

Bone Morphogenetic Protein-7 Suppresses TGF β 2-Induced Epithelial-Mesenchymal Transition in the Lens: Implications for Cataract Prevention

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PURPOSE. Epithelial-mesenchymal transition (EMT) of lens epithelial cells (LECs) is a key pathologic mechanism underlying cataract. Two members of the transforming growth factor- β (TGF β) superfamily, TGF β and bone morphogenetic protein-7 (BMP-7) have functionally distinct roles in EMT. While TGF β is a potent inducer of EMT, BMP-7 counteracts the fibrogenic activity of TGF β . We examine the modulating effect of BMP-7 on TGF β -induced EMT in LECs.

METHODS. Rat lens epithelial explants were treated exogenously with TGF β 2 alone or in combination with BMP-7 for up to 5 days. Expression levels of E-cadherin, β -catenin, α -smooth muscle actin (α -SMA), and phosphorylated downstream Smads were determined using immunofluorescence and Western blotting. Reverse transcriptase quantitative PCR (RT-qPCR) was used to study gene expression levels of EMT markers and downstream BMP target genes, including the Inhibitors of differentiation (*Id*).

RESULTS. Transforming growth factor- β 2 induced LECs to transdifferentiate into myofibroblastic cells. Addition of BMP-7 suppressed TGF β 2-induced α -SMA protein levels and mesenchymal gene expression, with retention of E-cadherin and β -catenin expression to the cell membrane. Addition of BMP-7 prevented lens capsular wrinkling and cellular loss associated with TGF β 2-induced EMT over the 5-day treatment period. The inhibitory effect of BMP-7 was accompanied by an early induction of pSmad1/5 and suppression of TGF β 2-induced pSmad2/3. Treatment with TGF β 2 alone suppressed gene expression of *Id2/3* and addition of BMP-7 restored *Id2/3* expression.

CONCLUSIONS. Exogenous administration of BMP-7 abrogated TGF β 2-induced EMT in rat lens epithelial explants. Understanding the complex interplay between the TGF β - and BMP-7-associated Smad signaling pathways and their downstream target genes holds therapeutic promise in cataract prevention.

Keywords: bone morphogenetic protein (BMP), transforming growth factor-beta (TGF β), epithelial-mesenchymal transition (EMT), cataract

Cataract, an opacification of the ocular lens, is the leading cause of blindness worldwide.¹ Although cataract surgery restores vision initially, a common postoperative complication is posterior capsular opacification (PCO). Advances in surgical techniques and intraocular lens design have reduced the prevalence of PCO, yet it remains a significant and uneradicated problem.² Currently, cataract surgery and Nd:YAG laser capsulotomy are the only effective treatments for cataract and PCO, respectively; however, they carry vision-related surgical complications and place major personal and financial burden on the aging population.² Hence, there is interest in the development of noninvasive pharmacologic alternatives to maintain lens transparency.

There is compelling evidence implicating epithelial-mesenchymal transition (EMT) in the pathogenesis of anterior subcapsular cataract (ASC) and PCO. During EMT, polarized lens epithelial cells (LECs) relinquish their cobblestone morphology and transdifferentiate into spindle-shaped myofibroblastic cells that elongate and migrate across the lens

capsule.³ Transforming growth factor- β (TGF β) has been studied extensively as a key inducer of this lens EMT *in vitro*^{3,4} and *in vivo*.⁵⁻⁷ While TGF β induces aberrant lens cell behavior, other growth factors found in the ocular media, such as bone morphogenetic protein-7 (BMP-7) regulate normal cellular processes in the lens. Specifically, BMP-7 and its receptors have been identified as key regulators of lens induction,⁸⁻¹⁰ gap-junction mediated communication,¹¹ and lens fiber differentiation¹²⁻¹⁴—all critical elements for proper lens development, maintenance, and function. Moreover, human genetic mutations in BMP-7 have resulted in severe eye defects, such as anophthalmia, in addition to kidney and skeletal defects.¹⁵

There is a growing body of literature supporting the role of BMP-7 in antagonizing TGF β -driven EMT.¹⁶ Several studies unequivocally demonstrate that exogenous administration of BMP-7 enhanced the recovery rate of TGF β -induced renal fibrosis.¹⁷⁻¹⁹ Adenoviral gene transfer of BMP-7 suppressed TGF β -driven corneal scarring in a mouse alkali burn-induced injury model.²⁰ In a human model of primary open angle

glaucoma, BMP-7 antagonized TGF β -induced expression of extracellular matrix proteins in trabecular meshwork cells.²¹ In the lens, however, there is a paucity of literature investigating the potential inhibitory role of BMP-7. Adenoviral-mediated overexpression of BMP target genes, Inhibitors of differentiation (Id)-2 and Id3, suppressed TGF β -induced EMT in a murine lens epithelial cell line.²² However, adenoviral-mediated expression of BMP-7 in situ delayed but did not completely inhibit EMT in a mouse lens injury-induced model.²³

We describe experiments in vitro investigating the role of BMP-7 in modulating TGF β -induced EMT in the lens. This is the first time exogenous administration of BMP-7 has been used to counteract TGF β -induced EMT in the lens epithelial explant system. To establish further evidence of the opposing effects of BMP-7 and TGF β we examined differences in activation of their respective downstream Smad signaling proteins. We found that inhibition of EMT by BMP-7 resulted in an increased phosphorylation of BMP-7-responsive Smads (Smad1/5) and a concurrent reduction in phosphorylation of TGF β -responsive Smads (Smad2/3). Moreover, we showed that TGF β suppresses expression of BMP-7 target genes, *Id2/3*, and the addition of BMP-7 restores these *Id2/3* levels. Taken together, we highlighted the contrasting roles of different TGF β superfamily members in the lens, in which tight regulation of BMP-7-responsive Smad-signaling and downstream target genes maintain the normal lens epithelial phenotype, and aberrant TGF β -responsive Smad-signaling leads to the EMT underlying fibrotic cataract formation.

METHODS

Animals

Ocular tissues were collected from postnatal-day-21 albino Wistar rats (*Rattus norvegicus*) that were euthanized by carbon dioxide asphyxiation and cervical dislocation. All animal procedures conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research (United States) and the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australia). All studies were approved by the Animal Ethics Committee of the University of Sydney (Australia).

Preparation of Lens Epithelial Explants

Lens epithelial explants were prepared as described previously.²⁴ All tissue culture was performed in Medium 199 with Earle's salts (Life Technologies, Waltham, MA, USA), supplemented with 0.1% BSA (Sigma-Aldrich Corp., St. Louis, MO, USA), 0.1 μ g/ml L-glutamine (Life Technologies), 100 IU/ml penicillin/100 μ g/ml streptomycin (Life Technologies), and 2.5 μ g/ml Amphostat B (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified 5% CO₂ incubator.

The effects of BMP-7 on TGF β -induced EMT were initially investigated by adding increasing doses (1, 5, 10, or 50 ng/ml) of recombinant human BMP-7 (R&D Systems, Minneapolis, MN, USA) to explants concurrently in the presence or absence of 200 pg/ml of recombinant human TGF β 2 (R&D Systems). Subsequent experiments were performed using 5 ng/ml of BMP-7 as this was the lowest inhibitory dosage of BMP-7. Lens epithelial cells were cultured for up to 5 days. For short-term exposure studies, TGF β 2 was added alone or in combination with BMP-7 for 2 hours after which the culture media was removed and replaced with fresh unsupplemented media. Cells in explants were viewed with phase-contrast microscopy

(CK2; Olympus, Tokyo Japan) and photographed (Leica DFC-280; Leica Camera, Wetzlar, Germany) before being harvested for immunofluorescence, Western blot or RT-qPCR analysis.

Assessment of Capsular Wrinkling

Following a 5-day culture period, explants were rinsed in water for 1 hour to remove the overlying cells, exposing the underlying lens capsule. The bare lens capsule was viewed using phase-contrast microscopy and photographed as described above.

Immunofluorescence

Explants were fixed in 100% methanol for 45 seconds and rinsed four times in phosphate-buffered saline (PBS). This was followed by rinsing (3 \times 5 minutes) in PBS supplemented with 0.1% (wt/vol) BSA (PBS/BSA). Explants then were incubated in 10% normal goat serum (NGS) diluted in PBS/BSA for 1 hour. Primary antibodies were diluted in PBS/BSA with 1.5% NGS and incubated overnight at 4°C in a humidified chamber. Anti-mouse antibodies specific for α -smooth muscle actin (α -SMA; A2547; monoclonal; Sigma-Aldrich Corp.), E-cadherin (4A2; monoclonal; Cell Signaling Technology, Danvers, MA, USA) and anti-rabbit antibodies specific for β -catenin (H-102, polyclonal; Santa Cruz Biotechnology, Dallas, TX, USA), and total-Smad2/3 (8685; monoclonal; Cell Signaling Technology) were all diluted at 1:100.

Following application of the primary antibody, explants were equilibrated to room temperature and any unbound primary antibody was removed by rinsing in PBS/BSA (3 \times 5 minutes). The appropriate secondary antibody was diluted 1:1000 in PBS/BSA and applied to the explants for 2 hours at room temperature in a dark humidified chamber. E-cadherin and α -SMA were detected using goat anti-mouse Alexa-Fluor 488 (Cell Signaling Technology). Beta-catenin was detected using goat anti-rabbit Alexa-Fluor 594 (Abcam, Cambridge, MA, USA). Explants were rinsed (3 \times 5 minutes) in PBS/BSA before counterstaining with 3 μ g/ml bisbenzimidazole (Hoechst 33342; Sigma-Aldrich Corp.) for 2 minutes. Explants then were rinsed in PBS/BSA (3 \times 5 minutes). Explants were mounted with 10% PBS (vol/vol) in glycerol and viewed with a Zeiss LSM-5Pa confocal microscope (Carl Zeiss AG, Jena, Germany).

SDS-Page and Western Blotting

Explants were rinsed in cold PBS and cell proteins were extracted in cold lysis buffer containing 2.5 mM EDTA, 25 mM Tris-HCl (pH 7.5), 0.375 NaCl, 250 mM sodium orthovanadate, 10 mM sodium deoxycholate, a protease inhibitor cocktail tablet (Complete; Roche Applied Science, Penzberg, Germany) and a phosphatase inhibitor tablet (PhosStop; Roche Applied Science). Homogenized samples were rotated for 2 hours at 4°C to facilitate lysis. Lysates were precleared by centrifugation at 15,500g for 15 minutes at 4°C. Protein content of the supernatant was quantified using the Micro BCA protein assay reagent kit (Thermo Fisher Scientific).

Protein lysates were mixed in a 1:1 ratio with Laemmli sample buffer (BioRad Laboratories, Hercules, CA, USA). Up to 10 μ g of lens explant protein extract was loaded onto 10% SDS-PAGE gels for electrophoresis for 1.5 hours at 200 V before being transferred onto an Immobilon polyvinylidene fluoride membrane (Merck Millipore, Billerica, MA, USA) for 2 hours at 100 V. The membrane then was incubated for 1 hour with a blocking solution of 5% (wt/vol) nonfat skim milk powder in 0.1% Tween-20 in Tris-buffered saline (TBST) for nonphosphorylated proteins and 2.5% BSA in TBST for phosphorylated proteins. Membranes were incubated over-

night at 4°C with the primary antibody. Anti-mouse antibodies specific against α -SMA (A2547; monoclonal; Sigma-Aldrich Corp.), E-cadherin (4A2; monoclonal; Cell Signaling Technology), GAPDH (G8795; Sigma-Aldrich Corp.), and anti-rabbit antibodies specific against β -catenin (H-102; Santa Cruz Biotechnology), phospho-Smad2/3 (D27F4; monoclonal; Cell Signaling Technology), total-Smad2/3 (8685; monoclonal; Cell Signaling Technology), and phospho-Smad1/5 (41D10; monoclonal; Cell Signaling Technology) were all diluted at 1:1000 with the exception of α -SMA and β -catenin, which were diluted at 1:2000.

Membranes were rinsed with TBST (3 \times 5 minutes) and incubated for 2 hours with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, either goat anti-mouse HRP-conjugated IgG or goat anti-rabbit HRP-conjugated IgG (both diluted 1:5000 in TBST; Cell Signaling Technology). Membranes then were rinsed (3 \times 10 minutes) in TBST and incubated for 2 minutes in Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore). Chemiluminescence signals were captured using the ChemiDoc MP imaging system (BioRad Laboratories) and densitometric analysis was performed using ImageLab software (BioRad Laboratories).

Total RNA Extraction and cDNA Synthesis

Following a 24-hour treatment period, explants were rinsed in cold PBS and total RNA was extracted using the Isolate II RNA Micro Kit (Bioline, Alexandria, NSW, Australia) according to the manufacturer's instructions. Concentration and purity of RNA was measured using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Integrity of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with 260/280 ratios greater than 2 and RNA integrity numbers greater than 8 were used for reverse transcription quantitative PCR (RT-qPCR) analysis.

Reverse Transcriptase-Quantitative PCR (RT-qPCR)

Total RNA (200 ng) was reverse-transcribed using the SensiFAST cDNA synthesis kit (Bioline) as per the manufacturer's instructions. Complementary DNA (cDNA) samples then were diluted 1:12 with nuclease-free water. Oligonucleotide primers (Table) were designed using Primer-BLAST to span the exon-exon junction.

All RT-qPCR reactions were performed using the SensiFAST SYBR No-ROX kit (Bioline). Reactions (10 μ l) were set up in a LightCycler 480, 384-well plate (Roche Diagnostics Ltd., Forrenstrasse, Switzerland) using a Freedom EV075 robotic station with Freedom EVOware Standard 3.2 software (Tecan, Port Melbourne, VIC, Australia) consisting of 4 μ l cDNA, 5 μ l SYBR, and 300 nM forward and reverse primers. Reverse transcriptase qPCR analysis was done using the Roche LightCycler 480 (Roche Diagnostics Ltd.) under the following thermal cycling conditions: 95°C for 2 minutes followed by 45 cycles consisting of denaturation (95°C, 5 seconds), annealing (60°C, 10 seconds), and extension (72°C, 15 seconds). At the end of each run, melting curve profiles (95°C for 5 minutes, 60°C for 1 minute, and then slowly heating at 0.11°C/s up to 98°C with continuous measurement of fluorescence per 5°C) to confirm amplification of specific transcripts. Standard curves were generated by serially diluting (1:2), covering an appropriate concentration range. Relative gene expression was determined as a ratio of the gene of interest and *GAPDH* using the second derivative maximum method of the LightCycler software (Roche Diagnostics Ltd.). All reactions,

TABLE. Oligonucleotide Primers

Gene Symbol	Gene Name	NCBI Accession Number	Forward Primer	Reverse Primer
<i>α-SMA</i>	Alpha-smooth muscle actin	NM_031004.2	5'-CTATGCTCTGCTTCATGCCA-3'	5'-CTCAGCGCTCAGCAGTAGTCA-3'
<i>COL1A1</i>	Collagen type I alpha 1 chain	NM_053304.1	5'-TGACTGGAAAGAGCGGAGAGT-3'	5'-GATAGCGACATCGGCAGGAT-3'
<i>Fln</i>	Fibronectin	NM_019143.2	5'-CCATCACTGGTCTGGAGCC-3'	5'-ACCAGTTGGGAAGCTCATC-3'
<i>CTGF</i>	Connective tissue growth factor	NM_022266.2	5'-GCGTGTGCACTGCCAAAAGAT-3'	5'-TGGCTCGCATCATAGTTGGG-3'
<i>N-Cadherin</i>	Neural-cadherin	NM_031333.1	5'-CTGCCATGACCTTCTACCGGA-3'	5'-GGGTCTGTTCAGGATGGCAAA-3'
<i>E-cadherin</i>	Epithelial-cadherin	NM_031334.1	5'-CTGGACCAGAGAGTTACCC-3'	5'-GGCACCGACCTCATTTCAA-3'
<i>Id2</i>	Inhibitor of differentiation-2	NM_013060.3	5'-CGACTGCTACTCCAAGTCA-3'	5'-ATTTCAGACCGCTGCAAGGAC-3'
<i>Id3</i>	Inhibitor of differentiation-3	NM_013058.2	5'-AGCCTCTTGGACGACATGAA-3'	5'-CTCAGCTGTCTGGATCGGGAG-3'
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	NM_017008.4	5'-AGACAGCCGCATCTTCTTGT-3'	5'-CTTTCCTGGGTAGAGTCAT-3'

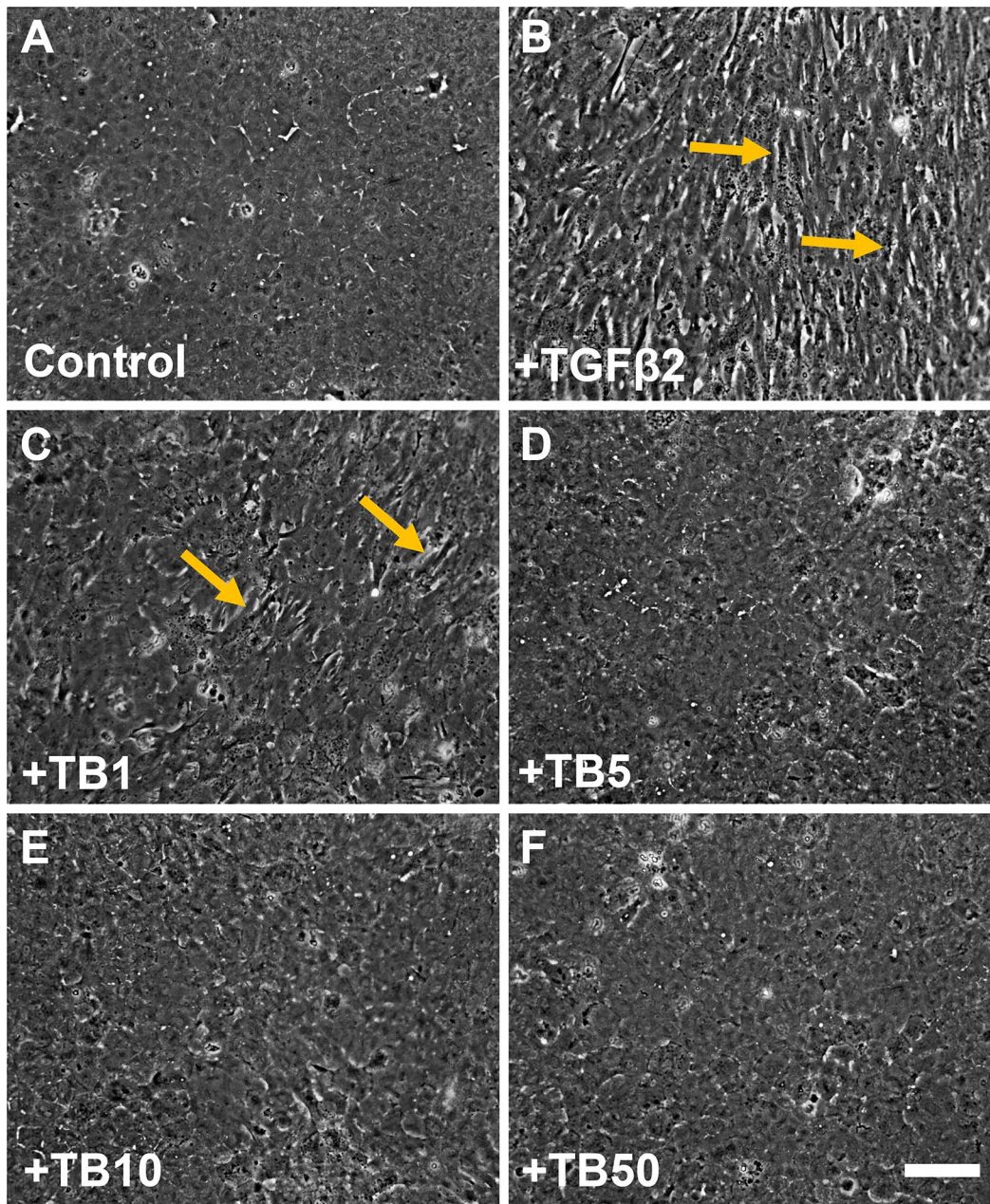


FIGURE 1. Bone morphogenetic protein-7 application inhibits TGF β 2-induced EMT in a dose-dependent manner. Following 48 hours of culture, untreated control explants (A) retained a normal cuboidal layer of epithelial cells, whereas cells in explants treated with TGF β 2 alone (B) displayed an elongate, spindle-shaped morphology (*yellow arrows*). Cotreatment with TGF β 2 and 1 ng/ml BMP-7 (C), +TB1) also resulted in elongate cells (*yellow arrows*), whereas higher doses of BMP-7 (5, 10, and 50 ng/ml; +TB5, +TB10, +TB50) retained the normal lens epithelial phenotype (D-F). Images are representative of 3 independent experiments. Scale bar: 100 μ m.

including no-template controls and minus RT controls, were run in duplicate.

Statistical Analysis

Each experiment was performed at least three times and graphing was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Western blot data were analyzed using GraphPad Prism version 6.0 (GraphPad Software, Inc.) by 1- or 2-way ANOVA with post hoc Tukey's multiple comparisons test. Reverse transcriptase PCR data were analyzed using the Light Cycler software (Roche Diagnostics Ltd.) and GenEx version 6.0 (MultiD Analyses AB,

Goteborg, Sweden) using an unpaired 2-tailed *t*-test. All data are presented as mean \pm SEM and *P* < 0.05 was considered statistically significant.

RESULTS

BMP-7 Blocks TGF β 2-Induced EMT of LECs in a Dose-Dependent Manner

Using phase-contrast microscopy, LECs in untreated explants remained as a cuboidal monolayer with a characteristic cobblestone-packed arrangement, indicative of a normal epithelial phenotype (Fig. 1A). At 48 hours, explants treated

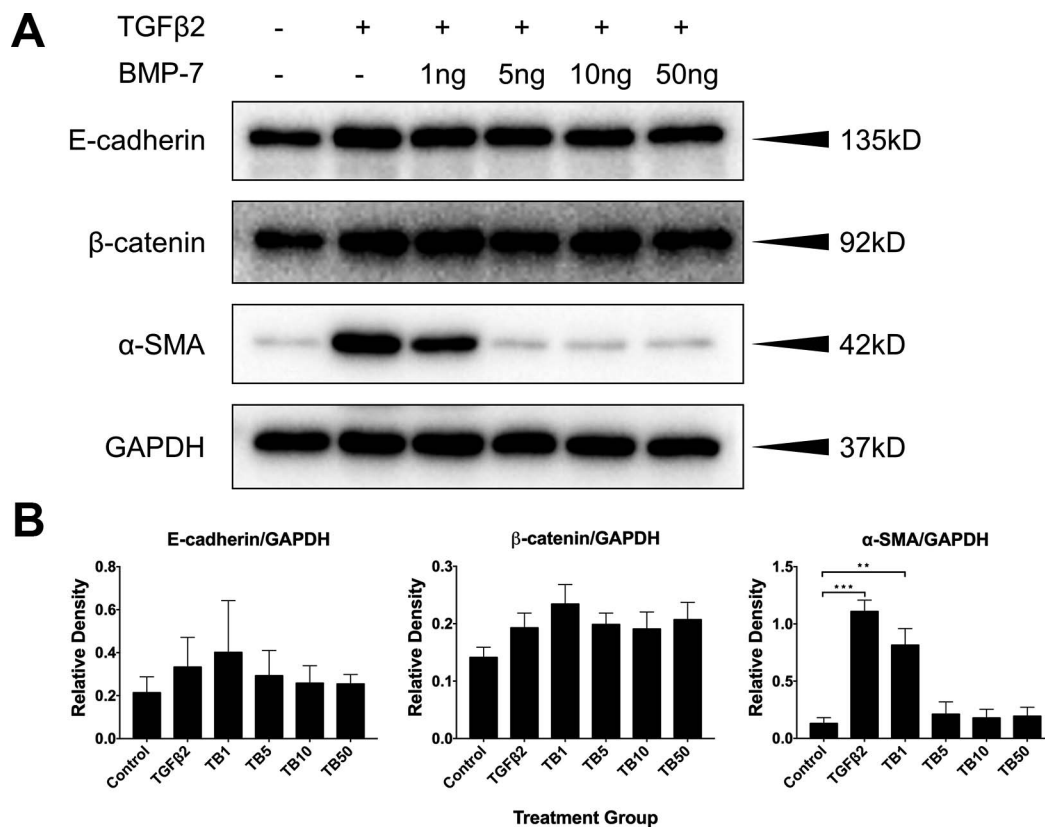


FIGURE 2. Bone morphogenetic protein-7 application inhibits TGFβ2-induced upregulation of α-SMA in a dose-dependent manner. Expression levels of epithelial and mesenchymal markers at 48 hours by Western blotting (**A**) and corresponding, relative densitometry analysis (**B**). In untreated control explants, lens epithelial cells showed minimal expression of the mesenchymal marker, α-SMA. Transforming growth factor-β2 alone and in combination with 1 ng/ml of BMP-7 significantly upregulated α-SMA expression compared to control explants. Complete inhibition of TGFβ2-induced α-SMA expression was evident with co-treatment of BMP-7 at higher doses (5 ng/ml and up), displaying no significant difference to control levels. No significant difference was observed in the levels of epithelial markers, E-cadherin, and β-catenin between all treatment groups. Representative Western blots from 3 independent experiments, with data presented relative to GAPDH, as the mean ± SEM. One-way ANOVA, Tukey's post hoc test $**P < 0.01$, $***P < 0.001$.

with TGFβ2 exhibited an elongate, spindle-shaped morphology, indicative of an EMT response (Fig. 1B). While the lowest dose of BMP-7 (1 ng/ml) in the presence of TGFβ2 resulted in similar elongation in many cells (Fig. 1C), higher doses of BMP-7 (from 5 ng/ml) displayed a retention of the normal epithelial phenotype, demonstrating suppression of TGFβ2-induced transdifferentiation (Figs. 1D–F).

To further explore the suppressive effect of BMP-7 on TGFβ2-induced EMT, we examined the expression of epithelial and mesenchymal markers in these treatment groups at 48 hours using Western blotting (Fig. 2A). Addition of TGFβ2 to LECs significantly upregulated α-SMA expression compared to cells in control explants ($P = 0.0001$, Fig. 2B). Cotreatment of LECs with TGFβ2 and 1 ng/ml of BMP-7 significantly upregulated α-SMA levels compared to that seen in control explants ($P = 0.0034$, Fig. 2B), and exhibited a slight, but not significant reduction in α-SMA levels compared to TGFβ2 alone-treated LECs ($P = 0.3314$, Fig. 2B). Cotreatment of higher doses of BMP-7 with TGFβ2 resulted in no difference in α-SMA levels compared to cells in control explants ($P = 0.9899$ for 5 ng/ml, $P = 0.9991$ for 10 ng/ml, $P = 0.9967$ for 50 ng/ml; Fig. 2B). As a result of this, the lowest suppressive dose of BMP-7 (5 ng/ml) was chosen for all subsequent blocking experiments. There was no significant effect in the expression of epithelial markers, E-cadherin, and β-catenin between all treatment groups ($P = 0.9293$ for E-cadherin, $P = 0.3341$ for β-catenin; Fig. 2B).

BMP-7 Application Blocks TGFβ2-Mediated Incorporation of α-SMA Into Stress Fibers

We examined the localization of α-SMA in LECs using immunofluorescence confocal microscopy. Cells in control explants displayed low basal levels of α-SMA immunoreactivity (Fig. 3A), while cells in explants treated with TGFβ2 exhibited strong immunoreactivity for α-SMA-labeled stress fibers, characteristic of an EMT response (Fig. 3B). Cotreatment with BMP-7 (5 ng/ml) inhibited TGFβ2-mediated labeling of α-SMA to stress fibers and resembled cells in control explants (Fig. 3C). Similarly, treatment with BMP-7 (5 ng/ml) alone showed no difference in α-SMA-labeling compared to cells in control explants (Fig. 3D).

BMP-7 Application Facilitates Retention of Epithelial Membrane Markers

Since the protein levels of E-cadherin and β-catenin remained similar in all treatment groups, we next investigated for changes in their spatial localization. In untreated cells, E-cadherin (Fig. 4A) and β-catenin (Fig. 4B) were localized to the cell membranes highlighting the cobblestone-packed arrangement. Although E-cadherin remained localized to the cell membrane in TGFβ2-treated explants, the expression pattern lacked the normal cobblestone-packed arrangement, appearing unevenly distributed in an elongate pattern (Fig. 4C). This was

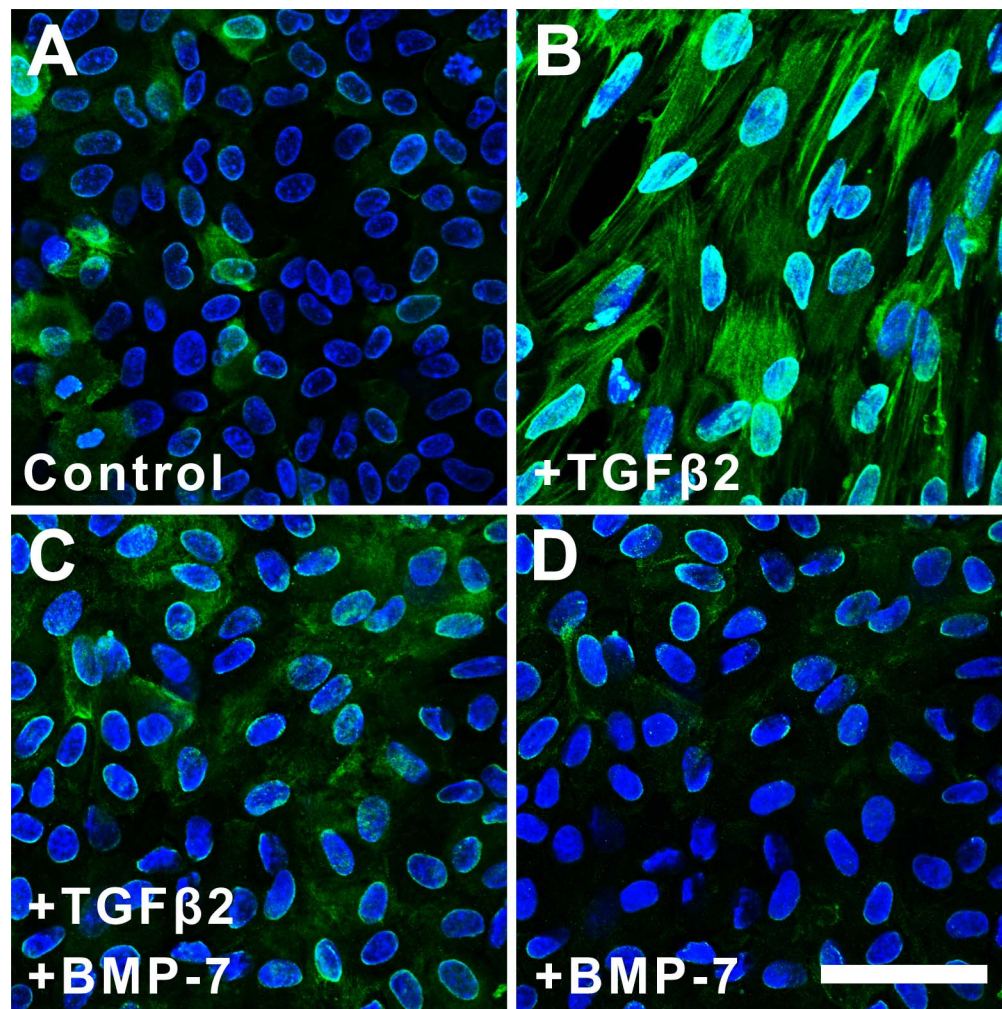


FIGURE 3. Bone morphogenetic protein-7 application blocks TGFβ2-mediated α-SMA. Immunofluorescence confocal microscopy of control explants at 48 hours showed low levels of basal cytoplasmic α-SMA immunoreactivity (A), while explants treated with TGFβ2 exhibited strong immunoreactivity for α-SMA, colabeling to stress fibers (B). Cotreatment with BMP-7 (5 ng/ml) and TGFβ2 (C) and treatment with BMP-7 alone (D) showed no difference in α-SMA labeling compared to control explants. Images are representative of 3 independent experiments. Scale bar: 50 μm.

in contrast to the lack of membranous localization of β-catenin in the same cells of these TGFβ2-treated explants (Fig. 4D). Specifically, β-catenin displayed a pronounced punctate labeling in the cell nuclei and cytoplasm following treatment with TGFβ2. Treatment with BMP-7 inhibited TGFβ2-induced dysregulation of these epithelial markers with retention of E-cadherin (Fig. 4E) and β-catenin (Fig. 4F) labeling to the membrane, again highlighting the cobblestone-packed arrangement of the LECs.

BMP-7 Application Reduces TGFβ2-Induced Mesenchymal Gene Expression

Following treatment of LECs with TGFβ2 for 24 hours, RT-qPCR showed a significant upregulation of mesenchymal gene expression compared to control explants: α-SMA ($P < 0.0001$), *Col1A1* ($P < 0.0001$), *fibronectin* ($P < 0.0001$), *CTGF* ($P < 0.0001$), and *N-cadherin* ($P = 0.0003$; Fig. 5). The addition of BMP-7 also significantly upregulated mesenchymal gene expression compared to control explants: α-SMA ($P = 0.016$), *Col1A1* ($P = 0.014$), *fibronectin* ($P = 0.0063$), *CTGF* ($P = 0.015$), and *N-cadherin* ($P = 0.0012$); however, this was to a significantly lesser extent when compared to TGFβ2 alone: α-

SMA ($P = 0.0094$), *Col1A1* ($P = 0.014$), *fibronectin* ($P = 0.0018$), and *CTGF* ($P = 0.0011$, Fig. 5). Interestingly, the addition of BMP-7 did not significantly reduce *N-cadherin* expression ($P = 0.86$) compared to TGFβ2 alone. It should be noted that TGFβ2 only weakly upregulated *CTGF* and *N-cadherin* showing changes less than 2-fold. Gene expression of the epithelial marker, *E-cadherin*, did not change significantly among all treatment groups, corroborating the Western blot data.

BMP-7 Application Blocks TGFβ2-Induced EMT and Apoptosis

To investigate whether the inhibitory effect of BMP-7 extended beyond 2 days, we followed the progression of TGFβ2-induced EMT over a five-day culture period. Progressive cell loss was evident from day 3 of culture indicated by cellular blebbing (dashed-line arrows) and exposed areas of bare lens capsule (asterisks, Fig. 6A). The exposed lens capsule also accentuated the presence of lens capsular wrinkling (solid-line arrows, Fig. 6A). By day 5 of culture with TGFβ2, there was extensive cell loss and lens capsular wrinkling (Fig. 6B). Explants cotreated with BMP-7 (5 ng/ml)

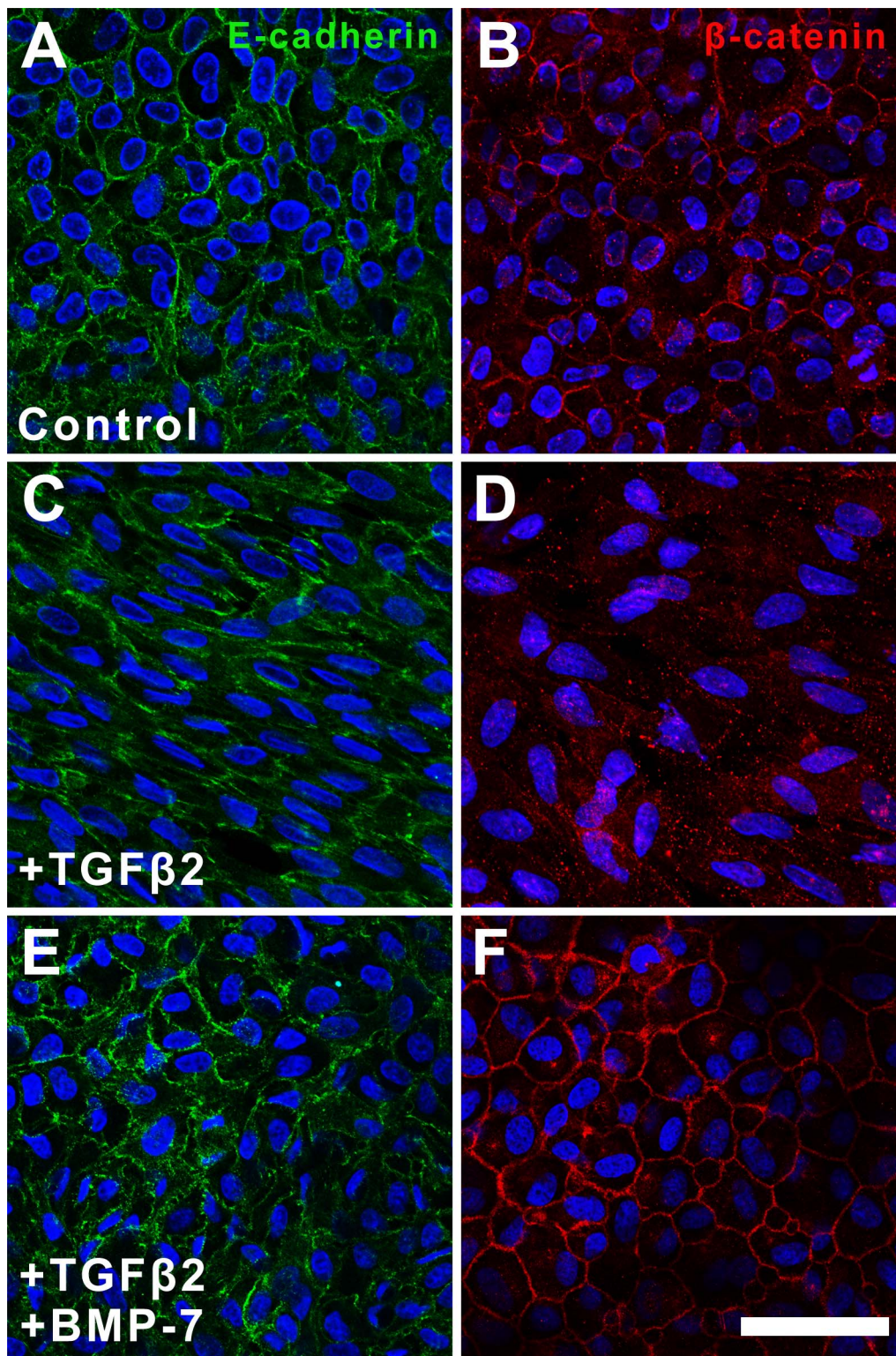


FIGURE 4. Bone morphogenetic protein-7 application facilitates retention of epithelial membrane markers. Immunofluorescence confocal microscopy at 48 hours showed that E-cadherin (A) and β -catenin (B) were localized to the cell membrane in control explants. In TGF β -treated explants, the expression pattern of E-cadherin (C) and β -catenin (D) appeared dysregulated and unevenly distributed. Treatment with BMP-7 inhibited TGF β -induced dysregulation of epithelial markers and restored E-cadherin (E) and β -catenin (F) labeling to the membrane. Images are representative of 3 independent experiments. Scale bar: 50 μ m.

and TGF β 2 retained a confluent epithelial monolayer throughout the 5-day culture period (Figs. 6C, 6D), with no evidence of capsular wrinkling (Fig. 6F) when we removed the overlying cells, compared to the extensive lens capsular

wrinkling in TGF β 2-treated alone explants (Fig. 6E). Untreated explants (Fig. 6G) and explants treated with BMP-7 alone (Fig. 6H) retained a monolayer of epithelial cells with no indication of cell loss (Figs. 6G, 6H).

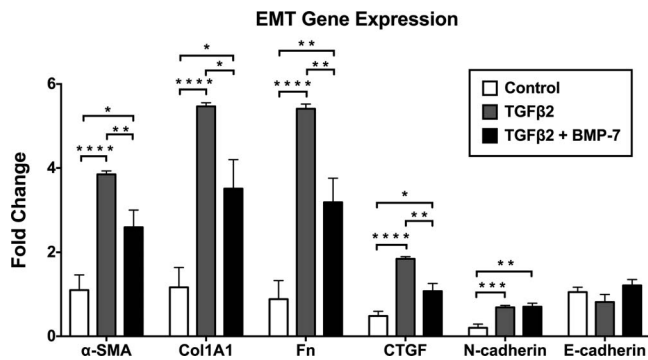


FIGURE 5. Bone morphogenetic protein-7 application blocks TGFβ-induced mesenchymal gene expression. Using RT-qPCR, treatment with TGFβ2 alone for 24 hours resulted in a significant upregulation of mesenchymal gene expression (*α-SMA*, *Col1A1*, *fibronectin*, *CTGF*, *N-cadherin*) compared to control. The addition of BMP-7 significantly reduced mesenchymal gene expression compared to TGFβ2 alone; however, a significant difference to control cells was still evident. Bone morphogenetic protein-7 did not significantly reduce *N-cadherin* expression compared to TGFβ2 alone. Gene expression of *E-cadherin*, also did not change significantly amongst all treatment groups. Data from 4 independent experiments are presented relative to GAPDH as fold change \pm SEM. Unpaired *t*-test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Immunofluorescence labeling of occasional cells remaining in explants treated with TGFβ2 at 5 days showed α-SMA-labeled stress-fibers (Fig. 7A) and punctate cytosolic β-catenin (Fig. 7B). In contrast, cells in explants cotreated with BMP-7 and TGFβ2 retained an epithelial phenotype, with little to no α-SMA label (Fig. 7C), and membranous β-catenin expression, highlighting their packed arrangement (Fig. 6D).

BMP-7 Application Modulates TGFβ2-Induced Smad Signaling

To examine the signaling pathways involved in mediating the inhibitory effect of BMP-7 on TGFβ2-induced EMT, we monitored for changes in the respective phosphorylated Smads during the first 6 hours following treatment (Fig. 8A). Treatment of LECs with BMP-7 alone induced a sustained upregulation of pSmad1/5 that was significantly higher than treatment with TGFβ2 alone at 2 ($P = 0.0280$), 4 ($P = 0.0114$), and 6 ($P = 0.0161$) hours (Fig. 8B). Cotreatment of cells with BMP-7 and TGFβ2 exhibited an early peak of pSmad1/5 signaling at 20 minutes, that was significantly higher than with TGFβ2 alone at this same time point ($P = 0.0112$; Fig. 8B). Lens epithelial cells treated with BMP-7 alone did not have altered basal pSmad2/3 levels. Treatment of LECs with TGFβ2 showed a marked increase of pSmad2/3, peaking at 2 hours compared to BMP-7 alone ($P = 0.0313$; Fig. 8C). Cotreatment with BMP-7 significantly reduced this 2-hour peak of TGFβ2-induced pSmad2/3 in LECs ($P = 0.0048$; Fig. 8C).

These findings were further corroborated with immunofluorescence analysis of total Smad2/3 (tSmad2/3) localization. Control explants displayed cytoplasmic labeling of tSmad2/3 at 20 minutes and 2 hours (Figs. 9A, 9B). Exposure of LECs to TGFβ2 for 20 minutes and 2 hours demonstrated localization of tSmad2/3 labeling to cell nuclei (Figs. 9C, 9D). The addition of BMP-7 at 20 minutes or 2 hours reduced TGFβ2-induced nuclear translocation of tSmad2/3, with retention of cytoplasmic tSmad2/3, similar to control explants (Fig. 9E); however, at 2 hours, some nuclear translocation of tSmad2/3 still was apparent (Fig. 9F).

Short-Term Exposure to BMP-7 Blocks TGFβ2-Induced EMT and Apoptosis

Given that BMP-7 induced a peak of pSmad1/5 signaling at 20 minutes and suppression of TGFβ2-induced pSmad2/3 at 2 hours, we next asked whether the inhibitory effect of BMP-7 was dependent on this early time point. Lens epithelial cells exposed to TGFβ2 alone for 2 hours continued to undergo EMT over the 5-day culture period with characteristic cell elongation and cell loss, but interestingly, minimal lens capsular wrinkling (Figs. 10A, 10B). The addition of BMP-7 for 2 hours blocked TGFβ2-induced EMT with retention of a monolayer of epithelial cells over the entire culture period (Figs. 10C, 10D).

Immunofluorescence labeling of control explants showed minimal α-SMA label (Fig. 11A) and membranous β-catenin expression (Fig. 11B). Explants treated with TGFβ2 at 5 days showed α-SMA-reactive stress-fibers (Fig. 11C) and punctate cytosolic β-catenin (Fig. 11D). Interestingly, LECs cotreated with BMP-7 and TGFβ2 for 2 hours displayed an upregulation in cytoplasmic α-SMA expression (Fig. 11E) despite retaining membranous β-catenin labeling highlighting the cobblestone cell arrangement (Fig. 11F).

BMP-7 Inhibits TGFβ2-Induced Suppression of Id2 and Id3 Gene Expression

As the Id proteins are reported to be downstream effectors of BMP signaling, we investigated the gene expression of *Id2* and *Id3* using RT-qPCR. Treatment with TGFβ2 alone significantly downregulated the expression of *Id2* ($P = 0.00014$) and *Id3* ($P = 0.0001$) compared to levels in control explants. The addition of BMP-7 significantly inhibited TGFβ2-induced suppression of *Id2* ($P = 0.0023$) and *Id3* ($P = 0.014$), showing no significant difference from control cells for *Id2* ($P = 0.50$) and *Id3* ($P = 0.62$) (Fig. 12).

DISCUSSION

The present study showed, for the first time to our knowledge, the direct ability of BMP-7 in blocking TGFβ2-driven EMT in the rat lens explant system. We demonstrated that exogenous administration of BMP-7 inhibited TGFβ2-induced myofibroblastic transdifferentiation by blocking cell elongation and incorporation of α-SMA into stress fibers. Addition of BMP-7 suppressed TGFβ2-induced upregulation of mesenchymal gene expression of ECM markers including *collagen 1A1*, *fibronectin*, and *connective tissue growth factor*. Although *N-cadherin* was not suppressed by the addition of BMP-7, the fold change in expression of this gene was below 2 for all treatment groups. Furthermore, BMP-7 promoted membranous localization of epithelial markers, E-cadherin, and β-catenin, maintaining the cobblestone-packed arrangement of cells, compared to the dysregulated and punctate cytosolic expression of these markers in LECs treated with TGFβ2 alone. Hence, BMP-7 promotes the epithelial phenotype, emphasizing its role in maintaining lens cell integrity and polarity.

We showed that the inhibitory effect of BMP-7 extends over the entire culture period examined, with a monolayer of cuboidal LECs retained by day 5. The progressive cell elongation, cell blebbing, extensive cell loss, and capsular wrinkling observed in TGFβ2-induced EMT over 5 days has been well characterized in the rat lens epithelial explant system and mimics morphologic features of human ASC and PCO.³ The cell loss induced by TGFβ2 has been reported to occur through apoptosis, corroborating the apoptotic features typically observed in human ASC and PCO, such as chromatin

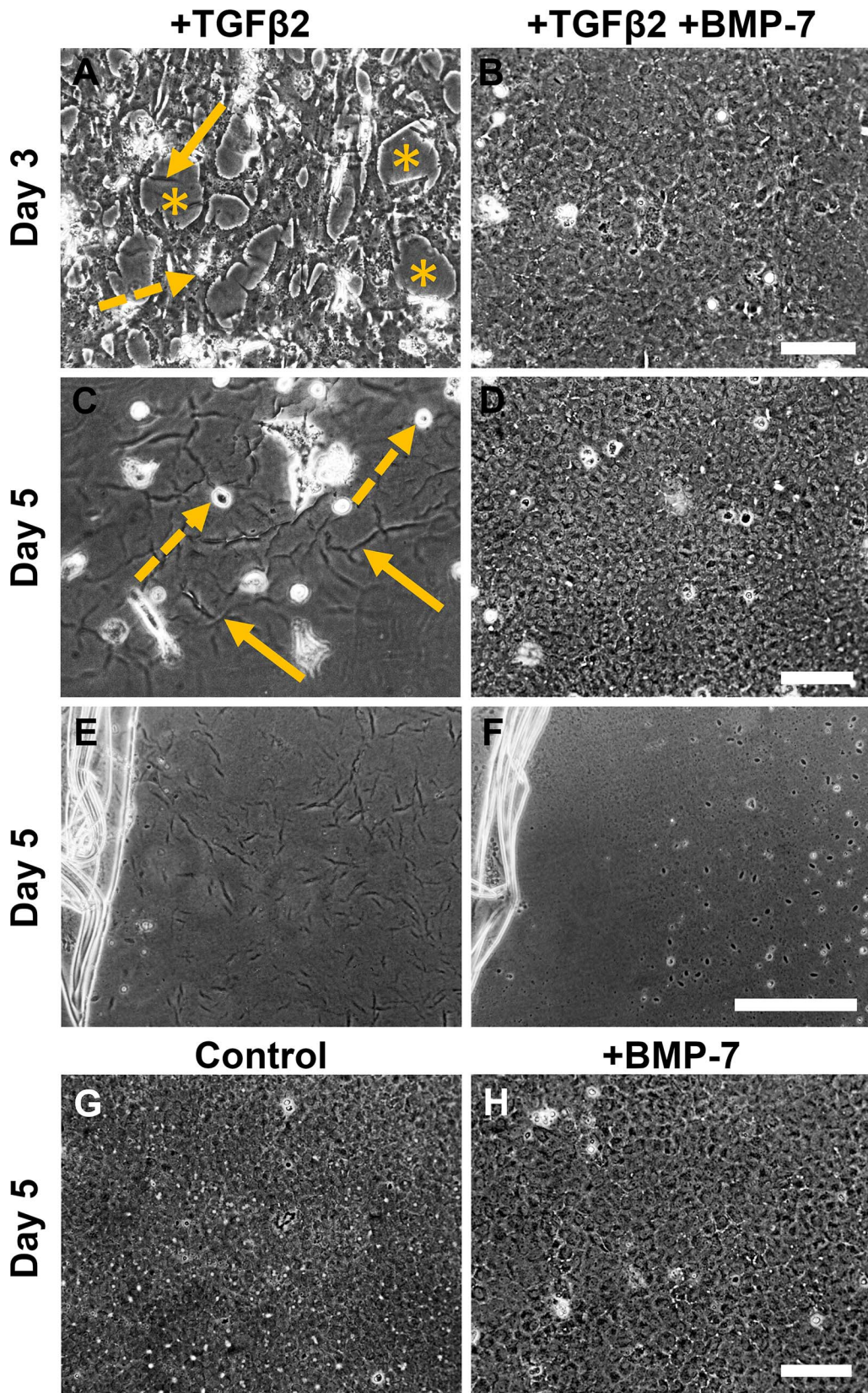


FIGURE 6. Bone morphogenetic protein-7 application blocks TGFβ2-induced EMT and apoptosis. Phase-contrast microscopy showed that treatment with TGFβ2 resulted in progressive cell loss from day 3 of culture (A), indicated by cellular blebbing (*dashed-line arrows*), exposed areas of bare lens capsule (*asterisks*), and resultant lens capsular wrinkling (*solid-line arrows*). By day 5 of culture (B), there is marked cell loss and more apparent lens capsular wrinkling (*solid-line arrows*). Explants cotreated with BMP-7 and TGFβ2 retained a confluent epithelial monolayer throughout the 5-day culture period (C–D), appearing similar to untreated control explants (E) as well as explants treated with BMP-7 alone (F). Images are representative of 3 independent experiments. *Scale bar:* 100 μm.

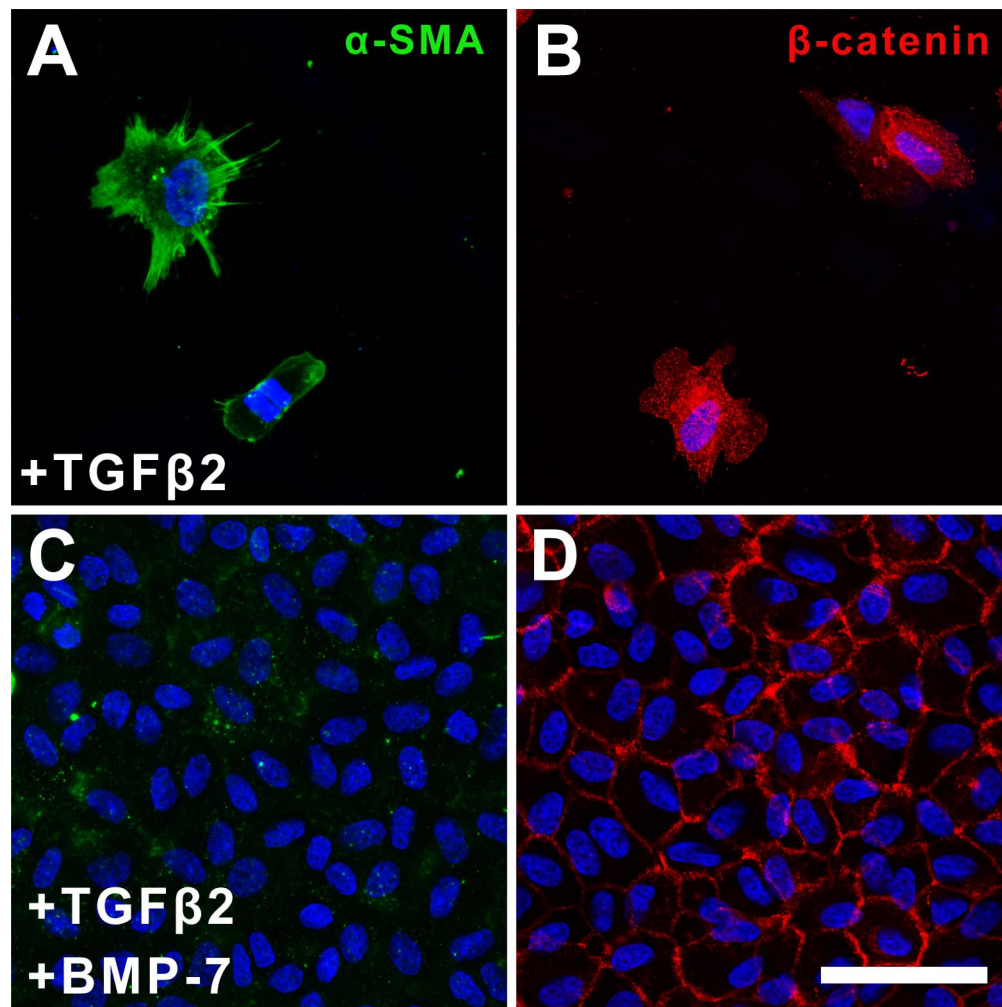


FIGURE 7. Bone morphogenetic protein-7 application blocks TGF β 2-induced α -SMA expression at 5 days of culture. Immunofluorescence confocal microscopy of the occasional remaining cells in TGF β 2-treated explants at day 5 of culture showed stress-fibers labeled for α -SMA (A) and punctate cytosolic β -catenin labeling (B). Cells in explants cotreated with BMP-7 and TGF β 2 showed minimal α -SMA expression (C) and membranous β -catenin labeling (D). Images are representative of 3 independent experiments. Scale bar: 50 μ m.

margination and cytoplasmic condensation.²⁵ In the present study, BMP-7 promoted cell survival by preventing TGF β 2-induced apoptosis. Moreover, BMP-7 prevented EMT-associated lens capsular wrinkling, a key feature of human PCO, highlighting its role in blocking myofibroblastic cell trans-differentiation whose contractility is believed to disrupt the surface integrity and optical properties of the lens capsule.²⁶

To elucidate putative elements of the signaling cascade used by BMP-7 to antagonize TGF β 2-induced EMT, we investigated the expression of phosphorylated Smads. Both BMP-7 and TGF β bind to distinct type II serine-threonine kinase receptors, which then form a complex with specific type I receptors.²⁷ The activated receptor complex relays the signal to the cytoplasm by phosphorylating Smad2/3 in TGF β signaling and Smad1/5/8 in BMP signaling.²⁷ Phosphorylated Smads then hetero-oligomerize with Smad4, shared by TGF β and BMP signaling, that translocates to the nucleus to regulate transcription of respective target genes.²⁷

In this study, treatment with BMP-7 alone increased phosphorylated Smad1/5 with protein levels sustained over the 6-hour culture period, indicating that the Smad signaling pathway can be activated in rat LECs by exogenous BMP-7. Furthermore, in explants cotreated with BMP-7 and TGF β 2, we showed that BMP-7 induces a significant increase in pSmad1/5

signaling, and concurrently reduces TGF β 2-induced Smad2/3 phosphorylation. These findings were corroborated by immunofluorescence data demonstrating that the addition of BMP-7 suppressed TGF β 2-induced nuclear translocation of tSmad2/3 at least early in the culture period. This is consistent with previous studies demonstrating that the mechanism underlying the antagonistic effect of BMP-7 is through direct Smad-dependent inhibition of the TGF β pathway, both in renal¹⁷ and pulmonary fibrosis.²⁸ Interestingly, in the present study, activation of pSmad1/5 is evident only at 20 minutes and falls to basal levels thereafter, suggesting that TGF β 2 also may abrogate BMP-7 signaling to some extent, albeit at a later time-point.

In the present study, short-term treatment of LECs with TGF β 2 for 2 hours was sufficient to induce an EMT response over the 5-day treatment period. Our data supported human studies where LECs exposed to TGF β 2 for a brief 2-day period resulted in significant and persistent long-term fibrotic events over a long 28-day culture period.²⁹ Cotreatment with TGF β 2 and BMP-7 for 2 hours blocked the EMT process with retention of an epithelial monolayer of LECs displaying membranous β -catenin localization. Interestingly, α -SMA expression still was upregulated despite the addition of BMP-7; however, it was not incorporated into stress fibers, hence, possibly unable to exert

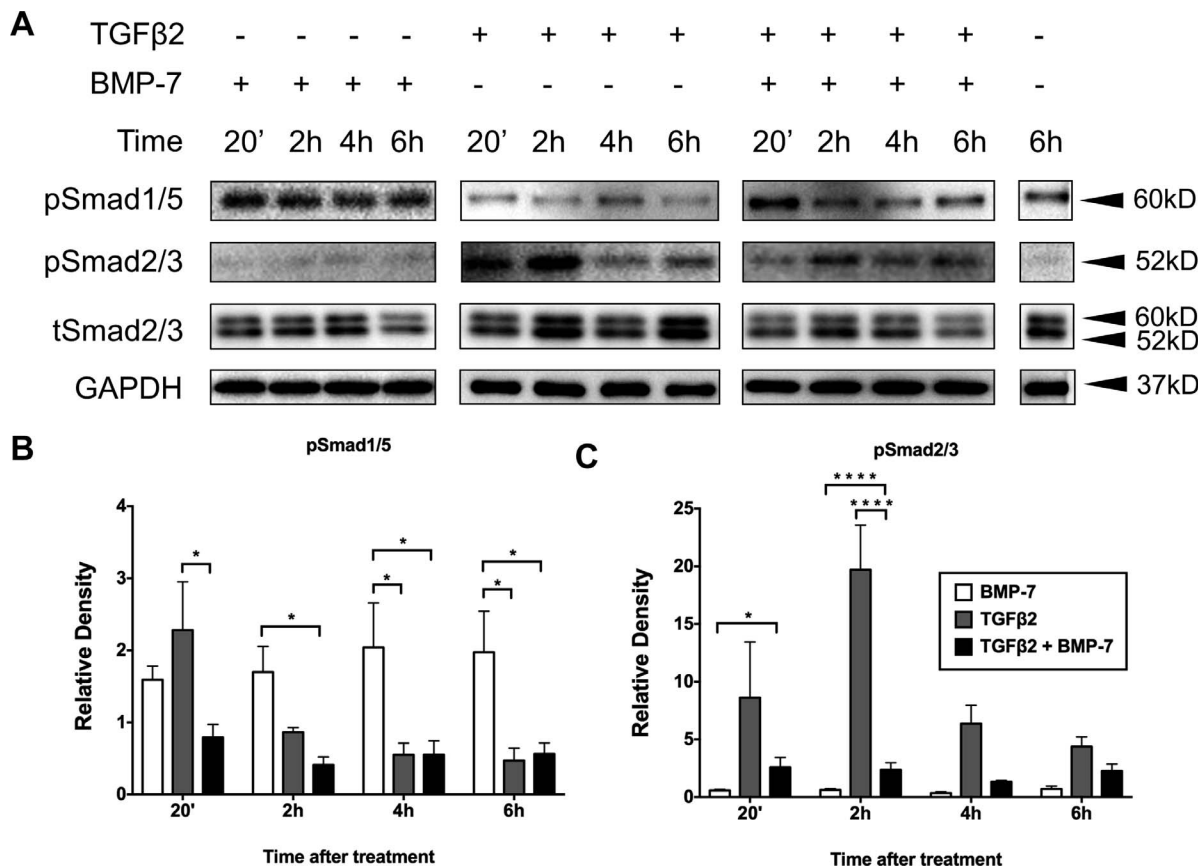


FIGURE 8. Bone morphogenetic protein-7 application modulates TGFβ2-induced Smad signaling. Treatment with BMP-7 alone induced a sustained increase in pSmad1/5 that was significantly higher than treatment with TGFβ2 alone at 2, 4, and 6 hours (B). Cotreatment with BMP-7 and TGFβ2 exhibited an early peak of pSmad1/5 signaling at 20 minutes (B). Treatment with TGFβ2 showed a marked increase of pSmad2/3, peaking at 2 hours (C). Cotreatment with BMP-7 significantly reduced TGFβ2-driven activation of pSmad2/3 at 2 hours (C). Representative Western blots from 3 independent experiments (A) with data presented as mean \pm SEM. Two-way ANOVA, Tukey's post hoc test * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

contractile activity. This suggests that the early surge in pSmad1/5 signaling may be sufficient to initiate downstream BMP-associated gene transcription, thus preventing subsequent activation of TGFβ2-mediated Smad signaling and resultant EMT. This finding has important ramifications in the prevention of PCO as it is the transient elevation of TGFβ during cataract surgery that gives rise to postoperative PCO many years later.³⁰ It is promising to see that even a short exposure to BMP-7 is able to abrogate the potent effects of TGFβ2 over an extended culture period and may be a novel treatment for the prevention of PCO.

Communication between TGFβ and BMP-7 pathways also occurs further downstream of Smad proteins at the level of specific target genes. Using a renal epithelial cell line, Zeisberg et al.¹⁷ showed that TGFβ-induced phosphorylation of Smad2/3 and EMT led to reduced E-cadherin expression. Exogenous addition of BMP-7 increased pSmad1/5/8 and this directly blocked pSmad2/3, restoring E-cadherin promoter activity to control levels.¹⁷ Interestingly, in the present study, expression levels of epithelial markers, E-cadherin, and β-catenin did not reduce with TGFβ2 treatment. It has been reported that instead of being downregulated, E-cadherin and β-catenin can relocate from the membrane to the cytoplasm during EMT in human corneal epithelial cells.³¹ This is consistent with our study that shows a dysregulated cellular distribution of these epithelial markers during TGFβ2-mediated EMT. Since E-cadherin and β-catenin are not localized to the membrane in their normal cobblestone-packed arrangement, they can no longer fulfil their functional role as cell-cell adhesion

molecules in maintaining epithelial cell integrity and intercellular junctions, hence promoting the EMT response.

In addition to the E-cadherin promoter gene, Id proteins also have been identified as downstream target genes mediating the antagonistic effect of BMP-7 on TGFβ-induced EMT.^{22,23,28} Id proteins have a crucial role in development and cancer biology, permitting proliferation and preventing premature terminal differentiation.³² Downregulation of Id proteins has been detected during TGFβ-induced EMT in a prostate cancer model.³³ Bone morphogenetic protein-7 also has been shown to antagonize TGFβ-induced pulmonary fibrosis by inducing Id2 and Id3,²⁸ while adenoviral gene transfer of Id2 and Id3 delayed injury-induced EMT of LECs in a mouse model.²³ Mouse mutants deficient in Id2 and Id3 enabled BMP-7 to induce EMT, further highlighting the importance of BMP-induced Id proteins in antagonizing TGFβ-induced EMT.²² Interestingly, in our present study, BMP-7 does not upregulate *Id2/3* gene expression but rather, is able to inhibit TGFβ2-induced *Id2/3* gene suppression, maintaining *Id2/3* gene expression at basal levels. Future research will focus on the importance of BMP-induced Id proteins in the lens, and examine the role of dysregulated Id expression in promoting EMT to potentially exacerbate cataract formation.

Using an in vivo capsular injury-induced mouse model, Saika et al.²³ reported that BMP-7 upregulated pSmad1/5/8 signaling and inhibited pSmad2 signaling. Although adenoviral gene transfer of BMP-7 suppressed the expression of α-SMA, the EMT process was merely delayed and LECs eventually

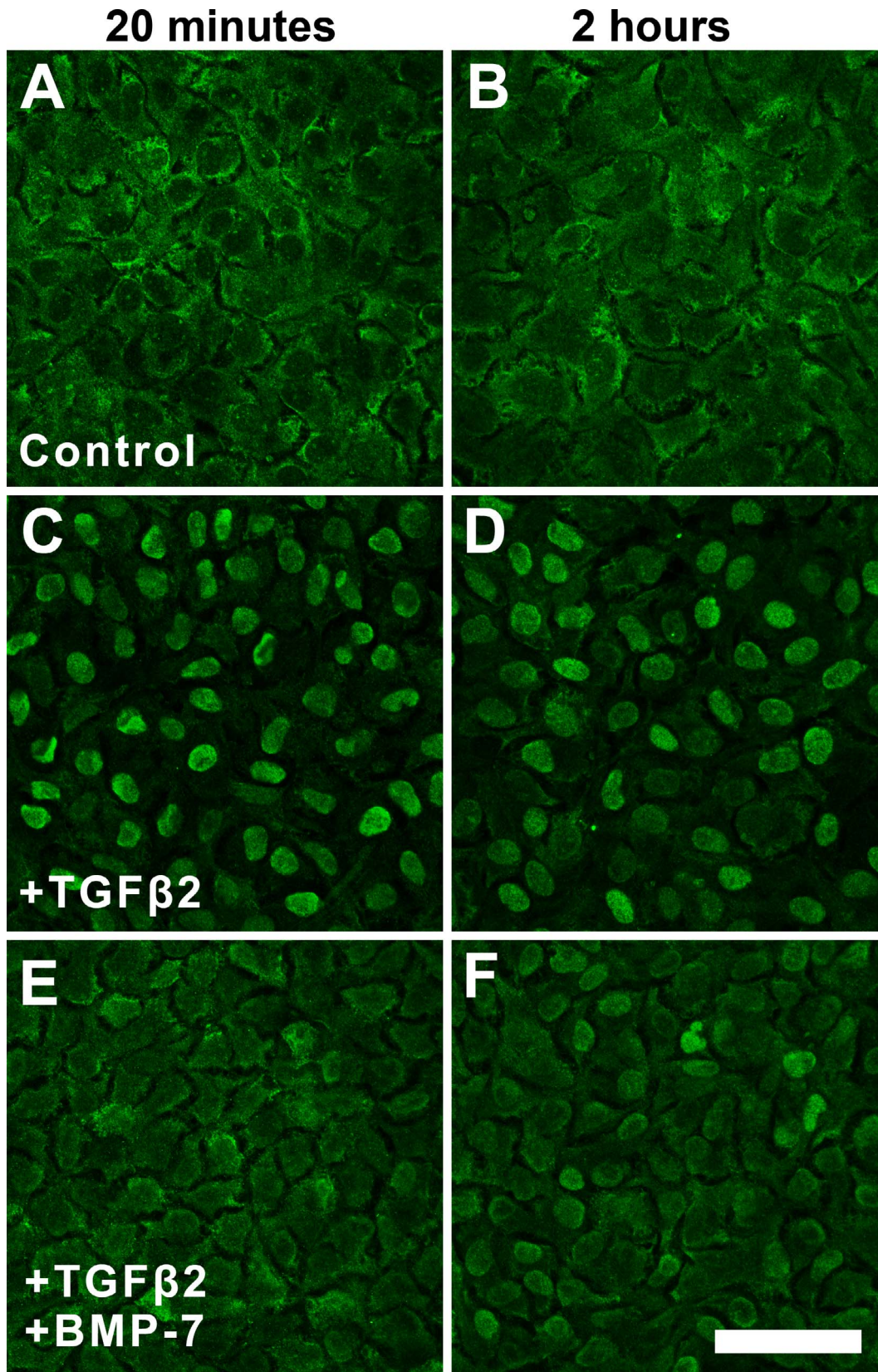


FIGURE 9. Bone morphogenetic protein-7 application modulates nuclear localization of tSmad2/3. Immunofluorescence confocal microscopy showed cytoplasmic labeling of tSmad2/3 at 20 minutes (A) and 2 hours (B) in control explants. Exposure of LECs to TGF β 2 for 20 minutes (C) and 2 hours (D) demonstrated nuclear localization of tSmad2/3. The addition of BMP-7 at 20 minutes retained cytoplasmic tSmad2/3 (E); however, some nuclear translocation of tSmad2/3 is evident at 2 hours (F). Images are representative of 3 independent experiments. *Scale bar:* 50 μ m.

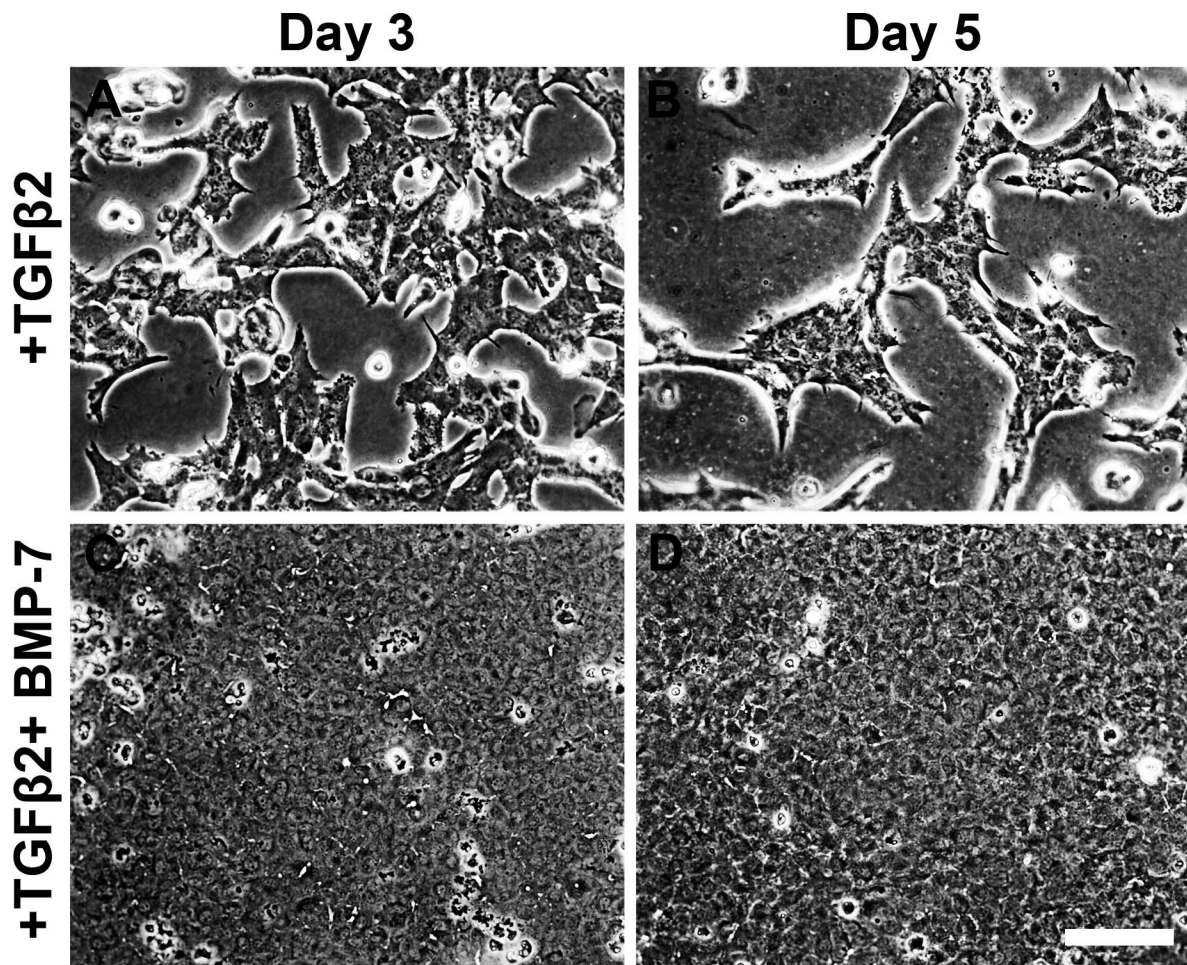


FIGURE 10. Short-term exposure to BMP-7 blocks TGF β 2-induced EMT and apoptosis. Phase-contrast microscopy showed that LECs exposed to TGF β 2 alone for 2 hours underwent EMT over the 5-day culture period with characteristic cell elongation and cell loss (A, B). Cotreatment of TGF β 2 and BMP-7 for 2 hours retained a monolayer of epithelial cells over the 5-day culture period (C, D). Images are representative of 3 independent experiments. *Scale bar:* 100 μ m.

continued to undergo myofibroblastic transdifferentiation.²³ In contrast, our study showed that the inhibitory effect of BMP-7 was maintained over the entire culture period. This may be attributed to differences in the model of EMT induction, as well as the administration of BMP-7. In our study, we induced EMT by exogenous addition of TGF β 2, whereas, the lens capsular injury-induced model of EMT relies on indirect activation of endogenous TGF β in situ through physical damage to the lens that may activate more complex signaling pathways. It also is possible that the method of enhancing BMP-7 signaling may have a role; where exogenous administration of BMP-7 may be more effective at counteracting the effects of TGF β compared to adenoviral-mediated overexpression of BMP-7.

The *in vivo* situation, no doubt, differs markedly from our lens explant system given LECs are in constant contact with a myriad of growth factors derived from the aqueous humor. These growth factors may interact with TGF β and/or BMP-7 in an antagonistic or synergistic manner; thus, adding to the complexity of effectively inhibiting EMT. For example, fibroblast growth factor has been found to exacerbate the cataractogenic effect of TGF β 2 in cultured rat lenses.³⁴ The major advantage of our rat lens epithelial explant system is that it enables the controlled application and study of growth factors in isolation and/or in combination, compared to the *in vivo* system. Our future research will involve characterizing the combined actions of other growth factors in the aqueous

humor with TGF β 2 and/or BMP-7 in regulating the EMT process.⁵⁵

Bone morphogenetic protein signaling is regulated by several secreted extracellular agonists and antagonists of BMPs.³⁶ While molecules, such as chordin, noggin, gremlin, and DAN/Cerberus, inhibit the binding of BMP to its receptor, other molecules, such as Kielin/chordin-like protein, are enhancers of BMP signaling, increasing BMP binding to its receptor.³⁶ Gremlin, an extracellular and intracellular BMP antagonist, has been shown to increase ECM synthesis in trabecular meshwork cells.^{37,38} In human lens epithelial cells, silencing gremlin effectively inhibited TGF β 2-induced EMT and ECM synthesis.³⁹ It is possible that inhibition of gremlin may abolish the inhibitory effect of endogenous BMP on TGF β 2, thereby blocking EMT. By investigating the role of these extracellular regulators of BMP signaling in the lens, we can potentially augment the antagonistic effect of BMP-7 on TGF β -induced EMT. Future studies should also aim to explore the role of other BMP isoforms found in the lens, including BMP-2 and BMP-4,¹⁴ in inhibiting EMT, to investigate whether the inhibitory capacity of BMP is unique to BMP-7. The expression levels of BMP agonists/antagonists, BMP receptors, and other BMP isoforms may influence the efficacy of BMP-7 in inhibiting TGF β -induced EMT. Hence, further investigation is required to understand the tight regulation of BMP signaling before there is potential for clinical application.

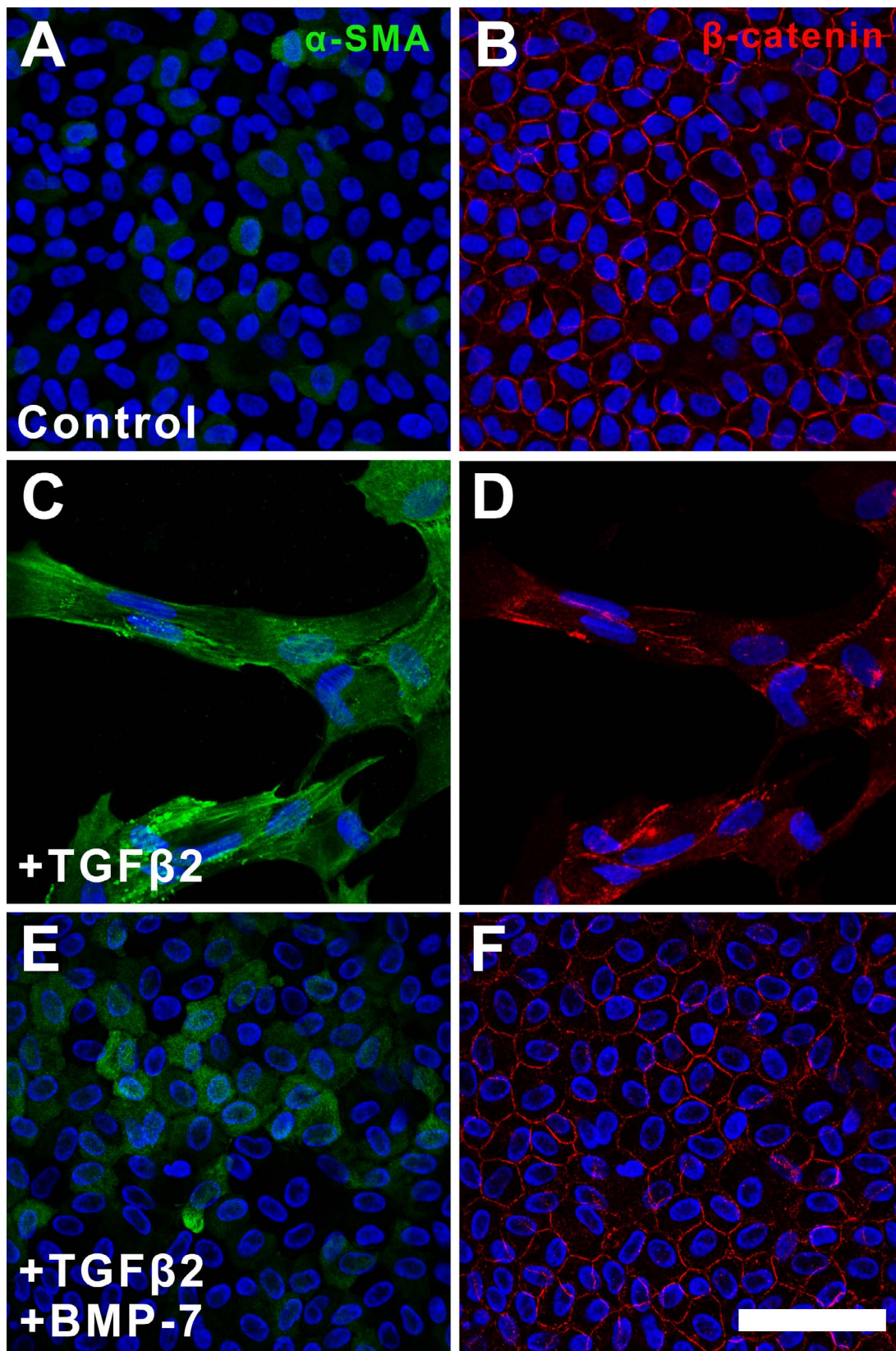


FIGURE 11. Short-term exposure to BMP-7 modulates TGF β 2-induced markers of EMT. Immunofluorescence confocal microscopy of control explants showed minimal α -SMA label (A) and membranous β -catenin expression (B). At day 5, explants exposed to TGF β 2 alone for 2 hours showed α -SMA-reactive stress-fibers (C) and punctate cytosolic β -catenin (D). Cotreatment of TGF β 2 and BMP-7 for 2 hours displayed an upregulation in cytoplasmic α -SMA expression (E) despite retaining membranous β -catenin expression (F). Images are representative of 3 independent experiments. Scale bar: 50 μ m.

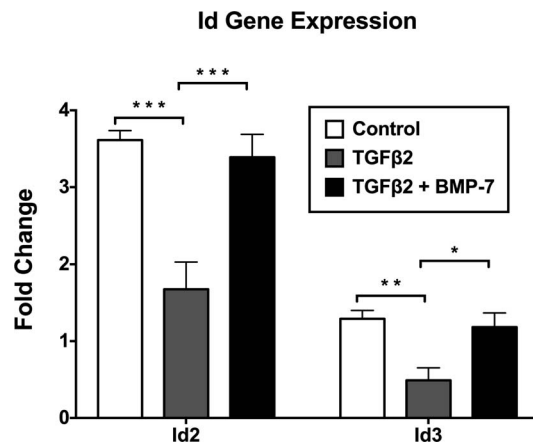


FIGURE 12. Bone morphogenetic protein-7 inhibits TGFβ2-induced suppression of *Id2* and *Id3* gene expression. Using RT-qPCR, treatment with TGFβ2 alone significantly downregulated expression of *Id2* and *Id3* compared to control explants. The addition of BMP-7 significantly inhibited TGFβ2-induced suppression of *Id2* and *Id3* showing no significant difference to control explants for *Id2* and *Id3*. Data from 4 independent experiments are presented relative to GAPDH as fold change \pm SEM. Unpaired *t*-test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Bone morphogenetic protein-7 is an endogenous growth factor that has a crucial role during lens embryogenesis and development. By exploiting this pre-existing BMP pathway in the lens through exogenous administration of recombinant human BMP-7 to lens epithelial explants, we have shown that TGFβ-driven EMT leading to cataract can be inhibited. Recombinant human BMP-7 already has been approved by the United States Food and Drug Administration for bone regeneration, and clinical trials of a BMP-7 analog have been launched to evaluate its efficacy on diabetic nephropathy.⁴⁰ Bone morphogenetic protein-7, hence, shows promise as a novel pharmaceutical agent in the prevention of ASC and PCO. Future studies will focus on extending these findings to in vivo models, characterizing the complex regulation of BMP signaling and identifying specific BMP-7 target genes mediating its protective effects.

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