

# Osthole Reduces Mouse IOP Associated With Ameliorating Extracellular Matrix Expression of Trabecular Meshwork Cell

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**PURPOSE.** Elevation of IOP in POAG is thought to involve excessive accumulation of extracellular matrix in the trabecular meshwork (TM), leading to an increase in outflow resistance of the aqueous humor. Osthole, a coumarin derivative extracted from the fruit of a variety of plants, such as *Cnidium monnieri*, is reported to prevent profibrotic responses by inhibiting Smad signaling pathway activated by TGF- $\beta$  in liver, kidney, and cardiac tissues. We tested if osthole can (1) inhibit TGF- $\beta$ 2-induced extracellular matrix expression in cultured human TM (HTM) cells, and (2) lower TGF- $\beta$ 2-induced ocular hypertension in the mouse.

**METHODS.** Cultured HTM cells were treated with 5 ng/mL TGF- $\beta$ 2 for 48 hours, then with osthole for 24 hours. The expressions of fibronectin, collagen type IV, and laminin were assessed by quantitative PCR, Western blot, and immunocytochemistry. BALB/cJ mice were injected intravitreally with an adenoviral vector encoding a bioactive mutant of TGF- $\beta$ 2 (Ad.hTGF- $\beta$ 2<sup>226/228</sup>) in one eye to induce ocular hypertension, with the uninjected contralateral or Ad.Empty-injected eye serving as controls. Mice were then treated with a daily intraperitoneal injection of 30 mg/kg osthole. Conscious mouse IOP values were measured using a TonoLab rebound tonometer.

**RESULTS.** In cultured HTM cells, stimulation with TGF- $\beta$ 2 increased expressions of fibronectin, collagen IV, and laminin. These in vitro changes were significantly and completely mitigated by osthole (10  $\mu$ M). Daily intraperitoneal injections of 30 mg/kg osthole, starting either at day 0 (same day as Ad.hTGF- $\beta$ 2226/228 injection) or at day 14, significantly decreased TGF- $\beta$ 2-induced ocular hypertension in the mouse. In contrast, osthole did not affect IOP of control eyes.

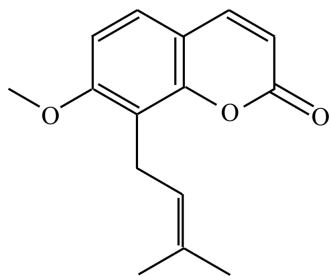
**CONCLUSIONS.** These results demonstrated that osthole is capable of reducing TGF- $\beta$ 2-induced extracellular matrix expression in cultured HTM cells. It also reduced TGF- $\beta$ 2-induced ocular hypertension in the mouse. These findings indicate that this natural product may be useful as a novel treatment for POAG.

**Keywords:** osthole, TGF- $\beta$ 2, trabecular meshwork, extracellular matrix, glaucoma, intraocular pressure

Glaucoma is the second leading cause of blindness around the world. Elevated IOP is the major risk factor of the development and progression in all glaucoma subtypes.<sup>1-3</sup> In patients with POAG, IOP elevation is primarily due to a decrease in aqueous humor outflow facility. The abnormal accumulation of extracellular matrix (ECM) in the trabecular meshwork (TM) is thought to play an important role in the pathogenesis of POAG.<sup>4,5</sup> This phenomenon will lead to increased AH outflow resistance, and elevated IOP.<sup>6,7</sup> The primary biological cause of ECM accumulation in the TM is not known. However, TGF- $\beta$ 2 is hypothesized to be a significant player. The level of TGF- $\beta$ 2 has been reported

to be elevated in the aqueous humor and TM of patients with POAG.<sup>8-12</sup> Furthermore, TGF- $\beta$ 2 has also been shown to increase ECM accumulation in the TM, and most importantly, to cause elevated IOP in both ex vivo human anterior segment perfusion<sup>13</sup> and in mouse eyes.<sup>14</sup>

At present, many therapeutic agents are effective in reducing IOP, but none are designed to address the underlying pathogenesis mechanisms, such as ECM accumulation, that lead to IOP elevation. Consequently, currently available glaucoma treatments decrease the IOP, but have no effect on the cause of the pathogenesis of POAG. They do not stop the disease progression. Thus,



**FIGURE 1.** Chemical structure of osthole ( $C_{15}H_{16}O_3$ ; formula weight = 244.29).

improvement in ECM accumulation and fibrotic changes in the TM may serve as a conceptually novel and promising direction that mitigate the disease progression for the treatment of glaucoma.<sup>15</sup>

Osthole (Fig. 1) is a coumarin derivative, which can be extracted from the fruit of *Cnidium monnieri*, *Angelica archangelica*, or *Angelica pubescens*. It has been shown to have various therapeutic effects and a good safety profile compared with other natural products, which makes it a promising compound for drug discovery. Importantly, osthole seems to interact with TGF- $\beta$  and has antifibrotic actions in many tissues. For example, Hou et al.<sup>22</sup> demonstrated that osthole inhibits the growth of hypertrophic scar fibroblasts through apoptosis and decrease in the expression of TGF- $\beta$ 1. Liu et al.<sup>23</sup> reported that osthole inhibits the TGF- $\beta$ 1 and Smad signaling pathway-stimulated expressions of collagen I and III in mouse cardiac fibroblasts. Chen et al.<sup>24</sup> showed that osthole reduces the level of TGF- $\beta$ 1 expression via the activation of peroxisome proliferator activated receptor  $\alpha/\gamma$  and subsequent inhibition of nuclear factor- $\kappa$ B in myocardial tissues to prevent isoprenaline-induced myocardial fibrosis in mice. Zhang et al.<sup>25</sup> showed that osthole prevents fibroblast activation and epithelial-mesenchymal transition to hinder renal fibrosis in UUO mouse. Liu et al.<sup>26</sup> showed that osthole alleviates TGF- $\beta$ 1 to improve the histologic architecture and decrease collagen and  $\alpha$ -smooth muscle actin accumulation, and improves hepatic fibrosis scores.

Based on these intriguing findings of the antifibrotic effects of osthole, we hypothesized that osthole may improve the fibrotic changes of the TM by reducing ECM accumulation, and consequently lower IOP. In the present studies, we found that indeed osthole inhibited TGF- $\beta$ 2-induced ECM expression, as represented by fibronectin (FN), collagen IV (COL-IV), and laminin (LN), in cultured human TM (HTM) cells, and lowered TGF- $\beta$ 2-induced ocular hypertension in the mouse.

## METHODS

### Cell Culture and Treatment

Primary HTM cells were isolated from fresh human volunteer donor eyes and characterized as previously described.<sup>27</sup> The cells were cultured at 37°C with 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Gibco, Halethorpe, MD) supplemented with glutamine, penicillin/streptomycin, and 10% fetal bovine serum (Invitrogen-Gibco, Carlsbad, CA).

Cells of passages 4 to 8 were used in the present study. For cell proliferation assay, cells were treated with osthole (purity = 99.4%, MedChemExpress, Monmouth Junction, NJ;

dissolved in dimethylsulfoxide, then diluted 1000 $\times$  with culture medium) at the indicated concentrations for 1 or 4 days. For RT-qPCR, Western blot, and immunocytochemistry studies, cells were treated with or without recombinant human TGF- $\beta$ 2 (5 ng/mL; R&D Systems, Minneapolis, MN) for 48 hours, followed by osthole treatment for 24 hours. During the TGF- $\beta$ 2 induction period, cell medium was replaced daily with fresh TGF- $\beta$ 2. All experiments were performed at least three separate times.

### Cell Proliferation Assay

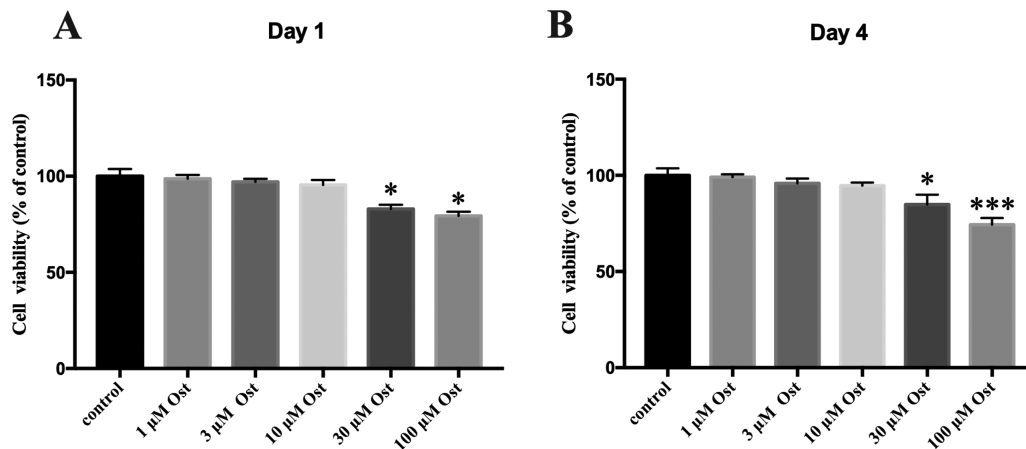
HTM cells were seeded at a density of  $5 \times 10^3$  cells/well in a 96-well plate with 100  $\mu$ L culture medium. At 1 day and 4 days after seeding, the culture medium was replaced by a culture medium containing 10  $\mu$ L Cell Counting Kit-8 reagent (MedChemExpress, Monmouth Junction, NJ). Optical density at A450 was measured. Cell proliferation was expressed as percentage of that of the vehicle control group.

### RT-qPCR

RNA isolation was performed with the RNeasy mini kit (Qiagen, Inc., Valencia, CA), 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: FN (forward: 5'-AAGACCAGCAGAGGCATAAGG-3'; reverse: 5'-TGTAGGGGTCAAAGCACGAG-3'), COL-IV (forward: 5'-TAGAGAGGAGCGAGATGTTCC-3'; reverse: 5'-GTGACATTAGCTGAGTCAGG-3'), LN (forward: 5'-AGCACTGTGGAGCAGTCAGC-3'; reverse: 5'-TTCGCTGCCAGTTCATCAAG-3'), GAPDH (forward: 5'-CGAGATCCCTCCAAAATCAA-3'; reverse: 5'-GTCTTCTGGTGCCAGTGAT-3').

### Protein Extraction and Western Blot Analysis

HTM cells were washed with prechilled PBS and harvested on ice. Total protein was extracted using a lysis buffer (pH 6.8, CWBIO, Beijing, China) supplemented with 5%  $\beta$ -mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (CWBIO). Western blots were performed as previously described.<sup>27</sup> Briefly, 20  $\mu$ g protein samples were denatured at 100°C for 10 minutes, separated on an 8% or 12% sodium dodecyl sulfate polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was washed, blocked with 5% nonfat milk for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibodies, including rabbit polyclonal FN antibody (1:1000; catalog #ab2413; Abcam, Cambridge, MA), rabbit polyclonal COL-IV antibody (1:1000; catalog #ab6586; Abcam), rabbit polyclonal LN antibody (1:1000; catalog #ab11575; Abcam), and rabbit polyclonal GAPDH antibody (1:2000; catalog #ab9485; Abcam). On the next day, the membrane was washed and incubated with corresponding horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; catalog #14708; Cell Signaling Technology, Danvers, MA) for 2 hours at room temperature. Following washes, the membrane was treated with enhanced chemiluminescence plus reagents (Millipore, Billerica, MA), then washed several



**FIGURE 2.** Effects of osthole on HTM cell survival. Different concentrations (1, 3, 10, 30, and 100  $\mu\text{M}$ ) of osthole was added to the HTM cells. At 1 day or 4 days after osthole treatment, cell viability was measured by CCK8 assay. Data are presented as mean  $\pm$  SEM ( $n = 5$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with controls. The mean value of control samples defines 100%. Ost, osthole.

times and scanned by hypersensitive chemiluminescence analyzer (Amersham Imager600; GE, Pittsburgh, PA). The protein density of every band was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD).

### Immunocytochemistry

HTM cells were seeded on coverslips in a 24-well plate with a density of  $8 \times 10^4$  cells/well. The cells were washed with PBS twice, fixed with 4% paraformaldehyde for 30 minutes at  $4^\circ\text{C}$ , permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 30 minutes, and blocked with 5% goat serum (Life Technologies, Grand Island, NY) for 2 hours at room temperature. The cells were then incubated overnight at  $4^\circ\text{C}$  with primary antibodies, including rabbit polyclonal FN antibody (1:200; Abcam), rabbit polyclonal COL-IV antibody (1:100; Abcam), and rabbit polyclonal LN antibody (1:200; Abcam). Cells were washed and incubated with Alexa 488-conjugated secondary antibodies (1:200; Invitrogen-Gibco) for 2 hours at room temperature. Afterward, the coverslips were mounted with ProLong Gold Antifade with DAPI (Life Technologies). Cell images were captured by the CellSens Standard electronic system (Olympus Optical Co. Ltd., Tokyo, Japan) under a fluorescence microscope (BX51, Olympus Optical Co. Ltd., Tokyo, Japan) using identical optical parameters.

### Animals

BALB/cj mice used in this study were obtained from Charles River Lab (Beijing, China). All mice were male and 3 to 4 months old at the start of the experiments, and 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.

All experiments were conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and the Shanghai Jiaotong University Animal Care and Use Committee guidelines.

### In Vivo Treatments

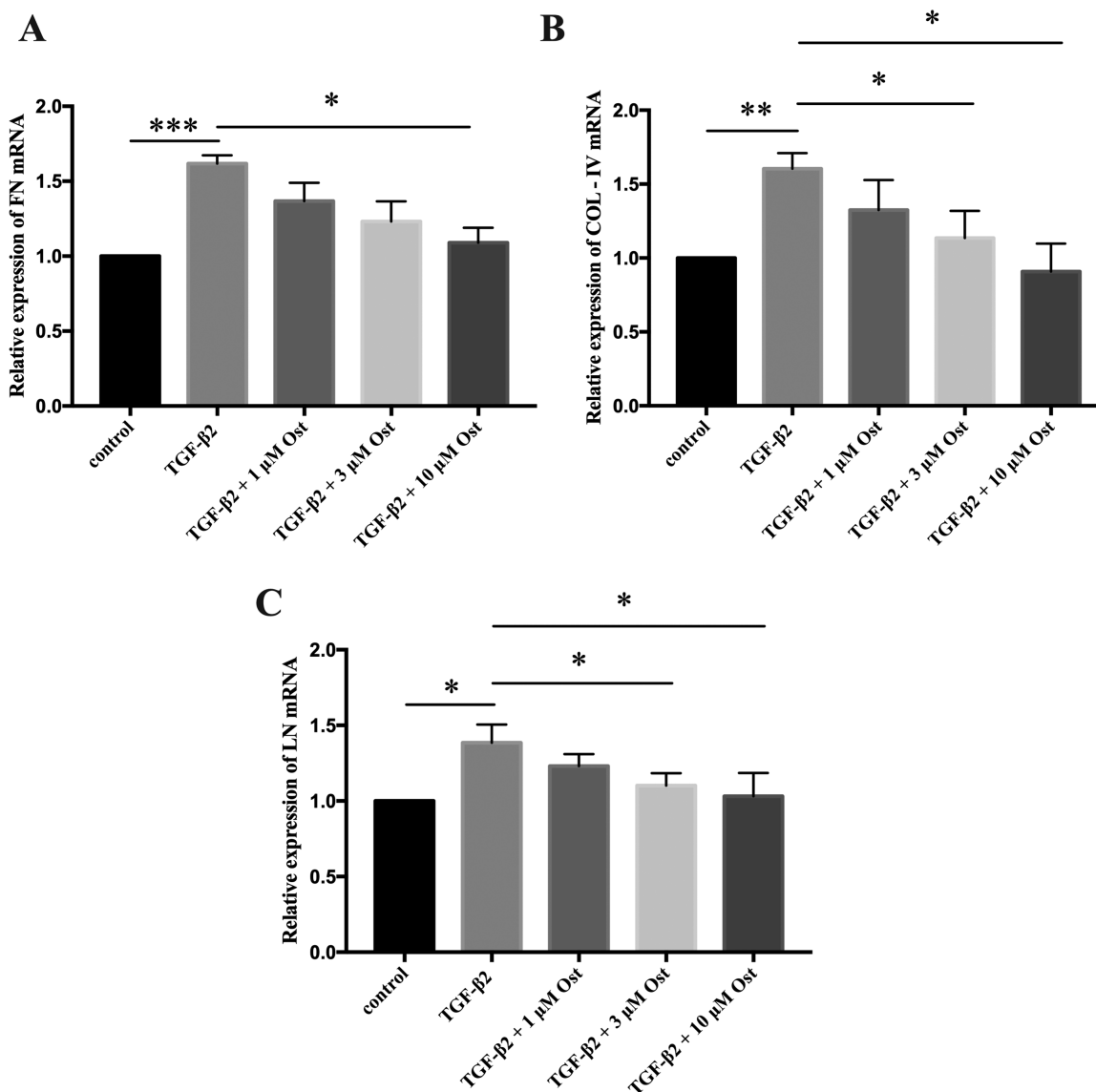
To induce ocular hypertension, one eye of each mouse was injected intravitreally with an adenoviral vector encoding a bioactive mutant of human TGF- $\beta 2$  (Ad.hTGF- $\beta 2^{226/228}$ ).<sup>14</sup> At the day of vector injection (day 0), mice were anesthetized by intraperitoneal injection of 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 (Mydracil; Alcon, Fort Worth, TX) to dilate the pupil, then topically anesthetized with 1 or 2 drops of 0.5% proparacaine (Alcaine; Alcon). A suspension of Ad.hTGF- $\beta 2^{226/228}$  ( $6 \times 10^7$  pfu) in a volume of 2  $\mu\text{L}$  was injected intravitreally using a Hamilton (Reno, NV) glass micro-syringe fitted with a 1-inch, 33G needle with a  $10^\circ$  bevel, as described previously.<sup>14</sup> 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 a noninjected control. An additional control group was injected intravitreally with a null adenoviral vector (Ad.Empty;  $6 \times 10^7$  pfu in 2  $\mu\text{L}$ ) in one eye.

The Ad.hTGF- $\beta 2^{226/228}$ -injected animals were further divided into three groups: (1) no osthole treatment, (2) osthole (30 mg/kg, intraperitoneal injection, daily) starting at day 0 (same day as Ad.hTGF- $\beta 2^{226/228}$  injection), and (3) Osthole (30 mg/kg, intraperitoneal injection daily) starting at day 14. The dose of 30 mg/kg was selected based a previous report,<sup>28</sup> and was confirmed efficacious in a pilot study.

All IOP measurements were conducted between 2 PM and 4 PM, right before Osthole treatment, using the TonoLab rebound tonometer (Icare, Finland), as described.<sup>29</sup> The researcher who measured IOP was masked regarding identities of study groups.

### Quantitative PCR and Western Blot Analysis

To detect RNA and protein changes at the peak of the IOP increase, eyes were harvested at day 60 after injection. Whole eyes from each group of mice were collected for qPCR analysis. The sclera rings were carefully dissected



**FIGURE 3.** Effects of TGF- $\beta$ 2 and osthole on the expression of ECM in HTM cells. Cells were treated with TGF- $\beta$ 2 (5 ng/mL) for 48 hours, followed by vehicle or osthole (Ost; 1–10  $\mu$ M) for 24 hours. Levels of mRNA of FN (A), COL-IV (B), and LN (C) were analyzed by RT-qPCR. GAPDH was used as normalizing control. Data are presented as mean  $\pm$  SEM ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with TGF- $\beta$ 2 alone group. Ost, osthole.

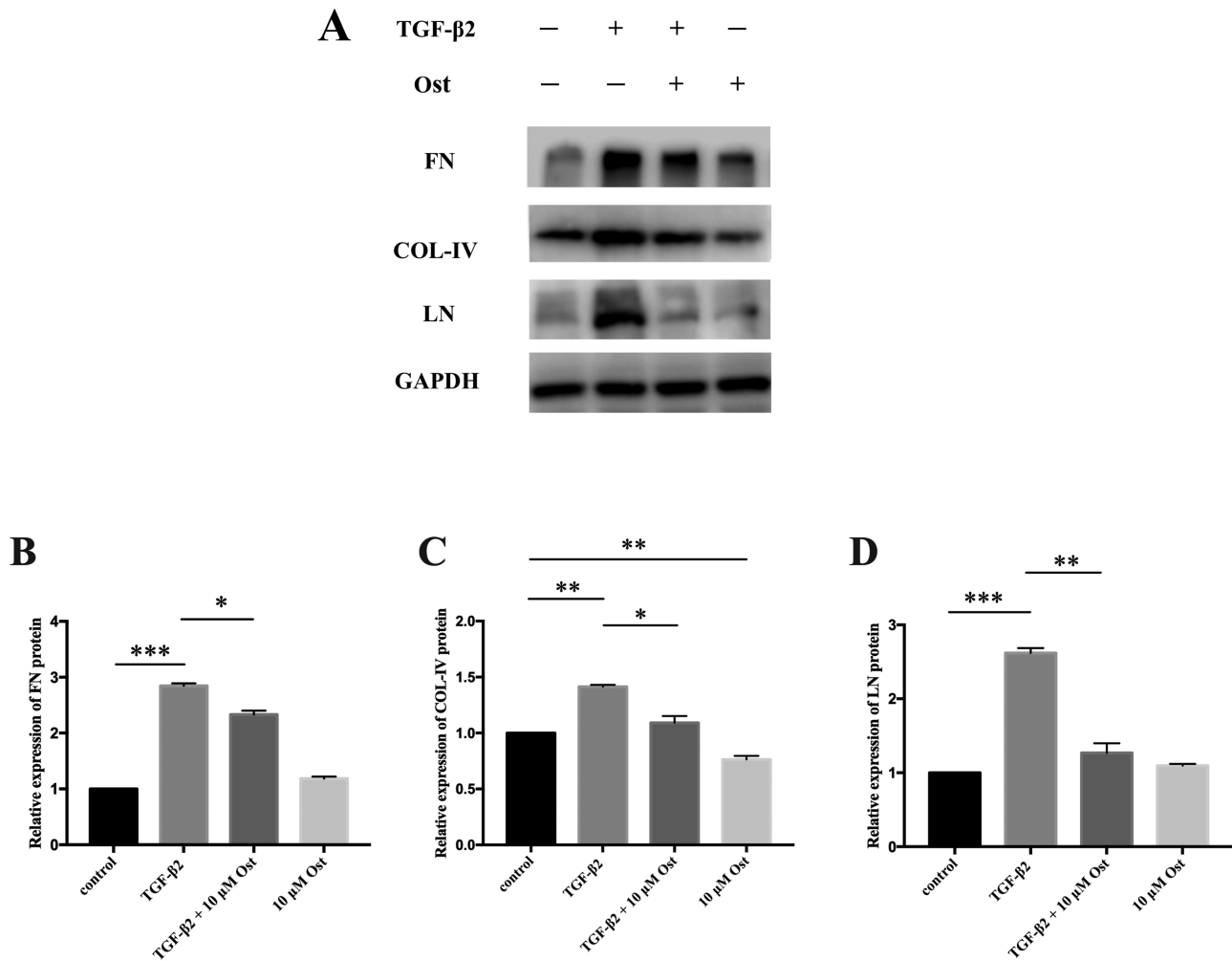
from the whole eye. Sclera rings contained mainly TM tissue and a small amount of sclera and cornea. Great effort was made to dissect away as much of the sclera and cornea as possible. Samples were homogenized and RNA extracted in Isol-RNA Lysis Reagent (5PRIME, Gaithersburg, MD) and reverse-transcribed to cDNA (Bio-Rad iScript cDNA synthesis Kit; Bio-Rad, Hercules, CA). Each PCR reaction contained 10  $\mu$ L 2 $\times$  iQ SYBR Green Supermix (Bio-Rad), 0.25  $\mu$ L forward primer (100  $\mu$ M), 0.25  $\mu$ L reverse primer (100  $\mu$ M), 8.5  $\mu$ L dH<sub>2</sub>O, and 1.0  $\mu$ L cDNA template (25 ng/ $\mu$ L). Primer pairs used in PCR reactions include  $\beta$ -actin (forward: 5'-CCATGTACGTAGCCATCC-3'; reverse: 5'-TCAGCTGTGGTG GTGAA-3'), FN (forward: 5'-ACAGTCCAGCAAGCAGCAAGC-3'; reverse: 5'-TGGTGGTCACTCTGTAGCCTGTGTC-3'), COL-IV (forward: 5'-TAGAGAGGAGCGAGATGTTTC-3'; reverse: 5'-GT GACATTAGCTGAGTCAGG-3'), LN (forward: 5'-TGC TCACAAGACGGCGAATAAGAC-3'; reverse: 5'-ATCGTAAT GCCTGTGCTGCTCCTC-3').

Similarly, tissue was collected for Western blot analysis by dissecting the sclera rings as described elsewhere in this article. The tissue was placed in lysis buffer and manually homogenized using a small plastic tissue homogenizer in a 1.5-mL microcentrifuge tube. Primary antibodies: rabbit polyclonal FN antibody (1:200; Abcam), rabbit polyclonal COL-IV antibody (1:100; Abcam), and rabbit polyclonal LN antibody (1:200; Abcam).  $\beta$ -Actin expression (mouse anti-actin; EMD Millipore) was used as a loading control for each blot.

#### Statistical Analysis

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





**FIGURE 4.** Effects of TGF- $\beta$ 2 and osthole on the expression of ECM in HTM cells. Cells were treated with TGF- $\beta$ 2 (5 ng/mL) for 48 hours, followed by vehicle or osthole (Ost, 10  $\mu$ M) for 24 hours. Levels of the ECM proteins were analyzed by western blotting. A representative immunoblot is shown (A). Protein levels of FN (B), COL-IV (C), and LN (D) were quantified using GAPDH as a normalizing internal control. Data are presented as mean  $\pm$  SEM ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with TGF- $\beta$ 2 alone group. Ost, osthole.

## RESULTS

### Osthole and TGF- $\beta$ 2 Had Minimal Effects on HTM Cell Viability

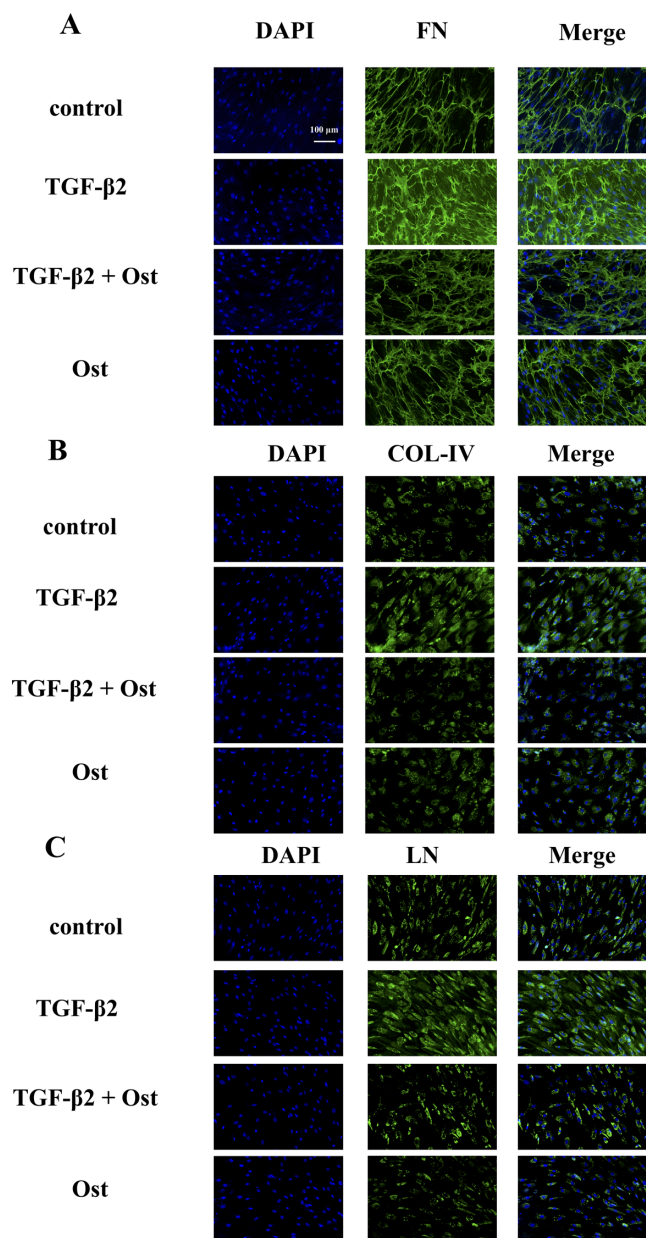
Osthole (up to 10  $\mu$ M) did not significantly affect HTM cell viability ( $95.5\% \pm 1.5\%$  compared with control  $100.0\% \pm 2.2\%$ , mean  $\pm$  SEM;  $n = 5$ ;  $P > 0.05$ ) after a 1-day incubation (Fig. 2). Similarly, at day 4, cell viability was not significantly changed by osthole at concentrations up to 10  $\mu$ M ( $94.7\% \pm 1.0\%$  compared with control  $100.0\% \pm 2.1\%$ , mean  $\pm$  SEM;  $n = 5$ ;  $P > 0.05$ ). These data suggest that osthole had minimal toxicity to the cells, and results of subsequent studies were not due to unexpected cell toxicity.

### Osthole Decreased TGF- $\beta$ 2-Induced Expressions of FN, COL-IV, and LN in HTM Cells

We used RT-qPCR to evaluate the effects of osthole on ECM expression in cultured HTM cells. After 48 hours of incubation with TGF- $\beta$ 2 (5 ng/mL), mRNA levels of FN, COL-IV, and LN were all significantly ( $P < 0.05$ ) elevated to

$1.62 \pm 0.06$ -fold (FN),  $1.60 \pm 0.06$ -fold (COL-IV), and  $1.39 \pm 0.07$ -fold (LN) (all  $n = 3$ ) of that of control group. These effects of TGF- $\beta$ 2 were decreased in a concentration-dependent manner by osthole treatment. For example, in the presence of 10  $\mu$ M osthole, the TGF- $\beta$ 2 effects were significantly ( $P < 0.05$ ) diminished, and ECM levels returned to close to their respective control level (relative to control level, FN:  $1.08 \pm 0.17$ -fold; COL-IV:  $0.91 \pm 0.11$ -fold; and LN:  $1.03 \pm 0.09$ -fold) (Figs. 3A–3C).

Similar to the mRNA level changes, protein levels of FN, COL-IV, and LN in HTM cells were also affected by both TGF- $\beta$ 2 and osthole treatments. Figure 4A shows a representative set of western blotting images indicating TGF- $\beta$ 2 (5 ng/mL) increased expressions of FN, COL-IV, and LN, which was reversed by osthole (10  $\mu$ M). Densitometry assessment showed that TGF- $\beta$ 2 increased levels of FN to  $2.95 \pm 0.05$  fold of control ( $n = 3$ ), COL-IV to  $1.41 \pm 0.01$  fold, and LN to  $2.62 \pm 0.05$  fold. These effects were partially or completely eliminated by osthole (10  $\mu$ M). When osthole was added to TGF- $\beta$ 2, the level of FN was significantly decreased to  $2.33 \pm 0.07$  fold of control, COL-IV to  $1.09 \pm 0.04$  fold, and LN to  $1.27 \pm 0.09$  fold (all



**FIGURE 5.** Representative images showing effects of TGF- $\beta$ 2 and osthole 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- $\beta$ 2 (5 ng/mL) for 48 hours or osthole for 24 hours (10  $\mu$ M) or both. Scale bar = 100  $\mu$ m. Studies were each repeated three times with similar results. Ost, osthole.

$P < 0.05$  compared with the respective TGF- $\beta$ 2-treated group). Interestingly, osthole itself significantly decreased the baseline levels of COL-IV ( $0.76 \pm 0.02$ ,  $P < 0.05$ ), but not FN ( $1.19 \pm 0.03$ ,  $P > 0.05$ ) or LN ( $1.10 \pm 0.02$ -fold,  $P > 0.05$ ) (Figs. 4B–4D).

The observed changes in mRNA and protein levels of these ECM molecules were confirmed by immunocytochemistry. As shown in Figure 5, the expression of FN, COL-IV, and LN immunoreactivities were upregulated by TGF- $\beta$ 2 (5 ng/mL), which was decreased by the treatment with osthole (10  $\mu$ M).

### Osthole Decreased Mouse Ocular Hypertension Induced by Ad.hTGF- $\beta$ 2<sup>226/228</sup>

As previously reported,<sup>24</sup> intravitreal injection of Ad.hTGF- $\beta$ 2<sup>226/228</sup> in BALB/cJ mice induced ocular hypertension. Significantly elevated IOP occurred starting at day 7 after vector injection and reached a plateau from approximately days 14 to 60. The IOP at day 14 was  $31.67 \pm 1.02$  mm Hg ( $n = 6$ ) in the Ad.hTGF- $\beta$ 2<sup>226/228</sup>-injected eyes, which was significantly higher ( $P < 0.001$ ) than the noninjected eyes ( $13.00 \pm 0.6$  mm Hg) or the Ad.Empty-injected eyes (Fig. 4A). Using a hand-held ophthalmoscope, we did not observe vector-induced ocular adverse effects, although we could not rule out the possibility of below-detectable level of immune-related responses.

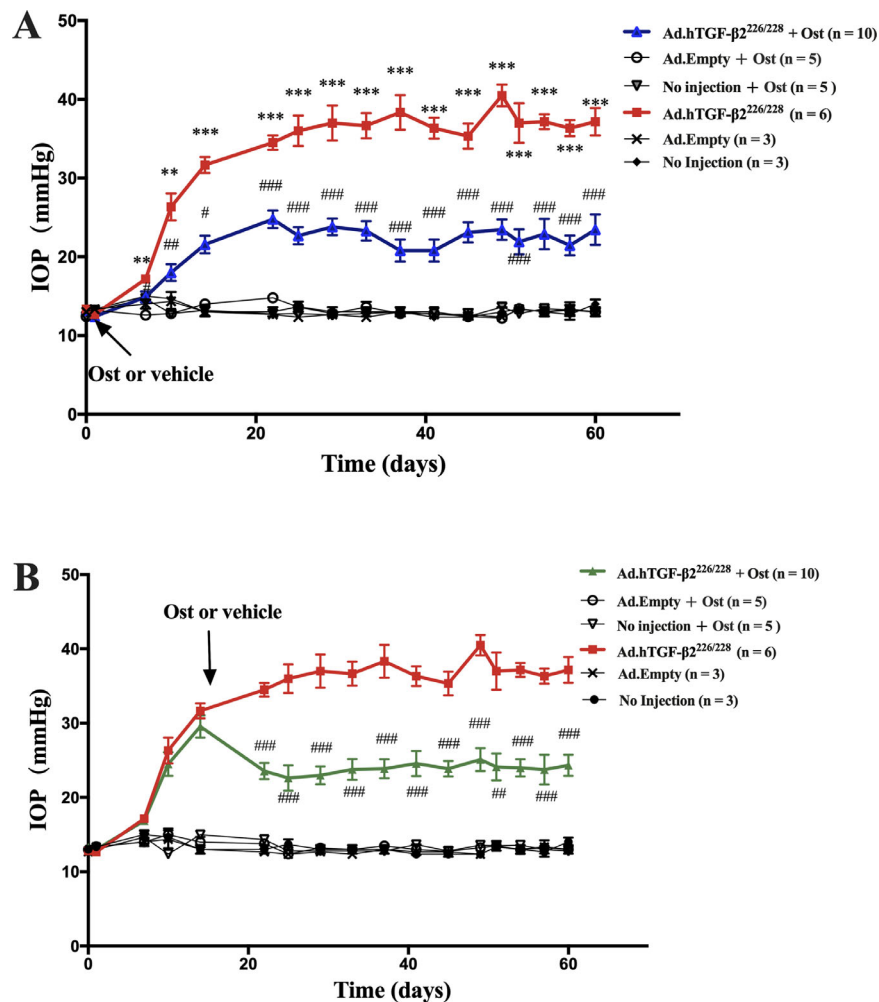
When the animals were also treated by daily intraperitoneal injections of osthole (30 mg/kg) starting at day 0, the Ad.hTGF- $\beta$ 2<sup>226/228</sup>-induced ocular hypertension was decreased. On day 7 after the injections of osthole and Ad.hTGF- $\beta$ 2<sup>226/228</sup>, IOP was  $14.88 \pm 0.71$  mm Hg ( $n = 10$ ), significantly ( $P < 0.05$ ) lower than that of the Ad.hTGF- $\beta$ 2<sup>226/228</sup> group ( $17.17 \pm 0.54$  mm Hg). This partial decrease of ocular hypertension lasted throughout the whole study period. On day 60, the IOP of the Ad.hTGF- $\beta$ 2<sup>226/228</sup> + osthole group was  $23.44 \pm 1.92$  mm Hg, which was significantly ( $P < 0.01$ ) lower than that of the Ad.hTGF- $\beta$ 2<sup>226/228</sup> group ( $37.17 \pm 1.74$  mm Hg) (Fig. 6A). Osthole did not affect IOP of the control eye. No systemic or ocular adverse effect was observed by gross examination in this study.

In addition to this study in which osthole was administered daily starting at day 0, which was before the onset of ocular hypertension, we also tested the drug effect on IOP after Ad.hTGF- $\beta$ 2<sup>226/228</sup>-induced ocular hypertension was established. In this experiment, daily intraperitoneal injection of osthole (30 mg/kg) was initiated on day 14 after Ad.hTGF- $\beta$ 2<sup>226/228</sup> injection. On day 22, IOP of the Ad.hTGF- $\beta$ 2<sup>226/228</sup> group was  $34.50 \pm 0.92$  mm Hg ( $n = 6$ ), significantly ( $P < 0.001$ ) higher than IOP of the control group of the same day. Osthole partially but significantly ( $P < 0.05$ ) lowered the IOP effect of Ad.hTGF- $\beta$ 2<sup>226/228</sup> to  $23.57 \pm 1.09$  mm Hg ( $n = 10$ ). Again on day 60, IOP of the Ad.hTGF- $\beta$ 2<sup>226/228</sup> + osthole group was  $24.33 \pm 1.42$  mm Hg, which was significantly ( $P < 0.01$ ) lower than that of the Ad.hTGF- $\beta$ 2<sup>226/228</sup> group ( $37.17 \pm 1.74$  mm Hg) (Fig. 6B). Again, no systemic or ocular adverse effect was observed.

These results indicate that systemic treatment of the mouse with osthole could partially prevent the ocular hypertension caused by the Ad.hTGF- $\beta$ 2<sup>226/228</sup>. More important, osthole also partially reversed the elevated IOP even after it was established 14 days after viral vector injection. The prolonged effect of osthole lasting the whole study period of 60 days suggests that no tachyphylaxis was involved.

### Effects of Osthole Treatment on FN, COL-IV, and LN in Mouse TM Tissue

To assess if the IOP effects of Ad.hTGF- $\beta$ 2<sup>226/228</sup> and osthole 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- $\beta$ 2<sup>226/228</sup> significantly ( $P < 0.05$ ) increased FN mRNA to  $3.71 \pm 0.26$  fold ( $n = 4$ ), COL-IV to  $2.37 \pm 0.15$  fold ( $n = 4$ ), and LN to  $3.54 \pm 0.25$  fold ( $n = 4$ ). These stimulatory effects were partially and significantly ( $P < 0.05$ ) decreased



**FIGURE 6.** Effects of Ad.hTGF- $\beta$ 2<sup>226/228</sup> and osthole on mouse IOP. One eye each of 32 mice was injected intravitreally with Ad.hTGF- $\beta$ 2<sup>226/228</sup> ( $6 \times 10^7$  pfu in 2  $\mu$ L). The contralateral eye of one-half of the animals was injected with Ad.Empty ( $6 \times 10^7$  pfu in 2  $\mu$ L) a few minutes later, and the contralateral eye of the remaining half was uninjected. **(A)** Starting at the day of Ad.hTGF- $\beta$ 2<sup>226/228</sup> treatment (day 0), animals received daily intraperitoneal injection of 30 mg/kg osthole ( $n = 10$ ) or vehicle ( $n = 6$ ). **(B)** Starting at day 14 of Ad.hTGF- $\beta$ 2<sup>226/228</sup> treatment when ocular hypertension was established, daily intraperitoneal injection of 30 mg/kg osthole ( $n = 10$ ) 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  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with control. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ , compared with Ad.hTGF- $\beta$ 2<sup>226/228</sup> without osthole by 1-way ANOVA and then least significant difference test. Ost, osthole.

by osthole treatments, either initiated at day 0 (FN:  $1.77 \pm 0.53$  fold of control,  $n = 3$ ; COL-IV:  $0.99 \pm 0.22$  fold,  $n = 3$ ; LN:  $0.96 \pm 0.22$  fold,  $n = 3$ ) or day 14 (FN:  $1.49 \pm 0.28$  fold,  $n = 3$ ; COL-IV:  $1.34 \pm 0.12$  fold,  $n = 3$ ; LN:  $1.28 \pm 0.31$  fold,  $n = 3$ ). (Figs. 7A–7C)

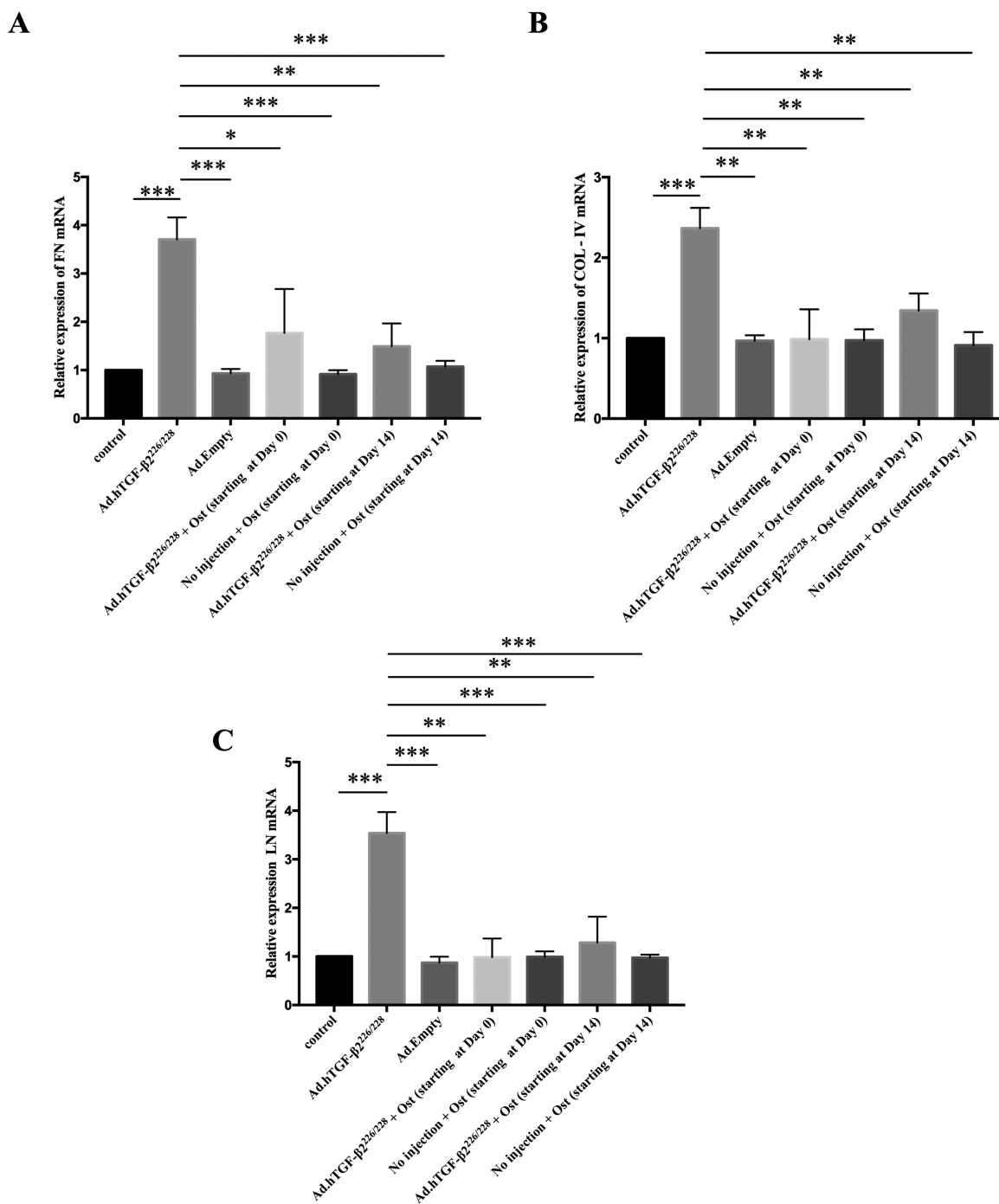
## DISCUSSION

Osthole, a pharmacologically active ingredient isolated from the fruit of *C monnieri* and other traditional Chinese herbs, has been shown to have various biological effects, such as hypotensive, antitumor, anti-inflammatory, antioxidative, and lipid modification actions.<sup>16–21</sup> Importantly, it seems to interact with TGF- $\beta$  and has antifibrotic actions in many tissues, such as scar fibroblasts,<sup>22</sup> heart,<sup>23,24</sup> kidney,<sup>25</sup> and liver.<sup>26</sup> TGF- $\beta$ 2 signaling pathway is an important contributor to the pathogenesis of POAG; its level is elevated in the aqueous humor and TM of patients with POAG<sup>10,30–32</sup>; it regulates ECM metabolism in the TM<sup>33–36</sup>; and it induces

ocular hypertension by decreasing trabecular outflow facility in both ex vivo perfused human anterior segments<sup>37,38</sup> and in vivo mouse eyes.<sup>14</sup> Because of the involvement of TGF- $\beta$  in POAG and the interaction between osthole and TGF- $\beta$ , we hypothesize that osthole may mitigate some of the TGF- $\beta$ 2 effects by interfering with the TGF- $\beta$ 2 signaling pathway in the TM, which may reduce the accumulation of ECM in the TM, and reduce the cytokine-mediated ocular hypertension. If this hypothesis is proven true, osthole should decrease ECM expression in the TM and lower IOP.

Findings from the present study support our hypothesis and show that osthole decreased TGF- $\beta$ 2-induced expressions of FN, COL-IV, and LN in cultured HTM cells. In addition, osthole decreased mouse ocular hypertension induced by Ad.hTGF- $\beta$ 2<sup>226/228</sup>, which was associated with reduced expressions of FN, COL-IV, and LN in the mouse TM.

Similar to our previous findings,<sup>27</sup> the current study demonstrated that TGF- $\beta$ 2 (5 ng/mL) increased both mRNA



**FIGURE 7.** Effects of Ad.hTGF- $\beta 2^{226/228}$  and osthole on ECM expressions in mouse TM. Expressions of ECM, including FN (A), COL-IV (B), and LN (C) were analyzed by RT-qPCR.  $\beta$ -Action was used as internal control. Data are presented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with the Ad.hTGF- $\beta 2^{226/228}$ -alone group.

and protein levels of fibrosis-related ECM molecules, such as FN, COL-IV, and LN, in cultured HTM cells. In this study, we further demonstrated that the TGF- $\beta 2$ -induced expressions of FN, COL-IV, and LN were decreased by osthole in a concentration-dependent manner between 1 and 10  $\mu$ M. It is important to note that, at 10  $\mu$ M, osthole did not significantly affect cell viability or baseline protein levels of FN, COL-IV, and LN. However, at this concentration, osthole decreased

the TGF- $\beta 2$ -elevated mRNA levels of the ECM molecules to almost baseline levels. In contrast, under the same study conditions, TGF- $\beta 2$ -elevated protein levels of FN and COL-IV were only partially reduced by osthole, suggesting that the decrease in protein levels lagged behind their decrease in synthesis.

Previous reports indicate that in vitro changes in TGF- $\beta 2$ -associated ECM levels in TM cells correlate with in



vivo changes in trabecular outflow facility and consequently IOP.<sup>34</sup> In the present study, in vitro effects of TGF- $\beta$ 2 and osthole on the TM also correlated well with their in vivo effects. Increase in ocular expression of a biologically active TGF- $\beta$ 2 mutant by intravitreal injection of Ad.hTGF- $\beta$ 2<sup>226/228</sup> in BALB/cJ mice increased mRNA levels of FN, LN, and COL-IV in TM tissue, corresponding with ocular hypertension. Moreover, when the mice were also treated with daily intraperitoneal injections of osthole starting at the same day as Ad.hTGF- $\beta$ 2<sup>226/228</sup>, the vector-induced increase in mRNA levels of ECM molecules in the TM was completely or almost completely eliminated (Fig. 7). Similarly, vector-induced elevation of IOP was also significantly lowered (Fig. 6A). However, the IOP was only partially reduced and it did not return completely to baseline as before Ad.hTGF- $\beta$ 2<sup>226/228</sup> injection. At this time, we do not know the exact reason for the partial reduction, but it is likely that the decrease in ECM protein levels in the TM may lag behind their decrease in synthesis, as seen in results of cultured TM cells. Alternatively, TGF- $\beta$ 2 is known to affect expressions of other cytokines and signaling molecules, which may contribute to IOP elevation. Osthole may also affect these signaling mechanisms and partially counteract the ocular hypertension induced by TGF- $\beta$ 2. Regardless, these findings indicate that osthole was efficacious in ameliorating the ocular hypertensive effect of TGF- $\beta$ 2.

In addition, we demonstrated that osthole could partially reverse the TGF- $\beta$ 2 effect even after IOP elevation was established. As shown in Figure 6B, administration of osthole starting at 14 days after viral vector injection was efficacious in reducing the TGF- $\beta$ 2-induced ocular hypertension. This result is clinically relevant as it indicates that osthole treatment may be useful in glaucoma patients with an already elevated IOP.

Our results are consistent with previous findings that osthole suppresses TGF- $\beta$ -induced fibrotic activities and ECM accumulation in various tissues. However, osthole has been shown to have biological effects other than antifibrotic actions. For example, it may inhibit histamine-dependent cell signaling,<sup>39</sup> which may directly or indirectly modify IOP, because histamine is known to affect aqueous humor hydrodynamics and IOP.<sup>40</sup> Currently, the exact pharmacologic mechanism of the ocular hypotensive action of osthole is still not fully elucidated. Nonetheless, osthole treatment did not significantly affect IOP in the control, non-Ad.TGF-treated group. We feel that its effect in the Ad.TGF-induced ocular hypertensive eyes seemed to be specific and was unlikely a result of histamine antagonism. Future mechanistic studies will shed light on this important question.

The current study only assessed osthole's IOP effect after intraperitoneal injection, which is definitely not a practical clinical treatment method for glaucoma. Much work in research and development awaits. Formulations for ocular topical administration of this compound are being developed. If sufficient amount of osthole can penetrate the cornea to produce sufficient ocular hypotension with a suitable duration of action and with an acceptable adverse effect profile, it may become a novel addition to the armament of glaucoma therapy.

In summary, this study demonstrated that osthole reduces TGF- $\beta$ 2-induced expression of ECM proteins in cultured HTM cells, and partially lowers TGF- $\beta$ 2-induced elevation of mouse IOP. This natural product may be useful as a novel addition to the medical treatment for POAG.

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## 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. Kwon YH, Fingert JH, Kuehn MH, Alward WL. Primary open-angle glaucoma. *N Engl J Med*. 2009;360:1113–1124.
3. Weinreb RN, Khaw PT. Primary open-angle glaucoma. *Lancet*. 2004;363:1711–1720.
4. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. *Prog Retin Eye Res*. 1999;18:91–119.
5. Rohen JW, Witmer R. Electron microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch Klin Exp Ophthalmol*. 1972;183:251–266.
6. Gabelt BT, Kaufman PL. Changes in aqueous humor dynamics with age and glaucoma. *Prog Retin Eye Res*. 2005;24:612–637.
7. Clark AF, Wordinger RJ. The role of steroids in outflow resistance. *Exp Eye Res*. 2009;88:752–759.
8. Tovar-Vidales T, Clark AF, Wordinger RJ. Transforming growth factor-beta2 utilizes the canonical Smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells. *Exp Eye Res*. 2011;93:442–451.
9. 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.
10. 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.
11. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol*. 2004;25:19–22.
12. 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.
13. Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest Ophthalmol Vis Sci*. 2004;45:153–158.
14. Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming growth factor-beta2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci*. 2010;51:2067–2076.
15. Megumi Honjo, Nozomi I, Junko N, et al. Role of the autotaxin-LPA pathway in dexamethasone-induced fibrotic responses and extracellular matrix production in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2018;59:21–30.
16. Fusi F, Sgaragli G, Ha LM, et al. Mechanism of osthole inhibition of vascular Ca(v)1.2 current. *Eur J Pharmacol*. 2012;680:22–27.
17. Hung CD, Kuo DH, Chou CH, et al. Osthole suppresses hepatocyte growth factor (HGF)-induced epithelial-mesenchymal transition via repression of the c-Met/Akt/mTOR pathway in human breast cancer cells. *J Agric Food Chem*. 2011;59:9683–9690.

18. Zhang QY, Qin LP, He WD, et al. Coumarins from *Cnidium monnieri* and their antiosteoporotic activity. *Planta Med.* 2007;73:13–19.
19. Chiu PR, Lee WT, Chu YT, et al. Effect of the Chinese herb *tractosthol* on IL-4- induced eotaxin expression in BEAS-2B cells. *Pediatr Neonatol.* 2008;49:135–140.
20. Zhang JJ, Xue J, Wang HB, et al. Osthole improves alcohol-induced fatty liver in mice by reduction of hepatic oxidative stress. *Phytother Res.* 2011;25:638–643.
21. Du R, Xue J, Wang HB, et al. Osthole ameliorates fat milk-induced fatty liver in mice by regulation of hepatic sterol regulatory element-binding protein-1c/2-mediated target gene expression. *Eur J Pharmacol.* 2011;666:183–188.
22. Hou XH, Cao B, Liu HQ, et al. Effects of osthole on apoptosis and TGF-1 of hypertrophic scar fibroblasts. *J Asian N Prod Res.* 2009;11:663–669.
23. Liu JC, Wang F, Xie ML, et al. Osthole inhibits the expressions of collagen I and III through Smad signaling pathway after treatment with TGF- $\beta$ 1 in mouse cardiac fibroblasts. *Int J Cardiol.* 2017;228:388–393.
24. Chen R, Xue J, Xie ML. Reduction of isoprenaline-induced myocardial TGF- $\beta$ 1 expression and fibrosis in osthole-treated mice. *Toxicol Appl Pharmacol.* 2011;256:168–173.
25. Zhang SP, Huang Q, Cai XX, et al. Osthole Ameliorates renal fibrosis in mice by suppressing fibroblast activation and epithelial-mesenchymal transition. *Front Physiol.* 2018;9:1650–1663.
26. Liu YW, Chiu YT, Fu SL, et al. Osthole ameliorates hepatic fibrosis and inhibits hepatic stellate cell activation. *J Biomed Sci.* 2015;22:63–73.
27. Fan YC, Guo L, Wei JH, et al. Effects of salidroside on trabecular meshwork cell extracellular matrix expression and mouse intraocular pressure. *Invest Ophthalmol Vis Sci.* 2019;60:2072–2082.
28. Yan YH, Kong L, Xia Y, et al. Osthole promotes endogenous neural stem cell proliferation and improved neurological function through Notch signaling pathway in mice acute mechanical brain injury. *Brain Behav Immun.* 2018;67:118–129.
29. 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.
30. Sethi A, Mao W, Wordinger RJ, Clark AF. Transforming growth factor-beta induces extracellular matrix protein cross-linking lysyl oxidase (LOX) genes in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2011;52:5240–5250.
31. Tovar-Vidales T, Clark AF, Wordinger RJ. Transforming growth factor-beta2 utilizes the canonical Smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells. *Exp Eye Res.* 2011;93:442–451.
32. 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.
33. 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.
34. Fuchshofer R, Yu AH, Welge-Lussen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor-beta2 in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2007;48:715–726.
35. 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.
36. Sethi A, Jain A, Zode GS, Wordinger RJ, Clark AF. Role of TGF beta/Smad signaling in gremlin induction of human trabecular meshwork extracellular matrix proteins. *Invest Ophthalmol Vis Sci.* 2011;52:5251–5259.
37. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol.* 2004;25:19–22.
38. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci.* 2006;47:226–234.
39. Niu NY, Hao S, Guang Y, et al. Osthole inhibits histamine-dependent itch via modulating TRPV1 activity. *Sci Rep.* 2016;6:25657.
40. Paul LK, Ernst HB, Kristine AE. Effect of serotonin, histamine and bradykinin on outflow facility following ciliary muscle retrodisplacement in the cynomolgus monkey. *Exp Eye Res.* 1982;35:191–199.