AMP-Activated Protein Kinase Regulates Intraocular Pressure, Extracellular Matrix, and Cytoskeleton in Trabecular Meshwork

Ayan Chatterjee, Guadalupe Villarreal Jr, Dong-Jin Oh, Min Hyung Kang, and Douglas J. Rhee

Department of Ophthalmology, Massachusetts Eye & Ear Infirmary, Harvard Medical School, Boston, Massachusetts, United States

Correspondence: Douglas J. Rhee, Department of Ophthalmology, Massachusetts Eye & Ear Infirmary, 243 Charles Street, Boston, MA 02114, USA; DougRhee@aol.com.

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PURPOSE. In this study, we investigate how adenosine monophosphate-activated protein kinase (AMPK) affects extracellular matrix (ECM) and cellular tone in the trabecular meshwork (TM), and examine how deletion of its catalytic $\alpha 2$ subunit affects IOP and aqueous humor clearance in mice.

METHODS. Human TM tissue was examined for expression of AMPK $\alpha 1$ and AMPK $\alpha 2$, genomically distinct isoforms of the AMPK catalytic subunit. Primary cultured human TM cells were treated for 24 hours with the AMPK activator 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR), under basal or TGF- $\beta 2$ stimulatory conditions. Conditioned media (CM) was probed for secreted protein acidic and rich in cysteine (SPARC), thrombospondin-1 (TSP-1), and ECM proteins, and cells were stained for F-actin. Cells underwent adenoviral infection with a dominant negative AMPK α subunit (ad.DN.AMPK α) and were similarly analyzed. Intraocular pressure, central corneal thickness (CCT), and aqueous clearance were measured in AMPK $\alpha 2$ -null and wild-type (WT) mice.

RESULTS. Both AMPK $\alpha 1$ and AMPK $\alpha 2$ are expressed in TM. AICAR activated AMPK α and suppressed the expression of various ECM proteins under basal and TGF- $\beta 2$ stimulatory conditions. AICAR decreased F-actin staining and increased the phospho-total RhoA ratio (Ser188). Transforming growth factor- $\beta 2$ transiently dephosphorylated AMPK α . Infection with ad.DN.AMPK α upregulated various ECM proteins, decreased the phospho-total RhoA ratio, and increased F-actin staining. AMPK $\alpha 2$ -null mice exhibited 6% higher IOP and decreased aqueous clearance compared with WT mice, without significant differences in CCT or angle morphology.

CONCLUSIONS. Collectively, our data identify AMPK as a critical regulator of ECM homeostasis and cytoskeletal arrangement in the TM. Mice that are AMPK $\alpha 2$ -null exhibit higher IOPs and decreased aqueous clearance than their WT counterparts.

Keywords: AMPK, POAG, glaucoma, trabecular meshwork, intraocular pressure, SPARC, TGF- $\beta 2$, RhoA

Glaucoma is a leading cause of irreversible blindness.¹ Elevated IOP in eyes with POAG is caused by poor aqueous humor drainage and can lead to visual field loss due to progressive optic nerve damage.² The only rigorously proven treatment for POAG is to lower IOP.^{3,4} Thus far, single gene mutations account for less than 10% of POAG cases, with the other 90% likely having polygenic origins.⁵ Elucidating the molecular underpinnings of IOP regulation is crucial in the search for new treatments.

In humans, approximately 80% to 90% of aqueous outflow occurs through the trabecular meshwork (TM; conventional pathway) with the remaining 10% to 20% exiting through the ciliary body face (alternative pathway).⁶ In mice, a greater proportion of outflow occurs via the alternative pathway.^{7,8} The juxtacanalicular (JCT) region of the TM, an amorphous layer composed of endothelial cells and extracellular matrix (ECM), is thought to be where the regulation of aqueous outflow takes place.⁹ Under conditions of elevated IOP, the JCT has the highest outflow resistance¹⁰ and the ECM within the JCT is constantly being remodeled.¹¹

The regulation of IOP in the JCT region is a complex system. Some processes, such as the regulation of ECM homeostasis, have been shown to influence IOP.^{12–17} Modifications in the actin cytoskeleton and cellular tone of the JCT TM and inner wall of Schlemm's canal cells have also been shown to affect IOP¹⁸ by contributing to changes in aqueous outflow facility.^{19,20} In nonglaucomatous eyes, increasing ECM production or slowing its turnover alters IOP, and alterations of the JCT ECM constitute primary pathophysiologic events.^{14,15,21,22}

Matricellular proteins are nonstructural secreted glycoproteins that facilitate cellular control over the surrounding ECM. Secreted protein acidic and rich in cysteine (SPARC), the prototypical matricellular protein, is widely expressed in human ocular tissues, including TM endothelial cells.^{23,24} Overexpression of SPARC by TM cells increases IOP in perfused cadaveric human anterior segments derived from nonglaucomatous eyes.²⁵ This elevation of IOP coincides with an increase of certain ECM proteins within the JCT. Conversely, SPARC-null mice demonstrate 15% to 20% lower IOP than their wild-type (WT) counterparts as a result of increased aqueous clearance²⁶

due, in part, to greater areas of high flow TM.²⁷ Thrombospondin-1 (TSP-1), like SPARC, is also a matricellular protein expressed in the TM.^{28,29} Thrombospondin-1-null mice have 10% lower IOP than their WT counterparts.³⁰ Elucidation of upstream regulators of proteins such as SPARC and TSP-1 may lead to new therapeutic targets.

Adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase that regulates cellular metabolism, proliferation, and aging processes.^{31–33} It exists throughout the eukaryotic domain as heterotrimeric complexes uniting a catalytic α subunit with regulatory β and γ subunits.³⁴ Within the mammalian kingdom, each subunit has multiple isoforms, $\alpha 1$ and $\alpha 2$; $\beta 1$ and $\beta 2$; $\gamma 1$, $\gamma 2$, and $\gamma 3$; in humans, each encoded at a distinct genetic locus within the genome, yielding a total of 12 possible heterotrimeric combinations that appear to be distributed throughout the body in a tissue-specific manner.³⁵ The role of AMPK in aging^{36–39} and in diabetes, atherosclerosis, and cancer progression has made it an attractive pharmaceutical target.^{31,34} Although AMPK signaling has been studied in ocular diseases such as diabetic retinopathy,^{40–42} its potential role in ECM homeostasis in the TM, IOP regulation, and glaucoma progression is unknown.

Transforming growth factor- $\beta 2$ (TGF- $\beta 2$) is greatly increased in the aqueous humor of patients with POAG compared with age-matched controls,^{43,44} and several studies suggest that TGF- $\beta 2$ -mediated fibrosis contributes to POAG pathogenesis.^{45–47} We have shown that TGF- $\beta 2$ upregulates SPARC expression in human TM cells.⁴⁸ Adenosine monophosphate-activated protein kinase regulates matrix remodeling following injury to various nonocular tissues,^{31,49–51} and its signaling pathways interact with TGF- $\beta 2$ during inflammation,⁵² angiogenesis,⁵³ and fibrosis.⁴⁹ Pharmacologic activation of AMPK has been shown to suppress TGF- $\beta 2$ -induced fibrosis in liver.⁴⁹ We hypothesized that AMPK has functional relevance to IOP and that at least part of its mechanism involves altering SPARC, TSP-1, and other select ECM proteins. We evaluated IOP and aqueous humor clearance in mice harboring single gene deletions in the catalytic $\alpha 2$ subunit of AMPK and examined the effects of AMPK modulation on matricellular and ECM protein levels under basal and TGF- $\beta 2$ stimulatory conditions in TM endothelial cells.

MATERIALS AND METHODS

Animal Care and Husbandry

All experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and received approval from the Massachusetts Eye and Ear Infirmary animal care and use committee. Viollet and colleagues⁵⁴ generously provided AMPK $\alpha 2$ -null mice, and they were developed as described elsewhere. Briefly, a targeting construct corresponding to the AMPK $\alpha 2$ catalytic domain (amino acids 189–260) was electroporated into 129/Sy MPI-I embryonic stem cells and the resultant PCR-confirmed clones were injected into C57Bl/6 blastocysts. Germline-transmitting chimeric animals were mated with C57Bl/6 mice to produce heterozygous offspring, which were then crossed to produce control and mutant mice. All mice for these experiments were bred at our facility, fed ad libitum, and housed at 21°C in clear plastic rodent cages under 12-hour light/12-hour dark cycles (on 07:00, off 19:00). Wild-type and null colonies were maintained by breeding heterozygotes with subsequent genotyping of all progeny to prevent species drift. Confirmation of homozygosity was performed as previously described,²⁶ using the following PCR primer sequences: AMPK $\alpha 2$ -WT [5'-GCTTAGCACGT TACCCTGGATGG-3'] (forward) and [5'-GTTATCAGCCCAAC

TAATTACAC-3'] (reverse) versus AMPK $\alpha 2$ -null [same forward primer as above] (forward) and [5'-GCATTGAACCA CAGTCCTTCCTC-3'] (reverse). Polymerase chain reaction amplification yielded 200-bp fragments for WT and 600-bp fragments for null mice. All IOP measurements were taken between 6 and 7 weeks of age. The mouse iridocorneal angle and its structures reach maturity by 5 weeks.⁵⁵

Measurement of IOP

Mouse IOP was measured as previously described.²⁶ Mice were anesthetized by intraperitoneal (IP) injection of a ketamine/xylazine mixture (100 mg/kg and 9 mg/kg, respectively; Phoenix Pharmaceutica, St. Joseph, MO, USA). Per manufacturer recommendations, the rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH, USA) was fixed horizontally to allow perpendicular contact with the central cornea, and the tip of the probe was positioned between 2 and 3 mm from the eye. To reduce variability, the rebound tonometer was modified to include a pedal that activated the probe, obviating handling of the device. Target verification was performed under direct visualization at $\times 5.5$ magnification. A single measurement was accepted only if the device indicated that there was “no significant variability” (per protocol manual; Colonial Medical Supply). The average IOP was taken from three sets of six measurements of IOP in each eye, alternating right and left eye, with the starting eye picked at random.^{56,57} All measurements were taken between 4 and 7 minutes after IP injection, as previous studies have shown this to be a period of stable IOP.^{58,59} Previous studies have shown that weekly administration of this anesthesia mixture does not affect IOP.⁶⁰ Intraocular pressure was measured once per mouse, between 11 AM and 3 PM at 7 weeks of age, 1 week after CCT measurement.

Optical Coherence Tomography

Eyes of adult mice (at 6 weeks) were imaged using optical coherence tomography (OCT) (Stratus; Carl Zeiss Meditec, Inc., Dublin, CA, USA). Under general anesthesia by IP injection of a ketamine/xylazine mixture, mouse eyes were scanned to acquire images and were analyzed using OCT software (Stratus version 4.0.7; Carl Zeiss Meditec, Inc.). Central corneal thickness was determined by measuring the distance between two peaks representing the corneal epithelium and endothelium. Measurements were performed in triplicate for each eye by the same investigator who was masked to the mouse strain. Values were averaged and reported as means and SEMs. We have previously validated the use of OCT in mice to estimate CCT against high-frequency ultrasound and histology.²⁶

Light Microscopy

For light microscopy, mice were euthanized using CO₂, and then immediately enucleated. The eyes were fixed with 10% formalin for 2 days, dehydrated in 70% ethanol, then rehydrated in ascending concentrations of ethanol (70%, 95%, 100%) for 2 hours. The eyes were incubated with methacrylate (Technovit 7100; Heraeus Kulzer GmbH, Wehrheim, Germany) and Harder 1 and 2 (Technovit 7100; Heraeus Kulzer GmbH) for 2 hours. Fixed sections were cut at 3 μ m, and then stained with toluidine blue (Sigma-Aldrich, St. Louis, MO, USA).

Assessment of Aqueous Humor Clearance

To investigate the mechanism of the observed IOP difference between AMPK $\alpha 2$ -null mice and their WT counterparts, we noninvasively measured aqueous humor clearance using a

FIGURE 1. At 6 to 7 weeks of age, AMPK α 2-null mice exhibit increased IOP compared with their WT counterparts, with no significant difference in CCT or gross architecture of the iridocorneal angle. **(A)** Intraocular pressure was obtained under sedation by TonoLab in WT ($n = 35$) and AMPK α 2-null mice ($n = 44$). On average, AMPK α 2-null mice had 6.0% higher IOP than WT mice ($*P = 0.0265$ by Student's t -test), 18.3 ± 0.3 vs. 17.2 ± 0.4 mm Hg (mean $\pm$ SEM). **(B)** Central corneal thickness measurements were obtained under sedation by OCT in WT ($n = 35$) versus AMPK α 2-null mice ($n = 44$). Data expressed as mean $\pm$ SEM ($P = 0.6877$ by Student's t -test; NS, not significant). Representative light microscopic images of iridocorneal angles in **(C)** AMPK α 2-WT and **(D)** AMPK α 2-null mice appear grossly indistinguishable with similar Schlemm's canals, trabecular beams and cellularity, uveoscleral outflow pathway, and ciliary body location. Scale bars: 50 μ m. All tissues stained with toluidine blue. AC, anterior chamber; SC, Schlemm's canal; CP, ciliary processes.

modified approach to a previously published fluorophotometric technique.⁶¹ All measurements were made between 11 AM and 3 PM, to reduce potential variability related to diurnal variation of aqueous inflow or outflow. After anesthetizing each mouse with the same solution used for IOP measurement, 10 μ L of 0.02% benzalkonium chloride (BAC) in saline were applied to the right eye to permeabilize the cornea to fluorescein.⁶² After 5 minutes, the BAC solution was blotted at the lid margin without contacting the corneal epithelium and 10 μ L of 0.02% fluorescein in saline was applied to the eye for 5 minutes. The eye and lids were then carefully washed with 600 μ L saline. The microscope was focused to a depth intermediate to the iris and cornea, and images were captured in 10-minute intervals thereafter for 1 hour (AxioCam ICC 1 camera and Stemi SV11 microscope; Carl Zeiss Meditec, Inc.).

FIGURE 2. At 7 weeks of age, AMPK α 2-null mice exhibit decreased aqueous humor clearance. **(A)** Representative series of green channel images captured at 10-minute intervals after corneal permeabilization with 0.02% BAC followed by topical application of 0.02% fluorescein and saline wash. **(B)** Aqueous fluorescein levels relative to values at $t = 0$ for WT and AMPK α 2-null mice ($n = 7$ and $n = 5$, respectively). Least-squares fit for exponential decay yielded (% intensity) = $100e^{-0.1112 \times \text{time}}$, and (% intensity) = $100e^{-0.0854 \times \text{time}}$ for WT and AMPK α 2-null, respectively. *Significant differences were observed in relative intensities between WT and AMPK α 2-null mice at 10, 20, 30, and 40 minutes ($P = 0.044$, 0.037 , 0.049 , 0.049 , respectively). Data expressed as mean relative intensity (%) $\pm$ SEM.

FIGURE 3. In human TM, AMPKα1 and AMPKα2 are expressed. (A) Representative immunoblots show detection of p-AMPKα, AMPKα1, and AMPKα2 in cell lysates of primary cultured human TM cells ($n = 4$), each at approximately 62 kDa. (B) Representative immunofluorescent staining of AMPKα1 and AMPKα2 in sections of adult human cadaveric donor eyes ($n = 4$). Nuclei were stained with DAPI. Scale bars: 50 μm.

Using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), an area with no corneal defects was selected and analyzed for average pixel intensity in the green channel. All averages were normalized to the intensity calculated for the image taken at time 0.

Trabecular Meshwork Cell Culture

Primary human TM cells were isolated, in accordance with the Declaration of Helsinki, and maintained in culture as described previously.⁶³ Independent primary human TM cell lines were generated from donors ranging in age from 35 to 72 years and no known history of ocular disease. Cell cultures were maintained, unless otherwise stated, in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) containing 20% fetal bovine serum, 1% L-glutamine (2 mM), and gentamicin (0.1 mg/mL) at 37°C in a 10% CO₂ atmosphere. Only TM cells from third through fifth passage were used. All experiments were performed using at least three different primary human TM cell lines.

Immunoblot Analysis

At the conclusion of each experiment for matricellular and ECM protein detection, conditioned media (CM) from TM cell cultures was harvested and centrifuged at 2300g for 10 minutes at 4°C. The supernatant was then concentrated (Amicon Ultra-4 Filter Unit, 10 kDa; Millipore, Milford, MA, USA), and protein content quantified using the DC Protein Assay kit adhering to manufacturer's protocols (Bio-Rad, Hercules, CA, USA). For AMPK protein detection, cells were lysed for 3 minutes on ice with cold 1× radioimmunoprecipitation buffer containing 0.5% Aprotinin, 0.1% EDTA, 1% EGTA, 0.5% phenylmethylsulfonyl fluoride, and 0.01% Leupeptin. Samples were then centrifuged at 18,000g for 15 minutes at 4°C and protein content quantified. In all experiments, equal amounts of protein were treated with

FIGURE 4. Treatment with AICAR leads to phosphorylation and activation of AMPKα. Primary cultured human TM cells were lysed at the specified time intervals after treatment with 0.5 mM AICAR. (A) Representative immunoblots of cell lysates showing detection of p-AMPKα (Thr172), total AMPKα, p-ACC, and total ACC with β-actin loading control. Antibodies of AMPKα detect both α1 and α2 isoforms. (B) Integrated band intensities calculated from above immunoblots. Data expressed as mean phospho-total ratios (normalized to zero time point) ± SEM (* $P < 0.05$ versus zero time point by Student's t -test; $n = 4$).

FIGURE 5. AICAR suppresses ECM proteins in primary human TM cells under basal and TGF- β 2 stimulatory conditions. (A) Representative immunoblots of ECM proteins from CM of human TM cells treated for 24 hours with PBS vehicle or 0.5 mM AICAR and (B) integrated band intensities calculated from those immunoblots. (C) Representative immunoblots of ECM proteins from CM of human TM cells under stimulation with 2.5 ng/mL TGF- β 2. Cells were pre-incubated for 1 hour with PBS or 0.5 mM AICAR prior to full 24-hour treatment. (D) Mean integrated band intensities. Data in (B) and (D) are expressed as mean $\pm$ SEM (* P < 0.05 versus PBS vehicle by Student's t -test; n = 5–7). (E) Representative 10% acrylamide gels stained with Coomassie Brilliant Blue as a loading control.

6 $\times$ reducing buffer and boiled for 5 minutes. Samples were then electrophoresed in 10% SDS-PAGE, alongside a pre-stained protein marker (Cell Signaling Technology, Inc., Danvers, MA, USA). For CM loading control, the resultant gels were stained with 0.1% Coomassie Brilliant Blue G-250 (Bio-Rad) for 3 hours and were destained with fixing/destaining solution until clear bands were visible and contrasted well with the true blue background. Otherwise, proteins were transferred to nitrocellulose membranes (0.45- μ m pore size; Invitrogen, Carlsbad, CA, USA). Membranes were blocked for 1 hour at room temperature (RT) in a 1:1 mixture of 1 $\times$ TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween-20) and blocking buffer (Rockland, Inc., Gilbertsville, PA, USA), followed by overnight incubation at 4 $^{\circ}$ C with the indicated primary antibody at 1:10,000 for SPARC (Hematologic Technologies, Inc., Essex Junction, VT, USA), 1:1000 for TSP-1 (AF3074; R&D Systems, Inc., Minneapolis, MN, USA), 1:1000 for COL1 (600-401-103-0.5; Rockland, Inc., Gilbertsville, PA, USA), 1:1000 for COL4 (600-401-106-0.5; Rockland, Inc.), 1:200 for Laminin (L8271; Sigma-Aldrich), and 1:000 for p(Thr172)-AMPK α , AMPK α , AMPK α 1, AMPK α 2, p-ACC, and ACC (Cell Signaling). A 1:200 dilution was used for p(Ser188)-RhoA and for total RhoA (Santa Cruz Biotechnology, Dallas, TX, USA), and a 1:1000 dilution was used for Myc-Tag and for β -actin (Cell Signaling). Following incubation with primary antibody, the membranes were washed three

times with 1 $\times$ TBS-T and incubated for 1 hour at RT with dye-conjugated affinity purified 680 anti-mouse or 800 anti-rabbit immunoglobulin G (IgG) antibodies, respectively (IRDye; 1:10,000 dilution; Rockland, Inc.). The membranes were then washed three times with 1 $\times$ TBS-T, scanned, and integrated band intensities were calculated using an infrared imaging system (Odyssey; Li-Cor, Lincoln, NE, USA).

Immunofluorescent Staining of Human Anterior Segments

Human donor eyes (aged 21, 44, 65, and 84) were immersion-fixed in 10% neutral buffered formalin within 15 hours of enucleation, dehydrated in sequential ethanol solutions (75%, 85%, 95%, 100%), and then embedded in paraffin. Sections (6 μ m) were mounted on poly-L-lysine-coated glass slides and baked for 2 hours at 60 $^{\circ}$ C. Slides were then deparaffinized in xylene, sequentially rehydrated in ethanol solutions, and washed three times for 10 minutes in PBS containing 0.1% Tween-20 (PBS-T). After 1 hour of incubation in 10% goat serum, tissues were permeabilized for 5 minutes in 0.2% Triton X-100 in 1 $\times$ PBS and washed three times in PBS-T. Prepared sections were incubated overnight at 4 $^{\circ}$ C in either primary AMPK α 1 or AMPK α 2 antibody diluted 1:200 in PBS or in PBS alone. Slides were washed three times in PBS-T and then incubated in 1:200 goat anti-rabbit 594 Alex Fluor secondary

FIGURE 6. Under basal and TGF- β 2 stimulatory conditions, AICAR treatment leads to decreased F-actin cytoskeletal staining, and fewer actin stress fibers. Human TM cells were plated on 8-well slides, treated as in Figure 5, and then stained with phalloidin (F-actin) antibody. Nuclei were stained with DAPI. Representative immunofluorescent images shown above ($n = 3$). Scale bars: 50 μ m.

IgG (Invitrogen), followed by three additional washes. Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) anti-fade reagent (SlowFade Gold; Invitrogen). Labeled tissues were imaged and analyzed by fluorescent light microscopy using a Zeiss Observer3.1 (Carl Zeiss Meditec, Inc.).

Immunofluorescent Staining of Primary Cultured Human TM Cells

Trabecular meshwork cells in 8-well slides were fixed for 30 minutes with 4% paraformaldehyde in PBS (pH 7.4) at 4°C, then washed in PBS for 10 minutes twice at RT. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes and then washed in PBS and blocked in 3% bovine serum albumin (BSA) in PBS for 1 hour at RT. Primary 568 phalloidin (F-actin) Alexa Fluor antibody (Invitrogen) was applied at 1:100 dilution to each section and incubated overnight at 4°C. Slides were washed with 3% BSA-PBS for 10 minutes, three times. Nuclei were stained with DAPI (Invitrogen), and labeled tissues were analyzed by fluorescent light microscopy using a Zeiss Observer3.1 (Carl Zeiss Meditec, Inc.).

5-Aminoimidazole-4-Carboxamide Riboside (AICAR) Time-Course Experiments

Trabecular meshwork cells at 90% to 100% confluency were cultured in serum-free media (SF) for 8 hours, and then incubated for the indicated time intervals in SF media containing 0.5 mM AICAR (Calbiochem, San Diego, CA, USA) prior to lysis and immunoblot analysis as described above.

TGF- β 2 Time-Course Experiments

Trabecular meshwork cells at 90% to 100% confluency were serum starved for 8 hours and then incubated in SF media containing 2.5 ng/mL activated TGF- β 2 (R&D Systems) for the indicated time intervals prior to processing as above. Where noted, 4 mM HCl containing 0.1% human serum albumin served as the vehicle for TGF- β 2.

Adenoviral-Mediated Infection Experiments

Trabecular meshwork cells at 70% to 90% confluency were infected in 2% FBS media with either adenovirus expressing a

FIGURE 7. Treatment with AICAR leads to phosphorylation of RhoA. Human TM cells were lysed at the specified time intervals after treatment with 0.5 mM AICAR. (A) Representative immunoblots of cell lysates showing detection of p-RhoA (Ser188) and total RhoA, with β-actin loading control. (B) Integrated band intensities calculated from above immunoblots. Data expressed as mean phospho-total ratios (normalized to zero time point) ± SEM (* $P < 0.05$ versus zero time point by Student's t -test; $n = 3$).

dominant negative form of the AMPK α subunit (ad.DN.AMPK α) or control empty adenoviral vector (ad.null) at 25 multiplicity of infection (MOI; Eton Bioscience, Charlestown, MA, USA). Multiplicity of infection is the ratio of infectious units (viruses) to infection targets (cells).^{64,65} The ad.DN.AMPK α virus expresses an $\alpha 2$ subunit harboring a K45R mutation in the kinase domain, which competes for binding with the β and γ subunits but lacks kinase activity. After 18 hours, an equal volume of 10% FBS media was added to each well and cells were incubated for an additional 48 hours then lysed.

Statistics

GraphPad Prism 6 software (GraphPad, La Jolla, CA, USA) was used. A two-tailed Student's t -test was used for comparing differences between two groups, and differences were considered significant when P was less than 0.05. Throughout, n refers to the number of independent experiments performed using different primary human TM cell lines, established from separate donors.

RESULTS

AMPK $\alpha 2$ -Null Mice Exhibit Increased IOP and Decreased Aqueous Humor Clearance

AMPK $\alpha 2$ -null mice exhibited 6% higher IOP ($P = 0.0265$) than WT counterparts (Fig. 1A), with no significant difference ($P = 0.6877$) in CCT (Fig. 1B). They had mean IOP of 18.2 ± 0.28 mm Hg versus the WT mean IOP of 17.2 ± 0.36 mm Hg. By light microscopy, the iridocorneal angles in AMPK $\alpha 2$ -null mice appeared grossly indistinguishable from WT counterparts with similar outflow structures and cellularity (Fig. 1C).

Aqueous humor clearance in AMPK $\alpha 2$ -null mice was reduced compared with their WT counterparts (Fig. 2). Least-

FIGURE 8. Treatment with TGF-β2 leads to transient dephosphorylation of AMPK α in human TM cells. Trabecular meshwork cells were lysed at the specified time intervals after treatment with 2.5 ng/mL TGF-β2. (A) Representative immunoblots of cell lysates showing detection of p-AMPK α (Thr172) and total AMPK α , with β-actin loading control. Antibodies detect both $\alpha 1$ and $\alpha 2$ isoforms. (B) Mean integrated band intensities calculated from above immunoblots. Data expressed as mean phospho-total ratios (normalized to zero time point) ± SEM (* $P < 0.05$ versus zero time point by Student's t -test; $n = 4$). Analysis reveals that the p-AMPK α /AMPK α ratio is significantly decreased only at the $t = 15$ minutes time point ($P = 0.0067$).

squares fit analysis yielded exponential decay constants of 0.1112%/min ($r^2 = 0.91$) and 0.0854%/min ($r^2 = 0.91$) for WT and AMPK $\alpha 2$ -null mice, respectively. Fluorescent intensities were greater at each time point for AMPK $\alpha 2$ -null, and Student's t -tests revealed significant differences between AMPK $\alpha 2$ -null and WT at 10, 20, 30, and 40 minutes ($P < 0.05$).

AMPK $\alpha 1$ and AMPK $\alpha 2$ Isoforms Are Expressed in Human TM and AICAR Treatment Leads to Activation

In its active form, AMPK exists as a heterotrimer with two regulatory β and γ subunits joined with a catalytic α subunit that has two distinct isoforms ($\alpha 1$ and $\alpha 2$).³³ Both isoforms were detectable by immunoblot (Fig. 3A). Immunofluorescent microscopy revealed that both isoforms were prominent in the TM, lining the trabecular beams and inner and outer walls of Schlemm's canal (Fig. 3B).

An adenosine analog, AICAR reproduces the effects of extracellular AMP and activates AMPK α via increased phosphorylation at Thr172.^{40,41,66} To examine whether AICAR phosphorylates and activates AMPK α in the TM, TM cells were incubated with 0.5 mM AICAR for a 24-hour time course and then lysed for immunoblot analysis (Fig. 4A). The ratio of phospho-total AMPK, normalized to the zero time point, increased by 77% within 1 hour of AICAR treatment ($P = 0.0323$) and peaked with a greater than 2-fold increased ratio at

FIGURE 9. Adenoviral transfer of a dominant negative form of the AMPKα subunit (ad.DN.AMPKα) increases matricellular and ECM expression, decreases the phospho-total RhoA ratio (Ser188), and increases F-actin cytoskeletal staining and disarray. (A) Representative immunoblots of ECM proteins from CM of human TM cells treated for 66 hours with null adenoviral vector (ad.null) versus ad.DN.AMPKα at 25 MOI. (B) Mean $\pm$ SEM integrated band intensities calculated from those immunoblots (* $P < 0.05$ by Student's t -test; $n = 4-6$). (C) Representative 10% acrylamide gels stained with Coomassie Brilliant Blue as a loading control. (D) Representative immunoblots of lysates from cells treated as described in (A); probed for p-RhoA, RhoA, Myc-Tag for confirmation of adenoviral expression, and β-actin loading control. (E) Mean integrated band intensities, showing a 27% decrease in the phospho-total RhoA ratio ($P = 0.0053$; $n = 7$). (F) Representative images of primary cultured human TM cells plated on 8-well slides and treated as in panel (A), and then stained for F-actin. Nuclei were stained with DAPI. Representative immunofluorescent images shown above ($n = 3$). Scale bars: 50 μm.

2 hours (Fig. 4B). To determine whether phosphorylation of AMPK led to functional activation, the phospho-total ratio of the known downstream signaling target acetyl-CoA carboxylase (ACC)³⁴ was similarly analyzed (Figs. 4A, 4B). The phospho-total ACC ratio increased by a statistically significant degree within 15 minutes ($P = 0.0076$), peaking with a 6.6-fold increased ratio at 6 hours.

AICAR Suppresses ECM Proteins and Alters Cytoskeleton in TM Under Basal and TGF-β2 Stimulatory Conditions

To examine whether modulation of AMPK affects certain matricellular and ECM proteins, TM cells were treated with 0.5 mM AICAR or PBS vehicle and CM was probed for SPARC, TSP-1, collagen I, collagen IV, and laminin (Fig. 5A). Calculation of mean integrated band intensities revealed 70%, 52%, and 64%

decreases in collagen I, collagen IV, and laminin, respectively ($P < 0.001$) but no change in SPARC or TSP-1 (Fig. 5B). Under TGF-β2 stimulation with 2.5 ng/mL, AICAR treatment decreased SPARC, collagen I, collagen IV, and laminin levels by 64%, 26%, 34%, and 33%, respectively ($P < 0.001$) with no change in TSP-1 levels (Figs. 5C, 5D).

Evidence suggests that cellular tone within the TM can contribute to outflow facility.^{19,20} Under basal and TGF-β2 stimulatory conditions, AICAR-treated TM cells exhibited decreased F-actin staining and actin stress fiber formation (Fig. 6).

AICAR Treatment Leads to Phosphorylation of RhoA at Ser188

RhoA induces ECM deposition in TM cells, contributing to increased resistance to aqueous humor outflow.^{19,20} Phosphor-

ylation of RhoA at Ser188 uncouples the RhoA/RhoA-associated protein kinase (ROCK) pathway that mediates increased ECM deposition.^{67,68} A recent study demonstrated that activated AMPK directly phosphorylates RhoA at Ser188.⁶⁹ When TM cells were treated with AICAR, the phospho-total RhoA ratio increased approximately 10-fold within 1 hour and remained statistically significant through 24 hours (Fig. 7).

TGF- β 2 Treatment Leads to Transient Dephosphorylation of AMPK α in TM

To assess whether TGF- β 2 has an effect on AMPK signaling, TM cells were incubated with 2.5 ng/mL TGF- β 2. The phospho-total AMPK ratio was calculated and normalized to the zero time point (Fig. 8). The ratio decreased by 30% within 15 minutes ($P = 0.0067$), but the difference was no longer significant at 30 minutes, returning to baseline.

Adenoviral Transfer of Dominant Negative AMPK α Induces ECM Expression in TM

In the CM of cells expressing the dominant negative AMPK α subunit SPARC, TSP-1, collagen I, collagen IV, and laminin protein levels were increased 2.7-fold ($P = 0.0137$), 2.0-fold ($P = 0.0012$), 3.6-fold ($P = 0.0497$), 2.6-fold ($P = 0.0178$), and 2.4-fold ($P = 0.0239$), respectively (Fig. 9). A 27% decrease in the phospho-total RhoA ratio ($P = 0.0053$) was observed in the corresponding cell lysates (Fig. 9). Additionally, transfer of ad.DN.AMPK α led to marked increases in F-actin cytoskeletal staining as well as a more disorganized staining pattern (Fig. 9F).

DISCUSSION

Mice that are AMPK α 2-null have higher IOPs than their WT counterparts, which does not appear to be an artifact of CCT. The absence of gross structural differences in the iridocorneal angles implicates cellular or biochemical processes. Intraocular pressure elevation may be the result of two possible mechanisms, decreased aqueous outflow facility or increased aqueous production. The decreased aqueous humor clearance exhibited by AMPK α 2-null mice suggests that reduced outflow facility is the underlying mechanism behind the observed IOP elevation. Although decreased fluorescein disappearance could be the result of decreased aqueous production, in the setting of an elevated IOP, decreased outflow has to be part of the mechanism.

A greater proportion of outflow occurs though the pressure-independent alternative pathway in mice than in humans. This appears to vary significantly across mice strains, ranging from 20.5% in BALB/cJ mice⁷⁰ to 82% in National Institutes of Health Swiss White mice.⁸ The mice used in this study were derived from C57Bl/6 mice, which have demonstrated 66% outflow through the alternative pathway.⁷ The observed variability in alternative outflow may be due to strain-specific properties, such as the degree of scleral permeability, or simply due to differences in the enucleation methodologies employed by the laboratories.⁷ When considering the findings presented in this study, it is important to note that, in humans, a greater proportion of outflow is pressure-dependent.

The extent to which AMPK signaling regulates ECM homeostasis and cellular tone in TM cells may explain its apparent role in aqueous clearance (Fig. 10). We demonstrated that AMPK α 1 and AMPK α 2 are present throughout the TM and that activation of AMPK signaling decreases certain ECM components, while also resulting in narrower cells with decreased F-actin staining. RhoA is a protein downstream of

FIGURE 10. Theoretical model for the role of AMPK signaling in the regulation of ECM homeostasis and cellular tone in TM. Treatment with pharmacologic activators of AMPK results in phosphorylation of the α subunit at Thr172. Activation of AMPK leads to phosphorylation of RhoA at Ser188, as demonstrated previously in nonocular tissue.⁶⁹ Phosphorylation of RhoA at Ser188 results in decreased interaction with ROCK and subsequent decrease in ECM deposition. In addition, cells adopt more unidirectional cytoskeletal arrangements with less prominent F-actin staining. With decreased ECM deposition in the TM and weaker intracellular actin stress fibers, aqueous humor outflow facility is enhanced and IOP is consequently reduced.

AMPK that could potentially unify our findings. RhoA harbors an optimal AMPK recognition motif, and one recent investigation using controlled *in vitro* kinase assays provides strong evidence that AMPK directly phosphorylates RhoA in vascular smooth muscle cells.⁶⁹ Furthermore, the authors describe a role for AMPK α 1 in the phosphorylation of RhoA in mice, but the relative contributions of α 1 and α 2 isoforms has yet to be fully explored. In addition to altering cellular tone, RhoA induces ECM deposition in TM, thereby increasing resistance to aqueous humor outflow.^{19,20} In the prevailing model of RhoA protein activation, there is a dynamic cycle between active GTP-bound and inactive GDP-bound RhoA, and a variety of signal intermediaries favoring GTP-RhoA, which translocates to the cell membrane where it interacts with ROCK to affect ECM deposition.⁷¹ We found that activation of AMPK increases the phospho-total RhoA ratio (Ser188), most likely uncoupling the RhoA/ROCK pathway that normally mediates actin stress fiber formation and ECM deposition in the TM. These cytoskeletal changes are the converse of what has been reported in cells infected with adenovirus expressing constitutively active RhoA, namely more rounded morphology with increased F-actin staining.¹⁹ Furthermore, we have found that adenoviral transfer of dominant negative AMPK α results in cytoskeletal changes similar to those induced by RhoA overexpression. Taken together, these data suggest that AMPK, through its effects on RhoA, plays a role in both (1) ECM homeostasis and (2) cellular tone within the TM.

The 24-hour time frame of the results reported in Figure 5 could be consistent with either an AMPK-mediated alteration in the rate of ECM turnover or in the rate of ECM protein production. Our study did not measure mRNA levels of the ECM components, so we cannot state conclusively which mechanism contributes more. It is possible that in Figure 5 we are observing the result of an increase in the rate of ECM

TABLE. AMPK and Potential Glaucoma Clinical Correlations From Genetic Studies

Subunit	Locus	MIM	Potential Disease Associations	References
$\alpha 1$	5p12	602739	N/A*	
$\alpha 2$	1p31	600497	N/A*	
$\beta 1$	12q24.1-q24.3	602740	Familial high myopia	82
$\beta 2$	1q21.1	602741	POAG; JOAG; familial high myopia	78, 79, 80, 81
$\gamma 1$	12q12-q14	602742	N/A*	
$\gamma 2$	7q36.1	602743	PDS; pigmentary glaucoma; familial high myopia	83, 84
$\gamma 3$	2q35	604976	PEX	85

MIM, molecular interactions map; JOAG, juvenile open-angle glaucoma; PDS, pigment dispersion syndrome; PEX, pseudoexfoliation syndrome.

* To date, no known disease associations at these loci.

turnover. For example, AICAR-induced suppression of SPARC may diminish the matricellular protein's ability to act post translationally, perhaps as a chaperone molecule that stabilizes ECM components.⁷²⁻⁷⁵ Alternatively, we have shown that activation of AMPK with AICAR results in a greater than 5-fold increase in the RhoA phosphorylation ratio within 15 minutes (Fig. 7B), leaving open the possibility that these rapid alterations in RhoA activation levels could contribute to changes in ECM production levels during this 24-hour time frame. One previous study by Zhang et al.¹⁹ demonstrated that 24 hours is sufficient time for adenoviral infection of cultured human TM cells with a constitutively active form of RhoA to result in statistically significant increases in the mRNA levels of various ECM components, including a 3.9-fold increase in SPARC expression. Our present study, however, did not directly measure ECM mRNA levels, so no definitive conclusions can be drawn.

The catalytic α subunit of AMPK is expressed in human TM, but it is unknown whether AICAR preferentially activates one α isoform over the other in TM. Adenoviral transfer of dominant negative α subunits does not provide isoform-specific information, as the mechanism behind this approach involves competitive binding of the defective α subunit to native β and γ subunits, effectively exhausting the native supply of heterotrimer components for both $\alpha 1$ and $\alpha 2$ complexes. Further investigation is needed to uncover which α isoform makes the greater contribution to ECM homeostasis as well as cytoskeletal organization, and whether or not the regulatory β and γ subunits are necessary for these influences; it has been shown that the β subunit may play a role in subcellular localization through an N-terminal site that targets AMPK to the cytosolic membrane,^{76,77} where its proposed effects on RhoA and ECM deposition are likely to be carried out. It is intriguing that the genes encoding several AMPK subunits lie in exceedingly close proximity to, or even within, loci that have been associated with diseases such as POAG, juvenile open-angle glaucoma, familial high myopia, pigment dispersion syndrome, pigmentary glaucoma, and pseudoexfoliation syndrome (Table).⁷⁸⁻⁸⁵ Further investigation within these populations, including fine sequencing studies, may be warranted given the evidence presented in this report.

Activation of the TGF- $\beta 2$ signaling cascade promotes deposition of ECM proteins, in part through its induction of SPARC.^{25,48} Transforming growth factor- $\beta 2$ appears to deactivate AMPK in TM cells, suggesting one additional mechanism by which it exerts influence over ECM proteins. Further investigation is needed to clarify the extent to which deactivation of AMPK signaling contributes to TGF- $\beta 2$ -induced fibrogenesis. Given this proposed interaction, modulation of AMPK signaling has potential surgical applications that warrant experimentation. Fibrosis causes failure of glaucoma filtration surgery,⁸⁶⁻⁸⁸ and patients with increased TGF- $\beta 2$ levels in their aqueous humor have been found to exhibit decreased bleb

formation.⁸⁹ Adenosine monophosphate-activated kinase protein signaling affects ECM homeostasis in TM endothelial cells, but whether or not it influences Tenon's fibroblasts in a similar fashion remains to be determined.

This study proposes that AMPK signaling has functional relevance to IOP homeostasis. Future studies may help clarify this link. Known AMPK activators include drugs such as AICAR, metformin, thiazolidinediones, and salicylates.^{34,90} One potential future investigation would be to examine the effects of AICAR and other pharmacologic AMPK activators on IOP in perfused ex vivo human anterior segments, as has been performed similarly using the manipulation of matricellular protein expression.^{25,91} In addition, assessing the effects of topical administration of these agents in rabbits would yield a more complete understanding of the role of AMPK signaling in regulating aqueous humor outflow and may further strengthen the possibility of future therapeutic applications in humans.

Complex regulatory mechanisms govern ECM homeostasis, cellular tone, and aqueous outflow in the TM. Our data identifies AMPK as a regulatory element for IOP and possible novel therapeutic target for glaucoma. A variety of pharmacologic activators of AMPK exist and further testing is warranted to investigate their in vivo influences on the TM and on IOP.

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