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MicroRNA-411 plays a protective role in diabetic retinopathy through targeted regulating Robo4

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-411 in diabetic retinopathy (DR) and to further understand its mechanism of action.

MATERIALS AND METHODS: A rat model of diabetes (20 in the DM group) and a normal control group (20 in the control group) was established. The changes in blood glucose and body weight were compared between the two groups. At the same time, the expression changes in microRNA-411 and Roundabout 4 (ROBO4) in the two groups of rats were detected. The biological prediction of the potential binding sites of ROBO4 and microRNA-411 was verified by the Dual-Luciferase reporter gene assay, and the regulatory relationship of microRNA-411 to ROBO4 was verified in human retinal pigment epithelial cells (ARPE-19). Meanwhile, we investigated the effects of high glucose and hypoxia on the viability of ARPE-19 cells and explored whether microRNA-411 has a regulatory role in the effects. Finally, a cell reverse experiment was designed to verify whether microRNA-411 functions through ROBO4.

RESULTS: Compared with the NC group, the blood glucose of the DM group was significantly increased while the body weight was reduced. In addition, the expression level of microRNA-411 was markedly decreased in diabetic rats, and the mRNA and protein levels of ROBO4 were notably increased, which were all dependent on time. Biological prediction revealed that ROBO4 might be a potential target gene of microRNA-411, and the results of the Dual-Luciferase reporter gene assay confirmed that there is a binding relationship between them; meanwhile, microRNA-411 was proved to be able to inhibit the expression level of ROBO4 *in vivo* and *in vitro*. Both high glucose and hypoxia could inhibit the proliferation of ARPE-19 cells and increase the monolayer permeability. Additionally, up-regulation of microRNA-411 or down-regulation of ROBO4 could partially reverse this phenomenon. Cell reverse experiment showed that overexpression of ROBO4 partially reversed the protective effect of microRNA-411 on DR.

CONCLUSIONS: MicroRNA-411 was down-regulated in the rat model of diabetic retinopathy and played a protective role in this disease, which might be achieved by negatively regulating the expression level of ROBO4.

Key Words:
Diabetic retinopathy, MicroRNA-411, ROBO4, High glucose, Hypoxia.

Introduction

Diabetic retinopathy (DR) is one of the most serious microvascular complications of diabetes mellitus and is the most common risk factor leading to visual dysfunction and blindness among the working-age population (20-65 years)[1-3]. At present, the incidence of diabetes, as well as the occurrence and development of DR, are increasing year by year. As of 2016, there are 425 million adults with diabetes worldwide, and in all countries, China has the highest burden of diabetes, with the number of patients (114 million) accounting for nearly 27% of the world. Due to the aging of the population and changes in lifestyles, it is expected that in 2045 the number of diabetic patients worldwide will further increase, reaching 700 million.

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules of approximately 21-23 nucleotides in length encoded by endogenous genes. Gene expression is regulat-

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ed at the transcriptional or post-transcriptional level, including complete complementation with the 3'-untranslated region (3'-UTR) of the target gene to induce mRNA degradation, or incomplete binding to 3'-UTR to inhibit translation of target genes. They are expressed in all human cells and are involved in major biological processes such as cell growth, differentiation and apoptosis[4-6]. MiRNAs have become novel biomarkers, regulatory factors and therapeutic targets for diseases such as cancer, heart disease and diabetes[7,8]. Researchers[9-11] have examined the effects of miRNAs on diabetes and its complications, including endothelial and vascular smooth muscle cell dysfunction, diabetic cardiomyopathy and diabetic nephropathy, and demonstrated that certain specific types of miRNAs are widely involved in the regulation of inflammatory genes. McArthur et al[12] have shown that multiple miRNAs are associated with DM; for example, the expression of miR-200b is decreased in high glucose-treated endothelial cells and the retina of rats one month after STZ-induced diabetes. In addition, in diabetic rat retinal endothelial cells, NF-κB can activate miR-146a and increase its expression level[13].

Studies have confirmed that microRNA-411 is involved in the development of various tumors, such as bladder cancer[14], non-small cell lung cancer[15], liver cancer[16], etc., but its role in diabetic retinopathy remains unclear. The experiment aimed to explore the role of microRNA-411 in diabetic retinopathy and to further investigate its potential mechanism.

Materials and Methods

Establishment and Observation of a Diabetic Rat Model

This study was approved by the Animal Ethics Committee of Nanjing University Animal Center. Forty healthy male Sprague Dawley (SD) rats, weighing 180-210 g, 8 weeks old, were randomly divided into the normal control group (NC, 20) and diabetic group (DM, 20). They were housed in standard plastic squirrel cages and fed for 1 week at 24°C in an environment with 12 hours light and 12 hours dark in turn. Rats were fasted for 12 hours before administration and weighed. Rats in the diabetic group (DM) were given a single intraperitoneal injection of STZ (65 mg/kg) to prepare a diabetic model. Normal control (NC) rats were given a single intraperitoneal injection of an equal volume of sodium citrate buffer. After 72 hours and 1 week of STZ injection, fasting blood glucose was measured in the tail vein of rats. When the blood glucose level was over 16.7 mmol/L, it was considered that the rat DM model was successfully established. Experiments were performed at 4, 6 and 8 weeks after modeling, and the body weight of the rats was weighed before treatment. At 10 weeks, a microinjection needle (10 μL, 1800 series, Hamilton, Switzerland) was used to inject the corresponding adeno-associated virus containing microRNA-411 mimic or its control into the vitreous cavity of each group of rats. After 4 weeks, rat retinal tissue was extracted and the samples were stored in liquid nitrogen.

Isolation of Rat Retina

Rats were intraperitoneally injected with 10% chloral hydrate for general anesthesia and administered with oxybuprocaine hydrochloride eye drops for topical anesthesia; then, their eyeballs were collected and the conjunctiva was removed, and the eyeballs were rinsed with saline to be placed on a wet gauze under a stereo microscope. The cornea was carefully separated and cut off about 1 mm behind the corneoscleral margin. The lens was removed, the vitreous was carefully removed, the retina was separated under the retina and the optic nerve was cut, and the separated retina was dissected and chopped.

Cell Culture and Transfection

ARPE-19 cells were routinely cultured in Dulbecco's Modified Eagle's Medium/F12 medium (DMEM/F12; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and the cells were placed in a 37°C constant temperature incubator with 5% CO_2 for incubation. The high glucose culture was as follows: when the ARPE-19 cells were in a normal culture state, 1×10^5 cells per well were plated in a six-well culture plate. When the cells were adherent and in good condition, the original common medium was discarded and replaced with DMEM/F12 high glucose medium (glucose concentration: 25 mmol/l) containing 10% FBS. After the cells were cultured for 1 d, transfection experiment was performed and high glucose culture was continued. Subsequently, hypoxic culture was performed. ARPE-19 cells were placed in a hypoxic bag 16 h before cell detection or treatment. The cells in good growth state were transplanted into a 6-well plate for

1 day, and the cell density was observed to be 30% to 40% 24 hours later. The corresponding reagents were added in cells according to the Lipofectamine™ 2000 instructions (Invitrogen, Carlsbad, CA, USA), and replaced with the original medium after 6 hours. The culture was continued for 48 hours, and the cells were collected for subsequent experiments.

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

When extracting retinal RNA, 300 μL of lysate and shredded retina were added to the tissue homogenizer, the retinal tissue was homogenized for 15 min on ice until no visible solid tissue was visible to the naked eye. Then, the lysate was transferred to 1.5 ml Eppendorf tube (EP; Eppendorf, Hamburg, Germany), and the total RNA was extracted according to the instructions. When the cellular RNA was extracted, TRIzol (Invitrogen, Carlsbad, CA, USA) was added in the cells transfected for 48 hours. The RNA was then carefully manipulated following the experimental procedures provided by the TRIzol reagent. The concentration of RNA was determined by an ultra-micro UV spectrophotometer, and the purity of the A260/A280 of the RNA solution was 1.8 to 2.1. 1 μg of total RNA was taken and reverse transcription was performed to obtain complementary deoxyribose nucleic acid (cDNA). The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) reaction solution was prepared according to the instruction manual of the SYBR fluorescence quantitative premixing kit (TaKaRa, Otsu, Shiga, Japan), and the total system was 10 μL. The PCR reaction conditions were pre-denaturation at 95°C for 30 s, 95°C for 5 s, and 60°C for 31 s for a total of 40 cycles. The experiment was repeated three times. U6 was used as an internal reference for microRNA-411 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was for ROBO4. The primer sequences were as follows: microRNA-411: F: CCATGUAUGUAACACGGUC, R: GGUUAGUGGACCGGTC; U6: F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; ROBO4: F: CCCTGTGCTTGGAACTCAGTG, R: CGCTGATGTACCCATAGGTGG; GAPDH F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Western Blot Assay

When the retinal protein was extracted, 1 mL of Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and the shredded retina were added to the tissue homogenizer; the retinal tissue was homogenized on ice for 15 min until no solid tissue was visible to the naked eye, and the liquid was collected and centrifuged at 2000 rpm for 5 min. The supernatant was removed, and 160 μL of a protein lysate containing a protease inhibitor was added. When the cell protein was extracted, the cells were washed twice with PBS 48 h after transfection, and then the protein lysate was added to lyse the cells. The cells were fully lysed through ultrasound on ice, and the supernatant was collected to determine the protein concentration using the bicinchoninic acid (BCA) method (Abcam, Cambridge, MA, USA). After being separated by sodium dodecyl sulphate (SDS)-page electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) *via* wet transfer method, and 5% skim milk was used to block the antigens for 1 hour. Then, the corresponding primary antibody was added to incubate the PVDF membrane in a refrigerator at 4°C overnight. After the membrane was washed 3 times with Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA) the next day, the corresponding secondary antibody was added, and after 2 hours of incubation at room temperature, the proteins in membrane was illuminated using enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) and photographed.

Cell Counting Kit-8 (CCK-8) Assay

After 48 hours of transfection, the cells were seeded at a density of 2×10^3/100 μL in a 96-well plate, cultured for the time shown in the figure and then subjected to the Cell Counting Kit-8 detection (CCK-8; Dojindo Laboratories, Kumamoto, Japan). During the test, the serum-free medium was applied, and 10 μL of CCK-8 solution was added to each well. The incubation was continued for 2 h at 37°C in a 5% CO_2 incubator to detect the optical density (OD) value at a wavelength of 450 nm, and each group was subjected to 3 replicate wells.

Dual-Luciferase Reporter Gene Assay

Roundabout 4 (ROBO4) was predicted to be the potential target gene of microRNA-411. The wild-type ROBO4 3'UTR-Luciferase vector was named ROBO4-WT, and the mutant PBOV1 3'UTR-Luciferase reporter vector was named ROBO4-MUT. The Luciferase reporter vector and microRNA-411 mimic or its control sequenc-

es were co-transfected into ARPE-19 cell cells based on the experimental grouping according to the Lipofectamine™ 2000 instructions. After transfection for 4 h, the cells were collected after culturing in an incubator for 48 h. The Luciferase activity was measured using a detector according to the Promega Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA). Relative Luciferase activity (= Firefly Luciferase activity value/ Renilla Luciferase activity value) was calculated.

Cellular Permeability Detection of ARPE-19 Cells Under Different Conditions

Monolayer cell permeability of ARPE-19 was examined using fluorescein isothiocyanate (FITC)-dextran. 200 μL of 1×10^5 cell suspension was prepared and added to the upper chamber of a 24-well transwell plate coated with a 0.4 μm pore size polycarbonate membrane, and 500 μL of the same medium was added to the lower chamber. The cells in the high glucose group were continued to culture for 2 days, while cells in the hypoxia group culture were continued to culture for one day and placed in a hypoxic bag for hypoxia treatment for 16 h. Subsequently, the medium in the upper and lower chamber was discarded, and the cells were rinsed twice with PBS. 100 μL of FITC-dextran was added to the upper chamber while 500 μL of PBS solution was added to the lower chamber. After incubating the transwell plate in a constant temperature cell incubator at 37°C for 30 min, 100 μL of the medium in the lower chamber of each well of the transwell plate was aspirated and transferred to an opaque 96-well plate. The fluorescence intensity of FITC-dextran leaking to the lower chamber from the upper chamber was detected by a microplate reader, the excitation and emission wavelengths used for the detection were 490 nm and 520 nm, respectively. Finally, the ratio of the measured values of each experimental group to the measured value of the positive control group was calculated to obtain a relative FITC-dextran permeability of single layer cells.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation (Mean ± SD). Unpaired *t*-test was used for comparison between groups. $p<0.05$ was considered statistically significant.

Results

The Expression of MicroRNA-411 is Decreased in the Retina of the Diabetic Model, while that of ROBO4 is Increased

A DM rat model was constructed to monitor changes in blood glucose and body weight in rats. As shown in Figure 1A, the blood glucose of rats in the DM group maintained at a high level from 2 to 8 weeks after the model was established, and the blood glucose level was over 16.7 mmol/L, which was higher than the normal level of the NC group, suggesting successful modeling (Figure 1A). Compared with the NC group, the body weight of rats in the DM group was significantly reduced, but there was no tendency to increase with time (Figure 1B). To confirm the expression changes of microRNA-411 and ROBO4 in early diabetic retinopathy, we compared the levels of both in the retina of the two groups of rats. The results showed that, compared with the NC group, the expression of microRNA-411 in the DM group was significantly decreased with time (Figure 1C), while the mRNA and protein level of ROBO4 in the DM group was markedly up-regulated and increased with time (Figure 1D, 1E), suggesting that microRNA-411 and ROBO4 were associated with diabetic retinopathy.

ROBO4 is a Potential Target Gene for MicroRNA-411

MiRNAs induce mRNA degradation by transcriptionally or post-transcriptionally regulating gene expression, including complete or incomplete complementation with the 3'-untranslated region (3'-UTR) of the target gene to inhibit its translation[5]. Therefore, by biological prediction, we found that there existed potential binding sites between ROBO4 and microRNA-411 (Figure 2A), and then we designed the Dual-Luciferase reporter gene assay to verify the results. The fluorescence value of the wild-type group decreased, while that of the mutant group did not change significantly (Figure 2B). Furthermore, we detected whether microRNA-411 can regulate the expression of ROBO4 in tissues and cells, and the results showed that ROBO4 expression was decreased in ARPE-19 cells (Figure 2C) and DM rats (Figure 2D) after up-regulation of microRNA-411.

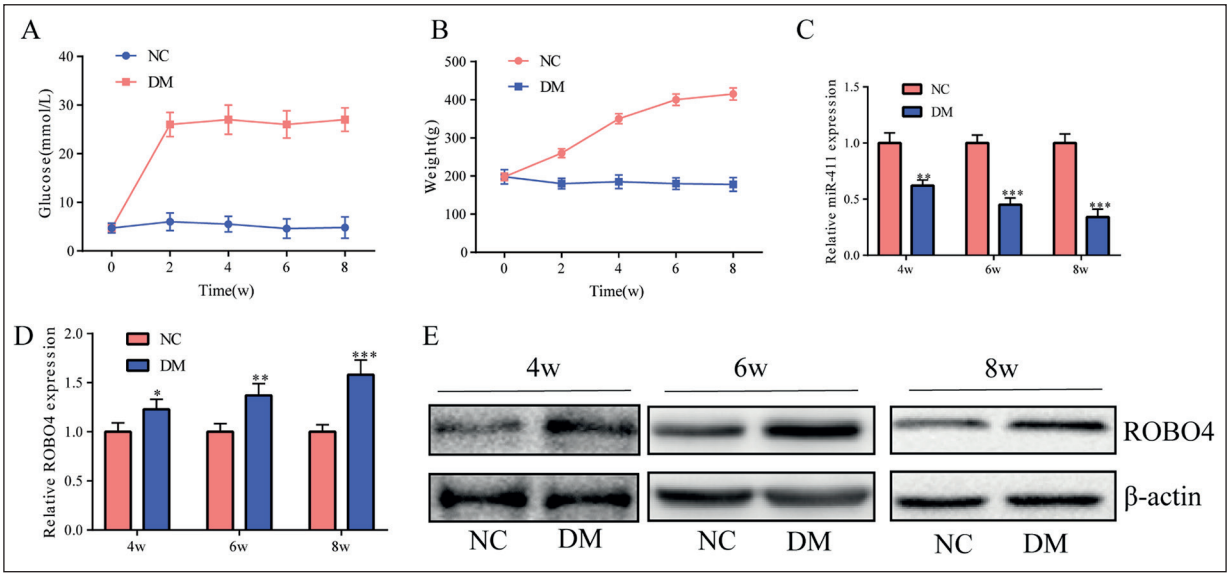

Figure 1. MicroRNA-411 is under-expressed in the retina of the diabetic model. **A,** Compared with normal rats, the blood glucose of diabetic model rats (DM) was significantly increased. **B,** Compared with normal rats, the weight of diabetic model rats (DM) was significantly reduced. **C,** In the DM model, microRNA-411 expression was significantly reduced and changed with the increase of time. **D,** In the DM model, the mRNA level of ROBO4 was significantly increased and correlated with the increase of time. **E,** In the DM model, the protein level of ROBO4 was significantly increased.

Effect of MicroRNA-411 on the Function of ARPE-19 Cells

To assess the functional effects of microRNA-411 on cells in high glucose or hypoxic conditions, we overexpressed microRNA-411 in ARPE-19 cells to observe changes in cell function under high glucose or hypoxia (Figure 3A, 3B). It was found that up-regulation of microRNA-411

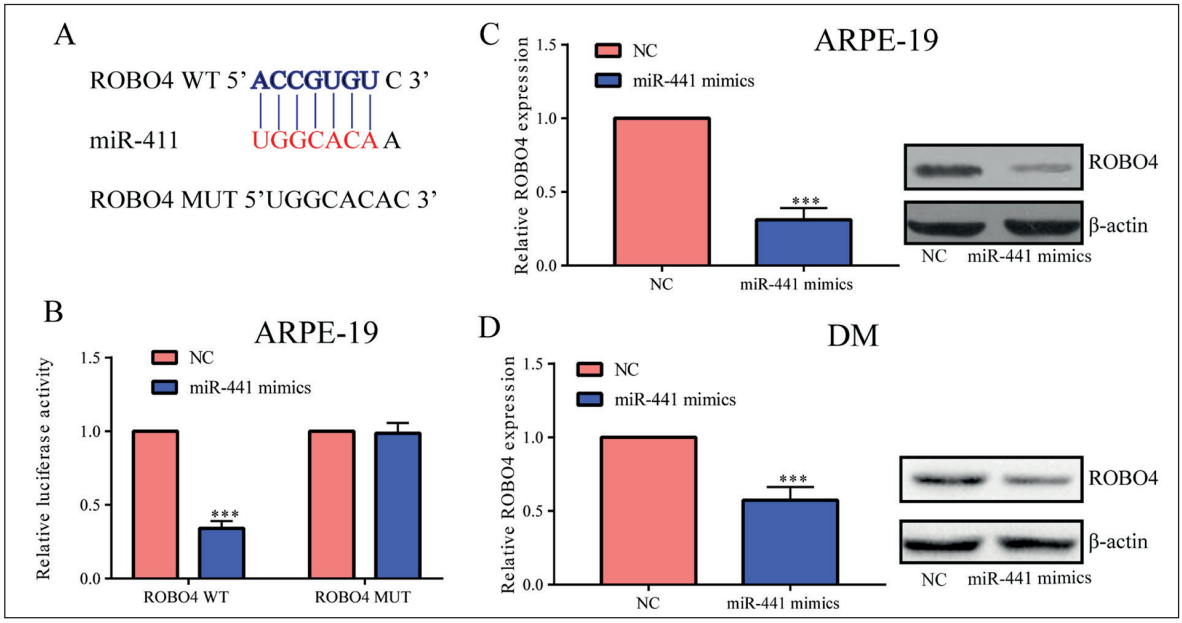

Figure 2. ROBO4 is a potential target gene for microRNA-411. **A,** ROBO4 and microRNA-411 have potential binding sites. **B,** Dual-Luciferase reporter gene results indicated the binding relationship. **C,** In human retinal pigment epithelial cells (ARPE-19), up-regulation of microRNA-411 significantly reduced the expression of ROBO4. **D,** In the DM rat model, up-regulated microRNA-411 markedly reduced the expression of ROBO4.

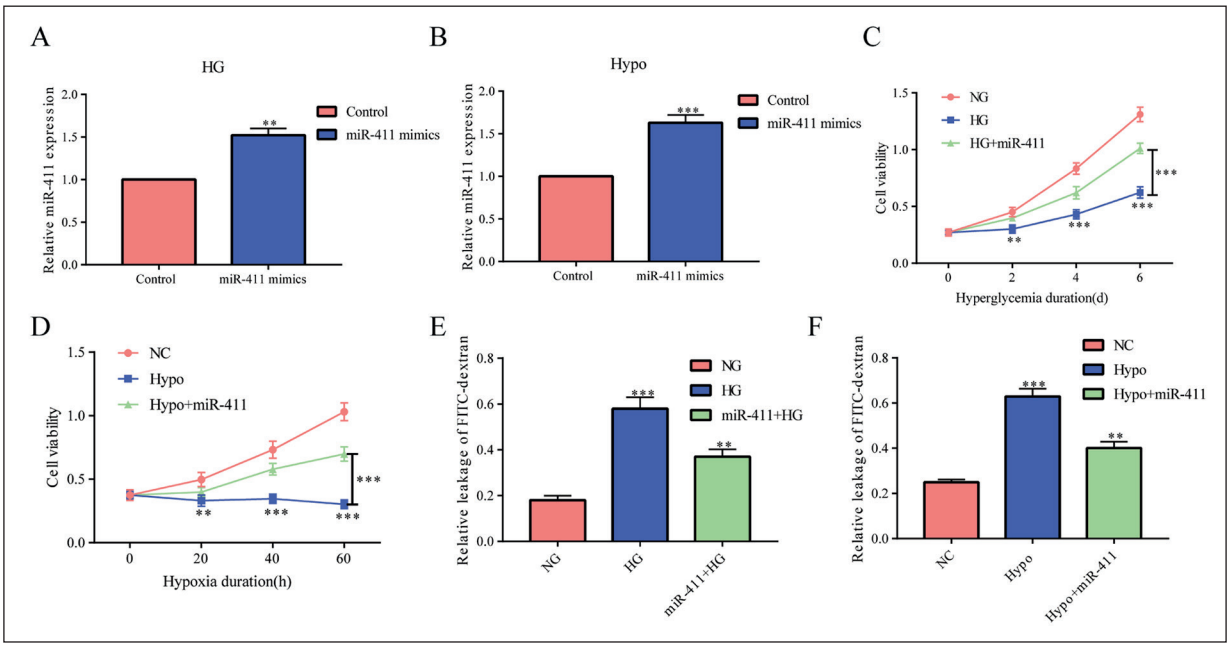

Figure 3. The effect of microRNA-411 on ARPE-19 cell function was shown. **A, B,** The transfection efficiency of microRNA-411 mimics in ARPR-19 cells treated with HG or hypoxia. **C,** Compared with the normal group (NG), high glucose (HG) significantly inhibited the proliferation of ARPE-19 cells, while up-regulation of microRNA-411 could remarkably improve the cell viability. **D,** Under hypoxic conditions, cell viability decreased with prolonged hypoxic time, while microRNA-411 could significantly increase cell viability. **E,** In high glucose environment, the monolayer permeability of ARPE-19 cells was markedly increased, while up-regulation of microRNA-411 partially reversed this phenomenon. **F,** In hypoxic environment, the monolayer permeability of ARPE-19 cells was significantly increased, while up-regulation of microRNA-411 could partially alleviate this phenomenon.

remarkably improved the inhibition of ARPE-19 cell viability and the promotion of monolayer permeability of ARPE-19 cells induced by high glucose and hypoxia (Figure 3C-3F).

MicroRNA-411 Works via Regulating ROBO4

The above experimental results suggested that microRNA-411 regulated the expression of ROBO4 and could partially reverse the effects of high glucose and hypoxia on ARPE-19 cell viability and monolayer cell permeability. We designed a cell reverse experiment to verify whether microRNA-411 can play a role in the function of ARPE-19 cells by regulating the expression of ROBO4. The results showed that, compared with the normal group, down-regulation of ROBO4 significantly improved the inhibition of ARPE-19 cell viability and the promotion of cell monolayer permeability induced by high glucose and hypoxia (Figure 4A-4D). Up-regulation of microRNA-411 and ROBO4 under high glucose or hypoxia conditions partially reversed the effect of overexpression of microRNA-411 alone on ARPE-19 cell viability (Figure 4E, 4F) and cell monolayer permeability (Figure 4G, 4H).

Discussion

DR is a metabolic disease caused by long-term retinal blood vessels in a high glucose environment, and its pathological mechanism is very complicated. Human retinal endothelial cells (HREC) and retinal pigment epithelial cells (RPE) constitute a blood-retinal barrier (BRB). The high glucose environment in the early stage of DR can induce increased permeability of retinal blood vessels, increased leakage of harmful substances, degeneration of capillaries, and ultimately leading to a hypoxic state of retina[17,18]. The interaction of high glucose and hypoxia will further exacerbate the progression of DR[19]. Biochemical and metabolic disorders in high-sugar environments, accompanied by neurological dysfunction, lead to increased vascular permeability, followed by macular edema and neovascularia-

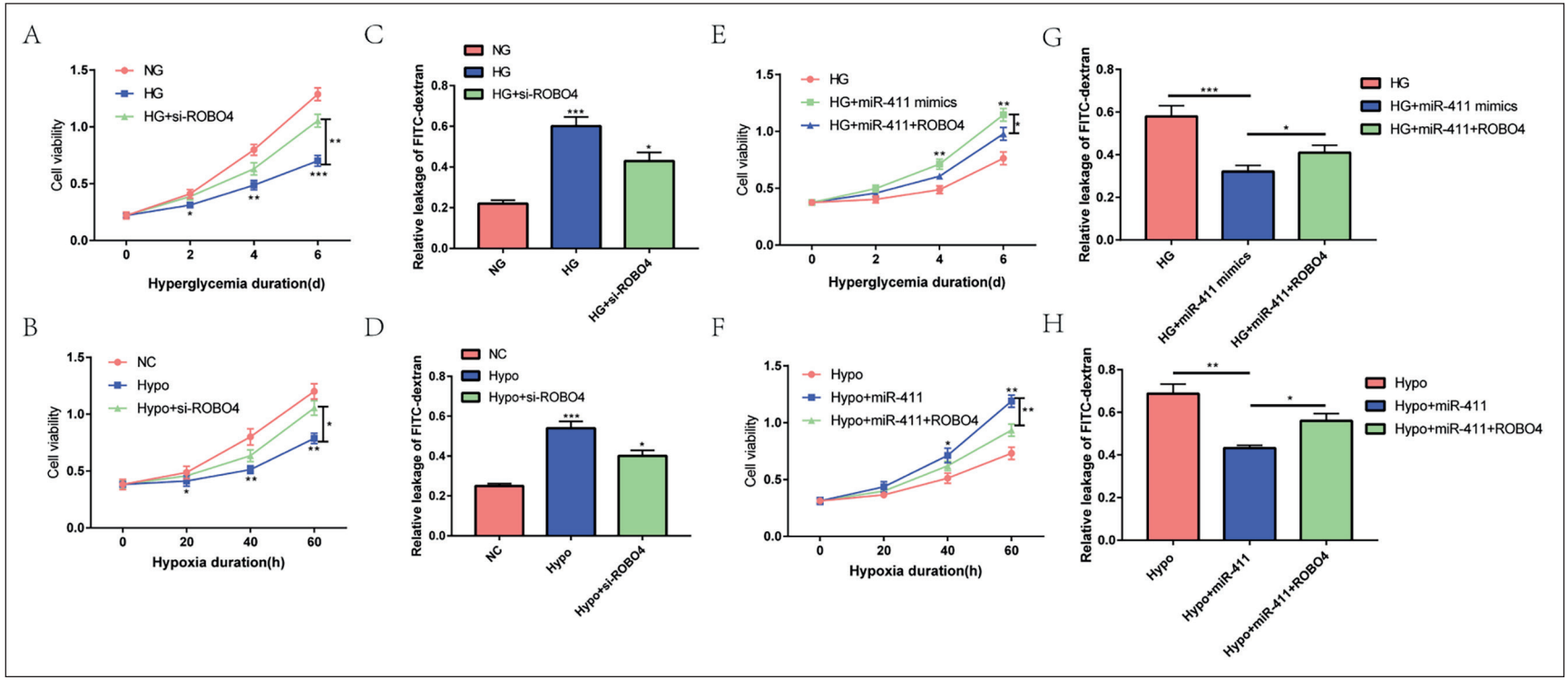

Figure 4. MicroRNA-411 works by regulating ROBO4. **A, B,** Compared with the normal group, high glucose and hypoxia treatment markedly inhibited the proliferation of ARPE-19 cells, while down-regulation of ROBO4 could significantly improve the cell viability. **C, D,** Under high glucose and hypoxia conditions, the monolayer permeability of ARPE-19 cells was remarkably increased, while down-regulation of ROBO4 partially reversed this phenomenon. **E, F,** The simultaneous up-regulation of ROBO4 under high glucose and hypoxia conditions can partially reverse the promotion of cell viability by microRNA-411. **G, H,** Under high glucose and hypoxia conditions, simultaneous up-regulation of ROBO4 could partially reverse the protective effect of microRNA-411 on cell monolayer permeability.

tion[2,20,21]. The disruption of tight junctions leads to dysfunction of BRB, which is the main cause of edema and neovascularization. Therefore, protecting or reversing the dysfunction of retinal microvasculature is currently the focus of most studies on DR. However, as a major component of the BRB outer barrier, the role of RPE in DR has not been sufficiently studied. Recently[22], RPE has been found to play an important role in the process of DR. MiRNAs play a crucial role in the proliferation, migration and apoptosis of human cells, including retinal cells. Moreover, miRNAs have been reported to play a vital role in the regulation of DR-associated neovascular diseases. A growing number of miRNAs have been proven to be abnormally expressed in the progression of DR, and some of them have been used in clinical treatment[23], or as a signature circulating miRNA for DR identification[24].

In this investigation, we constructed a rat model of diabetic retinopathy and detected and compared the expression levels of microRNA-411 and its target gene ROBO4. The results suggested that there was a difference in expression between microRNA-411 and ROBO4, which may be related to the occurrence of diabetic retinopathy. The biological prediction and the Dual-Luciferase reporting assay results showed that there was a binding relationship between the two. Animal and cell experiments revealed that microRNA-411 could negatively regulate the expression level of ROBO4. In addition, we indicated that microRNA-411 can promote the viability of ARPE-19 cells and the protective effect of monolayer permeability by negatively regulating ROBO4.

ROBO4 is a new member of the roundabout gene family discovered in 2002[25], also known as MRB (magic roundabout), which is specifically expressed on the surface of endothelial cells with active angiogenesis, regulates endothelial cell permeability, migration ability, etc., and affect neovascularization[26]. Studies have shown that in HREC cultured under normal conditions, ROBO4 can balance the permeability of single-layer HREC by regulating the expression levels of tight junction-associated proteins including ZO-1 and occluding and regulating the distribution of cytoskeletal actin F-actin. Targeted inhibition of ROBO4 expression leads to an imbalance in the above-mentioned homeostasis, resulting in increased cell permeability[27]. It can be seen that under normal physiological environment, ROBO4 plays an important role in maintaining the normal function of certain cells in the retina. ROBO4 expression was also detected in monkey choroidal retinal vascular endothelial cells (RF/6A) and RPE cells. Down-regulation of ROBO4 was effective in reducing the migration and lumen formation of RF/6A and RPE cells, indicating that inhibition of ROBO4 can be involved in the maintenance of blood vessels stability[28]. *In vitro* studies have shown that ROBO4 is always overexpressed in the pathological environment of DR, and the high expression level of ROBO4 can aggravate the functional damage of retinal cells, thereby promoting the development of retinal vasculopathy. To induce DR environment, HREC was cultured in high glucose or hypoxia and it was found that the expression level of ROBO4 increased with the prolongation of time[29,30], while inhibition of ROBO4 could significantly improve the hypoxia-induced increased HREC migration ability.

Conclusions

In this work, we found that microRNA-411 played a protective role in diabetic retinopathy, and this protective effect might be achieved by negatively regulating the expression level of the target protein ROBO4.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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