MicroRNA-29b participates in the epithelial-mesenchymal transition of retinal pigment epithelial cells through p-p65

MIN LI, HUI LI, SHUAI YANG, XIN LIAO, CHUN ZHAO and FANG WANG

Department of Ophthalmology, Shanghai Tenth People's Hospital, Shanghai 200072, P.R. China

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Abstract. Epithelial-mesenchymal transition (EMT) of retinal pigment epithelial (RPE) cells is considered to be the main mechanism of proliferative vitreoretinopathy (PVR). Our previous study demonstrated that microRNA-29b (miR-29b) and its target protein kinase B (Akt2) played vital roles in this process. miR-29b, a mesenchymal marker α-smooth muscle actin (α-SMA) and the epithelial marker E-cadherin were assessed in epiretinal membranes of patients with PVR. The potential mechanism of miR-29b and EMT was also investigated. The expression levels of miR-29b, E-cadherin, and α-SMA in PVR epiretinal membranes were measured using quantitative PCR. The expression levels of Akt2, phosphorylated (p)-Akt2, p65, p-p65, and Snail in ARPE-19 cells were assessed using western blotting. The expression levels of miR-29b were positively correlated with E-cadherin mRNA expression, while an inverse correlation was observed between miR-29b and α-SMA mRNA expression in epiretinal membranes of patients with PVR. When miR-29b was transfected into ARPE-19 cells, the expression levels of Akt2, p-Akt2, p-p65 and Snail were downregulated. shRNA-Akt2 inhibited p-p65 and Snail expression, while the NF-κB inhibitor BAY11-7082 reduced Snail expression. The Akt2/p-p65/Snail pathway may be the underlying mechanism of miR-29b in EMT of RPE cells. The results of the present study may provide a new strategy for prevention and therapy of PVR.

Introduction

Proliferative vitreoretinopathy (PVR) is characterized by the formation of fibrotic and tractive membranes that can lead to the reattachment of retina; it is a severe complication of rhegmatogenous retinal detachment (RRD) surgery (1). The formation mechanism of avascular epiretinal membrane and subretinal membrane in PVR remains unknown. It has been reported that retinal pigment epithelial (RPE) cells are the main cells in the epiretinal and subretinal membrane (2). After the RRD forms, RPE cells are exposed to environments full of cytokines and growth factors, upon which they undergo type 2 epithelial-mesenchymal transition (EMT), migrate to the epiretinal and subretinal spaces, proliferate and form avascular membranes (3). These findings, including our previous studies (4,5) indicated that EMT was involved in PVR progression.

MicroRNAs (miRNAs or miRs) are small non-coding RNAs that are involved in transcriptional and post-transcriptional regulation of gene expression (6). miRNAs play important roles in both the maintenance of homeostasis and the development of diseases (7,8). In the retina, more than 150 miRNAs have been determined to be involved in PVR (9).

In our previous study, an EMT model of RPE cell induction by transforming growth factor (TGF)-β1 was established and by using miRNA microarray, it was revealed that miR-29b was the most important miRNA. It was also demonstrated that miR-29b inhibited EMT in the RPE cell process through its target protein kinase B (Akt2) (10). Nevertheless, the mechanism of miR-29b function and its role in PVR clinical membranes remains unknown.

Akt and the nuclear factor-κB (NF-κB) signaling pathways play vital roles in regulating cell growth, survival, proliferation, metabolism, and inflammation (11,12). In NIH3T3 cells, TNF-α activated the NF-κB pathway and Akt was a downstream target of NF-κB (13). In oral squamous cell carcinoma cell lines (KB and KOSCC-25B), phosphorylated (p)-Akt was upstream of the NF-κB pathway (14). In our previous study, it was revealed that Snail was an important factor in TGF-β1-induced RPE cell EMT. EMT was inhibited when Snail was silenced (5). When Snail was overexpressed, RPE cell EMT was triggered, characterized by increased expression of α-smooth muscle actin (α-SMA) and decreased expression of E-cadherin and Zona occludin-1 (ZO-1) (15). The present study explored the interactions among Akt2, NF-κB, Snail expression, and miR-29b in RPE cell EMT.

The expression levels of Akt2, α-SMA, E-cadherin and miR-29b in epiretinal membranes of PVR patients were assessed. Furthermore, the downstream regulatory pathway of miR-29b with Akt2 in TGF-β1-induced ARPE-19 cell EMT

Correspondence to: Dr Fang Wang, Department of Ophthalmology, Shanghai Tenth People's Hospital, 301 Middle Yanchang Road, Shanghai 200072, P.R. China
E-mail: fwang7527@163.com

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was explored. The results of the present study may provide insights into the role of miR-29b in RPE cell EMT and epiretinal membrane formation in PVR.

Materials and methods

Ethical approval. All patients provided written informed consent for the preservation and analysis of their tissues for research purposes. The present study was approved by the Ethics Committee of Shanghai Tenth People's Hospital (Shanghai, China) and complied to the guidelines of the Declaration of Helsinki (version 2013) (16). The ethics approval no. was SHSY-IEC-KY-4.0/17-79/01.

Cell culture. ARPE-19 cells were obtained from the Eye Institute of Tongji University. ARPE-19 cells at passage 4-6 were cultured in DMEM/F12 (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in an incubator containing 5% CO_2. For TGF-β1 (HumanZyme, Inc.; ProteinTech Group, Inc.) treatment, ARPE-19 cells were subjected to serum-free medium at 37˚C for 16 h. TGF-β1 was added to the medium at 10 ng/ml. A total of 3.5 μM of the NF-κB inhibitor BAY11-7082 (Beyotime Institute of Biotechnology) was added to the medium at 37˚C for 1 h before TGF-β1 treatment.

RNA transfection. ARPE-19 cells were transfected with 100 nM miR-29b mimic (3'-UUGUGACUAAAGUUU ACCACGAU-5') and miR-negative control mimic (miR-NC mimic; 3'-AUGCAGUAUACUGUUAAUGACUC-5') (Guangzhou RiboBio Co., Ltd.) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 6 h following the manufacturer's instructions. ARPE-19 cells were starved overnight in serum-free medium before the addition of TGF-β1.

Plasmid DNA transfection. The plasmids of Akt2-targeting short hairpin RNA (sh)-Akt2 and sh-control (both from Shanghai GeneChem Co., Ltd.) were transfected into ARPE-19 cells (2.5 μg plasmids or controls in $2.5x10^5$ cells) at 37˚C for 48 h. The HET kit (Biowit Technologies Ltd.) was used for the transfections according to the protocols provided by the manufacturer.

Patients and samples. All patients with PVR (n=17; aged 45-66 years; 7 females and 10 males) were from the Shanghai Tenth People's Hospital (Shanghai, China) between January 2018 and August 2019. Patients with diabetes, acute inflammation or systemic immune diseases were excluded. After vitrectomy surgery, the epiretinal membranes were removed with internal limiting membrane (ILM) forceps. The epiretinal membranes were immediately stored at -80˚C.

Reverse transcription-quantitative PCR (RT-q)PCR. The total RNA of epiretinal membranes was extracted using the RNAprep pure Micro kit (cat. no. DP 420; Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. A reverse transcription kit (cat. no. RR036A; Takara Bio, Inc.) was used to synthesize cDNA according to the manufacturer's instructions. SYBR Green-based RT-qPCR was performed with a 7500 Fast RT-PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific primers (GENTEC Corporation) were used to determine the expression of EMT-related genes. GAPDH was used as an internal control. The thermocycling conditions were as follows: 94˚C for 30 sec, 40 cycles of 94˚C for 5 sec and 60˚C for 30 sec. The relative amount of each gene was measured using the $2^{-\Delta\Delta Cq}$ method as reported in a previous study (15). All experiments were repeated 3 times. Primer sequences, which were also used in a previous study (10), are presented in Table I.

Western blot analysis. Western blot analysis was performed as previously described (17). The antibodies and the dilutions were as follows: Rabbit anti-RPE-65 (1:1,000; cat. no. ab231782; Abcam), anti-Akt2 (1:500; cat. no. ab131168; Abcam), anti-p-Akt2 (ser474; 1:500; cat. no. ab38513; Abcam), anti-Snail (1:1,000; cat. no. ab216347; Abcam), anti-p65 (1:1,000; cat. no. AF1234; Beyotime Institute of Biotechnology), anti-p-p65 (ser536; 1:1,000; cat. no. AF5881; Beyotime Institute of Biotechnology), and anti-GAPDH (1:500; cat. no. AB-P-R001; Goodhere Biological Technology Group). Anti-rabbit IgG secondary antibodies (1:10,000; cat. no. W4011; Promega Corporation) were then applied and incubated at room temperature for 1 h. Image Quant LAS 4000 (GE Healthcare Life Sciences; Cytiva) and Image J analysis software (version. no. 1.52t; National Institutes of Health) were used to quantify band intensities.

Statistical analysis. At least three independent experiments were performed for each analysis. Data are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA), Dunnett's t-test and Tukey's post hoc test were used to analyze the data of multiple groups. The association between miR-29b and EMT biomarkers was evaluated using Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference. SPSS 16.0 (SPSS, Inc.) was used for significance analysis.

Results

Correlation between miR-29b and EMT markers in epiretinal membranes of patients with PVR. Epiretinal membranes are the characteristic of the PVR process that pulls the retina and leads to retinal detachment (1). To investigate the relationship between miR-29b and EMT during the formation process of epiretinal membranes, the correlation between miR-29b and the mRNA expression of E-cadherin, α-SMA and Akt2 in epiretinal membranes of patients with PVR was assessed. As demonstrated in Fig. 1A, miR-29b was positively correlated with E-cadherin mRNA expression, while there was a negative correlation between miR-29b and α-SMA mRNA expression (Fig. 1B), and between miR-29b and Akt2 mRNA expression (Fig. 1C). These results indicated that EMT played a vital role in the development of PVR, and that miR-29b and its target Akt2 participated in this process.

miR-29b inhibits the expression of p-p65 and Snail in ARPE-19 cells. The downstream pathways of miR-29b and Akt2 were further investigated. The NF-κB pathway has been widely

Table I. Primer sequences for reverse transcription-quantitative PCR amplification.

Gene	Forward (5'-3')	Reverse (5'-3')
E-cadherin	CAGTCTAGGCCAGTGCATCA	TTTGCCCTCTGCTTTGTTCT
α-SMA	AGCAGGCCAAGGGGCTATATAA	CGTAGCTGTCTTTTTGTCCCATT
Akt2	CGCCTGCCCTTCTACAACCA	ATGACCTCCTTGGCATCGCT
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

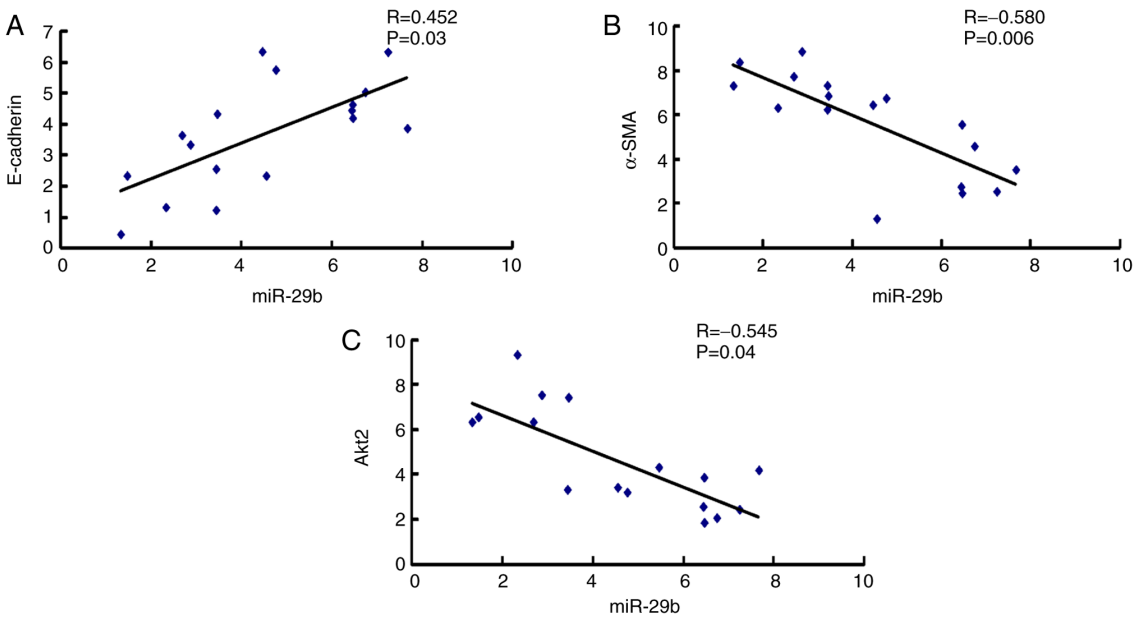

Figure 1. Correlation of miR-29b with E-cadherin, α-SMA, and Akt2 expression in epiretinal membranes of patients with PVR. (A) The positive correlation of miR-29b with E-cadherin by quantitative PCR. (B) The negative correlation between miR-29b and α-SMA. (C) The negative correlation between miR-29b and Akt2. Pearson correlation coefficients (R) and P-values (P) are indicated. All experiments were performed in triplicate. miR-29b, microRNA-29b; α-SMA, α-smooth muscle actin; PVR, proliferative vitreoretinopathy.

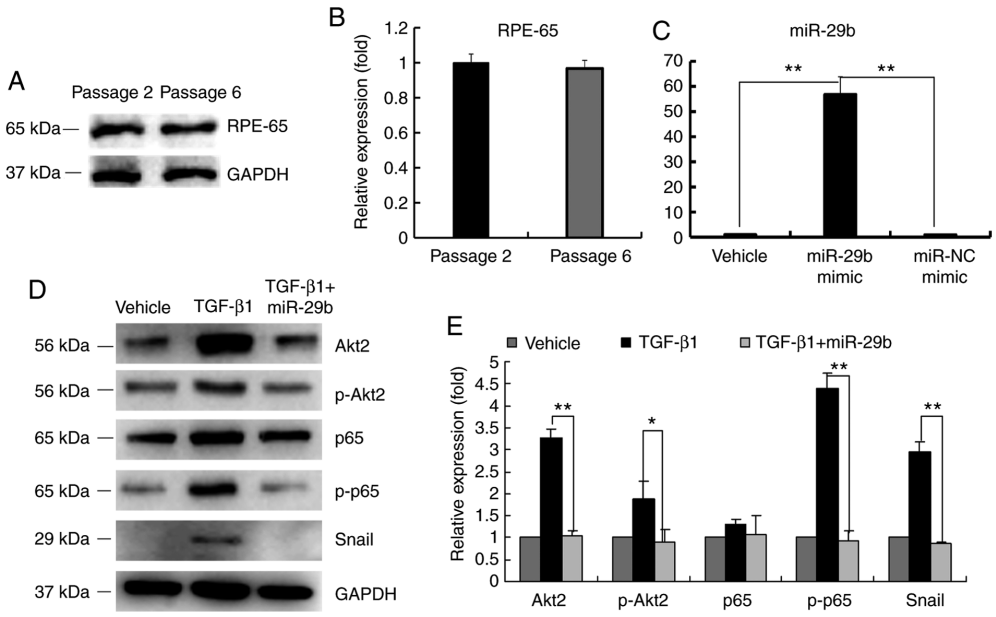

Figure 2. Effect of miR-29b on Akt2, p-p65 and Snail expression. (A) Western blot analysis of RPE-65. (B) Graphical representation of the relative abundance of RPE-65. (C) miR-29b expression after ARPE-19 cells were transfected with miR-29b mimic and miR-NC mimic. (D) Western blot analysis of Akt2, p-Akt2, p65, p-p65 and Snail expression with transfection of miR-29b before TGF-β1 treatment. (E) Graphical representation of the relative abundance of Akt2, p-Akt2, p65, p-p65 and Snail expression. All experiments were performed in triplicate. *P<0.05 and **P<0.01. miR-29b, microRNA-29b; p-, phosphorylated; RPE, retinal pigment epithelial; NC, negative control; TGF, transforming growth factor.

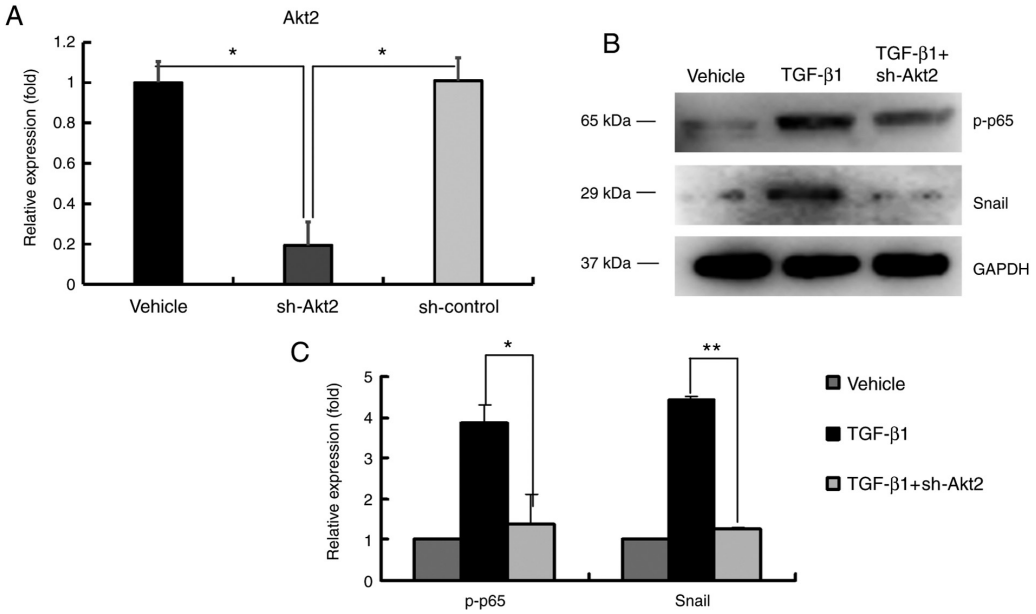

Figure 3. Expression of p-p65 and Snail after silencing of Akt2 in ARPE-19 cells. (A) Akt2 mRNA expression after ARPE-19 cells were transfected with sh-Akt2 and sh-control. (B) Western blot analysis of p-p65 and Snail expression with transfection of sh-Akt2 or sh-control plasmids before TGF-β1 treatment. (C) Graphical representation of the relative abundance of p-p65 and Snail protein expression. All experiments were performed in triplicate. *P<0.05 and **P<0.01. p-, phosphorylated; sh-, short hairpin; TGF, transforming growth factor.

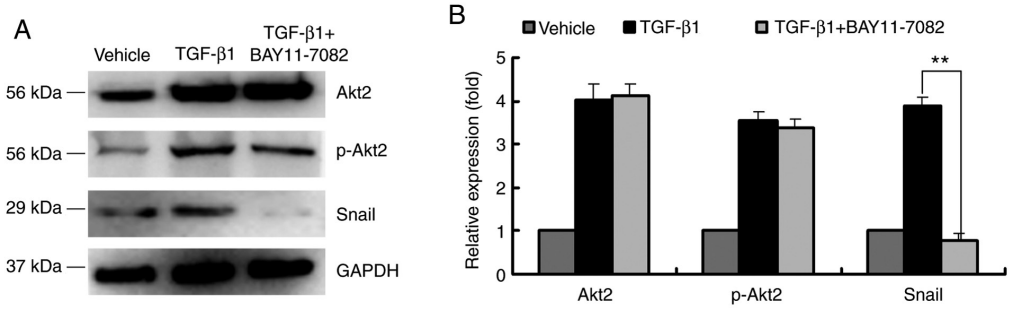

Figure 4. Effect of NF-κB inhibitor on the expression of Snail in ARPE-19 cells. (A) Western blot analysis of Akt2, p-Akt2 and Snail protein expression after inhibition of NF-κB signaling and TGF-β1 treatment. (B) Graphical representation of the relative abundance of Akt2, p-Akt2 and Snail protein expression. All experiments were performed in triplicate. **P<0.01. NF-κB, nuclear factor-κB; p-, phosphorylated; TGF, transforming growth factor.

studied in the EMT process of other cells (18,19). Snail was revealed to play a critical part in the EMT process of ARPE-19 cells in our previous study (5). It was concluded that p65, p-p65 and Snail were downstream of miR-29b and Akt2 in ARPE-19 cells. As demonstrated in Fig. 2A and B, the expression of RPE-65 did not decrease at passage 6 of ARPE-19 cells. Therefore, ARPE-19 cells at passages 4-6 were used in this study. miR-29b expression was significantly upregulated after miR-29b mimic was transfected into ARPE-19 cells (Fig. 2C). The results also revealed that TGF-β1 induced the expression of Akt2, p-Akt2, p-p65 and Snail while the addition of miR-29b significantly inhibited these expression levels (Fig. 2D and E). No significant difference in the expression of p65 was observed. Collectively, the expression levels of p-Akt2, p-p65 and Snail expression were affected by miR-29b. Additionally, p-p65 and Snail were probably involved in the EMT of ARPE-19 cells.

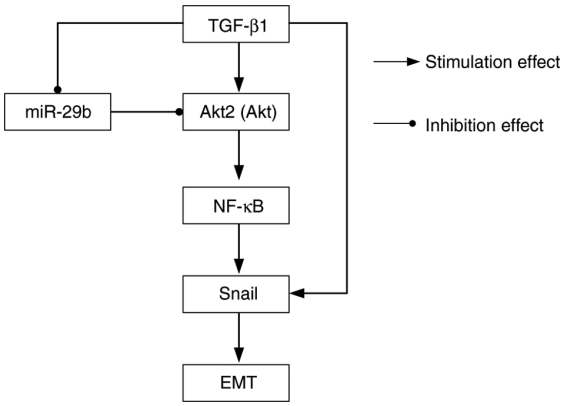

Figure 5. miR-29b inhibits TGF-β1-induced ARPE-19 cell EMT via the Akt2/p-p65/Snail pathway. miR-29b, microRNA-29b; TGF, transforming growth factor; EMT, epithelial-mesenchymal transition.

Downregulated Akt2 inhibits the expression of p-p65 and Snail in ARPE-19 cells. To further study the relationship of Akt2, p-p65 and Snail in ARPE-19 cells, the expression of Akt2 was silenced, while p-p65 and Snail expression was determined.

As demonstrated in Fig. 3A, Akt2 was significantly downregulated after sh-Akt2 transfection in ARPE-19 cells. TGF-β1 induced the expression of p-p65 and Snail; however, the expression levels of p-p65 and Snail were downregulated after Akt2 expression was inhibited by sh-Akt2 (Fig. 3B and C). The aforementioned results indicated that p-p65 and Snail participated in the downstream pathway of miR-29b and Akt2.

NF-κB inhibitor reduces the expression of Snail in ARPE-19 cells. It was revealed that Snail is an important zinc-finger transcription factor in ARPE-19 cell EMT (5). To investigate the regulatory effect of p-p65 on Snail, the NF-κB inhibitor BAY11-7082 was used to reduce p-p65 expression. TGF-β1 induced Akt2 and p-Akt2 expression and this was not affected by the addition of BAY11-7082 (Fig. 4A and B). Moreover, Snail expression was upregulated after ARPE-19 cells were treated with TGF-β1; however, this effect was inhibited by BAY11-7082. These results indicated that the function of Snail in EMT was regulated by NF-κB effector p-p65 (Fig. 4A and B).

Discussion

EMT of RPE cells in epiretinal membranes is a potential mechanism of PVR. TGF-β1 has been revealed to play a vital role in triggering EMT in fibrogenesis and tumor progression (20,21). In pulmonary fibrosis, a miR-29 family member was significantly reduced and this affected a subset of fibrosis-related genes including laminins and integrins (22). Arboleda *et al* reported that overexpression of Akt2 in breast cancer cells resulted in the formation of metastasis and suggested that Akt2 regulated the metastatic genes (23). In the present study, TGF-β1-induced ARPE-19 cells were used to establish the EMT model and the relationship between miR-29b and EMT was further investigated. It was demonstrated that miR-29b and its target Akt2 were closely related to PVR progression. In epiretinal membranes of patients with PVR, miR-29b expression was positively correlated with epithelial marker E-cadherin, while an inverse correlation was found between the expression of miR-29b, mesenchymal marker α-SMA, miR-29b and Akt2 mRNA expression. These results indicated that miR-29b and its target Akt2 participated in epiretinal membrane formation in PVR.

TGF-β1 is a well-recognized influencing factor of the NF-κB pathway (24). In the present study, it was demonstrated that TGF-β1 induced the expression of p-Akt2 and p-p65, but had no effect on p65. Specifically, TGF-β1 promoted p65 phosphorylation, but had little effect on total p65 expression. When Akt2 was silenced, the expression levels of the subunit of NF-κB (p-p65) and Snail protein were reduced. NF-κB inhibitor BAY11-7082 prevented TGF-β1-induced Snail protein expression; however, it had no effect on Akt2 and p-Akt2. These data indicated that Akt2 was upstream of p-p65 and Snail was downstream of p-p65. As reported previously, the activation of NF-κB subunit p65 was sufficient for induction of EMT (25), and the transcription factor Snail regulated EMT by affecting E-cadherin expression (26). Collectively, these data indicated that NF-κB and Snail are downstream signals of miR-29b in ARPE-19 cells (Fig. 5). The majority of experiments were performed using ARPE-19 cells due to their PVR membrane deficiency, which serves as a limitation to the current study.

In conclusion, the results of the present study indicated that miR-29b contributed to the progression of epiretinal membrane formation in patients with PVR. The effect of miR-29b on TGF-β1-mediated EMT in ARPE-19 cells was mediated by the Akt2/p-p65/Snail pathway. The aforementioned findings indicated a targeted therapeutic strategy for the treatment of PVR. The Akt2/p-p65/Snail pathway may be the underlying mechanism of miR-29b in EMT during PVR.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ML and FW designed the current study. ML, HL and SY performed the experiments. XL and CZ analyzed the data. ML wrote the original manuscript. HL and FW revised the final manuscript. CZ and FW confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent for the preservation and analysis of their tissues for research purposes. The present study was approved by the Ethics Committee of Shanghai Tenth People's Hospital (Shanghai, China) and complied to the guidelines of the Declaration of Helsinki (2013). The ethics approval no. was SHSY-IEC-KY-4.0/17-79/01.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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