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MiRNA-138-5p protects the early diabetic retinopathy by regulating NOVA1

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Abstract. – OBJECTIVE: To elucidate the function of miRNA-138-5p in the early diabetic retinopathy (DR) and the potential mechanism.

MATERIALS AND METHODS: DR model in rats was first established by streptozotocin (STZ) injection. MiRNA-138-5p expression in rat retinal tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Besides, its expression in retinal capillary endothelial cells (EC) and pericytes (RP) was also detected. Cell counting kit-8 (CCK-8) assay was performed to evaluate proliferative potentials of EC and RP cells. The target gene of miRNA-138-5p was predicted by bioinformatics and further confirmed by dual-luciferase reporter gene assay. Rescue experiments were carried out to verify whether the target gene could reverse the regulatory effect of miRNA-138-5p on the proliferation of EC and RP cells.

RESULTS: MiRNA-138-5p was lowly expressed in retinal tissues of DR rats, as well as in EC and RP cells. Overexpression of miRNA-138-5p suppressed the proliferative rate of EC and RP cells, and miRNA-138-5p knockdown obtained the opposite trends. NOVA1 was verified to be the target gene of miRNA-138-5p by dual-luciferase reporter gene assay and RIP assay, which was highly expressed in retinal tissues of DR rats, EC, and RP cells. MiRNA-138-5p knockdown markedly upregulated the mRNA and protein levels of NOVA1 in EC and RP cells. Of note, the inhibitory effect of miRNA-138-5p overexpression on proliferative potentials of EC and RP cells was reversed by NOVA1 overexpression. On the contrary, miRNA-138-5p knockdown accelerated their proliferative potentials and was further reversed by NOVA1 knockdown.

CONCLUSIONS: MiRNA-138-5p was lowly expressed in retinal tissues of DR rats, as well as in EC and RP cells. MiRNA-138-5p regulates the early DR by promoting cell proliferation via targeting NOVA1.

Key Words:
Diabetic retinopathy, MiRNA-138-5p, NOVA1, EC, RP.

Introduction

Diabetes mellitus (DM) is one of the most important non-communicable diseases that threaten human health throughout the world[1]. Diabetic retinopathy (DR) is the common microvascular complication of DM, which is a chronic process with a high specific[2]. It is believed that a series of metabolic abnormalities caused by persistent hyperglycemia are the major causes of DR. Pathologically, DR impairs the microvascular system of the retina, capillary permeability, and blood-retinal barrier. It further leads to retinal leakage, macular edema, vitreous hemorrhage of the retina, neovascularization, and retinal detachment, which severely impairs vision. Unfortunately, strict control of blood glucose level is unable to completely prevent the occurrence of DR. Moreover, DR has a tendency to family aggregation. The incidence of DR varies a lot in different ethnicities[3,4].

MicroRNAs are a class of endogenous, non-coding RNAs of about 22 nucleotides in length. Primary transcript (pri-microRNA) is firstly cleaved by RNase III (Drosha) in the nucleus to form a precursor microRNA (pre-microRNA), transported to the cytoplasm and produced to the mature microRNA cleaved by RNase III Dicer[5]. MicroRNA binds to the 3'-untranslated region (3'UTR) of the target mRNA through incomplete complementary pairing to form an RNA-induced silencing complex (RISC), which inhibits mRNA translation or directly induces mRNA degradation[6]. It is reported that microRNAs are widely involved in many biological activities, such as proliferation, apoptosis, migration, and invasion of tumor cells. In recent years, researches have focused on the biological functions of microRNAs in disease progression[7]. To date, approximately 1,100 microRNAs are iden-

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tified to be encoded by the entire human genome that regulate expressions of 60% protein-coding genes in mammals[8].

In this investigation, we mainly studied the role of miRNA-138-5p in DR and explored its mechanism.

Materials and Methods

Establishment of DM Model in Rats

This study was approved by the Animal Ethics Committee of Xi'an No. 4 Hospital Animal Center. Rats were fed with high-glucose and high-fat diet for 8 weeks. After fasting for 10 h, rats were intraperitoneally injected with 2% streptozotocin (STZ) solution at a dose of 50 mg/kg. Fasting blood glucose was determined one week later by extracting blood from rat tail vein. When the fasting blood glucose was higher than 16.7 mmol/L and stable for at least 5 days, the DR model was considered to be successfully established. Rats were continued to feed with high-glucose and high-fat diet until 12 weeks.

Isolation and Culture of EC Cells

Rats were anesthetized with 2% sodium pentobarbital and sterilized with 75% ethanol. Rat eyeball retaining 2 mm optic nerve was quickly harvested. The intact retinal tissue was bluntly dissected. After the retinal large vessel branches and pigment tissues were peeled off, tissues were digested in Ethylene Diamine Tetraacetic Acid (EDTA)-containing trypsin and 0.5% type II collagenase in a water bath, and filtered through a 300-mesh. The precipitate was incubated in 8 mL of Dulbecco's Modified Eagle's Medium (DMEM) (containing 10% fetal bovine serum (FBS), 50 μg/mL heparin, 1% ECGs, 1% penicillin-streptomycin) (Gibco, Rockville, MD, USA) in a flask pre-coated with 0.5% gelatin. Cell adherence was observed 24 h later, and the culture medium was replaced.

Isolation and Culture of RP Cells

Rat eyeballs were harvested for dissecting the intact retina under a microscope. Visible blood vessels were peeled off. Rat retina was cut into pieces, fully digested in phosphate-buffered saline (PBS) containing 0.05% IA collagenase and 0.025% bovine serum albumin at 37°C for 45 min. The mixture was repeatedly filtered using a 53 μm mesh. The eluate was centrifuged, and the precipitate was incubated in low-glucose DMEM with 20% FBS. Under the routine cell culture for 48 h, fresh medium was replaced.

Cell Transfection

EC and RP cells in logarithmic growth phase were seeded in 6-well plates. When the cell density reached 60-70%, cells were transfected with miRNA-138-5p mimic, miRNA-138-5p inhibitor, pcDNA-NOVA1, si-NOVA1 or negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 6 h, the fresh medium was replaced. Transfection efficacy was verified at 48 h. Transfection reagents were provided by GemePharma (Shanghai, China).

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

RNA extraction was conducted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 1 μL of RNA was harvested for determining its concentration and A260/A280 ratio by UV spectrophotometer. RNA samples with 1.8-2.1 of A260/A280 ratio were qualified and subjected to reverse transcription. The obtained complementary deoxyribose nucleic acid (cDNA) was diluted for 10 times and used for qRT-PCR with 3 replicates in each sample. U6 was utilized as the internal reference. QRT-PCR 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 Ct value was recorded as the average one from three independent experiments, and relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. Primer sequences were as follows: MiRNA-138-5p, F: CGCGGATCCTACCCACCCATGACCCCT, R: CCGGAATTCCAGCCCAGAACTGGAAAG; U6, F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; NOVA1, F: GGGTTCCCATAGACCTGGAC, R: CGCTCAGTAGTACCTGGGTAA.

Cell Proliferation Assay

Transfected cells for 48 h were cultured in a 96-well plate with 5×10^3 cells per well. After cell culture for 6, 24 h, 48, 72 h, and 96 h, 10 μL of cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, respectively. Absorbance at 450 nm wavelength was recorded after incubation for 2 h using a Microplate Reader (Bio-Tech Company, Minneapolis, MN, USA).

Western Blot

Total protein was extracted from retinal tissues for determining protein expression. The protein sample was quantified by bicinchoninic acid (BCA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (ECL).

RNA Binding Protein Immunoprecipitation (RIP)

RIP was performed using the Magna RIP RNA Binding kit (Millipore, Billerica, MA, USA). Cells were washed and cross-linked with 0.01% formaldehyde for 15 min. After centrifugation and cell lysis, cell extraction was incubated with RIP buffer containing protein A/G magnetic beads coated with anti-Ago2 or negative control anti-IgG antibody. After overnight incubation at 4°C, cells were incubated with Protein A Agarose for 1 h at 4°C, followed by the isolation and quantification of RNA.

Dual-Luciferase Reporter Gene Assay

EC and RP cells were inoculated in 24-well plates with 3×10^5 cells per well. Cells were co-transfected with wild-type NOVA1 or mutant-type NOVA1 vector with microRNA-138-5p mimics or miR-NC using Lipofectamine 2000. At 24 h later, luciferase activity was determined using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was utilized for statistical analysis. Normally distributed measurement data were represented as mean ± standard deviation ($\bar{x} \pm s$). The differences between the two groups were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

MiRNA-138-5p Was Lowly Expressed in Retina of DR Rats

DR model in rats was first established. We found that miRNA-138-5p expression was lower in retinal tissues of DR rats than controls (Figure 1A). Similarly, miRNA-138-5p was lowly expressed in EC and RP cells extracted from DR rats (Figure 1B). Subsequently, we constructed miRNA-138-5p mimics and inhibitor. Transfection efficacy was verified in both EC and RP cells by qRT-PCR

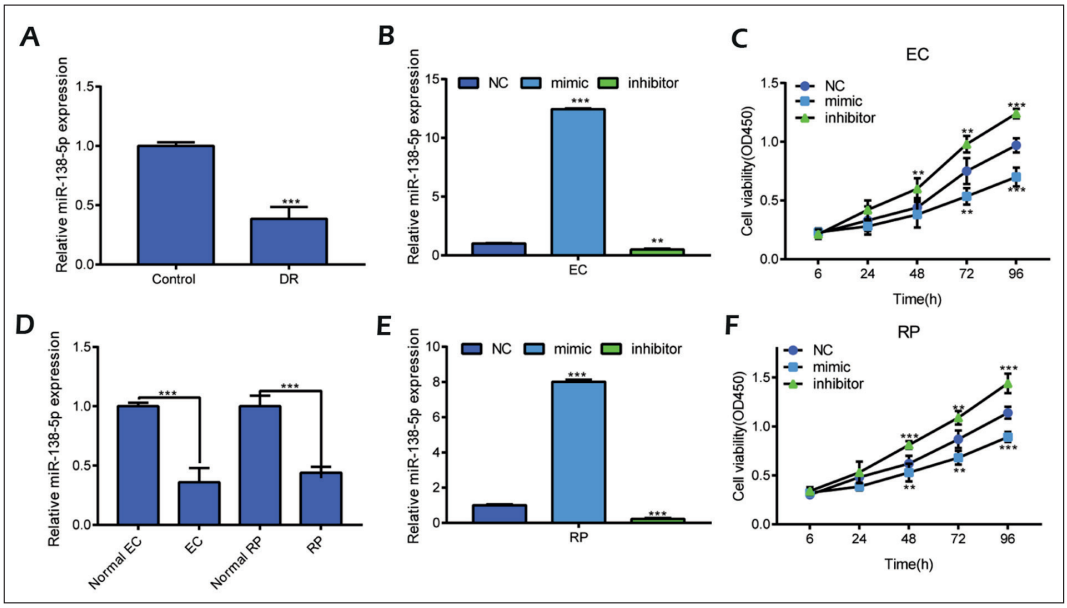

Figure 1. MiR-138-5p was lowly expressed in the retina of DR rats**. A,** MiR-138-5p expression was lower in retinal tissues of DR rats than controls. **B,** MiR-138-5p was lowly expressed in EC and RP cells extracted from DR rats. **C-D,** Transfection efficacy of miR-138-5p mimics and inhibitor was verified in both EC and RP cells by qRT-PCR. **E-F,** CCK-8 assay indicated that viability was inhibited in EC and RP cells overexpressing miR-138-5p. On the contrary, transfection of miR-138-5p inhibitor enhanced viability. **$p<0.01$, ***$p<0.001$.

(Figure 1C, 1D). CCK-8 assay indicated that viability was inhibited in EC cells overexpressing miRNA-138-5p. On the contrary, transfection of miRNA-138-5p inhibitor enhanced viability in EC cells (Figure 1E). Similar results were obtained in detecting viability of RP cells (Figure 1F).

NOVA1 Was Highly Expressed in Retina of DR Rats

Compared with controls, NOVA1 was highly expressed in retinal tissues of DR rats at the protein level (Figure 2A). Similarly, the protein level of NOVA1 was higher in EC and RP cells isolated from DR rats relative to controls (Figure 2B). Both mRNA and protein levels of NOVA1 were downregulated in EC and RP cells overexpressing miRNA-138-5p. On the contrary, NOVA1 expression was remarkably upregulated after transfection of miRNA-138-5p inhibitor (Figure 2C). To further elucidate the potential function of NOVA1 in the development of DR, pcDNA-NOVA1 and si-NOVA1 were constructed, showing sufficient transfection efficacies in both EC and RP cells (Figure 2D, 2E).

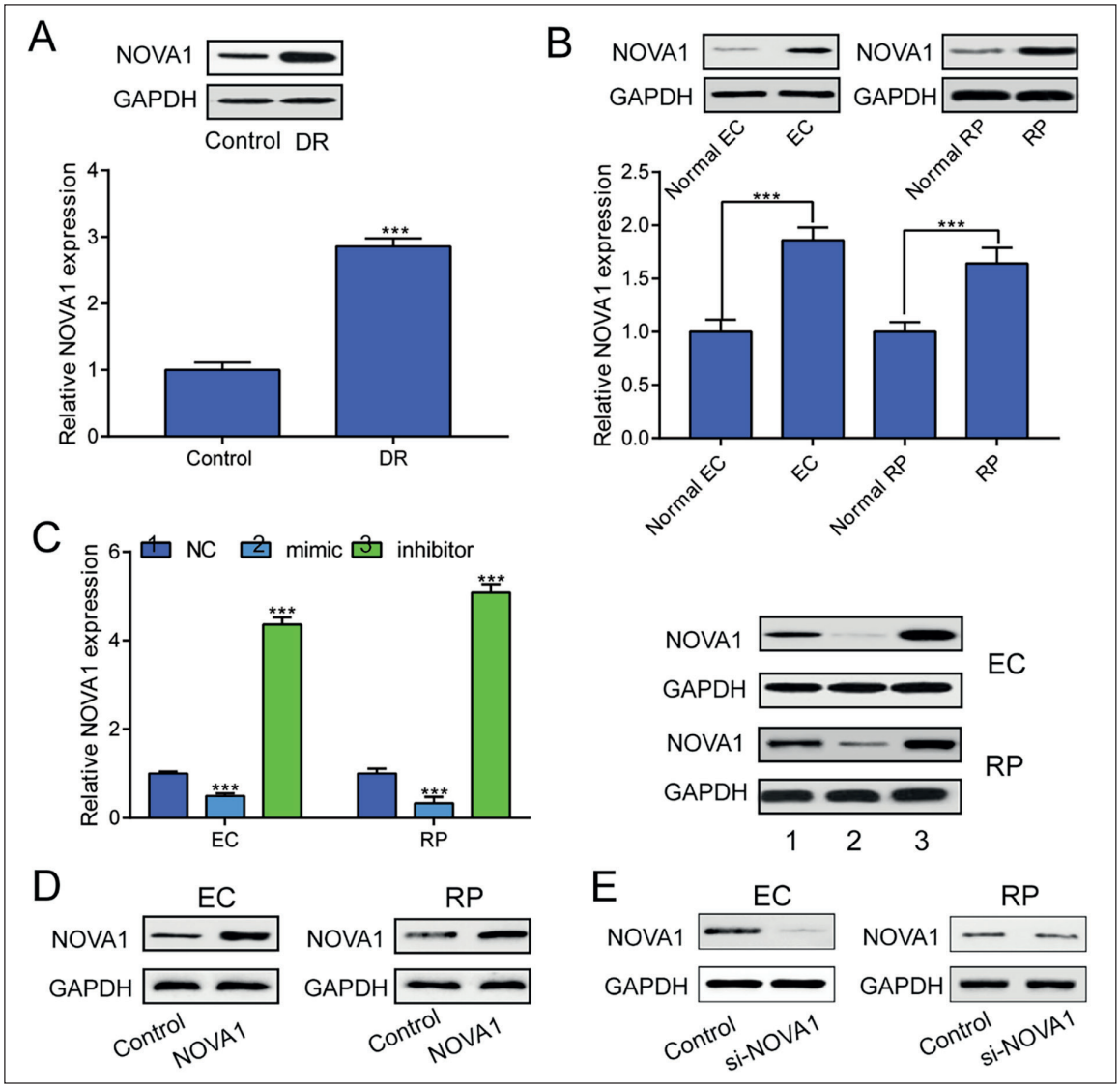

Figure 2. NOVA1 was highly expressed in the retina of DR rats. **A,** NOVA1 was highly expressed in retinal tissues of DR rats at protein level relative to controls. **B,** Protein level of NOVA1 was higher in EC and RP cells isolated from DR rats relative to controls. **C,** Transfection of miR-138-5p mimics downregulated NOVA1 expression in EC and RP cells, while transfection of miR-138-5p inhibitor upregulated NOVA1 expression. **D,** Transfection efficacy of pcDNA-NOVA1 in EC and RP cells. ***E*,** Transfection efficacy of si-NOVA1 in EC and RP cells. ****p*<0.001.

MiRNA-138-5p Could Bind to NOVA1

Through biological prediction, NOVA1 was found to be capable of binding to miRNA-138-5p (Figure 3A). Based on the binding sites between NOVA1 and miRNA-138-5p, we constructed wild-type and mutant-type NOVA1 vectors. RIP assay revealed a great abundance of miRNA-138-5p binding with anti-Ago2 in EC and RP cells (Figure 3B). Moreover, dual-luciferase reporter gene assay verified that cells co-transfected with miRNA-138-5p mimics and NOVA1-WT showed decreased luciferase activity, suggesting the binding of miRNA-138-5p to NOVA1 (Figure 3C, 3D). The above data all demonstrated that NOVA1 was the target gene to bind to miRNA-138-5p directly.

NOVA1 Reversed the Role of MiRNA-138-5p in Regulating the Viability of EC and RP Cells

We thereafter speculated whether miRNA-138-5p exerted its regulatory effect on DR *via* binding to NOVA1. Co-overexpression of miRNA-138-5p and NOVA1 attenuated the inhibitory effect of miRNA-138-5p on the viability of EC and RP cells (Figure 4A, 4B). Conversely, co-transfection of miRNA-138-5p inhibitor and si-NOVA1 attenuated the enhanced viability due to miRNA-138-5p knockdown (Figure 4C, 4D). It is suggested that miRNA-138-5p regulated proliferation of EC and RP cells by targeting NOVA1.

Discussion

The pathogenesis of DR is complicated, involving neovascularization, vascular endothelial dysfunction, and apoptosis. However, the exact pathogenesis of DR has not been fully elucidated. Kovacs et al[9] showed that there are 86 differentially expressed microRNAs in the retina of STZ-induced DR rats. Among them, target microRNAs of NF-κb, including miR-146a/B, miR-

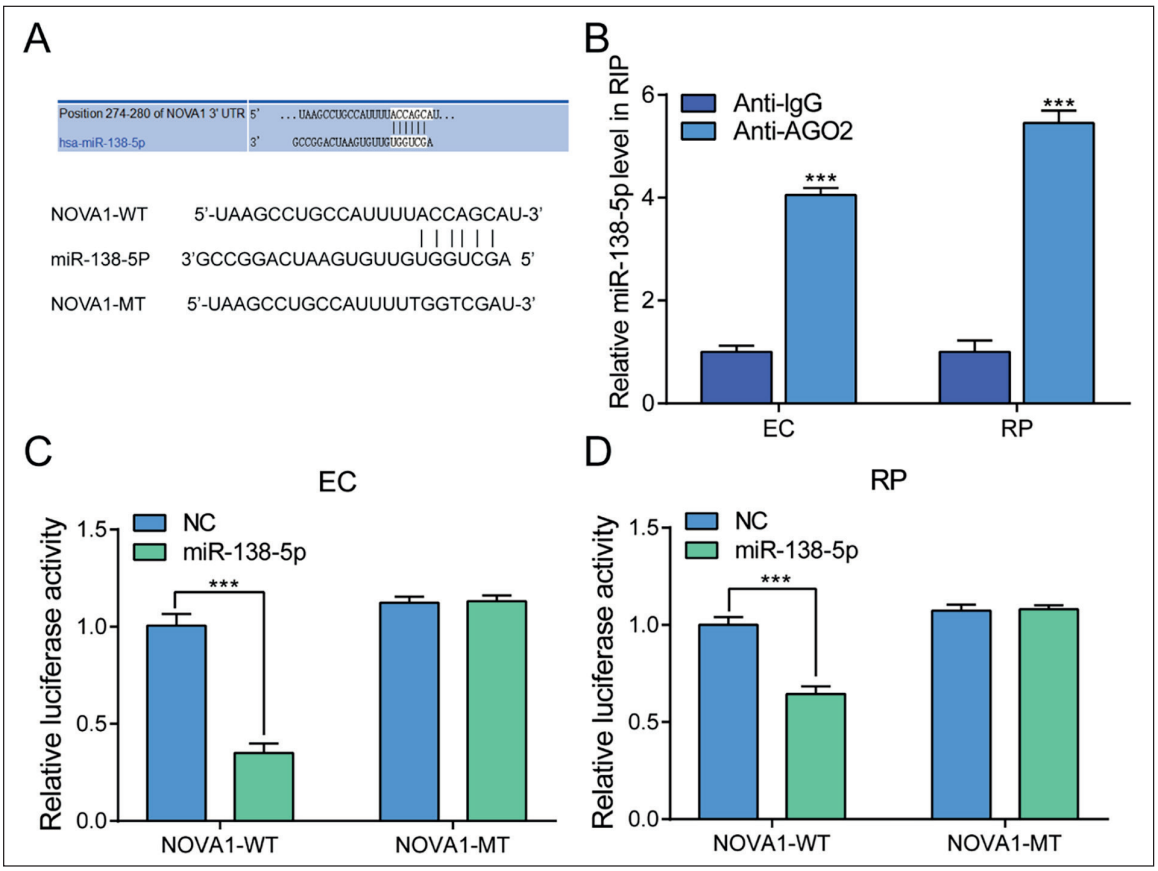

Figure 3. MiR-138-5p could bind to NOVA1. **A,** Potential binding sites between NOVA1 with miR-138-5p. **B,** RIP assay showed that miR-138-5p could bind to AGO2 in EC and RP cells. **C-D,** Luciferase activity was lower in EC and RP cells co-transfected with miR-138-5p mimics and NOVA1-WT relative to controls. ***$p<0.001$.

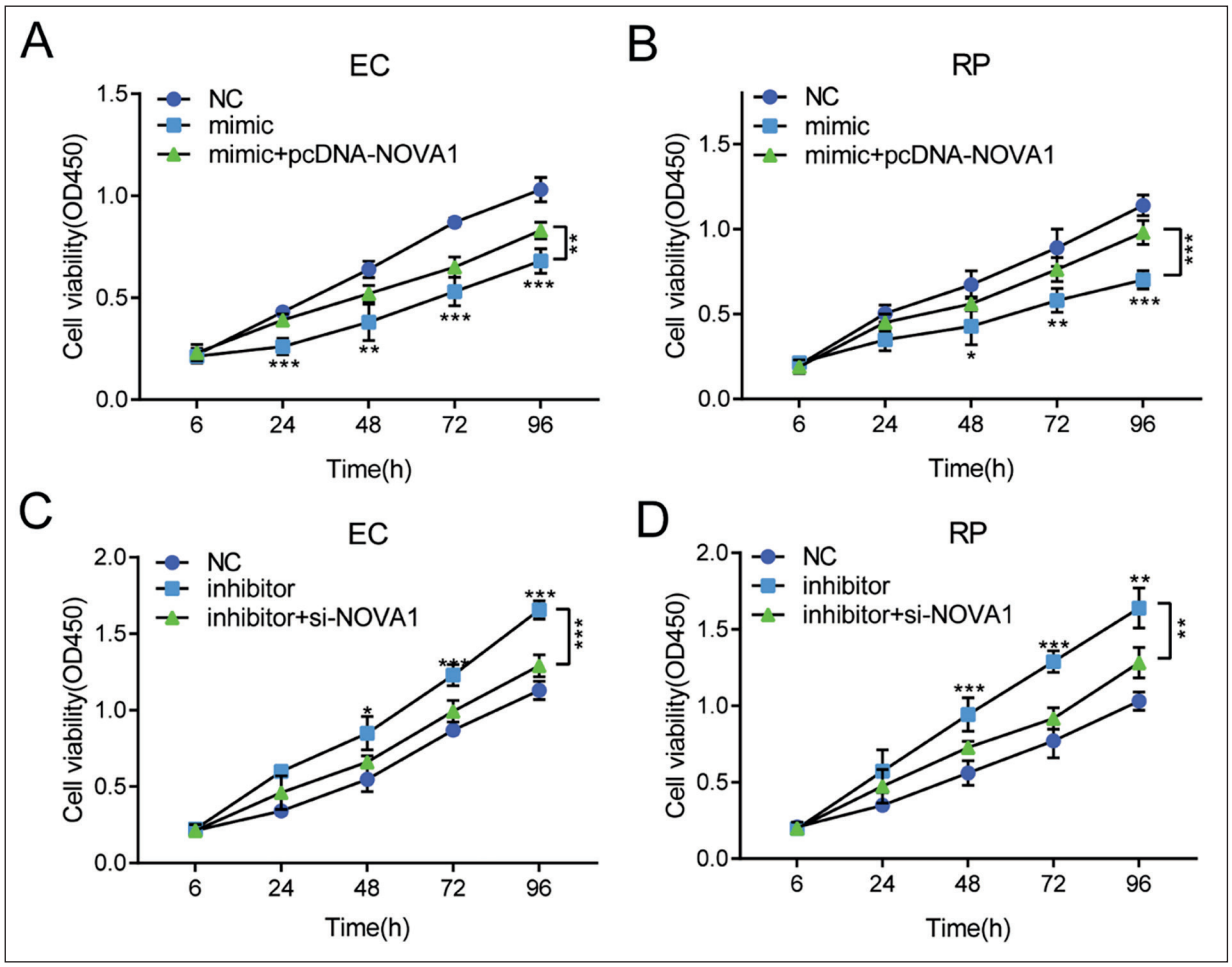

Figure 4. NOVA1 reversed the role of miR-138-5p in regulating the viability of EC and RP cells. **A-B,** Co-overexpression of miR-138-5p and NOVA1 attenuated the inhibitory effect of miR-138-5p overexpression on the viability of EC and RP cells. **C-D,** Co-overexpression of miR-138-5p and NOVA1 attenuated the promotive effect of miR-138-5p knockdown on the viability of EC and RP cells. **p*<0.05, ***p*<0.01, ****p*<0.001.

155, miR-132, and miR-21 are upregulated, which can be utilized as hallmarks for DR. Subsequent researches[10-13] identified that miR-155 is involved in immune signal regulation, miR-146a/B and miR-21 are involved in retinal fibrosis, and miR-132 is involved in angiogenesis. MiR-146a has also been shown to regulate fibronectin production in retinal epithelial cells[14]. A relevant study analyzed the differentially expressed microRNAs in microarrays of STZ-induced diabetic rats. In comparison with the rat retina of controls, a total of 21 microRNAs are upregulated, and 16 are downregulated in DR rats. In particular, 11 microRNAs are particularly upregulated, including miR-182, miR-96, miR-183, miR-211, miR-204, etc.[15]. In this study, we focused on the role of miRNA-138-5p in DR.

Previous studies have shown the vital role of miRNA-138-5p in a variety of tumors. In bladder cancer cells, miRNA-138-5p inhibits proliferative and invasive rates by interfering with the expression of survivin, a member of the inhibitor of apoptosis proteins[16]. MiRNA-138-5p arrests cell cycle progression and inhibits the proliferative rate of liver cancer cells by interfering with the expression of SOX9[17]. In this study, we demonstrated that miRNA-138-5p inhibited the proliferative potentials of EC and RP cells. As an RNA-binding protein, NOVA1 was first discovered in POMA patients, which is involved in alternative splicing of RNA at post-transcriptional level[18]. NOVA1-related primary tumors found nowadays include breast cancer, fallopian tube cancer, small cell lung

cancer, liver cancer, etc.[19-22]. In liver cancer, NOVA1 knockdown reduces the migratory and invasive abilities of hepatoma cells, while NOVA1 overexpression markedly enhances their abilities. Our study indicated that NOVA1 overexpression increased the proliferative potentials of EC and RP cells in the DR model.

Our study first constructed the DR model in rats. We determined the expressions of miRNA-138-5p and NOVA1 in rat retinal tissues, EC, and RP cells. *In vitro* experiments showed that miRNA-138-5p inhibited proliferative potentials of EC and RP cells. Through biological prediction, we found that NOVA1 may be a target gene of miRNA-138-5p. RIP and dual dual-luciferase reporter gene assay further confirmed their binding relationship. Besides, miRNA-138-5p negatively regulated NOVA1 expression. Through rescue experiments, we clarified that the regulatory role of miRNA-138-5p in the development of DR relied on targeting NOVA1.

Conclusions

MiRNA-138-5p was lowly expressed in retinal tissues of DR rats, as well as in EC and RP cells. MiRNA-138-5p regulates the early DR by promoting cell proliferation *via* targeting NOVA1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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