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GPR158 in the Visual System: Homeostatic Role in Regulation of Intraocular Pressure

Tatsuo Itakura,[1] Andrew Webster,[1] Shravan K. Chintala,[1] Yuchen Wang,[2] Jose M. Gonzalez, Jr.,[3] J.C. Tan,[3] Janice A. Vranka,[4] Ted Acott,[4] Cheryl Mae Craft,[5,6] Maria E. Sibug Saber,[7] Shinwu Jeong,[8] W. Daniel Stamer,[9] Kirill A. Martemyanov,[2] and M. Elizabeth Fini[1,*]

Abstract

Purpose: GPR158 is a newly characterized family C G-protein-coupled receptor, previously identified in functional screens linked with biological stress, including one for susceptibility to ocular hypertension/glaucoma induced by glucocorticoid stress hormones. In this study, we investigated GPR158 function in the visual system.

Methods: Gene expression and protein immunolocalization analyses were performed in mouse and human brain and eye to identify tissues where GPR158 might function. Gene expression was perturbed in mice, and in cultures of human trabecular meshwork cells of the aqueous outflow pathway, to investigate function and mechanism.

Results: *GPR158* is highly expressed in the brain, and in this study, we show prominent expression specifically in the visual center of the cerebral cortex. Expression was also observed in the eye, including photoreceptors, ganglion cells, and trabecular meshwork. Protein was also localized to the outer plexiform layer of the neural retina. *Gpr158* deficiency in knockout (KO) mice conferred short-term protection against the intraocular pressure increase that occurred with aging, but this was reversed over time. Most strikingly, the pressure lowering effect of the acute stress hormone, epinephrine, was negated in KO mice. In contrast, no disruption of the electroretinogram was observed. Gene overexpression in cell cultures enhanced cAMP production in response to epinephrine, suggesting a mechanism for intraocular pressure regulation. Overexpression also increased survival of cells subjected to oxidative stress linked to ocular hypertension, associated with TP53 pathway activation.

Conclusions: These findings implicate GPR158 as a homeostatic regulator of intraocular pressure and suggest GPR158 could be a pharmacological target for managing ocular hypertension.

Keywords: intraocular pressure, G-protein-coupled receptor, cAMP, trabecular meshwork, retina, homeostasis

[1]USC Institute for Genetic Medicine, Keck School of Medicine of USC, University of Southern California, Los Angeles, California.
[2]Department of Neuroscience, The Scripps Research Institute, Jupiter, Florida.
[3]Doheny Eye Institute and Department of Ophthalmology, University of California Los Angeles, Los Angeles, California.
[4]Casey Eye Institute, Oregon Health and Science University, Portland, Oregon.
[5]USC Roski Eye Institute, Department of Ophthalmology, Keck School of Medicine of USC, University of Southern California, Los Angeles, California.
[6]Department of Integrative Anatomical Sciences, Keck School of Medicine of USC, University of Southern California, Los Angeles, California.
[7]Department of Pathology, Keck School of Medicine of USC, University of Southern California, Los Angeles, California.
[8]USC Institute for Genetic Medicine, Department of Ophthalmology, USC Roski Eye Institute, Keck School of Medicine of USC, University of Southern California, Los Angeles, California.
[9]Department of Ophthalmology, Duke University, Durham, North Carolina.
*_Current affiliation:_ Department of Ophthalmology, Tufts University School of Medicine at New England Eye Center, Tufts Medical Center, Boston, Massachusetts.

Introduction

THE GLAUCOMAS ARE a group of optic neuropathies comprising the third most prevalent cause of visual impairment and blindness among Caucasian Americans and the leading cause among African Americans.[1] Ocular hypertension due to impaired aqueous humor outflow through the trabecular meshwork and Schlemm's canal is the major risk factor for glaucoma, and lowering intraocular pressure is the only proven treatment.[2] While effective pharmaceutical therapeutics exist, some patients do not reach the desired target pressure, even when maximally medicated, indicating a continuing unmet medical need.[3]

Of the current drugs, natural products, and drug candidates used to control ocular hypertension, most target members of a clan of cell surface proteins called G-protein-coupled receptors (GPCRs).[4–6] Represented by 30%–40% of marketed pharmaceuticals, GPCRs are by far the most successful targets for pharmacotherapy to date.[7,8] Many novel orphan GPCRs (without known ligands) were identified by human genome sequencing,[9] providing for potential new drug targets. One is GPR158, a distant member of GPCR family C, which includes receptors for the neurotransmitters glutamate and gamma-aminobutyric acid.[10]

We identified *GPR158* in a genomic screen for genes associated with susceptibility to ocular hypertension in humans caused by treatment with pharmaceutical forms of glucocorticoid stress hormones.[11–13] Gpr158, as well as the closely related Gpr179, was also identified in a biochemical screen for proteins that form complexes with the GTPase-activating protein RGS7 in brain and retinal neurons of mice.[14] *Gpr158* was recently shown to mediate effects of osteocalcin on cognition and anxiety-like behaviors in mice[15] and was most recently shown to play a causal role in chronic stress and depression.[16]

With relationship to steroid-induced ocular hypertension, glucocorticoids stimulate GPR158 expression by trabecular meshwork cells in culture.[11] In turn, when ectopically overexpressed in these cells, GPR158 itself stimulates cell proliferation. Correspondingly, knockdown of endogenously expressed GPR158 inhibits cell proliferation. GPR158 overexpression also increases barrier function of a cell monolayer, consistent with ocular hypertension. Similar effects on cell proliferation were demonstrated in prostate cancer cell lines, with expression stimulated by a second steroid hormone, androgen, leading to increased androgen receptor expression.[17] Alternatively, androgen receptor pathway inhibition, leads to a high level of GPR158 expression over a period of weeks, coincident with induction of neuroendocrine differentiation markers; high-level GPR158 alone induced neuroendocrine differentiation.[17] In the nervous system, GPR158 expression is likewise induced by glucocorticoid exposure.[16] Increased levels of GPR158 in the brain dampen signaling via second messenger cAMP, which controls ion channel function and production of neurotrophic factor BDNF.

GPR158 was an orphan receptor at the time the current study began, but 2 unconventional activities had been characterized. First, GPR158 is necessary for the plasma membrane recruitment of RGS7 in the brain, where it interacts with conventional GPCRs to modify their G-protein-coupled signaling.[18] It was also found to traffic to the plasma membrane like other GPCRs, but it is then rapidly endocytosed and translocates to the nucleus.[11] Mutation of the nuclear localization signal abrogates the enhancement of cell proliferation in both trabecular meshwork cells[11] and prostate cancer cell lines.[17]

In this study, we characterize GPR158 expression and activity in the visual system, demonstrating a functional role in homeostatic regulation of intraocular pressure.

Methods

GPR158 antibodies

Two rabbit polyclonal antibodies specific for human GPR158 were used, both purchased from Sigma-Aldrich Corp. (St. Louis, MO). The N-terminal-specific antibody was raised against AAs 24–74 of the human GPR158 extracellular domain (SAB4502509). The C-terminal-specific antibody was raised against AAs 914–1,052 of the human GPR158 intracellular domain (HPA013185). The use of these antibodies for immunohistochemistry, immunofluorescent localization, and immunoblot analysis in human and mouse cells and tissues has been described in several publications from our laboratories.[11,14,16–18] The specificity of the C-terminal-specific antibody was further validated by immunoblot analysis of brain extracts prepared from normal and *Gpr158* knockout (KO) mice (heterozygous and homozygous). N-terminal-specific antibody was used at a dilution of 1:100 for immunohistochemistry and immunofluorescent localization. The C-terminal-specific antibody was used at a dilution of 1:200 for immunoblot analysis.

Gpr158 *KO mice*

All studies were carried out in adherence to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and the recommendations of the NIH Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committees of The University of Southern California (USC) and the Scripps Florida Research Institute. The *Gpr158* KO mice [$Gpr158^{tm1(KOMP)Vlcg}$] were purchased from the University of California Davis Knockout Mouse Project (KOMP) Repository. Data on the KOMP website indicates that homozygotes for the KO allele are viable and anatomically normal. Mice were backcrossed onto a C57/Bl6 background for at least 4 generations. Heterozygous *Gpr158–/+* pairs were then bred to generate *Gpr158–/–* mice and *Gpr158–/+* and *Gpr158+/+* littermates. Littermates were used exclusively for all comparisons. All mice used were male. The single sex was used because the hypothalamic-pituitary-adrenal endocrine axis has sex-related differences. Mice were housed on a 12-h light–dark cycle with food and water available *ad libitum*.

Human brain and eye tissues

Research on human tissues was performed in conformance with the tenets of the Declaration of Helsinki. Human brain and eye autopsy specimens were obtained from the USC Alzheimer's Disease Research Center Neuropathology Core. Both were obtained from a 95-year-old male. The postmortem interval before fixation was 3 h. The

Institutional Review Board of the University of Southern California reviewed the protocols for histochemistry on human brain and eye sections, and designated them as nonhuman subject research. Human brain and eye tissue sections were prepared by the USC Pathology Core.

Beta-galactosidase (LacZ) histochemistry

Mouse eyes were dissected and then snap frozen in an OCT-filled mold. Frozen sections were made using a cryostat. For LacZ histochemistry, sections were briefly thawed from the freezer and then circled with pap pen. Section were then fixed with 0.2% glutaraldehyde for 10 min at 4°C, and the slides were washed 3 times in LacZ wash solution (2 mM $MgCl_2$, 0.01% Na Deoxycholate, 0.02% Nonidet P-40) for 5 min. Consequently, the sections are covered with LacZ staining solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 0.5 mg/mL X-gal in LacZ wash] and incubated at 37°C overnight. The slides are rinsed with phosphate-buffered saline (PBS), then Milli-Q water, and counterstained with Nuclear Fast Red (Sigma-Aldrich) for 5 min. Stained sections were dehydrated in 75% ethanol, then 100% ethanol, then cleared in xylenes, and cover-slipped with Permount (Fisher Scientific).

Immunohistochemistry

For immunohistochemistry on human tissue, we used a formalin-fixed human brain and a fresh human eye autopsy specimen. The latter was fixed in 4% paraformaldehyde, and then both were embedded in paraffin. For mice, brains and eyes were dissected after euthanization, then fixed in 4% paraformaldehyde and embedded in paraffin. Sections cut 4–8 μm thick were placed on slides, deparaffinized in xylene, and rehydrated using a decreasing ethanol gradient followed by rinsing with double-distilled H_2O. To retrieve antigenicity, slides were boiled for 5 min with 10 mM citrate buffer (pH 6.0) by pressure cooker in a microwave, washed with 0.1 M phosphate buffer, blocked with 0.3% hydrogen peroxide in methanol for 15 min and with 5% goat serum in PBS for 30 min at room temperature. The slides were incubated with the N-terminal-specific GPR158 antibody (1:100) in 5% goat serum at 4°C overnight followed by incubation with biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h and horseradish peroxidase streptavidin (Vector Laboratories) for 30 min, and then visualized using a 3,3′-diaminobenzidine chromogen kit (Vector Laboratories). In some cases, a red chromogen, 3-amino-9-ethilcarbasole (Abcam, Cambridge, MA), was used to enable a better distinction between staining and natural pigment in the tissue. The slides were subsequently counterstained with 5% (w/v) Harris hematoxylin.

Epinephrine treatment and intraocular pressure measurement in mice

Intraocular pressure was measured in *Gpr158*–/–, *Gpr158*–/+, *Gpr158*+/+ mice at ages 3, 6, and 9 months. Mice were anesthetized with isoflurane by placing them in a sealed chamber (VetEquip, Livermore, CA) with a supply of oxygen (oxygen flow rate of 1 L/min) and isoflurane (gauge reading at 1%) by Vaporizer (VetEquip) for 2–3 min. Then, an individual mouse was placed on the edge of a board with the Vaporizer tube in its mouth to keep it anesthetized, and the intraocular pressure measurement was carried out within 5 min using the TonoLab rebound tonometer (Colonial Medical Supply, Franconia, NH). Before measurement, mouse whiskers and eyelashes were trimmed to prevent interference with measurements. To open the eye, subtle pressure was applied to the skin below the eye with the eraser end of a pencil. The intraocular pressure was then measured 7 times in each eye with the Tonometer, and an average was taken. One measurement with the Tonometer is the average of 6 rebound measurements. When required, epinephrine (109 μM) was applied topically (2 μL) to the right eye of mice, and then intraocular pressure was measured at 30, 60, and 90 min and compared to the vehicle-treated left eye. Measurements are carried out between 10 a.m. and noon to control diurnal variation in intraocular pressure.

Electroretinography of mouse eyes

Electroretinograms were recorded by using the UTA system and a Big-Shot Ganzfeld (LKC Technologies). Mice ($\sim$4–8 weeks old) were dark-adapted ($\geq$6 h) and prepared for recordings using a red dim light. Mice were anesthetized with an intraperitoneal injection of ketamine and xylazine mixture containing 100 and 10 mg/kg, respectively. Recordings were obtained from the right eye only, and the pupil was dilated with 2.5% phenylephrine hydrochloride (Bausch and Lomb), followed by the application of 0.5% methylcellulose. Recordings were performed with a gold loop electrode supplemented with contact lenses to keep the eyes immersed in solution. The reference electrode was a stainless-steel needle electrode placed subcutaneously in the neck area. The mouse body temperature was maintained at 37°C by using a heating pad controlled by ATC 1000 temperature controller (World Precision Instruments). Electroretinography (ERG) signals were sampled at 1 kHz and recorded with 0.3-Hz low-frequency and 300-Hz high-frequency cutoffs.

Full-field white flashes were produced by a set of LEDs (duration <5 ms) for flash strengths $\leq$2.5 cd $\cdot$ s/m^2 or by a Xenon light source for flashes >2.5 cd $\cdot$ s/m^2 (flash duration <5 ms). ERG responses were elicited by a series of flashes ranging from 1×10^{-5} to 800 cd $\cdot$ s/m^2 in 10-fold increments. Ten trials were averaged for responses evoked by flashes up to 0.1 cd $\cdot$ s/m^2, and 3 trials were averaged for responses evoked by 0.5 and 1 cd $\cdot$ s/m^2 flashes. Single-flash responses were recorded for brighter stimuli. To allow for recovery, interval times between single flashes were as follows: 5 s for 1×10^{-5} to 0.1 cd $\cdot$ s/m^2, 30 s for 0.5 and 1 cd $\cdot$ s/m^2, 60 s for 5 and 10 cd $\cdot$ s/m^2, and 180 s for 100 and 800 cd $\cdot$ s/m^2 flashes.

ERG traces were analyzed using the EM LKC Technologies software and Microsoft Excel.

Doxycycline-inducible GPR158 *overexpression cell culture model*

The SV-40 large T antigen immortalized human trabecular meshwork cell line, TM-1,[19] was generously donated by Dr. Donna Peters, University of Wisconsin-Madison. The line was created in the laboratory of the late Dr. Jon Polansky from trabecular meshwork cells obtained from a 30-

year-old nonglaucomatous individual as previously described.[19] The original parental trabecular meshwork cells were characterized as previously described[20,21] and shown along with the TM-1 cells to upregulate MYOC in response to dexamethasone.[19,22] Cells were grown in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Atlanta Biologicals, Inc., Norcross, GA), 2 mM L-glutamine, 2.5 μg/mL amphotericin B, and 25 μg/mL gentamicin, as previously described.[19]

To create the doxycycline-inducible *GPR158* overexpression model, TM-1 cells were stably transduced with a lentiviral vector containing *GPR158*, in which expression was driven by a doxycycline-inducible promoter (pSlik-Hygro; Addgene, Cambridge, MA), as previously described.[17] The cell line was named lenti-*GPR158* TM-1. A matching control cell line stably transduced with empty vector was also created and named lenti-vector TM-1.

For induction of *GPR158* expression, cells of stably transduced lines were seeded in 6-well plates at 200,000 cells per well, then treated with doxycycline (100 or 500 μg/mL) for up to 96 h. *GPR158* transgene expression is leaky, thus the untreated lenti-*GPR158* TM-1 cell line was not the best comparison for all experiments. In such cases, comparison was made to the empty vector-transduced line that was doxycycline treated with the same concentration and duration.

Immunoblot analysis

Cell were lysed by suspension in 200 μL of radioimmunoprecipitation assay lysis buffer containing Tris 50 mM, NaCl 150 mM, sodium dodecyl sulfate (SDS) 0.1%, sodium deoxycholate 0.5%, and NP-40 1% and protease inhibitors cocktail. After 20 min, the lysates were centrifuged at 10,000 *g* for 10 min and supernatants were collected. Proteins in the extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Samples were prepared without boiling (which causes aggregation of GPR158); however, samples were reduced with beta-mercaptoethanol. Equal protein was loaded into each well based on bicinchoninic acid assay (Thermo Scientific, Rockford, IL).

Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes. Membranes were probed with primary antibody overnight at 4°C with gentle shaking, following the manufacturer's instructions. The membranes were then incubated for 1 h with secondary antibody–horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:10,000. Specific signals were developed for 1 min using the enhanced chemiluminescence kit components 1 and 2 (GE Health care UK Limited, Buckinghamshire, United Kingdom). Chemiluminescence was visualized by exposure of photographic film (LAS-4000; Fujifilm, Tokyo, Japan). Blots were probed with antibodies to GPR158 (described above) and some were also probed with a mouse monoclonal antibody to human TP53 (Cell Signaling; Cat No. IC12). Protein loading equivalence was monitored by probing of the membrane with ACTB antibody at 1:4,000 dilution (ab6276; Abcam).

Epinephrine treatment of cells and assay for cAMP production

GPR158 expression was induced with doxycycline at 500 ng/mL in lenti-*GPR158*-TM-1 or control lenti-vector-TM-1 cell lines overnight. The next day, cells were treated with 2% epinephrine in PBS (Sigma). Cell samples were then taken over a time course of 180 min with time points of 0, 30, 60, and 180 min. The cAMP in cell lysates was measured using a cAMP competitive ELISA kit (Pierce Thermo Scientific).

Cell survival assay

A metabolic assay was used to assess cytotoxicity due to oxidative stress and protection by *GPR158* overexpression. Both lenti-*GPR158* TM-1 and control lenti-vector TM-1 cell lines were seeded in the wells of a 96-well plate at 10,000 cells/well and left to adhere and spread overnight. The next day, cells were treated with tert-butyl hydroperoxide (t-BHP), serially diluted from 0.5 to 0.0 mM, for 24 h with or without doxycycline at 500 ng/mL. Then MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was initiated by removing half of the media in the wells (50 μL) and replacing with MTT solution (2.5 mg/mL). Superoxide anions generated by NADPH-dependent cellular oxidoreductase activity reduce MTT to water-soluble formazans which absorb visible light.[23] After the plate was incubated for 2 h at 37°C, the solution was removed, and the formazan was dissolved in 50 μL isopropanol with 1% HCl. Dye reduction was measured by absorbance at wavelengths of 590 nm.

Statistical analyses

All experiments were performed with appropriate positive and negative controls. All animals and cell cultures were randomly assigned to the experimental groups. All experiments were repeated at least twice and usually 3 times. All data are shown as means ± standard deviation. All cell culture assays and mouse assays were performed in triplicate ($n = 3$). The number of animals needed per experimental group was determined on the basis of previous statistical analyses by our group. The statistical significance of 2 data sets was assessed by the Student's *t*-test. The paired Student's *t*-test was used when comparing the 2 eyes of the same mouse, to take into account any possible correlation as described.[24] Statistical significance among 3 or more data sets was calculated using one-way analysis of variance. For the calculation of *P* values, all technical replicates from all biological replicates were used. A P value ≤ 0.05 was treated as significant.

Results

Gpr158 *expression in the visual center of the mouse cerebral cortex and GPR158 subcellular localization in human cortical neurons*

The *Gpr158* targeting approach for the KO mice used in this study utilized a knock-in strategy, inserting a bacterial beta-galactosidase (LacZ) transgene in the endogenous *Gpr158* locus. Histochemical evaluation of LacZ expression identified the brain as the primary *Gpr158* expression site, with high levels in the cortex.[25] A similar conclusion was made in 2 recent studies utilizing *in situ* hybridization.[15,16] In this study, we were specifically interested in determining if *Gpr158* was expressed in the visual center of the cerebral cortex, and its subcellular distribution. Gene expression was

investigated by LacZ histochemistry using tissue sections from the mouse brain, and protein localization was determined by immunohistochemistry in sections from the cortex of the human brain. Representative results are shown in Fig. 1. In one of the previous studies, *Gpr158* expression was specifically identified in neurons of the CA3 region of the hippocampus, but was not expressed substantially in the dentate gyrus.[15] We corroborate this finding here by LacZ histochemistry (Fig. 1A, B). We also make the novel observation of strong localized expression in the region of the visual center of the cerebral cortex (Fig. 1C). LacZ staining was not observed in wild-type mouse brains (Supplementary Fig. S1). Consistent with the expression analyses in mice, immunohistochemical analysis of sections from the human brain revealed high GPR158 protein levels in the cerebral cortex (Fig. 1D, E). The protein was localized subcellularly on both the plasma membrane and in the nucleus of neurons, consistent with our previous findings in studies of other types of cells in culture.[11,17]

Gpr158 *expression and GPR158 protein localization in the eye*

On the University of California Davis KOMP website, *Gpr158* expression was reported as negative in the eye, however, LacZ histochemical analysis was performed only at a gross level. We examined expression in eye tissue sections of *Gpr158* KO mice by LacZ histochemistry, and examined protein localization in wild-type mice by immunohistochemistry. Representative results are shown in Fig. 2.

Since GPR158 is highly expressed in neural tissues of the brain, we focused first on the neural retina. LacZ histochemistry on *Gpr158* KO mice revealed *Gpr158* expression in retinal photoreceptors (Fig. 2A–C) and ganglion cell bodies (Fig. 2C). LacZ staining was not observed in wild-type mouse retinas (Supplementary Fig. S1). Gpr158 immunohistochemistry revealed protein localized within the same cells identified by LacZ histochemistry, and also in a distinct line within the outer plexiform layer where the photoreceptors synapse with neurons of the inner nuclear layer (Fig. 2D, E).

The ciliary body that produces aqueous humor and the trabecular meshwork of the conventional aqueous outflow pathway are not neural tissues, but originate developmentally from the optic cup and neural crest.[26,27] Ciliary body and trabecular meshwork express neuroendocrine markers, and it has been suggested that they act as multifunctional neuroendocrine glands.[28–33] We could not detect Gpr158 expression or immunoreactive protein in the ciliary body. However, Gpr158 immunohistochemistry revealed protein within the trabecular meshwork cells of the aqueous outflow pathway (Fig. 2E, F).

GPR158 immunohistochemistry was also performed on tissue sections from the human eye. Representative results are shown in Fig. 3. The same distinct line of protein localization was seen in the outer plexiform layer of the human retina, as was seen in mice (Fig. 3A). In addition, protein was localized to cells of the trabecular meshwork (Fig. 3B), as was seen in mice. Again, there was no detectable immunoreactive protein in ciliary body, and primary cultures of ciliary epithelial cells were also negative (data not shown). These results indicate that GPR158 is expressed and localizes similarly in the eyes of humans and mice, supporting the use of mice as a model to investigate GPR158 function in the eye.

Effect of Gpr158 *on intraocular pressure and visual function in mice*

To learn whether Gpr158 might have an effect on intraocular pressure, we compared homozygous *Gpr158* KO

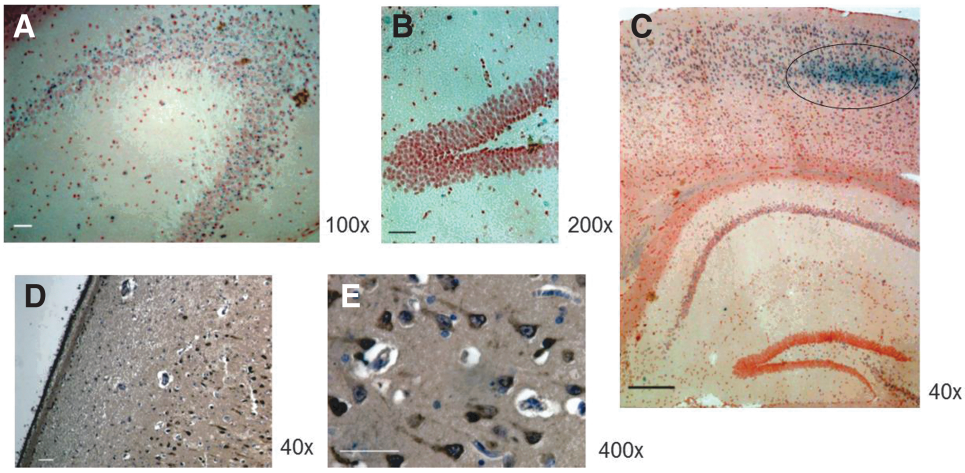

FIG. 1. *Gpr158* expression in the visual center of the mouse cerebral cortex and GPR158 subcellular localization in human cortical neurons. Tissue cross-sections from mouse and human brains. **(A)** Homozygous KO *Gpr158–/–* mouse brain hippocampus, CA3 region. LacZ histochemistry (*blue color*), nuclear fast red counterstain. Magnification: 100×, *white bar*: 100 μm. **(B)** Homozygous KO *Gpr158–/–* mouse brain hippocampus, DG region. LacZ histochemistry (*blue color*), nuclear fast red counterstain. Magnification: 200×, *black bar*: 100 μm. **(C)** Homozygous KO *Gpr158–/–* mouse brain cerebral cortex. The visual center is circled. LacZ histochemistry (*blue color*), nuclear fast red counterstain. Magnification: 40×, *black bar*: 500 μm. **(D)** Human brain cerebral cortex. Immunohistochemistry. Primary antibody specific for the GPR158 extracellular domain, 3,3′-diaminobenzidine chromogen (*brown color*). Magnification: 40×, *white bar*: 100 μm. **(E)** Human brain cerebral cortex. Immunohistochemistry. Primary antibody specific for the GPR158 extracellular domain, 3,3′-Diaminobenzidine chromogen (*brown color*). *Right panel* Magnification: 400×, *white bar*: 100 μm. DG, dentate gyrus; KO, knockout.

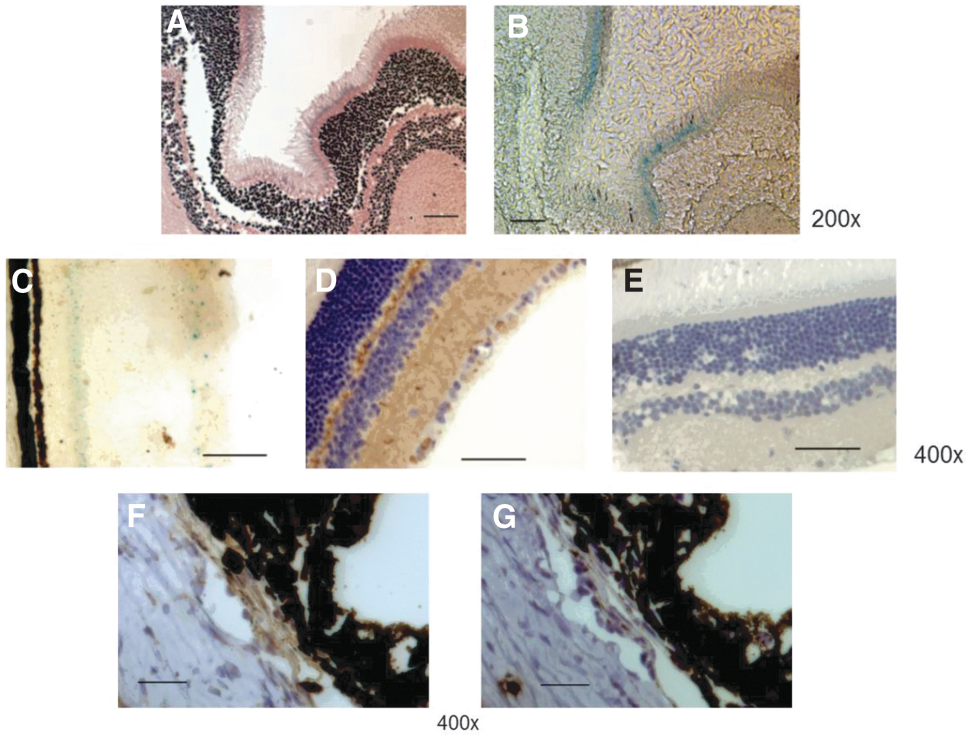

FIG. 2. *Gpr158* expression and Gpr158 localization in the mouse eye. Tissue cross-sections from mouse eyes. **(A)** Homozygous KO *Gpr158–/–* mouse eye, cross-section through the retina, zoomed into the photoreceptors, Hematoxylin and eosin stain. **(A, B)** Adjacent sections from the same block. Magnification: 200×, *black bar*: 100 µm. **(B)** Homozygous KO *Gpr158–/–* mouse eye, cross-section through the retina, zoomed into the photoreceptors. LacZ histochemistry. **(A, B)** Adjacent sections from the same block. Magnification: 200×, *black bar*: 100 µm. **(C)** Homozygous KO *Gpr158–/–* mouse eye, cross-section through the retina. LacZ histochemistry (*blue color*). Magnification: 400×, *black bar:* 100 µm. **(D)** Normal littermate control *Gpr158+/+* mouse eye, cross-section through the retina. Immunohistochemistry. Primary antibody specific for the GPR158 extracellular domain, 3,3′-diaminobenzidine chromogen (*brown color*). Magnification: 400×, *black bar*: 100 µm. **(E)** Homozygous KO *Gpr158–/–* mouse eye, cross-section through the retina. Immunohistochemistry. Primary antibody specific for the GPR158 extracellular domain, 3,3′-diaminobenzidine chromogen (*brown color*). Magnification: 400×, *black bar*: 100 µm. **(F)** Normal littermate control *Gpr158+/+* mouse eye, cross-section through the trabecular meshwork and Schlemm's canal. Immunohistochemistry. Primary antibody specific for the GPR158 extracellular domain, 3,3′-diaminobenzidine chromogen (*brown color*). Magnification: 400×, *black bar*: 100 µm. **(G)** Homozygous KO *Gpr158–/–* mouse eye, cross-section through the trabecular meshwork and Schlemm's canal. Immunohistochemistry. Primary antibody specific for the GPR158 extracellular domain, 3,3′-diaminobenzidine chromogen (*brown color*). Magnification: 400×, *black bar*: 100 µm.

mice (*Gpr158–/–*), heterozygous KO littermates (*Gpr158–/+*), and normal littermate controls (*Gpr158+/+*) at 3 month intervals after birth (Fig. 4A). An early protective effect against the small elevation in intraocular pressure with aging was observed with increasing *Gpr158* deficiency. The difference between normal littermate controls and homozygous *Gpr158* KO mice was statistically significant at 6 months of age ($P = 0.008$). This protection was no longer apparent at 9 months.

We next investigated whether GPR158 might affect intraocular pressure in response to stress. Mice and humans exhibit a slow and highly personalized response to the chronic glucocorticoid stress hormones[13]; however, response to the acute stress hormone, epinephrine, is more rapid and uniform.[34] Topical epinephrine significantly reduced intraocular pressure in *Gpr158* normal littermate control mice within 30 min (Fig. 4B), similar to results previously reported.[35] However, homozygous *Gpr158* KO mice were refractory to these effects of epinephrine. Specifically, at the 30-min time point, intraocular pressure in normal littermate control mice was decreased by 0.8-fold, while intraocular pressure in homozygous *Gpr158* KO mice was increased by 1.05-fold. Since general anesthesia has been reported to reduce intraocular pressure, intraocular pressure was measured in the opposite eye of each animal performed without the use of topical anesthetic over the same time course; no reduction due to isoflurane was seen over this short time course. These results suggest that Gpr158 is essential for intraocular pressure reduction in response to epinephrine.

To investigate a role for Gpr158 in visual function, we performed ERG on dark adapted *Gpr158* homozygous KO mice, comparing their phenotype with normal littermate controls (Fig. 4C). Flashes of light over the range of light intensities from dim scotopic to bright photopic elicited robust a- and b-waves indistinguishable between the genotypes. These results indicate that *Gpr158* deficiency does not overtly affect the ability of photoreceptors to generate light response and transmit it across the synapse to ON-bipolar cells.

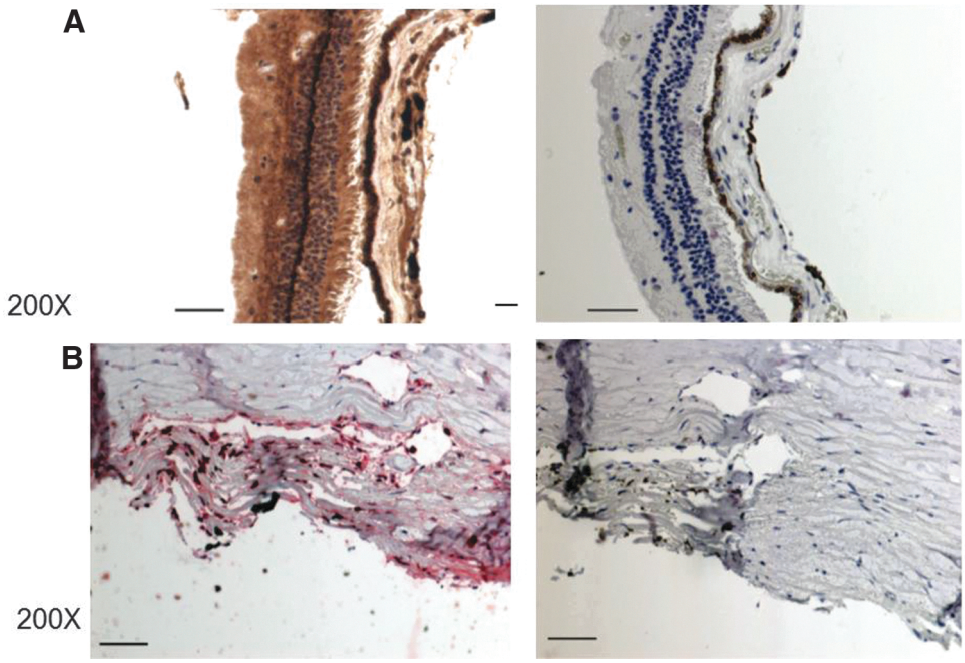

FIG. 3. *GPR158* expression and GPR158 localization in the human eye. Tissue cross-sections from human eyes. **(A)** Human eye, cross-section through the retina. Immunohistochemistry. The 2 *panels* are adjacent sections from the same block. *Left panel*: primary antibody specific for the GPR158 extracellular domain, 3,3′-diaminobenzidine chromogen (*brown color*). *Right panel*: secondary antibody only, hematoxylin counterstain. Magnification: 200×, *black bar*: 100 μm. **(B)** Human eye, cross-section through the trabecular meshwork and Schlemm's canal. Immunohistochemistry. The 2 *panels* are adjacent sections from the same block. *Left panel*: primary antibody specific for the GPR158 extracellular domain, 3-amino-9-ethilcarbasole chromogen (*red color*). *Right panel*: secondary antibody only, hematoxylin counterstain. Magnification: 200×, *black bar*: 100 μm.

Effect of GPR158 on intracellular signaling and survival of trabecular meshwork cells in culture

To investigate GPR158 effects on intracellular signaling, we developed a cell culture model for *GPR158* overexpression. A variety of tissues participate in the regulation of aqueous humor dynamics, including trabecular meshwork, ciliary epithelium, and ciliary muscle.[36] Of these, trabecular meshwork was positive for endogenous *GPR158* expression in our tissue section studies here, and in previous human cell culture studies.[11] To enhance reproducibility across experiments, we decided to create stable *GPR158* transfectants, which requires the use of an immortalized cell line. These considerations led us to choose the well-characterized TM-1 cell line.[19] The model we created is composed of matched cell lines stably transduced with a doxycycline-inducible lentiviral expression vector, one containing a *GPR158* insert and the other an empty vector control. The dose of GPR158 protein product received by the cell can be controlled by the concentration of doxycycline used.

In a prostate cancer cell version of our model, we used 100 and 500 ng/mL concentrations of doxycycline for low and high *GPR158*-induction, respectively.[17] In our first set of experiments, lenti-*GPR158* transduced cells were induced with these same doses (Fig. 5A). A set of cells was left uninduced (no doxycycline). The next day, a parallel set of doxycycline-treated and uninduced lenti-vector TM-1 cells were also treated with epinephrine. In this set of experiments, the positive control is uninduced cells treated with epinephrine and the negative control is uninduced cells (no doxycycline) not treated with epinephrine. After a short period (20 min), the level of cAMP was assayed in all experimental groups (Fig. 5B). Epinephrine robustly stimulated cAMP accumulation, as previously observed using trabecular meshwork cells in culture.[37] However, the level of cAMP was negligible for all other groups, whether or not *GPR158* expression was induced. Thus, it is unlikely that GPR158 signals directly via mechanisms that activate adenyl cyclase.

Next, we investigated possible indirect effects of GPR158 on cAMP accumulation in response to epinephrine, which signals through the adrenergic receptor subfamily of GPCRs. For the representative experiment shown in Fig. 5C, lenti-*GPR158* cells were left uninduced or induced with doxycycline at 500 μg/mL to stimulate *GPR158* expression; lenti-vector control cells were similarly treated. The next day, all cells were treated with epinephrine. As in the first set of experiments, the positive control is uninduced cells treated with epinephrine and the negative control is uninduced cells (no doxycycline), not treated with epinephrine. Cell samples were then taken for cAMP determination over a time course of 180 min, to observe a full response to the drug. It was observed that induced *GPR158* overexpression significantly enhanced the response to epinephrine. These results are consistent with the intraocular effects of *Gpr158* KO on response to epinephrine *in vivo*.

We next considered the other activity we identified for GPR158, that is, its effects on cell proliferation and neuroendocrine differentiation. We previously reported that low-

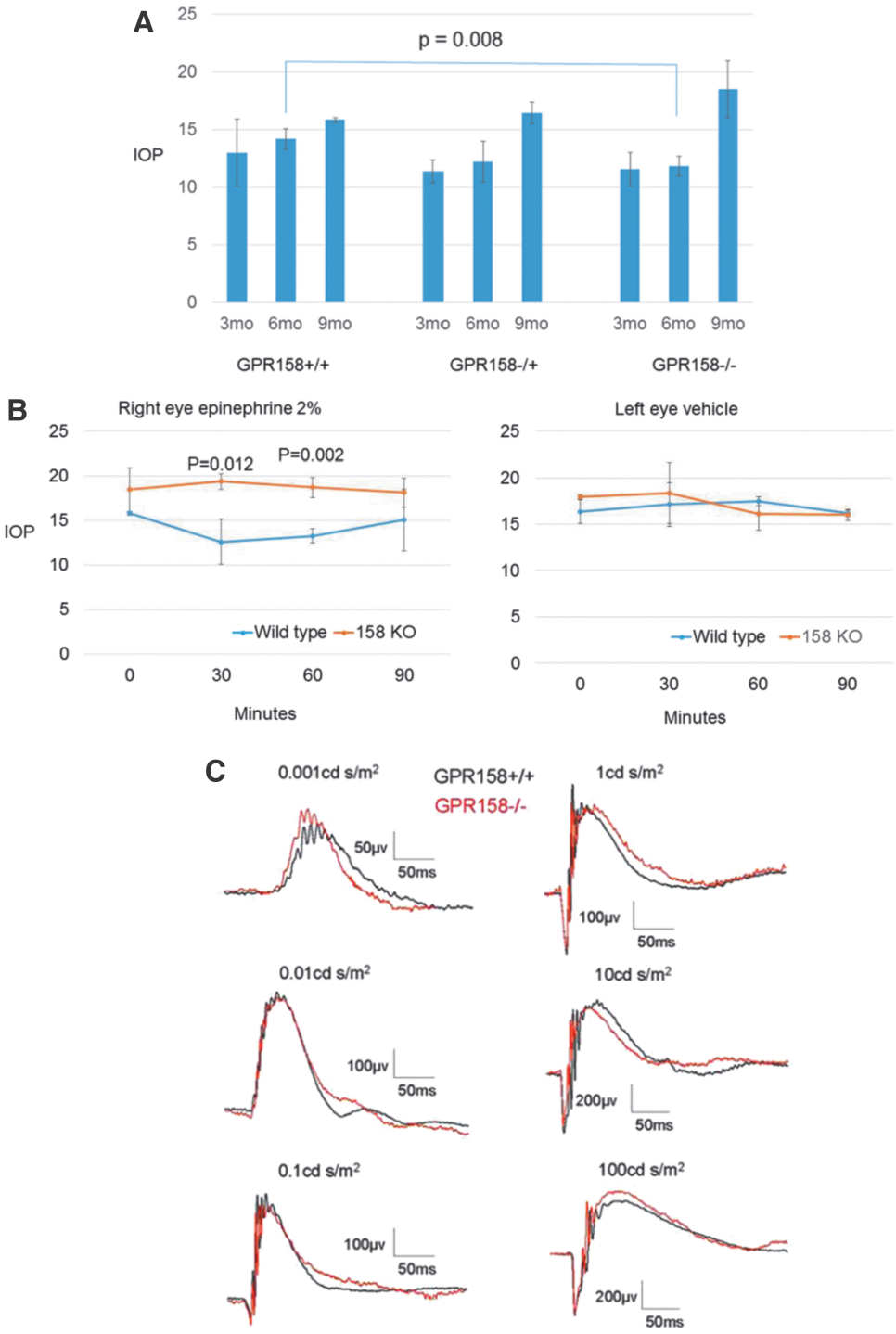

FIG. 4. Effect of GPR158 on intraocular pressure and visual function in mice. *Gpr158* homozygous and heterozygous KO mice and normal littermate controls. **(A)** Comparison of intraocular pressure changes over a 9-month time course in the different *Gpr158* KO mouse genotypes (normal littermate controls *Gpr158*+/+, heterozygous KO *Gpr158*–/+, homozygous KO *Gpr158*–/–). Significance of the data was determined by Student's *t*-test; $N=3$. **(B)** Intraocular pressure changes in response to epinephrine, comparing 9-month old *Gpr158* homozygous KO mice to normal littermate controls. Significance of the data was determined by the Student's *t*-test and the paired *t*-test; $N=3$. **(C)** Representative electroretinography traces comparing dark-adapted GPR158 homozygous KO mice and normal littermate controls stimulated with indicated flashes covering both scotopic and photopic ranges.

level overexpression of lenti-*GPR158* (100 ng/mL doxycycline treatment) in prostate cancer cells stimulated cell proliferation and androgen receptor expression, however, high-level overexpression (500 ng/mL doxycycline treatment) reversed this and induced expression of neuroendocrine differentiation markers.[17] The tumor suppressor TP53 is activated by a variety of stresses, arresting growth by holding the cell cycle at the G1/S regulation point through its action as a transcription factor.[38] This suggested TP53 as a candidate mediator of the observed reversals. Indeed, we found that TP53 protein accumulated in lenti-*GPR158* trabecular meshwork cells when induced with doxycycline at 500 ng/mL (Fig. 5D). This is consistent with the known mechanism of TP53 pathway activation, that is, TP53 protein stabilization in response to cellular stress.[38]

In our final set of experiments, we investigated possible effects of *GPR158* overexpression on trabecular meshwork cell survival. Since oxidative stress has been implicated in ocular hypertension,[39,40] this was utilized as the stress in our experiments. We treated the lenti-*GPR158* trabecular meshwork cells with increasing doses of the oxidant t-BHP in the absence or presence of doxycycline at 500 ng/mL to

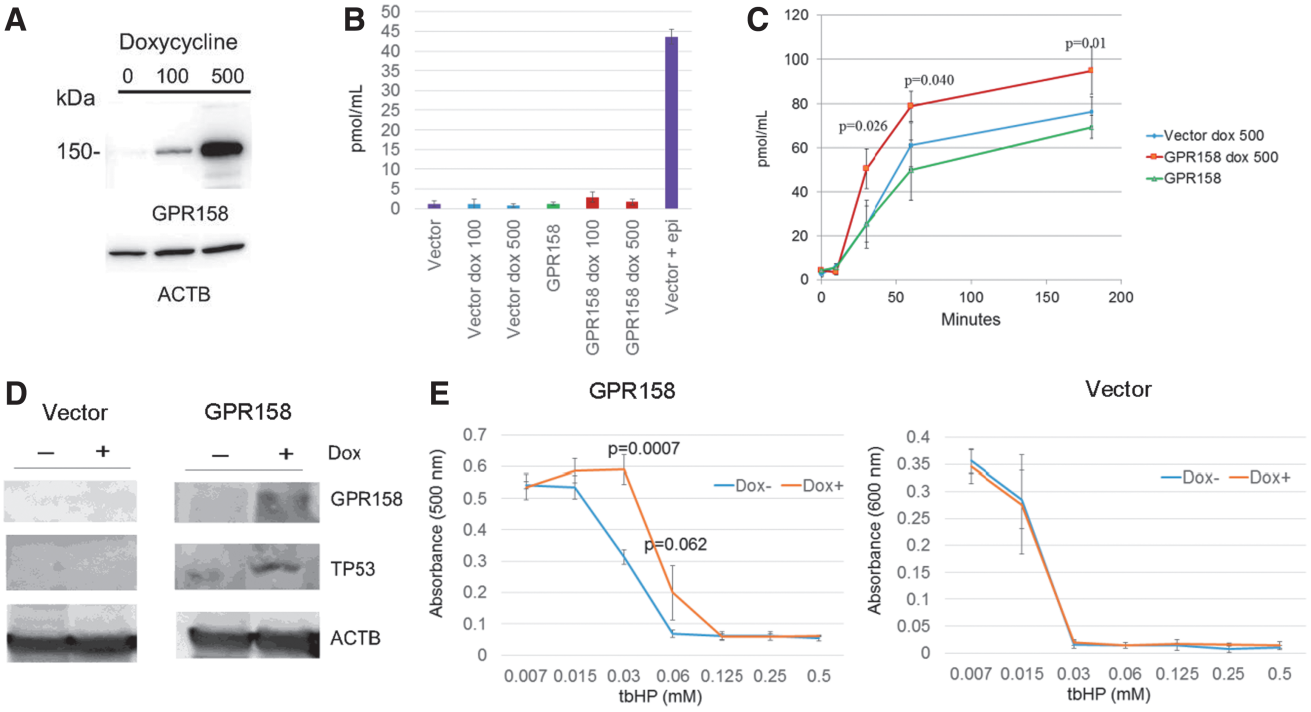

FIG. 5. Effect of GPR158 on intracellular signaling and survival in trabecular meshwork cell cultures. *GPR158* overexpression in cultured human trabecular meshwork cells. **(A)** Model. Western blot demonstrating induction of *GPR158* in the lenti-*GPR158* TM-1 cell line using low and high concentrations of doxycycline (100 and 500 μg/mL) for 24 h. **(B)** Accumulation of cAMP. Cells of the lenti-*GPR158* TM-1 line or the control lenti-vector TM-1 line were left uninduced or induced with doxycycline (100 or 500 ng/mL) to stimulate expression of the *GPR158* transgene. The next day, a parallel set of control lenti-vector TM-1 cells left uninduced or induced with doxycycline (500 ng/mL) were also treated with epinephrine. In this set of experiments, the positive control is uninduced cells treated with epinephrine and the negative control is uninduced cells (no doxycycline), not treated with epinephrine. Cell samples were then taken for cAMP determination after 20 min. Significance of the data was determined by Student's *t*-test; $N=3$. **(C)** Accumulation of cAMP in cells treated with 2% epinephrine. Cells of the lenti-*GPR158* TM-1 line or the control lenti-vector TM-1 line were treated with doxycycline at 500 ng/mL to induce the *GPR158* transgene. The next day, half the cultures were left untreated or treated with 2% epinephrine. Cell samples were then taken for cAMP determination over a time course of 180 min. Significance of the data was determined by analysis of variance; $N=3$. **(D)** TP53 pathway activation. *Left panel*: lenti-vector TM-1 cell line. *Right panel*: Lenti-*GPR158* TM-1 cell line. Cells were treated with doxycycline at 500 ng/mL to induce the *GPR158* transgene. After 24 h, cell lysates were prepared and proteins were separated by gel electrophoresis, immunoblotted, and probed for TP53 protein. The blot was reprobed with GPR158 antibody to demonstrate induction of expression, and with ACTB antibody to ascertain loading equivalence of gel lanes. **(E)** Survival of cells subjected to oxidative stress. *Left panel*: Lenti-*GPR158* TM-1 cell line. *Right panel*: lenti-vector TM-1 cell line. Cells were treated with various concentrations of the oxidant tert-butyl hydrogen peroxide (mM), with (*red line*) or without (*blue line*) doxycycline at 500 ng/mL to induce the *GPR158* transgene. After 24 h, cell lysates were prepared, and the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was performed and absorbance was read at 600 nm. The Student's *t*-test was used to determine significance of the results; resulting *P* values are indicated on the graph; $N=3$.

induce expression of *GPR158*; a set of lenti-vector control cells was also treated in this way. After 24 h, cell survival was quantified by metabolic assay. It was found that *GPR158* overexpression conferred significant resistance to the oxidant (Fig. 5E).

Discussion

GPR158 is a newly characterized family C GPCR, previously identified in functional screens linked with biological stress, including one for susceptibility to ocular hypertension/glaucoma induced by pharmaceutical analogs of glucocorticoid stress hormones.[11–13,17] Previously, we showed that glucocorticoids stimulate *GPR158* expression by human trabecular meshwork cells in culture,[11] and in this study, we demonstrate expression and protein localization in the trabecular meshwork of the aqueous outflow pathways of mice and humans. *Gpr158* deficiency in KO mice conferred short-term protection against the intraocular pressure increase that occurred with aging, but this was reversed over time. More strikingly, the pressure lowering effect of the acute stress hormone, epinephrine, was negated in *Gpr158* KO mice. Taken together, these findings implicate GPR158 as a homeostatic regulator of intraocular pressure.

Physiological homeostasis is maintained through a fine regulation of the many steps involved in the function of neuroendocrine axes. The hypothalamic–pituitary–adrenal axis is activated by stress, leading to a production of glucocorticoid stress hormones by the adrenal glands, as well as the more rapidly acting catecholamine stress hormones, such as epinephrine. The natural ligand for the adrenergic receptor subtype of GPCR family A, epinephrine lowers

intraocular pressure. This is due to the combined action on adrenergic receptors located in several tissues. Aqueous production is inhibited by the vasoconstrictive effect of epinephrine on blood vessels in the ciliary body. Epinephrine exerts this effect through ADRA2A, which couples with Galpha(s) to stimulate adenyl cyclase and cAMP accumulation.[37] In addition, an increase in aqueous outflow facility occurs, correlated in time with increased cyclic AMP production in the trabecular meshwork.[34,41] Trabecular meshwork cells express both ADRA2A and ADRB2; the latter couples with Galpha(i/o) to inhibit adenyl cyclase, however, ADRA2A activity appears to dominate.[36,37,42–44] The observation that epinephrine did not lower intraocular pressure in *Gpr158* KO mice suggested that Gpr158 is necessary to shift the balance of activity toward cAMP production.

To investigate GPR158 effects on cAMP accumulation, we used a human trabecular cell culture model for *GPR158* overexpression. Many GPCRs oscillate between inactive and active conformation, thus showing basal activity detectable upon overexpression, even in the absence of ligand binding.[45,46] However, GPR158 does not appear to utilize cAMP as a second messenger on its own, as we show here that its overexpression in the absence of epinephrine treatment did not lead to cAMP accumulation in cultured trabecular meshwork cells. Thus, an unconventional activity is suggested. As noted already, one of the unconventional activities of GPR158 is its formation of complexes with the GTPase-activating protein RGS7.[14] GTPase-activating proteins play an essential role in limiting signaling initiated by GPCRs by inhibiting G proteins. Interestingly, a similar complex also exists in retinal bipolar neurons, where closely related orphan receptor, GPR179 binds to RGS7 or its homolog RGS11. GPR179 recruits RGS7/11-GNB5 complexes to the plasma membrane at the dendritic tips of retinal bipolar cells.[14] In this way, it is positioned to inactivate Galpha(i/o) supplied by another class C GPCR, GRM6, thereby permitting depolarizing response of bipolar neurons to light. Similarly, GPR158 is necessary for the plasma membrane recruitment of RGS7 in the brain, and its activity in terminating signaling of conventional GPCRs.[18] If the RGS7 mechanism operates in TM-1 cells, then overexpression of *GPR158* would be predicted to enhance epinephrine-stimulated cAMP production by deactivating inhibitory Galpha(i/o) signaling. This is the result we observed in our cell culture model, supporting the hypothesis. Further studies will be needed to eliminate other possibilities.

Previously, we found that GPR158 has regulatory effects on cell proliferation: low-level expression is stimulatory,[11] but high-level expression is inhibitory.[17] In this study, we show that high-level overexpression of *GPR158* in trabecular meshwork cells activates a third stress pathway regulated by TP53. The tumor suppressor TP53 is activated by a variety of stresses, arresting growth by holding the cell cycle at the G1/S regulation point through its action as a transcription factor.[38] However, effects on proliferation are not particularly relevant to trabecular meshwork function in aqueous outflow, since the cells of this tissue are nonproliferative once eye development is complete. Importantly, we identified here, a second effect of high-level *GPR158* expression in trabecular meshwork cells that could also work through TP53: protection against oxidative stress. Accumulated oxidative stress has been suggested to be an important factor in failure of aqueous outflow,[39,40] and a protein that protects against the deleterious effects of oxidative stress is potentially very significant to homeostatic maintenance of the outflow pathways. We are currently investigating the possible causal link between oxidative stress protection by GPR158 and TP53 pathway activity.

We show in this study that *Gpr158* deficiency in KO mice does not overtly affect the ability of photoreceptors to generate light response and transmit it across the synapse to ON-bipolar cells. This makes it different from closely related family member *GPR179*. The mouse mutant, nob5, lacks an electroretinogram b-wave, the result of a large transposon-like DNA insertion in *Gpr179*. *Gpr179* is expressed by bipolar cells of the inner nuclear layer of the retina. The protein localizes to the dendritic tips of these neurons, which synapse with the photoreceptors in the outer plexiform layer of the retina.[14] In humans, *GPR179* is required for depolarizing bipolar cell function and is mutated in autosomal recessive complete congenital stationary night blindness. It serves as a model for a form of complete congenital stationary night blindness in humans also linked to *GPR179*.[47] We found that GPR158 protein also localizes to the outer plexiform layer of the retina; however, photoreceptors appear to be the major site of expression. We also found that GPR158 is localized to retina ganglion cells, whose axons bundle together in the optic nerve, and in the visual center of the cerebral cortex, where these axons terminate. The ERG cannot reliably detect electrical activity in ganglion cells; thus, we cannot rule out a role for GPR158 in visual perception at this time.

A subclass of retinal ganglion cells is intrinsically photosensitive due to the presence of melanopsin.[48] These light-sensing cells have been linked to entrainment of circadian rhythms to the master circadian oscillator,[49] and glaucoma alters circadian rhythms because of loss of these cells.[50] Intraocular pressure exhibits a robust circadian rhythm that is synchronized to the master circadian oscillator.[51,52] Glucocorticoid secretion is also under circadian control,[53] and was recently shown to act as a signal for entraining intraocular pressure rhythmicity.[54] Perhaps of significance with respect to mechanism, GPR158 was identified in a genome-wide siRNA screen as a potential modulator of the circadian clock.[55] One hypothesis is that GPR158 regulation by glucocorticoids might be part of the pathway for entraining the rhythm of intraocular pressure, linking ganglion cells with the trabecular meshwork. This and other possible roles for GPR158 in the eye will be explored in future studies.

Homeostatic mechanisms for control of intraocular pressure have been postulated, but remain poorly elucidated. Studies reported here implicate the newly-characterized family C GPCR, GPR158, as a homeostatic modulator of intraocular pressure in response to stress. Our results suggest that GPR158 could serve as a novel target for control of intraocular pressure in glaucoma, a common disease in need of alternative medications for many individuals. Further work will be directed toward defining direct and indirect signaling pathways regulated by GPR158 to determine the most effective agonists/antagonists for possible use as therapeutics.

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Authors' Contributions

T.I. was the lead experimentalist, designing and performing most experiments and analyzing resulting data. He also prepared first drafts of most figures and codrafted the article. A.W. managed the mouse colony and assisted with mouse experiments and analysis of resulting data. S.K.C. performed western blots documenting specific genes identified by IPA and analyzed resulting data. Y.W. performed the ERG experiment in mice and analyzed resulting data. J.M.G. advised on and assisted with execution of intraocular pressure measurement in mice, and analysis of resulting data. J.C.T. advised on and oversaw execution of intraocular pressure measurement in mice and analysis of resulting data. J.A.V. designed and executed some experiments using the lentiviral constructs and analyzed resulting data. T.A. designed and oversaw execution of some experiments using the lentiviral constructs and analyzed resulting data. C.M.C. advised on planning of mouse electroretinography experiments and data analysis. M.E.S.S. advised on human tissue immunohistochemistry and interpreted results as an ophthalmic pathologist. S.J. provided advice on experimental design, assistance in data analysis, and scientific discussion. W.D.S. provided GPCR and ocular hypertension conceptual framework and advised on experimental design and resulting data analysis. K.A.M. advised on GPCR conceptual framework, experimental design, and data analysis. He also provided breeding pairs of *Gpr158* KO mice for use in the intraocular pressure studies, oversaw the ERG experiments performed in his laboratory and data analysis, and wrote portions of the article first draft. M.E.F. conceived, guided, and oversaw the entire project, analyzed the resulting data, drafted the initial version of the article, and guided and assisted in figure preparation. All coauthors reviewed the article and figure drafts, provided edits, and approved the final article for submission.

Author Disclosure Statement

T.I. and M.E.F. are named as coinventors on a provisional patent application submitted by the University of Southern California claiming GPR158 as a drug target to control intraocular pressure in glaucoma. The other authors have no competing interests to declare.

Supplementary Material

Supplementary Figure S1

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Address correspondence to:
Dr. M. Elizabeth Fini
Department of Ophthalmology
Tufts Medical Center
800 Washington Street
Boston, MA 02111

E-mail: mefini@tuftsmedicalcenter.org