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Overexpression of Snail in retinal pigment epithelial triggered epithelial–mesenchymal transition

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ABSTRACT

Snail transcription factor has been implicated as an important regulator in epithelial–mesenchymal transition (EMT) during tumourigenesis and fibrogenesis. Our previous work showed that Snail transcription factor was activated in transforming growth factor β1 (TGF-β1) induced EMT in retinal pigment epithelial (RPE) cells and may contribute to the development of retinal fibrotic disease such as proliferative vitreoretinopathy (PVR). However, whether Snail alone has a direct role on retinal pigment epithelial–mesenchymal transition has not been investigated. Here, we analyzed the capacity of Snail to drive EMT in human RPE cells. A vector encoding Snail gene or an empty vector were transfected into human RPE cell lines ARPE-19 respectively. Snail overexpression in ARPE-19 cells resulted in EMT, which was characterized by the expected phenotypic transition from a typical epithelial morphology to mesenchymal spindle-shaped. The expression of epithelial markers E-cadherin and Zona occludin-1 (ZO-1) were down-regulated, whereas mesenchymal markers a-smooth muscle actin (a-SMA) and fibronectin were up-regulated in Snail expression vector transfected cells. In addition, ectopic expression of Snail significantly enhanced ARPE-19 cell motility and migration. The present data suggest that overexpression of Snail in ARPE-19 cells could directly trigger EMT. These results may provide novel insight into understanding the regulator role of Snail in the development of retinal pigment epithelial–mesenchymal transition.

1. Introduction

Epithelial–mesenchymal transition (EMT) is a fascinating phenotypic change that involves the loss of epithelial characteristics and acquires mesenchymal-like phenotype and migratory properties [1]. EMT was initially described in early embryogenesis; however, EMT has also recently been implicated in cancer progression, tissue repair and organ fibrosis [1–3]. Fibrotic diseases are characterized by the appearance of myofibroblasts, the key cell type involved in the fibrogenic reaction, and by excess accumulation of extracellular matrix with resultant tissue contraction and impaired function [4]. A multitude of studies have identified that myofibroblast and mature fibroblast in a significant portion of renal, ocular, peritoneal mesothelial, liver and pulmonary fibrosis arise from the conversion of epithelial cells through an EMT [2,5]. In one analysis, lineage-tagging experiments and bone marrow transplant studies demonstrated that during the course of kidney fibrosis in mice, about 30% of fibroblasts are derived via EMT from the tubular epithelial cells of the kidney [6]. Additionally, studies using fibrosis tissue from humans have also confirmed EMT. In a study of 133 patients with kidney fibrosis, an EMT was demonstrated in a substantial number of the samples, as evaluated using double labeling of the tubular epithelial cells with cytokeratin, vimentin, α-SMA, or zona occludens 1 (ZO-1) [7]. Similarly, in patients with proliferative vitreoretinopathy (PVR), an EMT was demonstrated in areas of fibrosis in the retina [8,9]. PVR is the leading cause of failure of retinal detachment surgery and sometimes results in the loss of visual function. Retinal pigment epithelial (RPE) cells dedifferentiate and undergo EMT following retinal detachment, playing a key role in formation of fibrous tissue on the detached retina and vitreous retraction [10]. However, the detailed information on the molecular and cellular events of EMT in RPE cells has not been fully clarified.

EMT can be induced by growth factors such as transforming growth factor β (TGF-β), epidermal growth factor (EGF) and transcription factors such as Snail, twist and slug [11,12]. Snail transcription factor, a member of the Snail superfamily, is a zinc finger protein that can mediate EMT through downregulation of cell adhesion molecules such as E-cadherin by binding several

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E-boxes located in the promotor region [13]. The expression of Snail is stimulated by signaling pathways of a number of growth factors including TGF-β [14]. Our previous data demonstrated that Snail was up-regulated when human RPE cells were induced by TGF-β1 to undergo EMT, and that inhibition of activated Snail could reverse TGF-β1-induced EMT in vitro [15]. We ask whether Snail was sufficient to trigger EMT when ectopically expressed in RPE cells.

Snail has been extensively studied in cancers and fibrosis disease such as renal fibrosis; however, its role in ocular fibrosis is not as widely studied, especially in PVR. Based on these findings, we sought to explore whether Snail overexpression in human RPE cells could directly induce EMT and to obtain additional insights into the role of Snail in retinal pigment epithelial–mesenchymal transition in vitro.

2. Materials and methods

2.1. Cell culture and transfection

Human retinal pigment epithelial cell lines ARPE-19 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) at 37 °C under 5% CO_2. For Snail overexpression, ARPE-19 cells were transfected with Snail expression vector (pReceiver; OmicsLink Expression Clones; GeneCopoeia, Inc., USA) or an empty vector as negative control. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to manufacturer's protocols.

2.2. Immunofluorescent staining

ARPE-19 cells were seeded and cultured in 24-well chamber slides in Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, USA) for 24 h and then were transfected with pReceiver-Snail or pReceiver-control plasmids respectively. After 48 h, cells were washed and fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature. Then the fixed cells were incubated with primary antibody against Snail (Abcam Ltd., Cambridge, USA) diluted 1:100 at 37 °C for 2 h, washed three times with PBS followed by treatment with FITC-conjugated secondary antibody (diluted in 1:200) at room temperature for 1 h. Nuclei were stained with 4′6′-diamidino-2-phenylindole hydrochloride (DAPI) for 5 min. Stained ARPE-19 cells were observed using OLYMPUS™ microscope.

2.3. Morphology observation

After ARPE-19 cells were transfected with pReceiver-Snail or pReceiver-control plasmids for 24 and 48 h, the morphology of cells were observed under an inverted phase-contrast microscope (Olympus, Tokyo, Japan) and photographed by a digital camera.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated using TRIzol reagent (Invitrogen-Gibco, Carlsbad, USA) following the manufacturer's protocol. QRT-PCR was performed using TaqMan Universal PCR Master Mix and the 7500 Sequence Detection System (Applied Biosystems, Foster, CA). 18S RNA was used to standardize the mRNA level of the target genes. The sequences of the primers were used as before [15]. The expression of each target gene was defined from the threshold cycle (Ct), and relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method [16] after normalization with reference to expression of 18S RNA. All experiments were performed in triplicate.

2.5. Immunoblotting assay

Preparation of whole cell extracts and immunoblotting assay was performed as previously described [17]. The primary antibodies used were as follows: 1:500 E-cadherin antibody and 1:1000 fibronectin antibody (R&D systems, Inc., USA), 1:1000 Snail antibody (Abcam Ltd., Cambridge, USA), 1:1000 α-SMA antibody (Sigma–Aldrich, MO, USA), 1:1000 ZO-1 antibody (Invitrogen, Carlsbad, CA), and 1:5000 GAPDH (Good HERE, Hangzhou, China). HRP-conjugated sheep anti-mouse or anti-rabbit antibodies were used as secondary antibodies (1:10,000, Jackson, USA). Image Quant LAS 4000 with Image Quant TL 7.0 software (GE Healthcare Life Sciences, Pittsburgh, PA, USA) was used to quantify band intensities.

2.6. RPE cell migration assay

Transwell chamber (8-μm pores, Costar, Conning, USA) was used to determine the effect of forced Snail expression on ARPE-19 cells migration. After ARPE-19 cells were transfected with pReceiver-Snail or pReceiver-control for 48 h, about 5×10^5 cells were plated into the insert in 100 μl DMDM/F12 containing 0.5% FBS and allowed to migrate from upper compartment to lower compartment toward a 10% FBS gradient. The chamber was then incubated at 37 °C for 24 h. After removing the non-migrating cells with a cotton swab, migrated cells on the lower surface of the culture inserts were fixed with 4% paraformaldehyde, stained with H&E, and photographed under a light microscope. Five random fields were chosen in each insert, and the cell number was counted. All the experiments were performed in triplicate.

2.7. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) from at least three independent experiments. Independent sample *t*-test was used for two-group comparisons. Statistical Product and Service Solutions 16.0 software (Chicago, IL) was used for statistical analysis. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Expression of Snail protein in transfected ARPE-19 cells

In order to determine whether Snail alone could induce EMT, ARPE-19 cells were transfected with specific Snail expression vector as described in methods. First, we confirmed the expression of Snail in ARPE-19 cells by the immunofluorescence stain. As shown in Fig. 1, after 48 h of transfection, an increase in the protein levels of Snail was detected in pReceiver-Snail transfected ARPE-19 cells comparing to pReceiver-control transfected cells. Moreover, by immunofluorescence we could observe Snail, as well as its delocalization from the cytoplasm to intracellular compartments.

3.2. Effects of Snail overexpression on ARPE-19 cells morphology

To investigate whether Snail overexpression was associated with ARPE-19 cells phenotype changes, the phase contrast microscope was used. As shown in Fig. 2, cultured normal ARPE-19 cells had a typical cobblestone-like epithelial morphology. After transfected with pReceiver-Snail, the epithelial cells morphology gradually changed to spindle fibroblast-like cells accompanied by loss of

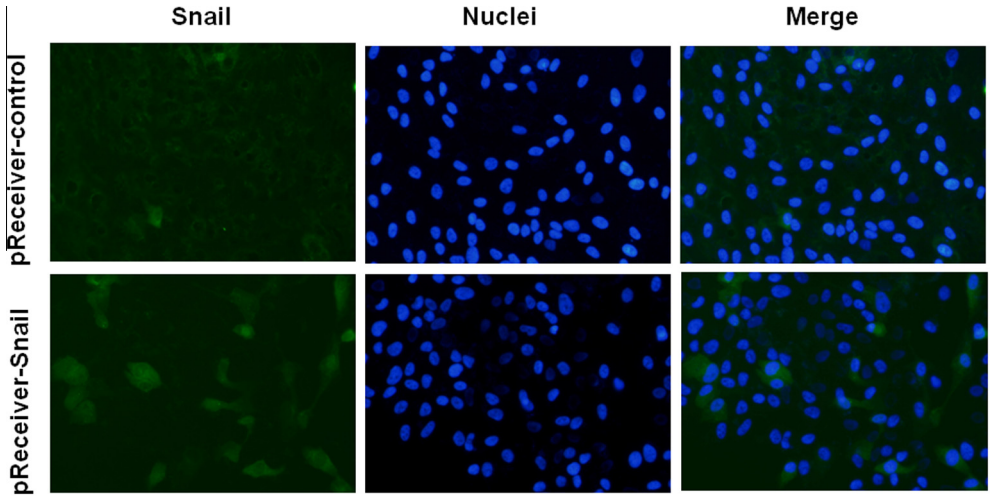

Fig. 1. Immunofluorescence staining for Snail. ARPE-19 cells were transfected with pReceiver-Snail or pReceiver-control for 48 h. Expression and distribution of Snail was assessed by immunofluorescence. The second antibody used for Snail was labeled by FITC (green) and nuclei were counterstained with DAPI (blue). Compared with pReceiver-control transfected cells, the expression of Snail was upregulated and positively expressed in the nuclei of pReceiver-Snail transfected cells (Original magnification 200×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

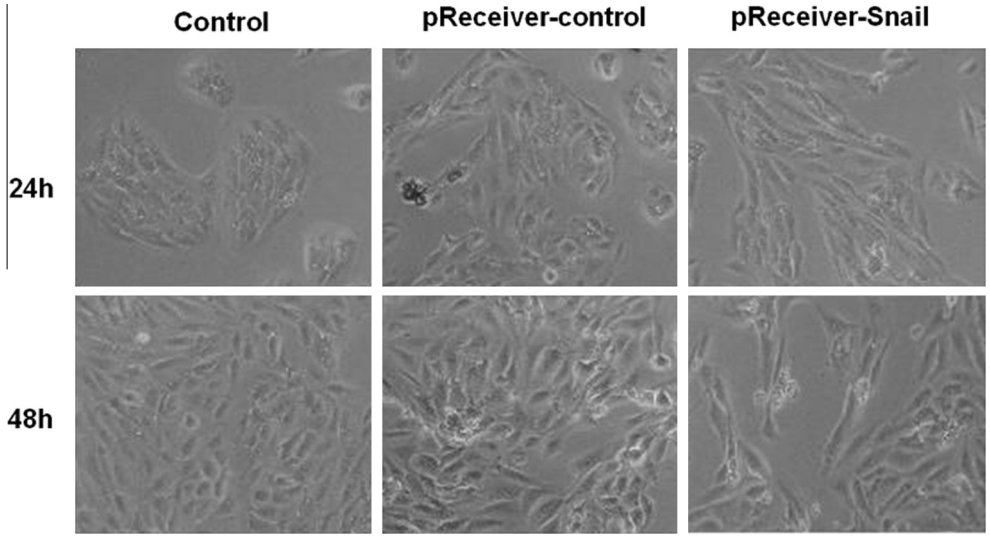

Fig. 2. Morphologic changes of ARPE-19 cells after Snail overexpression. ARPE-19 cells morphology was assessed after transfecting with pReceiver-Snail or pReceiver-control for 24 and 48 h. pReceiver-control transfected cells retained the oval cells morphology comparing to normal ARPE-19 cells. After transfected with pReceiver-Snail, ARPE-19 cells phenotype gradually changed to spindle mesenchymal morphology (Original magnification 100×).

cell-to-cell contacts. In contrast, pReceiver-control transfected cells retained their epithelial morphology. This result suggested that Snail overexpression in ARPE-19 cells is sufficient to induce the acquisition of a mesenchymal morphology.

3.3. Snail overexpression induced EMT in ARPE-19 cells

EMT is characterized by the loss of epithelial cell-to-cell contacts with a decrease in epithelial adhesion molecule E-cadherin, a major reorganization of the cytoskeleton, and an increase in mesenchymal marker expression [18]. Since we had observed a relation between Snail and phenotypic transition in ARPE-19cells, we sought to examine whether Snail overexpression could affect the expression of some epithelial and mesenchymal markers. QRT-PCR demonstrated that high Snail expression resulted in a significant decrease in E-cadherin and ZO-1 expression at mRNA level. Compared to control cells, overexpression of Snail in ARPE-19 cells also increased the expression of a-SMA and fibronectin. These changes in mRNA expression were accompanied by changes in the protein levels of epithelial and mesenchymal markers. Taken together, these data indicated that Snail overexpression is capable of inducing EMT in ARPE-19 cells in vitro.

3.4. Snail overexpression leads to increased ARPE-19 cells migration

We next analysis whether the ability of ARPE-19 cells migration was enhanced after up-regulation of Snail. As expected, we observed the significant changes of cell migration in pReceiver-Snail transfected cells. As shown in Fig. 4, there were no significant differences between cultured normal ARPE-19 cells and pReceiver-control transfected cells. Cells transfected with pReceiver-Snail, but not pReceiver-control, exhibited significantly increased ability of migration.

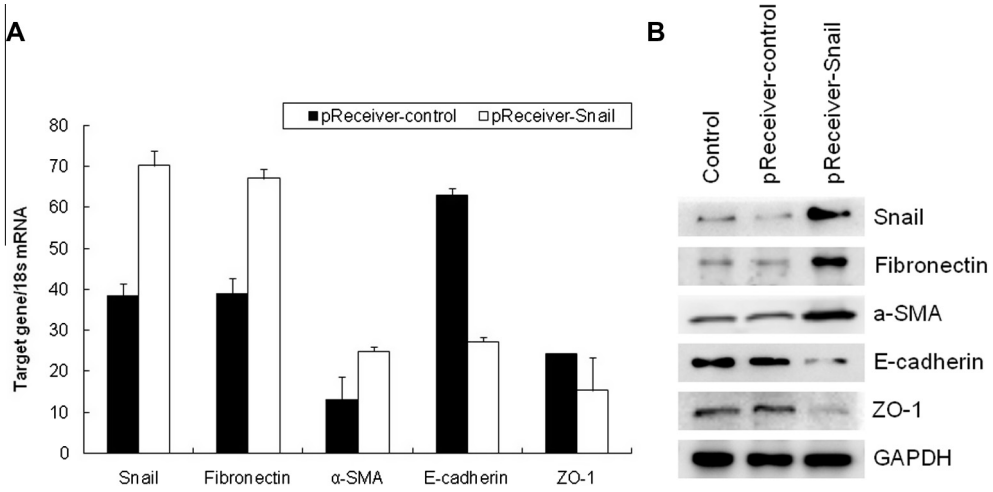

Fig. 3. Overexpression of Snail in ARPE-19 cells induced EMT. ARPE-19 cells were transfected with pReceiver-Snail or pReceiver-control for 48 h. QRT-PCR and Immunoblotting were used to examine the expression of Snail, E-cadherin, ZO-1, a-SMA and fibronectin. (A) QRT-PCR analysis showed the increased Snail, fibronectin and a-SMA mRNA expression and decreased E-cadherin and ZO-1 mRNA expression. $^{**}P < 0.01$ vs pReceiver-control. (B) Immunoblotting confirmed the expression of these EMT markers at protein levels.

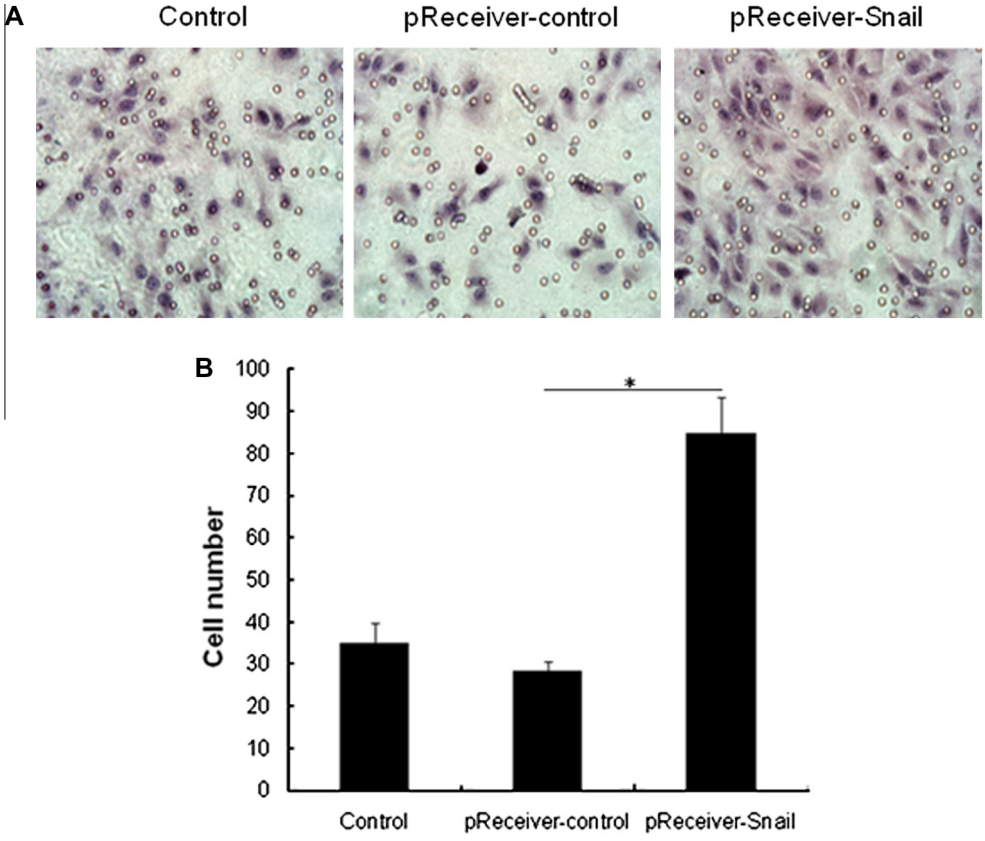

Fig. 4. Effects of Snail overexpression on ARPE-19 cells migration. After pReceiver-Snail or pReceiver-control transfection for 48 h, ARPE-19 cells were seeded into transwell chamber and allowed to migrate for 24 h. (A) Presentative pictures of migrated cells (Original magnification 400×). (B) The number of migrated cells in the group of normal ARPE-19 cells, pReceiver-control or pReceiver-Snail transfected cells. Compared with pReceiver-control group, Cells transfected with pReceiver-Snail showed enhanced migration. $^{*}P < 0.05$.

4. Discussion

In the present study, we provide evidence for the direct effect of exogenous Snail expression in ARPE-19 cells. The data indicate that forced Snail expression in ARPE-19 cells results in EMT, which was characterized by a phenotype transition from an epithelial to a fibroblastoid appearance, loss of E-cadherin and ZO-1 expression, gain α-SMA and fibronectin expression, and increased migration. This is the first report of the direct relationship between Snail transcription factor and retinal pigment epithelial–mesenchymal transition.

EMT is a well characterized process defined morphologically as the conversion of epithelial cells to a fibroblast or mesenchymal morphology and is known to be dependent on the Snail family of transcription factors [3,19,20]. During EMT, immobile epithelial

cells loose apicobasal polarity and intercellular junctions, acquire spindle cell morphology [21]. After confirmation the overexpression of Snail in ARPE-19 cells, we examined the phenotypic changes of cells. As Fig. 2 showed that ARPE-19 cells in the control group displayed an epithelial morphology and grew as epithelial colonies in culture. Snail overexpression lead to the cobblestone-like epithelial morphology change to spindle shaped.

Snail is known to bind E-box consensus sequences in the E-cadherin promoter and repress E-cadherin at the transcriptional level [22,23]. E-cadherin is a Ca2+-dependent cell adhesion molecule that plays an important role in the maintenance of epithelial phenotype, decreased expression of E-cadherin is considered as a hallmarker of EMT [24,25]. Snail can also lead to repression of tight junction proteins like ZO-1 [26]. We next investigated whether Snail overexpression could down-regulate the expression of E-cadherin and ZO-1 in ARPE-19 cells. Fig. 3 showed high Snail expression resulted in a significant decrease in E-cadherin and ZO-1 expression at mRNA and protein levels. These results suggest that Snail disrupts the function of adherens junctions as well as tight junctions. When epithelial cells transition to fibroblasts, they decrease expression of a number of epithelial proteins including E-cadherin and ZO-1, while increasing their synthesis of cytoskeletal proteins [27]. The expression of mesenchymal markers a-SMA and fibronectin in ARPE-19 cells were also upregulated after Snail overexpresson. Taken together, the data indicated that Snail overexpression in ARPE-19 cells could directly trigger EMT in vitro. Upon EMT, epithelial cells not only assume a more mesenchymal phenotype, but also acquire increased motility. We performed motility assays comparing pReceiver-Snail transfected cells to pReceiver-control cells. The results demonstrated that Snail overexpression significantly enhanced the ability of ARPE-19 cells migration, supporting retinal pigment epithelial–mesenchymal transition.

These findings complement our previous studies about that Snail involved in TGF-β1-induced ARPE-19 cells EMT. We recently reported that TGF-β1 could promote EMT in ARPE-19 cells in vitro and the expression of Snail was upregulated in this process. Furthermore, knock-down Snail efficiently attenuated TGF-β1-induced EMT. We now find that the role of Snail overexpressed in ARPE-19 cells is similar to TGF-β1 treatment. In addition, the ability of Snail triggered EMT may provide new insights into the mechanisms of RPE cells EMT. The signal transduction pathways that govern EMT are extensive, with cross-talk mechanisms forming a complex web of signaling. TGF-β is known to signal through phosphorylation-mediated ac tivation of Sma and Mad related family (Smad) transcription factors and in some cases by activation of the phosphoinositide 3-kinase and extracellular-signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways to induce EMT [28]. The transcriptional repressors Snail and Slug are sufficient to induce EMT when expressed in epithelial systems, and so is triggering the TGF-β signaling pathway, which can also stimulate Snail and Slug expression [29]. During RPE cells EMT, whether Snail could in turn activate TGF-β1 pathway in a positive feedback will need to be addressed.

In conclusion, the present work demonstrates that Snail overexpression in ARPE-19 cells is in itself sufficient to induce EMT. Based on our previous investigation, these results further confirmed the regulator role of Snail in retinal pigment epithelial–mesenchymal transition and might contribute to establish the pathophysiology of retinal fibro sis in PVR. Furthermore, these findings suggest Snail may be a potential therapeutic target to prevent the fibrosis of PVR.

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