Effects of Salidroside on Trabecular Meshwork Cell Extracellular Matrix Expression and Mouse Intraocular Pressure

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Purpose. Excessive accumulation of extracellular matrix (ECM) in the trabecular meshwork (TM) reduces aqueous humor outflow, which likely contributes to elevation of IOP in primary open-angle glaucoma (POAG). Salidroside, a phenolic glycoside isolated from *Rhodiola rosea* is reported to prevent profibrotic responses by inhibiting Smad signaling pathway activated by TGF-β in liver, lung, and kidney tissues. We tested if salidroside can (1) inhibit TGF-β2–induced ECM expression in cultured human TM cells, and (2) lower TGF-β2–induced ocular hypertension in the mouse.

Methods. Cultured human TM cells stimulated with 5 ng/mL TGF-β2 for 48 hours were treated with salidroside for 24 hours. The expressions of fibronectin (FN), collagen type IV (COL-IV), and laminin (LN) were evaluated by quantitative PCR, Western blot, and immunocytochemistry. BALB/cJ mice were injected intravitreally with an adenoviral vector encoding a bioactive mutant of TGF-β2 (Ad.hTGF-$\beta2^{226/228}$) in one eye to induce ocular hypertension, with the uninjected contralateral or Ad.Empty-injected eyes serving as controls. Mice were treated with a daily intraperitoneal injection of 40 mg/kg salidroside. Conscious mouse IOP values were measured using a TonoLab rebound tonometer.

Results. In cultured human TM cells, treatment with TGF-β2 increased expressions of FN, COL-IV, and LN, as assessed by quantitative PCR, Western blotting, and immunocytochemistry, all of which were significantly and completely ameliorated by 30 μM salidroside. Daily intraperitoneal injections of salidroside (40 mg/kg), starting either at day 0 (same day as Ad.hTGF-$\beta2^{226/228}$ injection) or at day 14, significantly lowered TGF-β2–induced ocular hypertension in the mouse. In contrast, salidroside did not affect IOP of control eyes.

Conclusions. These results demonstrated that salidroside is capable of minimizing TGF-β2–induced ECM expression in cultured human TM cells. It also reduced TGF-β2–induced ocular hypertension in the mouse. These findings indicate that this phenolic glycoside may be useful as a novel treatment for POAG.

Keywords: salidroside, TGF-β2, trabecular meshwork, extracellular matrix, glaucoma, intraocular pressure

Glaucoma is one of the major causes of blindness around the world.[1] Because elevated IOP is a major risk factor for the development and progression of glaucoma, reduction of IOP is an important therapeutic modality.[2] IOP is regulated by the equilibrium between production and outflow rates of aqueous humor. Elevated IOP in glaucoma is caused mainly by an increased resistance to aqueous outflow. TGF-β2 is thought to play an important role in the pathogenesis of primary open angle glaucoma (POAG). It has been shown to be elevated in POAG eyes.[3–10] It can increase aqueous humor resistance by upregulation of the extracellular matrix (ECM), such as fibronectin (FN), collagen IV (COL-IV), and laminin (LN) in the trabecular meshwork (TM),[11–15] suggesting that fibrotic changes in the TM may be related to the pathogenesis of POAG. Thus, reduction in fibrotic changes in the TM may be a useful therapeutic approach for the treatment of glaucoma.

Rhodiola rosea, a plant adapted to grow at high altitude and in cold regions, has long been used as a medicinal herb in Asian cultures due to its tissue protective and antioxidative activities.[16,17] Salidroside, an active ingredient isolated from *R. rosea*, is a phenolic glycoside that possesses various pharmacological actions. Studies demonstrated that salidroside can attenuate ischemic heart damage,[18] improve streptozotocin-impaired neurogenesis in rat hippocampus,[14] ameliorate lung injuries,[19,20] and mitigate acute lung injuries in lipopolysaccharide- or paraquat-challenged animals. It can reduce chronic hypoxia-induced pulmonary arterial hypertension in mice.[20] Most importantly, Tang et al.[21] showed that salidroside protects against bleomycin-induced pulmonary fibrosis by inhibiting TGF-β1/Smad-2/-3 pathways. Zhang et al.[19] reported that salidroside alleviates pulmonary symptoms of paraquat-induced rat by suppressing inflammatory cell infiltration and the

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expression of TGF-β1 resulting in delayed lung fibrosis.[20] Feng and colleagues[22] demonstrated that salidroside inhibits the production of ECM and affects its metabolism, which subsequently attenuates liver fibrosis via TGF-β1/Smad3 pathways. Furthermore, Ouyang et al.[23] recently showed that salidroside strengthens the therapeutic effects of rat mesenchymal stem cell transplantation on hepatic fibrotic lesions in rats by suppressing TGF-β1 expression.

These studies indicate that salidroside has an antifibrotic effect and inhibitory effect on TGF signaling. We hypothesized that similar effects may occur in the TM, which may affect ECM accumulation, and subsequently IOP. In the present studies, we found that indeed salidroside inhibited TGF-β2–induced ECM expression in cultured human TM cells, and lowered TGF-β2–induced ocular hypertension in the mouse.

Methods and Materials

Cell Culture and Treatment

Fresh human eyes were used to obtain primary human TM (HTM) cells. All experiments were conducted in accordance with the principles of the Declaration of Helsinki. Briefly, TM tissues were isolated from the corneal rim of three female donors (aged 55, 63, and 43 years; all due to accident-related deaths). They were not diagnosed with glaucoma or other ocular disorders. The tissues were carefully dissected and divided into approximately 1- to 3-mm sections, which were explanted into 10 cm^2 disposable plastic tissue culture dishes and cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco, Halethorpe, MD, USA) supplemented with glutamine, penicillin/streptomycin, and 10% fetal bovine serum (Invitrogen-Gibco, Carlsbad, CA, USA). The medium was changed three times in a week until the cells were approximately 80% confluent. Subsequently, cells were subcultured by digestion with Trypsin-EDTA (Invitrogen-Gibco) in culture medium for approximately 2 to 3 minutes, followed by centrifugation (135*g*) for 4 minutes. The cells were passed sequentially in a 1:3 ratio and maintained in the same conditioned medium. Cells of passage 3 were identified as TM cells by immunocytochemistry to detect the presence of aquaporin (AQP1), FN, COL-IV, and LN. Cultured HTM cells stimulated with 100 nM dexamethasone (DEX) (Sigma-Aldrich Corp., St. Louis, MO, USA) were added fresh every approximately 2 to 3 days to the media for up to 10 days. The level of myocilin (MYOC) was assessed by quantitative PCR and Western blotting.[24]

The cells of passages 4 to 10 were used in the present study. To evaluate drug effects, cells were treated with salidroside (MedChemExpress, Monmouth Junction, NJ, USA) with or without TGF-β2 (5 ng/mL; Sigma, St. Louis, MO, USA). During the TGF-β2 treatment period, cell medium was replaced daily with fresh TGF-β2. All experiments were performed at least three separate times.

Cell Viability

Cell Counting Kit-8 (CCK8; MedChemExpress) was performed to evaluate HTM cell viability. HTM cells (10^3 cells/well) were plated in 96-well plates and treated with the indicated compounds for the indicated time periods. CCK8 reagent was then added. The cells were incubated further for another 4 hours at 37°C. The microplate reader (ELX800; BioTeK, Winooski, VT, USA) was used to measure absorbance at 450 nm. The cell viability was expressed as the absorbance at 450 nm.

Real-Time Quantitative PCR (RT-qPCR)

RNA isolation was performed with the RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA), and the subsequent synthesis of cDNA was performed using the QuantiTect reverse transcription kit (Qiagen). RT-qPCR was performed using SYBR Green Master Mix (Qiagen). Results from HTM cells were normalized to the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences were as follows: MYOC (forward: 5′-ATACTGCCTAGGCCACTGGA-3′; reverse: 5′-CGTGTAGCCACCCCAAGAAT-3′), FN (forward: 5′-AAGAC CAGCAGAGGCATAAGG-3′; reverse: 5′-TGTAGGGGTCAAAG CACGAG-3′), COL-IV (forward: 5′-TAGAGAGGAGCGA GATGTTC-3′; reverse: 5′-GTGACATTAGCTGAGTCAGG-3′), LN (forward: 5′-AGCACTGTGGAGCAGTCAGC-3′; reverse: 5′-TTGCCTGCCAGTTCATCAAG-3′), GAPDH (forward: 5′-CGAGA TCCCTCCAAAATCAA-3′; reverse: 5′-GTCTTCTGGGTGGCAGT GAT-3′).

Protein Extraction and Western Blot Analysis

Total cellular protein was isolated from cultured HTM cells using M-PER extraction buffer (Pierce Biotech, Rockford, IL, USA) and Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Protein concentration was determined by using a BCA protein assay (Pierce, Rockford, IL, USA), according to the respective manufacturer's instructions.

A total of 20 μg of protein was loaded per well and separated by SDS-PAGE and then transferred by electrophoresis to polyvinyl difluoride (PVDF) membranes. The PVDF membranes were incubated in 5% BSA in Tris-Buffered Saline Tween (containing 20 mM Tris, 0.5 M NaCl, and 0.05% Tween 20, pH 7.4) for approximately 60 minutes to block nonspecific binding. The following antibodies were used for immunostaining. Primary antibodies: rabbit polyclonal myocilin antibody (1:500; catalog # ab41552; Abcam, Cambridge, MA, USA), rabbit polyclonal fibronectin antibody (1:1000; catalog # ab2413; Abcam), rabbit polyclonal COL-IV antibody (1:1000; catalog # ab6586; Abcam), rabbit polyclonal LN antibody (1:1000; catalog # ab11575; Abcam), rabbit polyclonal GAPDH antibody (1:2000; catalog # ab9485; Abcam) at 4°C for 8 to 16 hours. The membrane was washed three times for 10 minutes with TBS containing 0.1% Tween 20 followed by incubating with horseradish peroxidase–conjugated anti-rabbit IgG (1:2000; catalog # 14708; Cell Signaling Technology, Danvers, MA, USA) for 60 minutes at room temperature. The membrane was then washed several times and scanned by hypersensitive chemiluminescence analyzer (Amersham Imager600; GE, Pittsburgh, PA, USA). The protein density of every band was analyzed with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Immunocytochemistry

HTM cells were cultured on coverslips in 24-well plates. After the indicated treatment time was completed, cells were washed with PBS, and fixed in 4% paraformaldehyde at 4°C for 30 minutes. They were incubated with 0.5% Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA) in PBS at room temperature for 30 minutes, and then blocked with Superblock (ThermoFisher Scientific, Grand Island, NY, USA). Cells were incubated with the primary antibody at room temperature for 2 hours or 4°C overnight, and the corresponding secondary antibody for 1 hour at room temperature. After washing with PBS, cells were mounted with Anti-fade Prolong Gold containing 4′,6-diaminido-2-phenylindole (DAPI; Invitrogen Corporation, Carlsbad, CA, USA). The following antibodies

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were used for immunostaining. Primary antibodies: rabbit polyclonal AQP1 antibody (1:200, catalog # ab15080; Abcam), rabbit polyclonal FN antibody (1:200; Abcam), rabbit polyclonal COL-IV antibody (1:100; Abcam), and rabbit polyclonal LN antibody (1:200; Abcam). The secondary antibody was goat anti-rabbit alexa-488 (1:200; Invitrogen).

Animals

All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all protocols were approved and monitored by the Animal Care and Use Committee of Shanghai Jiaotong University. BALB/cJ mice (2–5 months old; Charles River Lab, Beijing, China) used in this study were maintained on a 12-hour light/12-hour dark cycle (lights on 6:00 AM). Food and water were available ad libitum. All mice were euthanized by cervical dislocation after anesthesia overdose.

In Vivo Treatments

To induce ocular hypertension, one eye of each mouse was injected intravitreally with an adenoviral vector encoding a bioactive mutant of human TGF-β2 (Ad.hTGF-$\beta2^{226/228}$).[15] At the day of vector injection (day 0), mice were anesthetized by intraperitoneal injection of a solution containing acepromazine (1.8 mg/kg), ketamine (73 mg/kg), and xylazine (1.8 mg/kg). A randomly assigned eye of each animal was pretreated with 1 or 2 drops 1% cyclopentolate (Mydriacyl; Alcon Laboratories, Fort Worth, TX, USA) to dilate the pupil, then topically anesthetized with 1 or 2 drops of 0.5% proparacaine (Alcaine; Alcon Laboratories). A suspension of Ad.hTGF-$\beta2^{226/228}$ (6×10^7 pfu) in a volume of 2 μL was injected intravitreally using a Hamilton (Reno, NV, USA) glass micro-syringe fitted with a 1-inch, 33-gauge needle with a 10° bevel, as described previously.[15,25,26] Each injection was made over the course of approximately 30 seconds. The needle was then left in place for an additional 1 minute before being rapidly withdrawn. The contralateral eye served as noninjected control. An additional control group were injected intravitreally with a null adenoviral vector Ad.Empty (6×10^7 pfu in 2 μL) in one eye.

The Ad.hTGF-$\beta2^{226/228}$-injected animals were further divided into three groups: (1) no salidroside treatment, (2) salidroside (40 mg/kg, intraperitoneal injection) starting at day 0 (same day as Ad.hTGF-$\beta2^{226/228}$ injection), and (3) salidroside (40 mg/kg, intraperitoneal injection) starting at day 14. The dose of 40 mg/kg was selected based a previous report,[27] and was confirmed efficacious in a pilot study.

All IOP measurements were conducted between 2 PM and 4 PM, right before salidroside treatment, using the TonoLab rebound tonometer (Icare, Helsinki, Finland), as described.[28] The researcher who measured IOP was masked regarding identities of study groups.

Histology and Immunofluorescence

Mouse eyes were enucleated and fixed overnight in freshly prepared 4% paraformaldehyde in PBS. Afterward, they were washed three times with PBS, dehydrated with ethanol, and embedded in paraffin. Samples were cut into 5-μm-thick sections and stained with hematoxylin and eosin for general evaluation of the anterior segment, including TM. For immunostaining, tissue sections were deparaffinized in xylene and rehydrated twice each with 100%, 95%, 70%, and 50% ethanol for 5 minutes. For antigen retrieval, the tissue sections were incubated in citrate buffer (pH 6.0) at 100°C for 13 minutes and then at room temperature for an additional 13 minutes. Tissue sections were blocked (10% goat serum) for 2 hours in a dark and humid chamber. Tissue sections were then washed briefly with PBS and immunolabeled with rabbit polyclonal FN antibody (1:200), rabbit polyclonal COL-IV antibody (1:100), or rabbit polyclonal LN antibody (1:200) and incubated overnight at 4°C. After incubation, tissue sections were washed three times with PBS and further incubated for 2 hours at room temperature with the appropriate secondary antibodies (1:500; Alexa goat anti-rabbit 568 or Alexa donkey anti-goat; ThermoFisher). Tissue sections were washed with PBS and mounted with mounting medium containing DAPI nuclear stain (Vector Labs, Inc., Burlingame, CA, USA). Images were captured by confocal microscope (Nikon A1, Tokyo, Japan).

Ocular Tissue Isolation for RT-qPCR

Mouse sclera rings containing the TM were carefully dissected, removing as much of the sclera and cornea as possible. Each ring was placed in a 1.5-mL Eppendorf tube containing 1 mL extraction solution (TRIzol; Invitrogen). After the tissue was homogenized with a homogenizer, RNA isolation was performed according to the manufacturer's instructions, and the subsequent synthesis of cDNA was performed using the QuantiTect reverse transcription kit (Qiagen). RT-qPCR was performed using SYBR Green Master Mix (Qiagen). Results of mouse samples were normalized to internal control β-Actin. Primer sequences were: β-Actin (forward: 5′-CCATGTACG TAGCCATCC-3′; reverse: 5′-TCAGCTGTGGTGGTGAA-3′), FN (forward: 5′-ACAGTCCAGCAAGCAGCAAGC-3′; reverse: 5′-TGGTGGTCACTCTGTAGCCTGTC-3′), COL-IV (forward: 5′-TAGAGAGGAGCGAGATGTTC-3′; reverse: 5′-GTGACATTAGCT GAGTCAGG-3′), LN (forward: 5′-TGCTCACAAGACGGCGAA TAAGAC-3′; reverse: 5′-ATCGTAATGCCTTGCTGCTTCCTC-3′).

Statistical Analysis

Data are presented as mean ± SEM. One-way ANOVA was used to compare results among three or more groups, followed by least significant difference (LSD) post hoc analysis. *P* values less than 0.05 were regarded as statistically significant.

Results

Isolation and Characterization of HTM Cells

After treatment with DEX (100 μM) at 10 days compared with vehicle-treated HTM cells, the protein expression of myocilin was significantly increased to 1.75 ± 0.18-fold of control ($P < 0.01$, $n = 3$). Similar to the protein level changes, mRNA levels of MYOC in the HTM cells was also elevated to 4.98 ± 0.77 ($P < 0.01$, $n = 3$) by DEX. In addition, we show that isolated HTM cell expressed fibronectin, collagen-4, and laminin, and these ECM proteins were upregulated by TGF-β2 compared with control cells. At the same time, the isolated HTM cells also expressed aquaporin-1 protein (Fig. 1D). These data demonstrated that HTM cells can be cultured and isolated in vitro.

Salidroside and TGF-β2 Had Minimal Effects on HTM Cell Viability

Even at a very high concentration (100 μM), salidroside did not affect HTM cell viability (95.8% ± 1.3% compared with control 100.0% ± 2.2%, mean ± SEM, $n = 5$) after a 1-day incubation (Fig. 2). Even at day 4, cell viability was not significantly changed by salidroside at concentrations up to 30 μM ($P > 0.05$). Only at 100 μM for 4 days did salidroside slightly reduce

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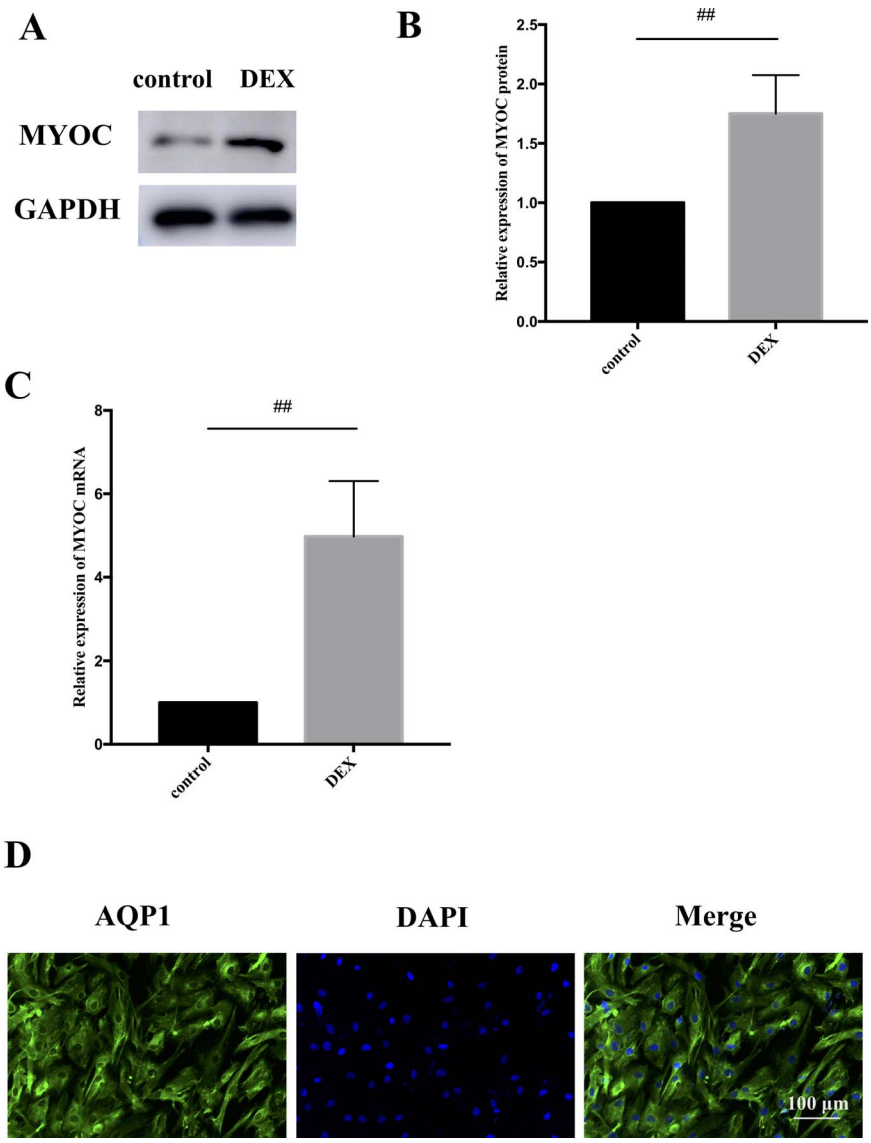

FIGURE 1. Characterization of human TM cells. Levels of the MYOC proteins after DEX (100 nM) for 10 days was analyzed by Western blotting. A representative immunoblot is shown (**A**). Protein levels of MYOC (**B**) were quantified using GAPDH as a normalizing internal control. Levels of mRNA of MYOC (**C**) were analyzed by RT-qPCR. Cells were treated with DEX (100 nM) for 10 days. GAPDH was used as normalizing control. Data are presented as mean ± SEM, $n = 3$. #$P < 0.05$ compared with control, ##$P < 0.01$ compared with control. The expression of aquaporin-1 protein of isolated HTM cells was evaluated by immunocytochemistry (**D**).

HTM cell viability (91.3% ± 0.7%, $P < 0.05$). Similarly, either 5 ng/mL TGF-β2 alone, or TGF-β2 together with salidroside (up to 30 μM) did not affect viability of the HTM cells after 1 to 4 days of incubation. These data suggest that both compounds had minimal toxicity to the cells, and results of subsequent studies were not due to unexpected cell toxicity.

Salidroside Decreased TGF-β2–Induced Expressions of FN, COL-IV, and LN in HTM Cells

We used RT-qPCR to evaluate the effects of salidroside on ECM expression in cultured HTM cells. After 48 hours of incubation with TGF-β2 (5 ng/mL), mRNA levels of FN, COL-IV, and LN were all significantly ($P < 0.05$) elevated to 1.77 ± 0.10-fold (FN), 1.75 ± 0.07-fold (COL-IV), and 1.63 ± 0.07-fold (LN) (all $n = 3$). These effects of TGF-β2 were reduced in a concentration-dependent manner by salidroside treatment; for example, in the presence of 10 μM salidroside, the TGF-β2 effects were significantly ($P < 0.05$) diminished (relative to control level, FN: 1.02 ± 0.11-fold, COL-IV: 1.03 ± 0.12-fold, and LN: 1.03 ± 0.05-fold). Similarly, 30 μM salidroside eliminated the stimulatory effects of TGF-β2 (relative to control level, FN: 1.03 ± 0.09-fold, COL-IV: 1.14 ± 0.17-fold, and LN: 0.96 ± 0.06-fold, all $n = 3$) (Figs. 3A–C).

Similar to the mRNA level changes, protein levels of FN, COL-IV, and LN in the HTM cells were also affected by both TGF-β2 and salidroside treatments. Figure 3D shows a representative set of Western blotting images indicating TGF-β2 (5 ng/mL) increased expressions of FN, COL-IV, and LN, which was reversed by salidroside (30 μM). Densitometry assessment showed that TGF-β2 increased levels of FN to 2.95 ± 0.11-fold of control ($n = 3$), COL-IV to 1.49 ± 0.05-fold, and LN to 1.51 ± 0.10-fold. These effects were partially or completely eliminated by salidroside (30 μM). When salidro-

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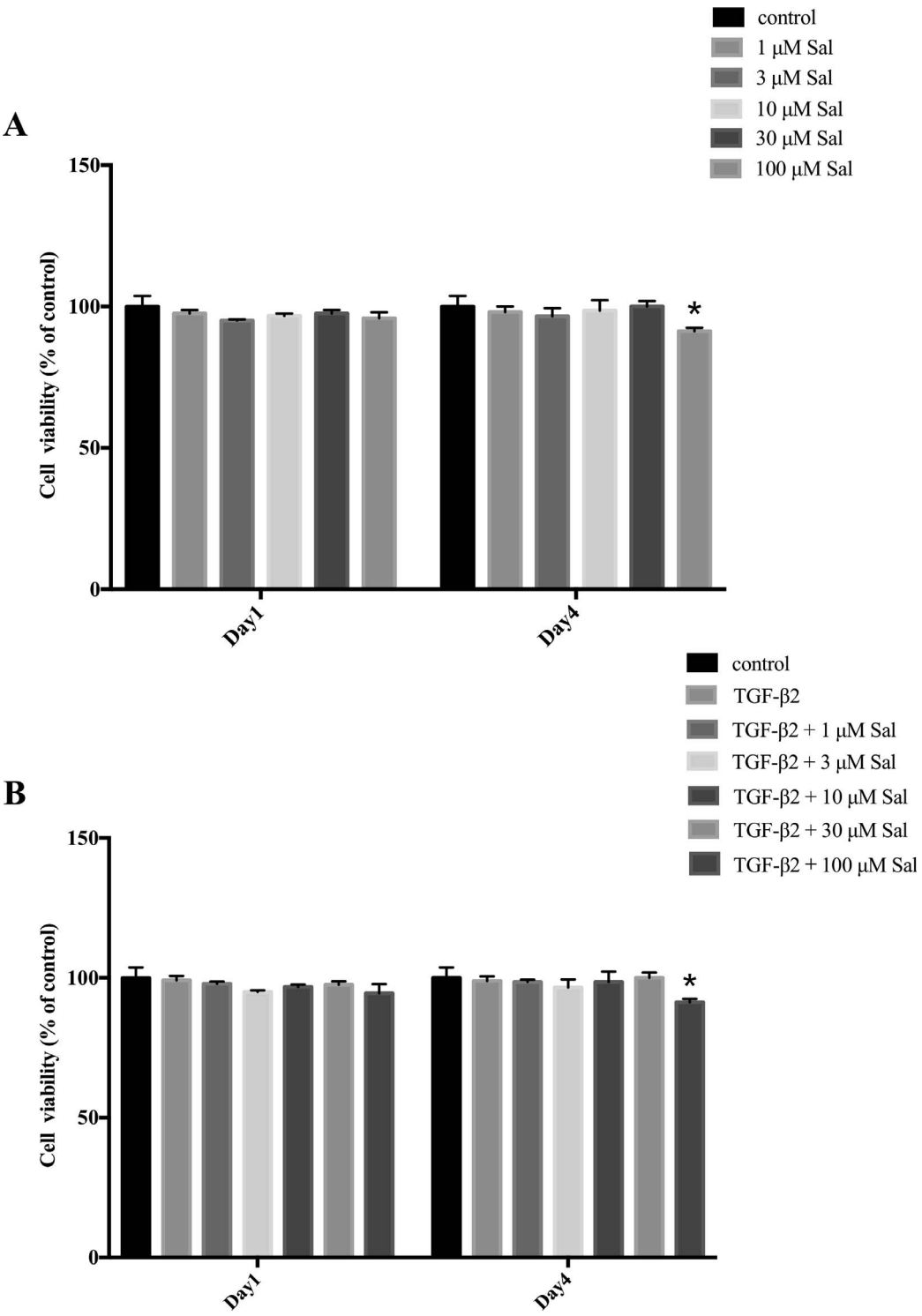

Figure 2. Effects of TGF-β2 and salidroside on HTM cell survival. Effects of salidroside (sal) at different concentrations (1, 3, 10, 30, 100 μM) without (**A**), or with (**B**) 5 ng/mL TGF-β2. Salidroside was added to the HTM cells after they were incubated with TGF-β2 for 48 hours. At 1 day or 4 days after salidroside treatment, cell viability was measured by CCK8 assay. Data are presented as mean ± SEM ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control. The mean value of control samples defines 100%.

side was added to TGF-β2, the level of FN was lowered to 1.61 ± 0.12-fold of control, COL-IV to 1.10 ± 0.03-fold, and LN to 0.80 ± 0.04-fold. Salidroside itself significantly lowered the baseline levels of LN (0.61 ± 0.05-fold, $P < 0.05$), but not FN (1.31 ± 0.13, $P > 0.05$) and COL-IV (1.14 ± 0.04, $P > 0.05$) (Figs. 3E–G).

The observed changes in mRNA and protein levels of these ECM proteins were confirmed by immunocytochemistry. As shown in Figure 4, the expression of FN (Fig. 4A), COL-IV (Fig. 4B), and LN (Fig. 4C) were upregulated by TGF-β2 (5 ng/mL), which was reduced by the concomitant treatment with salidroside (30 μM).

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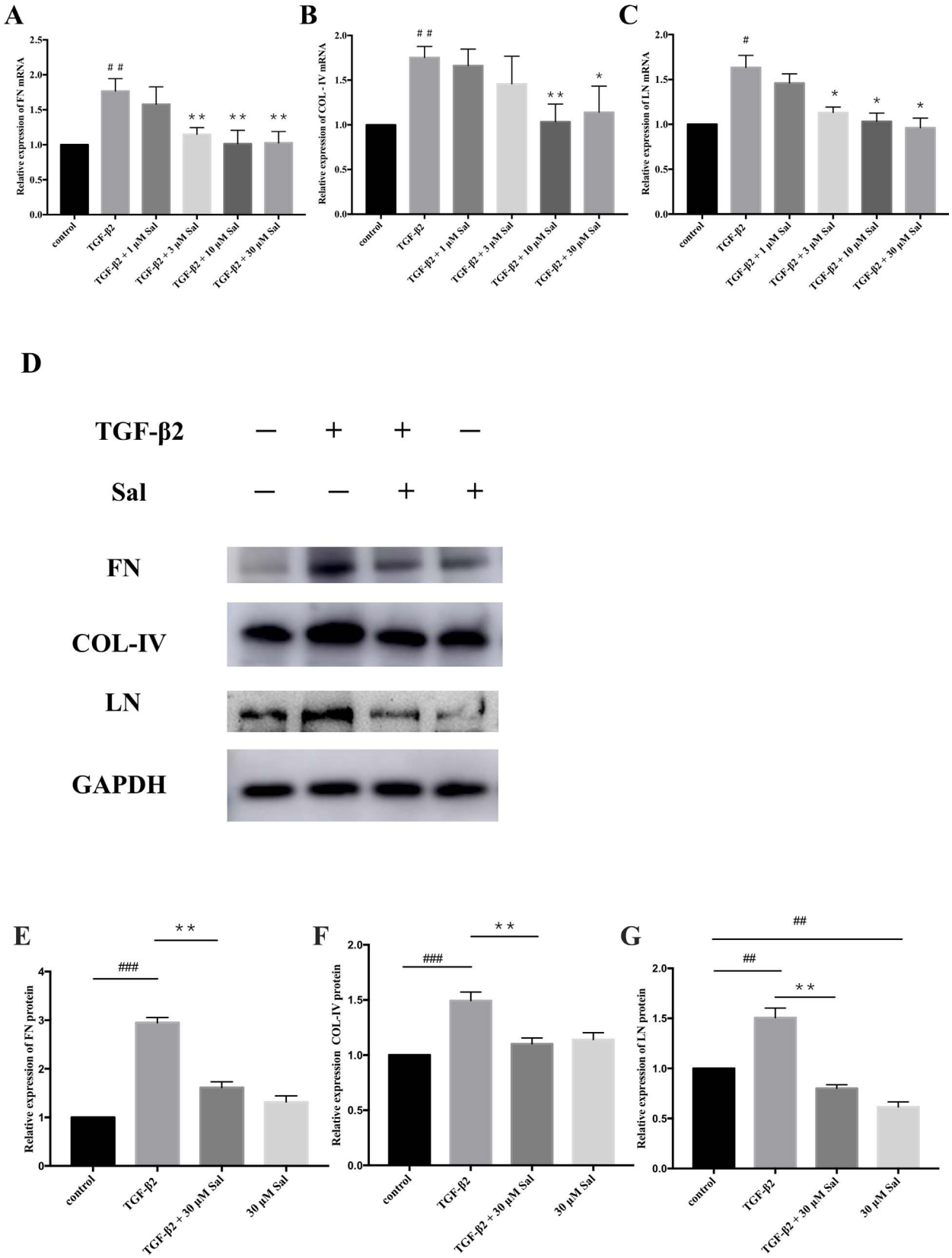

Figure 3. Effects of TGF-β2 and salidroside on the expression of ECM in HTM cells. Levels of mRNA of FN (**A**), COL-IV (**B**), and LN (**C**) were analyzed by RT-qPCR. Cells were treated with TGF-β2 (5 ng/mL) for 48 hours with or without salidroside (Sal; 1–30 μM) for 24 hours. GAPDH was used as normalizing control. Levels of the ECM proteins after TGF-β2 (5 ng/mL) for 48 hours and/or salidroside (30 μM) treatment for 24 hours were analyzed by Western blotting. A representative immunoblot is shown (**D**). Protein levels of FN (**E**), COL-IV (**F**), and LN (**G**) were quantified using GAPDH as a normalizing internal control. Data are presented as mean ± SEM ($n = 3$). #$P < 0.05$ compared with control, ##$P < 0.01$ compared with control, *$P < 0.05$ compared with TGF-β2 alone group; **$P < 0.01$ compared with TGF-β2 alone group.

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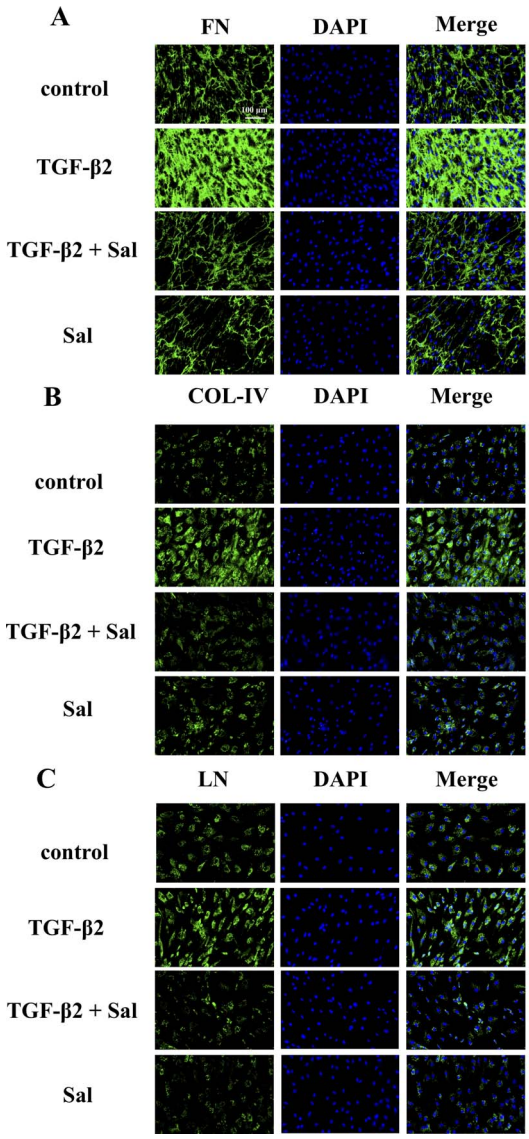

Figure 4. Effects of TGF-β2 and salidroside on ECM immunoreactivity in HTM cells. Cultured HTM cells were evaluated by immunocytochemistry of FN (**A**), COL-IV (**B**), and LN (**C**) after treatment with TGF-β2 (5 ng/mL) for 48 hours or salidroside for 24 hours (30 μM) or both. *Scale bar*: 100 μm. These studies were each repeated three times with similar results.

Salidroside Reduced Mouse Ocular Hypertension Induced by Ad.hTGF-β2$^{226/228}$

As previously reported,[24] intravitreal injection of Ad.hTGF-β2$^{226/228}$ in BALB/cJ mice induced ocular hypertension. Significantly elevated IOP occurred starting at day 3 after vector injection and reached a plateau from approximately days 11 to 60. The IOP at day 11 was 27.5 ± 2.0 mm Hg ($n = 6$) in the Ad.hTGF-β2$^{226/228}$–injected eyes, which was significantly higher ($P < 0.001$) than the noninjected eyes (13.8 ± 0.8 mm Hg) or the Ad.Empty-injected eyes (Fig. 5A). When the animals were also treated by daily intraperitoneal injections of salidroside (40 mg/kg) starting at day 0, the Ad.hTGF-β2$^{226/228}$–induced ocular hypertension was reduced. On day 11 after the injections of salidroside and Ad.hTGF-β2$^{226/228}$, IOP was 19.5 ± 0.7 mm Hg ($n = 10$), significantly ($P < 0.01$) lower than that of the Ad.hTGF-β2$^{226/228}$ group. This partial reduction of ocular hypertension lasted throughout the whole treatment

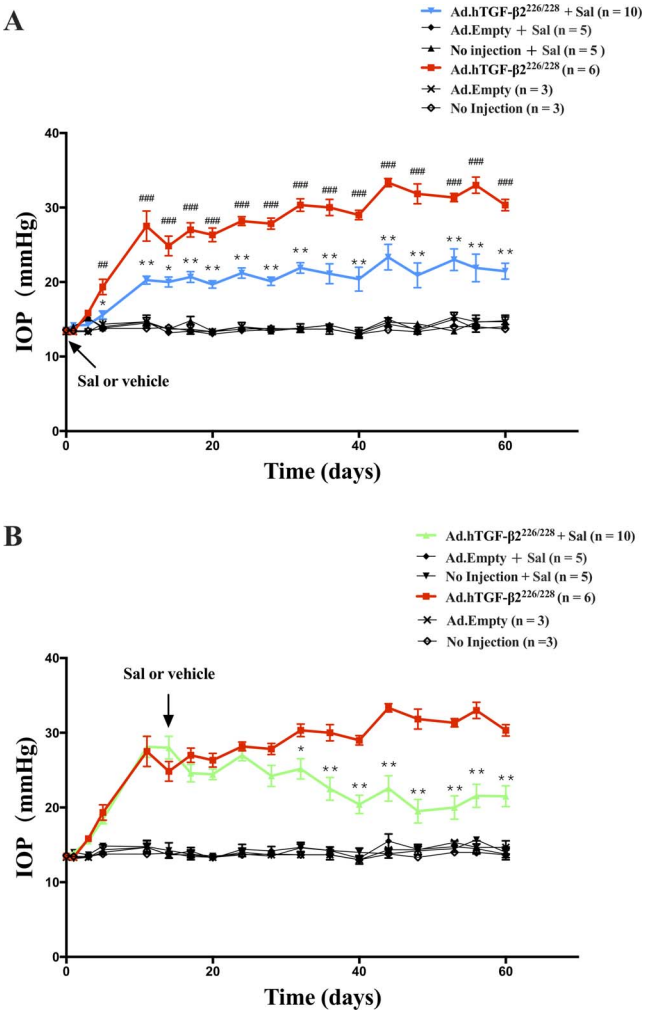

Figure 5. Effects of Ad.hTGF-β2$^{226/228}$ and salidroside on mouse IOP. One eye each of 32 mice was injected intravitreally with Ad.hTGF-β2$^{226/228}$ (6×10^7 pfu in 2 μL). The contralateral eye of half of the animals was injected with Ad.Empty (6×10^7 pfu in 2 μL) a few minutes later, and the contralateral eye of the remaining half was uninjected. (**A**) Starting at the day of Ad.hTGF-β2$^{226/228}$ treatment (day 0), animals received daily intraperitoneal injection of 40 mg/kg salidroside ($n = 10$) or vehicle ($n = 6$). (**B**) Starting at day 14 of Ad.hTGF-β2$^{226/228}$ treatment when ocular hypertension was established, daily intraperitoneal injection of 40 mg/kg salidroside ($n = 10$) or vehicle ($n = 6$) was initiated. Mouse IOP was measured between 2 PM and 4 PM, immediately before intraperitoneal injections. Data are presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with control. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with Ad.hTGF-β2$^{226/228}$ without salidroside by 1-way ANOVA and then LSD test. Sal, salidroside.

period. On day 60, IOP of the Ad.hTGF-β2$^{226/228}$ + salidroside group was 21.4 ± 1.1 mm Hg, which was significantly ($P < 0.01$) lower than that of the Ad.hTGF-β2$^{226/228}$ group (30.3 ± 0.7 mm Hg) (Fig. 5A). Salidroside did not affect IOP of the control eye. No systemic or ocular adverse effect was observed in this study.

In addition to the above study in which salidroside was administered at day 0, which was before the onset of ocular hypertension, we also tested the drug effect on IOP after Ad.hTGF-β2$^{226/228}$–induced ocular hypertension was established. In this experiment, daily intraperitoneal injection of salidroside (40 mg/kg) was initiated on day 14 after Ad.hTGF-β2$^{226/228}$ injection. On day 32, IOP of the Ad.hTGF-β2$^{226/228}$

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group was 30.3 ± 0.8 mm Hg ($n = 6$), significantly ($P < 0.001$) higher than IOP of the control group of the same day. Salidroside partially but significantly ($P < 0.05$) lowered the IOP effect of Ad.hTGF-β2$^{226/228}$ to 25.2 ± 1.4 mm Hg ($n = 10$). Again on day 60, IOP of the Ad.hTGF-β2$^{226/228}$ + salidroside group was 21.5 ± 1.4 mm Hg, which was significantly ($P < 0.01$) lower than that of the Ad.hTGF-β2$^{226/228}$ group (30.3 ± 0.7 mm Hg) (Fig. 5B). Again, no systemic or ocular adverse effect was observed.

These results indicate that systemic treatment of the mouse with salidroside could partially prevent the ocular hypertension caused by the Ad.hTGF-β2$^{226/228}$. More importantly, salidroside could also partially reverse the elevated IOP even after it was established 14 days after viral vector injection. The prolonged effect of salidroside lasting the whole study period of 60 days suggests that no tachyphylaxis was involved.

Effects of Salidroside Treatment on FN, COL-IV, and LN in the TM Tissue of the Mouse

To assess if the IOP effects of Ad.hTGF-β2$^{226/228}$ and salidroside were associated with changes in ECM proteins in the mouse TM, we evaluated mRNA levels of FN, COL-IV, and LN in sclera rings containing the TM of treated mice at day 60 after viral vector injection. As shown in Figure 6, Ad.hTGF-β2$^{226/228}$ significantly ($P < 0.05$) increased FN mRNA to 5.1 ± 0.9-fold ($n = 4$), COL-IV to 5.0 ± 0.8-fold ($n = 4$), and LN to 8.0 ± 1.4-fold ($n = 4$). These stimulatory effects were partially and significantly ($P < 0.05$) reduced by salidroside treatments, either initiated at day 0 (FN: 1.89 ± 0.4-fold of control, $n = 3$; COL-IV: 1.93 ± 0.1-fold, $n = 3$; LN: 3.44 ± 0.8-fold, $n = 3$) or day 14 (FN: 2.24 ± 0.5-fold, $n = 3$; COL-IV: 1.70 ± 0.3-fold, $n = 3$; LN: 3.12 ± 0.6-fold, $n = 3$).

The changes in mRNA levels were corroborated by immunohistochemistry. As shown in Figure 7 and Supplementary Figure S1, intravitreal injection Ad.hTGF-β2$^{226/228}$ appeared to increase the immunoreactivities of FN, COL-IV, and LN, which was partially reduced by salidroside treatment initiated at either day 0 or day 14. Hematoxylin and eosin staining revealed no apparent ocular abnormalities in every group mouse (Supplementary Fig. S2).

Discussion

A major risk factor for the development and progression of POAG is elevated IOP. Elevated IOP is primarily due to an increased aqueous humor outflow resistance within the juxtacanalicular region (JCT) of the TM,[29] associated with local biochemical changes. In POAG, the excessive accumulation of ECM/fibrotic proteins in the JCT is thought to reduce outflow facility and increase IOP.[30–32] The importance of TGF-β signaling pathway in the regulation of ECM proteins in the glaucomatous TM and the effect on IOP have been previously reported.[3,4,32–35]

In the current study, we observed that in the cultured HTM cells, treatment with 5 ng/mL TGF-β2 increased the expression of FN, COL-IV, and LN significantly, findings consistent with previous reports that the cytokine has profibrotic effects and increases ECM accumulation in the TM.[32] Most importantly, we further showed that salidroside reversed these effects of TGF-β2, which suggest that salidroside may interfere with the ocular hypertensive effect of TGF-β2.

To evaluate this hypothesis, we injected intravitreally an adenoviral vector encoding the bioactive version of TGF-β2, Ad.hTGF-β2$^{226/228}$, to induce ocular hypertension in the mouse. Elevation of mouse IOP was detected 3 days after vector injection and reached a plateau of 25 to 30 mm Hg, significantly higher than the baseline IOP value, at approximately 11 days. These results corroborate previously published information.[15] Moreover, when the mice were also treated with daily intraperitoneal injections of salidroside starting at the same day as Ad.hTGF-β2$^{226/228}$, the vector-induced elevation of IOP was partially and significantly reduced, albeit it was still significantly higher than the baseline IOP. These findings implicate that salidroside can prevent the hypertensive effects of TGF-β2.

In addition to prevention, we further demonstrated that salidroside can reverse the TGF-β2 effect even after IOP elevation was established and stabilized. As shown in Figure 5, administration of salidroside starting at 14 d after viral vector injection was also efficacious in mitigating the ocular hypertension induced by TGF-β2. The IOP effects of TGF-β2 and salidroside were associated with their effects on FN, COL-IV, and LN expressions and accumulations in the mouse TM as examined by postmortem assessments by RT-qPCR and immunohistochemistry (Figs. 6, 7). These results show that salidroside not only can prevent but also can reverse the TGF-β2–induced IOP increase as well as imply that this effect is likely mediated by affecting ECM accumulation in the TM, subsequently outflow facility. Nonetheless, it is interesting to note that the effect of salidroside was only partial; it did not bring the elevated IOP completely back to the baseline value. At this time, we do not know the underlying reason for the partial efficacy. Some of the reasons may involve suboptimal dose or suboptimal dosing frequency or both, which will be investigated in future studies.

At this time, the molecular mechanism of the biological functions of salidroside in the TM cells is yet to be elucidated. In other tissues, such as lung, kidney, and liver, salidroside was shown to interfere with the TGF-β/Smad signaling pathways. For example, it can lower the serum concentration of TGF-β1, decrease the mRNA and protein expressions of Smad4, increase both mRNA and protein levels of Smad7, reduce mRNA of the downstream effector CBP, and eventually suppress the expression of COL-1A2 and other ECM molecules.[18,19,21–23,36]

It is generally believed that TGF-β2 is an important contributor to the pathogenesis of glaucoma. Some of the key evidence includes the following: in the aqueous humor of glaucoma patients, TGF-β2 level is higher than in nonglaucomatous eyes; perfusion of TGF-β2 increases IOP by lowering outflow facility in ex vivo human ocular organ culture; and overexpression of TGF-β2 in the rodent eye produces ocular hypertension, again by lowering TM outflow. In the TM cells, TGF-β2 was shown to activate cytoplasmic Smad2/3, which leads to increased expression of ECM molecules, such as FN, COL-IV, and LN, which in turn leads to fibrotic changes in the tissue. These changes can explain the TGF-β2–induced impediment of aqueous humor outflow through the TM and increase in IOP.[35] Currently, we hypothesize that salidroside may mitigate some of these TGF-β2 effects by interfering the TGF-β2/Smad pathway. It should then reduce accumulation of ECM in the TM and reverse the cytokine-mediated ocular hypertension. Future investigations are definitely needed to answer this intriguing question.

It is also interesting to note that *R. rosea* has been used for centuries in traditional Chinese herbal medicines, as well as in Russia and Scandinavia. It is administered orally by concoction or pharmaceutical formulations. Systemic administration may not be optimal for the treatment of glaucoma. We are currently evaluating ocular dosage forms in order to improve its distribution and therapeutic efficacy in the eye.

In summary, this study demonstrated that salidroside plays a role in the production and regulation of the ECM in the TM.

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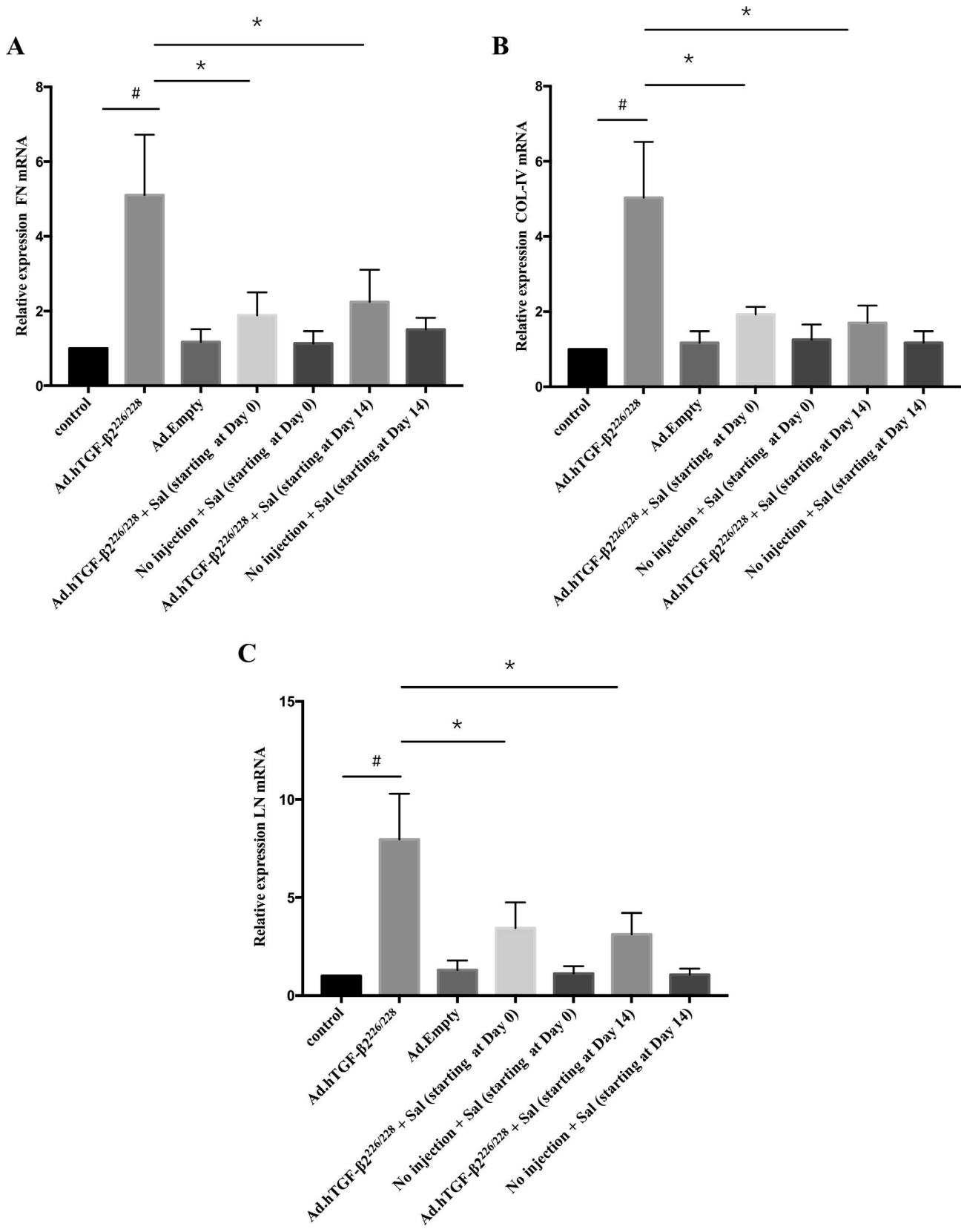

Figure 6. Effects of Ad.hTGF-$\beta 2^{226/228}$ and salidroside on ECM expressions in mouse TM. Expressions of ECM, including FN (**A**), COL-IV (**B**), and LN (**C**) were analyzed by RT-qPCR. β-Action was used as internal control. Data are presented as mean ± SEM. $^{\#}P < 0.05$ compared with control, $^{*}P < 0.05$ compared with Ad.hTGF-$\beta 2^{226/228}$-alone group.

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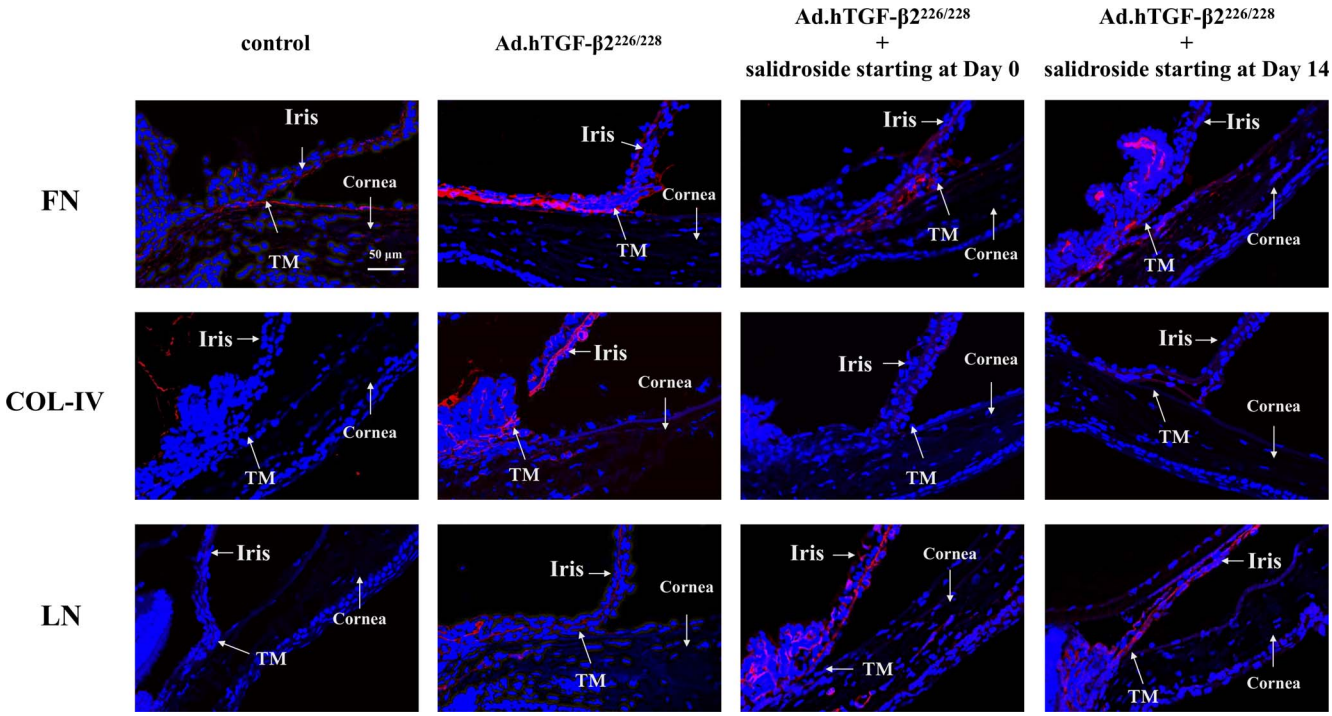

FIGURE 7. Effects of Ad.hTGF-β2$^{226/228}$ and salidroside on FN, COL-IV, and LN expressions in mouse anterior segment. Ad.hTGF-β2$^{226/228}$ (6×10^7 pfu) was intravitreally injected in one eye of each animal and the contralateral uninjected eye served as negative control. Daily intraperitoneal injection of 40 mg/kg salidroside was initiated at day 0 or day 14 relative to Ad.hTGF-β2$^{226/228}$ treatment. Eyes were harvested at day 60 and analyzed for FN, COL-IV, and LN expressions by immunohistochemistry. Blue fluorescence represents DAPI staining. Red fluorescence represents FN (*top row*), COL-IV (*middle row*), or LN (*bottom row*) immunoreactivity.

These data illustrate a potential novel drug that can reduce IOP by inhibiting the profibrotic effects of TGF-β2 in the TM.

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References

1. The AGIS Investigators. The Advanced Glaucoma Intervention Study (AGIS): 7. The relationship between control of intraocular pressure and visual field deterioration. *Am J Ophthalmol*. 2000;130:429–440.
2. Quigley HA. Glaucoma. *Lancet*. 2011;377:1367–1377.
3. Min SH, Lee TI, Chung YS, Kim HK. Transforming growth factor-beta levels in human aqueous humor of glaucomatous, diabetic and uveitic eyes. *Korean J Ophthalmol*. 2006;20:162–165.
4. Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol*. 2002;46:249–253.
5. Picht G, Welge-Luessen U, Grehn F, Lütjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch Clin Exp Ophthalmol*. 2001;239:199–207.
6. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res*. 1994;59:723–727.
7. Yamamoto N, Itonaga K, Marunouchi T, Majima K. Concentration of transforming growth factor beta 2 in aqueous humor. *Ophthalmic Res*. 2005;37:29–33.
8. Koliakos GG, Schlotzer-Schrehardt U, Konstas AG, Bufidis T, Georgiadis N, Dimitriadou A. Transforming and insulin-like growth factors in the aqueous humour of patients with exfoliation syndrome. *Graefes Arch Clin Exp Ophthalmol*. 2001;239:482–487.
9. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol*. 2004;25:19–22.
10. Medina-Ortiz WE, Belmares R, Neubauer S, Wordinger RJ, Clark AF. Cellular fibronectin expression in human trabecular meshwork and induction by transforming growth factor-β2. *Invest Ophthalmol Vis Sci*. 2013;54:6779–6788.
11. Vranka JA, Kelley MJ, Acott TS, Keller KE. Extracellular matrix in the trabecular meshwork: intraocular pressure regulation and dysregulation in glaucoma. *Exp Eye Res*. 2015;133:112–125.
12. Qu ZQ, Zhou Y, Zeng YS, Li Y, Chung P. Pretreatment with *Rhodiola rosea* extract reduces cognitive impairment induced by intracerebroventricular streptozotocin in rats: implication of anti-oxidative and neuroprotective effects. *Biomed Environ Sci*. 2009;22:318–326.
13. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGF-beta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci*. 2006;47:226–234.
14. Wordinger RJ, Fleenor DL, Hellberg PE, et al. Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: implications for glaucoma. *Invest Ophthalmol Vis Sci*. 2007;48:1191–1200.
15. Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming

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growth factor-beta2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci*. 2010;51:2067–2076.

16. Qu ZQ, Zhou Y, Zeng YS, et al. Protective effects of a *Rhodiola crenulata* extract and salidroside on hippocampal neurogenesis against streptozotocin-induced neural injury in the rat. *PLoS One*. 2012;7:e29641.
17. Zheng K, Sheng Z, Li Y, Lu H. Salidroside inhibits oxygen glucose deprivation (OGD)/re-oxygenation-induced H9c2 cell necrosis through activating of Akt-Nrf2 signaling. *Biochem Biophys Res Commun*. 2014;451:79–85.
18. Guan S, Xiong Y, Song B, Song Y, Wang D, Chu X. Protective effects of salidroside from *Rhodiola rosea* on LPS-induced acute lung injury in mice. *Immunopharmacol Immunotoxicol*. 2012;34:667–672.
19. Zhang Z, Ding L, Wu L, Xu L, Zheng L, Huang X. Salidroside alleviates paraquat-induced rat acute lung injury by repressing TGF-beta1 expression. *Int J Clin Exp Pathol*. 2014;7:8841–8847
20. Huang X, Zou L, Yu X, et al. Salidroside attenuates chronic hypoxia-induced pulmonary hypertension via adenosine A2a receptor related mitochondria-dependent apoptosis pathway. *J Mol Cell Cardiol*. 2015;82:153–166.
21. Tang H, Gao L, Mao J, He H, Liu J, Cai X. Salidroside protects against bleomycin-induced pulmonary fibrosis: activation of Nrf2-antioxidant signaling, and inhibition of NF-κB and TGF-β1/Smad-2/-3 pathways. *Cell Stress Chaperones*. 2015;21:239–249.
22. Feng J, Chen K, Xia Y, et al. Salidroside ameliorates autophagy and activation of hepatic stellate cells in mice via NF-κB and TGF-β1/Smad3 pathways. *Drug Des Devel Ther*. 2018;12:1837–1853.
23. Ouyang J, Gao Z, Ren Z, Hong D, Qiao H, Chen Y. Synergistic effects of rMSCs and salidroside on the experimental hepatic fibrosis. *Pharmazie*. 2010;65:607–613
24. Keller KE, Bhattacharya SK, Borrás T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. *Exp Eye Res*. 2018;171:164–173
25. McDowell CM, Hernandez H, Mao W, Clark AF. Gremlin induces ocular hypertension in mice through Smad3-dependent signaling. *Invest Ophthalmol Vis Sci*. 2015;56:5485–5492.
26. McDowell CM, Tebow HE, Wordinger RJ, Clark AF. Smad3 is necessary for transforming growth factor-beta2 induced ocular hypertension in mice. *Exp Eye Res*. 2013;116:419–423.
27. Chen T, Ma Z, Zhu L, Jiang W, Wei T, Zhou R. Suppressing Receptor-Interacting Protein 140: a new sight for salidroside to treat cerebral ischemia. *Mol Neurobiol*. 2015;53:1–11.
28. Wang WH, Millar JC, Pang IH, Wax MB, Clark AF. Noninvasive measurement of rodent intraocular pressure with a rebound tonometer. *Invest Ophthalmol Vis Sci*. 2005;46:4617–4621.
29. Rohen JW, Lutjen-Drecoll E, Flugel C, Meyer M, Grierson I. Ultrastructure of the trabecular meshwork in untreated cases of primary open-angle glaucoma (POAG). *Exp Eye Res*. 1993;56:683–692.
30. Johnson D, Gottanka J, Flugel C, Hoffmann F, Futa R, Lutjen-Drecoll E. Ultrastructural changes in the trabecular meshwork of human eyes treated with corticosteroids. *Arch Ophthalmol*. 1997;115:375–383.
31. Clark AF, Wilson K, de Kater AW, Allingham RR, McCartney MD. Dexamethasone-induced ocular hypertension in perfusion-cultured human eyes. *Invest Ophthalmol Vis Sci*. 1995;36:478–489.
32. Acott TS, Kelley MJ. Extracellular matrix in the trabecular meshwork. *Exp Eye Res*. 2008;864:543–561.
33. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol*. 2001;239:109–113.
34. Schlotzer-Schrehardt U, Zenkel M, Kuchle M, Sakai LY, Naumann GO. Role of transforming growth factor-beta1 and its latent form binding protein in pseudoexfoliation syndrome. *Exp Eye Res*. 2001;73:765–780.
35. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res*. 1994;59:723–727.
36. Wei Z, Ming P, Yang Y, Xiao Z, Song B, Lin Z. Protective effects of salidroside on mitochondrial functions against exertional heat stroke-induced organ damage in the rat. *Evid Based Complement Alt Med*. 2015;2015:504567.

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