Stanniocalcin-1 Is an Ocular Hypotensive Agent and a Downstream Effector Molecule That Is Necessary for the Intraocular Pressure–Lowering Effects of Latanoprost

Gavin W. Roddy,[1] Kimberly B. Viker,[1] Nelson S. Winkler,[1] Cindy K. Bahler,[1] Bradley H. Holman,[1] David Sheikh-Hamad,[2] Uttio Roy Chowdhury,[1] W. Daniel Stamer,[3] and Michael P. Fautsch[1]

[1]Department of Ophthalmology, Mayo Clinic, Rochester, Minnesota, United States
[2]Department of Medicine, Division of Nephrology, Baylor College of Medicine, Houston, Texas, United States
[3]Department of Ophthalmology, Duke University, Durham, North Carolina, United States

Correspondence: Michael P. Fautsch, Department of Ophthalmology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA; fautsch@mayo.edu.

Submitted: October 27, 2016
Accepted: April 25, 2017

Citation: Roddy GW, Viker KB, Winkler NS, et al. Stanniocalcin-1 is an ocular hypotensive agent and a downstream effector molecule that is necessary for the intraocular pressure–lowering effects of latanoprost. *Invest Ophthalmol Vis Sci*. 2017;58:2715–2724. DOI:10.1167/iovs.16-21004

Purpose. To identify downstream signaling molecules through which intraocular pressure (IOP) is lowered following treatment with the prostaglandin analog latanoprost.

Methods. Total RNA and protein isolated from primary human Schlemm's canal cells ($n = 3$) treated with latanoprost (free acid; 100 nM) were processed for quantitative PCR and Western blot analysis. IOP was evaluated in stanniocalcin-1 (STC-1$^{-/-}$) and wild-type mice following treatment with latanoprost or Rho kinase inhibitor Y27632. Human anterior segment pairs ($n = 8$) were treated with recombinant STC-1 (5, 50, or 500 ng/mL) and pressure was recorded using custom-designed software. The effect of recombinant STC-1 (0.5 mg/mL) on IOP was evaluated in wild-type mice. Tissue morphology was evaluated by light and transmission electron microscopy.

Results. Increased STC-1 mRNA (4.0- to 25.2-fold) and protein expression (1.9- to 5.1-fold) was observed within 12 hours following latanoprost treatment. Latanoprost reduced IOP in wild-type mice (22.0% ± 1.9%), but had no effect on STC-1$^{-/-}$ mice (0.5% ± 0.7%). In contrast, Y27632 reduced IOP in both wild-type (12.5% ± 1.2%) and in STC-1$^{-/-}$ mice (13.1% ± 2.8%). Human anterior segments treated with STC-1 (500 ng/mL) showed an increase in outflow facility (0.15 ± 0.03 to 0.27 ± 0.09 µL/min/mm Hg) while no change was observed in paired vehicle-treated controls. Recombinant STC-1 reduced IOP in wild-type mice by 15.2% ± 3.0%. No observable morphologic changes were identified between treatment groups when evaluated by microscopy.

Conclusions. Latanoprost-induced reduction of IOP is mediated through the downstream signaling molecule STC-1. When used by itself, STC-1 exhibits ocular hypotensive properties.

Keywords: Schlemm's canal, latanoprost, Rho kinase inhibitor Y27632, ocular hypotension, stanniocalcin-1

Glaucoma is the leading cause of irreversible visual impairment, projected to affect nearly 80 million people worldwide by the year 2020 and increasing to 110 million by 2040.[1–3] Glaucoma is characterized by loss of retinal ganglion cells, axonal attenuation, and optic nerve atrophy.[4] While there are no cures for the disease, treatment regimens that include medical, laser, and surgical therapies have proven useful in slowing disease progression. These therapies are all directed at reducing elevated intraocular pressure (IOP), the most prevalent and only treatable risk factor for glaucoma. Prostaglandin analogues such as latanoprost (Xalatan; Pfizer, Inc., New York City, NY, USA); bimatoprost (Lumigan; Allergan, Inc., Irvine, CA, USA); or travoprost (Travatan; Alcon Laboratories, Inc., Fort Worth, TX, USA) are typically the initial agents prescribed in the medical management of elevated IOP due to their high responder rate and IOP-lowering effects.[5–7]

Prostaglandin analogs reduce IOP primarily by increasing outflow facility via the uveoscleral pathway. This occurs by matrix metalloproteinase remodeling of the extracellular matrix leading to subsequent changes in the resistance of outflow in the ciliary muscle.[8–11] Several studies have also suggested that the conventional outflow pathway, which consists of the trabecular meshwork, Schlemm's canal, collector channels, and aqueous veins, may be a secondary pathway by which prostaglandin analogs reduce pressure.[7,9,12–18]

Studies using prostaglandin analogs in general, and latanoprost in particular, have found that binding of these drugs to the FP receptor leads to activation of the phosphatidylinositol and protein kinase C pathways, an increase in calcium release and subsequent phosphorylation of myosin light chain kinase in the iris.[7,19–21] While latanoprost binds to FP and FP-like receptors in cells of the conventional outflow pathway,[22] what happens following receptor activation and the identity of critical effector molecules necessary for increasing outflow facility are unknown. Identifying latanoprost-induced effector molecules will allow for a better understanding of the pathophysiology behind the treatment of ocular hypertension.

While generally well tolerated, prostaglandin analogs do exhibit notable side effects including conjunctival hyperemia, ocular surface irritation, pigmentation of the iris and

periocular skin, and hypertrichosis.[9] Therefore, elucidating downstream signaling partners of latanoprost will help identify targets for development of novel treatments potentially leading to improved IOP reduction with fewer side effects. In light of this, we sought to identify downstream signaling molecules following latanoprost treatment with reference to IOP reduction. In order to identify downstream effector molecules, we treated Schlemm's canal cells with latanoprost and analyzed gene and protein expression at several different time points. We identified stanniocalcin-1 (STC-1), a multifunctional phosphoglycoprotein hormone with anti-inflammatory,[23] antiapoptotic,[24] antioxidative damage,[25] and neuroprotective[26] properties, as one of the most differentially expressed molecules at the RNA level. In this study, we validated STC-1 upregulation and assessed its role as an effector molecule in latanoprost-mediated IOP regulation.

Methods

Validation of STC-1 Expression

Cell Culture. Primary human Schlemm's canal cell lines were grown to confluence in six-well plates (BD Falcon, Franklin Lakes, NJ, USA) containing Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA); 10% fetal bovine serum (Mediatech, Manassas, VA, USA); and 1% penicillin/streptomycin (Life Technologies) in 5% CO_2 at 37°C. Confluent cells were washed twice with phosphate-buffered saline and incubated in serum-free DMEM for 24 hours to synchronize the growth potential of the cells. Cells were incubated with latanoprost (de-esterified free-acid form at 100 nM final concentration; Cayman Chemical, Ann Arbor, MI, USA) or vehicle (ethanol, final dilution 1:1000) in DMEM containing 1% penicillin/streptomycin.

RNA Analysis. Primary Schlemm's canal cells were grown to confluence, treated with 100 nM latanoprost for 6 hours, and harvested. Total RNA was extracted using an RNA isolation kit (RNeasy Total RNA Isolation Kit; Qiagen, Hilden, Germany). Approximately 250 ng of total RNA was reverse transcribed into cDNA using a synthesis kit (iScript cDNA; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantitative real-time polymerase chain (qPCR) reaction using STC-1 (forward, 5′-AGGCGGAGCAGAATGACTC-3′; reverse, 5′-GTTGAGGCAAC GAACCACTT-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: forward, 5′-CCTCTGACTTCAACAGC-3′; reverse, 5′-GCTGTAGCCAAATTCGT-3′) primers were performed on a PCR system using a master mix (Roche Light Cycler 480 with SYBR Green I; Roche, Indianapolis, IN, USA). We performed qPCR amplification with a predenaturation step at 95°C followed by 45 cycles of denaturation at 95°C, annealing at 63°C, and extension at 72°C. Fold change was calculated after normalization with GAPDH.

Protein Analysis. Primary Schlemm's canal cells were grown to confluence, treated with 100 nM latanoprost for 15 minutes, 1, 2, 4, or 6 hours, and harvested. Schlemm's canal cell pellets were suspended in ice-cold lysis buffer (50 mM Tris pH 8.0, 0.5% sodium dodecyl sulfate, 0.5% Triton X-100, 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4-$7H_2O$, 1 mM KH_2PO_4, protease inhibitors; Roche) and passed repeatedly through a 21-gauge needle for homogenization. Lysate was centrifuged at 13,000*g* for 10 minutes, and total protein was quantified by the Bradford assay. Cell lysates containing 20 µg total protein were mixed with reducing lane marker sample buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing 15% 2-mercaptoethanol (Sigma-Aldrich Corp., St. Louis, MO, USA), heated and separated on a 4% to 15% SDS-PAGE gradient gel (Bio-Rad Laboratories, Inc.). Proteins were transferred to polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA, USA) in 1X transfer buffer (50 mM Tris, 384 mM glycine, 0.01% SDS, 20% methanol). Membranes were blocked in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.05% TWEEN-20, and 2% instant nonfat dry milk. Blots were probed with rabbit monoclonal anti-human STC-1 (Novus Biologicals, Littleton, CO, USA) and mouse monoclonal anti-human GAPDH (Novus Biologicals). Secondary antibodies used were horseradish peroxidase–linked anti-rabbit or anti-mouse, respectively (GE Healthcare, Piscataway, NJ, USA). Antibody/antigen complexes were detected using ECL Western blot signal detection reagent (GE Healthcare). Chemiluminescence film (BioMax XAR; Eastman Kodak, Rochester, NY, USA) was used to visualize protein signals. Each film was digitized with a photographic scanner (Epson Perfection 2400; Epson America, Inc., Long Beach, CA, USA). The band intensities for Western blot analysis were quantified using ImageJ software (http://rsb.info.nih.gov/ij/index.html in the public domain by the National Institutes of Health, Bethesda, MD, USA) and normalized to GAPDH.

Animal Experiments

All animal studies and treatment protocols were approved by the Mayo Clinic (Rochester, MN, USA) Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We obtained STC-$1^{-/-}$ and littermate wild-type mice from the Sheikh-Hamad laboratory, Baylor College of Medicine, and bred at Mayo Clinic. Mice, aged 5 to 8 months, were utilized in the experiments. A handheld rebound tonometer (Icare TonoLab; Colonial Medical Supply, Franconia, NH, USA) was used to measure IOP in conscious mice. For IOP measurements, the tonometer was held perpendicular to the cornea according to the manufacturer's instructions. The tonometer records six readings from the same eye, discards the highest and lowest values, and shows the average of the remaining four values as a single IOP reading. Three independent measurements were obtained daily at similar time points and were averaged to obtain the daily IOP value for each eye. After 1 week of baseline IOP measurements, STC-$1^{-/-}$ mice were treated with latanoprost ($n = 10$) or Rho kinase inhibitor Y27632 (Enzo Life Sciences, Farmingdale, NY; $n = 10$). Congenic wild-type controls were treated with latanoprost ($n = 8$) or Y27632 ($n = 10$). Treatments were daily in one eye for 7 consecutive days with 5 µL of latanoprost-free acid (100 µM dissolved in 1:1000 DMSO in PBS) or 10 mM Y27632 (dissolved in phosphate-buffered saline). In the contralateral eye, vehicle was added daily in the same proportion as the treated eye for 7 consecutive days. Additionally, wild-type mice ($n = 7$; Charles Rivers Laboratories, Wilmington, MA, USA) were treated with 5 µL of topically administered recombinant human STC-1 (0.5 mg/mL; Biovendor Research & Diagnostic Products, Asheville, NC, USA) or vehicle (phosphate-buffered saline) daily for 7 days to examine the effect of STC-1 on IOP. In all animals, the right eye served as the vehicle control eye while the left eye received study drug (latanoprost, Y27632, or STC-1). We recorded IOP in both eyes three times daily at 1, 4, and 23 hours following treatment.

Ex Vivo Human Anterior Segment Culture

Anterior segments from human donor eyes (age 75.5 ± 17.5 years, range: 51 to 98 years; $n = 8$) were perfused in culture with DMEM within 10.2 ± 4.4 hours of death as previously described.[27] After achieving a stable baseline pressure, one anterior segment from each pair received recombinant human STC-1 at concentrations of 5, 50, or 500 ng/mL (dissolved in

Investigative Ophthalmology & Visual Science

H_2O), while the fellow eye received vehicle and served as the control. We added STC-1 and vehicle using a gravity-driven constant pressure method of anterior chamber exchange followed by continuous perfusion. Hourly pressure readings were obtained from the average of 60, one-minute pressure measurements using a custom-designed software program. The experimental eye was typically the right eye and the control was the left eye.

Tissue Preparation and Microscopy

For human anterior segments, selected wedges of tissue 180° apart that included the trabecular meshwork and Schlemm's canal were isolated and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Tissue wedges were dehydrated in a series of ascending ethanol concentrations, cleared with 100% acetone, infiltrated and embedded in Epon araldite, and sectioned at 0.5 μm. Eye tissue sections were stained with toluidine blue and examined using a light microscope (Nikon Corp., Tokyo, Japan). Additional tissue wedges were sectioned at 100 nm, placed on copper film grids, and stained with uranyl acetate and lead citrate. Tissue sections on copper film grids were examined using a transmission electron microscope (JEOL-1400; JEOL USA, Inc., Peabody, MA, USA).

For mouse eye histopathology, whole eyes were enucleated from euthanized mice following termination of the experiment. Eyes were placed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight, and processed for light microscopy as described above for human anterior segment wedges. Additional 100-nm sections were placed on copper film grids, and stained with uranyl acetate and lead citrate. Digital images of toluidine blue–stained sections and transmission electron–processed sections were obtained following examination under a light microscope (Nikon Corp.) and a transmission electron microscope (JEOL USA, Inc.).

Statistics

Prior to initiation of statistical analysis, all data sets were evaluated for distribution assessment using the Shapiro-Wilk test. For animal studies, significance of IOP change was assessed between experimental and vehicle-treated control eyes using Student's paired *t*-test for data sets with normal distribution and Wilcoxon sign-rank test for nonparametric data sets. Variations in daily IOP are graphically presented as the mean daily IOP of the vehicle and treated eyes. All values are expressed as mean ± standard deviation. Statistical calculations were performed using a statistical software package (JMP; SAS Institute, Inc., Cary, NC, USA).

For human anterior segment studies, the effect of latanoprost was expressed as the change in outflow facility (C) for each anterior segment.[28] Results from each pair of anterior segments were combined into a group mean for each drug, and statistical significance was analyzed using a Student's 2-tailed paired *t* test. All values are expressed as mean ± standard deviation.

Results

STC-1 Is Upregulated in Latanoprost-Treated Primary Human Schlemm's Canal Cells

Preliminary findings from confluent primary human Schlemm's canal cell lines treated with latanoprost identified STC-1, a secreted phosphoglycoprotein hormone with multiple functions,[29] as a gene consistently upregulated following latanoprost treatment. Quantitative PCR studies in human Schlemm's canal cells showed an 18.1 ± 4.3-fold ($n = 3$) induction. Cell lysates isolated from latanoprost-treated primary human Schlemm's canal cells at various time points showed an increase of STC-1 protein expression of 1.9-fold at 2 hours and approximately 5.0-fold at 4 and 6 hours, correlating with increased mRNA expression levels identified by qPCR (Fig. 1).

IOP Recordings in STC-1 Knockout Mice Following Treatment With Latanoprost

Because of its multifunctional properties and ability to act in an autocrine and paracrine fashion similar to prostaglandin analogs,[9,30] we examined the role of STC-1 in downstream signaling following latanoprost treatment in vivo using STC-$1^{-/-}$ mice and congenic wild-type controls. Assessment of baseline IOPs for 6 days showed no significant difference between STC-$1^{-/-}$ mice (16.6 ± 0.5, $n = 10$) and wild-type controls (16.2 ± 0.3, $n = 8$; $P = 0.1$). Histologic examination of STC-$1^{-/-}$ mice by light and transmission electron microscopy showed a normal-appearing ocular anatomy, an open angle with appropriate trabecula within the trabecular meshwork, and intact inner and outer walls of Schlemm's canal, all similar to wild-type controls (Fig. 2). With no observable morphologic differences between STC-$1^{-/-}$ and wild-type mice, we proceeded to assess the effect of latanoprost treatment on IOP in these mice. Topical eye treatment with latanoprost (100 nM) reduced IOP in wild-type controls by 3.8 mm Hg ($P < 0.001$, $n = 8$), which correlated to a 22.0% ± 1.9% decrease in IOP when compared to the fellow contralateral eyes treated with vehicle alone (Fig. 3). Consistent IOP change was identified throughout the once daily dose as determined by IOP pressure monitoring at 1, 4, and 23 hours after treatment (Table). In contrast, STC-$1^{-/-}$ mice did not show any significant reduction in IOP after topical latanoprost treatment (0.5% ± 0.7%, $P = 0.34$, $n = 10$) at any time point throughout the 7-day treatment regime. To determine if this was unique to latanoprost, we treated STC-$1^{-/-}$ with Y27632, a Rho kinase inhibitor that increases outflow through both the uveoscleral and the conventional outflow pathway.[31–34] Treatment of either wild-type ($n = 10$) or STC-$1^{-/-}$ ($n = 10$) mice with Y27632 resulted in significant reductions of IOP, 12.5% ± 1.2% and 13.1% ± 2.8%, respectively ($P < 0.0001$, $n = 10$; Fig. 4; Table). These data suggest that STC-1 has a unique and key role in latanoprost signaling mediated IOP reduction.

STC-1 Perfusion in Ex Vivo Human Anterior Segment Culture

To determine if STC-1 would influence IOP reduction by itself, we perfused human anterior segments with several concentrations of recombinant STC-1 for 24 hours. Perfusion with either 5 ng/mL (0.15 ± 0.04 to 0.15 ± 0.04 μL/min/mm Hg, $n = 2$, $P = 0.20$) or 50 ng/mL (0.14 ± 0.05 to 0.18 ± 0.07 μL/min/mm Hg, $n = 4$, $P = 0.20$) had no significant effect on outflow facility. However, all anterior segments perfused with STC-1 at 500 ng/mL had decreased pressure and increased outflow facility (0.15 ± 0.03 to 0.27 ± 0.09 μL/min/mm Hg, $n = 5$, $P = 0.02$) compared to baseline (Fig. 5). Paired controls treated with vehicle showed no change in outflow facility from their baseline values. To determine whether the changes in pressure could be secondary to morphologic changes, microscopic analysis was performed. Morphologic analysis showed viable and healthy cells in the trabecular meshwork and Schlemm's canal of control and treated eyes (Fig. 6). No major disruptions of the juxtacanalicular tissue or the

Investigative Ophthalmology & Visual Science

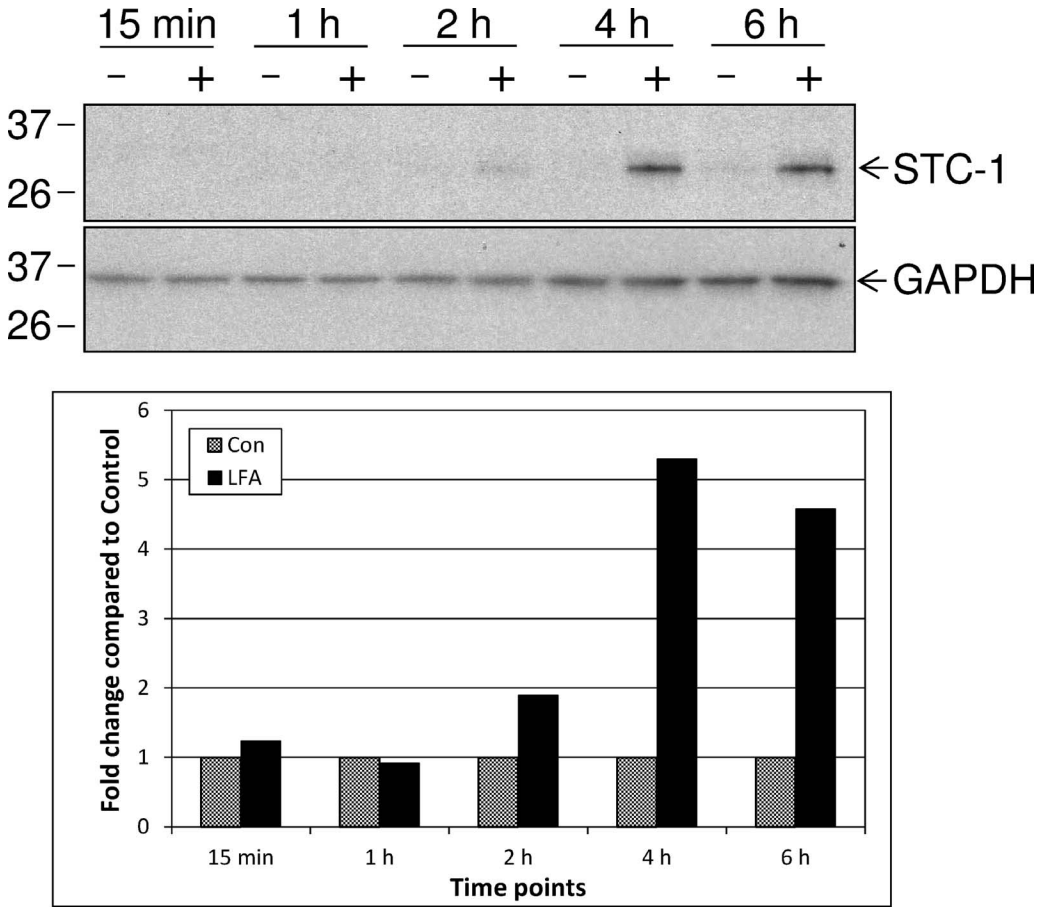

Figure 1. Induction of STC-1 in Schlemm's canal cells following treatment with latanoprost. *Top*: Western blot image showing induction of STC-1 following treatment with latanoprost. *Bottom*: Graphical depiction of STC-1 fold change following densitometry and normalization to GAPDH.

basement membrane of Schlemm's canal inner and outer walls were observed. These results indicate that STC-1 may be a molecule within the latanoprost signaling pathway that can be therapeutically targeted to lower IOP.

Effect of Recombinant STC-1 on IOP

To determine if STC-1 has ocular hypotensive activity in vivo, wild-type C57Bl/6 mice were treated with recombinant STC-1 (0.5 mg/mL) or vehicle daily for 7 days (Fig. 7). Stanniocalcin-1 reduced IOP by 15.2% ± 3.0% when compared to vehicle-treated contralateral eyes (range of IOP reduction from 5.1% ± 5.2% [day 1] to 21.4% ± 5.3% [day 7]). Following withdrawal of treatment, IOP returned to baseline. These results suggest that STC-1 has IOP-lowering properties when used as a standalone agent.

Discussion

Prostaglandin analogs like latanoprost are a first-line medical therapy for IOP reduction due to their once daily dosing,

Table. Intraocular Pressure in Wild-Type and STC-1$^{-/-}$ Mice Following Treatment With Latanoprost and Rho Kinase Inhibitor

	Latanoprost				Rho Kinase Inhibitor Y27632			
	Wild-Type, $n = 8$		STC-1$^{-/-}$, $n = 10$		Wild-Type, $n = 10$		STC-1$^{-/-}$, $n = 10$	
Time After Treatment	ΔIOP ± SD, mm Hg	% Change Compared to Control ± SD	ΔIOP ± SD, mm Hg	% Change Compared to Control ± SD	ΔIOP ± SD, mm Hg	% Change Compared to Control ± SD	ΔIOP ± SD, mm Hg	% Change Compared to Control ± SD
Baseline*	−0.02 ± 0.27‡	−0.10 ± 1.61‡	0.01 ± 0.13‡	0.07 ± 0.77‡	−0.06 ± 0.31‡	−0.33 ± 1.91‡	0.00 ± 1.18‡	−0.01 ± 1.18‡
1 h*	−3.39 ± 0.39†	−21.02 ± 2.26†	−0.07 ± 0.29‡	−0.40 ± 1.82‡	−2.15 ± 0.48†	−13.15 ± 2.91†	−2.18 ± 0.77†	−13.23 ± 4.57†
4 h*	−3.74 ± 0.32†	−23.11 ± 1.92†	−0.13 ± 0.19‡	−0.78 ± 1.15‡	−2.92 ± 0.54†	−17.86 ± 3.25†	−2.81 ± 0.52†	−17.20 ± 3.05†
23 h*	−3.58 ± 0.40†	−21.85 ± 2.47†	−0.06 ± 0.29‡	−0.36 ± 1.74‡	−1.09 ± 0.39†	−6.61 ± 2.31†	−1.43 ± 0.99†	−8.71 ± 6.13†
Average*	−3.57 ± 0.31†	−22.00 ± 1.90†	−0.09 ± 1.12‡	−0.52 ± 0.70‡	−2.05 ± 0.21†	−12.53 ± 1.18†	−2.14 ± 0.46†	−13.08 ± 2.81†

* IOP change for each time point is the average calculated from all days of treatment.
† $P < 0.002$.
‡ $P > 0.10$.

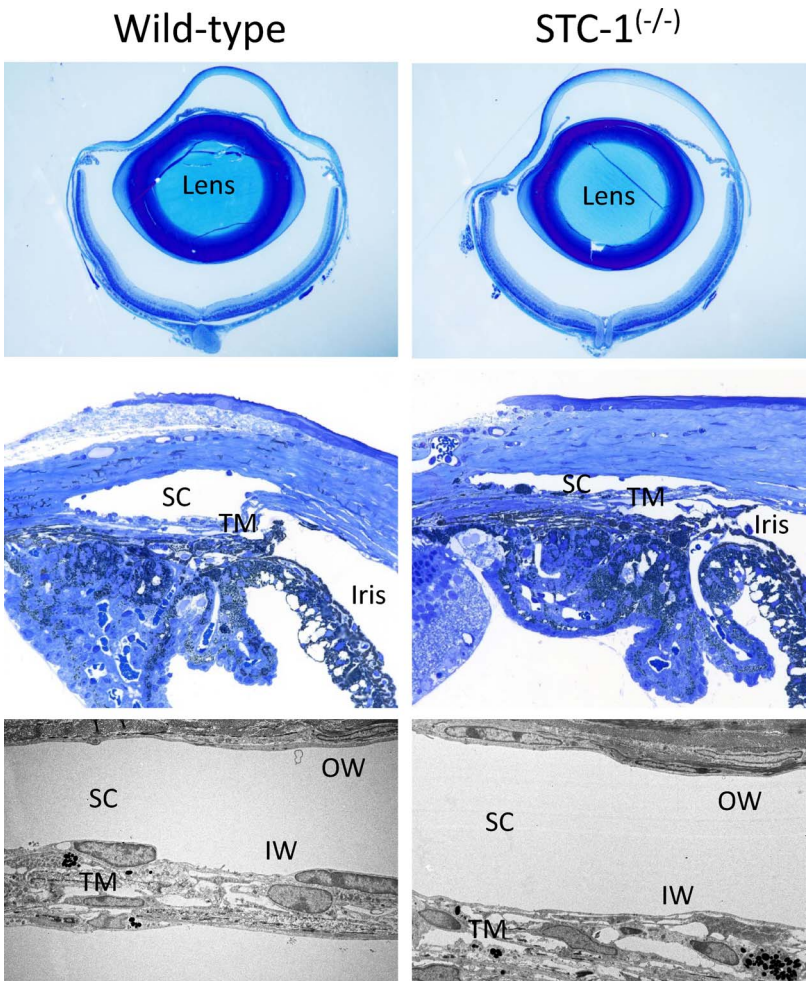

Figure 2. Histologic analyses of STC-1$^{-/-}$ mice and wild-type congenic controls. *Top*: Light microscopy images of whole eye sections from wild-type and STC-1$^{-/-}$ mice. Wild-type and STC-1$^{-/-}$ mice have similar-sized lens with both wild-type and STC-1$^{-/-}$ mice showing similar retinal morphology. *Middle*: Light microscopy images of wild-type and STC-1$^{-/-}$ mice showing conventional outflow pathway with open angles and similar number of trabecula, trabecular meshwork (TM) cells, and Schlemm's canal (SC) volume. No difference was noted in size or appearance of the iris. *Bottom*: Transmission electron micrographs of wild-type and STC-1$^{-/-}$ mice showing similar appearing trabecular meshwork and intact Schlemm's canal inner walls (IW) and outer walls (OW). Extracellular matrix appears morphologically similar between wild-type and STC-1$^{-/-}$ mice.

greater response rates, and greater amount of IOP reduction compared to other classes of pressure-lowering medications.[8] Like all IOP-lowering medications, latanoprost has notable side effects. Identifying the critical effector molecules in the signaling pathway of latanoprost will provide insights into additional molecules to target for IOP reduction, potentially with fewer side effects. In the current study, we identified STC-1 as a transcript and protein that is highly induced following treatment of human Schlemm's canal cells with latanoprost. Topical latanoprost significantly reduced IOP in wild-type controls while STC-1$^{-/-}$ mice demonstrated no IOP reduction to topical latanoprost. In contrast, Rho kinase inhibitor Y27632 demonstrated IOP reduction in both wild-type and STC-1$^{-/-}$ mice, indicating that STC-1 is a unique and important downstream signaling molecule necessary for the ocular hypotensive properties of latanoprost. Additionally, recombinant STC-1 by itself also increased outflow facility in human anterior segments and reduced IOP in vivo in wild-type mice. Together, these results suggest that STC-1 is a critical and unique effector molecule for the latanoprost signaling pathway that is necessary for latanoprost-induced IOP reduction and that STC-1 by itself can act as an ocular hypotensive agent.

Stanniocalcin was first described in fish as a 50-kDa homodimeric glycoprotein that is secreted from the corpuscles of Stannius into the bloodstream in the setting of hypercalcemia to regulate calcium excretion at the gills and gut.[29] In mammals, two homologues of STC have been identified, STC-1 and STC-2.[35–37] STC-1 is the most studied of the two mammalian forms, having a 50% amino acid homology with its fish counterpart. STC-1 is a secreted, homodimeric phosphoglycoprotein that has preserved protein structure similarity between mammals and fish including the conservation of 11 cysteine residues.[37] It is expressed in a wide variety of tissues most notably bone, skeletal muscle, heart, thymus, and spleen.[37] Functionally, STC-1 has been associated with calcium uptake,[38] hypoxic preconditioning,[39,40] and antioxidative stress properties through suppression of reactive oxygen species.[25,41–45] Additionally, STC-1 has also been shown to be neuroprotective for neurons, photoreceptors, and retinal ganglion cells,[26,46–48] and has been linked to anti-inflammatory effects by inhibiting macrophage chemotaxis,

Investigative Ophthalmology & Visual Science

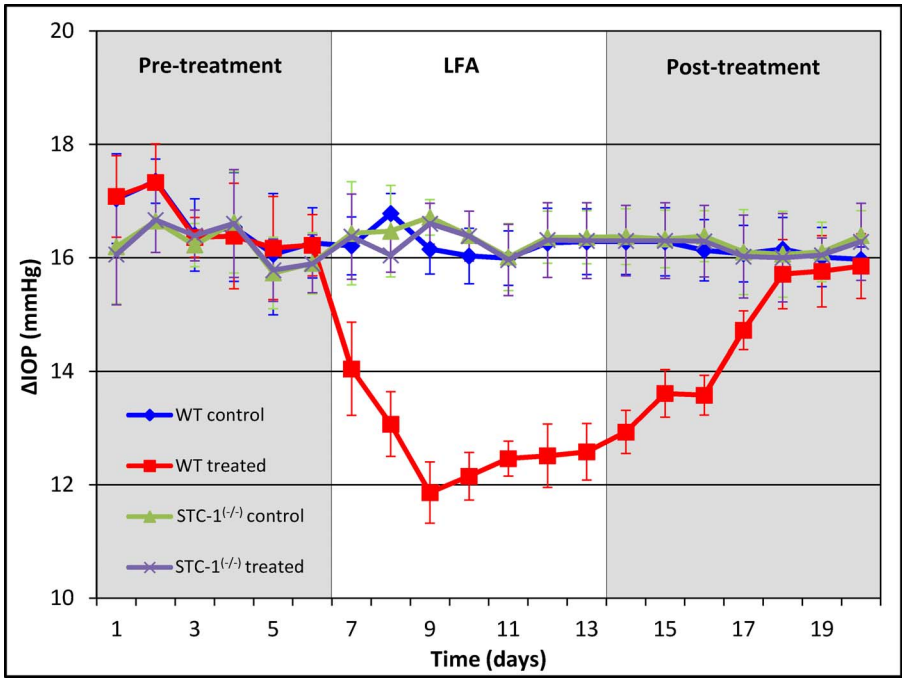

Figure 3. Effect of latanoprost treatment in STC-1$^{-/-}$ mice. STC-1$^{-/-}$ mice ($n = 10$) and congenic controls ($n = 8$) were treated once daily with 100 μM latanoprost. The daily IOP was recorded as the average of IOP measurements at 1, 4, and 23 hours following latanoprost treatment. While congenic control mice show IOP reduction with latanoprost, STC-1$^{-/-}$ do not, suggesting STC-1 is an important effector molecule for latanoprost-induced IOP reduction.

modulating transendothelial migration of leukocytes and reducing T cell infiltration.[23,45,49,50]

In treating wild-type mice with topical latanoprost, we observed a reduction in IOP by approximately 22.0% ± 1.9%, consistent with previous reports,[30] while the STC-1$^{-/-}$ mice showed no response (0.55% ± 0.7%). Mice that were STC-1$^{-/-}$ were responsive to Rho kinase inhibitor Y27632, which confirmed that STC-1$^{-/-}$ mice have normal functioning outflow pathways, but that elimination of STC-1 expression in these mice renders topical latanoprost ineffective.

The current study adds Schlemm's canal cells to the list of cells that express STC-1 and shows that its expression is highly

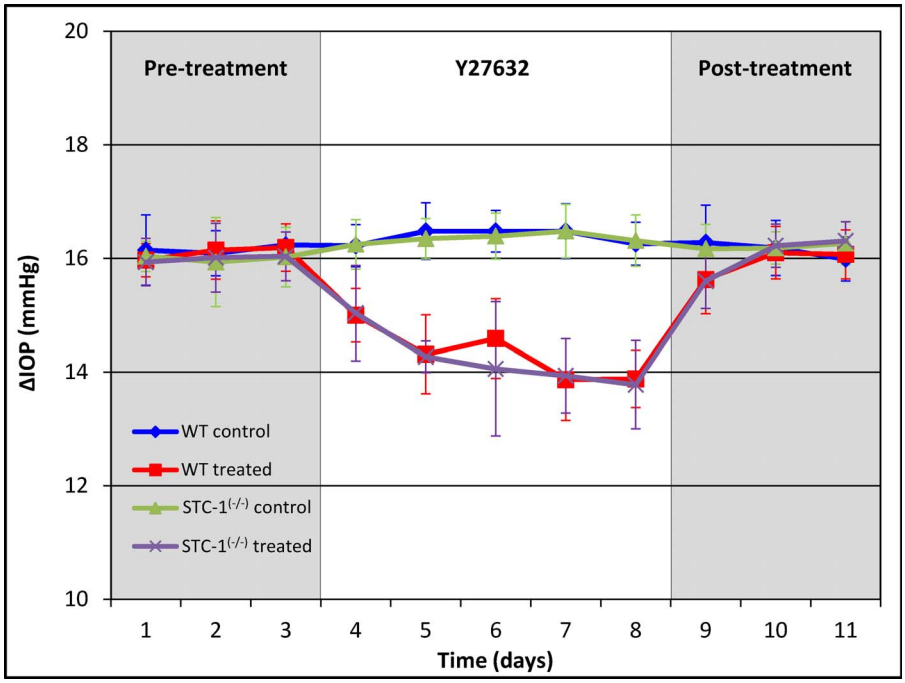

Figure 4. Rho kinase inhibitor Y27632 reduces IOP in STC-1$^{-/-}$ mice. STC-1$^{-/-}$ mice ($n = 10$) and congenic controls ($n = 10$) were treated once daily with 10 mM Y27632. The daily IOP was recorded as the average of IOP measurements at 1, 4, and 23 hours following Y27632 treatment. Both STC-1$^{-/-}$ mice and congenic controls showed IOP reduction following treatment with Y27632.

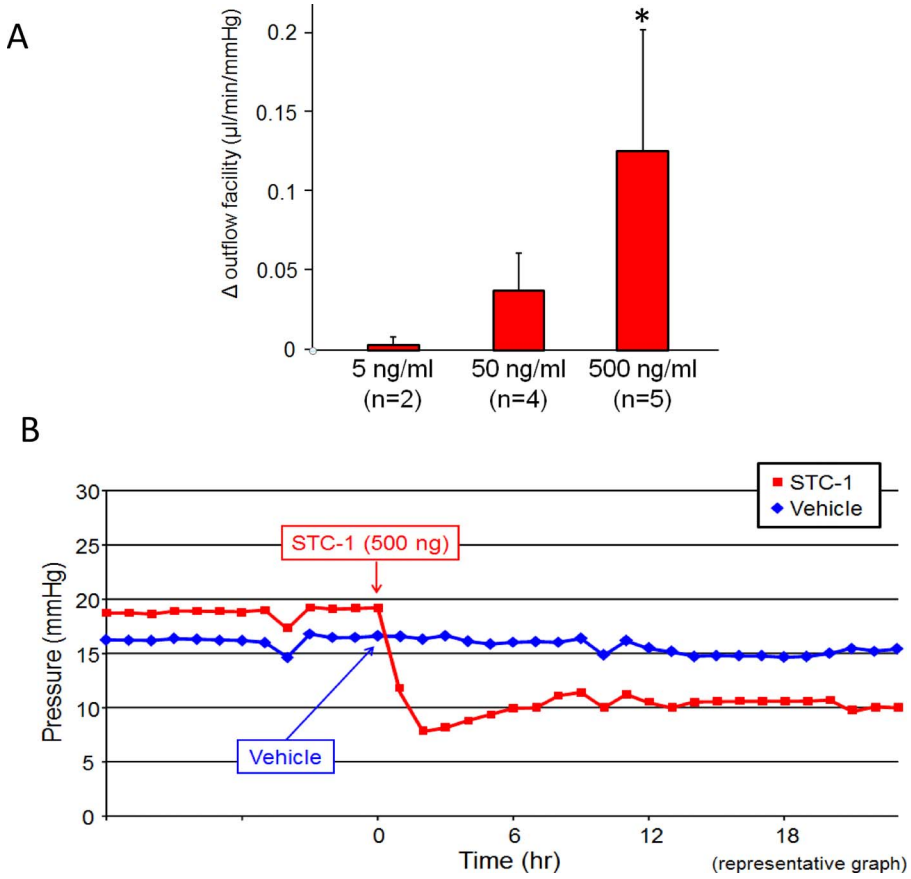

Figure 5. Perfusion with STC-1 shows increase in outflow facility and decrease in pressure. (**A**) Outflow facility of human anterior segments ($n = 8$) following perfusion with 5, 50, or 500 ng/mL of recombinant human STC-1. (**B**) Representative graph of an eye pair perfused with 500 ng/mL STC-1. $^{*}P \leq 0.05$.

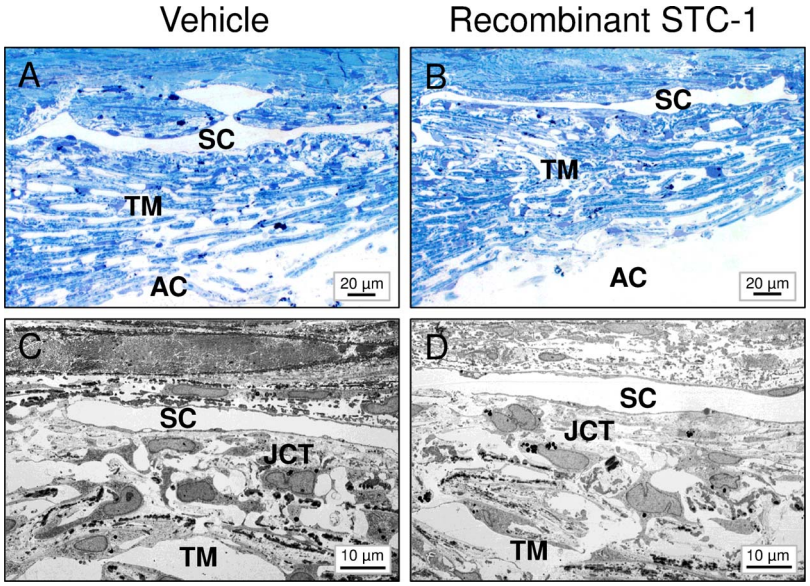

Figure 6. Histologic analysis of human anterior segment ocular tissue following treatment with recombinant STC-1. (**A**, **B**) Representative sections (3 µm) of recombinant STC-1 and vehicle-treated eyes that were stained with toluidine blue. (**C**, **D**) Transmission electron micrographs showing ultrastructure of recombinant STC-1 and vehicle-treated eyes. Recombinant human STC-1 and vehicle-treated eyes had similar morphology and ultrastructural appearance suggesting no apparent detrimental side effects of recombinant STC-1 treatment. AC, anterior chamber; JCT, juxtacanalicular region; TM, trabecular meshwork; SC, Schlemm's canal.

Investigative Ophthalmology & Visual Science

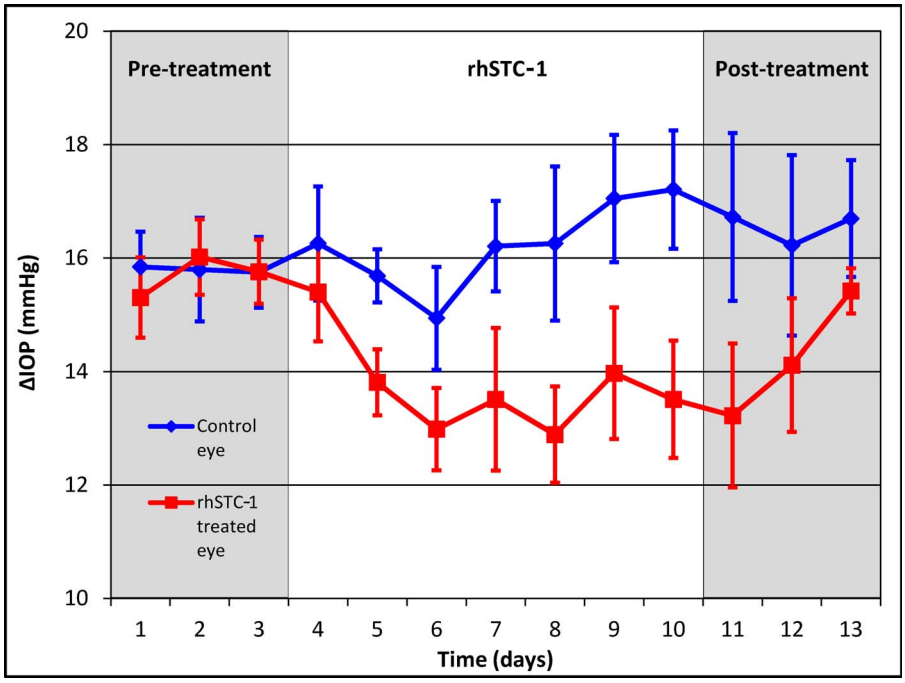

Figure 7. Topical STC-1 reduces IOP in C57BL/6 wild-type mice. Wild-type mice ($n = 10$) were treated topically with recombinant STC-1 (0.5 mg/mL) or vehicle (phosphate buffered saline). The daily IOP was recorded as the average of IOP measurements at 1, 4, and 23 hours following STC-1 treatment.

influenced by latanoprost. This is an important finding as little is known about the molecular events that connect latanoprost treatment with IOP reduction. Latanoprost has been shown to phosphorylate myosin light chain kinase in the iris,[19–21] indicating a role for latanoprost in cell relaxation. However, STC-1 is the first individual molecule that has been identified as a key downstream effector of latanoprost signaling. In addition to its variety of functional activities, our results also suggest that STC-1 has ocular hypotensive properties, since addition of recombinant STC-1 to human anterior segment cultures decreased pressure and increased outflow facility, and when applied topically to wild-type mice reduced IOP in vivo.

Despite the benefits of latanoprost in lowering IOP, long-term treatment with latanoprost can often cause significant side effects, which can be contraindicative for its prolonged usage. Identification of STC-1 as a downstream signaling molecule through which latanoprost executes its IOP-lowering effects, and the fact that recombinant STC-1 shows ocular hypotensive properties, makes this an attractive therapeutic target. Utilization of a specific downstream molecule such as STC-1 within the latanoprost signaling cascade may eliminate some of the major side effects while providing a specialized therapeutic strategy.

In summary, STC-1 is a necessary downstream signaling molecule for latanoprost-mediated lowering of IOP. Additionally, STC-1 demonstrates ocular hypotensive properties when used by itself, mimicking the effect of latanoprost. Given its novel role in latanoprost-mediated IOP reduction, STC-1 may be considered a promising candidate molecule for devising future therapeutic regiments to reduce IOP.

Acknowledgments

Supported by the National Eye Institute Grants EY21727 (MPF) and EY26490 (MPF); Veteran Administration (BX002006; DSH); Research to Prevent Blindness (MPF); American Society of Cataract and Refractive Surgery (GWR); Mayo Clinic CTSA through grant number UL1 TR000135 from the National Center for Advancing Translational Sciences (GWR, MPF); and Mayo Foundation (MPF).

Disclosure: **G.W. Roddy**, None; **K.B. Viker**, None; **N.S. Winkler**, None; **C.K. Bahler**, None; **B.H. Holman**, None; **D. Sheikh-Hamad**, None; **U. Roy Chowdhury**, None; **W.D. Stamer**, None; **M.P. Fautsch**, None

References

1. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol*. 2006;90:262–267.
2. Rudnicka AR, Kapetanakis VV, Wathern AK, et al. Global variations and time trends in the prevalence of childhood myopia, a systematic review and quantitative meta-analysis: implications for aetiology and early prevention. *Br J Ophthalmol*. 2016;100:882–890.
3. Tham YC, Li X, Wong TY, Quigley HA, Aung T, Cheng CY. Global prevalence of glaucoma and projections of glaucoma burden through 2040: a systematic review and meta-analysis. *Ophthalmology*. 2014;121:2081–2090.
4. Shahsuvaryan ML. Glaucomatous optic neuropathy management: the role of neuroprotective agents. *Med Hypothesis Discov Innov Ophthalmol*. 2013;2:41–46.
5. King A, Azuara-Blanco A, Tuulonen A. Glaucoma. *BMJ*. 2013;346:f3518.
6. Tanna AP, Lin AB. Medical therapy for glaucoma: what to add after a prostaglandin analogs? *Curr Opin Ophthalmol*. 2015;26:116–120.
7. Winkler NS, Fautsch MP. Effects of prostaglandin analogues on aqueous humor outflow pathways. *J Ocul Pharmacol Ther*. 2014;30:102–109.
8. Alm A. Latanoprost in the treatment of glaucoma. *Clin Ophthalmol*. 2014;1967–1985.

9. Toris CB, Gabelt BT, Kaufman PL. Update on the mechanism of action of topical prostaglandins for intraocular pressure reduction. *Surv Ophthalmol*. 2008;53(suppl 1):S107–S120.
10. Toris CB, Camras CB, Yablonski ME. Effects of PhXA41, a new prostaglandin F2 alpha analog, on aqueous humor dynamics in human eyes. *Ophthalmology*. 1993;100:1297–1304.
11. Ziai N, Dolan JW, Kacere RD, Brubaker RF. The effects on aqueous dynamics of PhXA41, a new prostaglandin F2 alpha analogue, after topical application in normal and ocular hypertensive human eyes. *Arch Ophthalmol*. 1993;111:1351–1358.
12. Alvarado JA, Iguchi R, Martinez J, Trivedi S, Shifera AS. Similar effects of selective laser trabeculoplasty and prostaglandin analogs on the permeability of cultured Schlemm canal cells. *Am J Ophthalmol*. 2010;150:254–264.
13. Bahler CK, Howell KG, Hann CR, Fautsch MP, Johnson DH. Prostaglandins increase trabecular meshwork outflow facility in cultured human anterior segments. *Am J Ophthalmol*. 2008;145:114–119.
14. Kalouche G, Beguier F, Bakria M, et al. Activation of prostaglandin FP and EP2 receptors differently modulates myofibroblast transition in a model of adult primary human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2016;57:1816–1825.
15. Richter M, Krauss AH, Woodward DF, Lutjen-Drecoll E. Morphological changes in the anterior eye segment after long-term treatment with different receptor selective prostaglandin agonists and a prostamide. *Invest Ophthalmol Vis Sci*. 2003;44:4419–4426.
16. Stamer WD, Piwnica D, Jolas T, et al. Cellular basis for bimatoprost effects on human conventional outflow. *Invest Ophthalmol Vis Sci*. 2010;51:5176–5181.
17. Toris CB, Zhan G, Fan S, et al. Effects of travoprost on aqueous humor dynamics in patients with elevated intraocular pressure. *J Glaucoma*. 2007;16:189–195.
18. Wan Z, Woodward DF, Cornell CL, et al. Bimatoprost, prostamide activity, and conventional drainage. *Invest Ophthalmol Vis Sci*. 2007;48:4107–4115.
19. Ansari HR, Davis AM, Kaddour-Djebbar I, Abdel-Latif AA. Effects of prostaglandin F2alpha and latanoprost on phosphoinositide turnover, myosin light chain phosphorylation and contraction in cat iris sphincter. *J Ocul Pharmacol Ther*. 2003;19:217–231.
20. Ansari HR, Kaddour-Djebbar I, Abdel-Latif AA. Effects of prostaglandin F2alpha, latanoprost and carbachol on phosphoinositide turnover, MAP kinases, myosin light chain phosphorylation and contraction and functional existence and expression of FP receptors in bovine iris sphincter. *Exp Eye Res*. 2004;78:285–296.
21. Husain S, Abdel-Latif AA. Effects of prostaglandin F(2alpha) and carbachol on MAP kinases, cytosolic phospholipase A(2) and arachidonic acid release in cat iris sphincter smooth muscle cells. *Exp Eye Res*. 2001;72:581–590.
22. Anthony TL, Pierce KL, Stamer WD, Regan JW. Prostaglandin F2 alpha receptors in the human trabecular meshwork. *Invest Ophthalmol Vis Sci*. 1998;39:315–321.
23. Huang L, Garcia G, Lou Y, et al. Anti-inflammatory and renal protective actions of stanniocalcin-1 in a model of anti-glomerular basement membrane glomerulonephritis. *Am J Pathol*. 2009;174:1368–1378.
24. Block GJ, Ohkouchi S, Fung F, et al. Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. *Stem Cells*. 2009;27:670–681.
25. Wang Y, Huang L, Abdelrahim M, et al. Stanniocalcin-1 suppresses superoxide generation in macrophages through induction of mitochondrial UCP2. *J Leukoc Biol*. 2009;86:981–988.
26. Zhang K, Lindsberg PJ, Tatlisumak T, Kaste M, Olsen HS, Andersson LC. Stanniocalcin: a molecular guard of neurons during cerebral ischemia. *Proc Natl Acad Sci U S A*. 2000;97:3637–3642.
27. Fautsch MP, Bahler CK, Jewison DJ, Johnson DH. Recombinant TIGR/MYOC increases outflow resistance in the human anterior segment. *Invest Ophthalmol Vis Sci*. 2000;41:4163–4168.
28. Johnson DH. The effect of cytochalasin D on outflow facility and the trabecular meshwork of the human eye in perfusion organ culture. *Invest Ophthalmol Vis Sci*. 1997;38:2790–2799.
29. Gerritsen ME, Wagner GF. Stanniocalcin: no longer just a fish tale. *Vitam Horm*. 2005;70:105–135.
30. Ota T, Aihara M, Narumiya S, Araie M. The effects of prostaglandin analogues on IOP in prostanoid FP-receptor-deficient mice. *Invest Ophthalmol Vis Sci*. 2005;46:4159–4163.
31. Tian B, Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. *Exp Eye Res*. 2005;80:215–225.
32. Tokushige H, Inatani M, Nemoto S, et al. Effects of topical administration of y-39983, a selective rho-associated protein kinase inhibitor, on ocular tissues in rabbits and monkeys. *Invest Ophthalmol Vis Sci*. 2007;48:3216–3222.
33. Wang RF, Williamson JE, Kopczynski C, Serle JB. Effect of 0.04% AR-13324, a ROCK, and norepinephrine transporter inhibitor, on aqueous humor dynamics in normotensive monkey eyes. *J Glaucoma*. 2015;24:51–54.
34. Williams RD, Novack GD, van Haarlem T, Kopczynski C; AR-12286 Phase 2A Study Group. Ocular hypotensive effect of the Rho kinase inhibitor AR-12286 in patients with glaucoma and ocular hypertension. *Am J Ophthalmol*. 2011;152:834–841.
35. Chang AC, Jellinek DA, Reddel RR. Mammalian stanniocalcins and cancer. *Endocr Relat Canc*. 2003;10:359–373.
36. Ishibashi K, Imai M. Prospect of a stanniocalcin endocrine/paracrine system in mammals. *Am J Physiol Renal Physiol*. 2002;282:F367–F375.
37. Yoshiko Y, Aubin JE. Stanniocalcin 1 as a pleiotropic factor in mammals. *Peptides*. 2004;25:1663–1669.
38. Radman DP, McCudden C, James K, Nemeth EM, Wagner GF. Evidence for calcium-sensing receptor mediated stanniocalcin secretion in fish. *Mol Cell Endocrinol*. 2002;186:111–119.
39. Westberg JA, Serlachius M, Lankila P, Andersson LC. Hypoxic preconditioning induces elevated expression of stanniocalcin-1 in the heart. *Am J Physiol Heart Circ Physiol*. 2007;293:H1766–H1771.
40. Westberg JA, Serlachius M, Lankila P, Penkowa M, Hidalgo J, Andersson LC. Hypoxic preconditioning induces neuroprotective stanniocalcin-1 in brain via IL-6 signaling. *Stroke*. 2007;38:1025–1030.
41. Huang L, Belousova T, Pan JS, et al. AKI after conditional and kidney-specific knockdown of Stanniocalcin-1. *J Am Soc Nephrol*. 2014;25:2303–2315.
42. Tang SE, Wu CP, Wu SY, et al. Stanniocalcin-1 ameliorates lipopolysaccharide-induced pulmonary oxidative stress, inflammation, and apoptosis in mice. *Free Radic Biol Med*. 2014;71C:321–331.
43. Nyamandi VZ, Johnsen VL, Hughey CC, et al. Enhanced stem cell engraftment and modulation of hepatic reactive oxygen species production in diet-induced obesity. *Obesity*. 2014;22:721–729.
44. Ohkouchi S, Block GJ, Katsha AM, et al. Mesenchymal stromal cells protect cancer cells from ROS-induced apoptosis and enhance the Warburg effect by secreting STC1. *Mol Ther*. 2012;20:417–423.

Investigative Ophthalmology & Visual Science

45. Sheikh-Hamad D. Mammalian stanniocalcin-1 activates mitochondrial antioxidant pathways: new paradigms for regulation of macrophages and endothelium. *Am J Physiol Renal Physiol*. 2010;298:F248–F254.
46. Durukan Tolvanen A, Westberg JA, Serlachius M, et al. Stanniocalcin 1 is important for poststroke functionality, but dispensable for ischemic tolerance. *Neuroscience*. 2013;229: 49–54.
47. Kim SJ, Ko JH, Yun JH, et al. Stanniocalcin-1 protects retinal ganglion cells by inhibiting apoptosis and oxidative damage. *PLoS One*. 2013;8:e63749.
48. Roddy GW, Rosa RH Jr, Oh JY, et al. Stanniocalcin-1 rescued photoreceptor degeneration in two rat models of inherited retinal degeneration. *Mol Ther*. 2012;20:788–797.
49. Chakraborty A, Brooks H, Zhang P, et al. Stanniocalcin-1 regulates endothelial gene expression and modulates transendothelial migration of leukocytes. *Am J Physiol Renal Physiol*. 2007;292:F895–F904.
50. Kanellis J, Bick R, Garcia G, et al. Stanniocalcin-1, an inhibitor of macrophage chemotaxis and chemokinesis. *Am J Physiol Renal Physiol*. 2004;286:F356–F362.

Investigative Ophthalmology & Visual Science