

In vitro protective effect of miR-181d-5p in high glucose-induced human retinal microvascular endothelial cells by targeting the angiogenic factor VEGFA

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Abstract. – OBJECTIVE: Previous studies show the downregulation of miR-181d-5p in the vitreous humor of patients with diabetic retinopathy (DR); however, it is not known whether miR-181d-5p is implicated in the development of DR. The present study aimed at evaluating the beneficial effects of miR-181d-5p on high glucose (HG)-induced human retinal microvascular endothelial cells (hRMECs), as well as the underlying mechanism.

MATERIALS AND METHODS: hRMECs treated with HG were used to induce an *in vitro* cell model of DR. The analysis of quantitative real-time PCR (qRT-PCR) and western blot were used to detect the expression of miR-181d-5p and vascular endothelial growth factor A (VEGFA). The cell viability was measured using CCK-8 assay. The migration was assessed by performing Transwell assay. The angiogenesis of hRMECs was evaluated using the tube formation assay. The binding between target genes was explored using bioinformatic prediction and the luciferase reporter assay.

RESULTS: HG treatment decreased miR-181d-5p but upregulated VEGFA expression in hRMECs. MiR-181d-5p inhibition augmented cell proliferation, migration and angiogenesis of hRMECs caused by HG. Upregulation of miR-181d-5p led to opposing effects, and miR-181d-5p directly targeted and negatively regulated VEGFA. The overexpression of VEGFA reversed the anti-proliferative, anti-migratory and antiangiogenic effects of miR-181d-5p in HG-treated hRMECs.

CONCLUSIONS: These findings indicate that miR-181d-5p ameliorates HG-stimulated proliferation, migration and angiogenesis of hRMECs through inhibition of VEGFA, which might provide a new target for the treatment of DR.

Key Words:

Angiogenesis, Diabetic retinopathy, MiR-181d-5p, VEGFA.

Introduction

High glucose (HG) is reported to cause endothelial hyperplasia and retinal neovascu-

larization^{1,2}, resulting in diabetic retinopathy (DR). DR is one of the most common complications resulting from diabetes mellitus (DM)^{3,4} and can lead to visual impairment and even blindness, seriously affecting a patients' quality of life⁵.

Increasing studies have indicated that certain few miRNAs regulate diabetic retinal vascular dysfunction by targeting angiogenic factors⁶⁻⁹. These miRNAs are a class of highly conserved, non-coding small RNA molecules, about 22 nucleotides in length¹⁰, which are involved in cell biological processes through the regulation of post-transcriptional translation by specifically binding to the 3'UTR of target genes^{11,12}. As an anti-oncogenic miRNA, miR-181d-5p is essential for tumor cell angiogenesis¹³⁻¹⁵ and is inhibited in the vitreous humor of DR patients¹⁶. However, the potential effect of miR-181d-5p in endothelial cell dysfunction has not yet been addressed. Therefore, HG-stimulated human retinal microvascular endothelial cells (hRMECs) were used to simulate the pathogenesis of DR *in vitro*, followed by the detection of cell biology.

Materials and Methods

Cell Treatment

Microvascular Endothelial Cell Basal Medium (Sigma-Aldrich, St. Louis, MO, USA) was used to culture hRMECs in a 5% CO₂ incubator at 37°C containing fetal bovine serum (FBS) and streptomycin/penicillin (Invitrogen, Carlsbad, CA, USA), which was replaced every three days. After 48 hours, the confluent cells were treated with D-glucose at a concentration of 5 mM (normal glucose; termed NG group) or 30 mM (high glucose; termed HG group), as previously

Table I. Primer sequences used for qPCR.

Name	Sequences (5'-3')	
miR-181d-5p	Forward	TGCGCAACATTCATTGTTGTCG
	Reverse	CTCAAGTGTCGTGGAGTCGGCAA
VEGFA	Forward	AGGGCAGAATCATCACGAAGT
	Reverse	AGGGTCTCGATTGGATGGCA
U6	Forward	CTCGCTTCGGCAGCACATATACT
	Reverse	ACGCTTCACGAATTTGCCGTGTC
GADPH	Forward	GGACCTGACCTGCCGTCTAG
	Reverse	GTAGCCCAGGATGCCCTTGA

described¹⁷⁻¹⁹. The miR-181d-5p mimics/corresponding negative controls (NC), as well as the VEGFA-overexpressed plasmid or empty pcDNA vector (GenePharma, Shanghai, China) were used for transfection through Lipofectamine 3000 (Invitrogen).

Dual-Luciferase Reporter Assay

hRMECs were co-treated with the VEGFA-WT/VEGFA-Mut (the pmirGLO vectors subcloned with the wild- or mutant- type sequences of VEGFA 3'UTR) and miR-181d-5p mimics/NC mimics. The measurement of the luciferase activities was performed at 48 hours post transfection using the corresponding detection system (Promega, Madison, WI, USA).

Quantitative Real-Time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) was used for extracting total RNA and synthesizing cDNA, respectively, followed by performing TaqMan PCR kit and SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, USA) on the ABI 7500 Fast Real-Time PCR system. Primer sequences of miR-181d-5p and VEGFA are listed in Table I.

Western Blot

RIPA buffer and a BCA Kit (Beyotime Biotechnology, Shanghai, China) were used to obtain total protein and to quantify protein concentration, respectively. After resolving by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) and transferring onto the polyvinylidene difluoride membranes (PVDF), the proteins were blocked and incubated with antibodies (Cell Signaling Technology, Boston, MA, USA), including VEGFA (#65373), β -actin (#4970) and IgG secondary antibody (#7074), followed by visualizing the immunoreactive bands using enhanced chemiluminescence (ECL).

CCK-8 Assay

HRMECs cells were seeded with 100 μ l medium into a 96-well plate (2×10^3 cells/well). After indicated treatments, the conditioned medium was replaced by 10 μ l CCK-8 solution (Beyotime Biotechnology). After another hour, the plate was detected using a microplate reader (450 nm, BioTek, Hercules, CA, USA).

Transwell Assay

The upper chamber was added with the resuspended hRMECs cells (100 μ L, 3×10^5 cells/mL), while the lower chamber contained 600 μ L medium containing 10% FBS. Twenty-four hours later, the migrated cells were fixed using paraformaldehyde, followed by crystal violet staining and visualization under microscopy (Olympus, Tokyo, Japan) of five random views.

Tube Formation Assay

Cells (1.5×10^4 /well) were pre-coated with 30 μ L Matrigel matrix in a 96-well plate and cultured for 24 hours. Then, the formed capillary-like structure was observed using a microscope and the endothelial tube number was calculated.

Statistical Analysis

Differences (mean \pm SD) were assessed using SPSS software (Version 20.0, IBM Corp., Armonk, NY, USA) and were analyzed by Student's *t*-test or ANOVA, followed by Tukey's post-hoc test. A *p*-value lower than 0.05 was considered significantly different.

Results

MiR-181d-5p is Downregulated While VEGFA is Upregulated in HG-Induced hRMECs

The expression of miR-181d-5p and VEGFA was evaluated in hRMECs under the NG or HG

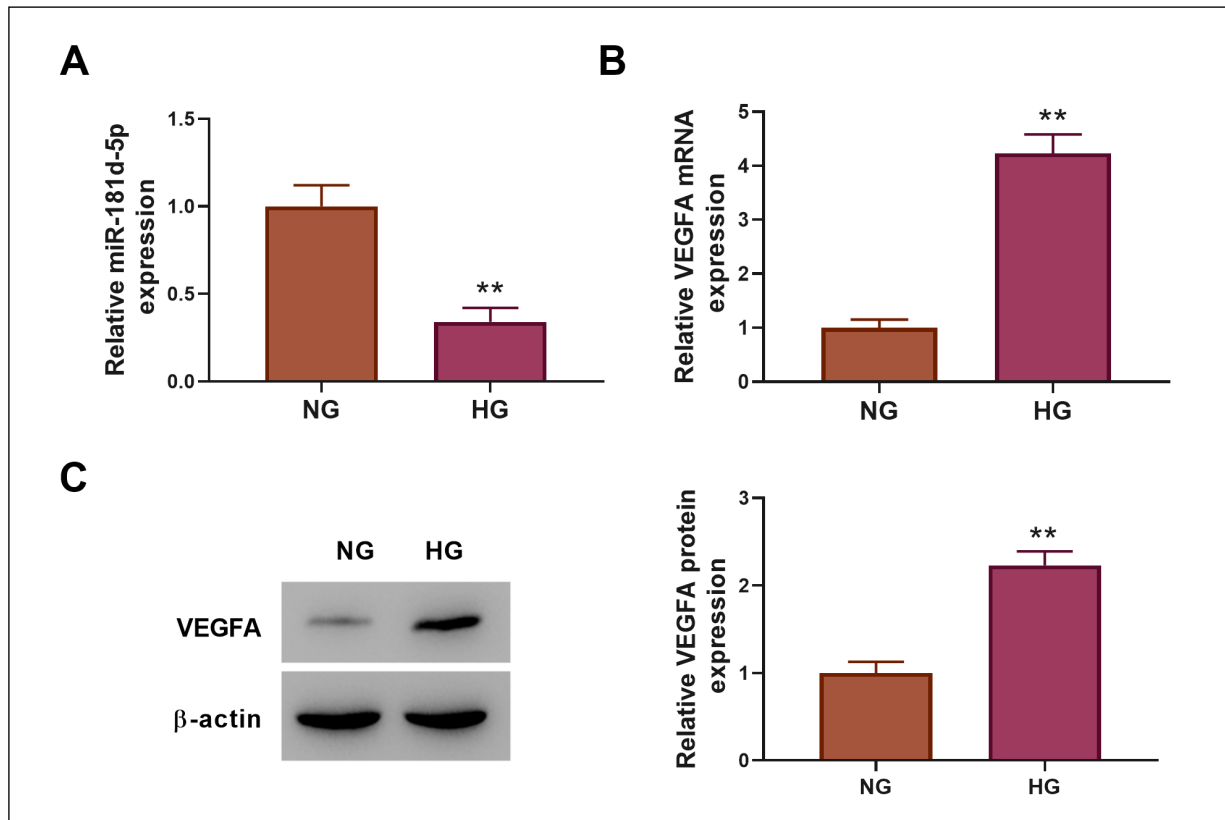


Figure 1. MiR-181d-5p is downregulated while VEGFA is upregulated in HG-induced hRMECs. **A**, miR-181d-5p and **(B)** VEGFA were detected in hRMECs exposed to normal- or high- glucose (NG or HG) using RT-qPCR analysis. **C**, Protein level of VEGFA was assessed by Western blot analysis in hRMECs exposed to normal- or high- glucose (NG or HG). Data (mean ± SD) were performed in triplicate from 3 different experiments. ** $p < 0.01$.

condition. According to qRT-PCR analysis, the HG condition resulted in a significant decrease in miR-181d-5p in hRMECs compared to those exposed to the NG condition (Figure 1A), while there was an increase in VEGFA gene expression (Figure 1B) and its protein level (Figure 1C).

In Vitro Effect of Mir-181d-5p on hRMEC Treated with HG

Transfection efficiency was determined by qRT-PCR in hRMECs, following treatment with the inhibitor (Figure 2A) and mimics (Figure 3A) for miR-181d-5p. The viability, migration and angiogenesis were elevated in hRMECs receiving HG treatment, which was further promoted by miR-181d-5p inhibitors (Figure 2B, C, D) but suppressed by the upregulation of miR-181d-5p (Figure 3B, C, D).

VEGFA Serves as a Target of MiR-181d-5p

MiR-181d-5p was predicted to have several potential binding sites to VEGFA using the star-Base V3.0 website (Figure 4A). The miR-181d-

5p mimics significantly suppressed the luciferase reporter activities in VEGFA-WT instead of VEGFA-Mut (Figure 4B). VEGFA was negatively regulated by miR-181d-5p, as demonstrated by qRT-PCR (Figure 4C) and western blotting (Figure 4D) analysis.

In Vitro Effect of Mir-181d-5p on HG-Treated hRMEC Treated with HG via Targeting VEGFA

VEGFA expression in HG-treated hRMEC was successfully increased by pcDNA-VEGFA (Figure 5A), resulting in the reversal of HG-induced miR-181d-5p overexpression and suppression on hRMEC viability (Figure 5B), migration (Figure 5C) and angiogenesis (Figure 5D).

Discussion

Abnormal retinal neovascularization is the main pathological feature of DR, which could

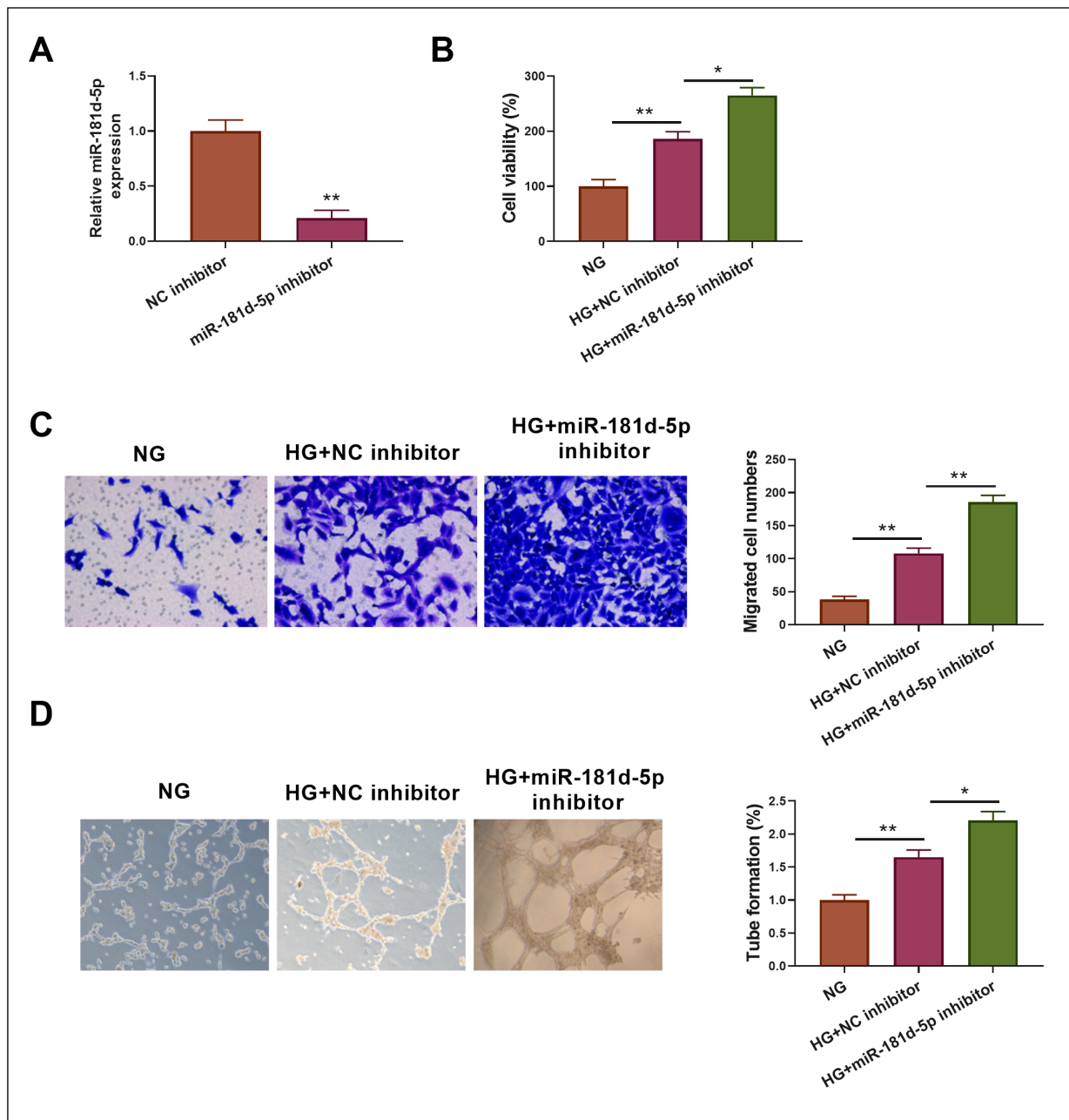


Figure 2. Knockdown of miR-181d-5p increases HG-induced hRMEC proliferation, migration and angiogenesis. **A**, miR-181d-5p were determined in hRMECs treated with its inhibitors using RT-qPCR analysis. **B–D**, Cell biology of hRMECs with miR-181d-5p knockdown under NG (normal glucose) or HG (high glucose) condition was detected using the following methods: CCK-8 (**B**), Transwell (**C**) and tube formation (200x) (**D**) assays (200x). Data (mean \pm SD) were performed in triplicate from 3 different experiments. The symbol of * and ** indicated $p < 0.05$ and $p < 0.01$, respectively.

result in vision loss^{20,21}. There is considerable evidence^{22,23} which indicates that endothelial cell proliferation, migration and capillary lumen formation are important processes of angiogenesis. HG treatment has been confirmed to increase the proliferation, migration and angiogenesis of hRMECs *in vitro*^{24–26}. In the pres-

ent study, HG treatment in hRMECs resulted in downregulation of miR-21-5p and upregulation of VEGFA, which triggered cell proliferation, migration and angiogenesis.

An extensive body of evidence suggests that several miRNAs are aberrantly expressed during the progression of DR and associat-

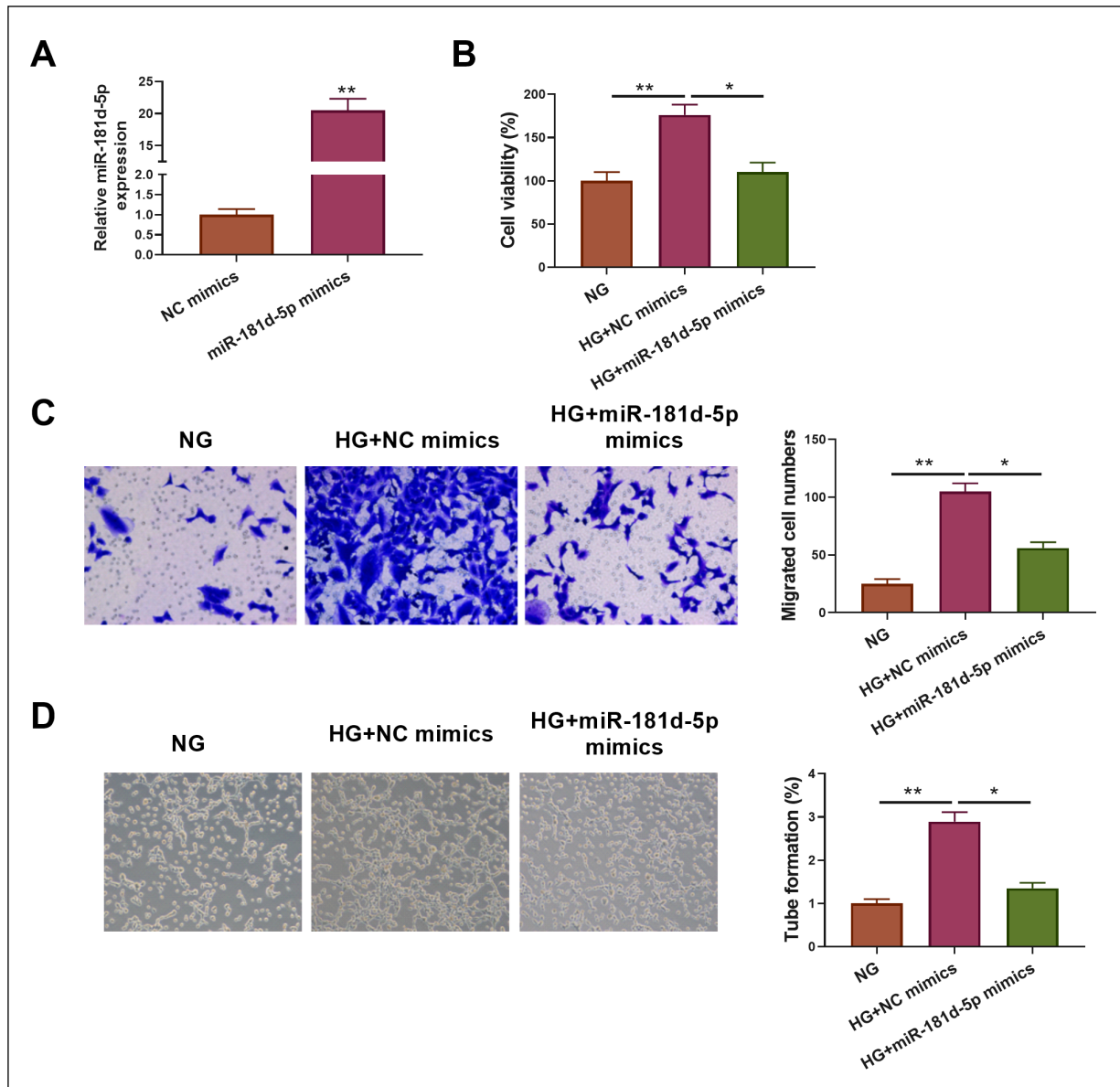


Figure 3. MiR-181d-5p alleviates proliferation, migration and angiogenesis in HG-treated hRMECs. **A**, MiR-181d-5p content was determined in hRMECs treated with its mimics using RT-qPCR analysis. **B-D**, Cell biology of hRMECs with miR-181d-5p knockdown under NG (normal glucose) or HG (high glucose) condition was detected using the following methods: CCK-8 (**B**), Transwell (**C**) and tube formation (200x) (**D**) assays (200x). Data (mean \pm SD) were performed in triplicate from 3 different experiments. The symbol of * and ** indicated $p < 0.05$ and $p < 0.01$, respectively.

ed with hRMEC dysfunction. For example, miR-21-5p inhibition attenuated HG-induced hRMEC proliferation, migration and angiogenesis *via* PI3K/AKT and ERK pathways²⁷. However, the cytoprotective effect of miR-199a-3p was revealed on HG-triggered hRMECs⁷. Research suggests that the miRNA tumor suppressive effects of miR-181d-5p in non-small-cell lung cancer further suppressed tumor growth

and metastasis¹⁴⁻²³. This suppression inhibited the angiogenic capacity of human umbilical vein endothelial cells induced by oxidative stress caused by the decreased cell proliferation and migration²⁸. In our study, we showed that HG treatment downregulated miR-181d-5p expression in hRMECs, in line with previous research, which demonstrated a decrease in miR-181d-5p in patients with DR¹⁶. In HG-exposed

hRMECs, miR-181d-5p inhibited cell viability, migration and angiogenesis.

VEGFA, a master regulator of angiogenesis, is reported to be forcefully upregulated in DR^{29,30}. Indeed, it has recently been shown that VEGFA was regulated by several miRNA, thus participating in HG-induced hRMEC dysfunction³¹⁻³³. In our study, VEGFA content within hRMECs was aversely modulated by miR-181d-5p, demonstrating their associated relationship. *In vitro* experiments confirmed that VEGFA overexpression reversed the protective effects of miR-181d-5p

against HG-challenged hRMECs, implying miR-181d-5p targeting of VEGFA may disturb angiogenesis during DR.

Limitations

The present study had some limitations. The results of the *in vitro* experiments need further *in vivo* investigations, such as an animal model of DR or clinical studies to confirm current conclusions and thoroughly understand the potential therapeutic possibility presented here. Whether there are other signaling pathways, downstream

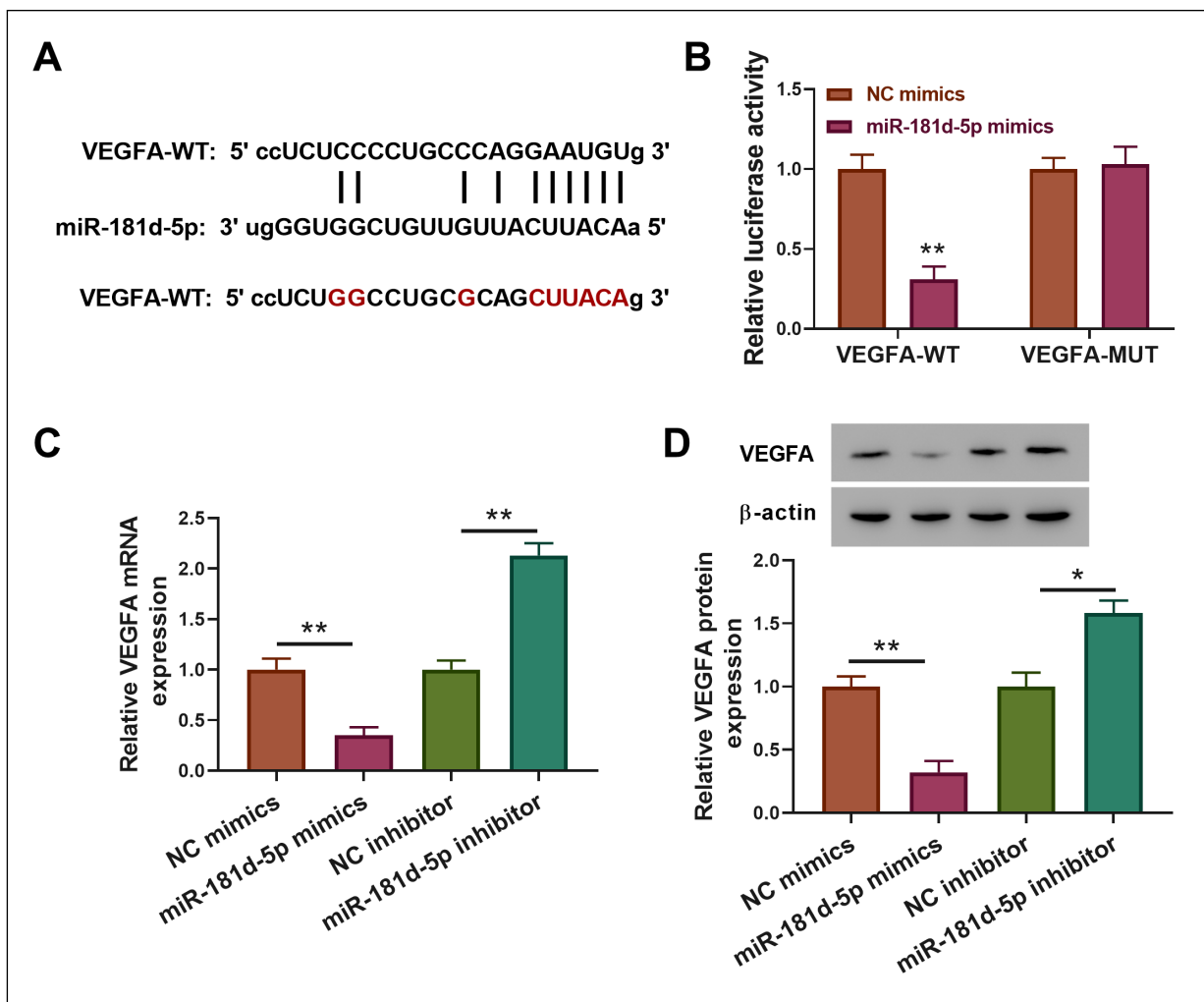


Figure 4. VEGFA in hRMECs served as a target of miR-181d-5p. **A**, The predicted sites between miR-181d-5p and its target gene VEGFA. **B**, Luciferase activity was determined in hRMEC after treatment with the VEGFA-WT/VEGFA-Mut and miR-181d-5p mimics/NC mimics. **C-D**, VEGFA content was determined in hRMECs with miR-181d-5p overexpression or inhibition. Data (mean \pm SD) was performed in triplicate from 3 different experiments. The symbol of * and ** indicated $p < 0.05$ and $p < 0.01$, respectively.

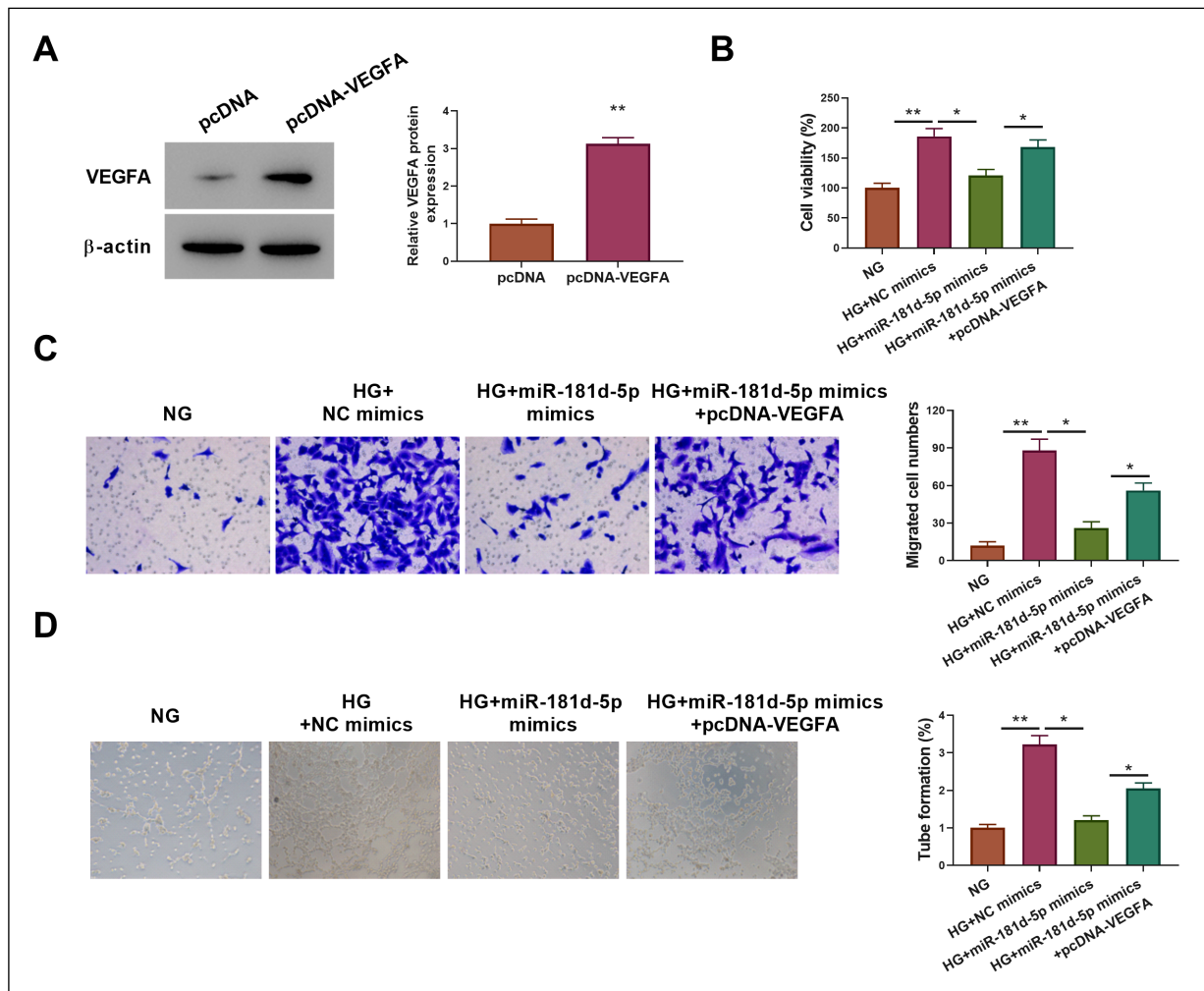


Figure 5. *In vitro* effect of miR-181d-5p on HG-treated hRMEC treated with HG *via* targeting VEGFA. **A**, Overexpression efficiency of VEGFA in hRMECs was examined using western blot analysis. **B-D**, Cell biology of hRMECs with miR-181d-5p knockdown under NG (normal glucose) or HG (high glucose) condition was detected using the following methods: CCK-8 (**B**), Transwell (**C**) and tube formation (200x) (**D**) assays (200x). Data (mean \pm SD) were performed in triplicate from 3 different experiments. The symbol of * and ** indicated $p < 0.05$ and $p < 0.01$, respectively.

of miR-181d-5p, involved in the process also need to be investigated.

Conclusions

The results presented within this study are the first to show the protective effects of miR-181d-5p against HG-triggered hRMECs *via* the direct targeting of VEGFA. Specifically, miR-181d-5p ameliorates HG-stimulated proliferation, migration, and angiogenesis of hRMECs through inhibition of VEGFA. These results further our understanding of DR progression

and provide possible therapeutic targets for future clinical investigation.

Conflict of Interests

None.

Consent for Publication

The submission and the policy of the journal were agreed by all authors.

Availability of Data

The data in this experiment can be requested from the corresponding authors.

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Authors' Contributions

The author contribution is listed as follows: conduction of the experiments: FW and CY; writing and revising of manuscript: FW and CY; data analyses: CY. The manuscript has been read and approved by all authors.

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