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Mice Homozygous for a Deletion in the Glaucoma Susceptibility Locus *INK4* Show Increased Vulnerability of Retinal Ganglion Cells to Elevated Intraocular Pressure

Shan Gao^{*†} and Tatjana C. Jakobs[†]

From the Department of Ophthalmology,* The First Affiliated Hospital, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi, China; and the Department of Ophthalmology,[†] Massachusetts Eye and Ear Infirmary/Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts

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Address correspondence to
Tatjana C. Jakobs, M.D.,
Schepens Eye Research Institute,
20 Staniford St., Boston,
MA 02114. E-mail: tatjana_jakobs@meei.harvard.edu.

A genomic region located on chromosome 9p21 is associated with primary open-angle glaucoma and normal tension glaucoma in genome-wide association studies. The genomic region contains the gene for a long noncoding RNA called *CDKN2B-AS*, two genes that code for cyclin-dependent kinase inhibitors 2A and 2B (*CDKN2A/p16^{INK4A}* and *CDKN2B/p15^{INK4B}*) and an additional protein (*p14^{ARF}*). We used a transgenic mouse model in which 70 kb of murine chromosome 4, syntenic to human chromosome 9p21, are deleted to study whether this deletion leads to a discernible phenotype in ocular structures implicated in glaucoma. Homozygous mice of this strain were previously reported to show persistent hyperplastic primary vitreous. Fundus photography and optical coherence tomography confirmed that finding but showed no abnormalities for heterozygous mice. Optokinetic response, electroretinogram, and histology indicated that the heterozygous and mutant retinas were normal functionally and morphologically, whereas glial cells were activated in the retina and optic nerve head of mutant eyes. In quantitative PCR, *CDKN2B* expression was reduced by approximately 50% in the heterozygous mice and by 90% in the homozygous mice, which suggested that the *CDKN2B* knock down had no deleterious consequences for the retina under normal conditions. However, compared with wild-type and heterozygous animals, the homozygous mice are more vulnerable to retinal ganglion cell loss in response to elevated intraocular pressure. (*Am J Pathol* 2016, 186: 985–1005; <http://dx.doi.org/10.1016/j.ajpath.2015.11.026>)

Family history is a major risk factor for the development of primary open-angle glaucoma (POAG),¹ but the search for genes that influence disease susceptibility has been difficult. Several genes that are known to cause rare early onset familial forms of glaucoma, such as myocilin (*MYOC*) on chromosome 1q21-q31² or optineurin (*OPTN*) on chromosome 10p14-p15.³ However, collectively these genes account for only about 5% of adult onset POAG.⁴ Most cases are believed to be polygenic in origin or caused by an interplay of genetic and environmental factors.^{5,6}

The recent decade has brought several systematic attempts to identify genetic loci that may account for most glaucoma cases by genome-wide association studies (GWASs) or other genome-surveying methods such as whole-genome (or whole-exome) sequencing of nuclear or

mitochondrial DNA.^{7–19} GWASs can generate false-positive findings that are not reproducible in further studies, so candidate genomic associations are treated with caution until they have been confirmed in different cohorts and different populations. Several candidate glaucoma-associated loci have stood this test and are now widely accepted to confer a relative risk of developing the disease.²⁰ One of these is located on human chromosome 9p21.3,

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Figure 1 Schematic of *CDKN2BAS* and adjacent regions on human and mouse. **A:** Human chromosome 9p21 region and SNPs found to be most significantly associated with POAG and NTG in GWASs. **B:** Orthologous region on mouse chromosome 4. A 70-kb deletion area of exon 4 to 6 in *AK148321* and adjacent intronic sequences is shown in the box. **C:** Chr4Δ70 kb after targeted deletion. Panel **A** is adapted from Ng et al²⁰ with permission from John Wiley & Sons Publishing. The localization of the glaucoma-relevant SNPs was partially adapted from Wiggs et al¹⁴ with permission from PLoS. Panel **B** is adapted from Visel et al⁵¹ with permission from Nature Publishing Group. Panel **C** is adapted from Chidlow et al⁵⁵ with permission from PLoS One. GWAS, genome-wide association study; NTG, normal tension glaucoma; POAG, primary open-angle glaucoma; SNP, single nucleotide polymorphism.

near cyclin-dependent kinase inhibitor 2B (*CDKN2B*). It was first reported to be associated with increased vertical cup-to-disc ratio (a known morphometric indicator of glaucoma²¹) in a European cohort,¹² and verified in an American study.²² These findings suggested that the same locus is associated with POAG, and several large GWASs on different continents have indeed shown highly significant associations between POAG and single nucleotide polymorphisms (SNPs) on chromosome 9p21.3 in different populations.^{8–11,14,23} The locus was also associated with normal tension glaucoma in several studies, suggesting that genetic variants in this region may primarily affect optic nerve susceptibility in glaucoma.^{10,14,24,25}

The Ink4 locus on chromosome 9p21.3 contains several genes (Figure 1). *CDKN2A* and *CDKN2B* are located next to each other in a region of about 80 kb in length. The *CDKN2A* gene also encodes an additional protein, p14^{ARF} (alternative reading frame), that uses an alternative first exon.²⁶ Partially overlapping with *CDKN2B* is the gene for a long noncoding RNA that is transcribed in the opposite direction, *CDKN2B-AS* (for antisense), often referred to as *ANRIL* (antisense noncoding RNA in the Ink4 locus), whose function is not yet well understood.²⁷ This arrangement is flanked by the genes for methylthioadenosine phosphorylase (*MTAP*) and doublesex and Mab3-related transcription factor A1 (*DMRTA1*). *CDKN2A* and *CDKN2B* encode tumor suppressor proteins (p16^{INK4A} and p15^{INK4B}, respectively) that inhibit cell cycle progression by forming complexes with cyclin-dependent kinase (CDK) 4 or CDK6. *CDKN2B* is up-regulated by transforming growth factor (TGF)-β and may mediate the growth-arresting activity of this cytokine.^{28,29}

Glaucoma was not the first disease to call attention to 9p21.3. Several GWASs have identified the same locus, albeit not usually the same SNPs, as being associated with cardiovascular disease, myocardial infarction, aneurysms, type 2 diabetes, glioma, and other types of cancer.^{20,30,31} Intriguingly, the SNPs

associated with POAG that were identified in GWASs localize to the antisense RNA *CDKN2B-AS* or its introns. This raises the question whether *CDKN2B-AS* plays a role in the pathogenesis of glaucoma, and, if so, what the mechanism might be. *CDKN2B-AS* can interact with components of the polycomb repressor complex 1 and 2 and can mediate transcriptional silencing of the Ink4 locus.³² Most SNPs fall into the intronic sequences of *CDKN2B-AS* and may influence the expression levels or the splicing pattern of the RNA.^{14,33} Several splice variants of *CDKN2B-AS* have been identified, but their function is as yet unclear.³⁴ One of these has been associated with POAG.¹⁴

Ink4 locus variants may result in dysregulation of *CDKN2B-AS*, and subsequent changes in TGF-β regulation. Up-regulation of TGF-β1 and TGF-β2 was reported in glaucomatous tissue from the anterior eye,^{35,36} in the aqueous humor,^{37–40} and in the optic nerve head.^{41,42} The TGF-β1 pathway is predicted to be activated in several models of glaucoma and optic nerve damage.^{43–48} Given that TGF-β induces the expression of *CDKN2B* and that *CDKN2B-AS* silences the transcription of the Ink4 locus, the two may be functional antagonists; in that case altered expression of *CDKN2B-AS* could lead to increased activity of the CDK inhibitor *CDKN2B*, making retinal ganglion cells more vulnerable to apoptosis in glaucoma.²⁰ However, other mechanisms are possible. First, *CDKN2B-AS* might also regulate genes outside the Ink4 locus that have an influence on ganglion cell fate. Second, SNPs in this chromosomal region could affect the binding of transcription factors and transcriptional regulators in a manner independent of *CDKN2B-AS*.^{31,49} Finally, SNPs could alter binding sites for microRNAs and thereby disrupt a post-transcriptional regulatory network.⁵⁰

To elucidate the role of the Ink4 locus in cardiovascular disease, Visel et al⁵¹ created a transgenic mouse model in which 70 kb of the murine chromosome 4, syntenic to human chromosome 9p21, are deleted.⁵¹ This leads to a loss

of exons 4 to 6 of the murine equivalent to *CDKN2B-AS* (in the mouse called *AK148321*) (Figure 1, B and C). The homozygous mice ($\text{Chr4}^{\Delta 70\text{kb}/\Delta 70\text{kb}}$) were susceptible to developing tumors and gained more weight than their wild-type (WT) littermates, and primary cultures from their aortic smooth muscle cells showed increased proliferation and delayed senescence.⁵¹ Furthermore, in cardiac tissue the expression levels of *CDKN2A* and *CDKN2B* were significantly reduced.⁵¹ The homozygous mice were later found to have an ocular phenotype that resembled persistent hyperplastic primary vitreous.⁵² This phenotype may be due to the lower expression of the p14^{ARF} (in the mouse p19^{ARF}) gene in the developing vitreous, because the $\text{Chr4}^{\Delta 70\text{kb}/\Delta 70\text{kb}}$ mouse phenocopies an *ARF* knockout.^{53,54}

We used the $\text{Chr4}^{\Delta 70\text{kb}/\Delta 70\text{kb}}$ mouse (hereafter referred to as $\Delta 4\text{C4-C5}$) to ask whether the deletion of parts of *CDKN2B-AS* (*AK148321*) leads to discernible phenotype in ocular structures implicated in glaucoma, including the anterior eye, retina, and optic nerve head. In addition, we tested whether mice homozygous or heterozygous (HET) for this deletion show increased susceptibility to elevated intraocular pressure (IOP).

Materials and Methods

Animals

All animal studies were performed in accordance to the Association for Research in Vision and Ophthalmology guidelines for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the Schepens Eye Research Institute. Mice were group housed under 12-hour dark and light cycles and received water and food *ad libitum*. The transgenic strain with a 70-kb deletion of murine chromosome 4 ($\text{Chr4}^{\Delta 70\text{kb}/\Delta 70\text{kb}}$, hereafter referred to as $\Delta 4\text{C4-C5}$) was obtained from Mutant Mouse Resource and Research Centers at the University of California at Davies [129S6/SvEvTac-Del (4C4-C5)1Lap/Mmucd, stock no. 032091-UCD]. The strain was created by a targeted deletion of 70 kb on murine chromosome 4 that eliminates exons 4 to 6 of the mouse ortholog of *CDKN2B-AS* (*AK148321*) and some adjacent intronic sequences.⁵¹ For a schematic overview see Figure 1, A–C.^{14,20,51,55} The mice are viable and fertile and were maintained as HET breeders to produce homozygous mutant (MUT), HET, and WT offspring. Male and female mice between 3 and 5 months were used.

Genotyping

PCR products were purified with a fast purification system Nucleospin Extract II (Macherey & Nagel, Dueren, Germany) for nucleic acids.⁵⁶ The primers used to amplify fragments of the *AK148321* gene were as follows: 5'-AAGGTATCC-TAAATTGTCTTCTTGCAG-3', 5'-CGAGTCAATTTTCT-TCATGTTTATCCTCCA-3', 5'-CGTAATGTCTATAGGG-

CG-3', and 5'-TATGAAAGCTTGTGGGCGTGT-3'. The sizes of the amplicons for WT and MUT mice were 180 and 236 bp, respectively.

Slit Lamp Photography

Slit lamp photographic images were captured with an IMAGENet EZ Lite Software system version 1 (TOPCON, Oakland, NJ). Mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine. The doses of ketamine and xylazine are calculated for the mouse's body weight. Their pupils were dilated with 1% tropicamide (Bausch and Lomb, Tampa, FL).

Fundus Photography

Fundus photographic images were captured with Micron III retinal imaging system (Phoenix Research Laboratories, Pleasanton, CA). Mice were anesthetized, and their pupils were dilated as described in *Slit Lamp Photography*. GenTeal gel drops (Alcon, Fort Worth, TX) were used to maintain corneal moisture and clarity.

OCT

Anterior and posterior segment ultrahigh-resolution optical coherence tomography (OCT) images were obtained with an SD-OCT system (Bioptigen, Morrisville, NC). Mice were anesthetized as described in *Slit Lamp Photography*. Both averaged single B scan and volume scans were obtained with images centered on optic nerve head (posterior segment) or cornea (anterior segment).

IOP Measurement

IOP was measured noninvasively in both eyes. After the mouse was anesthetized lightly with isoflurane (1.5% delivered via a nosecone), IOP was measured with a rebound tonometer (iCare, Espoo, Finland). The instrument takes five individual measurements and gives the mean as one reading. Five such readings were taken from each eye, and the average was calculated. For microbead injection, IOP was measured 1 day before injection and every 3 to 4 days afterward. Measurements were conducted at the same time in the morning for all genotypes.⁵⁶

Optokinetic Response

The optomotor reflex-based spatial frequency threshold test was used to estimate the visual acuity of mice.^{57,58} Individual freely moving mice were placed on a 2-inch diameter circular platform located in the middle of an arena surrounded by four computer monitors arranged in a quadrangle. The monitors displayed a moving vertical black and white sinusoidal grating pattern, which mimic a virtual rotating cylinder. Each eye could be tested separately. Clockwise and counterclockwise direction of rotation of the grating provided an independent

temporal to nasal stimulation of the left and right eye, respectively. Rotation speed ($12^\circ/\text{sec}$) and contrast (100%) were kept constant. Spatial frequencies ranged between 0.02 and 0.46 cycle/degree. Distance between bars ranged between 350 and 11 mm (Supplemental Table S1). One performer determined the direction of bar rotation and the distance between bars. One observer, who was unable to see the direction of the bars, monitored the tracking behavior of the mouse. Tracking was considered positive when there was a reproducible smooth pursuit of the head or rotation of the body in the direction concordant with the stimulus. The highest spatial frequency tracked in either direction was recorded as the visual acuity. Measurements were conducted blind to the genotype of the mice.

ERG

Full-field electroretinogram (ERG) recording was performed with Diagnosys LLC ColorDome system (Lowell, MA) under red dim light ($<600\text{ nm}$). Animals were dark adapted overnight ($>12\text{ hours}$) and anesthetized, and their pupils were dilated as described in *Slit Lamp Photography*. Corneal anesthesia was achieved with 0.5% proparacaine hydrochloride (Akorn, Lake Forest, IL). Signals were recorded in both eyes simultaneously with the use of gold wire loop electrodes (Diagnosys LLC). Active electrodes were precoated with GenTeal gel drops and placed on the center of each cornea. A reference electrode was inserted in the mouth, and a ground electrode was inserted into the tail. Mice were placed in front of a Ganzfeld bowl and subjected to a series of increasing-intensity light flashes.⁵⁹

For scotopic threshold response (STR) recordings, 50 to 60 responses were averaged per flash intensity. Intensities ranged from -5.5 to $-3\text{ log cd.s.m}^{-2}$ with an interstimulus interval of 2 seconds. For scotopic response recordings, 2 to 20 responses were averaged per intensity. Intensities ranged from -3.60 to $2.13\text{ log cd.s.m}^{-2}$ with an interstimulus interval of 5 to 60 seconds between flashes. After completion of the scotopic ERG intensity series, animals were light-adapted for 7 minutes to a steady white background. For photopic flash responses, 25 responses were averaged per flash intensity. Intensities ranged from -1 to $2.01\text{ log cd.s.m}^{-2}$ with an interstimulus interval of 2 seconds.⁶⁰

One month after IOP elevation induced by microbead injection, STR ERG was performed again under the same conditions to compare the positive STR (pSTR) amplitude loss.

Microbead Injection

A chronic elevation of IOP was achieved by unilateral anterior chamber injection of $15\text{ }\mu\text{m}$ polystyrene microbeads (Invitrogen, Carlsbad, CA). This method was previously used in several studies.^{61–63} After the cornea of anesthetized mice was gently punctured with a 30-1/2-gauge needle, a glass micropipette attached to a Hamilton syringe was

inserted into the initial puncture, and positive pressure was applied to create an air bubble. Then the micropipette was removed and reinserted to inject 2 to $3\text{ }\mu\text{L}$ of microbeads suspended in phosphate-buffered saline (PBS; final concentration, 2.7×10^7 beads per mL). The injected eye of the mouse was kept level for an even distribution of the microbeads within the anterior chamber during the recovery from anesthesia. The uninjected eye served as a contralateral control. PBS ($2\text{--}3\text{ }\mu\text{L}$) was injected into another batch of 11 mice as a sham control.

Tissue Harvest and Preparation

Mice were euthanized by carbon dioxide, followed by cervical dislocation, according to procedures approved by institutional animal care and use committee of the Schepens Eye Research Institute. To obtain the eye with the optic nerve without causing structural damage, the skull was opened, and the brain was carefully removed to expose the optic nerve and chiasm.⁶⁴

If the tissue was to be used for histology and immunohistochemistry, the head of the mouse was fixed in 4% paraformaldehyde for 2 hours at room temperature and then rinsed in PBS. After fixation, the orbital bones, retro bulbar tissue, and ocular muscles were removed to isolate the eyeball and attached optic nerve. The dura surrounding the optic nerve was delicately removed. For histology and hematoxylin and eosin staining, the whole eyeball together with the optic nerve was fixed in 4% paraformaldehyde at 4°C overnight. After rinsing, the tissue was embedded in methacrylate and was sectioned in the mid equatorial through optic disk level. For immunohistochemistry, the cornea was cut off along the limbus, the lens and vitreous were removed, and the sclera and pigment epithelium were peeled away from the neural retina. The optic nerve was gently detached from the retina, and the remaining sclera was removed. The cup-shaped retina and optic nerve head were embedded in 6% agarose. The unmyelinated portion of the optic nerve was sectioned at $100\text{-}\mu\text{m}$ thickness in transverse orientation. The retina was sectioned at $100\text{-}\mu\text{m}$ thickness in vertical orientation. For retinal cell counts, four small radial incisions were made in the retina, so that the retina could be flat-mounted ganglion side up on AA nitrocellulose filters (MF-Millipore, Billerica, MA). Sections or whole-mounted retinas were stored in a 24-well tissue culture plate (one section or retina per well) in PBS at 4°C until further use.⁴⁷

If the tissue was to be used for paraphenylenediamine staining, the optic nerve was fixed with half-strength Karnovsky's fixative (2% formaldehyde/2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4; Electron Microscopy Sciences, Hatfield, PA) overnight at 4°C . Tissue was then rinsed in 0.1 mol/L sodium cacodylate buffer and post-fixed with 2% osmium tetroxide. The tissue was dehydrated in a graded ethanol series and embedded in tEPON-812 resin (Tousimis, Rockville, MD). Semithin sections were cut at $1\text{ }\mu\text{m}$.

If the tissue was to be used for RNA extraction, the eyeball with optic nerve attached was enucleated and dissected in prechilled diethyl pyrocarbonate (Sigma-Aldrich, St. Louis, MO) treated PBS. The retina and the optic nerve were stored in RNAlater (Ambion, Austin, TX) at 4°C until further use.⁶⁵

Histology and Immunohistochemistry

Methacrylate-embedded retina sections were stained with hematoxylin and eosin (Sigma-Aldrich). Agarose retina and optic nerve head sections were incubated in blocking solution (5% donkey serum, 0.3% Triton X-100 in PBS) in a 24-well tissue culture plate with gentle shaking for 1 hour at room temperature. Afterward, primary antibodies were applied for 3 to 5 days at room temperature. The primary antibodies used were chicken anti–glial fibrillary acidic protein (GFAP; dilution 1:1000; Abcam, Cambridge, MA), rabbit anti–ionized calcium-binding adaptor molecule 1 (Iba1; dilution 1:200; Wako Pure Chemical Industries, Richmond, VA), rabbit anti– β -III-tubulin (dilution 1:200; Cell Signaling Technology Inc., Danvers, MA), mouse anti-Brn3a (dilution 1:200; EMD Millipore, Billerica, MA), rabbit anti-Calbindin (dilution 1:100; Swant, Marly, Switzerland), rabbit anti–glutamine synthetase (GS; dilution 1:5000; Sigma-Aldrich), goat anti–choline acetyltransferase (dilution 1:200; EMD Millipore), rabbit anti-PKC α (dilution 1:400; Biosource International, Camarillo, CA), rabbit anti-Recoverin (dilution 1:1000; EMD Millipore), rabbit anti-Synaptophysin (dilution 1:900; Abcam), and rat anti-endomucin (dilution 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Sections were washed in PBS and incubated with secondary antibodies conjugated to rhodamine (dilution 1:400; donkey anti-rabbit and donkey anti-mouse), fluorescein isothiocyanate (FITC; dilution 1:400; donkey anti-chicken, and donkey anti-goat), or FITC (dilution 1:400; goat anti-rat IgG; Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at room temperature. Finally, retinas were incubated with the nuclear dye DAPI (Life Technologies, Grand Island, NY) for 10 minutes, washed in PBS, and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

The hematoxylin and eosin-stained slides were imaged with 4 $\times$, 20 $\times$, and 40 $\times$ objectives of an Olympus BX51 microscope system (Olympus, Melville, NY). The immunohistochemistry slides were imaged with a 63 $\times$ glycerol immersion objective of a Leica TCS SP5 confocal microscope system (Leica Microsystems, Buffalo Grove, IL). The samples were imaged and processed in exactly the same way to ensure the final images between genotypes were comparable. Final images were prepared with the Photoshop CS6 program (Adobe, Waltham, MA).

Retinal Whole Mount Staining and Cell Counts

For whole retina immunohistochemistry, incubations were also conducted in 24-well tissue culture plates with gentle

shaking at 4°C. Retinas were first blocked in blocking solution (5% donkey serum, 0.3% Triton X-100) for 1 hour, followed by incubation in primary antibodies for 3 to 4 days. The primary antibodies used were rabbit anti– β -III tubulin (dilution 1:200; Cell Signaling Technology Inc.), mouse anti-Brn3a (dilution 1:200; EMD Millipore), rabbit anti-Iba1 (dilution 1:200; Wako Pure Chemical Industries), and rat anti-Endomucin (dilution 1:100; Santa Cruz Biotechnology, Inc.). Subsequently, retinas were washed in PBS and incubated in secondary antibodies conjugated to rhodamine (dilution 1:400; donkey anti-mouse IgG), rhodamine (dilution 1:400; donkey anti-rabbit IgG), FITC (dilution 1:400; donkey anti-rabbit IgG), or FITC (dilution 1:400; goat anti-rat IgG; Jackson ImmunoResearch Laboratories), for 2 to 3 days. Finally, retinas were incubated with the nuclear dye DAPI (Life Technologies) for 10 minutes, washed in PBS, and mounted in Vectashield (Vector Laboratories).^{62,66}

For ganglion cell counting, images of whole mount retinas were acquired on a 63 $\times$ glycerol immersion objective of Leica TCS SP5 confocal microscope system (Leica Microsystems). The retina was divided into quadrants, and two mid-peripheral regions (defined as equidistant to the optic nerve head and the border of the retina) were imaged per quadrant, for a total of eight images per retina (246.03 μ m by 246.03 μ m per region in size). Images were obtained as z-stacks (step size, 0.5 μ m). All cells in the ganglion cell layer that co-localized with both markers were counted, and the ganglion cell density per retina was obtained. For all eyes of the three genotypes, cells were counted manually by an individual blinded (T.C.J.) to the genotype of the animal in ImageJ software version 1.48v (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>).⁴⁷

For the counting of microglial cells, image stacks of whole-mounted retinas were acquired on a 20 $\times$ (Zoom 1.7) glycerol immersion objective of Leica TCS SP5 confocal microscope system. The retina was also divided into quadrants and one mid-peripheral region was imaged per quadrant, for a total of four images per retina (480 μ m by 480 μ m per region). Microglial cells were counted manually by an individual blinded (T.C.J.) to the genotype of the animal in ImageJ software.

PPD Staining of Optic Nerve Axons and Injury Evaluation

Paraphenylene diamine (PPD; MP Biomedicals, Hatfield, PA) stains degenerating and dying axons and has been used for optic nerve grading in glaucomatous animals.⁶⁷ An aqueous solution of 2% PPD was used to stain semithin cross-sections for 30 minutes. Images were acquired on an Olympus BX51 microscope with 20 $\times$ and 100 $\times$ objectives. Images from microbead-injected, saline-injected eyes and their contralateral eyes were masked and assessed by two independent observers. A graded scale of nerve injury that ranged from 1 (normal) to 5 (total degeneration) was used.⁶⁸

Table 1 Primers Used for Quantitative PCR

Primers	GenBank accession no.	Primer sequence (5'–3')	Amplicon size, bp
<i>CDKN2A</i> (Exon 1a-2)	NM_001040654	Forward: 5'-ATGGAGTCCGCTGCAGACA-3' Reverse: 5'-ATCATCACCTGAATCGGGGTA-3'	134
<i>ARF</i> (Exon 1b-2)	NM_009877	Forward: 5'-TTCTTGGTGAAGTTCGTGCGATCC-3' Reverse: 5'-CGTGAACGTTGCCCATCATCATCA-3'	149
<i>CDKN2B</i>	NM_007670	Forward: 5'-AGATCCCAACGCCCTGAAC-3' Reverse: 5'-TCGTGCACAGGTCTGGTAAG-3'	144
<i>MTAP</i>	NM_024433	Forward: 5'-TGGCAACCGACTATGATTGT-3' Reverse: 5'-CATTCCATCGACCCATCTCTGA-3'	139
<i>AK148321</i> (Exon 3)	AK148321	Forward: 5'-CAAGGCTGGCACCTGGGTAGATGTT-3' Reverse: 5'-TGGATGGGAGTGTGGTCTTCGTAGC-3'	127
<i>Tgfb1</i>	NM_011577	Forward: 5'-CTGCTGACCCCCACTGATAC-3' Reverse: 5'-AGCCCTGTATTCCGTCTCCT-3'	94
<i>Tgfb2</i>	NM_009367.3	Forward: 5'-AGTTCAGACACTCAACACACCAAAG-3' Reverse: 5'-CATTCCATCGACCCATCTCTGA-3'	107
<i>Tgfb3</i>	NM_009368	Forward: 5'-CCTGGCCCTGCTGAACCTTG-3' Reverse: 5'-TTGATGTGGCCGAAGTCCAAC-3'	75
<i>GAPDH</i>	NM_001289726	Forward: 5'-GGTTGTCTCCTGCGACTTCAA-3' Reverse: 5'-CCTGTTGCTGTAGCCGTATTCAT-3'	130

The Nucleotide database is available at <http://www.ncbi.nlm.nih.gov/nuccore>.

Each eye was then assigned a grade of injury determined by calculating the means of the two independent grade scores.

RNA Extraction and cDNA Preparation

Total RNA was extracted from retina with the use of the RNeasy plus mini kit (Qiagen, Valencia, CA), whereas the total RNA of the optic nerve was extracted with the RNeasy Plus Micro Kit (Qiagen) because of the small tissue volume, according to the manufacturer's instructions. RNA concentration and quality were assessed on BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). Only the RNA samples that had a RNA integrity number higher than 8 (mean, 9.40 ± 0.42) were used for cDNA synthesis. Twenty nanograms of RNA from the optic nerve and 1.2 μ g of RNA from the retina were reverse-transcribed with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. cDNA concentration and quality were assessed on NanoDrop 2000 (Fisher Scientific, Pittsburgh, PA).^{47,65} Quality and quantity of total RNA and cDNA are shown in [Supplemental Tables S2 and S3](#).

Quantitative PCR

The amplified cDNAs of optic nerves and retinas were diluted and used as a template for quantitative PCR. Quantitative PCR was performed with an Applied Biosystems Step One Plus Real-time PCR system and SYBR green master mix (Applied Biosystems, Foster City, CA) in a 10- μ L reaction volume according to standard procedures.⁶⁵ Melting curve analysis after SYBR green-based detection confirmed the specificity of amplification and the absence of nonspecific amplification and primer dimers. Genomic DNA was removed during RNA extraction with the use of a gDNA Eliminator spin column in the RNeasy

Plus Micro and Mini Kit, and primers that span intron/exon boundaries were used to avoid amplifying genomic DNA. *GAPDH* was used as a reference gene because the expression level in the retina and optic nerve are stable. The primer set for *ARF* was taken from a published study,⁵² and the other primers were designed with the primer design tool on the *Saccharomyces* Genome Database website (<http://www.yeastgenome.org/cgi-bin/web-primer>, last accessed October 30, 2015). Primer sequences are given in [Table 1](#).

Five biological replicates were used for each genotype in the normal control eye, and three biological replicates were used after microbead injection. All samples were run in triplicate with nontemplate controls. Because the concentration of total RNA from the retina and optic nerve are different because of the limited amount of tissue in the optic nerve, the dilution of cDNA is 1:10 for the retina and 1:5 for the optic nerve. For the genes in the 9p21 region, the concentration of primers was 1 μ mol/L for the retina and 5 μ mol/L for the optic nerve. For the *Tgfb1* to *Tgfb3* genes, the concentration of primers was 0.5 μ mol/L because of the low expression levels of the *Tgf* genes. The relative expression of target genes was obtained by the $2^{-\Delta\Delta C_T}$ method.

Data Analysis

Mean and SD were calculated, and data of the three types of mice were tested for statistically significant differences by one-way analysis of variance and *t*-tests.

After microbead injection, the IOPs in both eyes were measured twice weekly, and the area under the curve (IOP in mmHg versus time) was determined with a user-written Matlab routine. This area is referred to as the cumulative IOP (cIOP) because a measure of the total pressure insult the eye was subjected to during the time of the experiment (1 month). We

Figure 2 Clinical ocular features of PHPV phenotype in MUT mice. **A, D, G, and J:** WT; **B, E, H, and K:** HET; **C, F, I, and L:** MUT. **A–C:** Slit lamp images are normal in WT and HET eyes. The MUT eye is showing normal ocular surface and dense lens opacity. **D–F:** Fundoscopic images are normal in WT and HET eyes. Lens opacity obscures fundoscopic view in MUT eye. **G–I:** Anterior segment OCT images are normal in WT and HET eyes. CCT were measured for the whole layer of cornea in the central position (**G**, arrow), which show no significant difference among each genotype (**O**). Depth of anterior chamber was measured from the posterior of the cornea to the surface of the lens (**H**, arrow). The MUT mice have a deeper AC than WT and HET mice (**P**). **J–L:** Retinal OCT images are normal in WT and HET mice. Total retinal thickness measured the whole layer of retina (**J**, arrow). Inner retinal thickness combined ganglion cell layer, inner plexiform layer, and inner nuclear layer (**K**, arrow). The two thicknesses of the retina have no significant difference (**M** and **N**, *t*-test). The retinal detail of the MUT eye is blurred, and there is a membrane-like structure attached to the retina (**L**). **Q:** IOP of each genotype mice are normal and have no significant difference. Images are representative of 12 eyes per genotype. Data are expressed as means ± SD. *n* = 14 eyes in WT mice, 12 eyes in HET mice, 10 eyes in MUT mice. **P* < 0.05, ***P* < 0.01 (one-way analysis of variance). AC, anterior chamber; CCT, central corneal thickness; HET, heterozygous; IOP, intraocular pressure; MUT, mutant; OCT, optical coherence tomography; PHPV, persistent hyperplastic primary vitreous; WT, wild-type.

then calculated the Δ cIOP for each animal as the cIOP in the experimental eye minus the cIOP in the contralateral eye.⁶⁹ Ganglion cell loss was correlated with Δ cIOP to ensure that animals of all genotypes experienced the same amount of pressure injury. Ganglion cell losses after microbead injection were compared with a one-way analysis of variance.

Results

Clinical Features in Δ 4C4-C5 Mice

We compared the clinical ocular phenotype of 3- to 5-month-old WT (*n* = 14 eyes), HET (*n* = 12 eyes), and MUT (*n* = 10 eyes) Δ 4C4-C5 mice. The outward appearances of the WT and HET mice were normal. About

one-half of the MUT mice had microphthalmia. Eyes were assessed by slit lamp and fundus photography. Slit lamp examination demonstrated dense lens opacities in the MUT eyes (Figure 2C), whereas WT and HET eyes had normal ocular surface and lens structures (Figure 2, A and B). Lens opacity prevented fundoscopic analysis in MUT eyes (Figure 2F). The HET and WT eyes exhibited normal retinal pigmentation patterns, vascularization, and optic nerve head morphology (Figure 2, D and E).

In OCT, both anterior and posterior segments in the WT and HET eyes were normal (Figure 2, G, H, J, and K). The MUT eyes had normal corneas and irises (Figure 2I), but the retinas could not be imaged in detail because a membrane-like structure was attached to the retina and precluded visualization (Figure 2L). Total retinal thickness was

Figure 3 Pathologic features of PHPV phenotype in MUT mice. Photomicrograph of hematoxylin and eosin-stained eyes from HET (A and B) and MUT (C–E) mice. The lens architecture, vitreous space, and retina are normal in the HET eye. The MUT eye is showing misshapen lens (C, arrow). Retinal layers of MUT are normal (D) except some fibro tissue attached to the inner retina (D, arrowhead). The pigmented fibrovascular mass is within the posterior lens (E, arrow), and the membrane attached to the peripheral retina (E, arrowhead). Scale bars: 250 μ m (A and C); 25 μ m (B and D); 50 μ m (E). GCL, ganglion cell layer; HET, heterozygous; INL, inner nuclear layer; MUT, mutant; ONL, outer nuclear layer; PHPV, persistent hyperplastic primary vitreous.

measured on OCT images of the whole retinal layer. Inner retinal thickness combined ganglion cell layer, inner plexiform layer, and inner nuclear layer. Both were measured 500 μ m away from the optic nerve. The total retinal thickness was 211 ± 5 μ m for WT and 217 ± 7 μ m for HET (Figure 2M), the inner retinal thickness was 89.7 ± 3.3 μ m for WT and 89.6 ± 4.4 μ m for HET (Figure 2N). No significant difference was found between the WT and HET eyes for total and inner retinal thickness (*t*-test).

Because decreased central corneal thickness is a risk factor for developing glaucoma in patients with ocular hypertension,^{70,71} we measured the central corneal thickness in all genotypes by OCT (Figure 2O). Central corneal thickness was 116 ± 16 μ m for WT, 113 ± 18 μ m for HET, and 118 ± 5 μ m for MUT eyes (not significant, one-way analysis of variance). There was, however, a difference in the depth of anterior chamber as measured from the posterior of the cornea to the surface of the lens. In the MUT eyes the anterior chamber was 415 ± 131 μ m, deeper than in WT (321 ± 13 μ m) and HET (294 ± 53 μ m) eyes ($P < 0.05$ versus WT, $P < 0.01$ versus HET, one-way analysis of variance) (Figure 2P), which may be caused by the abnormal lens. Another risk factor for glaucoma is increased IOP,⁷² so we measured the IOP noninvasively in all three groups. The IOPs were 9.71 ± 1.86 mmHg for WT, 9.48 ± 1.61 mmHg for HET, and 8.44 ± 1.07 mmHg for MUT and showed no significant difference between genotypes (one-way analysis of variance) (Figure 2Q).

To correlate clinical findings with pathology, we obtained midline sagittal sections through the eyes of HET and MUT mice. In eyes taken from HET mice, the lens architecture,

posterior lens capsule, and vitreous space appeared normal (Figure 3A). As shown in the magnified image (Figure 3B), gross retinal anatomy was also normal. The lens of MUT eyes was misshapen, and the posterior lens capsular was not completely continuous (Figure 3C). Vascular-like structures were visible within the lens, and a pigmented fibro-vascular mass adhered to the posterior lens and the peripheral retina (Figure 3E). However, each layer of the retina was remarkably normal, and there were no overt signs of cell loss or degeneration (Figure 3D).

Ocular Gene Expression Changes in HET and MUT Mice

To investigate the effects of the 70-kb deletion on the neighboring genes, we extracted total RNA from retina and optic nerve and performed reverse transcription, followed by quantitative PCR, and compared the expression level of surrounding genes between WT, HET, and MUT mice. The relative expression levels were normalized to *GAPDH* and relative to the expression level of *MTAP* expression in the WT mice.

Compared with *MTAP*, the expression level of the *ARF* gene was extremely low in retina and optic nerve tissue (Figure 4). This suggests that contrary to its role in the developing vitreous, *ARF* probably does not play a major role in the posterior eye.⁵⁴

In the retinas of HET and MUT mice, the expression of *CDKN2B* was down-regulated (one-way analysis of variance: MUT versus WT, $P < 0.01$; HET versus WT, $P < 0.01$; HET versus MUT, $P < 0.05$), but no significant change was found in *CDKN2A* expression (Figure 4A). Likewise, in the optic nerve tissue in HET and MUT mice, the expression levels of

Figure 4 Deletion of the 70-kb interval affects expression of neighboring genes. **A:** In retina tissue, expression levels of *CDKN2B* in HET and MUT eyes are significantly reduced. Expression levels of *MTAP*, *CDKN2A*, and *ARF* genes are not significantly altered. **B:** In optic nerve tissue, expression levels of *CDKN2B* in HET and MUT eyes are significantly reduced; expression level of *CDKN2A* in MUT eyes is significantly increased. Expression levels of *MTAP* and *ARF* genes are not significantly altered. Data are normalized to *GAPDH* and relative to the expression level of the *MTAP* gene in WT eyes. Data are expressed as means $\pm$ SD. $n = 5$ eyes per genotype. * $P < 0.05$, ** $P < 0.01$ (one-way analysis of variance). HET, heterozygous; MUT, mutant; WT, wild-type.

CDKN2B were reduced (one-way analysis of variance: MUT versus WT, $P < 0.01$; HET versus WT, $P < 0.01$). However, *CDKN2A* was up-regulated significantly in MUT mice (one-way analysis of variance: MUT versus WT, $P < 0.05$; MUT versus HET, $P < 0.05$) (Figure 4B). When tested with primers that anneal in exon 3 of *AK148321* (proximal to the deletion), the expression of the noncoding RNA is not changed in HET and MUT retinas and optic nerves, agreeing with published results.⁵² In no case was the expression of the *MTAP* gene changed.

Retinas of the HET and MUT Eyes Are Anatomically Normal

We next asked whether the partial deletion of *AK148321* and the down-regulation of *CDKN2B* in the retina had any subtle effect on retinal anatomy. We stained vertical sections

of the retinas of HET and MUT eyes with various cell type-specific antibodies. Antibodies against recoverin labeled the outer nuclear layer (photoreceptors) and cone bipolar cells. Synaptophysin is a marker for the presynaptic elements of conventional and ribbon synapses. It labeled the outer and inner plexiform layers similarly in all genotypes. We also stained for horizontal cells (calbindin), rod bipolar cells (PKC α), cholinergic (starburst) amacrine cells (choline acetyltransferase), and ganglion cells (β -III-tubulin). In no case did we observe changes in the numbers or the morphology of these cell types (Figure 5, A–E, G–K, M–Q).

Because *CDKN2B-AS* and *CDKN2B* may be involved in the ganglion cell response to elevated IOP, we wanted to test whether the numbers of ganglion cells are reduced in HET and MUT mice compared with the WT mice. We double-labeled ganglion cells in retinal whole mounts with antibodies against β -III-tubulin and the transcription factor Brn3a. Ganglion cells were counted in WT, HET, and MUT eyes ($n = 7$ eyes for each genotype). The ganglion cell densities of WT, HET, and MUT eyes were similar ($4569 \pm 397/\text{mm}^2$, $4468 \pm 122/\text{mm}^2$, and $4512 \pm 339/\text{mm}^2$, respectively; no significant difference by one-way analysis of variance) (Figure 5, F, L, R, and S).

Because the 9p21 region also associated with cardiovascular disease,^{73,74} and because vascular smooth muscle cells from the MUT strain exhibit increased proliferation in tissue culture,⁵¹ we stained the retinal whole mounts with the use of an antibody against endomucin, which stains venous endothelium and capillaries,⁷⁵ and isolectin IB4, which stains all blood vessels, including arteries.⁷⁶ Blood vessels in WT, HET, and MUT retina appeared normal (Supplemental Figure S1).

Retinal Function of $\Delta 4C4$ -C5 Mice

The visual acuity of mice was estimated by optomotor reflex-based spatial frequency threshold test. The mean visual acuity of WT ($n = 14$ eyes) and HET ($n = 12$ eyes) mice was normal, with 0.36 ± 0.04 and 0.32 ± 0.05 cycle/degree, respectively. The MUT ($n = 10$ eyes) eyes had poorer visual acuity (0.11 ± 0.1 cycle/degree; $P < 0.01$ versus WT and HET, one-way analysis of variance) (Figure 6A). A large variation was found in the visual acuity in the MUT mice because five MUT eyes had poor acuity (<0.05 cycle/degree), whereas one MUT eye was almost normal (0.3 cycle/degree).

The finding that some MUT mice had almost normal acuity suggests that the visual acuity in this genotype is limited by the variation in lens opacity rather than a functional deficit of the retina itself. We therefore performed scotopic threshold ERG, scotopic ERG, and photopic ERG to investigate retinal and inner retinal function. The STR recently was shown to be directly dependent on intact retinal ganglion cell function.^{66,77–80} The STR contains a positive component, followed by a negative component, designated as the pSTR and nSTR,

Figure 5 The retinas of the HET and MUT eyes are anatomically normal. **A–F:** WT; **G–L:** HET; **M–R:** MUT. DAPI applied as nuclear dye. **A, G, and M:** Rabbit anti-Recoverin antibody labels retinal outer nuclear layer. **B, H, and N:** Rabbit anti-synaptophysin antibody labels retinal outer plexiform layer. **C, I, and O:** Rabbit anti-calbindin antibody labels horizontal cells. **D, J, and P:** Rabbit anti-PKC α antibody labels rod bipolar cells, goat anti-ChAT antibody labels cholinergic amacrine cells. **E, K, and Q:** Rabbit anti- β -III-tubulin antibody labels ganglion cells. **F, L, and R:** Images are representative of three eyes per genotype. Rabbit anti- β -III-tubulin antibody and mouse anti-Brn3a antibody was used to double label ganglion cells on the retinal flat mount. All of the above cells in HET and MUT retina have a normal cell morphology and distribution pattern. Images presented were single focal plane scans. **S:** The ganglion cell densities are normal and have no obvious difference among each genotype (one-way analysis of variance). Eight mid-periphery regions per retina were counted manually. Data are expressed as means $\pm$ SD. $n = 3$ eyes per genotype (**F, L, and R**); $n = 7$ eyes per genotype (**S**). CALB, Calbindin; ChAT, choline acetyltransferase; GC, ganglion cell; GS, glutamine synthetase; HET, heterozygous; Mut, mutant; RCVRN, Recoverin; SYP, Synaptophysin; TUBB3, β -III-tubulin; WT, wild-type. Scale bar = 50 μ m.

respectively. For scotopic response analysis, the a-wave amplitude was measured from baseline to the trough of the first negative wave, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the positive wave. For photopic response analysis, the b-wave amplitude was measured from the trough of the a-wave to the peak of the positive wave. The amplitudes of each wave are shown in Figure 6, B–E. In most cases the amplitudes of WT and HET eyes were similar and overlapped; however the amplitude of MUT eyes was lower. With the increase of the stimulus intensity, the difference of amplitudes between MUT eyes and the other two

groups was larger. We used one-way analysis of variance to test the differences of amplitudes at each stimulus intensity; for the pSTR wave, the differences were significant at the intensities larger than $-4.5 \log \text{cd.s/m}^2$; for scotopic a-wave, the differences were significant at intensities larger than $-2.4 \log \text{cd.s/m}^2$ ($P < 0.05$); for scotopic b-wave, the differences were significant at every stimulus intensity ($P < 0.05$); for photopic b-wave, the differences were significant at intensities larger than $0.8 \log \text{cd.s/m}^2$ ($P < 0.05$). The lower amplitude of MUT may result because the actual stimulus intensities were smaller due to the lens and the retrolental tissue.

Figure 6 Visual function features of the PHPV phenotype in MUT mice. **A:** Visual acuity assessed by OKR shows that the WT and HET mice have normal visual acuity and that MUT mice have much poorer visual acuity. **B–E:** Serial ERG amplitude versus stimulus intensity after dark and light adaptation. **B:** pSTR. **C:** Scotopic a-wave. **D:** Scotopic b-wave. **E:** Photopic b-wave. Amplitudes of each wave in WT and HET eyes are similar, whereas the MUT eyes have lower amplitudes. Data are expressed as means $\pm$ SD. $n = 14$ eyes in WT, 12 eyes in HET, 10 eyes in MUT. $^{**}P < 0.01$ (one-way analysis of variance). ERG, electroretinography; HET, heterozygous; MUT, mutant; PHPV, persistent hyperplastic primary vitreous; OKR, optokinetic reflex; pSTR, positive scotopic threshold response; VA, visual acuity; WT, wild-type.

Glial Cells Are Activated in the Retina and Optic Nerve Head of MUT Eyes

Activation of astrocytes in the optic nerve head was described in glaucoma.^{56,81} Therefore, we also looked into the glial cells in retina and optic nerve head area of three groups of mice.

GS is a Müller cell marker in the retina.⁸² In normal retinas, Müller cell fibers traverse the whole retinal thickness, and GS immunoreactivity is strongest within the cell soma (Figure 7A). The GS expression in HET retina looked similar to the WT retina (Figure 7D). In MUT retina, there was an increase in GS immunoreactivity in Müller glia endfeet, adjacent to ganglion cells (Figure 7G). In the WT retina, GFAP is localized to the Müller glia endfeet and astrocytes in the optic fiber layer (Figure 7B). The GFAP expression in HET retina appeared normal (Figure 7E). In MUT retinas, there was an increase in GFAP immunoreactivity in the optic fiber layer and in occasional thin processes of glia that spanned the inner plexiform layer (Figure 7H).

Iba1 immunoreactivity was used to examine the morphology and distribution of microglia in the retina.⁸³ In the retina of WT and HET eyes, Iba1 stained ramified microglia in the inner and outer plexiform layers (Figure 7, B and E). In the MUT retina, Iba1⁺ microglia showed more rounded cell morphology and thickened processes, features that are associated with microglial activation (Figure 7H). Cell densities of microglia were counted in the outer plexiform layer (mid-peripheral area) in WT ($n = 7$ eyes), HET

($n = 8$ eyes), and MUT ($n = 6$ eyes) eyes. Our results of WT and HET ($128.1 \pm 7.6/\text{mm}^2$, $136.4 \pm 6.7/\text{mm}^2$, respectively) were in agreement with a previous study.⁸⁴ The microglia densities of MUT eyes ($181.9 \pm 41.3/\text{mm}^2$) were higher than WT and HET (one-way analysis of variance, $P < 0.01$) (Figure 7, C, F, I, and J).

In the optic nerve head, GFAP⁺ astrocytes form the glial lamina and ensheath the unmyelinated axon bundles.⁶⁴ The GFAP expression in transverse optic nerve head sections of all three genotypes was normal (Figure 8, A, D, and G). In WT and HET mice, the Iba1⁺ cells of the nerve head showed the ramified morphology of surveilling microglia (Figure 8, B and E).⁸⁵ The Iba1-expressing microglia in optic nerve heads from MUT mice appeared to show a more rounded morphology and retraction of fine processes that is consistent with microglial activation (Figure 8H). We therefore took high-power image stacks through individual Iba1⁺ cells to show the morphology of individual microglial cells (Figure 8, C, F, and I).

Induction of Elevated IOP by Microbead Injection into the Anterior Chamber

We used the microbead occlusion model to induce a glaucomatous injury in mice of all three genotypes.^{61,62} Eleven WT, 12 HET, and 9 MUT mice were injected unilaterally (Supplemental Figure S2A). In one WT mouse, the IOP failed to increase, and one HET mouse developed an infection in the anterior chamber. These mice were removed from the study. In addition, a cohort of 11 mice was injected

Figure 7 Glial cells are activated in the retina of MUT eyes. **A–C:** WT; **D–F:** HET; **G–I:** MUT. **A, D,** and **G:** Rabbit anti-GS antibody labels Müller glia in the retina. **B, E,** and **H:** Chicken anti-GFAP antibody labels astrocytes and Müller glia, rabbit anti-Iba1 labels microglia in retina. **C, F,** and **I:** Rabbit anti-Iba1 labels microglia in retinal whole mounts (outer plexiform layer). The glia cells are normal in retinas of WT and HET eyes. **G** and **H:** In MUT retina, there is an increased GS intensity in Müller glia endfeet (**G**), increased GFAP immunoreactivity in the optic fiber layer (**H**), and in occasional thin processes of glia spanning the inner plexiform layer (**H**, arrowheads). The microglia labeled with Iba1 appears to be more rounded in cell morphology and has thickened processes (**H**, arrows). **C, F,** and **I:** Microglia in the outer plexiform layer in MUT retina shows smaller size but increased numbers. **J:** The microglia cell density of MUT is higher than WT and HET in the outer plexiform layer. Four mid-periphery regions per retina were counted manually in Image J. Data are expressed as means $\pm$ SD. $n = 7$ eyes in WT, 8 eyes in HET, 6 eyes in MUT. $**P < 0.01$, one-way analysis of variance. Images are representatives of single focal plane scans (**A, B, D, E, G,** and **H**) and of collapsed confocal image stacks (**C, F,** and **I**) (50 image planes, taken at 0.5- μ m z-step size). GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; HET, heterozygous; Iba1, ionized calcium-binding adaptor molecule 1; MUT, mutant; WT, wild-type. Scale bars: 50 μ m (**A, B, D, E, G,** and **H**); 100 μ m (**C, F,** and **I**).

with sterile saline solution as a control to exclude the possibility that any manipulation of the eye may cause an increase in pressure or ganglion cell loss. In no case did saline injection lead to an increase in IOP or ganglion cell loss (Supplemental Figure S2B).

The microbead occlusion model of IOP elevation in rodents has, since its inception, gained wide acceptance.^{61,69,86,87} However, a noted problem is that the IOP increase is relatively variable in amount and duration between individual animals in a cohort. It has therefore been suggested to use the cIOP (defined as the area under the curve of IOP over time, measured in mmHgDays) as an estimate of the total pressure insult to which the eye was subjected.⁶⁹ The Δ cIOP (cIOP in the experimental eye minus cIOP in the control eye) was 210.5 ± 89.1 mmHgDays ($n = 10$, means $\pm$ SD) for the WT, 236.9 ± 87.4 mmHgDays ($n = 11$) for the HET, and 232.1 ± 77.2 mmHgDays ($n = 9$) for the MUT (not significant, analysis of variance). We were concerned that an

excessively high IOP, even if sustained only for a few days, may cause more damage than would be predicted from the cIOP alone, so we compared the maximum IOPs that were measured during the experimental period in individual eyes for each strain. Maximum IOP for the WT was 25.4 mmHg (range, 19.8–29.6 mmHg), for the HET was 26.1 mmHg (range, 20–30.8 mmHg), and for the MUT was 23.8 mmHg (range, 20.6–37.4 mmHg). The differences in mIOP between strains were not significant (analysis of variance). Taken together, this indicates that the animals of all three genotypes received comparable pressure insults (Figure 9, A and B).

Ganglion Cell Loss and Axon Degeneration after IOP Elevation

We determined retinal ganglion cell loss by counting whole-mounted retinas after staining for Brn3a (Figure 10, A–F).

Figure 8 Microglial cells are activated in the optic nerve head of MUT eyes. **A–C:** WT; **D–F:** HET; **G–I:** MUT. **A, D,** and **G:** GFAP labeling of astrocytes in the optic nerve head shows no differences between genotypes. Single confocal image planes through the optic nerve head are shown. **B, E,** and **H:** Rabbit anti-Iba1 stains microglia in the optic nerve head. The images are representative of collapsed confocal image stacks (50 image planes, taken at 0.4 μm z-step size) to show the whole cell morphology. Microglia in MUT mice appear more rounded and have fewer fine processes (**H**). **C, F,** and **I:** High-power views of individual microglial cells stained for Iba1. The images represent collapsed confocal stacks (50 image planes, taken at 0.4- μm z-step size). GFAP, glial fibrillary acidic protein; HET, heterozygous; Iba1, ionized calcium-binding adaptor molecule 1; MUT, mutant; WT, wild-type. Scale bars: 50 μm (**A, B, D, E, G,** and **H**); 20 μm (**C, F,** and **I**).

It was reported recently that an increase in IOP in one eye also affects the contralateral eye to some extent, and especially that it causes activation of macroglial and microglial cells in the contralateral eye.^{88,89} Because we observed an increase in microglial cell counts and activation in MUT mice even in the absence of IOP elevation, we first determined whether we could detect ganglion cell loss in the contralateral eyes compared with naive controls for all three genotypes (Figure 9C). In WT mice, the ganglion cell counts in the contralateral eyes were 4412 ± 191 cells/ mm^2 (versus 4569 cells/ mm^2 in naive eyes; not significant), and in HET eyes they were 4547 ± 294 cells/ mm^2 (versus 4468 cells/ mm^2 in naive eyes; not significant). However, the contralateral MUT eyes showed a loss of ganglion cells with 3945 ± 375 cells/ mm^2 [versus 4512 cells/ mm^2 in naive eyes, corresponding to a 12.57% cell loss ($P < 0.01$, t -test)]. We therefore calculated all ganglion cell losses in the injected eyes relative to naive eyes of the same genotype.

Although the mean ganglion cell loss in the microbead-injected eyes was similar in WT and HET mice ($22.4\% \pm 6.5\%$ in RGC loss in WT and $20.1\% \pm 7.2\%$ in HET; not significantly different), the MUT eyes lost $35.8\% \pm 7.1\%$ of their ganglion cells. This difference was significant ($P < 0.001$, one-way analysis of variance) (Figure 9D). We then correlated the ΔcIOP for the 10 WT mice with the ganglion cell loss in the retina to test how well ΔcIOP would predict ganglion cell loss (Figure 9E). In

agreement with earlier studies,⁶⁹ we found ΔcIOP to correlate with ganglion cell loss ($R^2 = 0.5324$). HET mice followed the same trend as the WT mice, but MUT mice showed a larger ganglion cell loss than would be predicted from the pressure insult they sustained (Figure 9E and Figure 10, A–F).

We also evaluated the degree of the axon degeneration induced by IOP elevation by staining optic nerve cross-sections with PPD (Figure 10, G–I) and grading them on a scale from 1 to 5.⁶⁸ Optic nerves from saline-injected eyes had a mean score of 1.45 ± 0.37 (1.3 ± 0.24 in the contralateral eyes; not significant). Optic nerve grades were worse in injected eyes compared with the contralateral eyes (1.85 ± 0.63 versus 1.35 ± 0.39 for WT, 1.86 ± 0.74 versus 1.22 ± 0.33 for HET; $P < 0.05$, t -test). As expected from the observation that the MUT mice lose ganglion cells in the uninjected eyes, contralateral MUT optic nerves had a mean nerve score of 1.94 ± 0.86 . In the microbead-injected eyes the nerve score showed a tendency to further increase to 2.22 ± 0.85 , although this difference was not significant (Supplemental Figure S3).

Microglial Activation after IOP Elevation

As described in *Glial Cells Are Activated in the Retina and Optic Nerve Head of MUT Eyes*, the microglia in MUT optic nerve heads were activated with less ramified

Figure 9 IOP and retinal ganglion cell loss after microbead injection. **A:** $\Delta cIOP$ s were similar for mice from WT, HET, and MUT groups. **B:** mIOPs were similar in WT, HET, and MUT groups. **C:** Ganglion cell loss in the contralateral (non-injected) eye was determined by comparing Brn3a-labeled cells in the contralateral eye with naive controls. In WT and HET, no significant difference was found, but in the MUT the contralateral eye shows a loss of ganglion cells. **D:** Ganglion cell loss in the microbead-injected eyes compared with naive eyes in WT, HET, and MUT. MUT has a greater loss than WT and HET. **E:** $\Delta cIOP$ correlates with ganglion cell loss in WT and HET ($R^2 = 0.5342$ in WT), but MUT eyes are affected more severely than would be predicted from their $\Delta cIOP$. Data are expressed as means $\pm$ SD. $n = 10$ WT, $n = 11$ HET, and $n = 9$ MUT. ** $P < 0.01$ (t -test), *** $P < 0.001$ (one-way analysis of variance). HET, heterozygous; IOP, intraocular pressure; mIOP, maximum IOP that was recorded during the experimental period; MUT, mutant; RGC, retinal ganglion cell; WT, wild-type; $\Delta cIOP$, difference of cumulative IOP in the experimental and the control eyes.

appearance even under normal IOP. Because the severity of glaucomatous neurodegeneration can be predicted from the microglia alterations,⁹⁰ we next observed the morphology of microglia by staining cross-sections of optic nerve heads with Iba1 1 month after IOP elevation. The microglia of all three genotypes showed signs of activation; however, the degree of morphologic changes were different (Figure 10, M–O). Compared with WT and HET mice, the microglia in MUT mice have a more rounded morphology, with thicker and shorter processes. The grade of microglia activation had the same trend as ganglion cell loss and axonal degeneration.

Ganglion Cell Function Loss after IOP Elevation

One month after microbead injection, consistent with the greater loss of ganglion cells, MUT mice also showed greater visual function loss (Figure 11). Figure 11, A, B, and D, shows examples of individual waveforms from WT and MUT eyes. Amplitude loss percentage for individual mice was calculated as the reduction (baseline amplitude before injection minus amplitude after microbead injection) compared with its own baseline. The amplitude loss percentage in the pSTR ERG, which reflects ganglion cell function, was significantly greater ($n = 5$ eyes for each

Figure 10 Ganglion cell loss, axon degeneration, and microglia activation after microbead injection. **A, D, G, J, and M:** WT; **B, E, H, K, and N:** HET; **C, F, I, L, and O:** MUT. **A–C:** Mouse anti-Brn3a labeling of ganglion cells in retina of contralateral eyes. **D–F:** Brn3a labeling of ganglion cells in retina of microbead-injected eyes. Ganglion cell loss is more obvious in MUT eyes. The images are collapsed confocal image stacks (15 image plane, taken at 0.5 μm z-step size) in mid-periphery regions of retinal whole mounts. **G–I:** PPD staining of optic nerve axons of contralateral eyes. **J–L:** PPD staining of optic nerve axons of microbead-injected eyes. There are several focally degenerating axons present in WT and HET, and more degenerating axons with disorganized connective tissue in MUT, the contralateral MUT optic nerve also show focally degenerating axons. **M–O:** Rabbit anti-Iba1 stains microglia in the optic nerve head of microbead-injected eyes. The images are collapsed confocal image stacks (50 images planes, taken at 0.4- μm z-step size) to show the individual microglial cells. Microglia of the three genotypes all appear more rounded and have fewer fine processes compared with their naive eyes (Figure 8, C, F, and I). Microglia in MUT shows more reactivity than WT and HET. All images are representatives of three genotypes. HET, heterozygous; MUT, mutant; PPD, paraphenylenediamine; WT, wild-type. Scale bars: 50 μm (**A–F**); 20 μm (**G–L**); 30 μm (**M–O**).

genotype; $P < 0.01$, one-way analysis of variance) in MUT mice than in WT mice at the stimulus intensity of $-4.5 \log \text{cd.s/m}^2$ ($59.5\% \pm 14.3\%$ in WT, $70.3\% \pm 7.7\%$ in HET, $76.7\% \pm 12.2\%$ in MUT), $-4 \log \text{cd.s/m}^2$ ($58.7 \pm 13.6\%$ in WT, $72.1 \pm 10.2\%$ in HET, $84.2 \pm 4.9\%$ in MUT), and $-3.5 \log \text{cd.s/m}^2$ ($55.5\% \pm 11.8\%$ in WT, $70.2\% \pm 5.5\%$ in HET, $80.7\% \pm 16.0\%$ in MUT) (Figure 11C), whereas the loss

in HET mice was between that in WT and MUT mice, which was not significant.

Gene Expression Changes after IOP Elevation

Because *CDKN2B* is implicated in the *TGFB* signaling pathway,^{28,29} and changes in *TGFB* signaling were described

Figure 11 pSTR ERG wave form and amplitude loss after microbead injection. **A:** Serial pSTR ERG amplitude versus stimulus intensity in naive and microbead-injected eyes of three genotypes. MUT mice show a greater reduction in amplitude at all stimulus intensities. **C:** Statistical comparison of pSTR amplitude loss at the stimulus intensity of -4.5 , -4 , and $-3.5 \log \text{cd.s/m}^2$. A significant difference was found in pSTR amplitudes between WT and MUT. **B** and **D:** Representative individual waveforms of pSTR ERG in naive eyes (**black traces**) and microbead-injected (**gray traces**) eyes from WT (**B**) and MUT (**D**) mice. Stimulus flash intensities are listed above the wave forms. Post-stimulus time line is listed below waveforms. Data are expressed as means $\pm$ SD. $n = 5$ eyes per genotype. $***P < 0.01$ (one-way analysis of variance). ERG, electroretinography; HET, heterozygous; MUT, mutant; pSTR, positive scotopic threshold response; WT, wild-type.

in the $\Delta 4\text{C4-C5}$ mice,^{52,91} we tested the expression of *TGFB1*, *TGFB2*, and *TGFB3* in mice of all three genotypes. Consistent with earlier reports,^{35,41} we found *TGFB2* to be the most highly expressed isoform in the retina and the optic nerve head, but there were no significant differences in *TGFB2* expression between the three genotypes. The expression of *TGFB1* was higher in MUT retinas than in WT and HET retinas ($P < 0.01$, one-way analysis of variance) (Figure 12A), and the expression level of *TGFB3* was lower in HET and MUT optic nerves than in WT optic nerves ($P < 0.01$, one-way analysis of variance) (Figure 12B).

In the retinas (Figure 12C), but not the optic nerves, of WT, HET, and MUT mice 1 month after microbead injection, the expression of *CDKN2B* was down-regulated ($P < 0.01$, t -test). All *TGFB* isoforms were down-regulated variably in retinas and optic nerves after microbead injection, but the effect was most pronounced for *TGFB3* ($P < 0.01$, t -test) (Figure 12, D and E). No obvious differences were found in the degree of down-regulation among different genotypes.

Discussion

Several GWASs have identified glaucoma-associated SNPs in a hotspot on human chromosome 9p21.3 that has also been found in connection with other diseases.^{20,25,30} In particular, it appears that minor allele for SNPs in *CDKN2B-AS*, coding for

the long, noncoding RNA, confer relative resistance to POAG.¹⁴ However, the mechanism is not yet clear. Here, we used a transgenic mouse in which part of murine chromosome 4, including exons 4 to 6 of the *AK148321* gene, is deleted. This mouse strain is at present the best animal model to investigate the influence of genes in this locus on glaucoma. However, there are important caveats. First, it is not currently known whether murine *AK148321* is functionally equivalent to the human *CDKN2B-AS*. Second, the 70-kB deletion on murine chromosome 4 not only eliminates parts of the *AK148321* gene but also regulatory elements that are required for the normal expression of the protein-coding genes in the *INK4* locus.⁵¹ This mouse strain therefore does not allow one to directly assess the importance of the noncoding RNA for glaucoma. Finally, in the MUT state, the mice display persistent hyperplastic primary vitreous,^{52–54} making them more difficult to study and less desirable for glaucoma research.

We found no obvious histologic abnormalities of the neural retina in either HET or MUT mice. In particular, the number of ganglion cells was normal compared with WT mice. Functionally, as tested by ERG and optokinetic reflex measurement, WT and HET mice were indistinguishable. The ERG amplitudes and visual acuity of MUT mice are reduced, but our data are consistent with the interpretation that the defect lies not in the retina itself but in the lens opacity and the retrolental tissue. This is most clearly

Figure 12 Expression of *TGFβ* and *CDKN2B* after microbead injection. **A:** In retina tissue, expression level of *TGFβ1* are increased in MUT eyes compared with WT and HET. **B:** In optic nerve tissue, expression levels of *TGFβ3* in HET and MUT are decreased compared with WT. **C:** Expression levels of *CDKN2B* are down-regulated after microbead injection in retina tissue. Expression levels of *TGFβ3* are down-regulated after microbead injection in retina (**D**) and optic nerve (**E**) tissues. Data are expressed as means $\pm$ SD. $n = 3$ eyes in microbead-injected group and $n = 5$ in naïve group per genotype. $**P < 0.01$, one-way analysis of variance (**A** and **B**); $**P < 0.01$, t -test (**C–E**). HET, heterozygous; MUT, mutant; WT, wild-type.

demonstrated by the observation that some MUT mice showed only a mild manifestation of persistent hyperplastic primary vitreous and had near-normal visual acuity. The expression levels of *CDKN2B* are reduced in the retina and optic nerve of MUT and HET mice (Figure 4); similar to what was described for heart tissue.⁵¹ Taken together, our data indicate that neither the partial deletion of *AKI48321* nor the down-regulation of *CDKN2B* has a significant negative effect on ganglion cell function, at least under normal conditions.

We found signs of glial reactivity both in the retina and the optic nerve in MUT mice. The Müller cell and microglial reactivity in the retina may be due to the fibrovascular tissue behind the lens. The retrolental tissue was often found to be attached to the peripheral retina (Figure 3E). This may lead to traction on the retina that is not severe enough to cause permanent retinal detachment and photoreceptor degeneration, but it is sufficient to cause glial activation in the retina. However, the number of microglial cells in the MUT retinas is also increased compared with WT and HET retinas. An increase in the microglial cell numbers was reported in glaucoma.^{42,92,93} This may be due to mitosis of microglial cells⁹⁴ or to the entry of monocytes/macrophages from the blood stream that are difficult to distinguish from

the native microglia.⁹⁵ *CDKN2B* was described as one of those genes that were up-regulated in microglia after treatment with the plant compound 15-methoxypinusolidic acid that is under investigation as a potential inhibitor of neuroinflammation and microglial activation.⁹⁶ The expression levels of *CDKN2B* are reduced significantly in the MUT mice, which may have the opposite effect of inducing microglial activation even without IOP elevation. Microglial activation in the retina and the optic nerve is an early indicator of glaucoma that precedes ganglion cell loss,^{84,90,93} and neuroinflammation plays a role in the pathogenesis of glaucoma.⁹⁷ We therefore asked whether MUT mice would be more sensitive to an elevation of IOP, induced by the injection of microbeads into the anterior chamber. Ganglion cell loss and optic nerve pathology are significantly worse in the MUT mice. A possible explanation is that microglial cells are constitutively activated in MUT retinas and optic nerve heads and require less of an additional stimulus by IOP elevation to acquire a neurotoxic phenotype. If SNPs in the human *Ink4* locus have the potential to negatively affect *CDKN2B* (or *CDKN2B-AS*) expression in retinal and optic nerve microglia, it could be speculated that even a moderate elevation of IOP causes neuroinflammation in the affected patients and increases the risk of ganglion cell axon damage.

This might explain why particularly normal-tension glaucoma is associated with SNPs in chromosome 9p21.3.^{10,14,24,25}

The TGF- β 1 pathway was found to be activated in several models of glaucoma^{43–45,48} and traumatic optic nerve damage.⁴⁷ We were therefore surprised that *TGFB1* was not up-regulated after microbead injection. A possible explanation may be that a significant up-regulation of *TGFB1* was observed in a rat model of ocular hypertension only when considerable damage to the optic nerve had occurred (optic nerve grade 5),⁴⁴ and the injury to the nerve in our cohort of microbead-injected eyes may not have been sufficient to induce up-regulation. We did observe a pronounced down-regulation of *TGFB3* in the retina and optic nerve after microbead injection, although no differences were found between the genotypes.

Interestingly, in the MUT mice some ganglion cell loss also occurs in the uninjected contralateral eye. This is not normally observed in mouse models of ocular hypertension,⁹⁸ and neither did we find it in the WT and HET mice. Microglial activation in the contralateral eye was described before,⁸⁸ but the mechanism is as yet unclear. Several studies have reported that unilateral intravitreal injections of bevacizumab (an anti-vascular endothelial growth factor antibody used for the treatment of age-related macular degeneration) also have therapeutic effects on the contralateral eye. This effect is assumed to occur because some of the antibody escapes into the general circulation and reaches the fellow eye.⁹⁹ A similar mechanism may be at work in the MUT eyes after microbead injection. Reactive glial cells in the optic nerve secrete a variety of factors that may lead to increased microglial activation, inflammation, or macrophage entry in the contralateral eye, such as colony-stimulating factor 2, and chemokine C-X-C motif ligand 1.⁴⁷

Conclusion

Mutations in the glaucoma-susceptibility locus on chromosome 9p21.3 may predispose to increased vulnerability of ganglion cells to IOP at least partly by a stronger activation of microglia in the retina and the optic nerve. It would be interesting to test whether glaucoma patients bearing the high-risk alleles show signs of increased activation of microglia.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.11.026>.

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