

Original Article

The impact of miR-26b on retinal pigment epithelium cells in rhegmatogenous retinal detachment model

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Abstract: Rhegmatogenous retinal detachment (RRD) is a type of blind eye disease that seriously affects the physical and mental health. The early pathological changes are closely related to the migration of retinal pigment epithelium (RPE) cells to the vitreous body. It was showed miR-26b plays an important role in regulating lens epithelial cell growth and proliferation. However, the expression and role of miR-26b in RPE from RRD is still unclear. Rabbit RRD model was established. RPE cells were isolated and cultivated. MiR-26b inhibitor was transfected to RPE cells from model group. MiR-26b expression was tested by Real-time PCR. RPE cell proliferation was evaluated by MTT assay. Ki-67 and PCNA expressions were detected by Western blot. Caspase 3 activity was measured by the kit. RPE cell invasion was determined by Transwell assay. MiR-26b significantly increased in RPE cells from model group. It obviously promoted RPE cell proliferation and invasion, suppressed Caspase 3 activity, and upregulated Ki-67 and PCNA expression compared with control ($P < 0.05$). MiR-26b inhibitor transfection markedly restrained RPE cell proliferation and invasion, enhanced Caspase 3 activity, and inhibited Ki-67 and PCNA levels compared with model group ($P < 0.05$). MiR-26b expression was upregulated in RRD. Downregulation of miR-26b can postpone the occurrence and development of RRD through inhibiting Ki-67 and PCNA, regulating cell apoptosis, and restraining RPE cell proliferation and invasion.

Keywords: MiR-26b, rhegmatogenous retinal detachment, retinal pigment epithelium, proliferation, invasion

Introduction

Rhegmatogenous retinal detachment (RRD) is a common disease in ophthalmology. It is usually found in medium-elderly men, especially in patients with high myopia [1, 2]. In recent years, myopic population greatly increased following the aging of population and the change of lifestyle, leading to the rising incidence of RRD [3]. RRD results in shadow and diminution of vision that occurs successively in two eyes. It may cause blindness if left untreated, which seriously affects the life [4]. On the basis of hole formation in the retina, the separation between retinal pigment epithelium (RPE) layer and nerve fiber layer is the main cause of RRD [5]. RRD may lead to vitreous liquefaction to form enough tension, resulting in the detachment between the retina and RPE cells. It further leads to the ectoretina cannot obtain enough nutrition supply provided by choroid [6, 7]. RPE

is made up of single layer RPE in alignment. The cells are polygonal and divided into three parts, as the top, body, and base [8]. The detachment between retinal neurepithelium layer and RPE may aggravate the injury in macular region, which further exacerbates injury on retina and visual function damage [9, 10].

MiRNAs are types of small RNAs widely exist in animals and plants. They are made up of 20-30 nucleotides and cannot code protein [11]. MiRNAs can regulate mRNA degradation and protein translation through its complete or incomplete pairing with target genes to inhibit the downstream transcribed target protein expression [12]. MiRNAs have been widely investigated in tumors, such as lung cancer and colon cancer [12, 13]. Their roles in cardiovascular and cerebrovascular diseases and metabolic disease have been recently reported [14, 15]. MiR-26b is proved to be involved in the

growth and proliferation of lens epithelial cells [16]. However, the role and related mechanism of miR-26b in regulating RPE in RRD is still unclear. This study explored miR-26b expression in RPE from RRD and its potential impact, aiming to provide a reference for the pathogenesis and treatment of RRD.

Materials and methods

Experimental animals

Healthy male Wistar rats at 2-month old and weighted 250 ± 20 g were bought and raised in the experimental animal center in Sichuan University. The raising condition contained temperature at $21 \pm 1^\circ\text{C}$, relative humidity at 50-70%, and day-night cycle at 12 h. This study was approved by the ethics committee in West China Hospital, Sichuan University.

Main instruments and reagents

Pentobarbital sodium and lidocaine were got from Zhpharma (Shanghai, China). PVDF membrane was purchased from Pall Life Sciences. Western blot related reagents were obtained from Beyotime (Shanghai, China). ECL reagent was provided by Amersham Biosciences. Rabbit anti mouse Ki-67 and PCNA monoclonal antibodies and HRP labeled IgG secondary antibody were bought from Cell Signaling (USA). 0.1% atropine eye drops, 0.3% ofloxacin eye drops, and mydrin-P were supplied by Merck (USA). Caspase 3 activity detection kit was got from Cell Signaling (USA). Taqman miRNA reverse transcription kit was provided by Thermo (USA). MiR-26b inhibitor and negative control (NC) were synthesized by Genepharma (Shanghai, China). RNA extraction kit and reverse transcription kit were purchased from Axygen (USA). Transwell chamber was got from Corning (USA). Microinstrument was bought from Medical equipment factory (Suzhou, China). ABI 7700 Fast real time PCR was provided by ABI (USA). Benchtop was got from Sutai purification equipment engineering co., Ltd (Suzhou, China). Thermo Scientific Forma incubator was purchased from Thermo (USA). DNA amplifier was obtained from PE Gene Amp PCR System 2400 (USA).

Methods

Animal grouping and treatment: A total of 40 healthy male Wistar rats were randomly equally

divided into two groups, including control and RRD model group.

Rat RRD model establishment: After anesthetized by 0.1% pentobarbital sodium intraperitoneal injection, the rat was fixed on the operation in supine position. 0.1% atropine eye drops and 0.3% ofloxacin eye drops were used from 3 days before surgery. Mydrin-P was used for mydriasis. 2% lidocaine and 0.75% bupivacaine were used subconjunctivally for local anesthesia. After the bulbar conjunctiva was opened, three channels for vitrectomy, perfusion, and optical fiber illumination were made at 3 mm posterior to the corneoscleral limbus using a sclera stab knife. The vitreous body was removed and the retina was scratched to form 2-3 hiatus with the diameter as optic disc. Next, the cornea and bulbar conjunctiva incisions were closed. 10,000 units of gentamicin and dexamethasone were locally injected, while chloramphenicol eye drops was used postoperatively for anti-infection. Optical coherence tomography showed the interruption in retinal neurepithelium layer, significant apophysis and detachment on RPE, and opaque dark area of fluid, confirming the success modeling [17].

RPE cell cultivation: The rat was anesthetized by 2% lidocaine retrobulbar injection and the eyeballs were removed. After washed by gentamycin, the eyeball was put into DMEM. Next, the eyeball was cut open at 3 mm posterior to the corneoscleral limbus and the anterior segment and vitreous body were removed. Then the tissue was digested by 0.25% trypsin at room temperature for 10 min. The retinal neurepithelium layer was separated and removed. Next, the tissue was further digested by 0.25% trypsin at 37°C for 30 min. The cell suspension was centrifuged at 800 rpm for 10 min, and then the cells were cultured in DMEM medium containing 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. RPE cells in the 2-8th generation and logarithmic phase were used for experiment. The cells were randomly divided into three groups, including model group, inhibitor NC group, and miR-26b inhibitor group.

MiR-26b inhibitor transfection: MiR-26b inhibitor was transfected to RPE cells using lipofectamine. The sequences were as follows. MiR-26b inhibitor, 5'-AUUGUGGUGGACACCGUA-3'; negative control, 5'-UAGGCAGCAAGUUCGUUG-3'. When the cell fusion reached 70-80%, miR-26b inhibitor or NC was added to

MiR-26b regulates RPE in RRD

Table 1. Primer sequences

Gene	Forward, 5'-3'	Reverse, 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
Mir-26b	ATTAGCCCTGTCCCTCAATC	TAGATGGTTTATCTATGACC

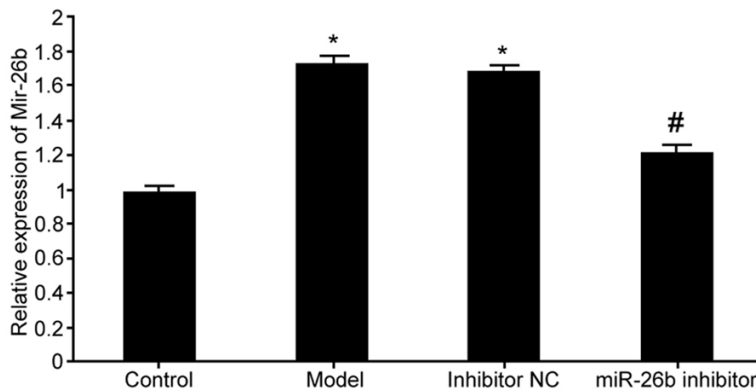


Figure 1. MiR-26b expression in RPE cells from RRD. *P < 0.05, compared with control; #P < 0.05, compared with model group.

200 μ l FBS free DMEM and incubated at room temperature for 15 min. Next, they were mixed with lipo2000 and incubated at room temperature for 30 min. At last, they were added to the cells and cultured for 6 h. The cells were further cultured for 48 h after changing to the complete medium for the following experiment.

Real time PCR: Total RNA was extracted using Trizol and reverse transcribed to cDNA. The primers used were designed by PrimerPrimer 6.0 software and synthesized by Invitrogen (Shanghai, China) (Table 1). Real time PCR reaction was performed as 95°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as the internal reference. The relative expression level was calculated using the $2^{-\Delta Ct}$ method.

MTT assay: RPE cells in logarithmic phase were seeded to 96-well plate at 5×10^3 cells/well and cultured for 24 h. The cells were divided into abovementioned three groups. After cultured for 48 h, 20 μ l MTT was added to each well and incubated for 4 h. Next, DMSO was added at 150 μ l/well and the plate was vibrated for 10 min. At last, the plate was read at 570 nm to obtain the absorbance value. Each experiment was repeated for at least three times.

Caspase 3 activity detection:

The cells were digested by trypsin and centrifuged at 600 g and 4°C for 5 min. Next, the cells were added with lysis on ice for 15 min. After centrifuged at 20000 g and 4°C for 5 min, the suspension was added with 2 mM Ac-DEVD-pNA and tested at 405 nm to obtain the absorbance value.

Transwell assay: According to the manual, Transwell chamber was coated by 50 mg/L Matrigel diluted at 1:5 and air dried at 4°C. A total of 500 μ l DMEM containing 10% FBS were added to the lower chamber, while serum free DMEM containing 100 μ l cell suspension was added to the upper chamber. After incubated

for 48 h, the membrane was washed and fixed using absolute alcohol. After stained by crystal violet, the membrane was observed under the microscope to calculate the penetrated cell number.

Western blot: Total protein was extracted from RPE cells using the RIPA on ice for 15-30 min. After ultrasonicated at 5 s \times 4 times and centrifuged at 4°C and 10,000 g for 15 min, the protein was moved to a new Eppendorf tube. The protein was stored at -20°C after quantification. The protein was separated by 10% SDS-PAGE and transferred to PVDF membrane at 100 mA for 1.5 h. After blocked in 5% skim milk at room temperature for 2 h, the membrane was incubated in primary antibody (Ki-67, 1:1000; PCNA, 1:2000) at 4°C overnight. After washed by PBST, the membrane was further incubated in goat anti rabbit secondary antibody at 1:2000 for 30 min. At last, the membrane was treated by ECL reagent for 1 min and developed. The membrane was scanned by image processing system and analyzed by Quantity One software. Each experiment was repeated for four times.

Statistical analysis

All data were depicted as mean \pm standard deviation ($\bar{x} \pm S$) and compared by t-test. SPSS

MiR-26b regulates RPE in RRD

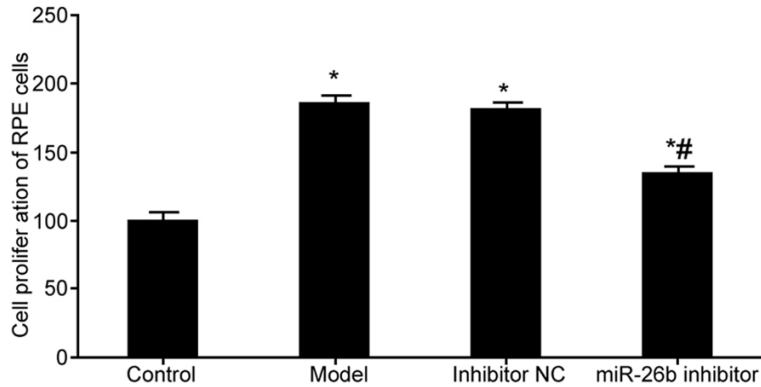


Figure 2. The impact of miR-26b on RPE cell proliferation in RRD. * $P < 0.05$, compared with control; # $P < 0.05$, compared with model group.

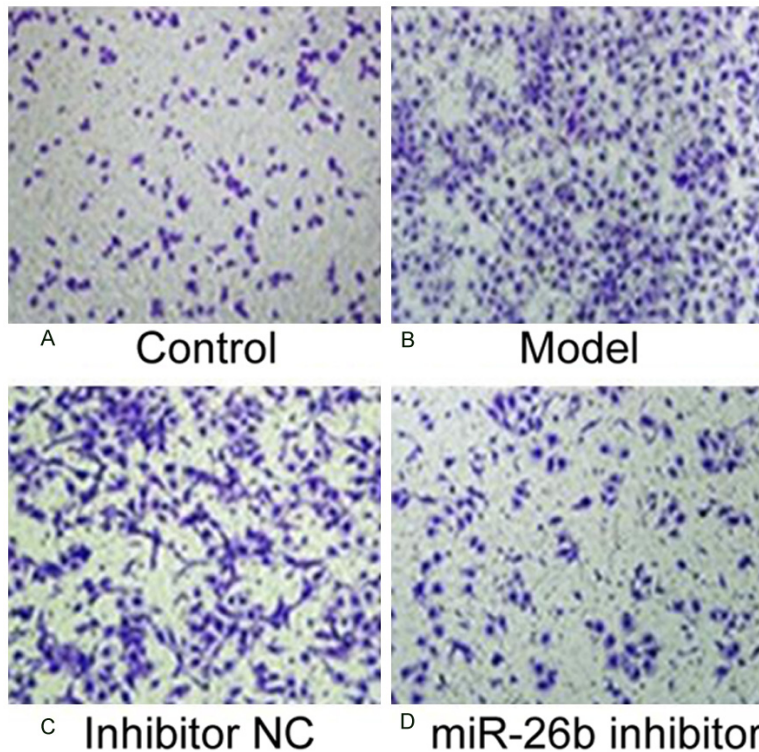


Figure 3. The impact of miR-26b on RPE cell invasion in RRD. A: control; B: model group; C: inhibitor NC group; D: miR-26b inhibitor group.

11.5 software was used for data analysis. Inter-group difference was analyzed by ANOVA. $P < 0.05$ was considered as statistical significance.

Results

MiR-26b expression in RPE cells from RRD

Real time PCR showed that miR-26b expression in RPE cells from RRD was significantly higher than the control ($P < 0.05$). MiR-26b

inhibitor transfection obviously suppressed miR-26b expression in model RPE cells compared with model group ($P < 0.05$) (**Figure 1**).

The impact of miR-26b on RPE cell proliferation in RRD

MTT assay was adopted to evaluate the impact of miR-26b on RPE cell proliferation in RRD. RPE proliferation significantly enhanced in RRD group compared with normal control ($P < 0.05$). MiR-26b inhibitor transfection markedly suppressed RPE cell proliferation compared with model group ($P < 0.05$) (**Figure 2**). It suggested that downregulation of miR-26b is in favor of regulating RPE cell abnormal proliferation in RRD.

The impact of miR-26b on RPE cell invasion in RRD

Transwell assay was used to test the impact of miR-26b on RPE cell invasion in RRD. RPE cell invasion markedly elevated in RRD group compared with normal control ($P < 0.05$). MiR-26b inhibitor transfection significantly inhibited RPE cell invasion compared with model group ($P < 0.05$) (**Figures 3, 4**). It indicated that downregulation of miR-26b is in favor of regulating RPE cell abnormal invasion in RRD.

The impact of miR-26b on Ki67 expression in RPE cell from RRD

It was showed that Ki67 upregulated in PRE cells from RRD compared with control ($P < 0.05$). MiR-26b inhibitor transfection obviously declined Ki67 expression in RPE cells compared with model group ($P < 0.05$) (**Figure 5**).

The impact of miR-26b on PCNA expression in RPE cell from RRD

It was revealed that PCNA increased in PRE cells from RRD compared with control ($P < 0.05$). MiR-26b inhibitor transfection signifi-

MiR-26b regulates RPE in RRD

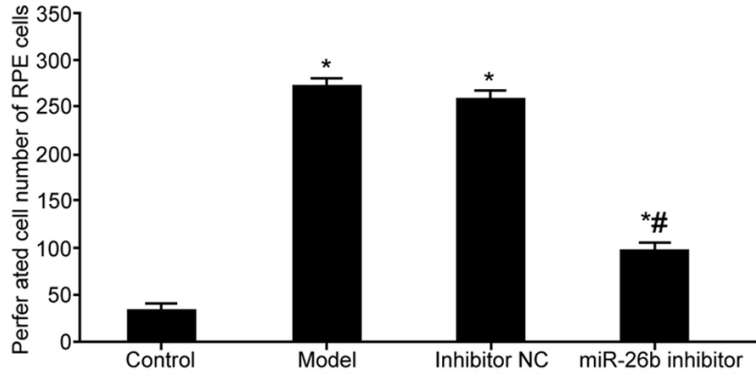


Figure 4. The impact analysis of miR-26b on RPE cell invasion in RRD. *P < 0.05, compared with control; #P < 0.05, compared with model group.

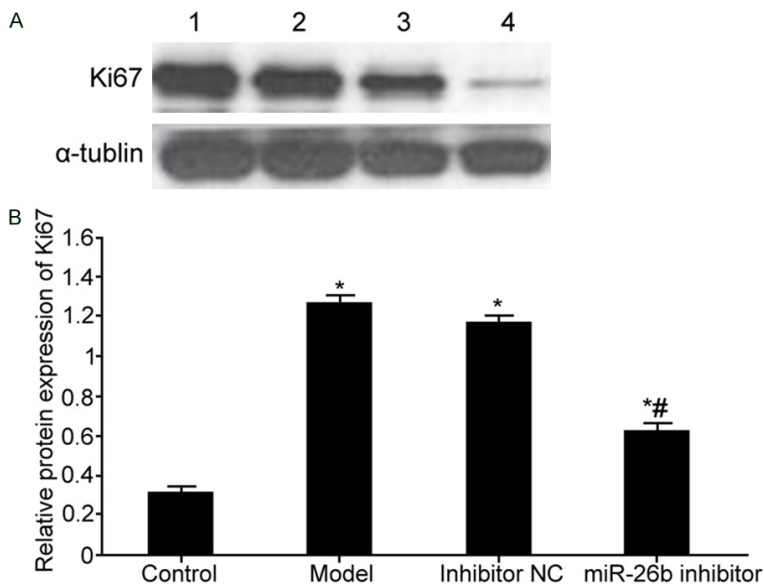


Figure 5. The impact of miR-26b on Ki67 expression in RPE cell from RRD. A. Western blot analysis of Ki67 expression. 1, model group; 2, inhibitor mimics group; 3, miR-26b inhibitor group; 4, control. B. The impact of miR-26b regulation on Ki67 expression. *P < 0.05, compared with control; #P < 0.05, compared with model group.

cantly reduced PCNA expression in RPE cells compared with model group (P < 0.05) (**Figure 6**).

The impact of miR-6b on Caspase 3 activity in RPE cells from RRD

It was demonstrated that Caspase 3 activity decreased in RPE cells from RRD compared with control (P < 0.05). MiR-26b inhibitor transfection apparently reduced Caspase 3 activity in RPE cells compared with model group (P < 0.05) (**Figure 7**). It indicated that downregulation of miR-26b promoted Caspase 3 activity to

facilitate RPE cell apoptosis in RRD.

Discussion

The earliest lesion of RRD is the migration of RPE cells to the vitreous body, leading to refactoring [18, 19]. RPE cell hyperplasia facilitates migration. After stimulation, RPE cells differentiate and migrate to the vitreous body. In addition, RPE proliferate and secrete to the extracellular matrix (ECM), therefore forming periretinal membrane with contraction ability and resulting in RD [18, 19]. It aggregates on the lower surface of retina or the interface between vitreous body and retina, and further secretes inflammatory cytokines. It promotes ECM secretion to form pathological deposition, which pulls the retina and causes RD [20]. MiRNAs exhibit various mechanisms, as participating in cell proliferation, apoptosis, signaling transduction, differentiation, hormone secretion, fat metabolism, and maintenance of the potential of embryonic stem cells, so as to regulate the growth and development and enhance the adaption to the environment [21]. In addition to lung cancer and colon cancer, miR-26b was confirmed to play a negative regulatory

role in the proliferation and migration of human lens epithelial cells [16]. However, the expression and role of miR-26b in RRD has not been clarified. This study found that miR-26b upregulated in the RPE cells of RRD, thus increasing RPE cell proliferation and invasion, declining Caspase 3 activity, and upregulating Ki67 and PCNA expressions.

We further transfected miR-26b inhibitor to RPE cells from RRD. It was showed that downregulation of miR-26b suppressed RPE cell proliferation and invasion. Caspase-3 is an important member of Caspase family. It plays a criti-

MiR-26b regulates RPE in RRD

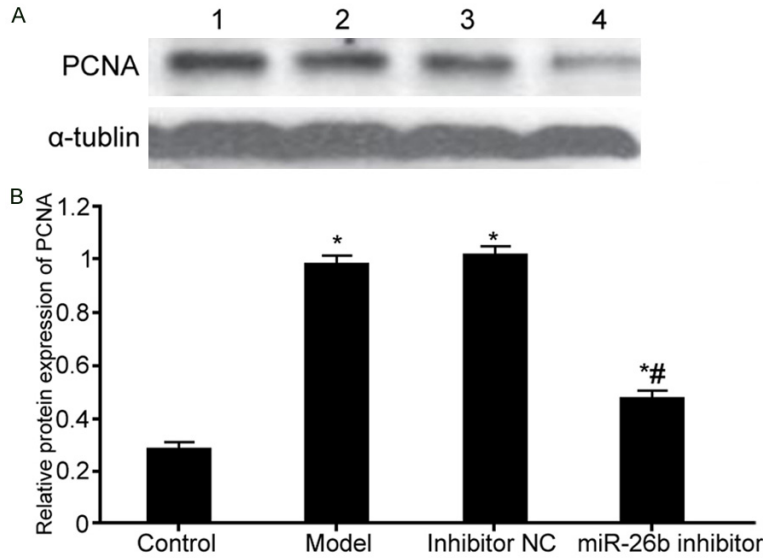


Figure 6. The impact of miR-26b on PCNA expression in RPE cell from RRD. A. Western blot analysis of PCNA expression. 1, model group; 2, inhibitor mimics group; 3, miR-26b inhibitor group; 4, control. B. The impact of miR-26b regulation on PCNA expression. *P < 0.05, compared with control; #P < 0.05, compared with model group.

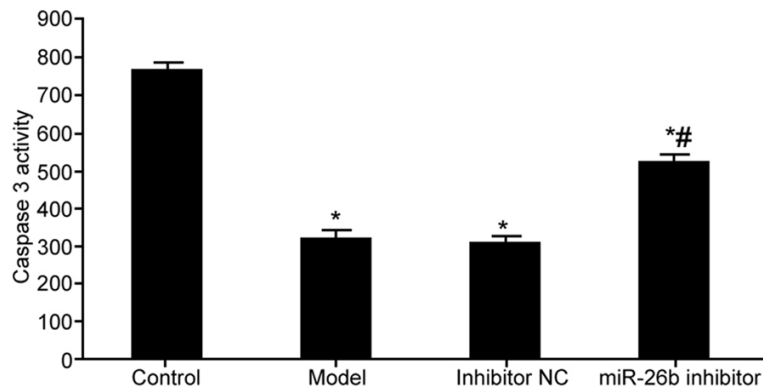


Figure 7. The impact of miR-6b on Caspase 3 activity in RPE cells from RRD. *P < 0.05, compared with control; #P < 0.05, compared with model group.

cal role in cell apoptosis by promoting cell apoptosis [22]. Ki67 represents the degree of cell proliferation, while PCNA is closely associated with DNA synthesis. They are good indicators to reflect the cell proliferation [23]. This study confirmed that downregulation of miR-26b can restrain RPE cell proliferation and invasion, increase Caspase 3 activity, and inhibit Ki67 and PCNA expressions.

Conclusion

MiR-26b expression increased in RRD. Downregulation of miR-26b can postpone the

occurrence and development of RRD through inhibiting Ki-67 and PCNA, regulating cell apoptosis, and restraining RPE cell proliferation and invasion.

Acknowledgements

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Disclosure of conflict of interest

None.

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MiR-26b regulates RPE in RRD

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