• Basic Research •

miRNA-21-5p targeting PTEN to regulate PI3K/Akt/mTOR pathway in retinal pigment epithelial cell photodamage

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Abstract

● **AIM:** To highlight the importance of microRNA (miRNA)-21-5p in directing the phosphatase and tensin homolog (*PTEN*) gene to control the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway in retinal pigment epithelial (RPE) cells in humans subjected to photodamage.

● **METHODS:** Human adult RPE cell line-19 (ARPE-19) was cultured *in vitro* and randomly divided into control, damage, overexpression, negative, and PI3K/Akt blocker groups to establish a photodamage model of ARPE-19 cells. The models were subjected to 24h of light exposure, after which the corresponding indices were detected. The cell counting kit-8 assay quantified cell viability, while flow cytometry determined apoptosis rates. The miRNA-21 mimics and miRNA mimic NC were transfected into ARPE-19 cells using a transient transfection technique. Quantitative reverse transcription polymerase chain reaction (SYBR Green) and Western blotting analyzed expression levels of miRNA-21-5p, PTEN, p-PI3K/PI3K, p-mTOR/mTOR, and p-Akt/Akt. Statistical analyses comprised one-way analysis of variance and the Student-Newman-Keuls test for multiple group comparisons.

● **RESULTS:** The photodamage group demonstrated reduced cell survival rates than the control group (P<0.01). The overexpression group exhibited higher cell survival rates than the injury group (P<0.01). The negative group showed no difference in viability (P>0.05). The PI3K/Akt blocker group demonstrated lower cell viability, compared with the overexpression group (P<0.01).

● **CONCLUSION:** miRNA-21-5p significantly increases ARPE-19 cell survival after photodamage and inhibits light-induced ARPE-19 cell apoptosis, suggesting that it may play a protective role in RPE by activating the PI3K/Akt/mTOR pathway while downregulating PTEN expression.

● **KEYWORDS:** retinal pigment epithelial cell; photodamage; apoptosis; PI3K/Akt/mTOR signaling pathway; miRNA-21-5p
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INTRODUCTION

Most people aged over 50y report age-related macular degeneration (AMD), a refractory eye disease that severely reduces vision. AMD is the principal cause of loss of sight in older individuals[1]. With global aging trends, its incidence is increasing annually, with reports estimating 288 million people with AMD by 2040[2]. Continued exposure to light can cause retinal photochemical damage. Diseases closely related to this damage, such as AMD[3], severely affect patients' lives. Therefore, the effective treatment of AMD has become an important focus for clinicians. MicroRNA (miRNA) is an endogenous, non-coding small molecule RNA that widely exists in animals and plants. In humans, miRNA-21 can act on its target gene on chromosome 10, the phosphatase and tensin homolog (*PTEN*), and subsequently regulate cell apoptosis, proliferation, and other processes[4]. Furthermore, as an important signaling pathway in cells, phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) governs important cell functions, including growth and division, differentiation,

and the signal transduction of apoptosis and other processes[5]. Activated PI3K catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to PIP3, allowing Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1) recruitment to bind to Akt downstream of the activation pathway, thereby regulating cell function[6]. Mammalian target of rapamycin (mTOR)—a downstream serine-threonine protein kinase—plays an essential part in promoting cell survival and proliferation[7]. PTEN is characterized by dual-specificity phosphatase activity and serves as a negative regulator of the PI3K/Akt pathway, while its loss can activate Akt and result in uninhibited cell proliferation and decreased apoptosis[8]. miRNA has been implicated in AMD onset and development[9-10]; however, minimal research has focused on the specific mechanisms and roles in AMD, which remain unclear. Our preliminary study has looked into the function of the PI3K/Akt/mTOR pathway in a human adult retinal pigment epithelial (ARPE-19) cell model subjected to photodamage. To further clarify the mechanism of action of this pathway[11], the objective of this research is to explore the function of miRNA-21-5p on PTEN and its impact on the PI3K/Akt/mTOR cascade in retinal pigment epithelial (RPE) cells subjected to photodamage in humans. This research may lead to improved management or prevention of AMD by providing new insight into further innovative research leading to the development of anti-AMD medications and other treatment measures.

MATERIALS AND METHODS

Ethical Approval Our research protocol was reviewed and permitted by the Medical Ethics Review Committee of Ningxia Medical University (No. 2020-070).

Cell Lines This study utilized ARPE-19 sourced from the China Beina Biologics Corporation (Beijing, China).

Reagents and Materials The key reagents and materials used in this study consisted of lipopolysaccharide of 75% purity (Qinghai CommScope Biotechnology Co., Ltd., KPGQZ-TQW-13-02, China), penicillin-streptomycin mixture (Procell, PB180120, China), RIPA lysate (Beijing Solabon, R0020, China), fetal bovine serum (FBS; Sijiqing, 11011-8611, China), DMEM/F12 (HyClone, SH30023.01, USA), Annexin V-FITC apoptosis detection kit (ClusterBio, AP101, China), phosphate buffer (HyClone, USA), cell counting kit-8 (CCK8; Jiangsu Keygen Biotechnology Co., Ltd., BB-4202-2, China), ECL luminescence kit (Beijing Nakasugi Jinqiao, SC-2048, China), polyvinylidene difouride (PVDF) membrane (MilliporeSigma, USA and Canada, IPVH00010), PI3K (AF6241), PTEN (AF6351), p-AKT (AF0016), AKT (AF6261), p-PI3K (AF4372), MTOR (AF6308), p-MTOR (AF3308), and β-actin (AF7018) antibodies (Affinity, USA), miRcute miRNA detection kit (Catb FP411), miRcute miRNA first-strand cDNA synthesis kit (Catb KR201), and miRcute miRNA isolation kit (Catb DP501; Beijing Tiangen Biotechnology Co., Ltd., China), fluorescence polymerase chain reaction (PCR) kit (Wuhan Mona Biotech, Catb MQ00401S, China), transfection reagent TransIntro EL transfection reagent (Beijing Quanzhijin, FT201-01, China), miR21 mimic kit (Guangzhou RiboBio, miR10000076-1-5, China), LY294002 (MCE, 154447-36-6, USA).

Main Instruments A Multiskan microplate reader (Thermo Company, USA), TES-1332A digital illuminance meter (Taipei Taishi Electronics Industry, China), FACSAria II flow cytometer (BD Company, USA), and a fluorescence quantitative PCR instrument (Bio-Rad, USA) were used.

Cell Culture ARPE-19 cells were incubated in a complete medium supplemented with 10% FBS and 1% double antibody at 37℃ with 5% CO_2. The medium was replaced every 2d until the cells reached approximately 90% density. These cells were subcultured at a 1:4 ratio, and second- to fourth-passage cells were selected for further experiments.

Cell Transfection For counting and inoculation, ARPE-19 cells showing stable growth were seeded at a density of 4×10^7 cells per well. When cells reached 80% to 90% confluency, they were transfected. Solutions A and B were prepared separately on an ultraclean workbench. Solution A was comprised of gently mixed diluted 250 pmol small interfering (si)RNA in 200 μL OPTI-MEM medium. Solution B comprised 10 μL TransIntro EL added to the diluted siRNA, which was lightly stirred and placed for 20min at room temperature. Finally, the siRNA-TransIntro EL complex was added to the cells and incubated at 37℃ with 5% CO_2. After 4–6h of transfection, the cell medium was changed, and after 48h of continuous culture, the cells from each group were collected for subsequent use and detection.

Experimental Grouping The experiment comprised five groups: control (ARPE-19 cells+TransIntro EL transfection reagent transfection solution group), injury (ARPE-19 cells+TransIntro EL transfection reagent transfection solution+light irradiation), overexpression (ARPE-19 cells+TransIntro EL transfection reagent+miRNA-21-5p mimics+light irradiation), negative (ARPE-19 cells+TransIntro EL transfection reagent+miRNA mimics NC+light irradiation), and PI3K/Akt blocker (ARPE-19 cells+TransIntro EL transfection reagent transfection solution+miRNA-21-5p mimics+LY294002+light irradiation). Indicators were detected after 24h of light exposure.

Construction of Cell Photodamage Model The second- to fourth-passage ARPE-19 cells were cultured in a six-well plate. They were subjected to vertical irradiation using an LED cold light lamp with an illumination intensity of (16 500±200) lx for 24h.

qRT-PCR to Detect miRNA-21-5p and PTEN mRNA Expressions Total RNA was isolated from ARPE-19

cells using the total RNA isolation reagent, and the RNA concentration was measured using a microspectrophotometer. Per the instructions provided in the reverse transcription polymerase chain reaction (RT-PCR) kit, cDNA was synthesized by reverse transcription and amplified via PCR. The resulting products were separated by 12 g/L agarose gel, stained with EB; the internal reference was U6. A melting curve analysis was performed after amplification. The sequences were as follows: miRNA-21-5P primer sequence: GGTAGCTTATCAGACTGATGTTG; PTEN primer sequence: upstream TTTGAAGACCATAACCCACCAC, downstream ATTACACCAGTTCGTCCCTTTC; and U6 primer sequence: TCGCTTCGGCAGCACATA. The $2^{-\Delta\Delta Ct}$ method quantified relative gene expression.

Detection of Cell Viability Using the CCK-8 Logarithmic growth phase cells were chosen. After enzymatic digestion, cells were seeded in a 96-well plate at a density of 5×10^3/well. After the cells were intervened, we followed the steps outlined in the CCK8 kit and used a microplate reader. A microplate reader measured optical density (OD) at 450 nm and calculated the cell viability accordingly.

Flow Cytometry to Quantify Cell Apoptosis Rate After light treatment, cells from each group were collected in centrifuge tubes. After the cells were washed with cold phosphate buffered saline (PBS) three times, 400 μL of AnnexinV binding solution was added to suspend the cells. Then, 5 μL of FITC and 10 μL of propidium (PI) were added to the cell suspension in sequence and mixed gently. The reaction was performed in the dark for 15min, and flow cytometry detection was completed within 1h.

Western Blotting Cellular proteins were extracted with RIPA lysate, quantitatively detected, electrophoresed, and transferred to a membrane. The membrane was incubated overnight at 4°C with the primary antibody. Secondary antibody incubation was conducted at 37°C for 2h on a shaker. A chemiluminescent solution was added in a darkroom, followed by exposure. Protein band intensities were analyzed using Image J software.

Statistical Analysis SPSS21.0 software (IBM, USA) was used for data analyses. A one-way analysis of variance was utilized for group comparisons. The mean±standard deviation(s) denote all data. Multiple comparisons were conducted using the SNK-*Q* test, with statistical significance set at a *P*-value <0.05 (α=0.05).

RESULTS

Cell Transfection Fluorescence microscopy showing the transfection efficiency. After repeated exploration, the relative optimal transfection time was 48h (Figure 1).

miRNA-21-5p and PTEN mRNA Expressions in Transfected Light-Induced ARPE-19 Cells Compared with the control group, the injury group showed a reduction in miRNA-21-5p expression and an increase in PTEN mRNA expression (P<0.01). However, no difference was observed in the expression of PTEN mRNA and miRNA-21-5p in the negative group (P>0.05). Hence, miRNA-21-5p was underexpressed in light-induced ARPE-19 cells, whereas PTEN mRNA expression was not affected by miRNA-21-5p (Table 1).

Table 1 Comparison of miRNA-21-5p and PTEN mRNA expressions in transfected light-induced ARPE-19 cells (n=3) mean±SD

Groups	miRNA-21-5p	PTEN mRNA
Control group	0.91±0.01	1.13±0.13
Injury group	0.30±0.04[a]	3.46±0.18[a]
Overexpression group	2.72±0.21[b]	3.11±0.14[c]
Negative group	0.25±0.04[c]	3.29±0.28[c]
PI3K/Akt blocker group	1.23±0.07[d]	3.34±0.27[c]

ARPE: Human adult retinal pigment epithelial; PTEN: Phosphatase and tensin homolog; PI3K: phosphoinositide 3-kinase; Akt: Protein kinase B. Contrasted to the control group, [a]P<0.01; Contrasted to the injured group, [b]P<0.01, [c]P>0.05; Contrasted to the overexpression group, [d]P<0.01.

Survival Rates of Transfected Light-Induced ARPE-19 Cells Compared with the control group, the injury group showed a decrease in cell survival (P<0.01). Nevertheless, cells in the overexpression group showed a higher survival rate than cells in the injury group (P<0.01). No difference was observed in cell survival in the negative group (P>0.05). The survival rate of cells in the PI3K/Akt blocker group was lower than that in the overexpression group (P<0.01). Hence, miRNA-21-5p significantly improved ARPE-19 cell survival after photodamage (Table 2).

Detecting Apoptosis Rates in Transfected Light-Induced ARPE-19 Cells Compared with the control group, the injury group showed higher apoptosis rates (P<0.01). Compared with the injury group, the overexpression group showed lower apoptosis rates (P<0.01). No difference in the apoptosis rate was observed in the negative group (P>0.05). PI3K/Akt blocker group showed higher apoptosis rates than the overexpression group (P<0.01). Hence, miRNA-21-5p inhibits apoptosis in light-induced ARPE-19 cells (Figure 2, Table 3).

PTEN, p-PI3K/PI3K, p-Akt/Akt, and p-mTOR/mTOR in Transfected Light-Induced ARPE-19 Cells Compared with the control group, the injury group showed lower phosphorylation levels of PI3K, Akt, and mTOR, whereas higher PTEN protein expression (P<0.01). Compared with the injury group, the overexpression group showed increased phosphorylation levels of PI3K, Akt, and mTOR, whereas decreased PTEN protein expression (P<0.01). No difference in the phosphorylation levels of PI3K, Akt, and mTOR and PTEN protein expression were observed in the negative group

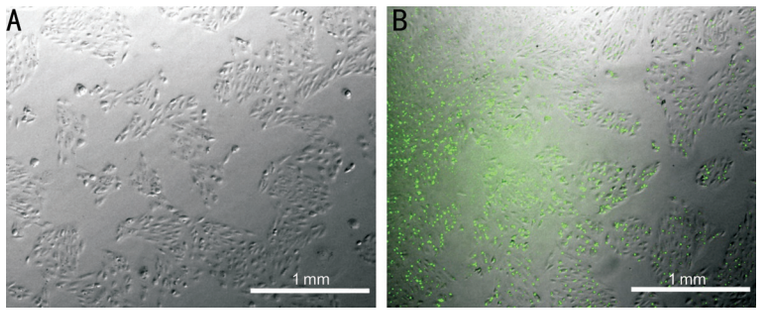

Figure 1 Transfecting ARPE-19 cells A: Untransfected ARPE-19 cells (magnification: 40×); B: ARPE-19 cells after transfection (magnification: 40×). ARPE: Human adult retinal pigment epithelial.

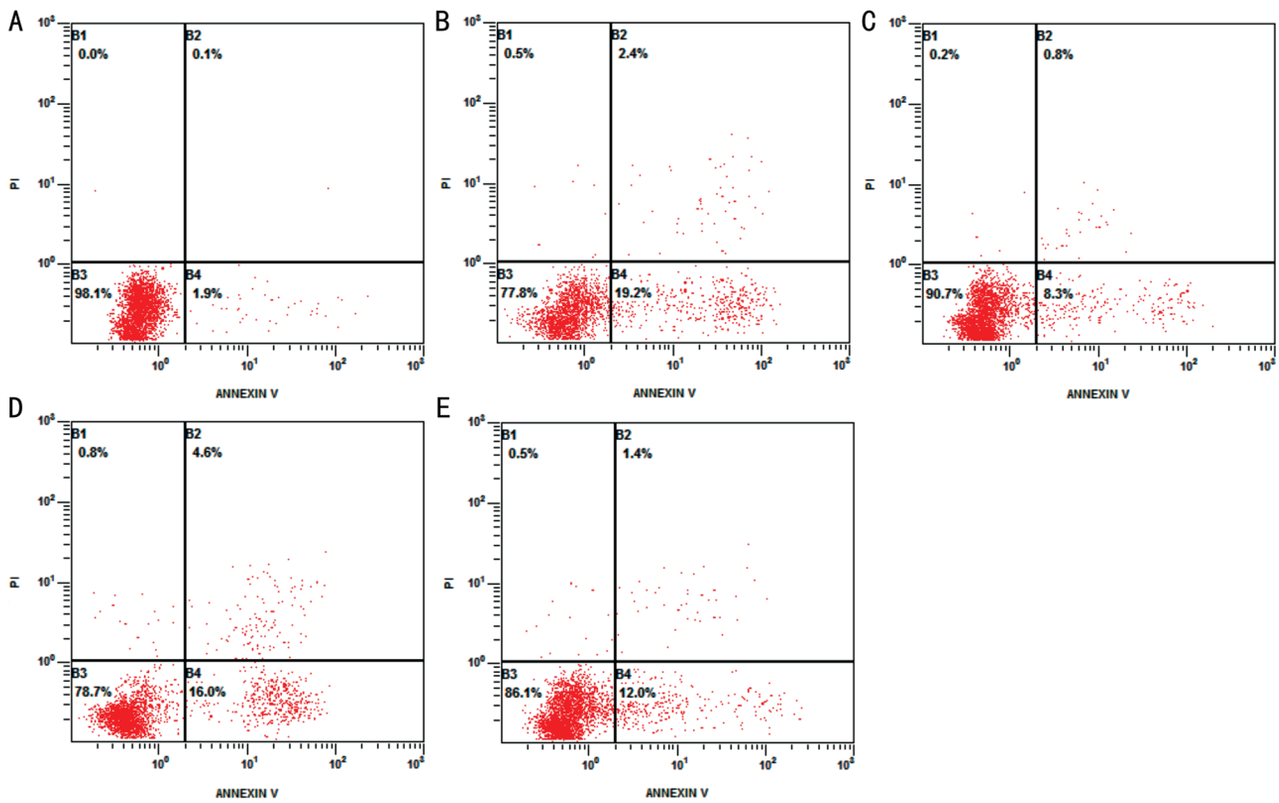

Figure 2 Apoptosis rates in transfected light-induced ARPE-19 cells A: Control group ; B: Injury group; C: Overexpression group; D: Negative group; E: PI3K/Akt blocker group. PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; ARPE: Human adult retinal pigment epithelial.

Table 2 Survival rates in transfected light-induced ARPE-19 cells (*n*=6) mean±SD, %

Groups	24h
Control group	99.96±3.91
Injury group	57.36±3.17[a]
Overexpression group	86.90±3.69[b]
Negative group	54.08±1.61[c]
PI3K/Akt blocker group	65.21±1.89[d]

ARPE: Human adult retinal pigment epithelial; PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B. Compared with the control group, [a]*P*<0.01; Contrasted to the injured group, [b]*P*<0.01, [c]*P*>0.05; Contrasted to the overexpression group, [d]*P*<0.01.

Table 3 Apoptosis rates in transfected light-induced ARPE-19 cells (*n*=6) mean±SD

Groups	Apoptosis rate (%)
Control group	2.63±0.57
Injury group	21.03±0.55[a]
Overexpression group	9.53±0.75[b]
Negative group	20.47±1.11[c]
PI3K/Akt blocker group	12.53±0.85[d]

Contrasted to the control group, [a]*P*<0.01; Contrasted to the injured group, [b]*P*<0.01, [c]*P*>0.05; Contrasted to the overexpression group, [d]*P*<0.01. ARPE: Human adult retinal pigment epithelial.

(*P*>0.05). Compared with the overexpression group, the PI3K/Akt blocker group showed decreased phosphorylation levels of PI3K, Akt, and mTOR, whereas increased PTEN protein expression (*P*<0.01). Hence, miRNA-21-5p protects ARPE-19 cells from light-induced damage by downregulating PTEN expression and activating the PI3K/Akt/mTOR cascade (Table 4, Figure 3).

Table 4 Light-induced PTEN and PI3K/Akt/mTOR cascade in transfected ARPE-19 cells (*n*=3) mean±SD

Groups	PTEN	p-PI3K/PI3K	p-Akt/Akt	p-mTOR/mTOR
Control group	0.22±0.02	1.17±0.04	0.92±0.01	1.61±0.08
Injury group	0.83±0.04[a]	0.31±0.02[a]	0.23±0.02[a]	0.55±0.03[a]
Overexpression group	0.33±0.06[b]	0.75±0.05[b]	0.68±0.02[b]	1.32±0.15[b]
Negative group	0.80±0.05[c]	0.27±0.03[c]	0.22±0.01[c]	0.53±0.05[c]
PI3K/Akt blocker group	0.45±0.05[d]	0.48±0.04[d]	0.41±0.01[d]	1.05±0.04[d]

PI3K: Phosphoinositide 3-kinase; PTEN: Phosphatase and tensin homolog; mTOR: Mammalian target of rapamycin; Akt: Protein kinase B; ARPE: Human adult retinal pigment epithelial. Contrasted to the control group, [a]*P*<0.01; Contrasted to the injured group, [b]*P*<0.01, [c]*P*>0.05; Contrasted to the PI3K/Akt blocker group, [d]*P*<0.01.

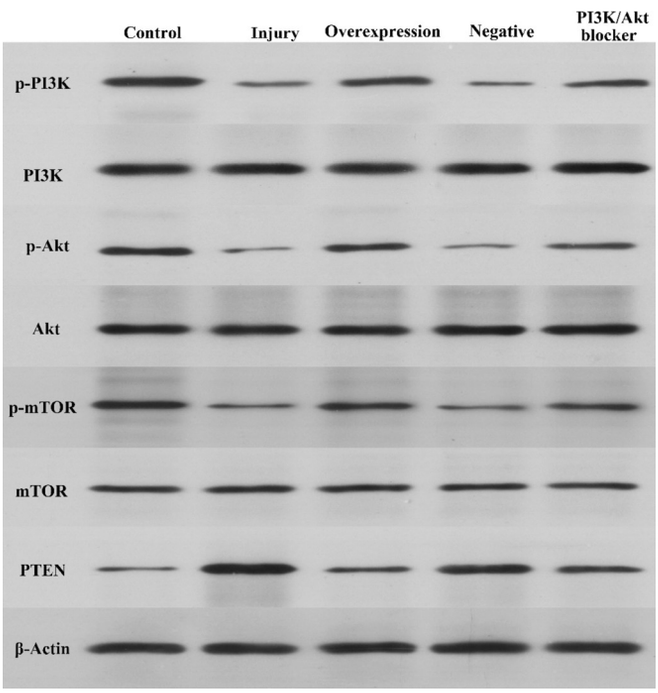

Figure 3 Effects of light-induced PTEN and PI3K/Akt/mTOR signaling pathways in ARPE-19 cells after transfection PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog gene; ARPE: Human adult retinal pigment epithelial.

DISCUSSION

The RPE is the outermost layer of the retinal structure, located between the choroid and retina. It encompasses a regularly arranged single-cell layer and maintains retinal function[12]. Degenerative changes of RPE cells have been implicated in AMD occurrence and development[13]. AMD is an age-related chronic disease characterized by the degeneration of photoreceptors and RPE in the retinal macular area and blood vessel formation. Because AMD mainly affects the foveal macular area, it can cause irreversible vision loss[14]. miRNAs are non-coding, single-stranded, endogenous, 22-nucleotide-long RNAs, which can negatively regulate approximately 60% of human genes through the RNA interference mechanism after transcription[15]. The expression of hundreds of miRNAs has been found in eye tissues such as the cornea, where they maintain the normal function of eye tissues by regulating downstream target genes[16]. miRNA-21 belongs to the miRNA family and is located on chromosome 17q23.2. miRNAs act as oncogenes. Tumors show high miRNA-21 expression, which influences apoptosis, invasion, differentiation, and proliferation of most tumor cells through the regulation of related target genes[17-18]. Some studies have shown that when miRNA-21 is knocked out in H358 lung cancer cells, PTEN expression in the tumor cells is significantly increased[19]. In recent years, research on miRNAs regulating RPE proliferation and apoptosis has gradually increased. miRNA-145 inhibits RPE proliferation and induces apoptosis[20], whereas miRNA-7 can inhibits human RPE proliferation and migration[21]. In our experiments, ARPE-19 cells were illuminated for 24h in all groups excluding the control group. Compared with the control group, the injury group showed decreased cell survival rates and increased apoptosis rates. Compared with the PI3K/Akt blocker group, the overexpression group showed increased cell survival rates and decreased apoptosis rates. This shows that miRNA-21-5p can significantly improve the survival rate of ARPE-19 cells after light damage. Furthermore, miRNA-21-5p inhibits light-induced ARPE-19 cell apoptosis.

Related studies have confirmed that miRNA-21 mediates PTEN expression in various tumor cells[22-23]. The research on liver cancer cells found that miR-21 inhibition increases PTEN expression, thus lowering cell proliferation, migration, and invasion. However, elevated miR-21 expression decreases PTEN expression, increasing cell proliferation, migration, and invasion[24-25]. mTOR acts downstream of Akt and PI3K. When its expression is altered, cell metabolism and cancer cell growth are affected[26]. The PI3K/Akt/mTOR signal cascade is well known for promoting cell proliferation, inhibiting apoptosis, and controlling tumorigenesis, proliferation, invasion, and metastasis[27]. The PTEN protein is related to drug sensitivity owing to its inhibitory effect on the PI3K/Akt pathway; miR-22, miR-222, and miR-21 target genes are all PTEN[28-29]. For example, in a study on cardiomyocytes, miRNA-21 was found to inhibit the activity of PTEN, participate in the biological behavior of cardiomyocytes by regulating PI3K/Akt, and

reduce the expression of endogenous and exogenous apoptotic signaling pathways to inhibit cardiomyocyte apoptosis and death[30]. Gastric cancer cells have low miRNA-21, which can induce tumor cell apoptosis. miRNA-21 activates the PI3K/Akt signaling cascade by inhibiting PTEN, finally promoting the phosphorylation of P13K and Akt[31]. To affirm that miRNA-21-5p functions via the PI3K/Akt signaling cascade, we used a PI3K/Akt blocker group (LY294002). LY294002 controls the acyl inositol triphosphate kinase transduction pathway. It is widely used in characteristic research and its function is to inhibit the phosphorylation of PI3K and Akt. LY294002 blocks the PI3K cell signal transduction pathway. The injury group showed decreased miRNA-21-5p expression, increased PTEN mRNA expression, decreased phosphorylation levels of PI3K, Akt, and mTOR, and increased PTEN protein expression than the control group. The overexpression group showed increased miRNA-21-5p expression, no change in PTEN mRNA expression, increased phosphorylation levels of PI3K, Akt, and mTOR, and decreased PTEN protein expression than the injury group. The treatment group showed decreased miRNA-21-5p expression, no change in PTEN mRNA expression, decreased phosphorylation levels of PI3K, Akt, and mTOR, and increased PTEN protein expression. This indicates that the mechanism by which miRNA-21-5p inhibits light-induced ARPE-19 cell injury may involve downregulating PTEN expression and activating the PI3K/Akt/mTOR signaling cascade, hence protecting human RPE cells.

In summary, miRNA-21-5p inhibits *PTEN* expression, thereby activating the PI3K/Akt/mTOR cascade, and finally inhibiting ARPE-19 cell apoptosis and reducing the photodamage to human RPE cells. Research on miRNA-21 target genes is increasing; however, most studies focus on tumors. Few clinical reports have investigated miRNA-21-5p expression and influence on AMD. The present study, along with further research, will provide important information for improved differential diagnosis of AMD, selection of treatment options, and development of effective medications against this condition.

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