• Basic Research •

JNK inhibitor SP600125 alleviates TGF-β2-induced epithelial-mesenchymal transition in RPE cell *via* TGF-βR2/Smad2/3 and JNK/c-Jun pathway

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Abstract

● **AIM:** To explore the effect of SP600125, a c-Jun N-terminal kinases (JNK) inhibitor, on epithelial-mesenchymal transition (EMT) in retinal pigment epithelial (RPE) cell caused by transforming growth factor-beta 2 (TGF-β2).

● **METHODS:** Human RPE cell line (ARPE-19) cells were treated with TGF-β2 and JNK inhibitor SP600125 *in vitro*. Cellular viability, migration and proliferation in ARPE-19 cells were examined by cell counting kit-8 (CCK-8) assay, wound scratch, and bromodeoxyuridine (BrdU) staining assay, respectively. Transforming growth factor-beta receptor 2 (TGF-βR2), Smad2/3, JNK, c-Jun, alpha-smooth muscle actin (α-SMA), N-cadherin, and vimentin proteins were analyzed by immunoblotting. Moreover, TGF-βR2 was detected by immunofluorescence assay.

● **RESULTS:** TGF-β2 significantly enhanced viability, migration, and proliferation in ARPE-19 cells, induced phosphorylation of TGF-βR2, Smad2/3, JNK, and c-Jun, and upregulated α-SMA, N-cadherin, and vimentin expression. SP600125 inhibited these cellular processes and reduced the expression/phosphorylation of the above proteins; notably, it blocked TGF-β2-induced effects, including cell viability, migration, proliferation, phosphorylation of TGF-βR2, Smad2/3, JNK, and c-Jun, as well as upregulation of α-SMA, N-cadherin, and vimentin.

● **CONCLUSION:** JNK inhibitor SP600125 suppresses TGF-β2-induced the increases in cell viability, migration, proliferation, and EMT in RPE cells *via* the TGF-βR2/Smad2/3 and JNK/c-Jun signaling pathways.

● **KEYWORDS:** epithelial-mesenchymal transition; transforming growth factor-beta 2; c-Jun N-terminal kinases; SP600125; retinal pigment epithelial cell

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INTRODUCTION

Proliferative vitreoretinopathy (PVR), a blinding retinal disease, is the most serious complication of the failure of rhegmatogenous retinal detachment operations and has become an enormous challenge for ophthalmologist in treatment[1]. The manifestations of PVR are mainly production and contraction of proliferative membranes and fibrosis on top of and beneath in the retina[2]. Some studies have found that epithelial-mesenchymal transition (EMT) caused by extracellular damage and abnormal growth factor stimulation in retinal pigment epithelial (RPE) cells is known as a crucial pathogenic mechanism of PVR[3-4]. The RPE cells are cobblestone-like monolayer polarized cells that exist between the neuroretina and choroid. These cells have the functions of phagocytosis, antioxidation, and material transport, participate in the forming a part of the blood-retinal barrier, and are crucial in maintaining the retina's structure and function[5]. When ocular trauma occurs or the balance of the intraocular environment is disrupted, RPE cells gradually lose their epithelioid morphology, obtain mesenchymal-like cell morphology, and migrate to the vitreous cavity, resulting in cellular membrane growth and contraction, tractional retinal detachment, and PVR[6]. Some studies show that transforming growth factor

beta (TGF-β) stimulates cellular EMT by the classical and non-classical signaling pathways[7]. However the mechanism of cellular EMT is complex, and it is worthwhile to consider whether the pathological progression of PVR can be alleviated by inhibiting the cellular EMT process in RPE cells.

The c-Jun N-terminal kinase (JNK), one of the mitogen-activated protein kinase family, can be activated by inflammatory cytokines, ultraviolet-visible radiation and hypoxia, and has prominent functions in biological processes such as inflammation, cellular proliferation and survival, and cell apoptosis and death[8]. Some studies found that JNK activation promoted cellular EMT[9-11]. Other studies showed that some drugs or molecules regulated the cellular EMT process through the JNK pathway[12-13]. Our previous study found that epidermal growth factor-induced RPE cell activation was accompanied by JNK activation[14], but the specific mechanism of affecting EMT through JNK inhibition was not clear. Some studies showed pharmacologic inhibition or knockdown of TGF-β or JNK signaling reduced cell proliferation, colony formation, and cell migration[15]. The SP600125, as an effective inhibitor of JNK, has been used to regulate JNK in different studies[16]. However, it remains unclear whether SP600125 can alleviate RPE cells EMT caused by TGF-β.

In the present study, the TGF-β2 and JNK inhibitor SP600125 were used to treat ARPE-19 cell and the effect of SP600125 was explored. Our data indicated that SP600125 alleviated TGF-β2-caused RPE cells EMT through the TGFβR2/Smad2/3 and JNK/c-Jun signaling pathway.

MATERIALS AND METHODS

Cell Line and Reagent A human RPE cell line (ARPE-19) was from the American Type Culture Collection (Manassas, VA, USA). TGF-β2 (human TGF-beta 2 recombinant protein, PeproTech®, USA), total and phosphorylated TGF-β receptor 2 (TGF-βR2) and phosphorylated Smad2/3 primary antibodies were from ThermoFisher Scientific (Cranbury, NJ, USA). SP600125 came from Selleck Chemiacls (Shanghai, China). Cell counting kit-8 (CCK-8) kit was purchased from EnoGene Biotech (Nanjing, China). Total Smad2/3, total and phosphorylated JNK and c-Jun, alpha-smooth muscle actin (α-SMA), vimentin, and N-cadherin primary antibodies, and biotin-horseradish peroxidase (HRP) labeled secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell Viability Assay ARPE-19 cells (8×10^3 cells/well) were treated with different reagents in 96-well cell culture plates. Then, CCK-8 solution was added in culture medium for 1h. Absorbance was assessed at 450 nm by using a microplate reader (Gene Company limited, Beijing, China). Cell viability was represented by the optical density of each well.

BrdU Staining Assay ARPE-19 cells were cultured in media with 5-bromo-2'-deoxyuridine (BrdU) solution (30 μmol/L) for 4h in 12-well cell culture plates following treatment with different reagents. Then, 4% paraformaldehyde solution was used to fix cells for 15min, eluted thrice with phosphate buffer solution (PBS), acidified by 2 mol/L hydrochloric acid for 5min, incubated in PBS with 5 % bovine serum albumin for 1h. Next step, the cells were immersed in BrdU antibody solution at 37℃ for 2h, washed thrice, and incubated with fluorescent secondary antibody solution for 1h in a dark environment. Finally, the cells were sealed with anti-fluorescence mounting medium with 4',6-diamidino-2-phenylindole (DAPI) and captured *via* a fluorescence microscope (Olympus, Tokyo, Japan).

Cell Migration Assay When cell density was nearly 90%–100% of cell fusion, the monolayer cell was scratched to form a wound injury by a pipette tip (200 μL). Then, cells were treated with reagent and the "wound injury" were captured and the cell migration was calculated and analyzed by Image J software (National Institutes of Health, Bethesda, USA).

Western Blotting Analysis After different treatments, a lysis buffer was used to lyse cells and the cell lysis solution was centrifuged, added to the loading buffer, and heated for 10min at 100℃. The proteins of the sample were isolated by using electrophoresis and transferred by electrophoretic transfer apparatuses to polyvinylidene fluoride membrane. The membrane was placed in a skimmed milk solution (5%) to block for 1h, rinsed thrice, and immersed in primary antibody solution in a 4℃ environment overnight. Next, at room temperature, the membrane was washed thrice and immersed in the secondary antibody solution for 1h, rinsed thrice, combined with a chemiluminescence reagent, and the specific protein band was displayed using a gel imaging system (Gene Company limited, Beijing, China).

Immunofluorescence Staining Assay ARPE-19 cells on glass slides in 12-well plates were treated with reagents. After 4% paraformaldehyde fixation for 15min, cells were rinsed three times in phosphate buffer solution, permeabilized for 10min, and rinsed thrice again. In the next step, cells were blocked with bovine serum albumin solution (5%) for 1h, rinsed thrice, incubated with primary antibody solution for 2h. Finally, the cells were immersed in fluorescently-labeled secondary antibody for 1h, rinsed thrice, sealed with anti-fluorescence mounting medium with DAPI, and captured with a fluorescence microscope.

Statistical Analysis All experimental data were presented as mean±standard error of the mean (SEM) and statistical analysis of differences was performed by one-way analysis of variance and Bonferroni test by GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered statistical significance.

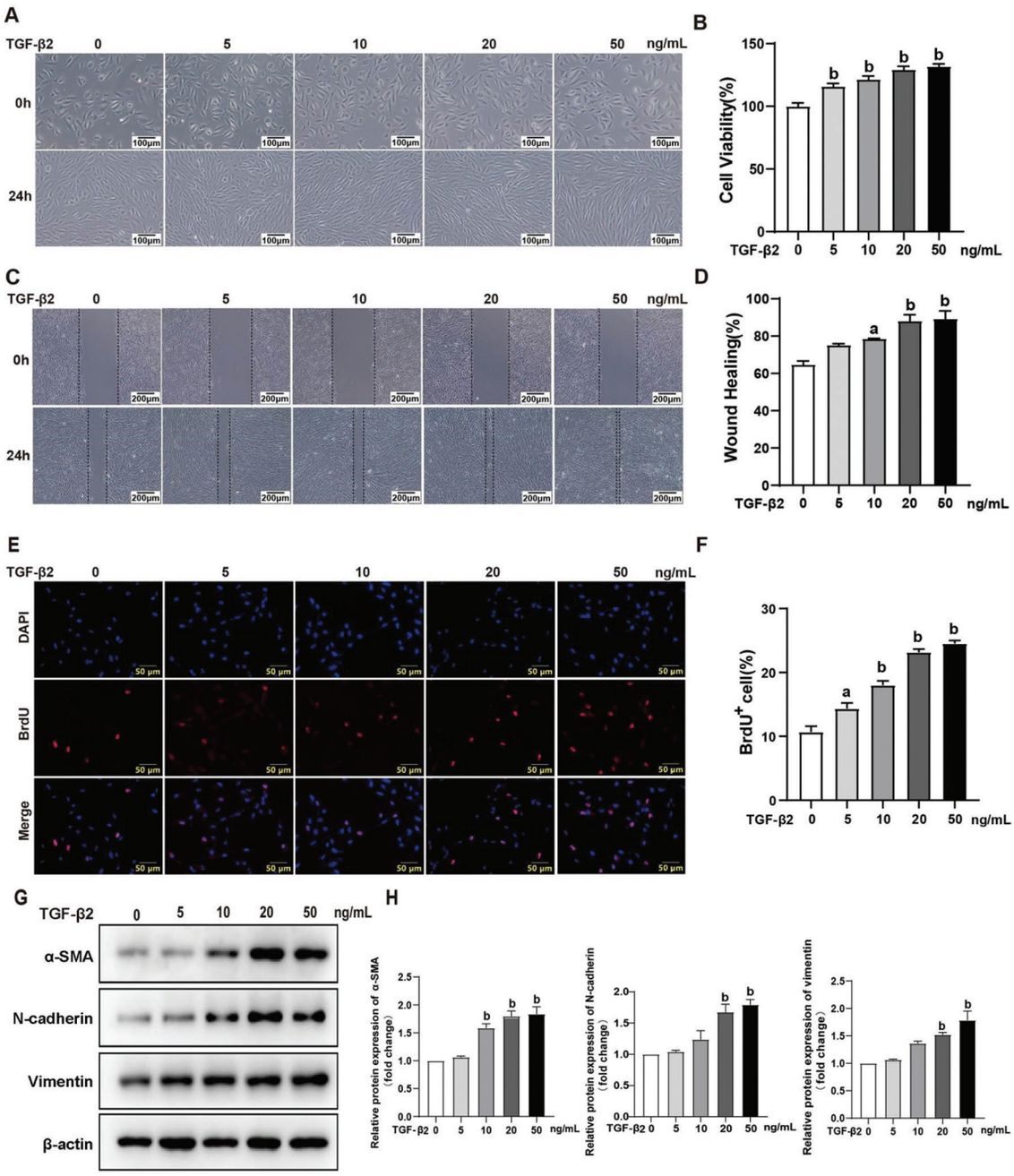

Figure 1 TGF-β2 caused EMT of ARPE-19 cells ARPE-19 cells were dealt with 0, 5, 10, 20, and 50 ng/mL TGF-β2 for 24h. A: An inverted phase contrast microscope was used to capture image of cell morphology, scale bar: 100 μm; B: CCK-8 assay was used to measure cell viability, *n*=4; C, D: Wound-healing assay was used to measure cell migration, *n*=3, scale bar: 200 μm; E, F: BrdU staining assay was used to analyze cell proliferation, *n*=3, scale bar: 50 μm; G, H: α-SMA, N-cadherin, and vimentin proteins were analyzed by using Western blot assay. [a]*P*<0.05, [b]*P*<0.01 *vs* 0 ng/mL TGF-β2. TGF-β2: Transforming growth factor-beta 2; EMT: Epithelial-mesenchymal transition; ARPE-19: Human retinal pigment epithelial cell line; CCK-8: Cell counting kit-8; BrdU: 5-bromo-2'-deoxyuridine; α-SMA: Alpha-smooth muscle actin; DAPI: 4',6-diamidino-2-phenylindole.

RESULTS

TGF-β2 Induced EMT of ARPE-19 Cells ARPE-19 cells' morphologic change, viability, migration, and proliferation were observed after treatment with 0, 5, 10, 20, and 50 ng/mL TGF-β2 for 24h. As Figure 1A showed, the ARPE-19 cells appeared as irregular polygonal paving stones in the control group, but, after treatment for 24h, the shape of the ARPE-19 cells was spindle-shaped in the 5, 10, 20, and 50 ng/mL TGF-β2 groups. The CCK-8 assay showed that, compared with the control, cell viability increased by 15.93%, 21.61%, 29.26%, and 31.80% in the 5, 10, 20, and 50 ng/mL TGF-β2 groups, respectively (Figure 1B). The cell migration assay data showed, compared with the control, cell migration increased by 10.42%, 13.80%, 23.34%, and 24.50% in the 5, 10, 20, and

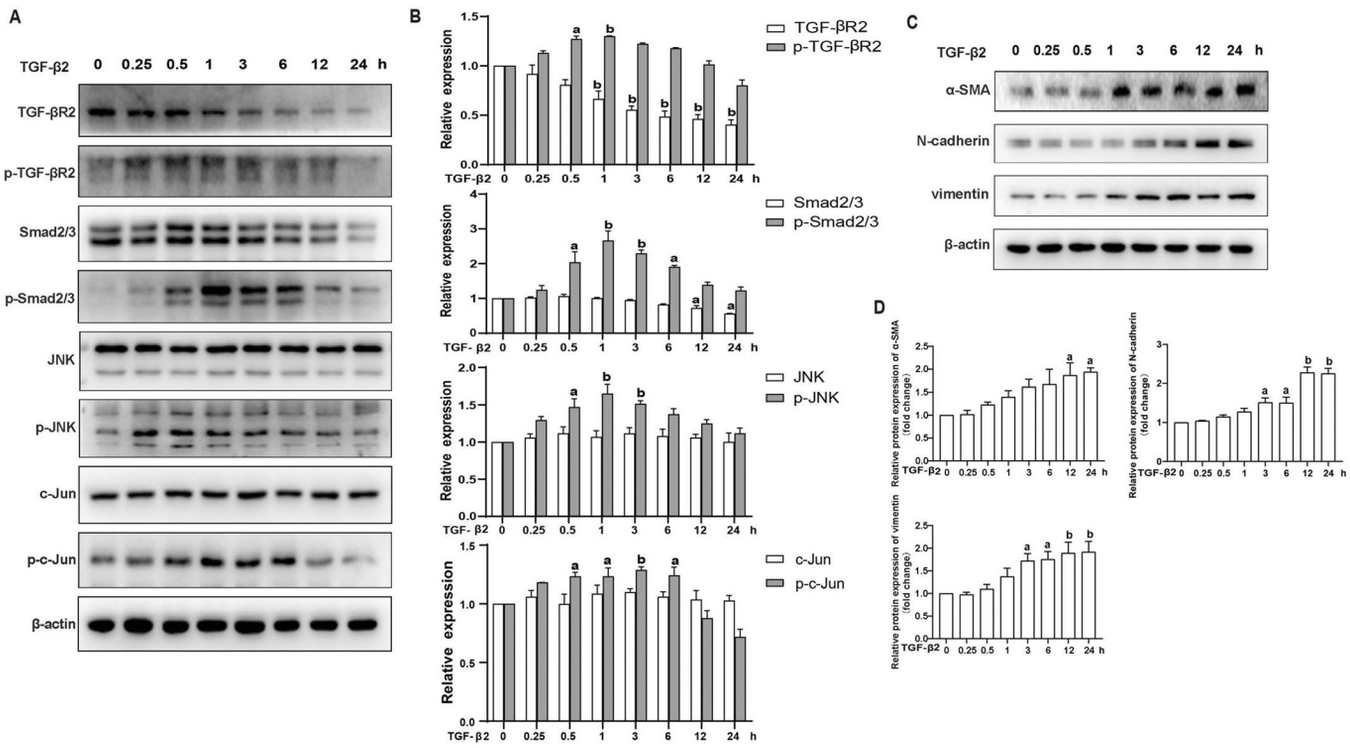

Figure 2 TGF-β2 affected expression of EMT relative proteins through TGF-βR2/Smad2/3 and JNK/c-Jun pathway ARPE-19 cells were dealt with 20 ng/mL TGF-β2 for 0, 0.25, 0.5, 1, 3, 6, 12, and 24h. A, B: The total and phosphorylated TGF-βR2, Smad2/3, JNK, and c-Jun proteins were quantified by Western blot assay; C, D: α-SMA, N-cadherin, and vimentin proteins were quantified by Western blot. $^{a}P<0.05$, $^{b}P<0.01$ *vs* 0h. TGF-β2: Transforming growth factor-beta 2; EMT: Epithelial-mesenchymal transition; TGF-βR2: Transforming growth factor-beta receptor 2; JNK: c-Jun N-terminal kinases; ARPE-19: Human retinal pigment epithelial cell line; α-SMA: Alpha-smooth muscle actin.

50 ng/mL TGF-β2, respectively (Figure 1C, 1D). The BrdU staining assay revealed that, compared with the control, the ratio of positively stained cells increased by 3.63%, 7.31%, 12.46%, and 13.81% in the 5, 10, 20, and 50 ng/mL TGF-β2 groups, respectively (Figure 1E, 1F). Western blot data indicated that treatment with TGF-β2 for 24h up-regulated expression levels of α-SMA, N-cadherin, and vimentin proteins (Figure 1G, 1H). TGF-β2 20 ng/mL treatment for different time caused down-regulation of the expression of total TGF-βR2 and Smad2/3 proteins, but did not affect the expression levels of total JNK/c-Jun proteins. It is worth noting that 20 ng/mL TGF-β2 induced significant phosphorylation of TGF-βR2, Smad2/3, JNK, and c-Jun proteins (Figure 2A, 2B). Meanwhile, treatment with 20 ng/mL TGF-β2 up-regulated expression levels of α-SMA, N-cadherin, and vimentin proteins as the processing time increased (Figure 2C, 2D).

SP600125 Inhibited Cellular Activity in ARPE-19 Cells By using inverted phase contrast microscope, we found that SP600125 did not cause significant changes in cell morphology (Figure 3A). The CCK-8 assay indicated that, compared with the control, cellular viability decreased by 9.32%, 16.76%, 31.68%, and 33.54% in the 5, 10, 25, and 50 μmol/L SP600125 groups, respectively (Figure 3B). The cell migration assay data indicated that, compared with the control, the relative migration decreased by 27.15%, 30.96%, 48.94%, and 60.8% in the 5, 10, 25, and 50 μmol/L SP600125 groups, respectively (Figure 3C, 3D). BrdU staining assay data indicated, compared with the control, the ratio of positively stained cells decreased by 1.74%, 3.46%, 6.89% and 8.94% in the 5, 10, 25, and 50 μmol/L SP600125 groups, respectively (Figure 3E, 3F). The data of Western blot assay indicated that SP600125 down-regulated the expression levels of TGF-βR2, Smad2/3, JNK, c-Jun, α-SMA, N-cadherin, and vimentin proteins after treatment with SP600125 for 24h (Figure 4A, 4B). The immunofluorescence assay displayed that SP600125 (50 μmol/L) decreased expression level of TGF-βR2 protein (Figure 4C).

SP600125 Alleviated TGF-β2-induced EMT in ARPE-19 Cells *via* TGF-βR2/Smad2/3 and JNK/c-Jun Pathways As Figure 5A showed, TGF-β2 caused a distinct spindle-shaped change of the ARPE-19 cells and SP600125 significantly prevented the change caused by TGF-β2. The CCK-8 assay displayed that cell viability decreased by 46.6% in the combined treatment with SP600125 and TGF-β2 group, compared to the TGF-β2 treatment (Figure 5B). The cell migration assay indicated that migration ability of ARPE-19 cell decreased by 33.63% in the combined treatment with SP600125 and TGF-β2 group, compared to the TGF-β2

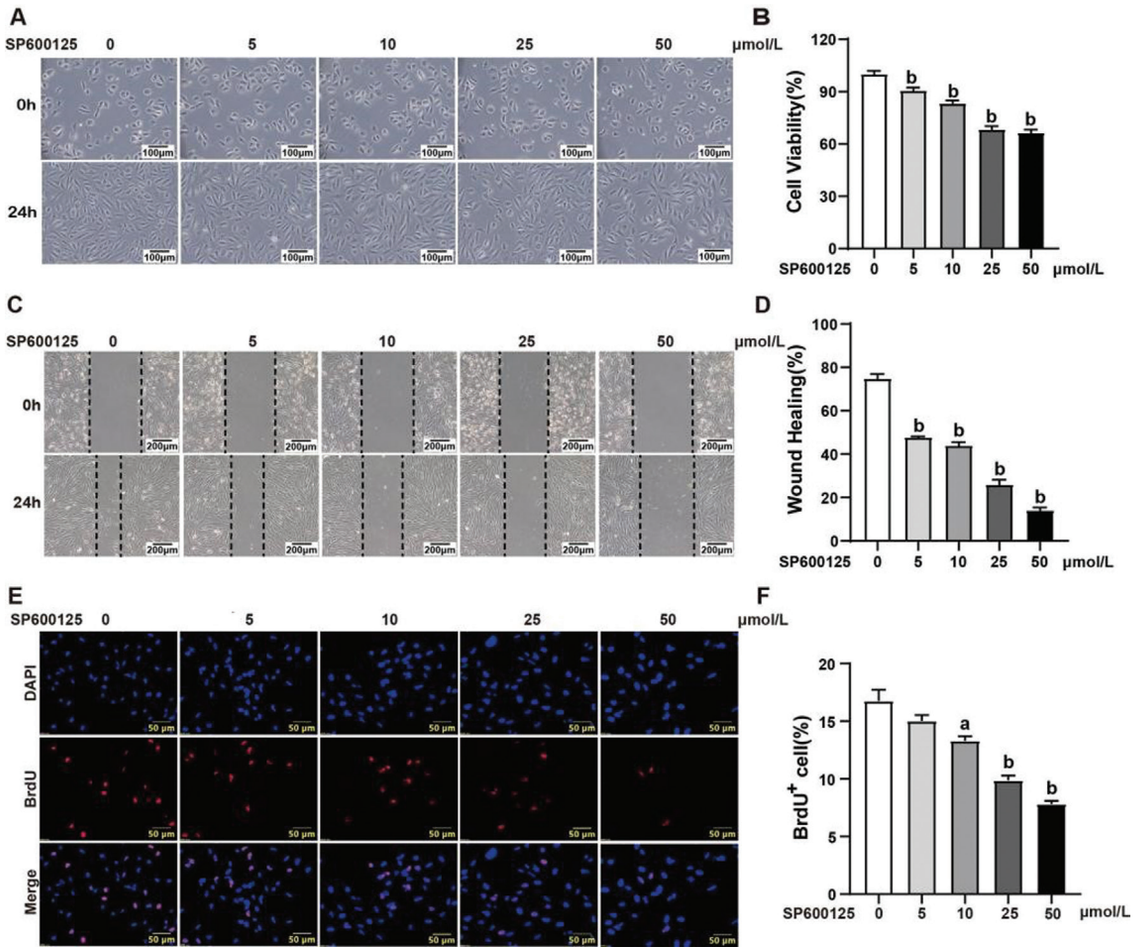

Figure 3 SP600125 affected ARPE-19 cells activity ARPE-19 cells were dealt with 0, 5, 10, 25, and 50 μmol/L SP600125 for 24h. A: Cell morphology was obtained from an inverted phase contrast microscope, scale bar: 100 μm; B: CCK-8 kit was used to test cell viability, *n*=3; C, D: Wound-healing assay was used to investigate cell proliferation, *n*=3, scale bar: 200 μm; E, F: BrdU staining assay was used to measure cell proliferation, *n*=3, scale bar: 50 μm. [a]*P*<0.05, [b]*P*<0.01 *vs* 0 μmol/L SP600125. CCK-8: Cell counting kit-8; BrdU: 5-bromo-2′-deoxyuridine; ARPE-19: Human retinal pigment epithelial cell line; DAPI: 4′,6-diamidino-2-phenylindole.

treatment (Figure 5C, 5D). The data from BrdU staining assay showed that the ratio of positively stained cells decreased by 6.36% in the combined treatment with SP600125 and TGF-β2 group, compared to the TGF-β2 treatment (Figure 5E, 5F). The Western blot assay data indicated that SP600125 significantly suppressed the phosphorylation of TGF-βR2, Smad2/3, JNK, and c-Jun proteins activated by TGF-β2 for 1h (Figure 6A, 6B). Additionally, pretreatment with SP600125 for 12h significantly attenuated TGF-β2-induced up-regulation of expression levels of α-SMA, N-cadherin, and vimentin proteins (Figure 6C, 6D).

DISCUSSION

PVR is a serious blinding ocular disease that is characterized by the proliferative membranes on the outer and inner of the retina following EMT of RPE cells[17]. The loss of polarity of RPE cells leads to EMT and myofibroblast characteristics because of the influence of some abnormal extracellular factors in PVR[6]. Various abnormal expression of cytokines, such as TGF-β, epidermal growth factor, hepatocyte growth factor, and fibroblast growth factor have been found in the vitreous cavity of PVR patients[1,4,18]. Therefore, investigating the induction factors and molecular mechanisms of RPE activation and EMT might show potential for the prevention and cure of PVR.

TGF-β, a pivotal multifunctional cytokine including TGF-β1, -β2, and -β3, can accelerate cell migration and proliferation, and induce cell fibrosis deformation[19-21]. TGF-β2 is regarded as a crucial cytokine in the pathogenesis process of PVR and can directly induced RPE cells EMT[22-24]. In the present study, to find the optimal concentration of TGF-β2 for inducing EMT, ARPE-19 cells were dealt with different doses of TGF-β2. Our data showed that RPE cells viability, migration and proliferation were positively correlated with TGF-β2 concentrations. To explore the specific mechanism of action of TGF-β2, we observed changes of TGF-βR2, Smad2/3, JNK, and c-Jun proteins and EMT marker proteins after treatment with TGF-β2. Our data indicated TGF-β2 significantly induced phosphorylation of TGF-βR2, Smad2/3, JNK, and c-Jun proteins, and gradually increased expression

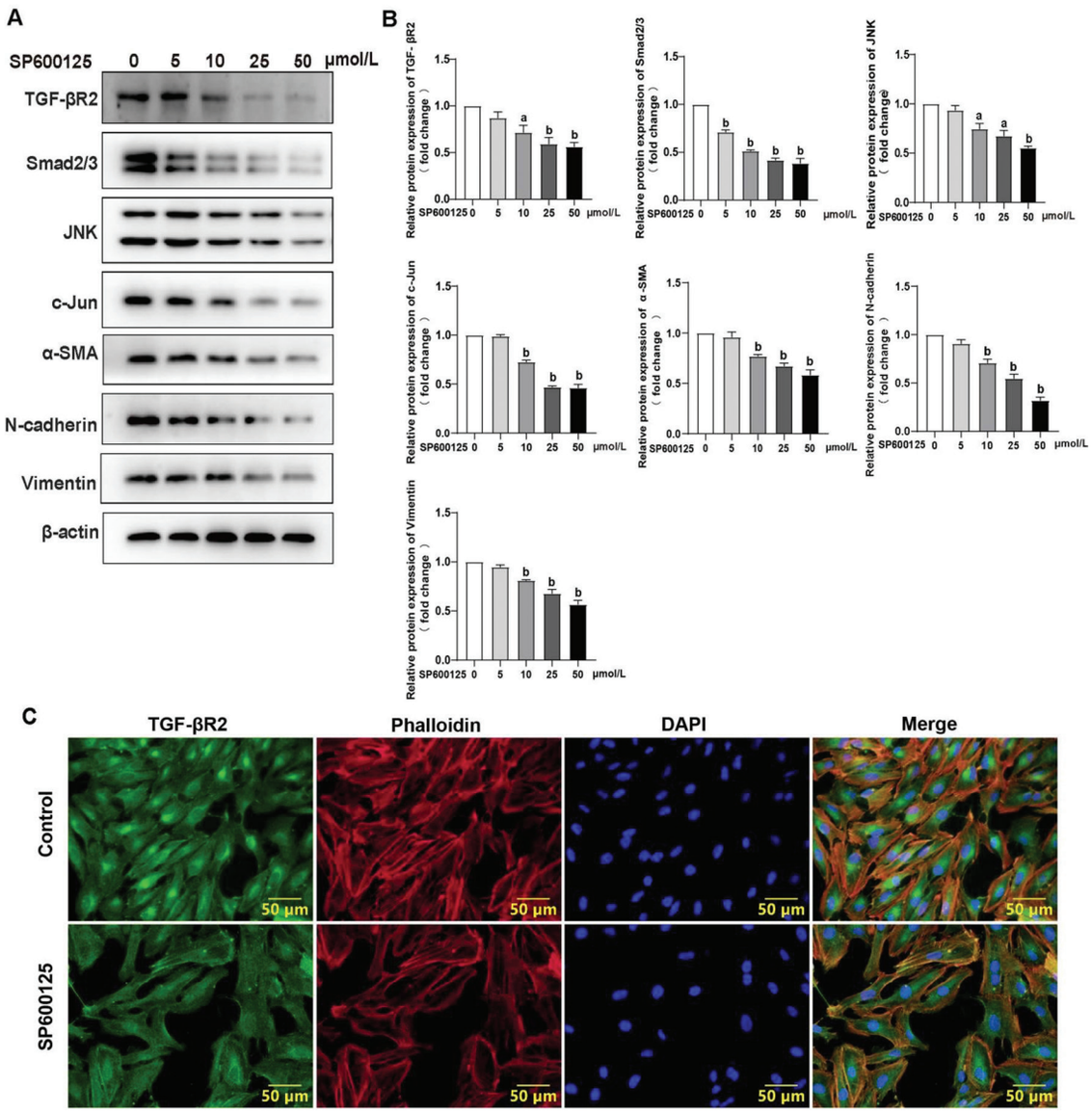

Figure 4 SP600125 affected expression levels of TGF-βR2, Smad2/3, JNK/c-Jun, α-SMA, N-cadherin, and vimentin proteins A, B: ARPE-19 cells were disposed with 0, 5, 10, 25, and 50 μmol/L SP600125 for 24h. TGF-βR2, Smad2/3, JNK, c-Jun, α-SMA, N-cadherin, and vimentin proteins was detected by using Western blot assay. [a]*P*<0.05, [b]*P*<0.01 *vs* 0 μmol/L SP600125. C: ARPE-19 cells were dealt with SP600125 (50 μmol/L) for 24h. TGF-βR2 was detected by immunofluorescence staining assay, scale bar: 50 μm. TGF-βR2: Transforming growth factor-beta receptor 2; JNK: c-Jun N-terminal kinases; α-SMA: Alpha-smooth muscle actin; ARPE-19: Human retinal pigment epithelial cell line; DAPI: 4',6-diamidino-2-phenylindole.

levels of α-SMA, N-cadherin, and vimentin proteins. These data showed, on the one hand, TGF-β2 had a noticeable effect on cellular viability, migration, and proliferation of RPE cells; on the other hand, TGF-β2 achieved the classical TGF-βR2/Smad and non-classical TGF-βR2/JNK signal pathway. Some researchers have reported that TGF-β2 promoted RPE cell migration, upregulated expression of α-SMA, fibronectin, and vimentin proteins, downregulated expression of E-cadherin protein[25], and induced phosphorylation of JNK and p38 mitogen-activated protein kinase proteins in RPE cells[26]. These researchers also found that inhibition of JNK or p38 caused partial alleviation of accelerating effects of bone morphogenetic protein 6 know down on the fibrosis of RPE cells caused by TGF-β2[26]. Therefore, we attempted to target JNK protein and treat the RPE cells with JNK inhibitor SP600125, and observed the effect and mechanism of SP600125 on EMT induced by TGF-β2 in RPE cells.

JNK, a serine threonine protein kinase, can be activated by stress or inflammatory factors. As a master protein kinase, JNK can regulate many physiological and pathological processes, including morphogenesis, cellular proliferation, differentiation, inflammatory responses, and death[27]. Some scholars have pointed out that JNK can be regarded as a potential target for anticancer therapy, but the sophisticated function of JNK protein is still unclear in current research[8]. SP600125, an anthrapyrazolone inhibitor of JNK, is widely used as an

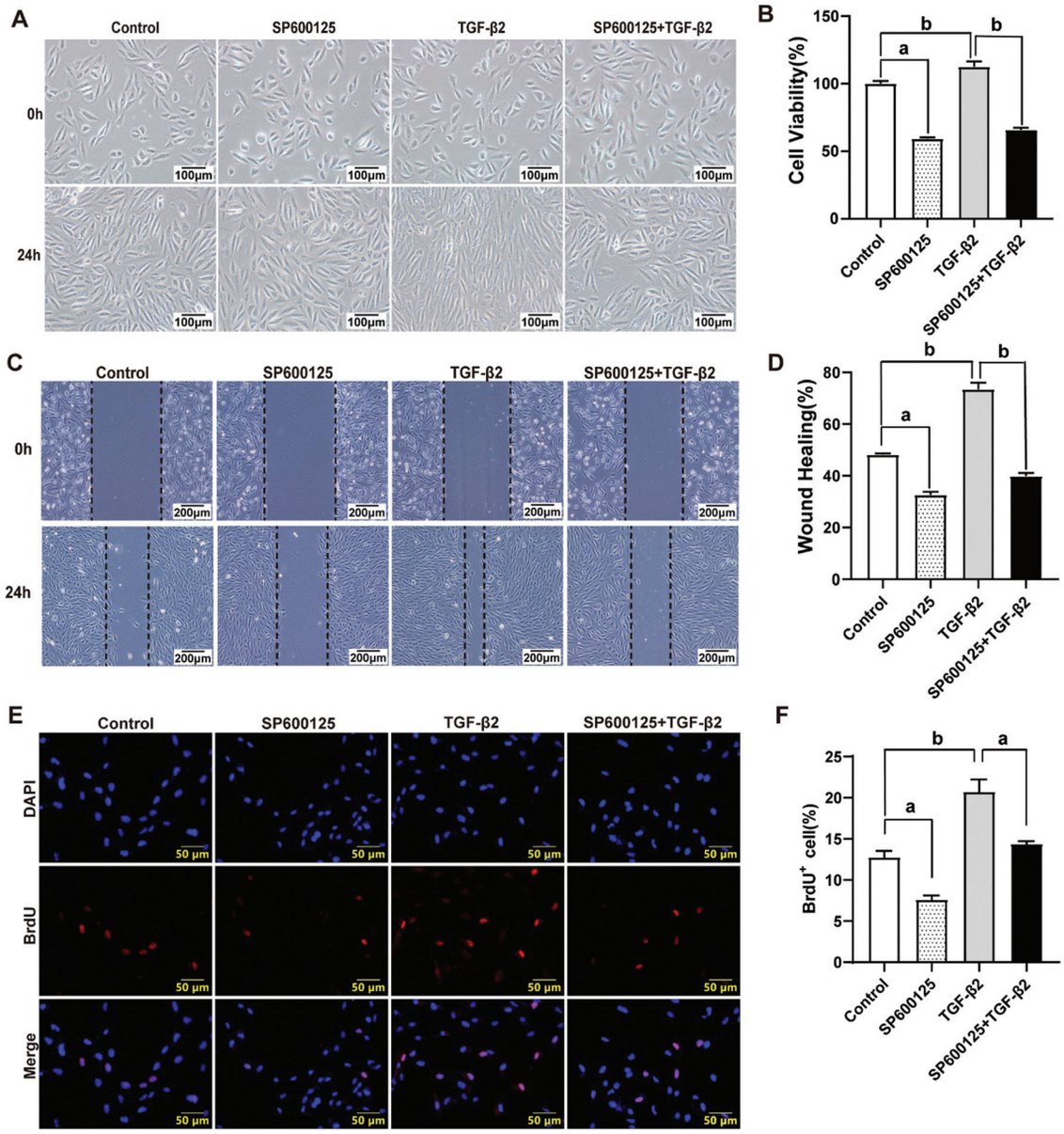

Figure 5 SP600125 inhibited activation induced by TGF-β2 in ARPE-19 cell After pretreatment with 10 μmol/L SP600125 for 12h, ARPE-19 cells were dealt with 20 ng/mL TGF-β2 for 24h. A: The cell morphology images were obtained from an inverted phase contrast microscope, scale bar: 100 μm; B: CCK-8 kit was used to detect cellular viability, *n*=3; C, D: Wound healing assay was used to measure cell migration, scale bar: 200 μm, *n*=3; E, F: BrdU staining assay was used to measure cell proliferation, *n*=3, scale bar: 50 μm. [a]*P*<0.05, [b]*P*<0.01. TGF-β2: Transforming growth factor-beta 2; ARPE-19: Human retinal pigment epithelial cell line; CCK-8: Cell counting kit-8; BrdU: 5-bromo-2′-deoxyuridine; DAPI: 4′,6-diamidino-2-phenylindole.

adenosine triphosphate-competitive selective inhibitor of JNK and has anticancer potential for multiple cancers[16,28-29]. Some researchers found SP600125 alleviated cleavage of caspase-3 and depolarization of matrix metallopeptidase, and augmented myeloid cell leukemia 1 protein level in silver nanoparticles-treated ARPE-19 cells[30], whereas SP600125 could not affect up-regulation of the level of interleukin-6 caused by MG132 in RPE culture medium[31]. In the present study, our results revealed that although the SP600125 did not cause significant changes in cell morphology, it can effectively inhibit RPE cells' viability, migration, and proliferation. To understand the specific action mechanism of SP600125, we observed its effect on JNK, c-Jun, TGF-βR2, and Smad2/3 proteins. Western blot assay data showed SP600125 down-regulated the expression of JNK/c-Jun and TGF-βR2/Smad2/3 proteins while decreasing the expression of α-SMA, N-cadherin, and vimentin proteins. These data suggest SP600125 not only targets the JNK/c-Jun and TGF-βR2/Smad signal pathway, but also can affect RPE cells activity through down-regulating expression of EMT relative proteins α-SMA, N-cadherin, and vimentin.

Based on above data, we speculated that SP600125 might affect TGF-β2-caused EMT of RPE cells. To confirm our speculation, we observed the effect of SP600125 pretreatment on TGF-β2-induced EMT in RPE cells. Our data indicated that

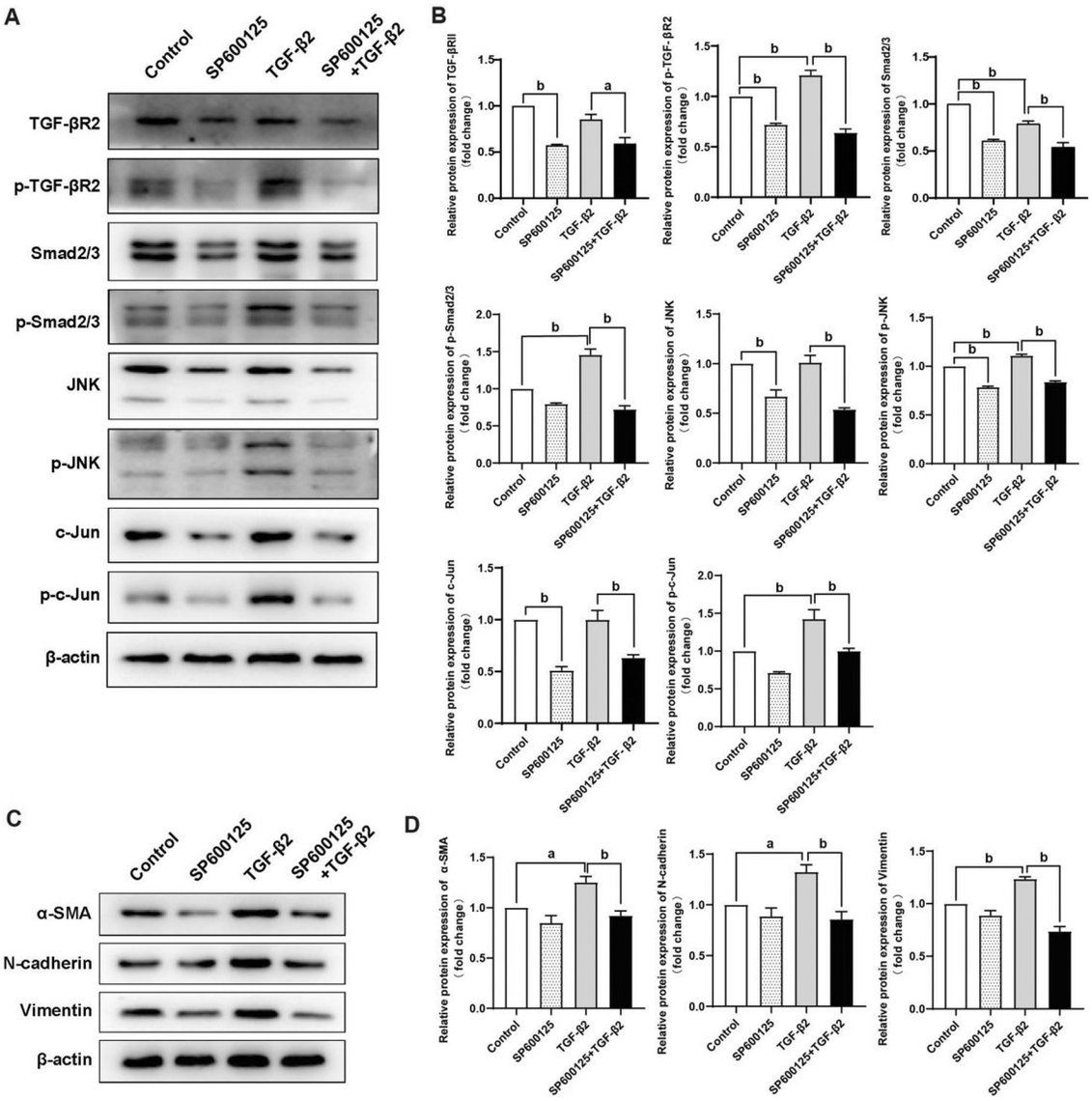

Figure 6 SP600125 inhibited EMT caused by TGF-β2 *via* the TGFβR2/Smad2/3 and JNK/c-Jun pathway in ARPE-19 cell TGF-β2 (20 ng/mL) was used to dealt with ARPE-19 cells for 1h following pretreatment with 10 μmol/L SP600125 for 12h. A, B: Western blot assay was used to analyze total and phosphorylated TGF-βR2, Smad2/3, JNK, and c-Jun proteins; C, D: Western blot assay was used to analyze the α-SMA, N-cadherin, and vimentin proteins. [a]*P*<0.05, [b]*P*<0.01. EMT: Epithelial-mesenchymal transition; TGF-β2: Transforming growth factor-beta 2; TGF-βR2: Transforming growth factor-beta receptor 2; JNK: c-Jun N-terminal kinases; ARPE-19: Human retinal pigment epithelial cell line; α-SMA: Alpha-smooth muscle actin.

SP600125 pretreatment significantly suppressed an increase in cell viability, migration, and proliferation induced by TGF-β2. SP600125 also significantly attenuated phosphorylation of JNK, c-Jun, TGF-βR2, and Smad2/3 proteins, and expression up-regulation of α-SMA, N-cadherin, and vimentin proteins caused by TGF-β2. Some studies have found that TGF-β2 can activate the classical or non-classical pathway downstream of the TGF-β/TGF-βR signaling pathway by binding to its specific receptor proteins on cell surface[7]. Some studies showed that TGF-β2 is a dominant isoform in the RPE layer and neural retina[32]. EMT of RPE cells caused by TGF-β2 is well known as an important mechanism responsible for the development of PVR[33]. As EMT-related markers, α-SMA, N-cadherin, and vimentin proteins are well established as RPE cells EMT and fibrosis-related proteins[34-35]. Based on above researches, our data explicitly suggested that SP600125 attenuated TGF-β2-caused EMT through TGF-βR2/Smad2/3 and JNK/c-Jun signal pathway in RPE cells, and might be an effective and potential reagent for preventing and treating PVR. Some studies have showed that SP600125 pretreatment alleviated TNF-α-induced inflammatory damage to bone mesenchymal stem cells in mice[36] and decreased tumor volume and induced spindle-shaped tumor cells *in vivo*[37]. However, the data in the present study still lack some results from experiments *in vivo*. Therefore, it is necessary for us to conduct further experiments of PVR *in vivo* to clarify specific mechanism of SP600125.

In conclusion, the JNK inhibitor SP600125 suppressed TGF-β2-caused activation and EMT of RPE cells through the TGF-βR2/Smad2/3 and JNK/c-Jun pathway which might be a promising approach to prevention and treatment of PVR.

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Data Availability Statement: The experimental data used and analyzed in this study were completed by authors themselves, and the data set can be obtained from the first authors or the corresponding author.

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Conflicts of Interest: Zhang HH, None; **Zhu YS,** None; **Guo JY,** None; **Chen XD,** None.

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