Am J Transl Res 2016;8(12):5723-5728
www.ajtr.org /ISSN:1943-8141/AJTR0036264

Original Article

Inhibitory effect of miR-145 on RPE cell proliferation

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Received December 22, 2015; Accepted November 27, 2016; Epub December 15, 2016; Published December 30, 2016

Abstract: Objective: This study aims to explore the impact of micro RNA miR-145 on retinal pigment epithelial cell proliferation and apoptosis. Methods: A stable culture and passage system of hPNE cells was first established, and its migration ability was determined. Then, miR-145 lentiviral vectors were constructed to transfect hPRE cells. Thereafter, hRPE cell proliferation was detected by MTT assay after they were transfected by lentivirus, cell cycle was analyzed by flow cytometry, and apoptosis was detected by Annexin V/PI double staining immunofluorescence. Results: Cultured hPRE cells had good migrating and metastatic ability, in which subsequent lentivirus infection experiments can be carried out. After transfection by miR-145 lentiviral vectors, hPRE cell proliferation slowed down and RPE cells in the G_1 phase was inhibited; thus, apoptosis rate increased. Conclusion: MiR-145 can slow down retinal pigment epithelial cell proliferation and increase their apoptosis rate. This has a certain therapeutic potential for diseases caused by RPE cell proliferation such as PVR.

Keywords: miR-145, RPE cells, lentiviruses, proliferation, apoptosis

Introduction

Retinal pigment epithelium (RPE) is a single layer of epithelial cells between retinal neurons and the choroid that has a supportive function to photoreceptors [1] and functions as biological barriers of the retina due to cellular polarity and close non-synaptic connection [1, 2]. It is essential for the inner balance of the retina such as the blood-retinal barrier, optical absorption, transportation of nutrients and immune privilege [3]. Thus, the injury and destruction of RPE cells would cause the degeneration of photoreceptors and vision loss, and ultimately lead to a variety of diseases such as proliferative vitreoretinopathy (PVR) and age-related macular degeneration [4]. Generally, PVR is one of the rare complications of retinal detachment, with an incidence of approximately 8-10%. Its occurrence is due to the vigorous proliferation of RPE cells, macrophages and fibroblasts [5]. Among them, RPE cells are considered as key, and the uncontrolled proliferation of RPE cells leads to their migration to the vitreous cavity, as well as to the junction between the vitreum and retina. This gradually forms an anterior epiretinal mold that retracts the retina, causing retinal detachment and visual impairment [6, 7]. Moreover, even before the development of PVR, migration- and proliferation-related phenotypes of RPE are difficult to return to a normal state [8]. Therefore, determining how to inhibit the occurrence of PVR in the early stage remains a problem.

MicroRNA (miRNA) was first discovered in Caenorhabditis elegans by Lee RC *et al.* in 1993. It is a group of small molecular non-coding RNAs of 18-25 nucleotides that influences biological processes by inhibiting protein translation and mRNA degradation, regulates more than 30% of genes, and participates in some key physiological processes such as cell growth, apoptosis and differentiation [9, 10]. Some small interfering mRNA such as siRNA acts on a single gene. Unlike these small mRNA, miRNA silences multiple genes, combines with the 3'-UTR end of mRNA, which eventually lead to mRNA degradation and protein translation termination [11, 12]. In recent years, microRNA145 (miR-145) has been commonly applied in studies on tumor cells such as cells of breast

cancer, colon cancer, lung cancer and so on. Studies have revealed that it has an inhibitory effect on a variety of tumors. However, its role in this field of ophthalmology remains unelucidated. By studying the impact of microRNA145 on RPE cell proliferation and apoptosis, we further explore its therapeutic significance in various diseases caused by RPE cell proliferation in this study.

Materials and methods

Material

DMEM/F12 medium (Gibco, USA); RPE cells (American Type Culture Collection, USA); Transwell kit (3422 Corning, USA); Trypan blue (Shanghai Genebase, China); 250 bp DNA ladder Marker (Shanghai Generay, China); Restriction endonuclease (New England Biolabs, USA); n-Fusion™ PCR Cloning Kit (Clontech); Taq polymerase (SinoBio); Plasmid Extraction Kit (Promega); MTT (Beijing Dingguo Biotechnology); DMSO (Shanghai Pharmaceutical Group); Annexin V/PI apoptosis kit (Multi-Sciences Lianke Biotechco).

Fluorescence microscope (Olympus, micropublisher 3.3RTV, Japan); Microplate reader (Biotek Elx800); 1 kp DNA ladder Marker (Fermentas); PCR machine (Applied Biosystems); positive clone sequencing (Megorbio biotech); Bacterial shaker (Hangzhou Hualida); Bacterial incubator (Shanghai Yiheng Scientific Instruments); Gilson pipette (Gilson Inc.); High-speed centrifuge (Hitachi, Japan); Flow cytometry (FACX Calibur, Becton-Dickinson, American).

Culture and passage of RPE cells

Recovered RPE cells were prepared into cell suspension, seeded in DMEM/F12 medium, placed in an incubator at 37°C with 5% CO_2, and the medium was replaced on the next day and culture continued. Thereafter, the medium was replaced every 3-4 days until cells fused and were digested by trypsin, then passaged.

Transfection and expression of miR-145 lentiviral vectors

After obtaining the sequence of miR-145, the target genes were amplified, and the plasmid was recombined, at last the recombined plasmid was identified by gene sequencing. The 293 T cells were transfect by lentivirus. After 48 hours, the supernatant was collected and the concentrated viral liquid was extracted by means of centrifugation and tested for titer. Cell suspension of RPE cells in the logarithmic phase was prepared and seeded in 6-well plates, and incubated until the fusion degree reached approximately 30%. According to cell MOI values, 3.0 ml of the virus was added in the experimental group (titer 8 E + 8 TU/ml), while 1.0 μl of the negative control virus was added in the negative control group (titer 3 E + 8 TU/ml). After 12 hours, cell state was observed. If the detected cytotoxic effect was not significant, culture was continued for 24 hours and the medium was replaced; while, if the cytotoxic effect was significant, the medium was immediately replaced. Five days after infection, the expression of the reporter gene in the lentivirus was observed via green fluorescent protein (GFP). Photos were taken when fluorescence rate was more than 80%.

Experimental groups were as follows. Blank control group: RPE cells, the cell group were not infected by any virus. Negative control group: RPE cells, the cell group were infected by negative control virus. miR-145 group: RPE cells, the cell group were infected by miR-145 virus. Cellular total RNAs of each cell group were extracted with Trizol RNA extraction reagent (Gibco, USA). The expression of intracellular mature miR-145 virus was detected via reverse transcription polymerase chain reaction (RT-PCR) after transfection, and compared with the control groups. Then, reliability of the transfection was evaluated. Relative quantification was conducted using the comparative Ct method: U6 was used as an internal reference, $\Delta\Delta Ct = Ct_{miR\text{-}145} - Ct_{U6}$, $\Delta\Delta Ct = \Delta Ct_{experimental\ group} - \Delta Ct_{control\ group}$. The fold of the gene expression of miR-145 in the experimental group can be calculated according to the formula: Fold = $2^{-\Delta\Delta Ct}$.

MTT detection of RPE cell proliferation after transfected with lentivirus

Five days after lentiviral transfection, cells were resuspended into cell suspension in each experimental group, counted and plated. A total of five 96-well plates were seeded, and tests were continuously conducted for five days. Four hours before culture termination, 20 μL of 5 mg/mL MTT was added and followed by 150 μL of DMSO. OD value was detected by a microplate reader at 490 nm.

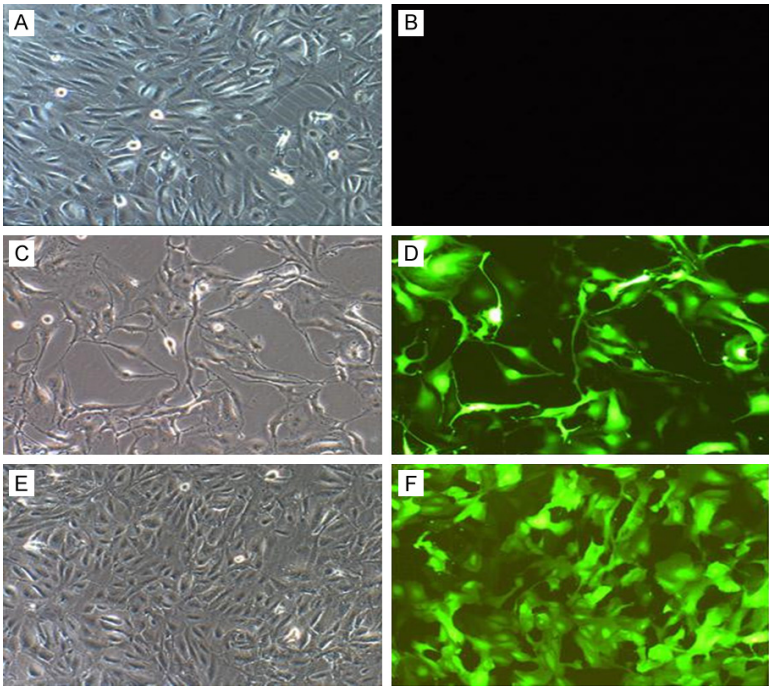

Figure 1. Microscopic view and fluorescence expression of RPE cells in each group (×100). A: Microscopic view of the control group; B: Fluorescence expression of the control group; C: Microscopic view of the negative control group; D: Fluorescence expression of the negative control group; E: Microscopic view of the transfected group by miR-145 lentivirus; F: Fluorescence expression of the transfected group by miR-145 lentivirus. Without lentivirus transfection control group showed non-colored cells. And negative and miR-145 groups with green fluorescent cells stood for successful transfection that the difference was negative group with blank vehicle transfection.

Detection of cell cycle and apoptosis of RPE cells after transfected with lentivirus

After 48 hours of transfection, cells in each group were obtained via centrifugation, resuspended in PBS, fixed by 70% cold ethanol at 4°C, stood for 30 minutes, and cells were suspended in PBS after centrifugation. RNase A was added until a final mass concentration of 50 mg/ml, and incubated at 37°C for one hour, Subsequently, 50 μl of propidium iodide (PI) solution was added, placed in the dark, stained at 4°C for 30 minutes, then the cell cycle was detected by flow cytometry. An Annexin V/PI apoptosis kit was used in the following steps. Cells were centrifuged 48 hours after transfection in each group. Five-hundred μl of cell suspension was taken from each group after being resuspended in PBS. Then, 5 μl of Annexin V-FITC and 10 μl of PI were added, placed in the dark, and incubated at room temperature for 15 minutes. The suspension was dropped onto a clean glass slide and observed under fluorescent microscope. Photos were taken and apoptosis was detected by flow cytometry.

Statistical methods

The data was analyzed using SPSS 11.5 statistical software. Each experiment was repeated three times, and data were averaged. All data were expressed as $\bar{x} \pm s$. Data are evaluated using paired samples *t*-test, $P<0.05$ wasconsideredstatisticallysignificant.

Results

The expression of RPE cells after transfected with miR-145 lentiviral vectors

The successfully constructed lentiviral vector miR-145 was transfected into hRPE cells. Cells infected with miR-145 presented obvious deformations, and their drawings became long, compared with cells in the negative control and control groups (**Figure 1**). Based on RT-PCR detection results on the content of miR-145, miR-145 expression in miR-145 transfected cells significantly increased; and the difference was statistically significant with the control group (0.53 ± 0.035; $t = 20.47$, $P<0.05$). Compared with the negative control group (1.01 ± 0.13), the difference in the miR-145 group (1,003.08 ± 84.80) was statistically significant ($t = 20.44$, $P<0.05$).

MTT detection of RPE cell proliferation after transfected with lentivirus

Compared with the control and negative control groups, miR-145 revealed a significant inhibitory effect on RPE cell proliferation. Compared to the other two groups, RPE cell proliferation in the miR-145 group was slow (**Figure 2**).

Cell cycle of RPE cells after transfected with lentivirus

The percentage or proportion of cells in each cell cycle phase in the miR-145 group changed, cells in the G_0/G_1 phase decreased, cells in the

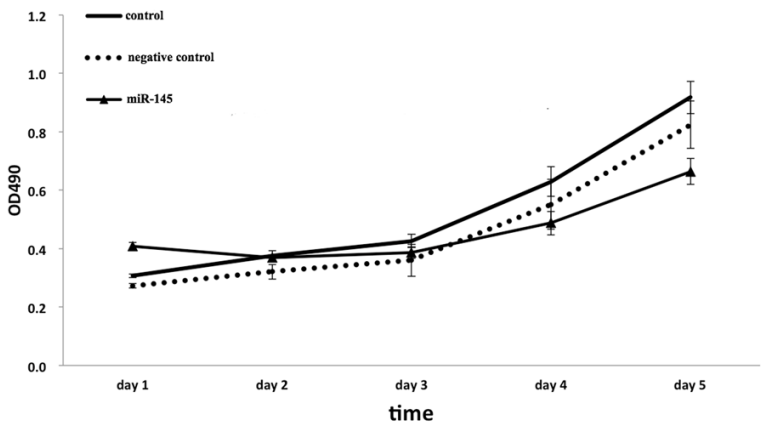

Figure 2. Proliferation curve of each group. The cellular proliferating ability was assessed by the cell growth curve. MiR-145 delayed RPE cell growth in miR-145 group in comparision with negative and control groups.

Table 1. Influences of miR-145 on RPE cell cycle

Group	Cell numbers in different cycles (%)		
	G_0/G_1	S	G_2/M
NC	92.19 ± 4.21	26.45 ± 2.24	3.62 ± 0.53
Micro up	74.20 ± 1.93*	32.35 ± 2.93*	6.70 ± 0.69*
CON	90.73 ± 4.56	27.38 ± 3.47	4.12 ± 0.57
F	5.43	7.46	2.17

*P < 0.05, significantly different from the control group and negative groups.

S phase increased, and cells in the G_2/M phase increased; revealing that miR-145 arrests RPE cells in the G_1 phase (P<0.01, **Table 1**).

Detection of RPE cell apoptosis after transfected with lentivirus

Annexin V-FITC/PI staining in normal living cells were low, and the cell membrane and nucleus were not colored. In the miR-145 group: Annexin V and PI staining were both high. Green fluorescence was presented in the cell membrane, while red fluorescence was presented in the nucleus, suggesting that apoptotic characteristics have appeared. Negative control and blank control group: Annexin V and PI staining were both low, suggesting that cells were active. These trends were consistent with the results of the flow cytometric analysis of apoptosis (**Figures 3** and **4**).

Discussion

Currently, researches on miR-145 have focused mostly on various types of tumor cells. It has been proven that miR-145 shows an inhibitory effect on the proliferation of cells of breast cancer, lung cancer, prostate cancer, meningiomas and other tumors [13]. In recent years, studies on the association between small RNA and RPE cells in ophthalmology gradually become hot topic. For example, Yoon C *et al.* considered that miR-155 is related with RPE cell angiogenesis [14]. Several studies have revealed that miR-125b, miR-24, miR-320, miR-23b, miR-204, miR-211, miR-204 and miR-302s were confirmed to be related with the growth and differentiation of RPE cells [4, 15, 16]. In addition, miR34a expression in the retina and RPE increases with age, and is consistent with the degree of mitochondrial DNA damage, suggesting that miR-34a is related with the apoptosis of the retinal and RPE cells [17], and that miR34a, miR-133a-3p, miR-17-5p, miR-21-5p and miR-221-3p are also related to RPE apoptosis [18]. It is worth mentioning that miR-29b shows an inhibition to TGF-β1-mediated epithelial-mesenchymal transition, while epithelial-mesenchymal transition is the main cause for the formation of PVR [19].

Nevertheless, few studies have been reported on miR-145 in the field of ophthalmology. In this study, we investigated the impact of miR-145 on RPE cell proliferation and apoptosis, in which when miR-145 expression in RPE cells continued, it was involved in gene regulation, blocked protein translation, prevented cell proliferation, and accelerated apoptosis. Thus, after its expression in RPE cells via lentiviral transfection, miR-145 changed the distribution in cell number in each cell cycle, reduced the number of cells in the G_1 phase, increased RPE cell apoptosis, rendered the cell number to become fewer compared with normal situations, and slowed cell proliferation.

After successfully transfecting RPE cells using lentivirus as vectors to carry genetic information of miR-145, in RPE cells overexpressing miR-145, the content of miR-145 was significantly higher than in normal cells, resulting in a

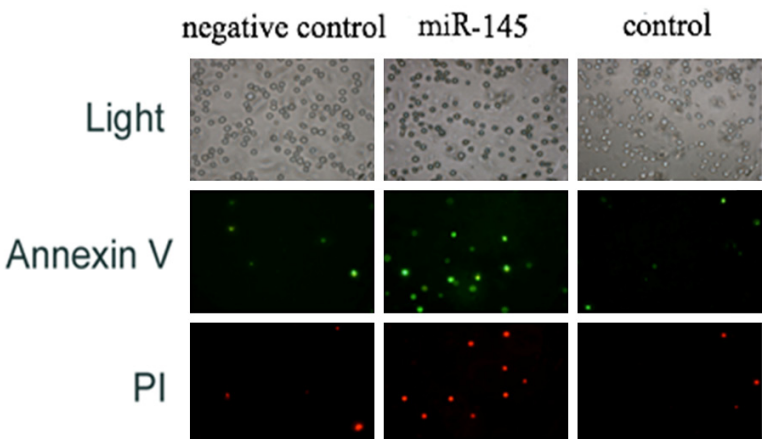

Figure 3. Observation of apoptosis under an inverted fluorescence microscope after Annexin V/PI double staining (×100). First flow showed the morphology of RPE cells in light field. Via Annexin V/PI double staining, it was obvious miR-145 group had many Annexin V (green fluorescence) and PI (red fluorescence) positive cells while the other two groups had little.

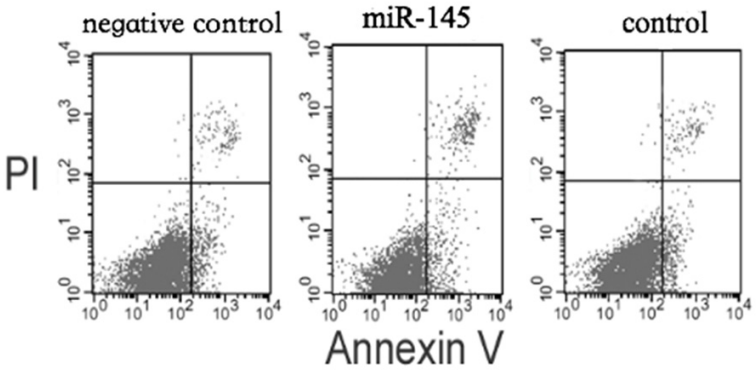

Figure 4. Detection of RPE cell apoptosis in each group via FCM. On the right hand side down of the frames displayed Annexin V positive cells also on behalf of apoptotic cells were more in miR-145 group than others.

much higher apoptosis rate than in normal cells. It is possible that in the normal physiological process of cells, miR-145 expression is at a constant low state, causing it to fail to play its role. However, in the process of progress in PVR, a variety of cells including RPE cells actively proliferate and form proliferative membranes, and retract the retina, causing proliferative membranes to lead to a sharp loss of vision and poor prognosis. Proliferative membranes are mainly composed of fibrous tissue. Collagen fibers can be seen under electron microscopy, and RPE cells have been proven to produce a variety of subtypes of collagen fibers *in vivo*; thus, it is considered that RPE produces a large amount of collagen fibers in proliferative membranes [20]. It is clear that the expression of miR-145 remains low in the whole process of the formation of proliferative membranes. If miR-145 expression in this case increases, cell proliferation decreases; and it is able to block the further deterioration of the disease. Therefore, the mechanism or substance at the right time to stimulate the miR-145 expression is to prevent the proliferation of diseased cells and accelerate its apoptosis; which may be a potential therapy for PVR and other diseases.

Disclosure of conflict of interest

None.

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