Resveratrol Inhibits Wound Healing and Lens Fibrosis: A Putative Candidate for Posterior Capsule Opacification Prevention

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Purpose. Posterior capsule opacification (PCO) is a common complication of cataract surgery. In addition to improved surgical methods and IOL designs, it is likely additional agents will be needed to improve patient outcomes. Presently no pharmacological agent is in clinical use to prevent PCO. Here we investigate the putative ability of resveratrol (RESV), a naturally occurring polyphenol, as a therapeutic agent.

Methods. The human lens epithelial cell line FHL124, a human lens capsular bag model, and central anterior epithelium were used as experimental systems. Standard culture was in 5% fetal calf serum Eagle's minimum essential medium; 10 ng/mL transforming growth factor-β2 (TGFβ2) was used to induce fibrotic changes. A scratch wound assay was used to measure cell migration and the patch assay was used to assess matrix contraction by FHL124 cells. Protein expression was assessed by immunocytochemistry and Western blot and gene expression by quantitative RT-PCR. In capsular bags, cell growth across the posterior lens capsule, capsular wrinkling, and epithelial-to-mesenchymal transition were determined by image analysis.

Results. In FHL124 cells, addition of 30 μM RESV significantly impeded cell migration in a wound-healing assay. RESV significantly inhibited TGFβ2-induced expression of the myofibroblast marker alpha-smooth muscle actin (α-SMA) at both the message and protein levels, as well as significantly inhibiting matrix contraction induced by TGFβ2. In human capsular bags, 30 μM RESV significantly inhibited cell growth. TGFβ2-induced α-SMA expression and capsular wrinkling were also significantly inhibited by RESV treatment. RESV significantly suppressed expression of TGFβ2-induced genes associated with fibrotic disease, including matrix metalloproteinase-2 in FHL124 cells, capsular bags, and central anterior epithelium.

Conclusions. RESV can counter PCO-related physiological events in two human lens model systems. RESV therefore has the potential to be used as a candidate agent for the prevention of PCO, which in turn could benefit millions of cataract patients.

Keywords: human, lens, fibrosis, resveratrol, posterior capsule opacification

Cataract is the leading cause of blindness globally.[1] Defined as any opacity of the eye lens, it is treatable only by surgical intervention. Initially extremely successful in restoring vision, this surgical procedure is the most commonly performed operation in the developed world hugely burdening health care budgets.[2] Surgery involves removal of the cataractous lens fiber mass and its replacement with an artificial IOL to restore free passage of light along the visual axis.[3] However, a significant proportion of patients develop a secondary loss of vision termed posterior capsule opacification (PCO) within 5 years of surgery.[2,4] In these patients, residual lens epithelial cells that remain in the capsular bag following surgery, rapidly grow to recolonize the anterior lens capsule and also migrate to the previously cell-free posterior lens capsule. As the cells advance, they also undergo epithelial-to-mesenchymal transition (EMT), to become myofibroblastic cells expressing increased alpha-smooth muscle actin (α-SMA) and produce excessive extracellular matrix components. This aberrant extracellular matrix deposition and capsule contraction/wrinkling, can also cause light scatter and distort vision by impeding the free passage of light along the visual axis.[5]

PCO is a fibrotic disease resulting from a wound-healing response initiated by surgical trauma and breaching of the blood-aqueous barrier. The profibrotic cytokine, TGFβ, and its signaling via the canonical (Smad) signaling pathway, is a requirement for lens epithelial cells to undergo EMT, and it has been strongly implicated in PCO development as well as fibrotic responses in numerous other tissue types.[6–8] The predominant isoform in the human eye is TGFβ2,[9] and under normal physiological conditions TGFβ is present in an inactive, latent form. Following the breaching of the blood-aqueous barrier at cataract surgery, active levels of TGFβ increase with subsequent inflammation, elevating levels of activators of latent TGFβ, such as matrix metalloproteinases (MMPs) and thrombospondin and αV integrins.[10,11] These events lead to a sustained TGFβ activation, driving fibrotic events that lead to PCO.

PCO is treated by Nd:YAG capsulotomy, whereby a laser is used to perforate the posterior lens capsule to create an opening, again restoring the free passage of light to the retina.

This procedure also risks complication and places further burden on health care resources.[6] PCO is therefore a significant concern, with strategies to prevent or decrease its occurrence required urgently. Remarkably, there is currently no pharmacological agent with which to manage or prevent PCO.

Resveratrol (RESV) is a naturally occurring polyphenolic phytoalexin compound identified as being present in red wine and to a lesser extent other foods, including peanuts and berries, and has been long considered to have potential health benefits.[12] Previous studies have interestingly demonstrated an ability of RESV to inhibit fibrotic events in various disease models,[13–17] and thus in the present study we aimed to evaluate whether RESV could inhibit fibrotic events associated with PCO, that is, cell proliferation, EMT, matrix production, and matrix contraction.

Methods

FHL124 Cell Cultures

FHL124 human lens epithelial cells were routinely cultured at 35°C with 5% CO_2, 95% air in Eagle's minimum essential medium (EMEM) (Gibco, Paisley, UK) supplemented with 5% vol/vol fetal calf serum (FCS) (Gibco) and 50 μg/mL gentamicin (Sigma-Aldrich, Dorset, UK). FHL124 cells were seeded at 50,000 cells per 35-mm cell culture dish for Western blot and quantitative RT-PCR (QRT-PCR), 5000 cells per patch in 35-mm cell culture dish for patch contraction assays, 50,000 cells per patch in 35-mm cell culture dish for wound-healing assays, and 5000 cells per coverslip for immunocytochemistry.

Experimental conditions for FHL124 cells involved the simple addition of RESV (Stratech Scientific Limited, Ely, UK) versus control for 48 hours for the wound-healing assay. To assess the effects of RESV (30 μM) on EMT, gene expression, and contraction, the following protocol was adopted. Cells were maintained in EMEM containing 5% FCS ± 30 μM RESV for 24 hours. Following this period, TGFβ2 was applied directly to the selected cultures (half of each group) to give a final concentration of 10 ng/mL. This resulted in four experimental groups, which were control, RESV alone, TGFβ2 alone, or RESV and TGFβ2 treated. Cultures were maintained in these conditions for 24 hours (QRT-PCR) or 48 hours (Western blot and patch contraction assay). Coverslips were maintained in conditions for 2 hours.

Wound-Healing Assay

FHL124 cells were seeded on a 35-mm tissue culture dish at 50,000 cells in 200 μL 5% FCS-EMEM and allowed to establish over a 72-hour period, such that a distinct patch of cells was observed. Following this period, a scratch was made through the middle of the confluent sheet using a plastic pipette tip. The medium was then replaced with fresh 5% FCS-EMEM to remove cellular debris. Cells were then exposed to experimental conditions. Indentations within the plastic dish were made as a reference mark. The cells were then exposed to experimental conditions and maintained for 48 hours. Images were captured at 0-, 24-, and 48-hour time-points and wound closure determined using image analysis software (ImageJ 1.48v; http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Quantitative RT-PCR

Total RNA was extracted from FHL124 cells, central anterior lens epithelial samples, and human capsular bags cells using the ReliaPrep RNA cell Miniprep System (Promega, Madison, WI, USA) or the ReliaPrep RNA tissue Miniprep System (Promega) as appropriate, following the manufacturer's instructions. RNA was quantified using a NanoDrop spectrophotometer and cDNA generated using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (Promega) following the manufacturers' instructions. Gene expression was quantified by real-time PCR with 18S used as an endogenous control gene using predesigned TaqMan probes (Life Technologies, Carlsbad, CA, USA) (Table) and TaqMan PCR master mix (PCR Biosystems, London, UK).

Table. Predesigned TaqMan Probe Sets for Genes of Interest

Gene Name	Protein Encoded	TaqMan Primer/ Probe Set
ACTA2	Alpha-smooth muscle actin (α-SMA)	Hs00426835_g1
FN1	Fibronectin-1 (FN1)	Hs01549976_m1
ITGA5	Integrin subunit alpha 5 (ITGA5)	Hs1547673_m1
ITGAV	Integrin subunit alpha V (ITGAV)	Hs00233808_m1
MYLK	Myosin light chain kinase (MLCK)	Hs00364926_m1
MMP2	Matrix metalloproteinase 2 (MMP2)	Hs01548727_m1
TGFB2	Transforming growth factor beta 2 (TGFβ2)	Hs00234244_m1

Western Blot Analysis

FHL124 cell lysates were obtained using M-PER buffer (Thermo Scientific, Basingstoke, UK) supplemented immediately before use with 10 μL/mL phosphatase and protease inhibitors and 0.5 M EDTA (Thermo Scientific). The bicinchoninic acid assay (Thermo Scientific) was used to measure total protein content to enable equal loading of protein onto 10% SDS-polyacrylamide gels. Proteins were transferred onto polyvinyl difluoride (PVDF) membranes using a Trans-Blot Turbo Transfer System (Bio-Rad, Watford, UK). PVDF membranes were blocked with PBS containing 0.5% wt/vol nonfat dry milk and 0.05% vol/vol Tween-20, hybridized with primary antibody against α-SMA (Sigma-Aldrich) or β-actin (Cell Signaling Technology, Danvers, MA, USA), followed by incubation with secondary antibody conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, UK). Proteins were detected using Clarity Western ECL Substrate (Bio-Rad) and visualized with a ChemiDoc imaging system (Bio-Rad).

Patch Contraction Assay

FHL124 cells were seeded as patches (four per 35-mm culture dish) and allowed to grow until approximately 5 mm in diameter and confluent. Cells were then placed in experimental conditions. On appearance of cell-free holes within patches, cells were fixed with 4% formaldehyde for 30 minutes at room temperature and washed three times for 5 minutes in PBS. Patches were then stained with Coomassie brilliant blue (Sigma-Aldrich) and washed with PBS to remove excess stain. Patches were then imaged with a charge-coupled device camera with (Synoptics, Cambridge, UK) and measured with image analysis software (ImageJ, 1.48v).

Human Capsular Bag Model

Following the removal of corneo-scleral disks for transplantation processes, a simulated cataract operation was performed on postmortem human donor eyes in a laminar flow hood as previously described.[18] Donor eyes were obtained with written informed consent obtained from the next of kin. The

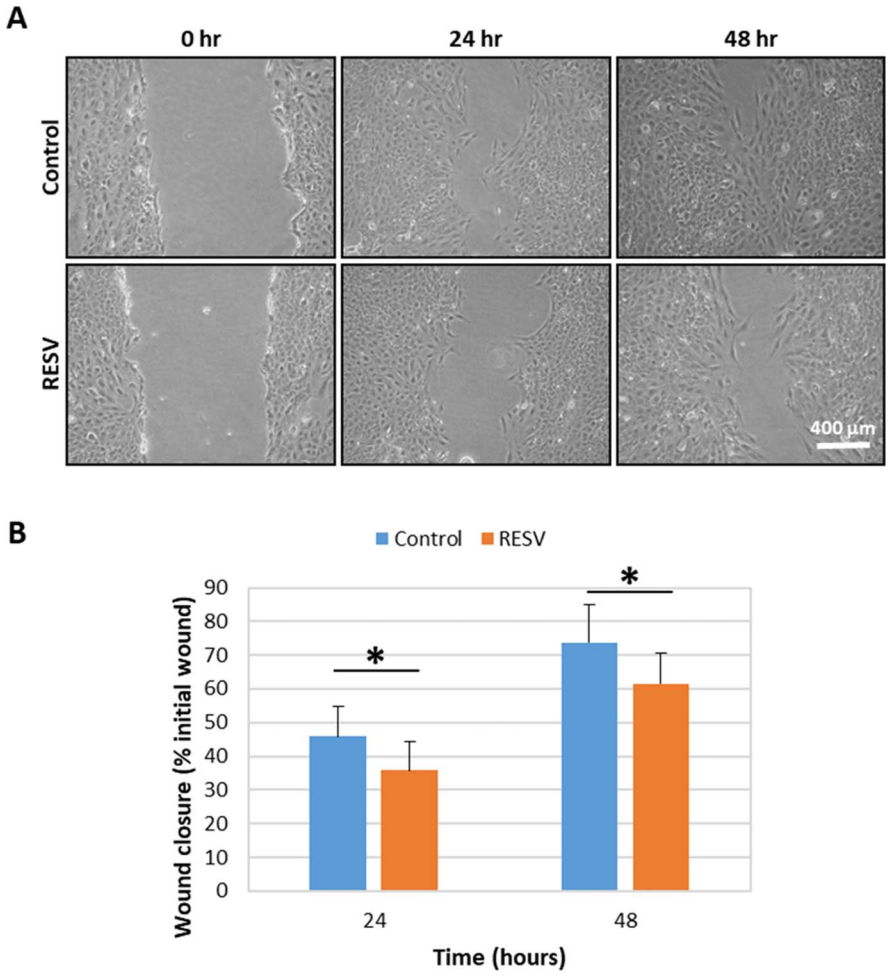

Figure 1. Effect of RESV on wound closure by FHL124 cells. FHL124 cells were scratched to induce a wound before washing to remove debris and addition of experimental conditions. Wound closure was assessed at 24 and 48 hours following the initial wound and maintained under control (5% FCS-EMEM) conditions or treated with 30 µM RESV. (**A**) Representative images and (**B**) quantitative data established from multiple experiments ($n = 4$). Wound closure was quantified as % initial wound. The data are presented as mean ± SEM. *Significant differences between experimental conditions (Student's *t*-test; $P \leq 0.05$).

research was approved by the UK National Research Ethics Committee (REC 04/Q0102/57) and followed the tenets of the Declaration of Helsinki regarding the use of human donor material. Match-paired capsular bags were maintained in 35-mm tissue culture dishes, in the following match-paired experimental conditions: 5% FCS-EMEM (control) versus 10 ng/mL TGFβ2; control versus 30 µM RESV; 10 ng/mL TGFβ2 versus 30 µM RESV and 10 ng/mL TGFβ2, culture medium and conditions were replaced at day 4 with ongoing observations of cell growth performed using Nikon phase-contrast microscope (Nikon, Tokyo, Japan) and a digital camera (Nikon). Quantification of cell growth across the posterior lens capsule was performed using image analysis software (ImageJ, 1.48v). Capsular wrinkling/matrix contraction was assessed at experimental endpoint (day 7) by analyzing modified dark-field images of the central posterior capsule. Images were placed through the find edges function on ImageJ. Edges associated with wrinkles appear bright against a dark background. The image is then subjected to thresholding, which allows quantification.

Central Anterior Lens Epithelial Samples

Human central anterior lens epithelium samples (capsule and cells) removed by capsulorhexis during simulated cataract surgery were isolated. Each sample was halved and the four pieces of tissue from the same donor were secured to individual 35-mm tissue culture dishes using entomological pins, cell-side up. Preparations were maintained at 35°C in a 5% CO_2 atmosphere in either control conditions (EMEM supplemented with 5% vol/vol FCS and 50 µg/mL gentamicin), 30 µM RESV alone, 10 ng/mL TGFβ2 alone, or 30 µM RESV and 10 ng/mL TGFβ2. Preparations were maintained in experimental conditions until endpoint (day 7), when samples were fixed in 4% formaldehyde for immunocytochemistry or maintained in experimental conditions for 48 hours for gene expression analysis by QRT-PCR.

Immunocytochemistry

FHL124 cells, human capsular bags, or central anterior lens epithelial samples were fixed with 4% formaldehyde in PBS for 30 minutes followed by three washes in PBS for 5 minutes. Samples were permeabilized with 0.5% vol/vol Triton X-100 (Sigma-Aldrich) for 30 minutes. Three washes in PBS containing 0.02% wt/vol BSA and 0.05% vol/vol commercial detergent (IGEPAL; Sigma-Aldrich) were performed before blocking for nonspecific binding sites with either normal donkey or normal goat serum (1:50 in 1% wt/vol BSA in PBS) for 1 hour. Primary antibodies against α-SMA (Sigma-Aldrich), fibronectin (Sigma-

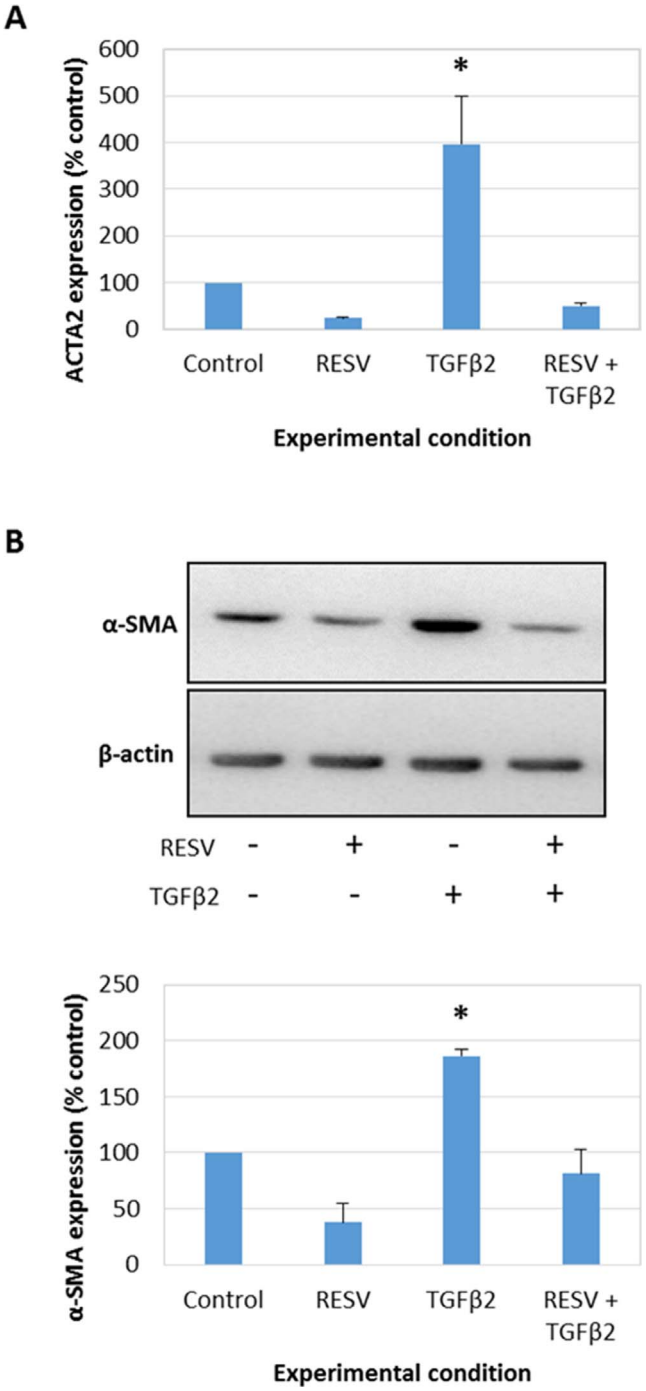

Figure 2. Effect of RESV treatment on EMT. FHL124 cells were maintained under control conditions (5% FCS-EMEM), treated with 30 μM RESV, 10 ng/mL TGFβ2 or 30 μM RESV + 10 ng/mL TGFβ2. Cells were treated with RESV 24 hours before addition of TGFβ2. (**A**) ACTA2 gene expression was assessed by quantitative real-time RT-PCR, 24 hours post-TGFβ treatment ($n = 5 \pm$ SEM). (**B**) α-SMA protein expression was determined by Western blot 48 hours post-TGFβ treatment ($n = 3$). The data are presented as mean ± SEM. *Significant difference between experimental conditions (ANOVA with Tukey's post hoc test; $P \leq 0.05$).

Aldrich), and Smad2/3 (Cell Signaling Technology) were diluted 1:200 in 1% BSA in PBS and applied overnight at 4 °C. Three further washes in PBS containing 0.02% wt/vol BSA and 0.05% vol/vol IGEPAL were performed followed by addition of secondary antibody (Alexa-Fluor 488-conjugated donkey anti-rabbit or Alexa-Fluor 488-conjugated goat anti-mouse) (Invitrogen) diluted 1:100 in 1% BSA in PBS) for 1 hour protected from light at 37°C in a humidified atmosphere. Samples were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and Texas red-x-phalloidin (Invitrogen). A further three washes in PBS containing 0.02% wt/vol BSA and 0.05% vol/vol IGEPAL were performed followed by mounting of samples onto glass microscope slides. Samples were viewed with fluorescence microscopy (wide-field microscope Zeiss Axioplan 2ie; Carl Zeiss Microscopy Ltd, Cambridge, UK). Image quantification was performed using image analysis software (ImageJ, 1.48v).

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's test was performed to determine statistical differences between multiple groups (SPSS 16.0; SPSS Inc., Chicago, IL, USA) and Student's *t*-test was used to determine statistical differences when two experimental groups were being compared (Excel; Microsoft, Redmond, WA, USA). A P value of ≤ 0.05 was considered significant.

Results

FHL124 Cell Line

To establish a working concentration of RESV, a Celltiter-Glo viability assay (Promega) was performed (data not shown). No loss in viability was observed at concentrations up to 30 μM RESV, over a 72-hour culture period. Toxicity was, however, observed with 100 μM. In the current study, we aimed to assess the functional capabilities of RESV at noncytotoxic concentrations in preventing PCO-related events, and thus 30 μM was used as a working concentration. In the first instance, FHL124 cells were used as an initial model system.

Wound-Healing Assay

A wound-healing assay was used to assess FHL124 cell migration and growth. RESV treatment significantly impeded wound closure by FHL124 cells when compared with FHL124 cells maintained under control conditions (5% FCS-supplemented EMEM) (Fig. 1).

Epithelial-to-Mesenchymal Transition

To assess the effect of RESV on EMT, the myofibroblast marker, α-SMA, was assessed at the message and protein level. FHL124 cell cultures were maintained in 5% FCS-EMEM ± 30 μM RESV for 24 hours before TGFβ2 was applied directly to the selected cultures to give a final concentration of 10 ng/mL. The four experimental groups were therefore control, RESV alone, TGFβ2 alone, or RESV and TGFβ2 treated. Using quantitative real-time PCR, it was found that the expression of ACTA2, the gene encoding α-SMA protein, was significantly increased following a 24-hour application of TGFβ2, relative to non-stimulated controls (Fig. 2A). Addition of RESV alone appeared to reduce ACTA2 expression, but was not significantly different from nonstimulated control. Notably, when cells were treated with RESV in the presence of TGFβ2, ACTA2 levels were suppressed relative to TGFβ2-treated cells, such that they were equivalent to the control group (Fig. 2A).

At 48 hours, Western blots for α-SMA were performed (Figs. 2B, 2C). Protein levels of α-SMA were significantly elevated in the TGFβ2-treated group compared with those maintained in control conditions. Again RESV alone appeared to suppress α-

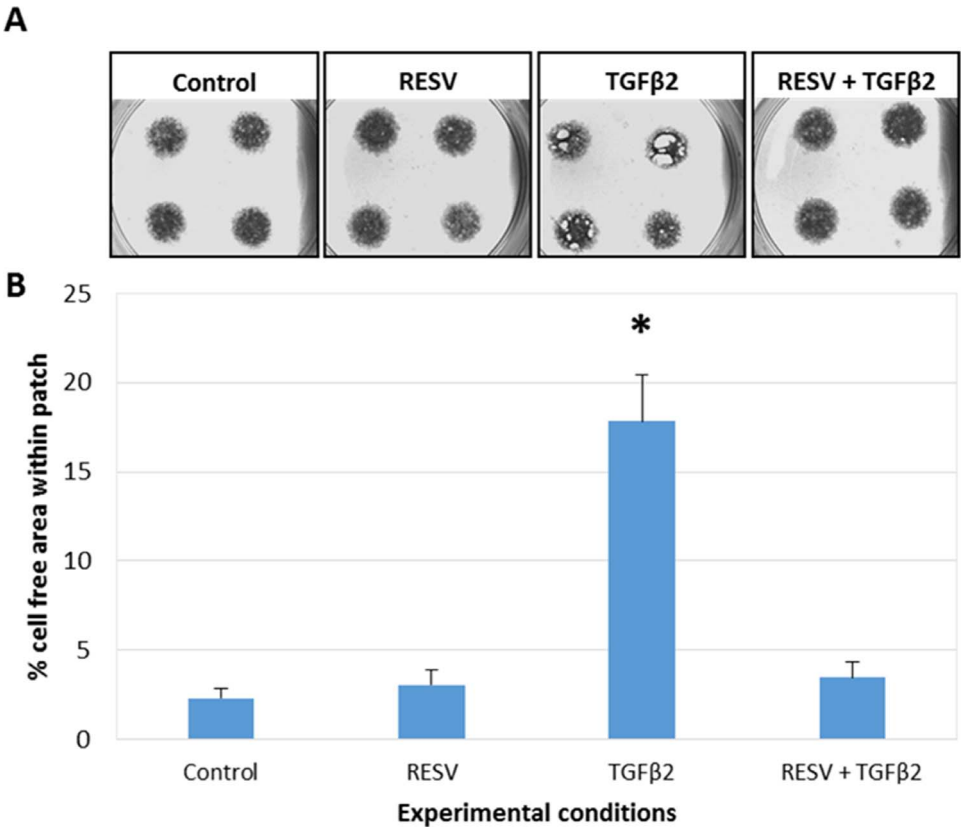

FIGURE 3. RESV inhibits TGFβ2-induced matrix contraction in FHL124 cells. FHL124 cells were maintained under control conditions (5% FCS-EMEM), treated with 30 μM RESV, 10 ng/mL TGFβ2 or 30 μM RESV + 10 ng/mL TGFβ2. Cells were treated with RESV 24 hours before addition of TGFβ2 with matrix contraction assessed by patch contraction assay. (**A**) Representative images of patches in different experimental conditions. (**B**) Quantitative data established from multiple experiments ($n = 3$). The data are presented as mean ± SEM. *Significant difference between experimental conditions (ANOVA with Tukey's post hoc test; $P \leq 0.05$).

SMA protein, but as with gene expression, was not statistically different. Treatment of cells with RESV countered the effects of TGFβ2 when applied together, such that levels were equivalent to control treatment.

Matrix Contraction

FHL124 cell patches treated with TGFβ2 demonstrated a significantly greater proportion of cell-free areas within the patch than observed under control conditions (Fig. 3). Treatment with RESV alone did not generate cell-free regions within the patch area. RESV treatment was found to suppress TGFβ2-induced patch contractions, which did not differ significantly from controls (Fig. 3).

Gene Expression Changes

To assess the effect of RESV on genes associated with fibrosis, FHL124 cell cultures were maintained in 5% FCS-EMEM ± 30μM RESV for 24 hours before TGFβ2 was applied to selected cultures for a further 24 hours. Gene expression of FN1, ITGA5, ITGAV, MYLK, MMP2, and TGFB2 was assessed. Treatment of FHL124 cells with TGFβ2 significantly elevated expression of FN1, ITGA5, ITGAV, MMP2, and TGFB2 from levels observed in cells maintained under control conditions (Fig. 4). No significant increase in MYLK was observed. Treatment with RESV did not alter expression of any genes studied when compared to controls (Fig. 4). Treatment of FHL124 cells with TGFβ2 in the presence of RESV resulted in significantly less expression of ITGAV, ITGA5, MYLK, MMP2 and TGFB2 compared to cells treated with TGFβ2 alone, such that levels detected did not significantly differ from the control group (Fig. 4). No significant reduction in FN1 expression was observed in FHL124 cells treated with RESV and TGFβ2 compared with TGFβ2 treatment alone (Fig. 4).

Canonical TGFβ/Smad Signaling Pathway

To assess the effect of RESV on TGFβ2-induced Smad signaling, FHL124 cells were maintained in 5% FCS-EMEM ± 30 μM RESV for 24 hours before 10 ng/mL TGFβ2 was applied to selected cultures for a further 2 hours. Immunocytochemistry was performed to assess Smad2/3 nuclear translocation in response to these treatments (Fig. 5). FHL124 cells treated with RESV alone did not demonstrate any change in nuclear translocation of Smad2/3 compared with control treatments; however, a significant increase in Smad2/3 nuclear translocation was observed in TGFβ2-treated cells compared with control and RESV-treated cells. Cells treated with RESV and TGFβ2 also demonstrated a significant increase in Smad2/3 nuclear translocation compared with control and RESV-treated cells; these levels were not significantly different from cells treated with TGFβ2 alone (Fig. 5).

Human Capsular Bag Model

Human lenses were dissected from donor eyes and subjected to simulated cataract surgery to create capsular bags that were then cultured for 7 days in experimental conditions. Match-paired experiments were conducted to compare untreated

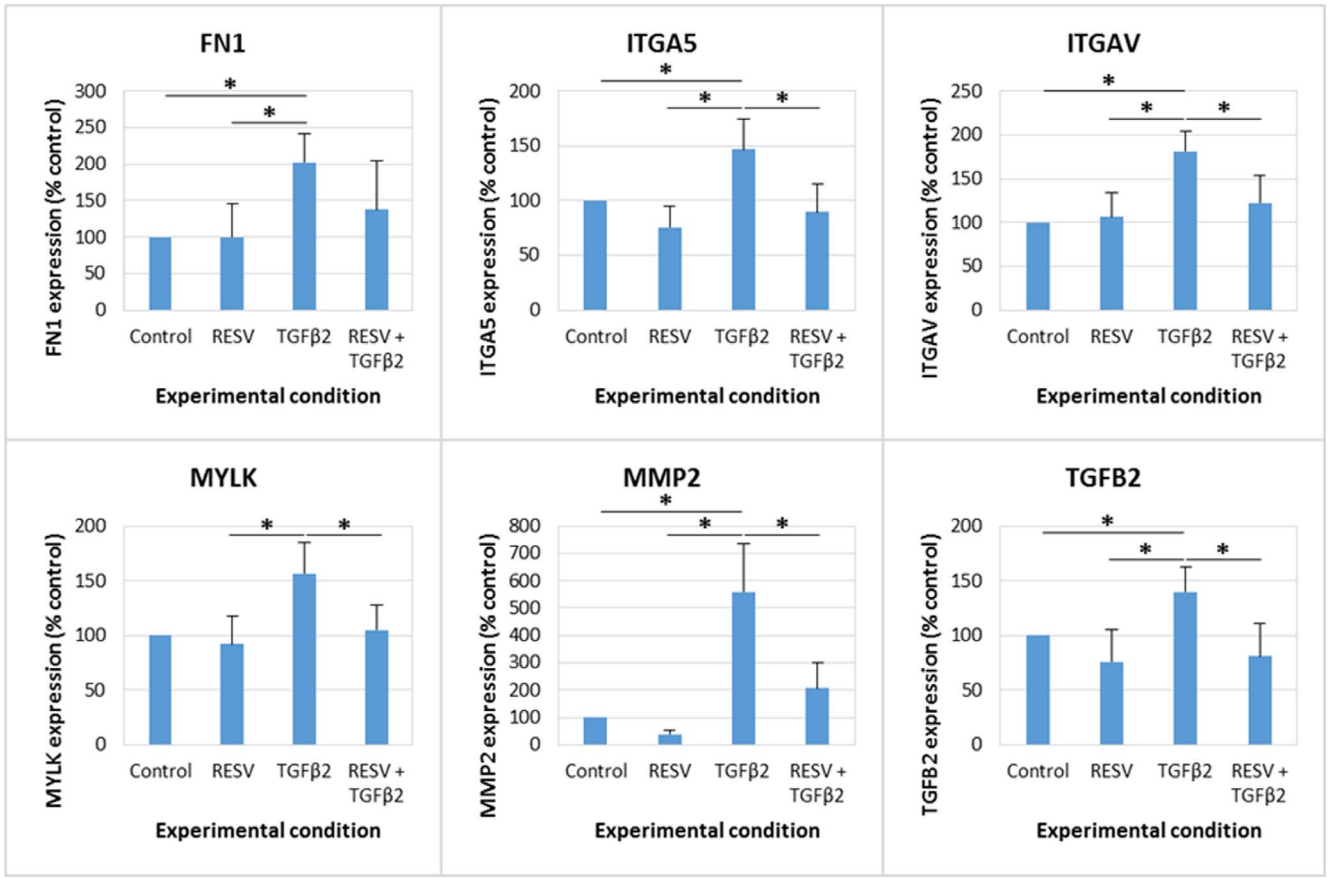

Figure 4. Effect of RESV on gene expression. FHL124 cells were maintained under control conditions (5% FCS-EMEM), treated with 30 μM RESV, 10 ng/mL TGFβ2 or 30 μM RESV + 10 ng/mL TGFβ2. Cells were treated with RESV 24 hours before addition of TGFβ2 with expression of FN1, ITGA5, ITGAV, MYLK, MMP2, and TGFB2 assessed by real-time quantitative RT-PCR 24 hours posttreatment. The data are presented as mean ± SEM; $n = 5$. *Significant difference between experimental conditions (ANOVA with Tukey's post hoc test; $P \leq 0.05$).

capsular bags maintained in control conditions versus those treated with 10 ng/mL TGFβ2, untreated versus 30 μM RESV and capsular bags treated with 10 ng/mL TGFβ2 versus 30 μM RESV + 10 ng/mL TGFβ2. Ongoing observations of cell growth across the posterior lens capsule were performed, with matrix contraction and α-SMA expression as a marker of EMT assessed at experimental endpoint (7 days).

Cell Growth Across Posterior Lens Capsule

Match-paired human capsular bags were first cultured in the presence or absence of 10 ng/mL TGFβ2. Cell coverage across the posterior lens capsule in capsular bags maintained under control conditions (EMEM containing 5% FCS) was 100% at day 7. Capsular bags treated with 10 ng/mL TGFβ2 demonstrated a retardation of cell growth across the posterior lens capsule, which was significant from day 6 until experimental endpoint at day 7 (Fig. 6).

To assess the effects of RESV treatment on cell growth across the posterior lens capsule, match-paired capsular bags were either maintained under control conditions or treated with 30 μM RESV for a 7-day period. Cell coverage was again measured daily. RESV treatment was found to suppress cell growth across the posterior lens capsule, such that coverage was significantly less than the untreated control from day 2 until experimental end point at day 7 (Fig. 6).

To investigate the effect of RESV treatment on TGFβ2-induced cellular changes in the capsular bag model, preparations were either cultured in the presence of 10 ng/mL TGFβ2 or 30 μM RESV and 10 ng/mL TGFβ2 with cell growth across the posterior lens capsule observed. Cell growth across the posterior lens capsule was found to be significantly attenuated in capsular bags treated with RESV and TGFβ2 compared with those maintained in TGFβ2 alone from day 3 through to endpoint (day 7) (Fig. 6).

EMT/Matrix Deposition

Immunocytochemistry was used to assess the myofibroblast marker α-SMA, and to assess matrix deposition using fibronectin as a marker in cells present on the posterior lens capsule at experimental endpoint (day 7). A significant increase in α-SMA and fibronectin expression was noted in cells present on the posterior capsule of capsular bags treated with 10 ng/mL TGFβ2 compared with untreated match-paired controls, whereas no differences in α-SMA or fibronectin were observed in capsular bags treated with 30 μM RESV compared with untreated controls. Capsular bags treated with RESV and TGFβ2 demonstrated significantly less α-SMA and fibronectin expression in cells present on the posterior capsule compared with those treated with TGFβ2 alone (Fig. 7).

Matrix Contraction/Capsular Wrinkling

Wrinkling of the posterior lens capsule (matrix contraction) was assessed at experimental endpoint. In TGFβ2-treated capsular bags, a significant increase in capsular wrinkling/matrix contraction was observed compared with untreated,

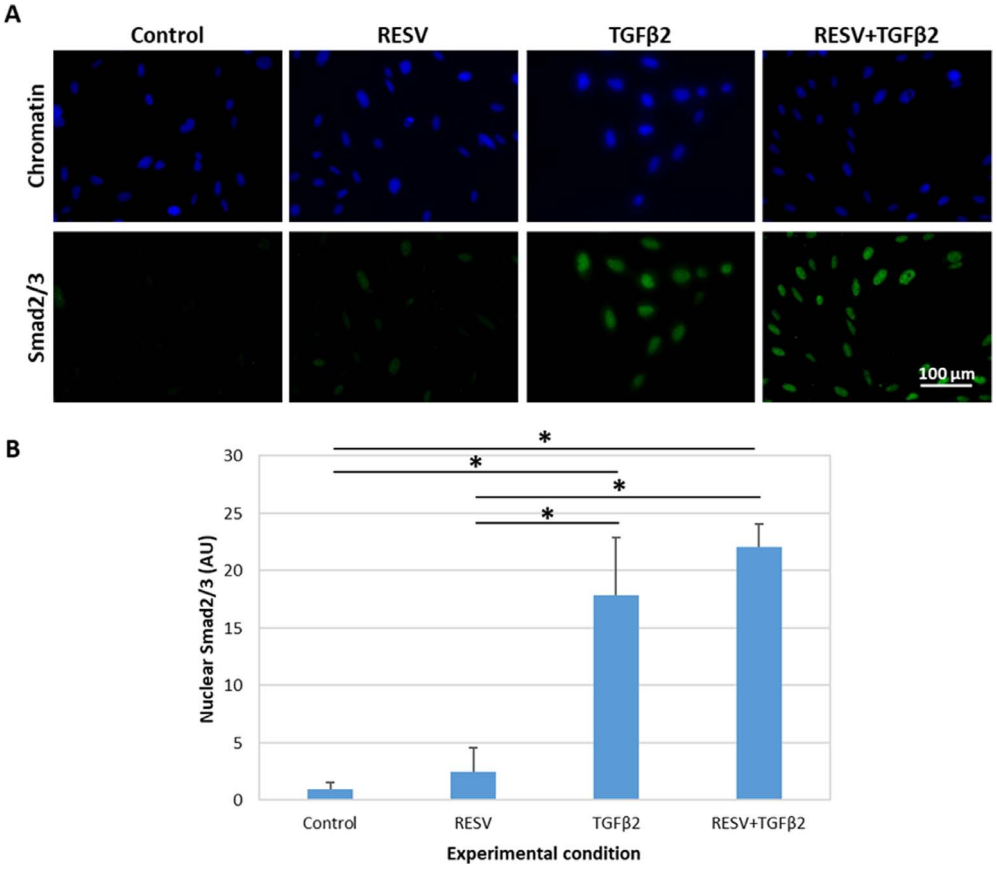

FIGURE 5. RESV treatment does not prevent TGFβ2-induced Smad2/3 nuclear translocation. FHL124 cells were maintained under control conditions (5% FCS-EMEM), treated with 30 µM RESV, 10 ng/mL TGFβ2 or 30 µM RESV + 10 ng/mL TGFβ2 for 2 hours. (**A**) Immunocytochemistry was performed to assess nuclear translocation of Smad2/3 (*green*). Cells were counterstained for chromatin with DAPI (*blue*). (**B**) The data are presented as mean ± SEM; $n = 3$. *Significant differences between experimental conditions (ANOVA with Tukey's post hoc test; $P \leq 0.05$).

match-paired control preparations. No significant differences in capsular wrinkling/matrix contraction was observed at endpoint between RESV-treated and untreated capsular bags, whereas treatment with 30 µM RESV in the presence of TGFβ2 significantly reduced capsular wrinkling/matrix contraction compared with that observed in capsular bags treated with TGFβ2 alone (Fig. 8).

Gene Expression Changes

Quantitative real-time PCR was used to assess whether RESV treatment could influence TGFβ2-induced changes in gene expression in human capsular bags. Match-paired experiments were performed with capsular bags cultured in 5% FCS-supplemented EMEM and treated with 30 µM RESV and/or 10 ng/mL TGFβ2, with ACTA2, FN1, MMP2, and TGFB2 gene expression assessed 48 hours posttreatment (Fig. 9). Treatment with 10 ng/mL TGFβ2 induced a significant increase in ACTA2, FN1, and MMP2 expression compared with untreated controls. No difference in TGFB2 expression was noted with TGFβ2 treatment compared with untreated controls. Treatment with 30 µM RESV caused a significant decrease in MMP2 and TGFB2 expression compared with untreated controls, with no difference observed between RESV-treated capsular bags and untreated controls in terms of ACTA2 or FN1 expression. In match-paired capsular bags treated with TGFβ2 or RESV + TGFβ2, RESV treatment significantly reduced TGFβ2-induced expression of ACTA2, FN1, MMP2, and TGFB2.

Central Anterior Lens Epithelium

Central anterior lens epithelium samples consisting of a circular portion of lens epithelial cells and central anterior capsule measuring approximately 0.5 cm in diameter were obtained during simulated cataract surgery on human donor eye tissue. Central lens epithelium samples were cultured in the presence of 30 µM RESV and/or 10 ng/mL TGFβ2 or maintained under control conditions for 7 days. α-SMA expression was assessed by immunocytochemistry (Fig. 10). Treatment with TGFβ2 caused a significant increase in α-SMA expression compared with all other treatment groups. No changes in α-SMA expression were noted in central lens epithelium samples treated with RESV alone compared with controls or those treated with RESV + TGFβ2 compared with those maintained under control conditions. The general organization of the F-actin cytoskeleton also differed with culture conditions, such that control samples presented a regular cobblestone appearance, whereas TGFβ treatment increased the presence of stress-fibers. Treatment with RESV in the presence or absence of TGFβ resulted in a pattern similar to controls.

Quantitative real-time PCR was also used to assess whether RESV treatment could influence TGFβ2-induced gene expression changes in central anterior lens epithelium samples. Match-paired experiments were performed with central anterior lens epithelium samples cultured in 5% FCS-supplemented EMEM and treated with 30 µM RESV and/or 10 ng/mL TGFβ2, with ACTA2, FN1, and MMP2 gene expression assessed 48 hours posttreatment (Fig. 11). Treatment with 10 ng/mL

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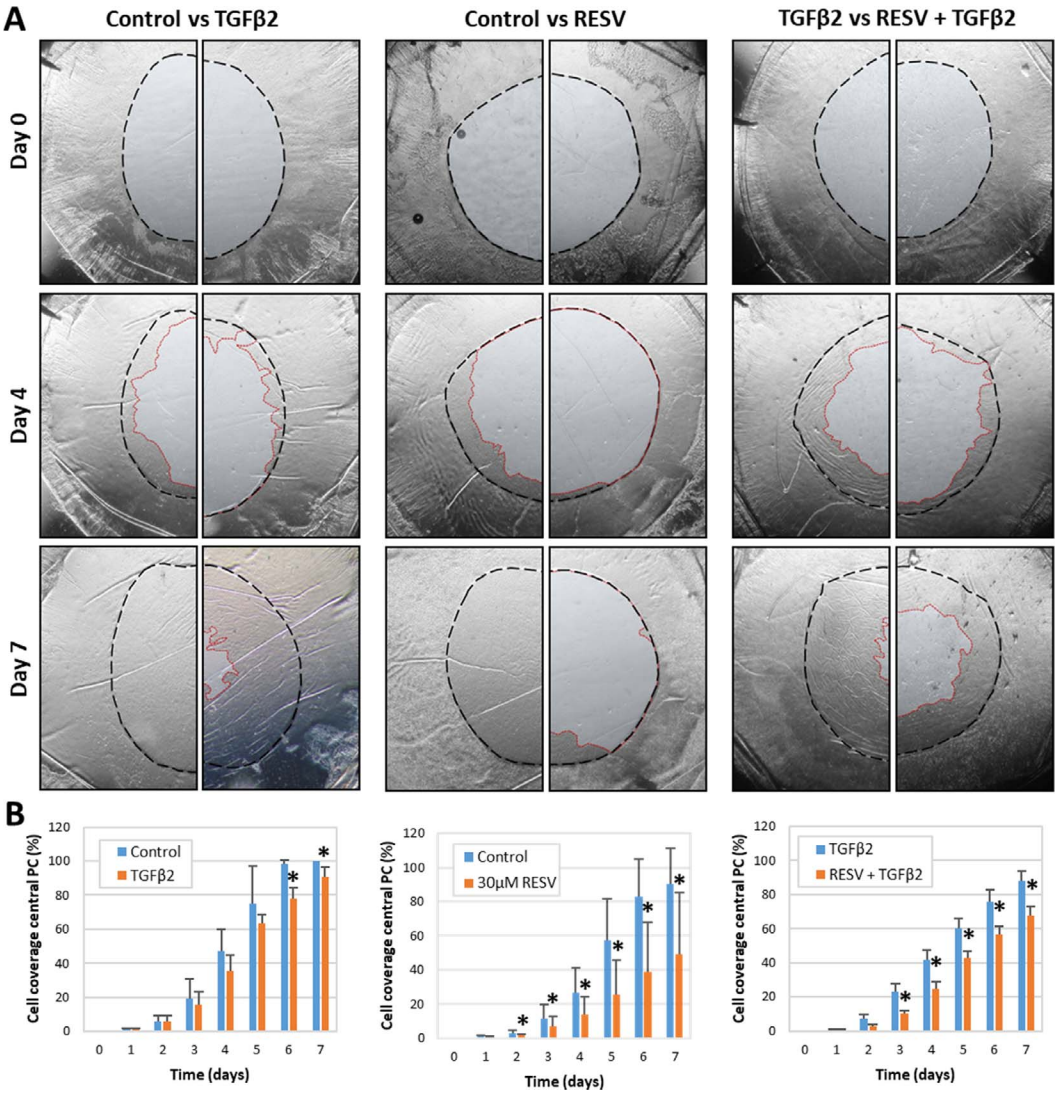

Figure 6. RESV inhibits lens cell growth across the posterior lens capsule of human capsular bags. (**A**) Representative images and (**B**) quantitative data established from multiple experiments. Match-paired capsular bags were placed into experimental conditions comparing control (5% FCS-EMEM) versus 10 ng/mL TGFβ2, control versus 30 μM RESV and 10 ng/mL TGFβ2 versus 30 μM RESV + 10 ng/mL TGFβ2. Experimental conditions were replaced at day 4 and cell growth across the posterior lens capsule analyzed daily by phase-contrast microscopy and image analysis software. The data are presented as mean ± SEM; $n = 6$. *Significant difference between experimental conditions (Student's *t*-test; $P \leq 0.05$).

TGFβ2 induced significant increases in ACTA2, FN1, and MMP2 expression compared with untreated controls. Treatment with 30 μM RESV caused a significant decrease in ACTA2 and MMP2 expression compared with untreated controls, with no difference noted in FN1 expression. In central anterior lens epithelium samples treated with TGFβ2 or RESV + TGFβ2, RESV treatment significantly reduced TGFβ2-induced expression of ACTA2, FN1, and MMP2 expression.

Discussion

PCO remains an important and common complication of cataract surgery.[2] Numbers undergoing surgery will increase as the population ages, and the need for better management of PCO is heightened. Improved IOL design has restricted PCO progression to some degree, but the problem is far from resolved.[19] Application of agents that can disrupt PCO in addition to improved IOL designs will provide the best opportunity to maintain a good level of visual quality after cataract surgery. The present body of work has demonstrated that the naturally occurring polyphenol, RESV, can retard growth/migration, reduce EMT, and suppress matrix contraction in human cell and tissue culture models of PCO, and thus indicates that RESV could serve as a useful therapeutic agent for this prevalent condition.

RESV was first isolated from hellebore roots and more recently found to be present in red wine.[20] RESV has since been identified as having therapeutic potential in the prevention of various diseases, including cancers, cardiovascular disease, and neurological disorders.[21] In addition to its reported antioxidant and anti-inflammatory properties,[21] RESV has been demonstrated to inhibit fibrosis in various disease models ranging from the liver,[13] gastrointestinal tract,[14] lung,[22] pancreas,[15] skin,[23] urinary tract,[16] and retina.[17] Evaluating its potential ability to prevent PCO, which is a fibrotic disorder, was therefore a logical step. PCO is characterized by growth and migration of lens epithelial cells that remain following cataract surgery to the previously cell-free posterior lens capsule. These cells can undergo EMT, secrete excessive

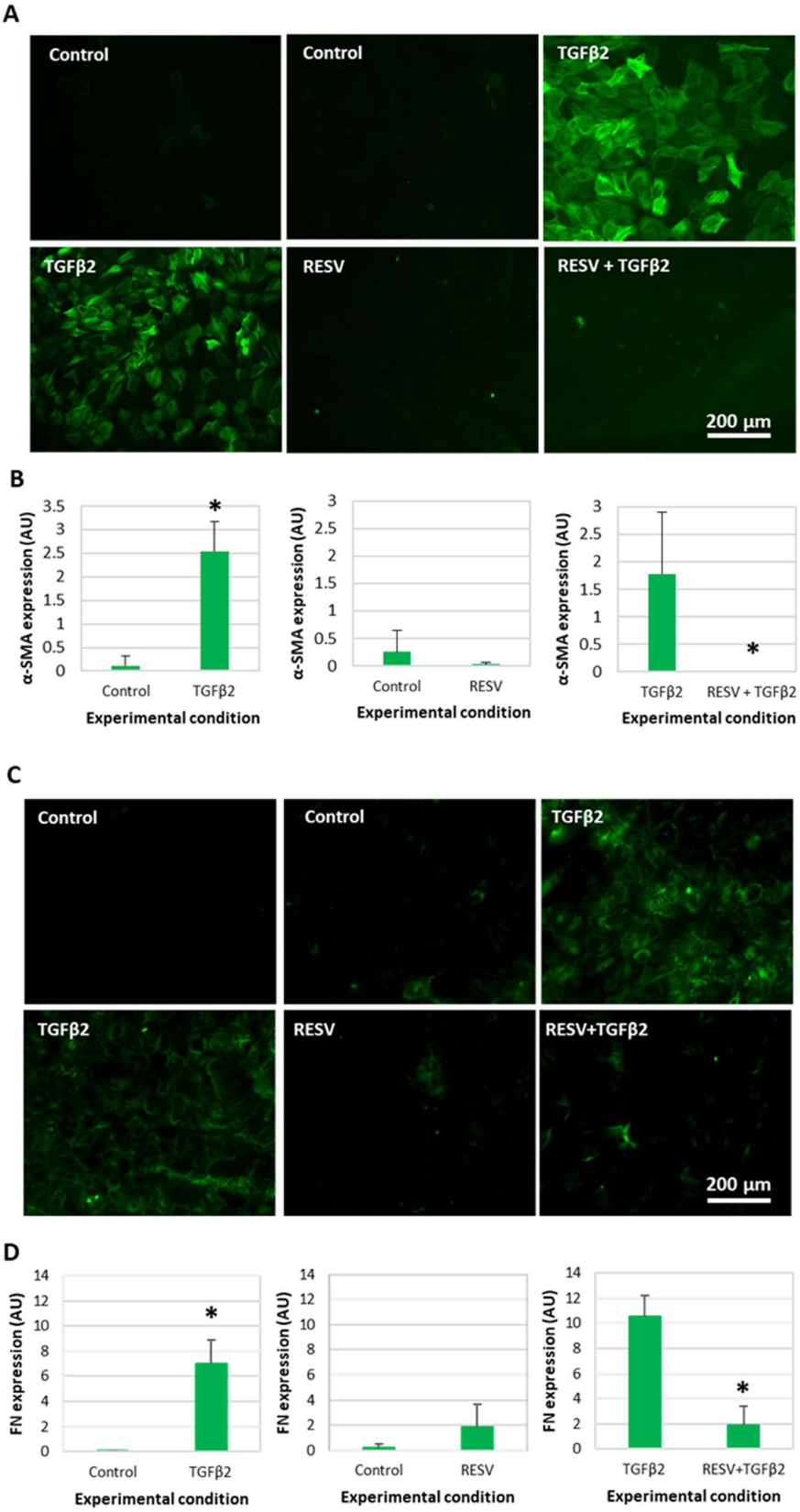

FIGURE 7. RESV suppresses TGFβ2-induced α-SMA and fibronectin expression in cells present on the posterior lens capsule of human capsular bags. (**A**, **C**) Representative images and (**B**, **D**) quantitative data established from multiple experiments. α-SMA expression or fibronectin (*green*) was assessed at experimental endpoint (day 7) by immunocytochemistry. Match-paired capsular bags were placed into experimental conditions comparing control (5% FCS-EMEM) versus 10 ng/mL TGFβ2, control versus 30 µM RESV, and 10 ng/mL TGFβ2 versus 30 µM RESV + 10 ng/mL TGFβ2. Experimental conditions were replaced at day 4. The data are presented as mean ± SEM; $n = 3$. *Significant difference between experimental conditions (Student's *t*-test; $P \leq 0.05$).

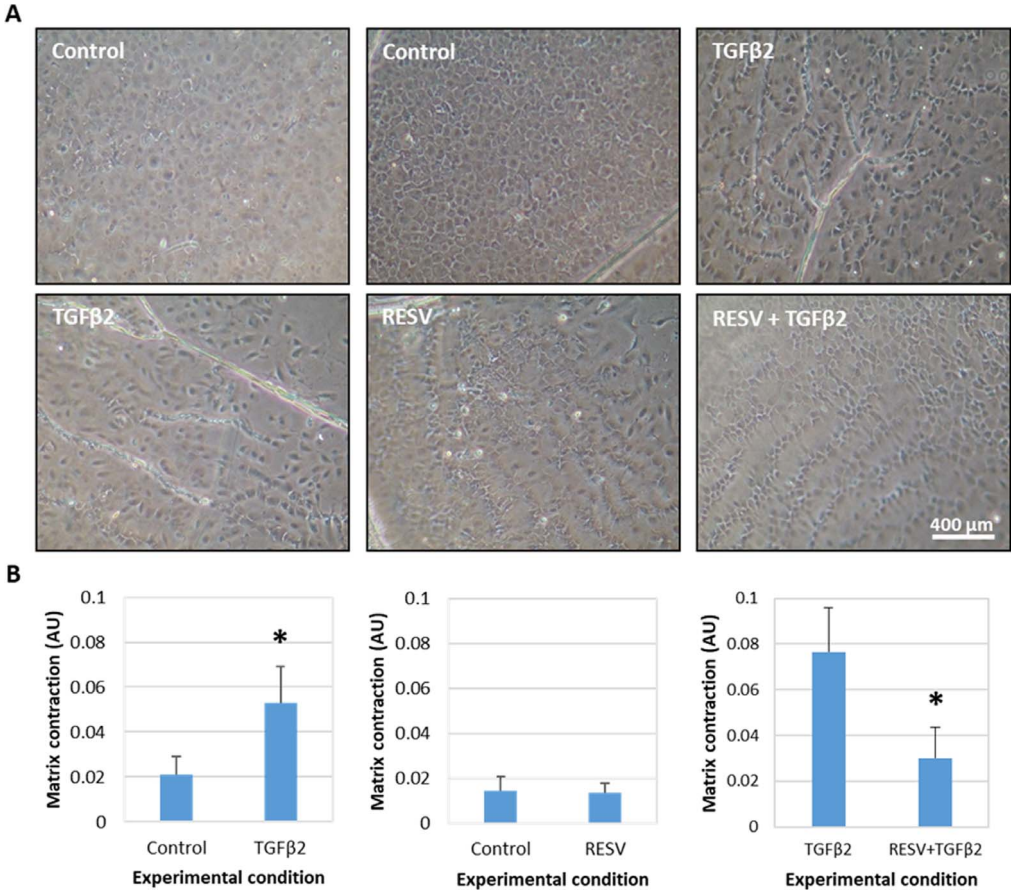

Figure 8. RESV suppresses TGFβ2-induced matrix contraction/posterior capsule wrinkling. (**A**) Representative images and (**B**) quantitative data established from multiple experiments. Capsular wrinkling was assessed at experimental endpoint (day 7) by image analysis. Match-paired capsular bags were placed into experimental conditions comparing control (5% FCS-EMEM) versus 10 ng/mL TGFβ2, control versus 30 μM RESV, and 10 ng/mL TGFβ2 versus 30 μM RESV + 10 ng/mL TGFβ2. Experimental conditions were replaced at day 4. The data are presented as mean ± SEM; $n = 7$ (5% FCS-EMEM versus 10 ng/mL TGFβ2 and control versus 30 μM RESV) or $n = 6$ (10 ng/mL TGFβ2 versus 30 μM RESV + 10 ng/mL TGFβ2). *Significant difference between experimental conditions (Student's *t*-test; $P \leq 0.05$).

extracellular matrix components, and cause the posterior lens capsule to contract or wrinkle.[5,10] Using human cell and tissue culture models allowed the effects of RESV on these features of PCO to be assessed.

The present body of work has revealed RESV is able to impede lens cell migration and wound healing. This is important in the context of PCO progression, as the invasion of cells within the visual axis provides the platform on which further light-scattering events can result. It should be pointed out that antimigratory actions of RESV were less pronounced in our cell-line assays than in the tissue culture capsular bag model. Previous work also has indicated that RESV can affect migration of various cancer cells,[24,25] and it has been suggested that RESV may inhibit migration of ARPE-19 retinal epithelial cells.[26]

RESV treatment resulted in a significant reduction in transdifferentiation from lens epithelial cells to myofibroblasts. The profibrotic cytokine, TGFβ is heavily involved in the development of PCO and EMT in particular.[5,10] We have demonstrated the ability of RESV to significantly inhibit the TGFβ2-induced expression of the myofibroblast marker, α-SMA, in a human lens cell line (FHL124) and human capsular bags following simulated cataract surgery, indicating the ability of RESV to prevent the EMT associated with PCO. EMT is also believed to play an important role in another lenticular condition, anterior subcapsular cataract (ASC).[10] This is linked to modification of the lens epithelium and is characterized by a fibrotic plaque that obscures the light path. TGFβ is also implicated in this condition, and it has been reported that ASC samples express increased TGFβ and TGFβ receptors along with the matrix components fibronectin and type I collagen and α-SMA.[27] Addition of TGFβ to cultured human anterior lens epithelium resulted in a dramatic increase in EMT. This TGFβ-induced change was ablated by RESV treatment. These results in the human lens reflect findings in other ocular tissues. For example, Ishikawa et al.,[17] investigating proliferative vitreoretinopathy, showed that RESV inhibited TGFβ2-induced EMT of RPE cells, countering a reduction of epithelial markers E-cadherin and ZO-1 by TGFβ2 and TGFβ2-induced increase in α-SMA expression.

Deformation of the lens capsule can lead to light scatter. It has been demonstrated in previous works that TGFβ can promote matrix modifications of the capsular bag that generate matrix contraction/wrinkles and are reflective of those observed in postmortem specimens.[28] Contraction or wrinkling of the posterior lens capsule ultimately results in the obstruction of the free passage of light to the retina and secondary loss of vision. RESV importantly was found to prevent matrix contraction/wrinkling of the posterior capsule and in a human lens cell contraction assay. Other works also have demonstrated effects of RESV on TGFβ-induced contraction of RPE cells using a collagen gel contraction assay.[26]

The canonical Smad signaling pathway plays an important role regarding EMT in the lens. Saika et al.[29] demonstrated that

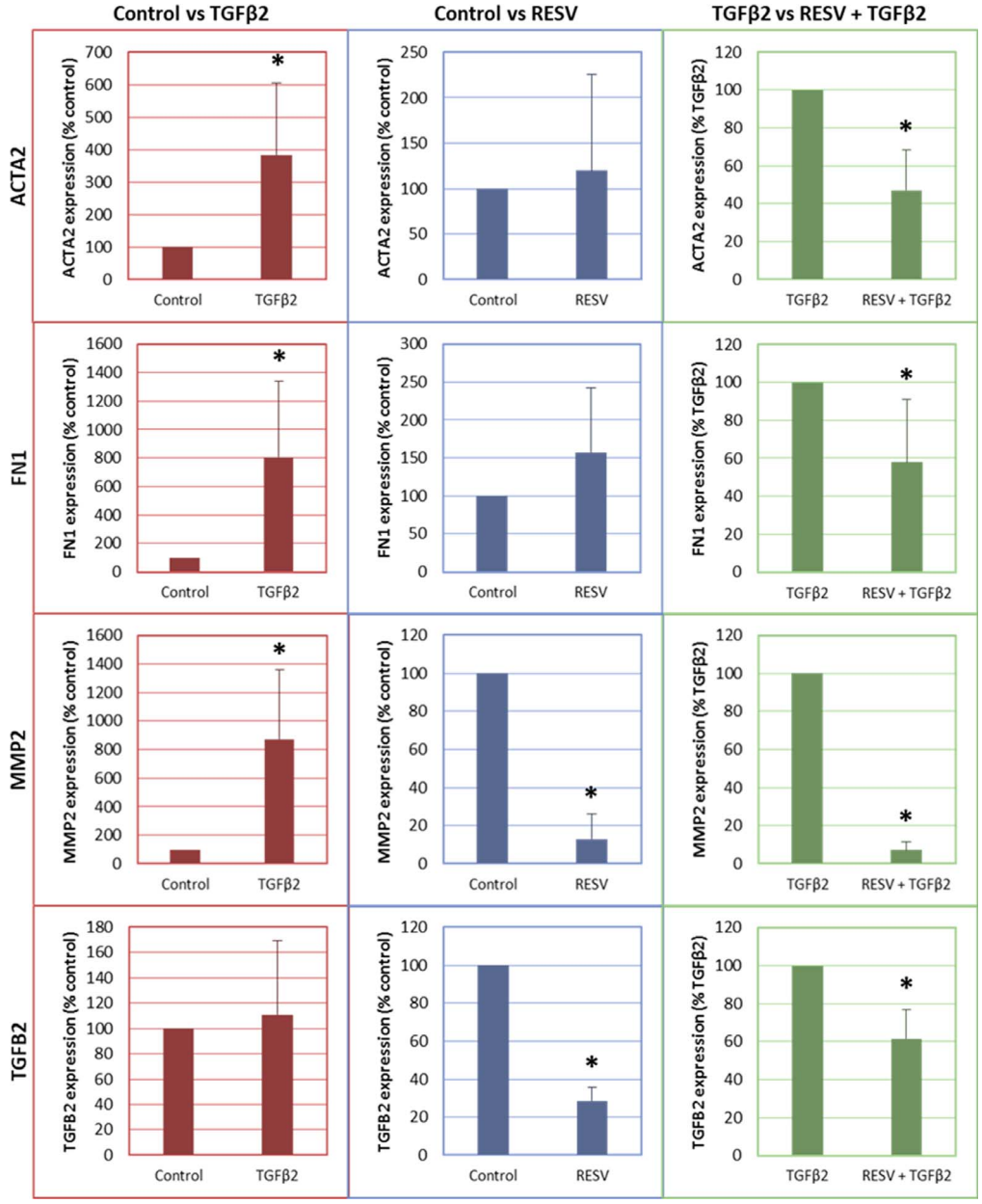

FIGURE 9. RESV suppresses expression of genes associated with TGFβ2 signaling in human capsular bags. Human capsular bags were treated comparing control (5% FCS-EMEM) versus 10 ng/mL TGFβ2, control versus 30 μM RESV, and 10 ng/mL TGFβ2 versus 30 μM RESV + 10 ng/mL TGFβ2 for 2 days. Real-time quantitative PCR was performed to assess changes in gene expression. The data are presented as mean ± SEM; $n = 4$. *Significant difference between experimental conditions (Student's *t*-test; $P \leq 0.05$).

Smad3 knockout mice presented significantly smaller anterior subcapsular plaques relative to wild-type. In the present study, we did not find a significant inhibition of Smad2/3 translocation to the nucleus in response to TGFβ in the presence or absence of RESV. This suggests that RESV does not affect initiation of the Smad signaling pathway, but may affect transcriptional activity. It is known that both Smad2 and 3 recruit p300 histone acetyltransferase to the MHII domain, which is believed to facilitate transcriptional activity to take place.[30] RESV is reported to increase Sirtuin activity,[31] and it is reported that Sirt1 and 2 can inhibit p300 function,[32] which could explain the downregulation in Smad-associated fibrotic genes observed in the current study.

TGFβ is also known to signal through Smad-independent pathways. These pathways, involving ERK and p38 for example, are stimulated in human lens cells by TGFβ.[33] Work in nonhuman systems has also suggested that ERK plays an important role in the initiation of EMT in the lens.[34] Moreover, it has been suggested that matrix contraction can be regulated by Smad-independent pathways without the requirement for EMT.[35] To test the putative therapeutic benefit of RESV in our studies, we maintained cells in serum-culture medium as a baseline to drive growth and migration. Although this provides an excellent test for therapeutic assessment, the ability to identify changes in TGFβ-induced Smad-independent signaling is confounded, as serum contains many factors that can drive these pathways. This does not diminish the potential involvement of Smad-independent signaling pathways in the PCO-related events observed, but extensive inhibition experiments will be required and will form the basis for future studies.

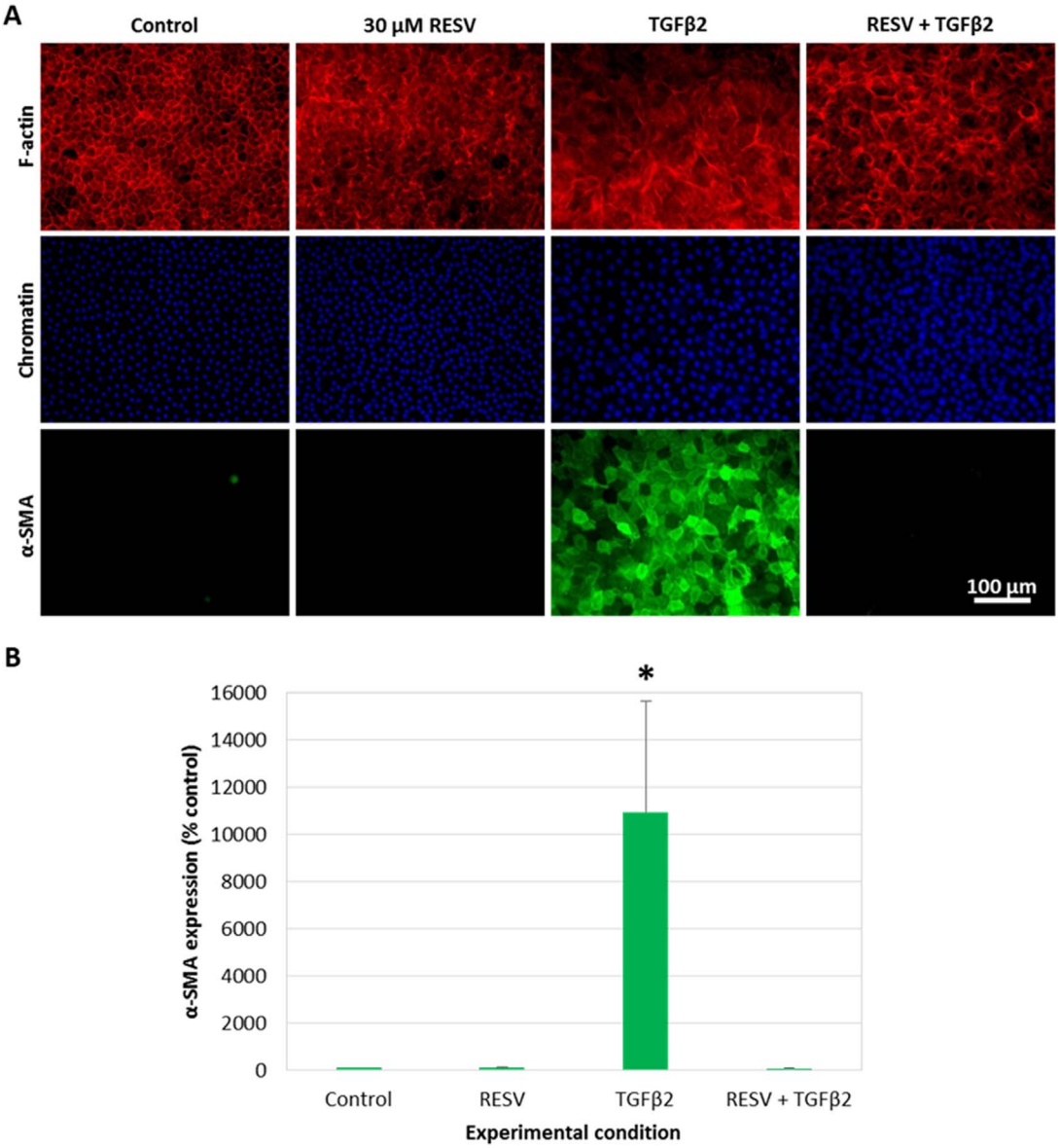

Figure 10. RESV suppresses EMT in human central lens epithelial cells. (**A**) Representative images and (**B**) quantitative data established from multiple experiments. Central lens epithelium samples were maintained in control conditions or treated with 30 µM RESV, 10 ng/mL TGFβ2, or 30 µM RESV + 10 ng/mL TGFβ2 with expression of α-SMA (*green*) assessed by immunocytochemistry. Expression was quantified using image analysis software. The data are presented as mean ± SEM; $n = 3$ *Significant difference between experimental conditions (ANOVA with Tukey's post hoc test; $P \leq 0.05$).

To further ascertain a mechanism that underpins the therapeutic benefit of RESV, we chose to observe changes in gene expression of the FHL124 cell line, central anterior lens epithelium, and capsular bag cultures under different culture conditions. A number of these genes were markers for specific PCO-related events. However, one of the genes investigated, MMP2, could feasibly play a role in multiple physiological events attributed to PCO.

A normal healthy lens epithelium within an intact lens has a relatively low-level expression of MMP2. This is also a general pattern observed for other secreted MMP family members.[36] However, culture or injury can provoke changes in expression level.[36] Previous work[37] and the present study have shown MMP2 to be upregulated in FHL124 cells following TGFβ2 treatment. The work in the current study demonstrates increased MMP2 gene expression in human capsular bag preparations in response to TGFβ2. This finding complements previous work that showed MMP2 protein was secreted at a greater level by capsular bags treated with TGFβ2.[28] Moreover, ex vivo cultures (removed from donors who had previously had surgery and developed PCO) are also known to secrete MMP2 when cultured.[28] MMPs are known to cleave extracellular matrix (ECM) components, such as collagen IV, a key component of the lens capsule, altering the ECM to expose sites of cellular attachment,[37–39] which could allow migration of lens epithelial cells. RESV-mediated suppression of MMP2 expression could provide an explanation as to how RESV is able to inhibit lens epithelial cell growth across the posterior lens capsule. In support of the present study's findings, previous work[40] demonstrated that inhibition of MMPs with a broad-spectrum MMP inhibitor prevented the migration of lens epithelial cells onto the posterior lens capsule of cultured human lens capsules. In addition, it also has been proposed that the lens capsule acts as a reservoir for growth and survival

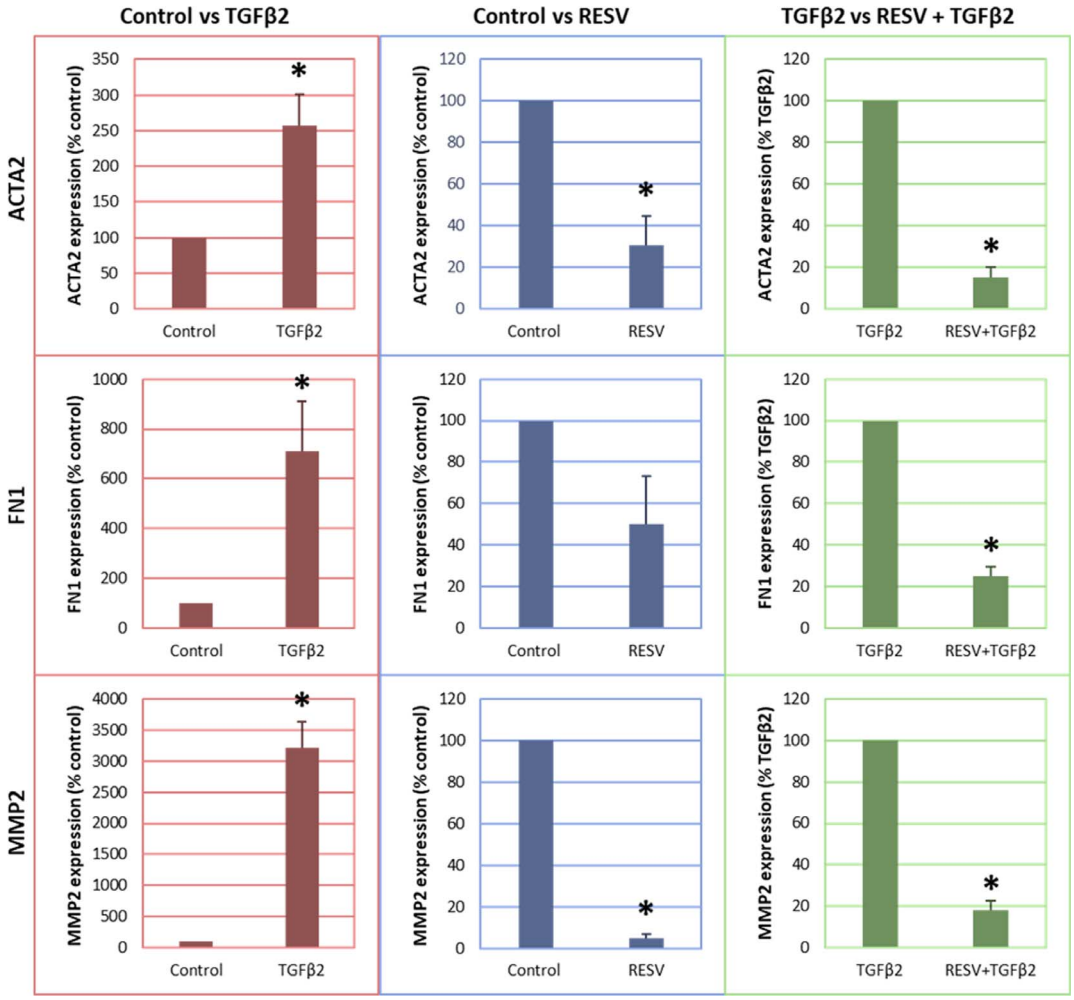

Figure 11. RESV suppresses expression of genes associated with TGFβ2 signaling in central anterior lens epithelium. Samples were treated comparing control (5% FCS-EMEM) versus 10 ng/mL TGFβ2, control versus 30 μM RESV, and 10 ng/mL TGFβ2 versus 30 μM RESV + 10 ng/mL TGFβ2 for 2 days. Real-time quantitative PCR was performed to assess changes in gene expression. The data are presented as mean ± SEM; $n = 4$ (control versus 10 ng/mL TGFβ2) or $n = 3$ (control versus 30 μM RESV or TGFβ2 versus RESV + TGFβ2). *Significant difference between experimental conditions (Student's t-test; $P \leq 0.05$).

factors, such as FGF-2, which are required for lens epithelial cell proliferation, migration, and differentiation. MMP2 has been found to facilitate the release of FGF-2 from the lens capsule[41] and thus provides another potential route by which RESV may influence lens epithelial cell migration across the posterior lens capsule. FGF2 also has been demonstrated to exacerbate TGFβ2-induced ASC formation in cultured rat lenses,[42] suggesting how suppression of MMP2 expression by RESV may be further involved in subduing events associated with PCO.

MMPs, principally MMP2 and MMP9, have been implicated in the activation of latent TGFβ to its active form, thus enabling TGFβ signaling to occur and drive the EMT process.[43] TGFβ is produced by FHL124 and lens cells within the capsular bag. This de novo pool of TGFβ could therefore further advance changes observed in the capsular bag cultures. If this autocrine source of TGFβ is to contribute, then activation will be required. It is therefore possible that RESV could lead to lower total and active levels of TGFβ within the capsular bag, which could contribute to the therapeutic benefits of RESV in PCO prevention in our models.

Furthermore, the importance of MMP2 in contraction of the extracellular matrix, specifically in the human lens, has been demonstrated. Eldred and colleagues[37] revealed that MMP2 levels are increased with TGFβ2 treatment, and that MMP2 activity is critical for TGFβ2-induced matrix contraction in human lens cells and the human capsular bag model, importantly suggesting an important role of MMP2 in PCO.

The contribution of MMPs in EMT and fibrotic disease also has been further highlighted in mouse models of ASC. Korol at al.[44] found that in mouse, MMP9, rather than MMP2 is important for TGFβ-induced ASC formation. This appears to differ from the human system in which MMP2 plays a greater role, but nevertheless it would appear that the gelatinases (MMP2 and 9) contribute to both ASC and PCO. Consequently the suppression of MMP2 by RESV is likely to have therapeutic benefit as a result of reduced EMT. It is important, however, to consider how RESV could be used to treat or prevent lens pathologies. In the case of ASC, this will require long-term application and most likely will require eye drops or be taken as a dietary supplement. RESV is a compound that exhibits rapid metabolism following oral consumption with limited bioavailability,[45] with one study finding no detectable RESV present in the aqueous or vitreous humors following oral consumption.[46] Despite much being made of RESV content in certain foods and red wine,[47] the authors consider that RESV

would have little therapeutic benefit as an oral therapy to prevent events associated with PCO. It would therefore appear that delivery to treat ASC would require eye drops, but the dosage and treatment regimen would require detailed pharmacological profiling and development to ensure adequate levels reach the lens, while preserving the integrity of other ocular tissues, in particular the cornea.

In the case of PCO, the scope for drug delivery is greater. Cataract surgery is invasive, and drugs can be applied directly to the lens cells during surgery using closed drug delivery systems[48,49] or through modification of the IOL or a tension ring,[50–52] and these options will be explored in the future.

The present study has shown that RESV can prevent three events pivotal for PCO development: cell proliferation/migration, EMT, and contraction of the posterior lens capsule.

RESV is an exciting therapeutic candidate to better manage PCO after cataract surgery, which could improve the well-being of millions.

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