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MicroRNA-15b participates in diabetic retinopathy in rats through regulating IRS-1 via Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: To explore the role of microRNA-15b in diabetic retinopathy (DR) and its underlying mechanism.

MATERIALS AND METHODS: Diabetic retinopathy rat model was first constructed. Retinal endothelial cells (EC) and retinal pericytes (RP) in DR rats were extracted. The mRNA expression of microRNA-15b in EC and RP cells was detected by qRT-PCR (quantitative Real Time-Polymerase Chain Reaction). Protein expression of insulin receptor substrate 1 (IRS-1) in EC and RP cells was detected by Western blot. After altering microRNA-15b expression by plasmid transfection, cell viability was detected by CCK-8 (cell counting kit-8) assay. Furthermore, the target gene of microRNA-15b was predicted by TargetScan analysis and the binding condition was verified by luciferase reporter gene assay. Finally, rescue experiments were carried out to explore the regulatory effect of microRNA-15b on IRS-1.

RESULTS: MicroRNA-15b was lowly expressed, whereas IRS-1 was highly expressed in EC and RP cells. After overexpression of microRNA-15b, viabilities of EC and RP cells were decreased and β-catenin expression was inhibited. TargetScan predicted that IRS-1 was the downstream gene of microRNA-15b, which was further verified by luciferase reporter gene assay. Rescue experiments indicated that microRNA-15b was capable of regulating IRS-1 via Wnt/β-catenin signaling pathway.

CONCLUSIONS: MicroRNA-15b participates in the development of diabetic retinopathy by targeting IRS-1 via Wnt/β-catenin signaling pathway.

Key Words:
Diabetic retinopathy, MicroRNA-15b, IRS-1, Wnt/β-catenin.

Introduction

With the rapid development of society and significant changes in lifestyle, diabetes mellitus has become one of the most common chronic diseases[1]. Diabetic retinopathy (DR) is a severe complication of diabetes, which is frequently seen in middle-aged and elderly diabetic patients[2,3]. About one-third of these patients present symptoms of DR[4]. Since the prevalence of DR has been astonishingly increased in recent years, it brings a heavy burden on DR patients and the whole society.

However, the pathogenesis of DR is not fully elucidated. Studies have shown that blood glucose is regulated by four classical pathways, including activation of the polyol pathway, advanced glycation end products, protein kinase C pathway, and hexosamine pathway. The above pathways interact with each other. Unfortunately, efficacies of treatments based on these pathways are far from satisfactory. In recent years, microRNAs have been well recognized. MicroRNAs are a class of non-coding, single-stranded RNA molecules with approximately 22 nucleotides in length. They exert the regulatory role by binding the 3' UTR of target RNAs to inhibit the gene transcription[5].

Abnormalities in insulin content and cellular metabolism are the main pathophysiological changes in diabetes, thereafter resulting in ophthalmic microcirculation alteration. Insulin exerts its physiological role mainly through binding to an insulin receptor (IR) located on the cell membrane. IR is widely expressed in the entire retina[6]. Insulin receptor substrate 1 (IRS-1) is a signaling adapter protein, which plays a key role in transmitting signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors. IRS-1 is overexpressed in diabetic patients[6,7], which is closely related to insulin signaling and insulin metabolism[8,9]. In the present work, IRS-1 was predicted as the target gene for microRNA-15b by TargetScan analysis. We aim to investigate the role of microRNA-15b in DR and its underlying mechanism.

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Materials and Methods

DR Rat Model

Sprague-Dawley rats were obtained from Experimental Animal Center, Jiamusi University. This study was approved by the Medical Ethics Committee. For the construction of DR rat model, rats were fed with high-sugar and high-fat diet for 8 weeks. 2% streptozotocin (STZ) was injected 10 h before diet. Blood samples from tail vein after STZ injection for 1 week were harvested for blood glucose detection. Blood glucose higher than 16.7 mmol/L for at least 5 days was considered as the successful construction of DR rat model. High-sugar and high-fat diet was continuously given to rats for another 4 weeks. This study was approved by the Animal Ethics Committee of Jiamusi University Animal Center.

EC Cell Extraction and Culture

Diabetic rats were anesthetized by intraperitoneal injection of pentobarbital sodium. Pupils were removed with preservation of 2 mm optic nerve. The retinal tissue was then isolated, and macroscopic branches of the retinal vessels and pigmented tissues were resected. The remaining retinal tissues were then fully smashed, digested and centrifuged. Type II collagenase was added and filtrated using a screen mesh. EC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA), and incubated in a 5% CO_2 incubator at 37°C. Culture medium was replaced 24 h later.

RP Cell Extraction and Culture

The extraocular muscles, eye fascia, and the optic nerve were cut off under the microscope. The anterior ocular segment and the vitreous body were then cut off under an aseptic condition, and the intact retina was peeled off. Macroscopic branches of the retinal vessels and pigmented tissues were resected. Retina tissues were fully smashed, digested and centrifuged. Type IA collagenase and PBS (phosphate buffer saline) containing 10% FBS (fetal bovine serum) were added and cell supernatant was filtrated using a 53 μm-screen mesh. RP cells were cultured in DMEM (Gibco, Grand Island, NY, USA) containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA), and incubated in an incubator with 5% CO_2 and 21% O_2 at 37°C. Culture medium was replaced 48 h later.

Cell Transfection

Cells in good growth condition were selected and seeded in the 6-well plates. Cell transfection was performed when the cell confluence was up to 60%-70% according to the instructions of Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids (microRNA-15b mimic, microRNA-15b inhibitor, IRS-1 overexpression plasmid, si-IRS-1) were purchased from GenePharma (Shanghai, China).

Dual-Luciferase Reporter Assay

IRS-1 was predicted as the target gene of microRNA-15b by TargetScan. Corresponding reporter plasmids of IRS-1 were constructed by GenePharma (Shanghai, China). Mutant-type (IRS-1 wt) or wild-type of IRS-1 (IRS-1 mut) and microRNA-15b mimic or microRNA-15b inhibitor were co-transfected into EC and RP cells, respectively. After transfection for 48 h, relative luciferase activity was detected based on the recommendations of Dual-Glo®Luciferase Assay System (Promega, Madison, WI, USA).

RNA Extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

The mRNAs of cells were extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed to complementary DNAs (cDNAs). The reaction conditions were as follows: denaturation at 95°C for 30 s, followed by annealing at 95°C for 5 s, and extension at 60°C for 31 s, for a total of 40 cycles. Each sample was repeatedly performed for 3 times. Primers used in this study were as follows: MicroRNA-15b, F: ATCCAGTGCGTGTCGTG, R: TGCTTAGCAGCACATCATG; U6, F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; IRS-1, F: CAGGCAGAATGAAAGACCTAAAT, R: CAAAGTAAACAAACTGTAAGGGATG; GAPDH, F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

CCK-8 (Cell Counting Kit-8) Assay

Transfected cells were seeded into 96-well plates with 2×10^3 per well. 10 μL of the CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blot

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) so-

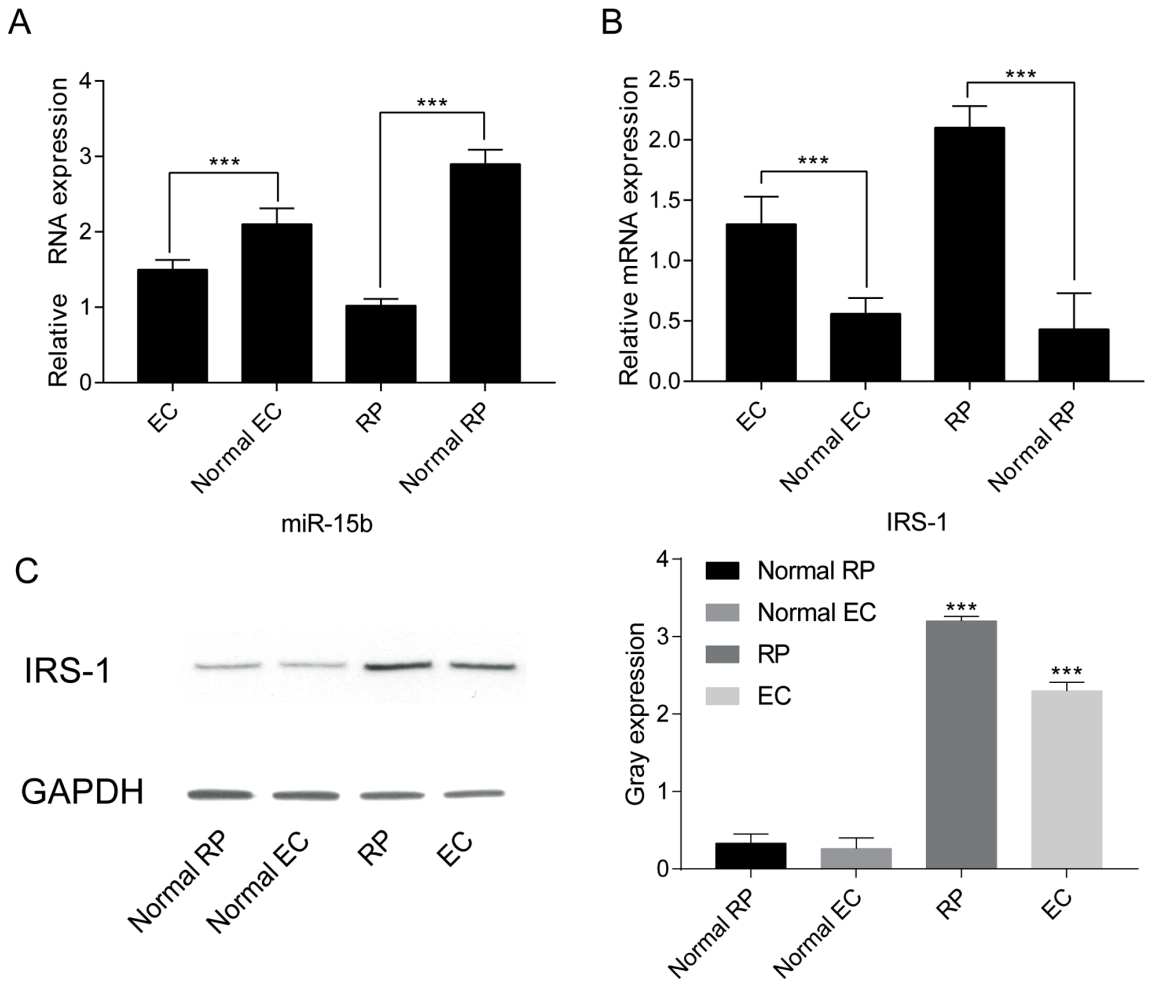

Figure 1. Expressions of microRNA-15b and IRS-1 in EC and RP cells. *A,* MicroRNA-15b was downregulated in EC and RP cells of diabetic rats than that of normal controls. *B,* IRS-1 was upregulated in EC and RP cells of diabetic rats than that of normal controls. *C,* Protein expression of IRS-1 was increased in EC and RP cells of diabetic rats than that of normal controls.

lution (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene difluoride) membrane (Roche, Basel, Switzerland). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBS-T (Tris-buffered Saline with Tween 20) (Beyotime, Shanghai, China) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by chemiluminescence.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables were shown as mean ± standard deviation. The *t*-test was used to compare the data between the two groups. $p<0.05$ indicated that the difference was statistically significant.

Results

Expressions of microRNA-15b and IRS-1 in EC and RP Cells

After the construction of DR rat model, we extracted EC and RP cells to detect expressions of microRNA-15b and IRS-1. Our results suggested that microRNA-15b was downregulated in EC and RP cells (Figure 1A), whereas both mRNA (Figure 1B) and protein (Figure 1C) levels of IRS-1 were upregulated.

MicroRNA-15b Inhibited Proliferation of EC and RP Cells

MicroRNA-15b mimic or microRNA-15b inhibitor was transfected into EC and RP cells, respectively. The transfection efficacy was verified by qRT-PCR (Figure 2A). Decreased mRNA level of IRS-1 was found after microRNA-15b overexpression, suggesting that IRS-1 might be negatively regulated by microRNA-15b (Figure 2B). The CCK-8 assay was performed and decreased viabilities of EC and RP cells were found after microRNA-15b overexpression (Figure 2C and 2D). It is reported that β-catenin is involved in DR[10]. Therefore, Western blot was utilized to detect protein level of β-catenin after transfection of corresponding microRNA-15b plasmids. The data indicated that overexpression of microRNA-15b led to a reduction of β-catenin expression (Figure 2E).

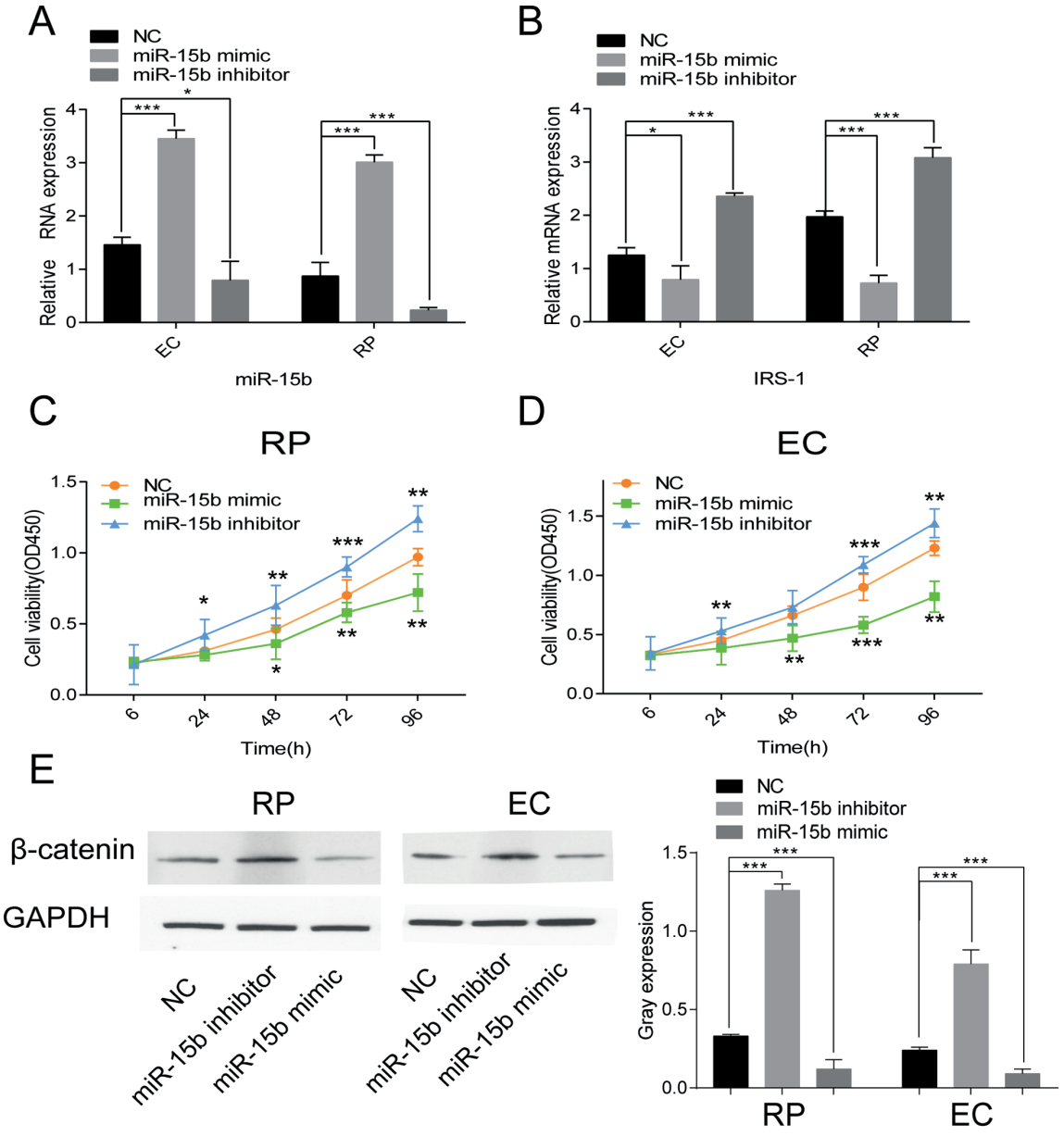

Figure 2. MicroRNA-15b inhibited proliferation of EC and RP cells. *A,* Transfection efficacies of microRNA-15b mimic and microRNA-15b inhibitor. *B,* The mRNA level of IRS-1 after overexpression or inhibition of microRNA-15b. *C, D,* Cell viability in EC and RP cells after microRNA-15b overexpression. *E,* Protein expression of β-catenin was negatively correlated with microRNA-15b expression.

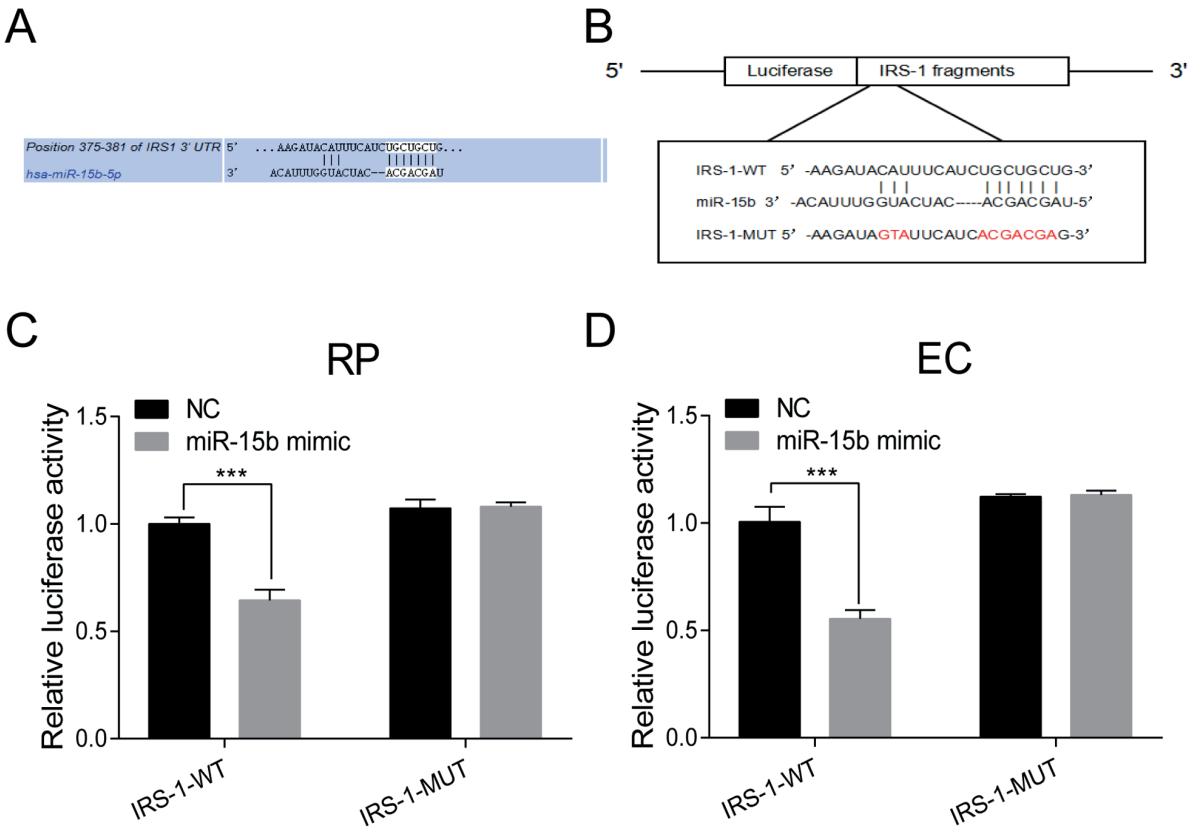

Figure 3. MicroRNA-15b targeted to IRS-1. *A,* Binding locations of microRNA-15b and IRS-1 were predicted by TargetScan. *B,* Construction of IRS-1 wt and IRS-1 mut. *C, D,* Luciferase activities of EC and RP cells co-transfected with IRS-1 wt and microRNA-15b mimic were decreased.

MicroRNA-15b Targeted to IRS-1

Here we used TargetScan to predict the downstream genes of microRNA-15b, and IRS-1 was screened out to be complementarily paired (Figure 3A). Corresponding reporter plasmids of IRS-1 were then constructed. Mutant-type (IRS-1 wt) or wild-type of IRS-1 (IRS-1 mut) and microRNA-15b mimic or microRNA-15b inhibitor were co-transfected into EC and RP cells, respectively. After transfection for 48 h, relative luciferase activity was detected. We found the lowest luciferase activity in cells co-transfected with IRS-1 wt and microRNA-15b mimic. Meanwhile, microRNA-15b had no effect on regulating IRS-1 mut, indicating that IRS-1 is the downstream factor of microRNA-15b (Figure 3C and 3D).

IRS-1 Reversed the Effect of microRNA-15b on EC and RP Cells

To further verify whether IRS-1 was regulated by microRNA-15b, we designed rescue experiments using EC and RP cells. Our data indicated that decreased viabilities of EC and RP cells after microRNA-15b overexpression were reversed by overexpression of IRS-1 (Figure 4A and 4B). On the contrary, elevated viabilities induced by microRNA-15b inhibition were also reversed by knockdown of IRS-1 (Figure 4C and 4D). The above results demonstrated that microRNA-15b exerts its specific role in DR through regulating IRS-1.

Discussion

DR is a serious complication of diabetes mellitus[11]. Destruction of blood-retinal barrier directly leads to increased vascular permeability, macular edema, thus eventually leading to decreased vision and even blindness[12,13]. Retinal endothelial cells are connected by adhesion in the retinal capillary network. VE-cadherin is a transmembrane protein that mediates calcium-dependent homogenous adhesion of endothelial cells. VE-cadherin is linked to actin through α-catenin and β-catenin, so as to maintain cell-cell stability and cell gap

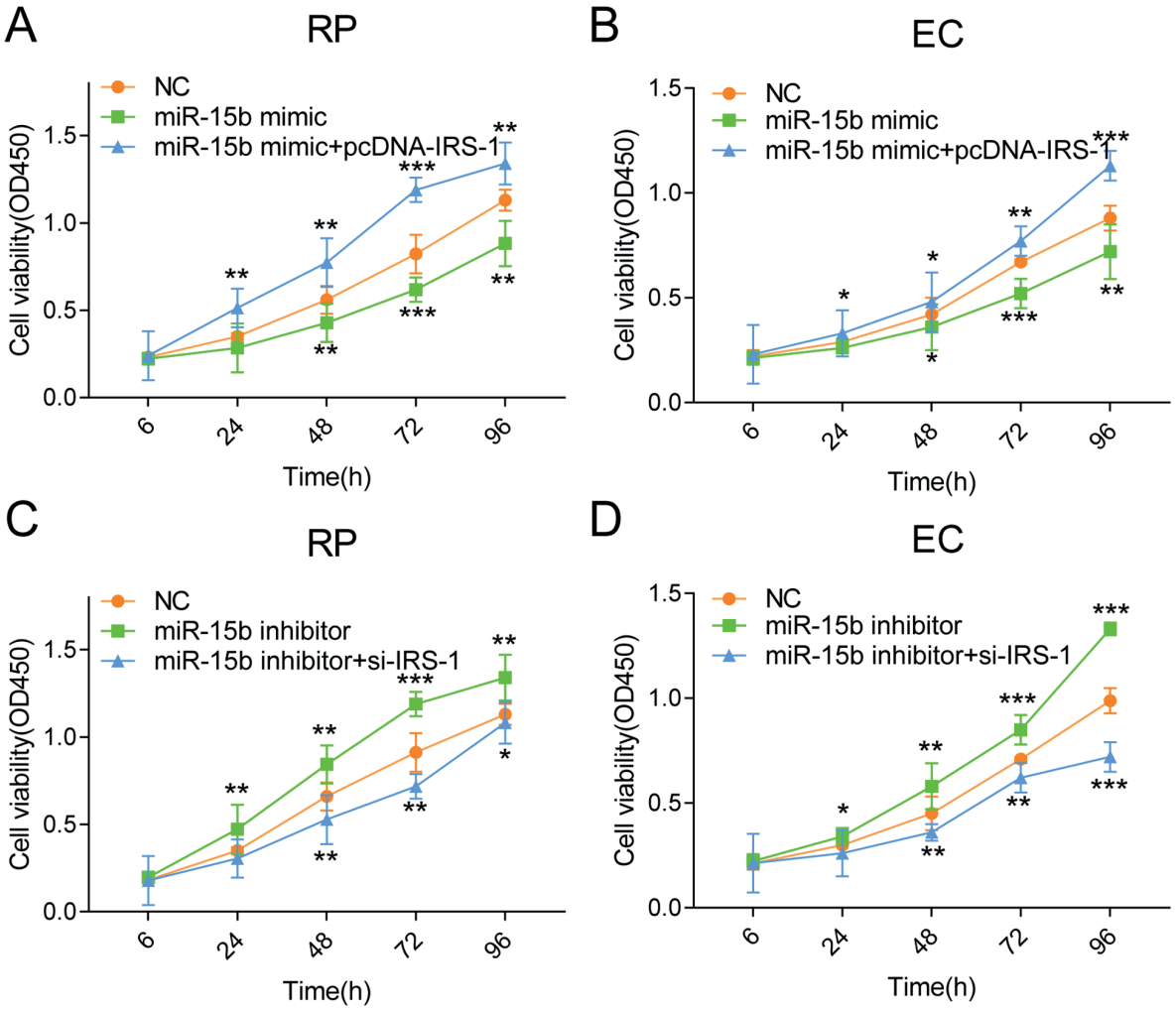

Figure 4. IRS-1 reversed the effect of microRNA-15b on EC and RP cells. *A, B,* Decreased cell viability of EC and RP cells after microRNA-15b overexpression was rescued by IRS-1 overexpression. *C, D,* Increased cell viability of EC and RP cells after microRNA-15b knockdown was rescued by the IRS-1 knockdown

permeability[14]. Overexpressed β-catenin in DR is involved in the disruption of blood-retinal barrier and retinal damage *via* regulating adhesive attachment, especially the interaction between retinal microvascular endothelial cells and pericytes. Reorganization of the actin cytoskeleton eventually leads to the destroyed retinal vascular barrier. It is also reported that β-catenin is associated with dysfunction and loss of pericytes[10]. Functionally, β-catenin exerts its role by binding to Wnt receptor *via* the classical Wnt pathway. During the pathological neovascularization induced by hypoxia, Wnt/β-catenin pathway was remarkably activated to destroy the integrity of blood vessel network[15]. Hence, Wnt/β-catenin pathway exerts an essential role in the formation of retinal neovascularization[16,17].

Abnormalities in insulin content and cell metabolism are the main pathogenesis of diabetes. Insulin is secreted by pancreatic islet beta cells, which is the only hormone in the body that could downregulate blood glucose level through binding to IR on the cell membrane[18]. IR and IRS-1 are widely expressed in the entire retina[6,19]. Expression levels of insulin receptors in retinal cells are upregulated in the diabetic patients[20], indicating their crucial roles in the development of diabetic retina. Insulin specifically binds to the retinal vascular endothelial cells and pericytes to promote cell mitosis. Moreover, insulin can also stimulate the production of oxygen free radicals in human fibroblasts[21,22], which further disrupts the blood-retinal barrier, changes retinal blood flow and secretes various cytokines. As a consequence, ab-

normal insulin level stimulates the development of diabetic retinopathy. IRS-1 is greatly involved in promoting angiogenesis[23], which exerts an important role in the development of type 2 diabetes and DR[24].

In this study, microRNA-15b was downregulated in EC and RP cells of DR rats, suggesting that microRNA-15b is involved in the development of DR. IRS-1 was predicted to be the downstream target of microRNA-15b, which was further verified by luciferase reporter gene assay. For *in vitro* experiments, microRNA-15b was capable of regulating viabilities of EC and RP cells through directly targeting IRS-1. Furthermore, we found that microRNA-15b exerts its role in DR *via* regulating β-catenin.

Conclusions

We have found that microRNA-15b participates in the development of diabetic retinopathy by targeting IRS-1 *via* Wnt/β-catenin signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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