MYOC with an ER stress molecule, GRP78 (BiP), provided additional support for the hypothesis that ER stress may be involved in the mechanisms by which mutant *MYOC* promotes glaucoma.

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No animal subjects were included in this study.

Author Contributions:

Conception and design: van der Heide, Alward, Flamme-Wiese, Riker, Anderson, Stone, Mullins, Fingert

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Abbreviations and Acronyms:
ER = endoplasmic reticulum; **JOAG** = juvenile-onset open-angle glaucoma; **MYOC** = myocilin; **PBS** = phosphate-buffered saline.

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