

# Quercetin inhibits transforming growth factor $\beta$ 1-induced epithelial–mesenchymal transition in human retinal pigment epithelial cells via the Smad pathway

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**Purpose:** The purpose of this study was to evaluate the effect and mechanism of quercetin on TGF- $\beta$ 1-induced retinal pigment epithelial (RPE) cell proliferation, migration, and extracellular matrix secretion.

**Materials and methods:** Cell counting kit-8, transwell, wound-healing assays, and ELISA were used to assess viability, migration, and collagen I secretion, respectively. Western blot analysis and qPCR were employed to detect mRNA and protein expression levels, respectively.

**Results:** Quercetin suppressed TGF- $\beta$ 1-induced cell proliferation, migration, and collagen I secretion. The results also showed that mRNA and protein expression of epithelial–mesenchymal transition (EMT)-related markers such as alpha-smooth muscle actin and N-cadherin was downregulated by quercetin in TGF- $\beta$ 1-treated RPE cells; conversely, quercetin upregulated the expression of E-cadherin and tight junction protein 1 (ZO-1). In addition, quercetin could inhibit mRNA and protein expression of matrix metalloproteinases. Quercetin may reverse the progression of EMT via the Smad2/3 pathway.

**Conclusion:** Our results demonstrate the protective effects of quercetin on RPE cell EMT, revealing a potential therapeutic agent for proliferative vitreoretinopathy treatment.

**Keywords:** proliferative vitreoretinopathy, quercetin, epithelial–mesenchymal transition, transforming growth factor- $\beta$ 1

## Introduction

Proliferative vitreoretinopathy (PVR) is a vision-threatening disease commonly associated with rhegmatogenous retinal detachment (RD). RD is the separation of the neurosensory retina from the linked retinal pigment epithelium. The intravitreal growth factors and cytokines after occurrence of RD may influence postoperative outcomes. Statistically, PVR occurs in 5%–10% of RD patients, especially postoperatively.<sup>1</sup> Progression of PVR involves several steps, such as the proliferation and migration of retinal pigment epithelial (RPE) and glial cells, the formation and contraction of the proliferative membrane, the production of extracellular collagen, and the formation of retinal folds.<sup>2</sup> The epithelial–mesenchymal transition (EMT) plays a vital role in the progression of PVR. Following the stimulation by several factors, polarized epithelial cells switch to a mesenchymal cell phenotype, producing an extracellular matrix (ECM) and exhibiting changes in morphological and molecular characteristics.<sup>3</sup>

Some studies have indicated that multiple growth factors and cytokines are involved in the vitreous body of PVR patients, including tumor necrosis factor- $\alpha$ ,

interleukin-(IL-) 6, transforming growth factor-beta (TGF- $\beta$ ) and epidermal growth factor (EGF).<sup>4</sup> In our previous studies, TGF- $\beta$ 1 was found to have an essential role in EMT in RPE cell lines (ARPE-19).<sup>5,6</sup> TGF- $\beta$ 1-induced EMT triggers epithelial cells to alter their epithelial phenotype to one with mesenchymal characteristics, and the proliferation, migration, and collagen generation of TGF- $\beta$ 1-induced RPE cells are enhanced, accelerating EMT progression. Some therapeutic methods have recently been proposed for reversing EMT development both in vitro and in vivo, such as the application of flavonoids or gene silencing. For example, Ren et al reported that curcumin inhibits RPE cell proliferation via downregulation of EGF and thus effectively inhibits the development of PVR.<sup>7</sup> In 2013, protein kinase C $\alpha$  silencing was demonstrated to have effect on suppressing RPE cell proliferation and migration, which was crucial against PVR disease.<sup>8</sup> The degradations of collagen and other ECM proteins were closely associated with matrix metalloproteinases (MMPs). The MMP-2 and MMP-9 were expressed in higher levels in PVR patients, which played a vital role in the subretinal membrane formation and cell migration.<sup>9,10</sup> However, despite the large number of studies of EMT in PVR treatment, no therapeutic drugs have been developed in the last few decades to effectively prevent PVR.

Quercetin is a natural polyphenolic flavonoid compound extracted from plants such as *Phyllanthus emblica*.<sup>11</sup> Some studies have reported that quercetin has many beneficial properties such as antioxidant,<sup>12,13</sup> anti-inflammation,<sup>14</sup> antimicrobial,<sup>15</sup> anti-angiogenesis,<sup>16,17</sup> and anticancer properties.<sup>18,19</sup> Wang et al found that quercetin was able to upregulate certain oxidative stress-related genes such as Cu/Zn superoxide dismutase (SOD-1) and catalase (CAT) in vivo and in vitro.<sup>13</sup> Quercetin also downregulates vascular endothelial growth factor receptor (VEGFR) expression, blocking angiogenesis in retinoblastoma.<sup>17</sup> According to another report, quercetin conjugated with nanoparticles exhibited an anti-angiogenic effect on breast cancer via the epidermal growth factor receptor/VEGFR-2 (EGFR/VEGFR-2)-mediated pathway.<sup>16</sup> In addition, quercetin appears to have effects in several ocular diseases such as age-related macular degeneration,<sup>20</sup> diabetic cataract (DC),<sup>21</sup> dry eye,<sup>22</sup> and retinoblastoma.<sup>17</sup> Xu et al demonstrated that quercetin could protect oxidative damage via activation of the Nrf2 pathway.<sup>23</sup> In 2017, Du et al reported that quercetin had a potential therapeutic effect on DC, alleviating EMT by inhibiting the TGF- $\beta$ /PI3K/Akt pathway.<sup>21</sup> In 2013, Stoddard et al indicated that quercetin could protect the corneal epithelium

from oxidative damage by decreasing reactive oxygen species production.<sup>24</sup>

Nonetheless, it remains unclear whether quercetin could inhibit TGF- $\beta$ 1-induced EMT progression and associated signaling in RPE cells.

## Materials and methods

### Cell culture and treatment

Human retinal pigment epithelium (ARPE-19) cells were purchased from iCell Bioscience Inc. (Shanghai, China) and cultured in DMEM/F-12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 100 U/mL penicillin and streptomycin. The cells were grown at 37°C in 5% CO<sub>2</sub>-air. The cells with good shape and in good growth status were used in our experiments. The culture medium was changed every 2–3 days. Upon reaching 60%–70% confluence, the cells were treated with FBS-free DMEM/F-12 culture medium for 24 hours to simulate starvation conditions before experiments. The ARPE-19 cells were incubated with 0, 2.5, 5, 7.5, 10, 12.5, and 15 ng/mL TGF- $\beta$ 1 (PeproTech Inc., Rocky Hill, NJ, USA) and 0, 25, 50, 60, 75, and 100  $\mu$ mol/L quercetin (Sigma-Aldrich Co., St Louis, MO, USA) for 24 and 48 hours. The following four groups were established: Group A, control; Group B, 10 ng/mL TGF- $\beta$ 1; Group C, 10 ng/mL TGF- $\beta$ 1+25  $\mu$ mol/L quercetin; and Group D, 10 ng/mL TGF- $\beta$ 1+50  $\mu$ mol/L quercetin.

### Cell viability assay

Cell viability was measured using cell counting kit-8 (CCK-8 assay; Yeasen, Shanghai, China). ARPE-19 cells were seeded in 96-well plates at a density of 2,000 cells/well in 100  $\mu$ L cultural medium and then starved in FBS-free DMEM/F-12 culture medium for 12 hours before stimulation with TGF- $\beta$ 1 at various concentrations of TGF- $\beta$ 1 and treatment with quercetin. After incubation as described above for 24 and 48 hours, 10  $\mu$ L of CCK-8 reagent was added to each well for another 2 hours. Cell viability was analyzed spectrophotometrically at 450 nm, with the numbers of living, metabolically active cells being reflected in the absorbance values.

### Wound healing assay

The above four groups of cells were plated in six-well plates at 1 $\times$ 10<sup>5</sup> cells per well. At 80% confluence, wounds were created with a 200  $\mu$ L pipette tip in the middle of the monolayer, and the wells were washed with PBS. Images were acquired at 0 hours as a starting point, and the cells were treated as described above. Wound closure was recorded after incubation for 24 and 48 hours by measuring the

scratch widths, and migration rates were calculated based on the formula (distance/scratch width) $\times$ 100%. Duplicated wells for each group were used, and the experiments were repeated three times.

## Transwell assay

The four groups of cells were treated as mentioned above. A total of  $5\times 10^4$  cells in 200  $\mu$ L of FBS-free DMEM/F-12 culture medium were seeded in the upper compartment of a transwell system (8 mm pore size; Corning Incorporated, Corning, NY, USA); culture medium with 10% FBS was added to the lower compartment. After incubation for 18 hours, non-migratory cells were removed with cotton swabs. After washing with PBS, the migrated cells attached to the bottom membrane were fixed with ethanol for 20 minutes and stained with 1% crystal violet for 15 minutes after drying in air. Images in three fields were acquired under phase-contrast microscopy (100 $\times$ ).

## Type I collagen ELISA

The human Col I ELISA kit (Shanghai Saige Biotechnology Co Ltd, Shanghai, China) was used according to the manufacturer's protocol to assess cell supernatant type I collagen (Col I) secretion. Cells were seeded in six-well plates at a density of  $2\times 10^5$  cells per well and exposed to four different treatments. All groups were cultured for 48 hours. The supernatants were collected, and Col I concentrations were measured at 492 nm; values were calculated using a standard curve.

## RNA extraction and qRT-PCR

Total cellular RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol, and a NanoDrop 2000 spectrophotometer was employed to quantify RNA concentrations. PrimerScript RT reagent Kit (TaKaRa Bio Inc., Osaka, Japan) was used to synthesize cDNA. The relative expression level of each gene was detected according to 7500 Fast Real-time PCR System (Thermo Fisher Scientific), as normalized with GAPDH in three replications. The primer sequences are presented in Table 1.

## Western blotting

RIPA lysis buffer was used to lyse cells to extract total protein. The supernatant was collected after centrifugation (12,000 rpm, 4°C, 10 minutes), and the total protein concentration was quantified using a BCA Protein Assay kit (Beyotime, Shanghai, China). Cytosolic and nuclear protein were isolated using a Nuclear and Cytoplasmic Protein Extraction Kit, respectively, according to

**Table 1** Nucleotide sequences of primers used for PCR

Gene		Primer sequence (5'-3')
ZO-1	Forward	CAACATACAGTGACGCTTCACA
	Reverse	CACTATTGACGTTTCCCCTACTC
$\alpha$ -SMA	Forward	AAAAGACAGCTACGTGGGTGA
	Reverse	GCCATGTTCTATCGGGTACTTC
E-cadherin	Forward	CGAGAGCTACACGTTTCACGG
	Reverse	GGGTGTCGAGGGAAAAATAGG
N-cadherin	Forward	TCAGGCGTCTGTAGAGGCTT
	Reverse	ATGCACATCCTTCGATAAGACTG
MMP-2	Forward	TACAGGATCATTGGCTACACACC
	Reverse	GGTCACATCGCTCCAGACT
MMP-9	Forward	TGTACCGCTATGGTTACTACTCG
	Reverse	GGCAGGGACAGTTGCTTCT
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

the manufacturer's instructions (Beyotime) and also quantified using a BCA Protein Assay kit. Proteins were separated by 8%–10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked in non-fat milk dissolved in Tween-20/PBS buffer for 1 hour at room temperature and incubated overnight with primary antibodies including anti-ZO-1, - $\alpha$ -smooth muscle actin (- $\alpha$ -sma), -E-cadherin, -N-cadherin, -Smad2/3, -MMP-9, and -GAPDH antibodies (1:1,000, #8193, #19245, #3195, #13116, #3102, #8828, #2270, #5174, respectively, Cell Signaling Technology, Danvers, MA, USA) and anti-MMP-2 (1:1,000, ARG55236; Arigo Biolaboratories, Shanghai, China). The membranes were washed three times for 10 minutes each in Tween-20/PBS buffer, followed by incubation of secondary goat anti-rabbit antibodies for 45 minutes at room temperature in the dark. An Odyssey two-color infrared laser imaging system (LI-COR Biosciences, Lincoln, NE, USA) was used to scan the membranes.

## Immunofluorescence staining

ARPE-19 cells were seeded and cultured in 35 mm confocal dishes (glass-bottom dishes) and then fixed with 4% paraformaldehyde for 10 minutes. The cells were permeabilized and blocked for 1 hour at room temperature with 0.1% Triton X-100 and 5% BSA dissolved in PBS and then incubated overnight at 4°C with primary antibodies (all 1:100 dilution). The cells were incubated with Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 40 minutes after washing three times with PBS. Next, nuclei were stained with DAPI (1:1,000 dilution) for 20 minutes. Images were obtained by confocal microscopy (LSM710; Carl Zeiss, Jena, Germany).

## Statistical analyses

Statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 19.0 (IBM Corporation, Armonk, NY, USA) software packages. Data are expressed as mean  $\pm$  standard deviation (SD), based on results from three separate experiments. One-way ANOVA was performed for multiple groups. *P*-values  $<0.05$  were considered statistically significant.

## Results

### Effects of quercetin on TGF- $\beta$ 1-induced cell proliferation in ARPE-19 cells

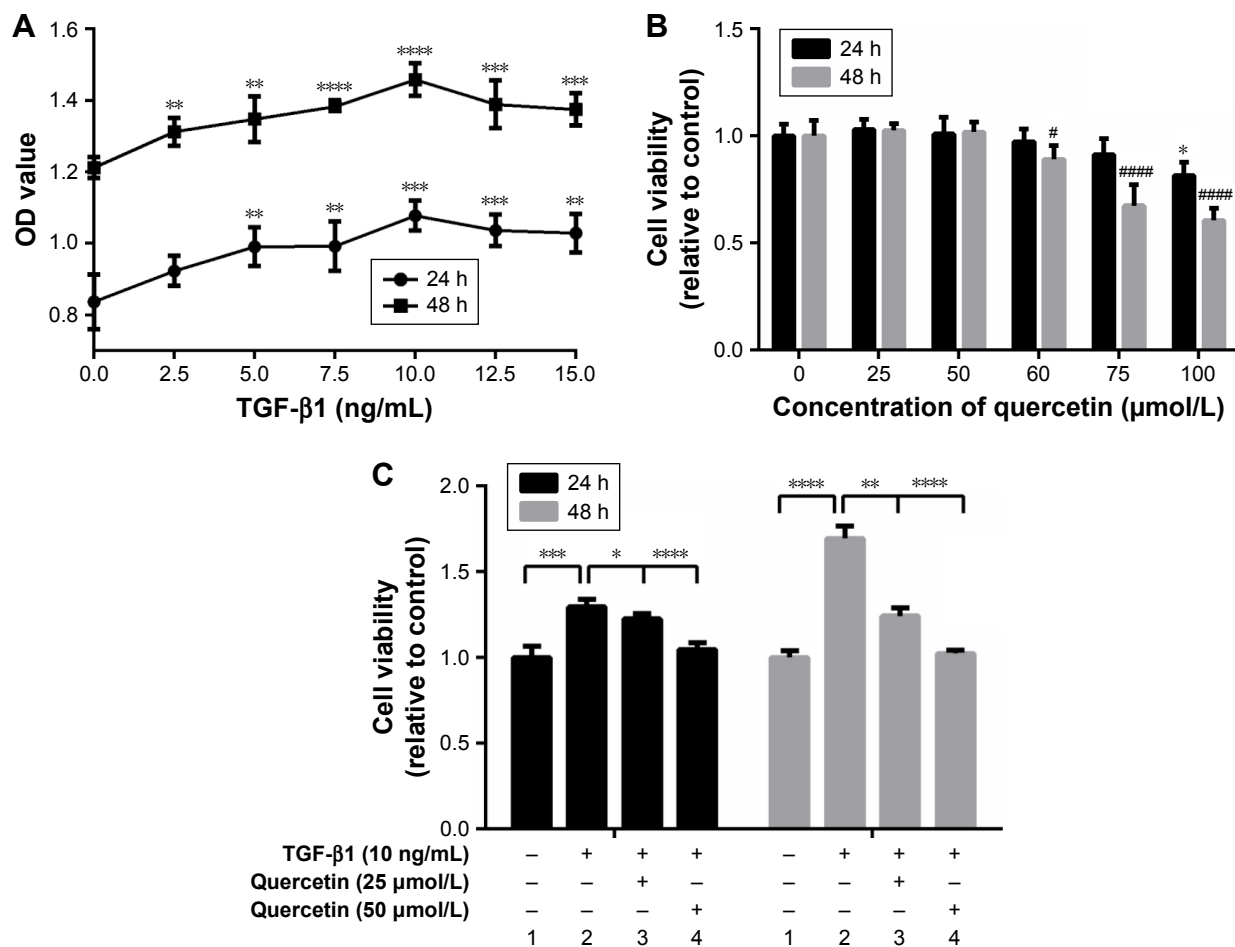
ARPE-19 cells were treated with different concentrations of TGF- $\beta$ 1 (0, 2.5, 5, 7.5, 10, 12.5, and 15 ng/mL). As shown in Figure 1A, cell proliferation was stimulated, except at 12.5 ng/mL, by TGF- $\beta$ 1 at 24 and 48 hours in a concentration-dependent manner, especially after 48 hours (Figure 1A).

Next, the cells were treated with various concentrations of quercetin to observe effects on cell viability. No cytotoxicity in ARPE-19 cells was found for quercetin for concentrations  $<100$   $\mu$ mol/L at 24 hours and 60  $\mu$ mol/L at 48 hours, as shown in Figure 1B. Moreover, quercetin inhibited TGF- $\beta$ 1-induced cell proliferation at 25 and 50  $\mu$ mol/L after 24 and 48 hours, respectively (Figure 1C).

### Effects of quercetin on TGF- $\beta$ 1-induced ARPE-19 cell migration

#### Quercetin-suppressed wound closure in TGF- $\beta$ 1-induced ARPE-19 cells

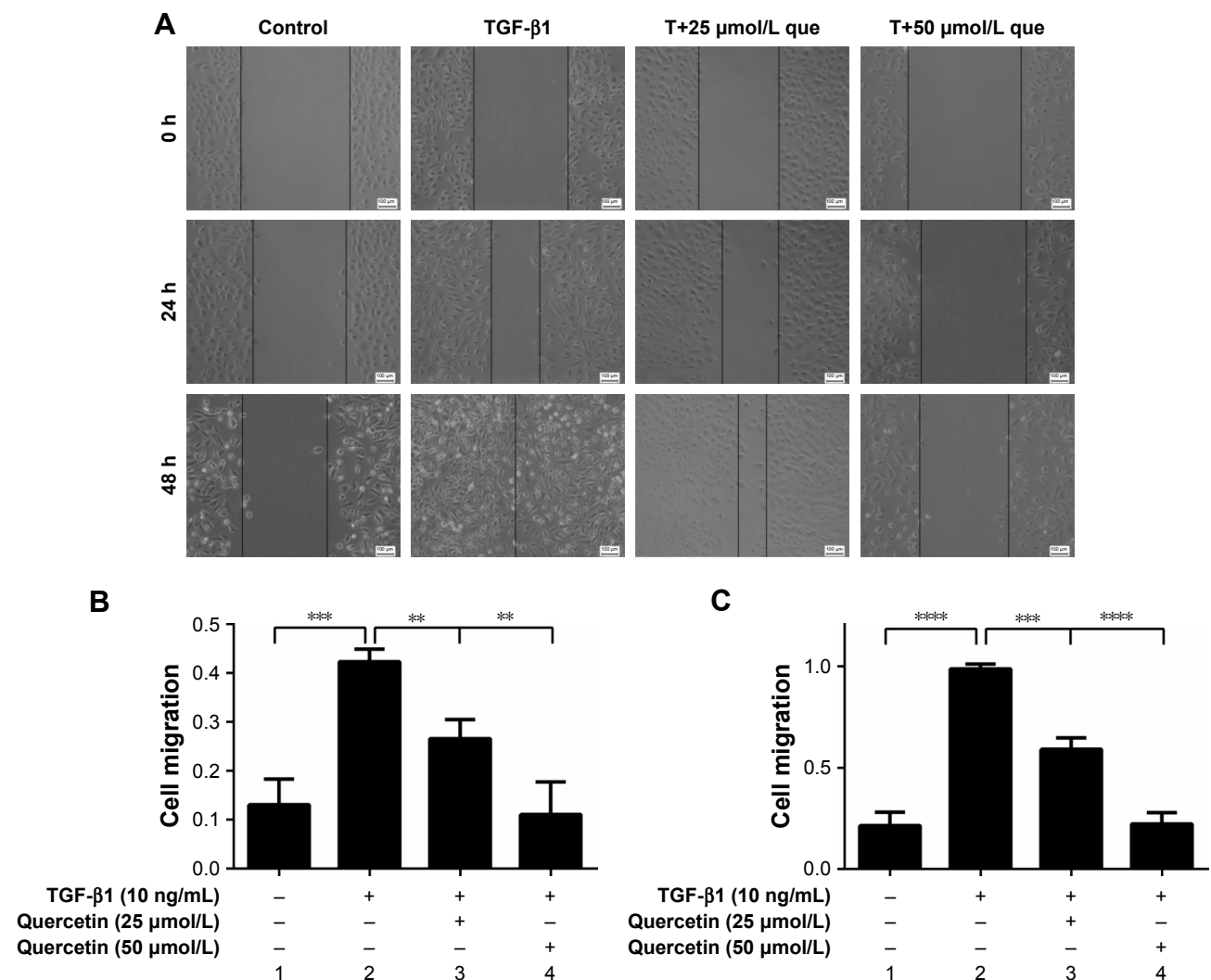
For the wound-healing test, the width of the opening was measured at 0, 24, and 48 hours, as shown in Figure 2, and the ratios of (24 h-0 h)/0 hour and (48 h-0 h)/0 hour were calculated. Migration by TGF- $\beta$ 1-treated cells was greater than that of cells not treated with TGF- $\beta$ 1 at both 24 and



**Figure 1** Effects of quercetin on the cell viability of ARPE-19 cells stimulated by TGF- $\beta$ 1.

**Notes:** (A) The cells were treated with different concentrations of TGF- $\beta$ 1 (0, 2.5, 5, 7.5, 10, 12.5, and 15 ng/mL, respectively) for 24 and 48 hours, and the cell viability was determined by CCK-8 assay. \*\**P* $<0.01$ , \*\*\**P* $<0.001$ , \*\*\*\**P* $<0.0001$  vs the OD value of cells cultured without TGF- $\beta$ 1. (B) Different concentrations of quercetin (0, 25, 50, 60, 75, and 100  $\mu$ mol/L, respectively) were incubated in ARPE-19 cells for 24 and 48 hours. The cell viability was detected. \**P* $<0.05$  vs the control group without quercetin treatment for 24 hours, #*P* $<0.05$ , ####*P* $<0.0001$  vs the cells treated without quercetin for 48 hours. (C) The cells were treated with or without quercetin (50 and 100  $\mu$ mol/L) in TGF- $\beta$ 1-stimulated ARPE-19 cells for 48 hours, and cell viability was measured using a CCK-8 assay. The data are shown as mean  $\pm$  SEM; *n*=3.

**Abbreviations:** CCK-8, cell counting kit-8; TGF- $\beta$ , transforming growth factor- $\beta$ .



**Figure 2** Effects of quercetin on wound closure in TGF- $\beta$ 1-treated RPE cells.

**Notes:** (A) RPE cells were pretreated with or without 10 ng/mL of TGF- $\beta$ 1 in the absence of 25 or 50  $\mu$ M quercetin after a scratch. The images were captured at 0, 24, and 48 hours in four groups. The migratory length was calculated according to the scratch at 0 hour. The data are representative of at least three independent experiments. Magnification:  $\times 100$ . (B) Cell migration data at 24 hours were quantified in wound closure via recording migration length. (C) Cell migration data at 48 hours were quantified in wound closure via recording migration length. The data are shown as mean  $\pm$  SEM;  $n=3$ .  $^{***}P<0.01$ ,  $^{****}P<0.0001$ ,  $^{****}P<0.0001$ .

**Abbreviations:** que, quercetin; RPE, retinal pigment epithelial; TGF- $\beta$ , transforming growth factor- $\beta$ .

48 hours ( $^{***}P<0.001$  and  $^{****}P<0.0001$ , respectively). Quercetin attenuated TGF- $\beta$ 1-induced cell migration in a concentration-dependent manner. The migration rates at 24 hours decreased from  $0.42\pm 0.03$  in TGF- $\beta$ 1-treated cells to  $0.27\pm 0.04$  and  $0.11\pm 0.07$  at 25 and 50  $\mu$ mol/L in quercetin-treated cells, respectively. The migration rates of the four groups were  $0.21\pm 0.067$ ,  $0.99\pm 0.02$ ,  $0.59\pm 0.06$ , and  $0.22\pm 0.05$  at 48 hours.

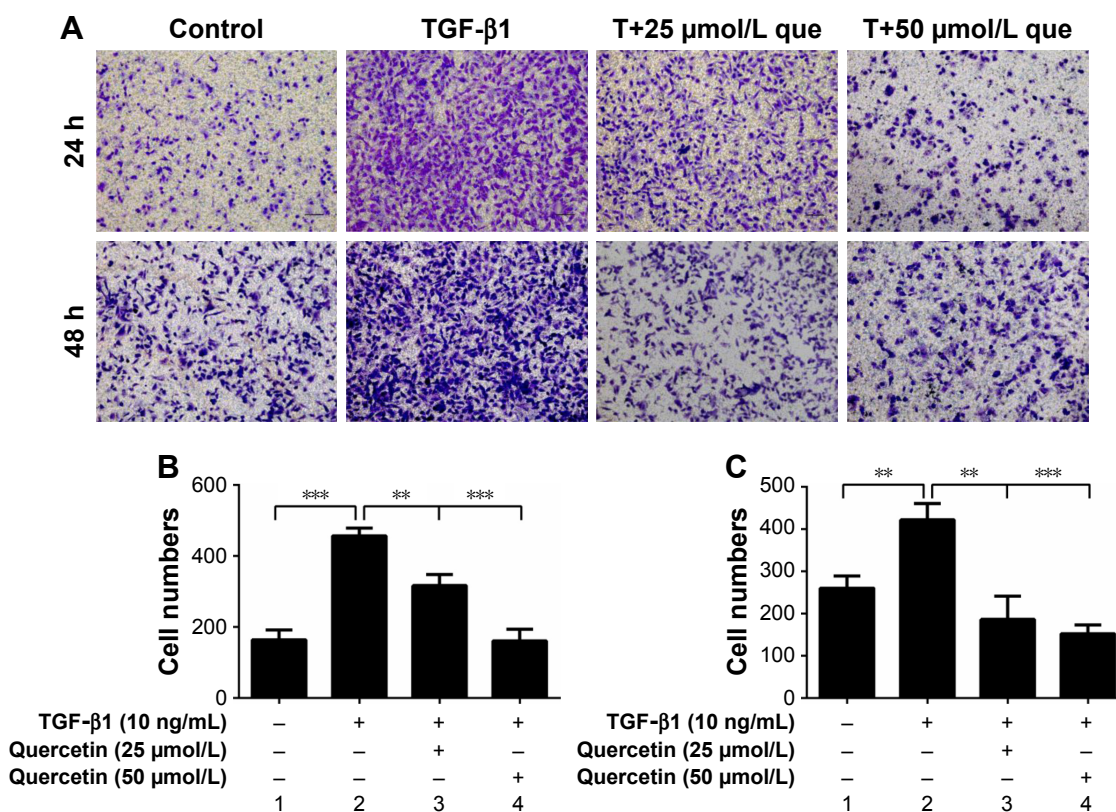
### Quercetin-suppressed TGF- $\beta$ 1-induced cell migration

The transwell assay was employed to evaluate cell migration. The numbers of cell migrating after stimulation with TGF- $\beta$ 1 were increased from  $164.00\pm 28.48$  to  $457.00\pm 22.11$  at 24 hours ( $^{***}P<0.001$ ), and quercetin reduced these numbers to  $316.67\pm 30.55$  ( $^{**}P<0.01$ ) and  $161.33\pm 32.96$

( $^{***}P<0.001$ ) at concentrations of 25 and 50  $\mu$ mol/L, respectively. The numbers for the four groups after incubation for 48 hours were  $259.67\pm 29.09$ ,  $422.00\pm 38.00$ ,  $186.00\pm 55.57$ , and  $152.33\pm 20.65$ . These results showed that quercetin significantly inhibited TGF- $\beta$ 1-induced cell migration in a concentration-dependent and time-dependent manner (Figure 3).

### Effects of quercetin on TGF- $\beta$ 1-treated cell collagen I secretion in ARPE-19 cells

As shown in Figure 4, 10 ng/mL of TGF- $\beta$ 1 promoted cell collagen I secretion at 48 hours. The concentration of collagen I in the culture supernatant increased from  $44.16\pm 0.54$  to  $51.20\pm 0.48$  ng/mL but was significantly reduced after treatment with quercetin.



**Figure 3** Effects of quercetin on cell migration in TGF-β1-treated RPE cells.

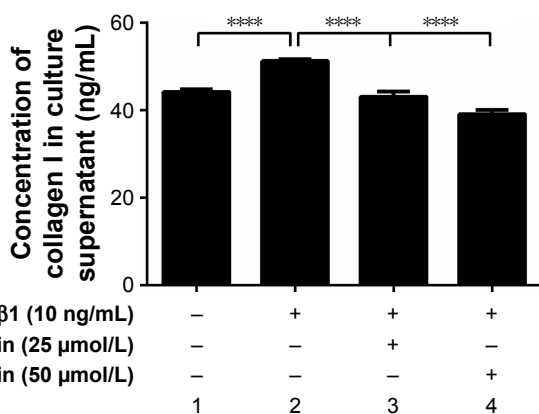
**Notes:** (A) After treatment with 10 ng/mL of TGF-β1 with or without quercetin (50 and 100 μM) for 48 hours, the migration of RPE cells with different treatments were detected via transwell migration analysis. Migrated cells at 24 h (B) and 48 h (C) were quantified by counting three random vision fields under a microscope. Magnification  $\times 100$ . The data are shown as mean  $\pm$  SEM.  $n=3$ . \*\* $P<0.01$ , \*\*\* $P<0.001$ . Scale bar: 100 μm.

**Abbreviations:** que, quercetin; RPE, retinal pigment epithelial; TGF-β1, transforming growth factor-β1.

## Effects of quercetin on TGF-β1-induced MMP-2 and MMP-9 expressions in ARPE-19 cells

As shown in Figure 5, 10 ng/mL of TGF-β1 promoted the mRNA and protein expression of MMP-2 and MMP-9 at

48 hours. Meanwhile, quercetin significantly decreased the levels of expression of MMP-2 and MMP-9 in a concentration-dependent manner.



**Figure 4** Effect of quercetin on collagen I secretion in TGF-β1-treated RPE cells.

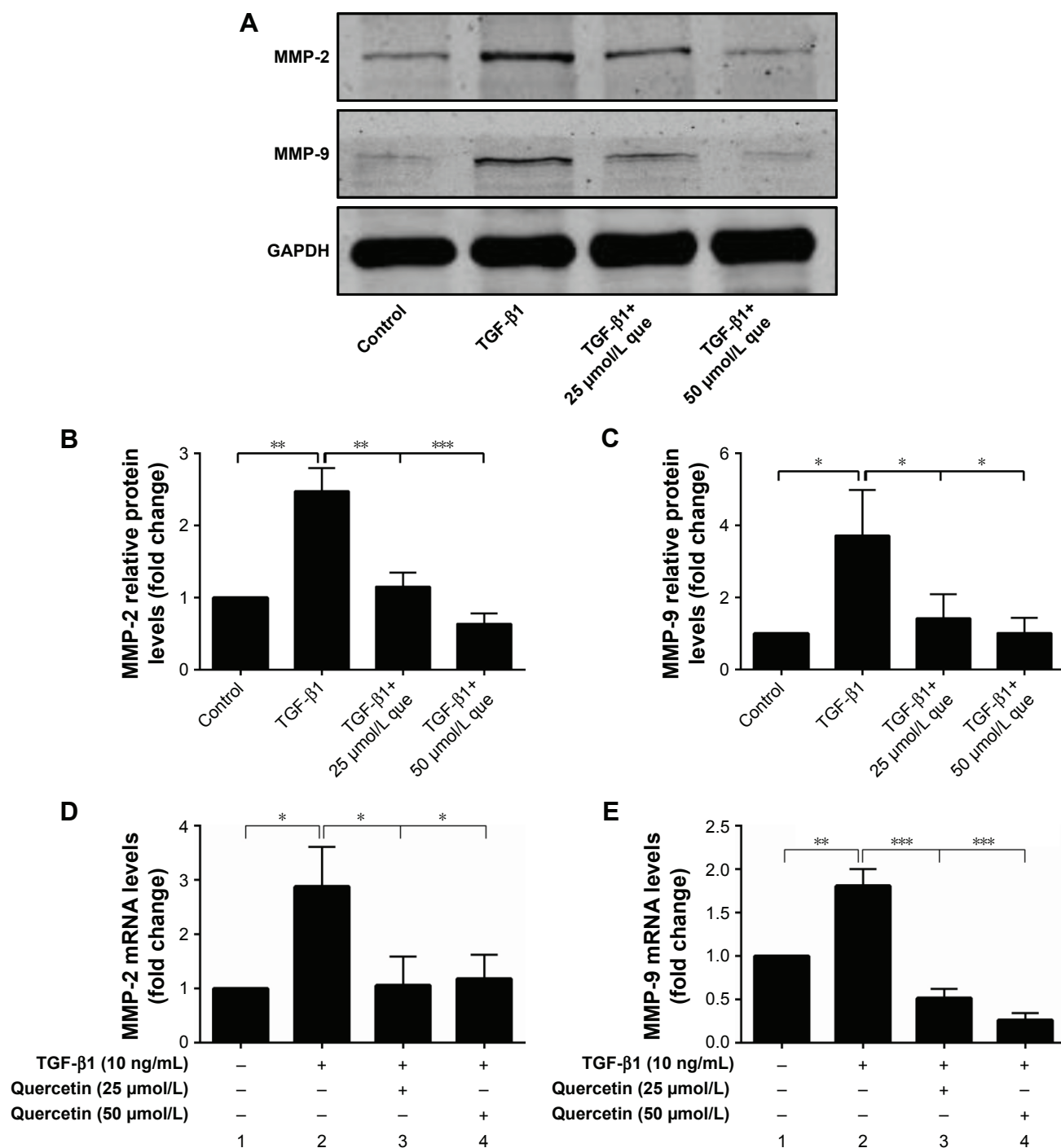
**Notes:** The supernatants were collected after incubation with or without quercetin and TGF-β1 for 48 hours. The concentration of collagen I was quantified via ELISA. \*\*\*\* $P<0.0001$ .

**Abbreviations:** RPE, retinal pigment epithelial; TGF-β1, transforming growth factor-β1.

## Effects of quercetin on expression of EMT markers in TGF-β1-induced RPE cells

Quercetin reversed mRNA and protein expression of ZO-1, E-cadherin, N-cadherin, and α-SMA during TGF-β1-induced EMT in ARPE-19 cells

As shown in Figure 6, qPCR and Western blot analyses demonstrated an increase in the expression levels of the mesenchymal markers N-cadherin and α-SMA after TGF-β1 incubation; however, quercetin significantly decreased the levels of expression of mesenchymal markers in a concentration-dependent manner. Moreover, expression of the epithelial marker ZO-1 and E-cadherin was significantly decreased after treatment with TGF-β1, whereas that of ZO-1 and E-cadherin was increased by exposure to quercetin. Based on immunofluorescence staining, quercetin significantly upregulated the expression of ZO-1 and E-cadherin while downregulating N-cadherin and α-SMA (Figure 7).



**Figure 5** Quercetin suppressed TGF- $\beta$ 1-induced MMP expression in RPE cells.

**Notes:** (A) The protein levels of MMP-2 and MMP-9 were detected by Western blot in TGF- $\beta$ 1 and quercetin-treated RPE cells for 48 hours. (B and C) Relative protein expression (normalized to GAPDH) was quantified in Western blots via recording gray scale values. (D and E) The mRNA levels of MMP-2 and MMP-9 were detected by real-time PCR. The data are presented as mean  $\pm$  SEM.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

**Abbreviations:** MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; que, quercetin; RPE, retinal pigment epithelial; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

### Quercetin-suppressed expression of phosphorylated-Smad2/3 and nuclear translocation of Smad4 during TGF- $\beta$ 1-induced EMT in ARPE-19 cells

We then investigated the effect of quercetin on the Smad signaling pathway, which has been identified as an important factor for EMT. Phosphorylation of Smad2/3 was

significantly increased after TGF- $\beta$ 1 treatment, contributing to EMT progression. In addition, cytosolic protein of Smad4 was decreased while nuclear protein of Smad4 was increased after TGF- $\beta$ 1 incubation, which indicated that Smad4 was transported into the nucleus. Interestingly, incubation with quercetin inhibited Smad2/3 phosphorylation

and translocation of Smad4. In summary, these results suggest that quercetin inhibits TGF- $\beta$ 1-induced EMT in ARPE-19 cells by suppressing phosphorylation of Smad2/3 (Figure 8).

## Discussion

The results of our study show that quercetin inhibits TGF- $\beta$ 1-induced EMT in RPE cells by regulating phosphorylation of Smad2/3 pathway components. Based on our data, we

propose that quercetin might serve as a therapeutic agent in the treatment of PVR.

PVR, which involves a wound-healing process, is the most common cause of anatomic failure in RD surgery and a challenge in the clinic. Several cells such as RPE cells, Müller glia cells, macrophages, and fibroblasts are known to participate during the development of PVR.<sup>25</sup> RPE cells have been reported to de-differentiate, proliferate, and migrate through retinal holes onto the retinal surface.<sup>26,27</sup> Müller glia release

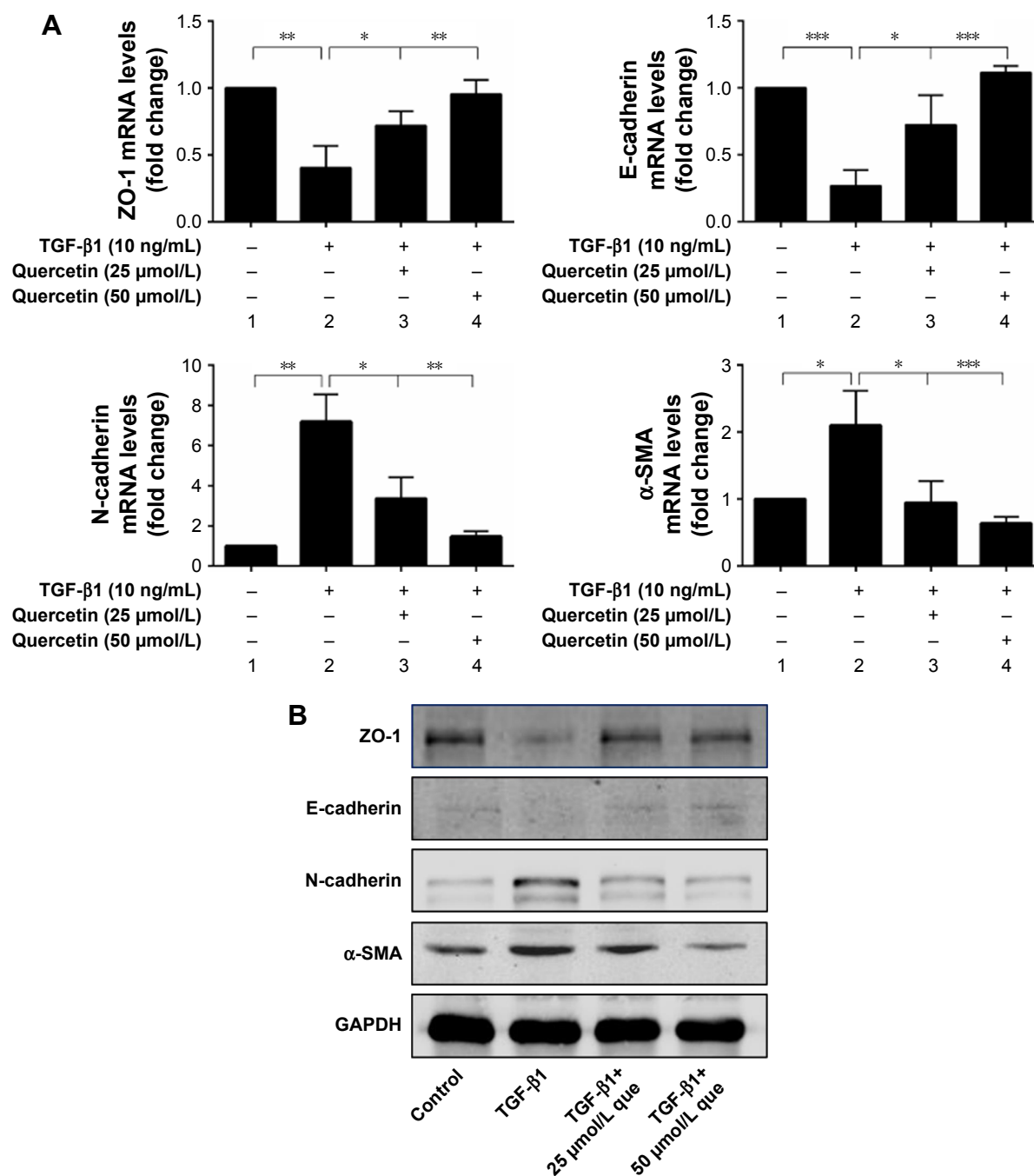
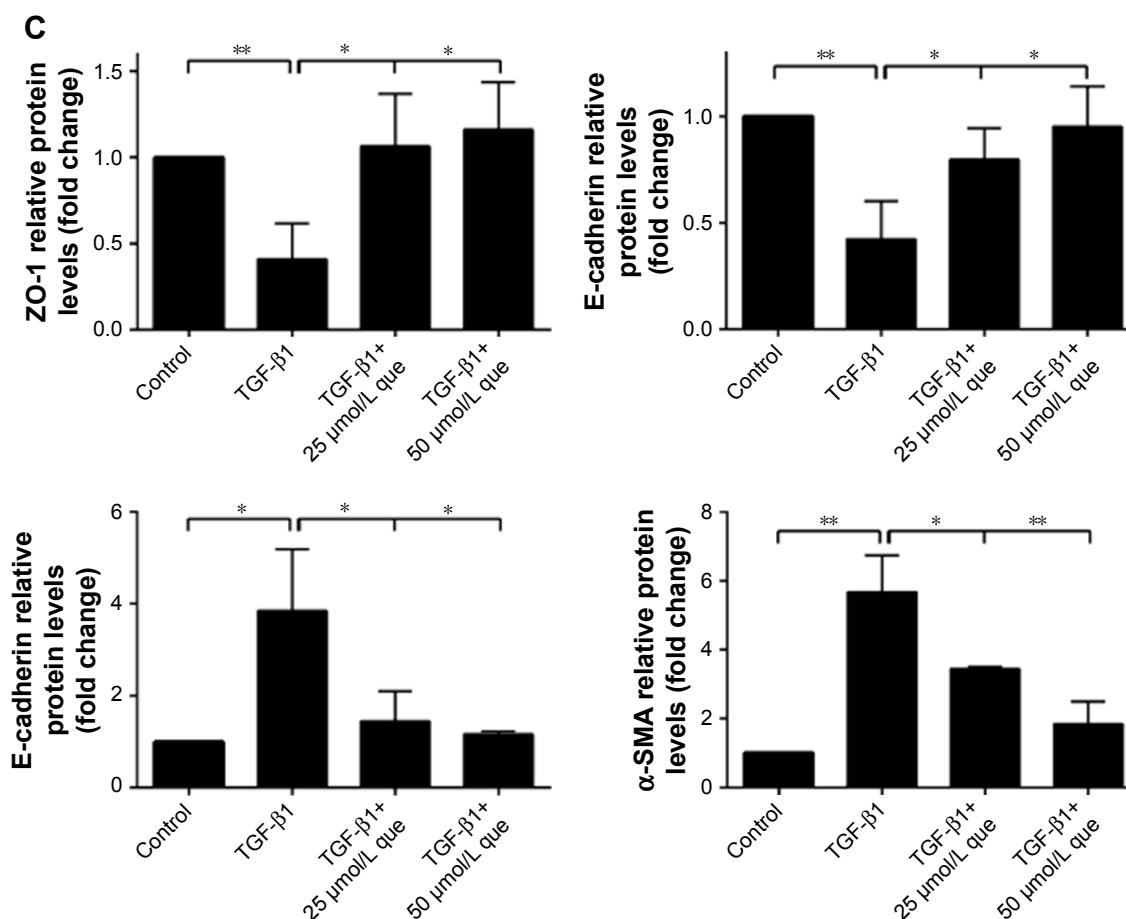


Figure 6 (Continued)





**Figure 6** Quercetin suppressed TGF- $\beta$ 1-induced EMT in RPE cells.

**Notes:** (A) The mRNA levels of EMT marker genes (ZO-1, E-cadherin, N-cadherin, and  $\alpha$ -SMA) were detected by real-time PCR. (B) The protein levels of EMT marker (ZO-1, E-cadherin, N-cadherin, and  $\alpha$ -SMA) were detected by Western blot in TGF- $\beta$ 1 and quercetin-treated RPE cells for 48 hours. (C) Relative protein expression (normalized to GAPDH) was quantified in Western blots via recording gray scale values. The data are presented as the mean  $\pm$  SEM.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

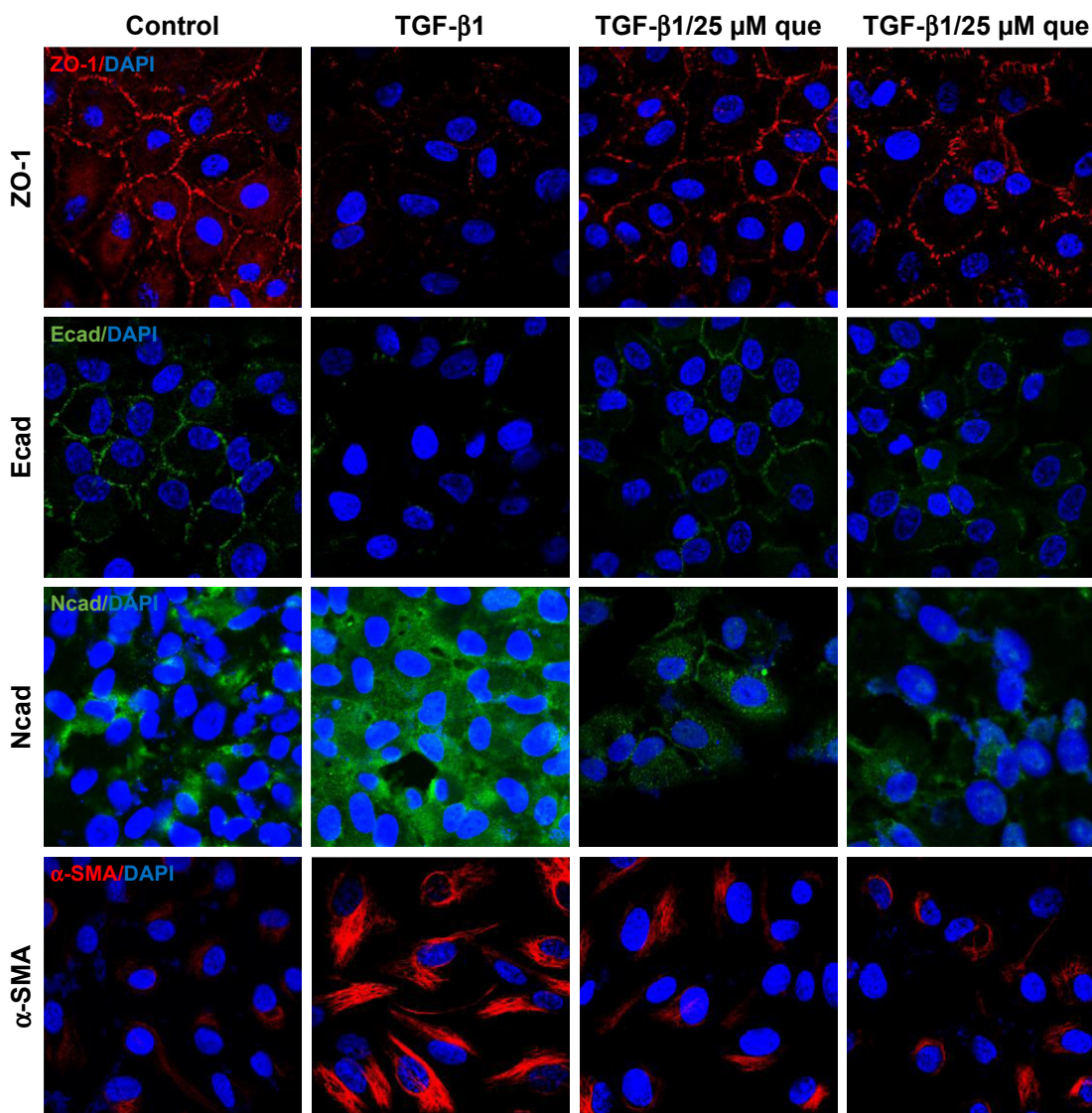
**Abbreviations:**  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; EMT, epithelial–mesenchymal transition; que, quercetin; RPE, retinal pigment epithelial; ZO-1, zonula occludens-1.

inflammatory factors and cytokines such as Granulocyte colony-stimulating factor (Platelet-derived growth factor-bb G-CSF), Platelet-derived growth factor-bb (PDGF-bb), and VEGF,<sup>28</sup> and macrophages release inflammatory cytokines, which trigger cell proliferation and migration.<sup>29</sup> Fibroblasts might be involved in the contraction of epiretinal membranes, which would accelerate the progression of PVR.<sup>30</sup> Various studies have illuminated that certain growth factors, cytokines, MMPs, and chemokines are present in the vitreous or subretinal fluid of PVR patients,<sup>31</sup> and Hoerster et al indicated that several factors, such as TGF- $\beta$ 1 and 2, IL-6 and 8, and CC-chemokine ligand 2/monocyte chemoattractant protein, are involved in PVR development.<sup>32</sup>

There have been many efforts to prevent the occurrence or progression of PVR in the last few decades.<sup>2</sup> Some therapeutic strategies, such as vitreous surgery or the use of pharmacologic agents, have been studied in PVR treatment.<sup>1</sup> In 1984,

corticosteroids were first studied in the rabbit vitreous in an experimental model of PVR.<sup>33</sup> However, complications with secondary ocular hypertension after intravitreal injection of triamcinolone have been reported,<sup>34</sup> and patients under PVR treatment had a poor response.<sup>35</sup> Pharmacologic agents such as anti-inflammatory, anti-proliferative, or antioxidant agents have been investigated for PVR treatment. Anti-proliferative agents used in previous studies include curcumin, resveratrol, and epigallocatechin gallate.<sup>36–38</sup> However, none of these compounds have been incorporated into clinical treatments. Thus, it is necessary to investigate additional pharmacologic agents in the treatment of PVR.

Quercetin exhibits beneficial properties such as antioxidative, anti-inflammatory, anti-angiogenic, and anticarcinogenic activities in various diseases. In 2011, Kwiecinski et al reported that by acting as a free radical scavenger, quercetin effectively inhibited oxidative stress.<sup>39</sup> Feng et al found that quercetin could



**Figure 7** Immunofluorescence analysis of EMT-related proteins in ARPE-19 cells.

**Notes:** After RPE cells were treated with 10 ng/mL of TGF- $\beta$ 1 with or without quercetin (50 and 100  $\mu$ M) for 48 hours, ZO-1, E-cadherin, N-cadherin, and  $\alpha$ -SMA were detected using the primary antibody. DAPI was incubated to detect nuclei. The photos were recorded by confocal microscopy. Original magnification: 630 $\times$ , oil. Scale bar: 10  $\mu$ m. Top to bottom: ZO-1.

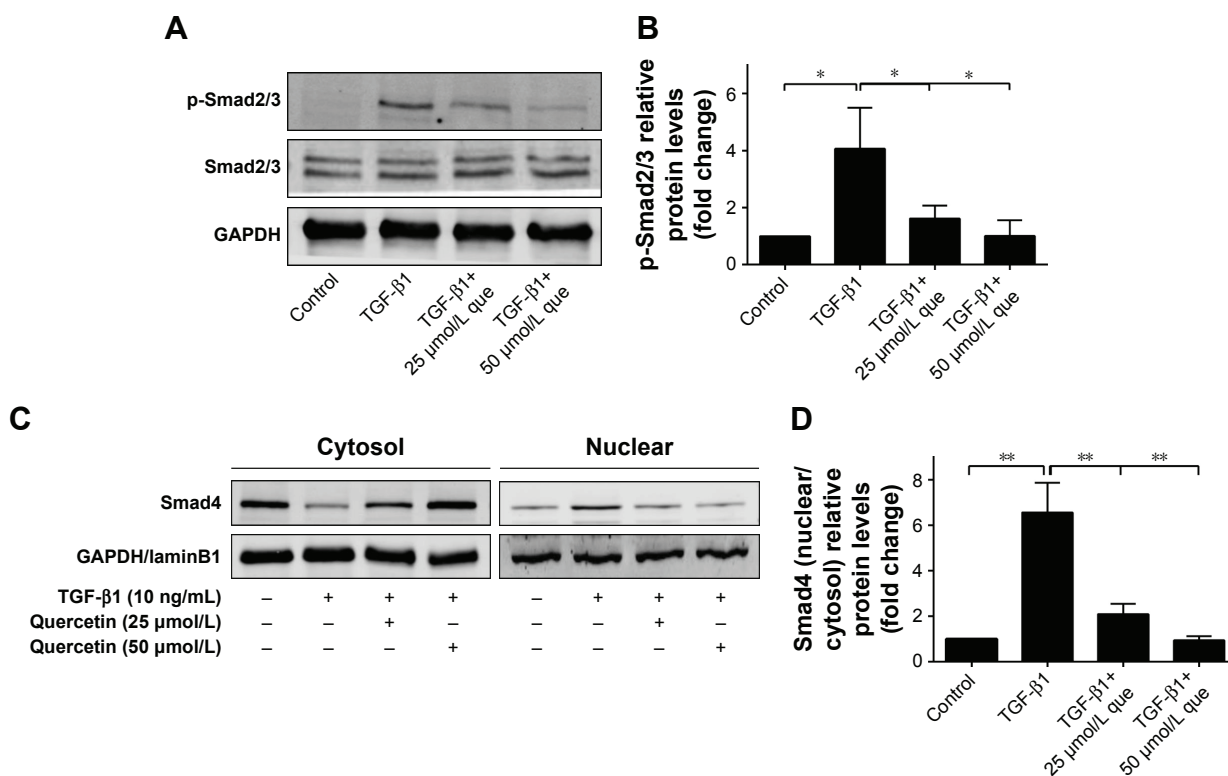
**Abbreviations:**  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; Ecad, E-cadherin; EMT, epithelial–mesenchymal transition; Ncad, N-cadherin; que, quercetin; RPE, retinal pigment epithelial; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; ZO-1, zonula occludens-1.

restrain EMT by regulating E-cadherin expression, as enhancing E-cadherin expression restored cell–cell adhesion to prevent the progression of EMT.<sup>40</sup> In ocular diseases, quercetin can protect the lens from oxidative damage and prevent cataract development due to its strong antioxidant and chelating properties.<sup>41</sup>

In our study, we investigated the effect of quercetin on ameliorating TGF- $\beta$ 1-induced RPE cell proliferation, migration, and collagen secretion. First, RPE cells were exposed to various concentrations of TGF- $\beta$ 1 for 24 and 48 hours to determine the most appropriate concentrations. As 10 ng/mL of TGF- $\beta$ 1 could effectively accelerate proliferation, this was used in our experiments. Next, we treated these cells

with quercetin to observe the effects on proliferation, and the results showed that quercetin could effectively inhibit TGF- $\beta$ 1-induced cell proliferation at both 24 and 48 hours. TGF- $\beta$ 1 caused the epithelial cells to transform their epithelial morphology to a mesenchymal morphology. RPE cell migration has a crucial role in the progression of PVR, and TGF- $\beta$ 1 promoted cell migration, whereas quercetin suppressed migration in a concentration-dependent manner.

MMP-2 and MMP-9 were correlated with collagen and ECM protein production, which induced the development and progression of PVR. Wang et al reported that repression of MMP-2 and MMP-9 could markedly reverse EMT



**Figure 8** Quercetin attenuates TGF- $\beta$ 1-induced Smad2/3 phosphorylation and nuclear translocation of Smad4 in RPE cells.

**Notes:** (A) The protein levels of p-smad2/3 were detected via Western blot in RPE cells treated with or without quercetin and TGF- $\beta$ 1 for 48 hours. (B) Quantitative data for p-Smad2/3 relative protein levels are calculated based on the expression of Smad2/3. (C) The protein levels of Smad4 in both cytoplasm and nucleus were detected via Western blot. (D) Quantitative data for Smad4 (nuclear vs cytosol ratio) relative protein levels are calculated. The results are presented as mean  $\pm$  SEM. \* $p$ <0.05, \*\* $p$ <0.01.

**Abbreviations:** que, quercetin; RPE, retinal pigment epithelial; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

in human non-small-cell lung cancer (NSCLC).<sup>42</sup> Lin et al showed that inhibition of MMP-9 could reduce RPE cell migration so that ameliorated the PVR progression.<sup>10</sup> In our studies, quercetin could markedly suppress the MMP-2 and MMP-9 expression. Thus, quercetin effectively decreased extracellular collagen production.

TGF- $\beta$ /Smad signaling pathway is involved in many diseases such as PVR,<sup>43</sup> multiorgan fibrosis,<sup>44–46</sup> and cancers.<sup>47,48</sup> TGF- $\beta$  induces the activation and phosphorylation of Smad2/3, forming trimers with Smad4. Then the complex is transported into the nucleus. Wu et al have shown that quercetin could inhibit Smad signaling pathway in the hepatic fibrosis animal model, and our results were consistent with it.<sup>49</sup> Xin et al reported that incubation with quercetin of 1, 3, and 10  $\mu$ M concentration for 48 hours could effectively inhibit Smad2/3 activation, which protected renal fibrosis of diabetic rats.<sup>50</sup> Lu et al found that quercetin ameliorated kidney injury via the inactivation of TGF- $\beta$ 1/Smad2/3 signaling, which decreased the ECM production.<sup>51</sup>

In the present study, expression of the tight junction protein ZO-1 and E-cadherin was upregulated after incubation

with quercetin, which contributed to maintaining epithelial cell properties. Loss of cell–cell adhesion plays a vital role in the progression of EMT, and E-cadherin is involved in EMT as a key mediator of cell–cell adhesion. The mesenchymal markers  $\alpha$ -SMA and N-cadherin were upregulated during EMT progression, and the ability of quercetin to modulate these markers would promote EMT. Our results show significant increases in phosphorylated Smad2/3 under TGF- $\beta$ 1 stimulation, which was restrained by quercetin. These findings indicate that quercetin may prevent the development of EMT by regulating the Smad2/3 pathway.

There are some limitations to our study. First, we investigated the effect and mechanism of quercetin in cultured RPE cells. However, we did not establish an animal model of PVR, such as RPE cell-induced PVR in pigmented rabbits. Second, we elucidated the therapeutic effect of quercetin on TGF- $\beta$ 1-induced EMT via the Smad-dependent signaling pathway, although other signaling pathways are involved in EMT. Thus, further studies should be applied to reveal the mechanism of quercetin in cell lines and PVR animal models to determine the underlying effects.

## Conclusion

We found that quercetin significantly suppressed TGF- $\beta$ 1-induced proliferation and migration without influencing cell viability. Our findings suggest that quercetin is a potential therapeutic agent in PVR therapy. Further studies are needed to investigate the mechanisms in detail before the clinical application of quercetin.

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## Disclosure

The authors report no conflicts of interest in this work.

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