• Basic Research •

Cross-talk between microRNA-let7c and transforming growth factor-β2 during epithelial-to-mesenchymal transition of retinal pigment epithelial cells

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Abstract

● **AIM:** To explore the roles of microRNA-let7c (miR-let7c) and transforming growth factor-β2 (TGF-β2) and cellular signaling during epithelial-to-mesenchymal transition (EMT) of retinal pigment epithelial cells.

● **METHODS:** Retinal pigment epithelial (ARPE-19) cells were cultured with no serum for 12h, and then with recombinant human TGF-β2 for different lengths of time. ARPE-19 cells were transfected with 1×10^6 TU/mL miR-let7c mimcs (miR-let7cM), miR-let7c mimcs negative control (miR-let7cMNC) and miR-let7c inhibitor (miR-let7cI) using the transfection reagent. The expression of keratin-18, vimentin, N-cadherin, IKB alpha, p65 were detected by Western blot, quantitative polymerase chain reaction and immunofluorescence.

● **RESULTS:** The expression of miR-let7c was dramatically reduced and the nuclear factor-kappa B (NF-κB) signaling pathway was activated after induction by TGF-β2 (P<0.05). In turn, overexpressed miR-let7c significantly inhibited TGF-β2-induced EMT (P<0.05). However, miR-let7c was unable to inhibit TGF-β2-induced EMT when the NF-κB signaling pathway was inhibited by BAY11-7082 (P<0.01).

● **CONCLUSION:** The miR-let7c regulates TGF-β2-induced EMT through the NF-κB signaling pathway in ARPE-19 cells.

● **KEYWORDS:** microRNA-let7c; transforming growth factor-β2; epithelial-to-mesenchymal transition; human retinal pigment epithelial cells; nuclear factor-kappa B pathway

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INTRODUCTION

Epiretinal membranes (ERMs) develop as a result of cellular proliferation and connective tissue formation on the surface of the retina. ERMs are commonly observed in various chronic intraocular proliferative disorders, especially proliferative vitreoretinopathy (PVR), which is the leading cause of severe visual impairment in patients[1-2]. During the evolution of PVR, the contraction of the ERM causes a marked distortion of the retina, resulting in a complex tractional retinal detachment that is difficult to repair and eventually leads to blindness[1]. Until now, the surgical removal of the ERM is still the most effective treatment option for PVR[3]. However, patients that are surgically treated often have limited visual recovery due to retinal damage from recurrent detachment and from the PVR process itself. Thus, more effective preventative or treatment methods are desperately needed.

Recent studies have shown that several cell types are stimulated in PVR, particularly retinal pigment epithelial (RPE) cells[4-5]. RPE cells can undergo cell-type switching once injured, which could directly lead to retinal diseases and even loss of vision[6]. In PVR, RPE cells can become exposed to serum factors and lose their epithelial morphology[7]. This dramatic change in RPE cells is related to a complex and multifaceted process that is defined as epithelial-to-mesenchymal transition (EMT). During the EMT, RPE cells lose their epithelial characteristics and gain mesenchymal characteristics both in appearance and function[3,5-6]. Subsequently, these mesenchymal-like cells arising through the EMT significantly contribute to the formation of an ERM[8]. It has been clearly demonstrated that

RPE cells undergoing EMT play a key role in the pathogenesis of PVR. Consequently, the suppression of EMT might be beneficial in preventing the formation of ERMs[6].

EMT can be initiated by multiple extracellular cues modulated by various transcription and growth factors[5,9-11]. Among these, transforming growth factor-β2 (TGF-β2) is considered to be the predominant cytokine in the aqueous humor and the dominant isoform related to the pathogenesis of fibro-proliferative diseases through various ocular physiological or pathological processes[6,10,12]. Therefore, it is believed that TGF-β2 plays a major role in retinal fibrosis.

microRNAs are non-coding single-stranded RNA molecules that post-transcriptionally silence the expression of target genes by highly specific binding to the 3' or 5' UTRs of the target mRNA[13-14]. microRNAs may act as potent regulators in fibrotic diseases through their ability to directly target multiple EMT transcription factors[10]. However, the molecular mechanisms underlying this transcriptional regulation are still unknown. Here, microRNA-let7c (miR-let7c), a member of the microRNA let7 family, which is known EMT regulators, was selected to control TGF-β2 induction in RPE cells.

Nuclear factor-kappa B (NF-κB) is a ubiquitous transcription factor of many genes[15-16] that is presumed to regulate a wide spectrum of biological processes, including inflammation, cell proliferation, and apoptosis[17]. In normal epithelial cells (in inactive status), NF-κB family members exist as p65/p50 heterodimers and are sequestered in the cytoplasm by inhibitors of κB proteins (IκB) in the absence of activation signals. However, when such signals are present, *i.e.*, cytokines, IκB proteins are phosphorylated and degraded in a proteasome-dependent manner, leading to the release and translocation of NF-κB dimers and the activation of downstream genes[16]. Recent studies also demonstrated that the interaction of TGF-β2 and NF-κB is critical for EMT, in addition to playing an important role in cancer invasion and metastasis[18].

According to previous studies, miR-let7c is rarely expressed in TGF-β2-induced EMT in PVR[2], but otherwise, little is known regarding the function of microRNAs in PVR. In this new work, we explored the potential role of miR-let7c as a regulator of TGF-β2 during EMT in PVR. Furthermore, we also assessed the role of miR-let7c in the activation of the NF-κB pathway in ARPE-19 cells to determine the mechanisms of miR-let7c in TGF-β2-induced EMT in PVR. The present study is the first to indicate that the regulation of miR-let7c is associated with the NF-κB signaling pathway in TGF-β2-induced RPE cells.

MATERIALS AND METHODS

Cell Culture The human RPE cell line, ARPE-19, was cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12; Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), under standard cell culture conditions, at 5% CO_2 and 37℃. Cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Reagents, Antibodies and Transfection ARPE-19 cells were cultured with no serum for 12h, and then with recombinant human TGF-β2 (15 μg/L; Peprotech, Rocky Hill, NJ, USA)/ BAY11-7082 (5, 10, 20 μmoL; Sigma, St. Louis, MO, USA) for different lengths of time based on the needs of the experiments. Subsequently, the expression of various proteins and genes was analyzed by Western blot analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Antibodies against keratin-18 (Mab, #4548T), vimentin (Mab, #5741T), N-cadherin (Mab, #13116), and GAPDH (#5174) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against IκBα (Mab, ab76429), IκBα (Mab, phospho S36 ab133462), IκBα (Mab, phospho S32 ab92700) and NF-κB p65 (Mab, ab76311) were from Abcam (Cambridge, MA, USA). miR-let7c mimcs (miR-let7cM), miR-let7c mimcs negative control (miR-let7cMNC) and miR-let7c inhibitor (miR-let7cI) were obtained from GnenChem Co. (Shanghai, China).

Transfection of microRNA To introduce microRNA, 3×10^4 ARPE-19 cells were incubated overnight and transfected with 1×10^6 TU/mL miR-let7cM, miR-let7cMNC and miR-let7cI using the appropriate transfection reagent according to the manufacturer's instructions. Cells were cultured for 12h in a 37℃ incubator under a 5% CO_2 atmosphere, and then, the medium was replaced with fresh medium supplemented with 10% FBS.

Western Blotting Analysis RPE cells were washed and lysed with radioimmunoprecipitation buffer, and cell lysates were then centrifuged at 4℃. Samples were denatured, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were then incubated with primary antibodies (keratin-18, vimentin, N-cadherin, GAPDH, NF-κB p65 (1:1000), IκBα (ab76429), IκBα (phospho S36), IκBα (phospho S32; 1:500) at 4℃ overnight. After the membranes were incubated with secondary antibody (1:10 000, Boster, Pleasanton, CA, USA) for 1h, the protein expression was visualized with the Image Quant LAS4000 system (GE Healthcare, Chicago, IL, USA). The relative levels of each protein were quantified with Image J software (National Institutes of Health, Bethesda, MD, USA).

Real-time Quantitative Polymerase Chain Reaction Total RNA was isolated from ARPE-19 cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription reactions were accomplished using a PrimeScript RT Master Mix kit (TaKaRa, Kusatsu, Japan). qRT-PCR analysis was

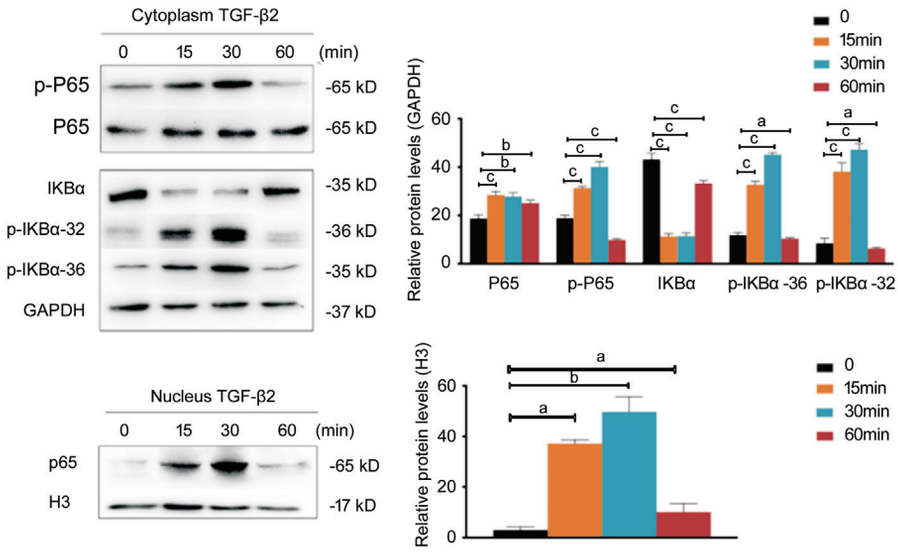

Figure 1 NF-κB is activated in RPE cells after treatment with TGF-β2 p65 nuclear translocation and IκBα phosphorylation was determined using antibodies specific to p65 and phospho-IκBα after incubation of RPE cells with 15 μg/L TGF-β2. The translocation and protein phosphorylation were more pronounced at 15 and 30min, as indicated. [a]P<0.05, [b]P<0.01, and [c]P<0.001, respectively.

performed using the ABI PRISM 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the SYBR1 Premix Ex TaqTM kit (TaKaRa). GAPDH and U6 primers served as internal controls. Real-time PCR was performed in triplicate, and statistics are included as standard deviations (SDs). The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change.

Cell Immunofluorescence After being treated with TGF-β2 and microRNAs, cells were fixed in 4% paraformaldehyde, and stored in 0.5% Triton X-100 (Sigma) and 5% goat serum at room temperature. Slides were immersed in a solution of primary antibodies overnight at 4°C. The coverslips were then maintained in a solution of fluorescent dye-labeled antibody. Cells were finally double-stained with DAPI (4', 6-diamidino-2-phenylindole 1:1000; Invitrogen) for 2min and observed using a confocal laser scanning microscope (ZEISS, Oberkochen, Germany).

Statistical Analysis The results in this study were obtained from triplicate experiments, independently repeated more than 3 times. The results are presented as the mean±SD and were analyzed using SPSS 17.0 (SPSS, Chicago, IL, USA) software. The *t*-test was used for two-group comparisons. Statistical significance was defined as P<0.05.

RESULTS

Detection of p65, p-p65, IκBα, and p-IκBα Expression in TGF-β2-induced EMT In previous studies, we found that after stimulation with TGF-β2, RPE cells exhibited a classical spindle-shaped appearance and presented with upregulation of mesenchymal markers fibronectin and N-cadherin[19]. In the present study, we explored the role of the NF-κB signaling pathway in TGF-β2-induced EMT in RPE cells. Consistent with studies on other fibrosis-based diseases, the induction of RPE cells with TGF-β2 gradually activated the NF-κB signaling pathway, as p65 was translocated into the nucleus and the expression of phosphorylated IκBα (p-IκBα) was upregulated. Specifically, this activation effect was more pronounced at 15 and 30min after the RPE cells were induced by TGF-β2 (Figure 1). These results strongly suggest that TGF-β2 promotes the EMT process, and the activation of the NF-κB pathway is essential in this process.

Detection of Keratin-18, N-cadherin and Vimentin Expression After Increasing miR-let7c Expression in TGF-β2-Induced RPE Cells The microRNA-let7 family has been shown to be altered by TGF-β2 treatment in RPE cells. To verify whether miR-let7c plays a critical role in TGF-β2-induced EMT in RPE cells, we assayed the expression of miR-let7c in RPE cells with and without TGF-β2 treatment. The results revealed that the expression of miR-let7c significantly decreased with the increase in TGF-β2 concentration (Figure 2A), suggesting that TGF-β2 stimulation induced the EMT process and also attenuated miR-let7c expression in RPE cells. Subsequently, miR-let7cM was transfected into RPE cells to assess the effect of overexpressed miR-let7c on TGF-β2 in RPE cells. For this purpose, the optimum concentration of miR-let7c was initially determined in a preliminary study. As shown in Figure 2C, keratin-18 expression was significantly decreased, and the expression of N-cadherin and vimentin increased when RPE cells were induced by TGF-β2 without microRNA transfection. However, miR-let7cM in combination with the TGF-β2 treatment significantly enhanced the expression of epithelial-specific marker keratin-18, while reducing the expression of mesenchymal markers N-cadherin

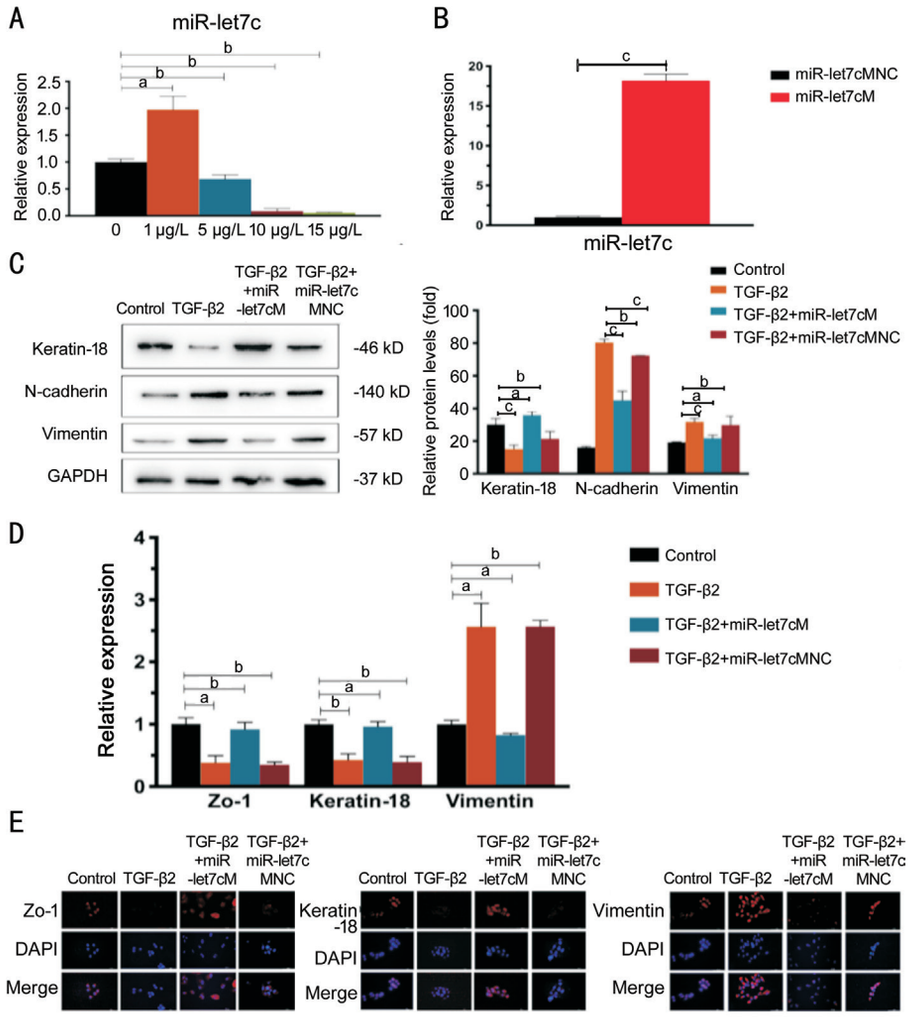

Figure 2 miR-let7c exerts an EMT-suppressive effect with abrogation of cellular response to TGF-β2 A: miR-let7c was inversely correlated with TGF-β2 during the EMT process in a concentration-dependent manner; B: RPE cells were treated with miR-let7cM or negative control (NC) for 48h, and the relative expression levels of miR-let7c in TGF-β2-induced human RPE cells were measured by qRT-PCR. U6 was used as the control; C: Compared with the vehicle, the TGF-β2 group showed hypo-expression of keratin-18 and hyper-expression of vimentin and N-cadherin. However, RPE cells were transfected with miR-let7cM, the expression of keratin-18 increased and the expression of vimentin and N-cadherin decreased, as shown by Western blotting; D, E: RT-PCR and immunofluorescence analysis also showed that miR-let7cM transfected RPE cells still maintained epithelial characteristics despite being induced by TGF-β2. $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$, respectively. miR-let7cM: miR-let7c mimcs; miR-let7cMNC: miR-let7c mimcs negative control.

and vimentin in RPE cells. This suggests that miR-let7c almost completely reversed the inhibitory effect of TGF-β2. Similarly, RT-PCR analysis and cell immunofluorescence also showed that miR-let7cM disrupted the EMT process induced by TGF-β2 (Figure 2D, 2E). Furthermore, as a comparison group, RPE cells were treated with miR-let7cMNC and TGF-β2. The result showed that the miR-let7cMNC did not inhibit the TGF-β2-induced EMT (Figure 2). These data demonstrate that miR-let7c is a valid inhibitor of TGF-β2-induced EMT in RPE cells. Therefore, we experimentally demonstrated that miR-let7c is expressed in normal RPE cells, but poorly expressed in TGF-β2-induced RPE cells. Strikingly, TGF-β2-induced EMT was dramatically suppressed due to the overexpression of miR-let7c, further emphasizing that miR-let7c could exert an EMT-suppressive effect with abrogation of cellular response to TGF-β2. However, the underlying molecular mechanisms remain unclear and should be further explored.

Detection of p65, p-p65, IκBα, and p-IκBα Expression in RPE Cells Induced by TGF-β2 and Transfected with miR-let7c Because research has increasingly shown that miR-let7c in various cell types is often associated with the NF-κB signaling pathway, and several studies have linked NF-κB-dependent pathways to fibrotic diseases, we focused on the roles of NF-κB and NF-κB-dependent pathways as regulators of miR-let7c expression. We further investigated the effects of miR-let7c overexpression on the NF-κB pathway in RPE cells. After RPE cells were exposed to 15 μg/L TGF-β2 for 15 and 30min, a significant increase was observed in p-p65 and

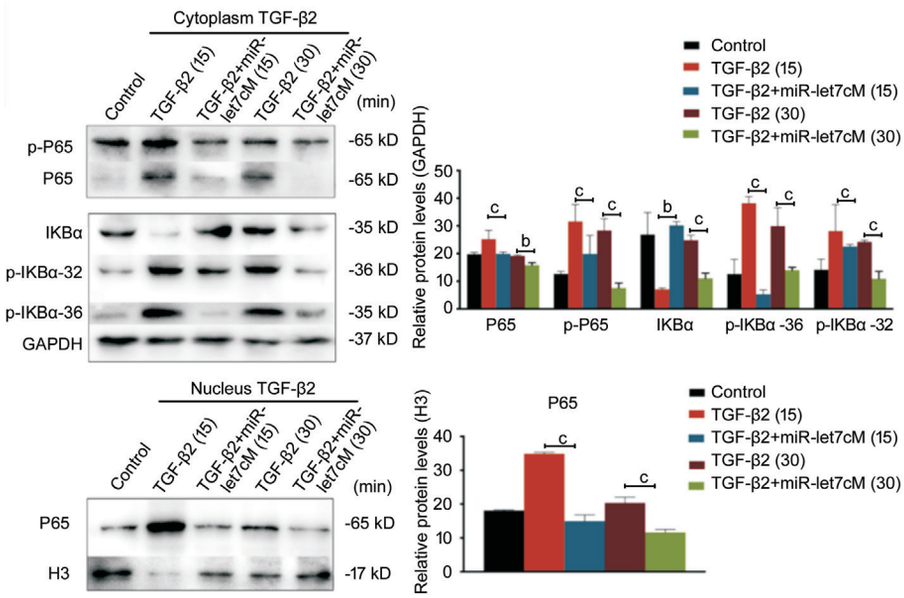

Figure 3 miR-let7c inhibited the TGF-β2-activated NF-κB signaling pathway Cells were treated with TGF-β2 for 15 and 30min, and then transfected with miR-let7cM. p65, p-p65, IkBα, and phospho-IkBα levels were measured by Western blotting. GAPDH served as a loading control. [a]P<0.05, [b]P<0.01, and [c]P<0.001, respectively. miR-let7cM: miR-let7c mimcs.

p-IκB levels (Figure 3). However, the protein levels of p-p65 and p-IκB were totally reversed at both 15 and 30min after RPE cells were transfected with miR-let7cM (Figure 3). These results show that in RPE cells, the NF-κB signaling pathway was activated during the TGF-β2-induced EMT process, and this TGF-β2-activated NF-κB signaling pathway was blocked by miR-let7c overexpression.

Regulation of miR-let7c on TGF-β2-Induced RPE Cells Through the NF-κB Signaling Pathway To further explore how miR-let7c affects TGF-β2-induced EMT in RPE cells, we next sought to determine the role of the NF-κB signaling pathway as a factor responsible for the miR-let7c-mediated suppression of TGF-β2-induced EMT in human RPE cells. First, the RPE cells were treated with TGF-β2 and different concentrations of BAY11-7082 (which selectively or specifically blocks the NF-κB pathway) to determine the concentration at which the inhibition of BAY11-7082 was optimum (Figure 4A). Then, RPE cells were treated with TGF-β2, miR-let7cM, and 20 μmol/L BAY11-7082, which revealed that once the NF-κB signaling pathway was inhibited by BAY11-7082, the inhibitory effect of miR-let7c on TGF-β2-induced EMT was decreased. As a comparison, the group of RPE cells treated with TGF-β2 and miR-let7cM without BAY11-7082 still exhibited high expression of keratin-18 and low expression of N-cadherin and vimentin on Western blots (Figure 4B). These data further indicate that miR-let7c cannot fulfill its normal function in TGF-β2-induced RPE cells when the NF-κB signaling pathway is blocked.

In addition, we detected the effects of the NF-κB signaling pathway on the miR-let7cI. TGF-β2 and miR-let7cI treatment downregulated the level of keratin-18 in the Western blotting assay, while further upregulating the protein levels of N-cadherin and vimentin. These results strongly implied that TGF-β2 and the miR-let7cI acted together in RPE cells, further inducing the transformation of RPE cells into mesenchymal-like cells. Moreover, compared with the TGF-β2 and miR-let7cI treatment group, the expression of EMT markers N-cadherin and vimentin was relatively reduced and that of epithelial-specific marker keratin-18 was relatively increased after treatment with TGF-β2, miR-let7cI, and BAY11-7082 (Figure 4C).

DISCUSSION

We previously established that TGF-β2 plays a potent and prominent role in the induction of EMT, possibly acting as a transcription factor of EMT in RPE cells[19]. Herein, we assessed the use of a microRNA (miR-let7c) in suppressing the TGF-β2-induced EMT process in RPE cells.

According to fibrosis-related experimental studies, the expression levels of more than 300 microRNAs have been shown to be altered by TGF-β2 treatment in RPE cells, including the microRNA-let7 family, which are known EMT regulators[2,9,11,13-14]. Let7 family members participate in regulating TGF-β2-induced fibrosis as negative regulators of profibrotic processes in various cell types and diseases[13]. Additionally, multiple studies suggested that miR-let7c expression is associated with cell proliferation and fibrillation in fibrotic diseases[20-21]. These data suggest that the alteration of miR-let7c regulation could play an important role in TGF-β2-induced EMT. As with cell EMT assay results described in other systems, upon TGF-β2 treatment, changes in the

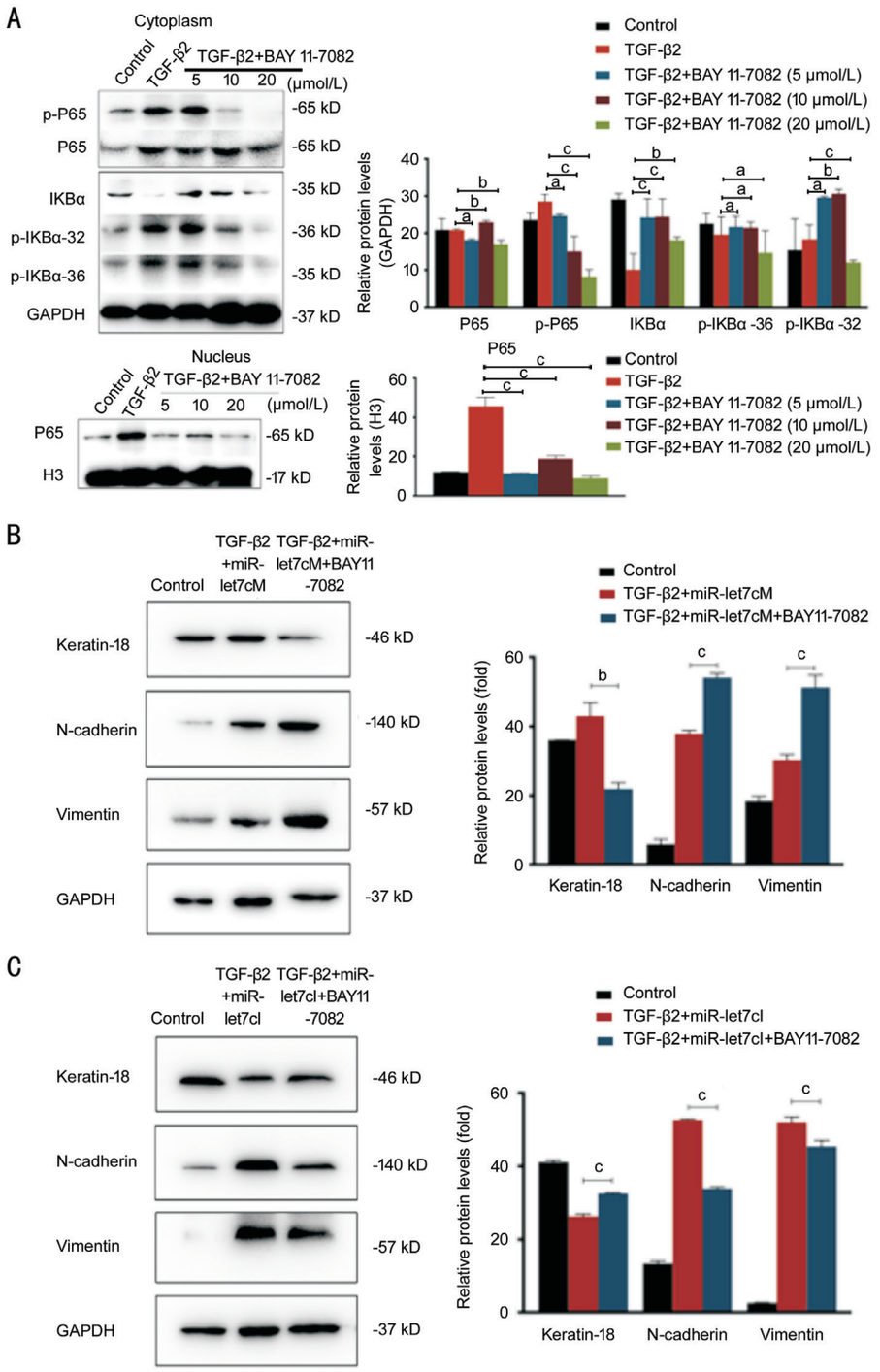

Figure 4 miR-let7c inhibited the TGF-β2-activated NF-κB signaling pathway A: The NF-κB pathway was inhibited after treatment with BAY11-7082, and the inhibitory effect was most obvious at 20 μmol/L; B: Western blot analysis of EMT in miR-let7cM transfected RPE cells treated with 15 μg/L TGF-β2 for 24h with or without pretreatment with BAY11-7085. The BAY11-7085 treatment decreased the inhibitory effect of miR-let7c by blocking NF-κB signaling; C: BAY11-7085 decreased miR-let7cI-induced EMT, but the appearance and function of cells still presented mesenchymal characteristics because BAY11-7082 could not inhibit the induction by TGF-β2, or it showed little effect on TGF-β2-induced EMT. GAPDH served as a loading control. $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$, respectively. miR-let7cM: miR-let7c mimcs, miR-let7cI: miR-let7c inhibitor.

expression of miR-let7c were detected in our RPE cells. Specifically, TGF-β2 directly decreased the level of miR-let7c in RPE cells (Figure 2A).

After the first study on microRNA in human B-cell chronic lymphocytic leukemia in the early 2000s[22], microRNAs were then studied under many conditions, especially in various fibrotic diseases. For example, experiments exploring fibrotic lung diseases demonstrated that miR-21 targeting the negative regulator Smad7 can enhance TGF-β2 signaling[23]. Moreover, mutations of the HMGA2 gene were associated with many

fibrotic diseases regulated by miR-let7c[24-26]. All these studies focused on the implication of microRNA in fibrotic diseases by directly regulating the expression of members of the TGF-β2 signaling cascade and TGF-β2 target genes. However, in our study, we confirmed that TGF-β2 itself could also be altered by microRNAs to inhibit EMT in PVR (Figure 2). Studies also reported that TGF-β2 is required for the development and progression of EMT and fibrosis, and that TGF-β2 could be directly inhibited by miR-141 and miR-200a in rat proximal tubular epithelial cells (NRK52E)[27]. Therefore, we strongly support the assertion that miR-let7c acts as an EMT suppressor in PVR.

According to Wynn[28], ongoing inflammation is required for the successful resolution of fibrosis, and there are now clinical, epidemiological, and molecular links between inflammation and EMT. Recent research has also shown that the reactivation of EMT-inducing transcription factor TGF-β2 can cause the phosphorylation of IκBα, thereby mediating its degradation, which leads to the activation of NF-κB target gene transcription[29]. NF-κB has been found to suppress the expression of the mesenchymal-specific gene vimentin and induce the expression of the epithelial-specific gene E-cadherin. Moreover, in cells that have undergone EMT, blocking NF-κB activity leads to a partial reversal of the mesenchymal phenotype[30]. These findings led us to believe that NF-κB signaling is also a significant contributor to EMT.

In our study, this idea was supported by a series of experimental results (Figures 1 and 3). As expected, despite being transfected with miR-let7cM, high levels of mesenchymal cytoskeletal proteins were still expressed in RPE cells, depending on the inhibition of NF-κB (Figure 4B). In contrast, the miR-let7cI successfully induced EMT, although NF-κB signaling was inhibited by BAY11-7082 (Figure 4C). This may have occurred because BAY11-7082 could not inhibit the TGF-β2-mediated induction, and therefore, TGF-β2 still mediated the EMT of RPE cells even though the induction by the miR-let7cI was blocked by BAY11-7082. This suggests that TGF-β2 induces EMT in an NF-κB-dependent manner.

Taken together, our results imply that miR-let7c acts as an EMT suppressor gene mainly through the NF-κB signaling pathway. The microRNA-mediated repression of TGF-β2 is another type of EMT-inactivating event that could aid in the prevention and treatment of PVR. However, at present, microRNA-based therapeutic approaches are only in experimental, preclinical, or early clinical phases, as treatment proves difficult even when a local delivery approach might be appropriate to ensure the cell-specific effect. Therefore, further studies are needed to fine-tune microRNA-based therapies for various diseases, including PVR.

The present results demonstrate that miR-let7c might inhibit TGF-β2-mediated EMT *via* the NF-κB signaling pathway. These findings could aid in the exploration of new molecular mechanisms of ERM and in the development of novel strategies for the prevention or treatment of PVR. Certainly, further studies are needed to explore the utility of miR-let7c as a predictive biomarker and therapeutic target for PVR.

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