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MiR-7 regulates the PI3K/AKT/VEGF pathway of retinal capillary endothelial cell and retinal pericytes in diabetic rat model through IRS-1 and inhibits cell proliferation

Y.-L. CAO[1], D.-J. LIU[2], H.-G. ZHANG[3]

[1]Department of Ophthalmology, Weifang Medical University, Weifang, China
[2]Department of Ophthalmology, Yantai Yeda Hospital, Yantai, China
[3]Department of Ophthalmology, Liaocheng Traditional Chinese Medicine Hospital, Liaocheng, China

Yongliang Cao and Dejie Liu contributed equally to this work

Abstract. – OBJECTIVE: To investigate the role of miR-7 in diabetic retinopathy and the underlying mechanism.

MATERIALS AND METHODS: The rat model of diabetic retinopathy (DR) was established. After that, the endothelial cell (EC) and retinal pericyte (RP) were isolated. QRT-PCR was used to detect the expression of miR-7 and insulin receptor substrate-1 (IRS-1) in ECs and RPs cells while the protein level of IRS1 was detected by Western blot. miR-7 mimic and miR-7 inhibitor were transfected to achieve miR-7 overexpression or knockdown. Cell viability was detected by Cell Counting Kit-8 (CCK-8) assay after miR-7 overexpression or knockdown. Besides, the expression levels of PI3K, AKT, and VEGF were detected by Western Blot. The luciferase reporter assay was performed to investigate whether miR-7 could be combined with IRS-1. Conversely, whether miR-7 could affect IRS-1 was also verified.

RESULTS: miR-7 expression was significantly decreased in ECs and RPs of the experimental group compared with the control group, while the mRNA and protein levels of IRS-1 were increased. The CCK-8 assay showed that overexpression of miR-7 decreased the cell activity in ECs and RPs. In contrast, knock-down of miR-7 could increase the cell viability. Besides, Western blot showed that after overexpression of miR-7, the expressions of PI3K, AKT, and VEGF in ECs and RPs cells were down-regulated. Meanwhile, miR-7 knockdown upregulated the protein levels of PI3K, AKT, and VEGF. The luciferase reporter assay suggested that the 3'UTR region of IRS-1 could be combined with miR-7, which may be the downstream target gene for miR-7. Moreover, knockdown of IRS-1 could reverse the effect of the miR-7 inhibitor on cell proliferation in the diabetic model.

CONCLUSIONS: MiR-7 was lowly expressed in ECs and RPs cells. Overexpression of miR-7 can down-regulate the expression levels of PI3K, AKT, and VEGF by down-regulating its downstream target gene IRS-1, and ultimately inhibit the proliferation of retinal cells.

Key Words:
miR-7, IRS-1, PI3K/AKT, Diabetic retinopathy.

Introduction

Diabetic retinopathy (DR) is one of the most important eye diseases that endangers the visual acuity of the middle-aged and the elderly. It is characterized by the proliferation of retinal neovascularization[1]. The longer the course of diabetes mellitus progress goes, the higher the prevalence of diabetic retinopathy may occur[2]. The main pathophysiological features of diabetic retinopathy are the formation of retinal neovascularization and the destruction of the blood retinal barrier[3]. Vascular endothelial growth factor (VEGF) is an important factor for neovascularization in patients with diabetic retinopathy retinal formation, which plays a key role in all aspects of angiogenesis, including the proliferation and migration of endothelial cells, changes of endothelial cell gene activation and the increase of plasminogen activating factor[4]. However, the regulation mechanism of diabetic retinopathy is not clear at present. A large number of patients could not be cured by existing therapies and the lesions continue to progress. Therefore, we need to explore the pathogenesis of diabetic retinopathy in order to find more effective and feasible treatments.

microRNA (miRNA) is a highly conserved, non-protein encoded endogenous small RNA[5],

Corresponding Author: Hongguo Zhang, MM; e-mail: fangm5121165@163.com

which consists of about 22 bases. The transcription and synthesis of miRNA are a complex process which takes place in the nucleus and cytoplasm with the functions of many enzymes. It can inhibit target gene transcription by selectively binding to target gene mRNA, and then plays a biological effect[6,7]. MiRNA is involved in many kinds of life activities, including cell proliferation, differentiation, apoptosis, and other pathophysiological processes, and is closely related to the occurrence and development of fundus tumors[8].

A research[8] shows that miR-7 is located on the seventh chromosome, which is the most highly expressed miRNA in pancreatic endocrine department. Studies have confirmed that miR-7 could inhibit the microvascular endothelial cells (ECs) in 11 glioblastoma[9]. In addition, the expression level of miR-7 has a long-term close relationship with the islet hormone increase index[10]. The silence of miR-7 in early mouse embryos can reduce insulin secretion and lead to islet cell development disorder or even apoptosis[11]. However, the role of miR-7 in diabetic retinopathy remains unknown. Therefore, this study would explore the role of miR-7 in diabetic retinopathy and investigate the underlying mechanism.

Materials and Methods

Establishment of Animal Model

Rats were fed with high-glucose and high-fat diet for 8 weeks. After that, the rats were fasted for 10 hours and intraperitoneally injected with 2% streptozotocin (STZ) solution (50 mg/kg). After 1 week, the fasting blood glucose was measured by caudal vein blooding. Rats with fasting glucose >16.7 mmol/L for at least 5 days were considered successfully modeled. These rats continued on high-glucose and high-fat diet for 12 weeks. This study was approved by the Animal Ethics Committee of Weifang Medical University Animal Center.

Extraction and Culture of ECs Cells

Rats were anaesthetized with 2% pentobarbital sodium and disinfected with 75% ethanol. The rat head was routinely sterilized on the operating board of the ultra-clean table; then, the eyeballs were removed with a 2 mm optic nerve left. The structures of extraocular fascia should be removed and placed in a dish containing ice D-Hank liquid gauze. The anterior segment, lens and vitreous body should be cut off while the intact retinal tissue was blunt separated, and the retinal branches and pigmented tissues were removed. After that the remaining tissues were fully cut into pieces and filtered by 200 mesh screen. 3 mL trypsin containing Ethylene Diamine Tetraacetic Acid (EDTA) was added and the lysate was centrifuged after water bath. The trypsin was abandoned and 5 mL 0.5% of type II collagenase was added. After the water bath, the cells were filtered with 300 mesh screens. The supernatant was then discarded and 8 mL Dulbecco's Modified Eagle Medium (DMEM) culture medium (containing 10% fetal bovine serum, 50 μg/mL heparin, 1% ECGs, 1% green chain double antibody) was added to the cells. The cells were inoculated in 0.5% gelatin-coated culture flasks in the 21% O_2, 5% CO_2, 37°C incubator for 24 h. After that cells were cultured as adherent cell lines

Extraction and Culture of Retinal Pericytes (RsP) Cells

Extraocular muscle, eyeball fascia, and optic nerve were removed under microscope after removal of the eyes of rat models. The residual tissue was disinfected repeatedly under sterile conditions. The anterior segment of eye was removed and the whole retina was peeled and washed repeatedly. Visible blood vessels were removed, and then the retina was shredded into a paste. The tissue was incubated for 45 min in phosphate buffered saline (PBS) added with 0.05% IA collagenase and 0.025% bovine serum albumin at 37°C for fully digestion, then lightly mixed and washed repeatedly with 53 μm stainless steel filter screen. The eluent was centrifuged, and the supernatant was abandoned. Low glucose DMEM medium containing 20% fetal bovine serum (FBS) cell culture medium was added and the cells were inoculated in the culture dish. The fresh medium was replaced after 48 h culture in the incubator of 37°C, 21% O_2 and 5% CO_2, and the routine culture was the same as ordinary cells.

Quantitative Real-Time Polymerase Chain Reaction

RNA extraction was in strict accordance with the operation procedures of TRIzol instructions to extract total RNA. RNA concentration was measured by UV spectrophotometry. The cDNA was synthesized according to the corresponding reverse transcription kit. The qRT-PCR reaction

system was prepared according to the instructions. 3 replicates were set in each sample. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as the internal controls. The Ct value of each well was recorded and the average value was used for analysis. The results were analyzed by $2^{-\Delta\Delta Ct}$ method. The primer sequences are as follows: IRS-1 F: CTTCTCAGACGTGCGCAAGG; R: GTTGATGTTGAAACAGCTCTC. U6 F: CGCTTCGGCAGCACATATAC; R: CAGGGGCCATGCTAATCTT. GAPDH F: TGCACCACCAACTGCTTAGC; R: GGCATGGACTGTGGTCATGAG.

Cell Transfection

The ECs and RPs cells were seeded in 6 well plates 1 days before the transfection. When the density reached 30%-40%, cells were transferred with miR-7 mimic, mimic control, miR-7 inhibitor, inhibitor control, si-IRS-1, or miR-7 inhibitor + si-IRS-1 according to the instructions of lipofectamine™2000. After 6h, the medium was replaced. Cells were further cultured for 48 h at 37°C. Then the cells were collected for subsequent experiments.

Western Blotting Analysis

Cells were collected on the ice by the lysis buffer. After centrifugation, the supernatant was collected, and the protein concentration was determined by bicinchoninic acid (BCA) colorimetry. 80 μg total protein sample was separated for electrophoresis with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride (PVDF) membrane. The immunoblots were blocked with 5% skim milk, and then incubated with primary antibodies for overnight at 4°C. Then, the membrane was washed with Tris-buffered saline and Tween 20 (TBS-T) for 3 times and incubated with the secondary antibodies. Then, the membrane was washed 3 times with TBS-T again. Enhanced chemiluminescence (ECL) was used for the detection of protein bands.

Cell Counting Kit-8 (CCK-8) Assay

48 h after transfection, the cells of each group were inoculated into 96 well plate at a density of $5 * 10^3$/100 μL per well. 3 replicates were set up in each group. 10 μL CCK-8 solution was added to each well at different time points (6, 24, 48, 72, 96 h). Then cells were further incubated for 4 h in 5% CO_2 37°C incubator. The absorbance at the wavelength of 450 nm was measured by a microplate reader.

Luciferase Reporter Assay

MiR-7 and IRS-1 recombinant vectors were co-transfected into ECs and RPs cells. The cells were divided into the following groups: miR-7 control+IRS-1-WT, miR-7+IRS-1-WT, miR-7 control+IRS-1-MUT, miR-7+IRS-1-MUT. The dual luciferase assay was used to detect the luciferase activity of transfected cells.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS16.0, SPSS Inc., Chicago, IL, USA) software was used to analyze the data. The measurement data were expressed as mean ± standard deviation ($\overline{x}\pm s$), and the differences between two groups were compared through group *t*-test. $p<0.05$ was considered statistically significant.

Results

The Expression of miR-7 in ECs and RPs Cells Was Low, While IRS-1 Was Highly Expressed

Compared with the normal control group, the expression level of miR-7 in ECs and RPs cells of diabetic rats was significantly decreased (Figure 1A), suggesting that the abnormal expression of miR-7 may be involved in the occurrence and development of diabetic retinopathy. Previous studies have shown that IRS-1 is involved in the development of diabetic retinopathy[23]. The results of this study showed that the expression of IRS-1 in ECs and RPs cells of diabetic rats was significantly up-regulated at mRNA and protein levels (Figure 1B, 1C).

The Relationship Between the Expression Level of miR-7 and the Activity of ECs and RPs Cells

miR-7 mimic and inhibitor were transfected respectively to achieve miR-7 overexpression or knockdown in ECs and RPs cells of diabetic rat model. qRT-PCR results showed that miR-7 mimic could overexpress miR-7 (Figure 2A), while miR-7 inhibitor could effectively reduce the expression level of miR-7 (Figure 2B). Then, we detected the change of cell viability in the above two states. The results of CCK-8 assay showed that cell viability decreased significantly after overexpression of miR-7, while

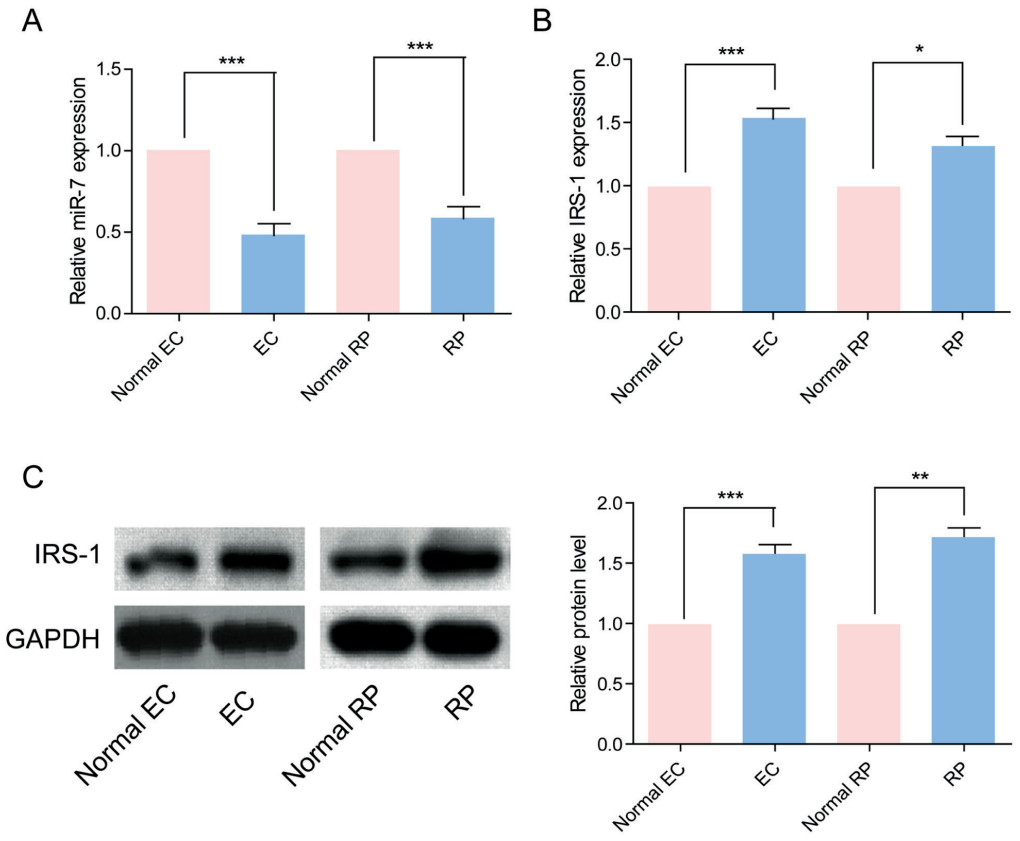

Figure 1. The level of expression of miR-7 and IRS-1 in ECs and RPs. ***A,*** Compared with the normal control group, the expression level of miR-7 in ECs and RPs was significantly lower. ***B,*** Compared with the normal control group, the gene expression level of IRS-1 in ECs and RPs was significantly higher. ***C,*** The protein expression level of IRS-1 in ECs and RPs was significantly higher than that in the normal control group.

miR-7 activity was significantly increased after knocking down miR-7 (Figure 2C, 2D). These above results had preliminarily found that high expression of miR-7 may be involved in diabetic retinopathy by inhibiting the proliferation of EC and RP cells.

MiR-7 Can Regulate the PI3K/AKT Pathway

The expression of PI3K/AKT pathway and the expression of VEGF protein were detected by Western blot test after overexpressing or silencing miR-7 in ECs and RPs cells of diabetic rats. We showed that after overexpression of miR-7, the protein expression levels of PI3K, AKT, and VEGF in ECs and RPs cells were all decreased (Figure 3A). In contrast, the protein expression level of PI3K, AKT, and VEGF were all up-regulated after miR-7 knockingdown (Figure 3B). This suggested that miR-7 may play a role in the regulation of the PI3K/AKT pathway.

IRS-1 is the Target Gene of miR-7, and Knockingdown IRS-1 Can Weaken the Role of miR-7 in ECs and RPs Cells of Diabetic Model Rats

The results of the luciferase reporter gene experiment showed that miR-7 could be combined with IRS-1 3'UTR (Figure 4A). To further investigate the relationship between miR-7 and IRS-1, cells were divided into the following groups: si-IRS-1 group, miR-7 inhibitor group, miR-7 inhibitor+si-IRS-1 group, and inhibitor control group, respectively. So, we detected the change of cell viability in each group. The results showed that the cell viability of miR-7 inhibitor group was significantly higher than that of the control group, and the activity of si-IRS-1 group was the weakest. The cell viability of miR-7 inhibitor and si-IRS-1 co-transfected group was higher than that of group si-IRS-1. However, the cell viability was significantly reduced compared

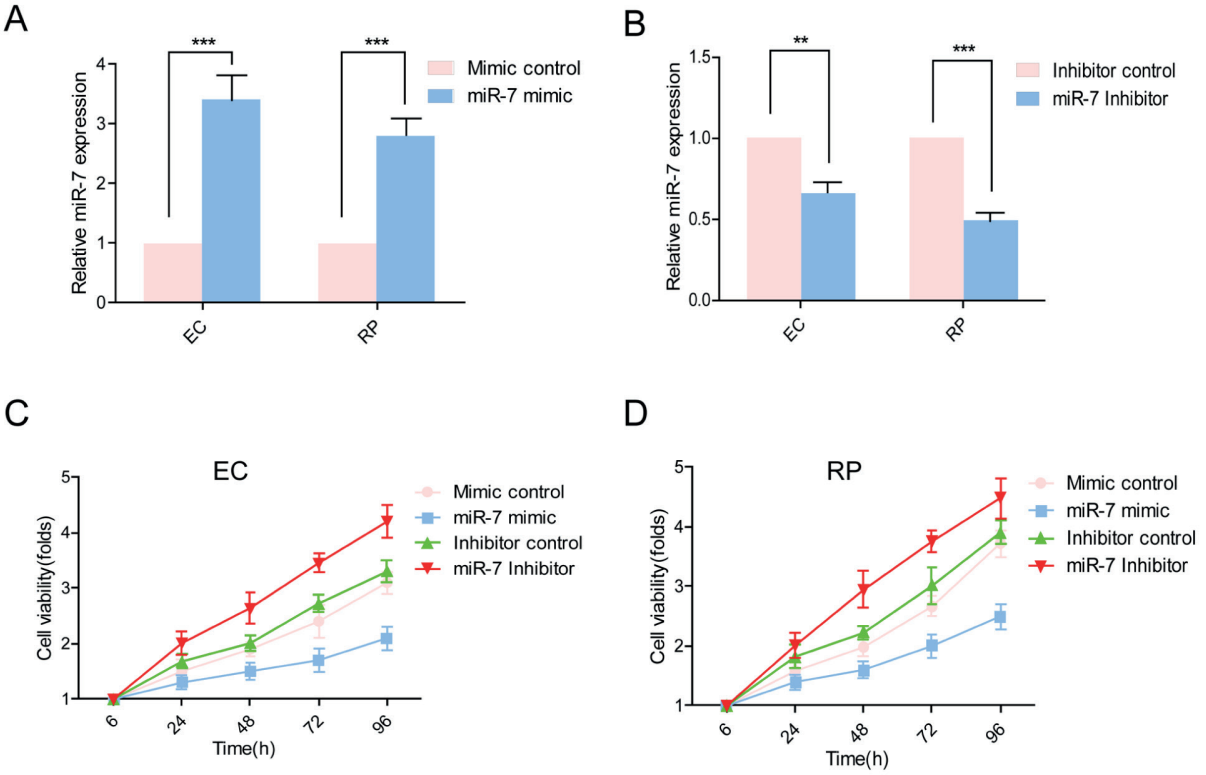

Figure 2. The relationship between the expression level of miR-7 and the activity of ECs and RPs cells. ***A,*** After overexpression of miR-7, the expression level of miR-7 was significantly increased. ***B,*** When miR-7 was knocked down, the expression level of miR-7 decreased significantly. In ECs (***C***) and RPs (***D***), CCK8 was performed to detect cell viability. After overexpression of miR-7, cell viability decreased significantly. While after knockdown of miR-7, cell viability was significantly increased.

with the miR-7 inhibitor group (Figure 4B), which was consistent in the ECs of diabetic model rats (Figure 4C). These results demonstrated that knockdown of IRS-1 could weaken the role of miR-7 in ECs and RPs cells in diabetic rats.

Discussion

Diabetic retinopathy is a disease with complex pathogenesis. Many factors and growth factors are involved in the development of this disease. Chronic hyperglycemia is the main pathogenesis of diabetic retinopathy[12]. Insulin mediates the utilization of glucose through the insulin signal system. The combination of insulin and insulin receptor (INSR) phosphorylates IRS-1 and initiates the insulin signaling system[13]. The phosphorylated IRS-1 activates the PI3K pathway by binding to the SH2 of the PI3K[14,15].

One of the important mechanisms of diabetic retinopathy is overexpression of VEGF[16-18]. VEGF can stimulate the proliferation of vascular endothelial cells by activating some signal pathways and promote the formation of in the neovascularization[19-21]. PI3K/AKT signal transduction system is involved in glucose transport, the synthesis and decomposition of glycogen and protein, endothelial cell proliferation and angiogenesis, cell cycle regulation, and apoptosis process[22]. It has been confirmed that the PI3K/AKT pathway regulates the role of VEGF, and the PI3K/AKT pathway is regulated by IRS-1[23,24].

Studies[25-27] showed that many miRNAs are expressed specifically in the retina. Many miRNAs participated in the development of diabetic retinopathy. MiR-146a/b, miR-155, miR-132, and miR-21 were up-regulated in the retina of diabetic retinopathy rats, and miR-132 was confirmed to participate in angiogenesis[28,29]. MiR-126 has a close relationship with type 2 diabe-

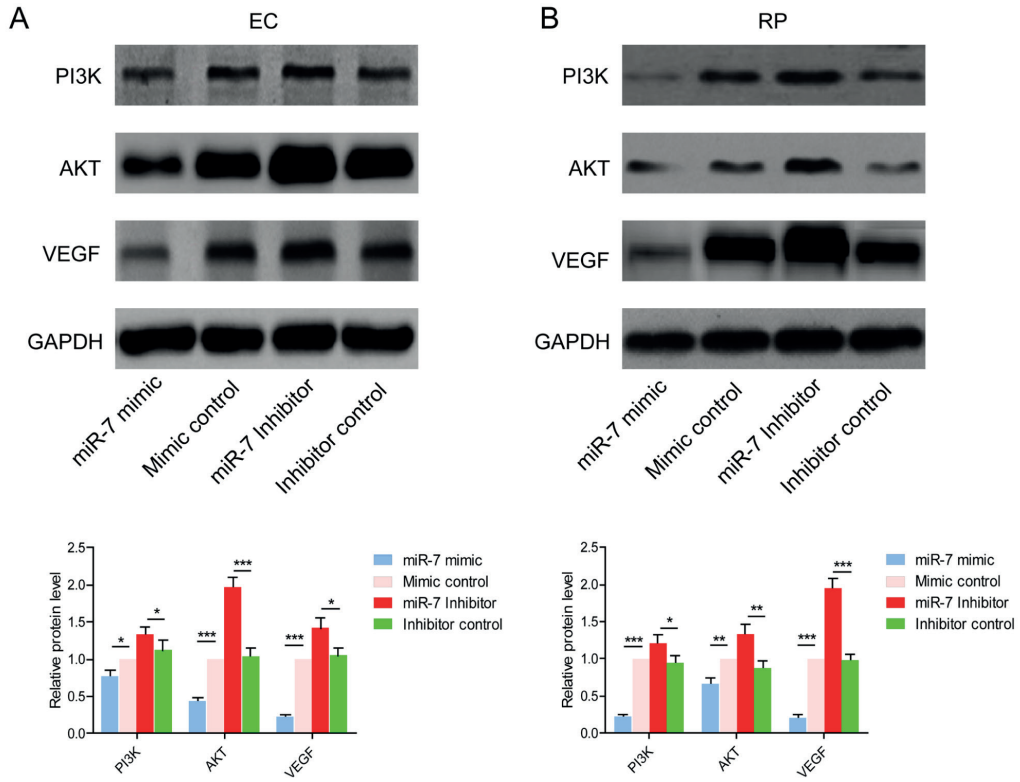

Figure 3. MiR-7 regulates the PI3K/AKT pathway by targeting IRS-1. Over expression and knockdown of miR-7 respectively, expression level of PI3K, AKT, and VEGF in ECs (***A***) and RPs (***B***) changed.

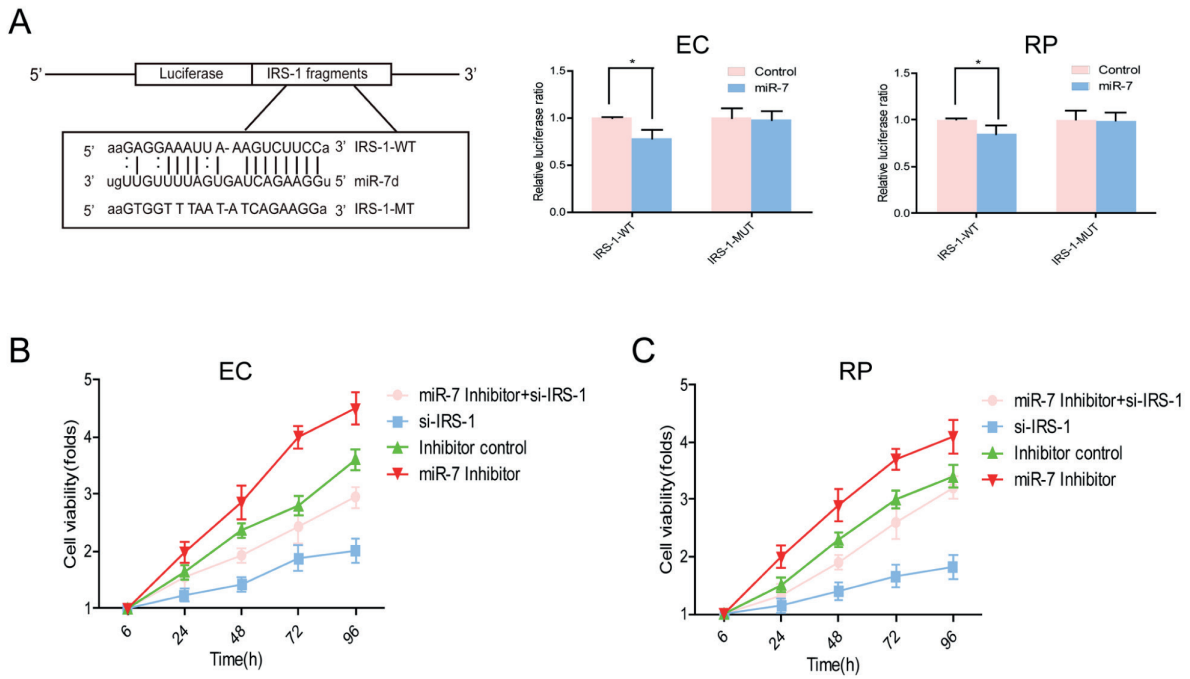

Figure 4. Knocking down IRS-1 can weaken the role of miR-7 in RPs and ECs. ***A,*** The binding site of miR-7 with IRS-1 was detected by luciferase reporter gene assay. ***B,*** After over expression of miR-7, cell activity in ECs (***B***) and RPs (***C***) decreased, while knockdown of IRS-1 can reduce the up regulation of cell viability induced by miR-7.

tes[30], which is involved in diabetic retinopathy[23]. Recent evidence have shown that many miRNAs have the functions of anti-angiogenesis, including miR-31, miR-200b, miR-150, miR-221/222, miR-184 and miR-20a[31,32]. MiR-17-92, miR-92a, miR-23-27, and miR-17-5p can promote angiogenesis[33].

In this investigation, we first constructed a diabetic retinopathy rat model. Retinal endothelial cells (ECs) and pericytes (RPs) were used as experimental cells. The expression of IRS-1 in diabetic cells was increased. Luciferase reporter assay confirmed that miR-7 could bind to IRS-1 3'UTR specifically, suggesting that miR-7 may be involved in the development of diabetic retinopathy through IRS-1. After overexpressing or knocking down of miR-7, CCK-8 assay was used to detect ECs and RPs cell viability in diabetic retinopathy rats. In addition, it was found that cell viability was weakened when miR-7 was overexpressed. Further experiments indicated that after overexpression of miR-7, the expression of PI3K, AKT, and VEGF decreased, which was consistent with the CCK-8 results. Torsion tests showed that knockdown of IRS-1 weakened the level of miR-7 ECs and RPs cells proliferation. In combination with the results of luciferase experiment, we hypothesized that miR-7 played a role in diabetic retinopathy by regulating the expression of IRS-1. Further experiments preliminarily discussed the mechanism. When miR-7 was overexpressed, the expression of PI3K, AKT, and VEGF decreased. The torsion test suggested that after knocking down the level of IRS-1, low level of miR-7 could reduce the proliferation of ECs and RPs cells. In sum, we concluded that miR-7 could play a role in diabetic retinopathy by regulating the expression of IRS-1.

Conclusions

We demonstrated that MiR-7 was lowly expressed in ECs and RPs cells. Overexpression of miR-7 can down-regulate the expression levels of PI3K, AKT, and VEGF by downregulating its downstream target gene IRS-1, and ultimately inhibit the proliferation of retinal cells.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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