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Original Article

Upregulation of miR-181a-5p represses VEGF pathway in high glucose treated human retinal endothelial cells

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Abstract: Purpose: In this study, we examined the function of microRNA 181a-5p (miR-181a-5p) in regulating vascular endothelial growth factor (VEGF) signaling pathway in human retinal endothelial cells (hRECs) under high glucose (HG) conditions. Methods: HRECs were cultured *in vitro*, and treated with 25 mM D-glucose to induce HG condition. Quantitative RT-PCR (QRT-PCR) was used to evaluate the gene expressions of VEGF, and its receptor VEGF-R1 and VEGF-R2, and cell viability was examined by MTT assay in hRECs under HG condition. Possible binding of miR-181a-5p on VEGF-R1 was evaluated by dual-luciferase reporter assay. The effect of HG on miR-181a-5p expression in hRECs was examined by qRT-PCR. MiR-181a-5p was ectopically upregulated in hRECs by lentiviral transduction. The effect of miR-181a-5p upregulation on VEGF signaling pathway and cell viability was re-examined in hRECs under HG condition. Results: HG upregulated VEGF and its receptor VEGF-R1/R2, as well as reduced cell viability in hRECs. MiR-181a-5p was confirmed to be an upstream regulator of VEGF-R1, and was shown to be downregulated in hRECs under HG condition. Lentivirus-induced miR-181a-5p upregulation had no effect on VEGF or VEGF-R2 expressions, whereas suppressed VEGF-R1 gene expression in hRECs. Though only affected VEGF-R1, miR-181a-5p upregulation was sufficient to rescue viability in hRECs under HG condition. Conclusions: MiR-181a-5p suppressed VEGF-R1 expression, thus rescued HG-induced viability reduction in hRECs. Out data suggests miR-181a-5p, through the interaction on VEGF-R1, played an important role in regulating diabetic retinopathy.

Keywords: Diabetic retinopathy, hRECs, miR-181a-5p, high glucose, VEGF

Introduction

Diabetic retinopathy (DR) is the major factor leading to blindness in working-age diabetic patients caused by high glucose level or intraocular hypertension [1]. The underling pathologic mechanisms of DR are very complex, involving multiple signaling pathways involved, such as inflammatory pathways, neural pathways, or vascular pathways [2]. Studies have found that vascular endothelial growth factor (VEGF) is closely associated with ocular pathology, often upregulated in retinal tissues in diabetic patients [3, 4], and anti-VEGF treatment, such as intravitreal pegaptanib or ranibizumab could improve visual acuity in patients with DR [5, 6]. Under experimental diabetic retinopathy, high level of glucose was found to upregulate VEGF and its receptors, VEGF-R1 and VEGF-R2 in retinal microvascular cells or endothelial cells, thus reducing cell viability or inducing apoptosis [4, 7]. However, the complete understanding toward the molecular mechanism of high glucose (HG) induced VEGF signaling pathway upregulation in retinal tissues is lacking and much more efforts are needed to unravel the molecular profile of DR.

MicroRNAs (miRNAs) are groups of short-length (18~22 nt long) non-coding RNAs that post-transcriptionally suppress gene expression or protein production by binding the 3' untranslated region (UTR) of target genes [8]. In recent decades, emerging evidence has shown that aberrant miRNA expressions were closely associated with diabetes or DR [9-11]. In a study evaluating the miRNA expression in streptozotocin induced diabetic rats, both upregulated and downregulated miRNAs were found during the process of DR [11]. Furthermore, miRNAs were found to play functional roles in DR regulation. For example, in one study on streptozotocin induced diabetic rats, MacArthour and colleagues discovered that miR-200b inhibited

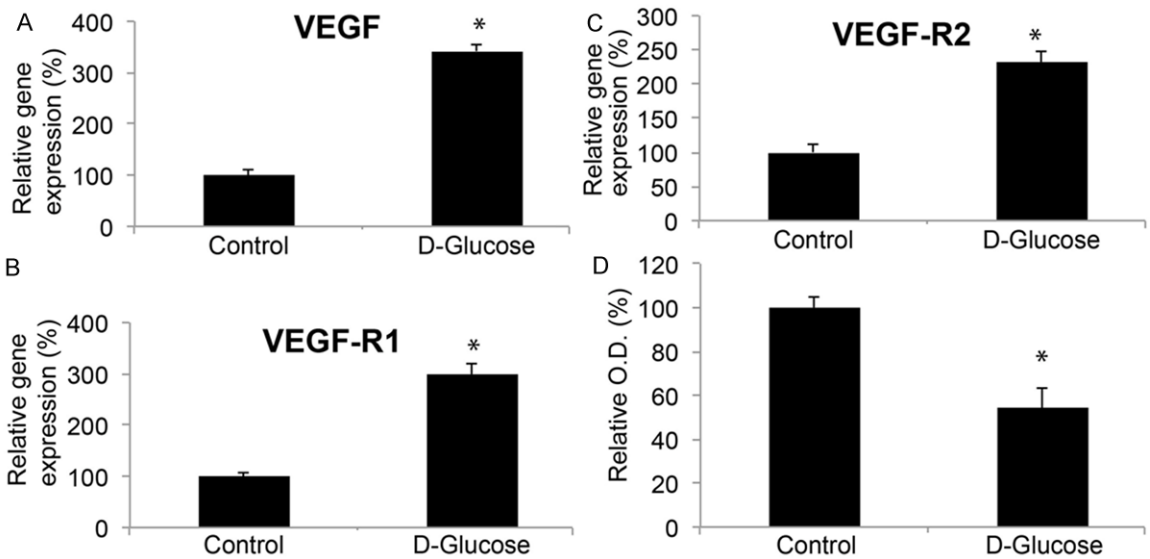

Figure 1. The effect of high glucose on hRECs. Human retinal endothelial cells (hRECs) were cultured in vitro and treated with 25 mM D-glucose for 48 hours. Control hRECs were treated with mock solution (RPE medium). QRT-PCR was used to measure the mRNA levels of VEGF (A), VEGF-R1 (B) and VEGF-R2 (C) ($^*P<0.05$). (D) After high glucose treatment, hRECs were continuously cultured for another 72 h, followed by a MTT assay to measure cell viability ($^*P<0.05$).

VEGF production in DR [12]. In another study on human retinal endothelial cells (hRECs), Haque and colleagues demonstrated that miR-152 regulated (pro)renin receptor to mediate HG-induced VEGF and BEGF-R2 elevation [13]. However, little is known whether miRNAs may direct bind the other VEGF receptor, VEGF-R1, to regulate HG induced VEGF regulation in DR.

Family of miR-181a was found to be abundantly expressed in retina [14]. It was recently shown that miR-181a was downregulated in pathologic eye, and its upregulation inhibited the epithelial-mesenchymal transition in of postoperative residual lens epithelial cells [15]. In the present study, we cultured hRECs *in vitro* and introduced HG in the culture to induce DR-related VEGF upregulation. We then evaluated the expression of microRNA 181a-5p (miR-181a-5p), a putative VEGF-R1 upstream regulator, under the influence of HG in hRECs. Furthermore, we used lentiviral transduction to ectopically upregulate miR-181a-5p in hRECs to examine its effect on HG-induced VEGF signaling pathway regulation.

Materials and methods

HREC culture and high glucose treatment

Human retinal pigment endothelial cells (hRECs) were obtained from Angio-Proteomie (Angio-Proteomie, Boston, MA, USA), and maintained *in vitro* according to the method described previously [16]. Briefly, after thawing, hRECs were cultured in 6-well plates (5×10^5 cells/well) with RPE medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) over-night at 37°C with 5% CO_2. On second day, floating and unhealthy cells were aspirated. Culture was replenished with fresh RPE medium with 5% FBS and maintained for 4 weeks. HRECs between passages 3 and 5 were used for the experiments. For high glucose (HG) treatment, hRECs were treated with 25 mM D-glucose (Sigma-Aldrich, USA) for 48 h.

RNA extraction and quantitative RT-PCR

HRECs were washed three times with ice-cold PBS. Total RNA was extracted with an RNeasy kit (Invitrogen, USA), and cDNA was reverse-transcripted with SuperScript II reverse transcriptase (Invitrogen, USA) at 37°C for 1 h. To detect mRNA expression levels VEGF, VEGF-R1 and VEGR-R2, quantitative real-time PCR (qRT-PCR) was carried out using SYBR Green Real-Time PCR Master Mix Kit (Applied Biosystems, USA) with 0.2 μM TaqMan primers. GAPDH was the control probe. In addition, to detect miR-181a-5p gene expression, a TaqMan MicroRNA Assays (Applied Biosystems, USA) was per-

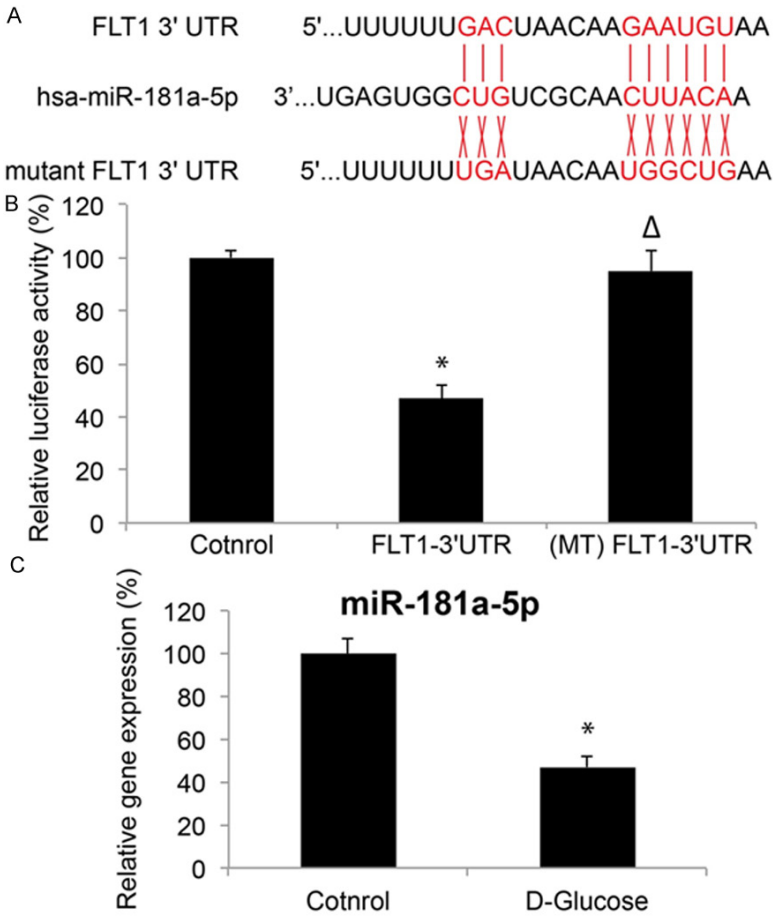

Figure 2. MiR-181a-5p targets VEGF-R1 and was regulated by HG in hRECs. A. The schematic cartoon was shown for the binding site of human miR-181a-5p on the 3'-UTR of VEGF-R1 gene FLT1. The mutant 3'-UTR of FLT1 with abolished binding by miR-181a-5p was also cartooned. B. HEK293T cells were co-transfected with a luciferase reporter containing wild type FLT1-3'UTR, a luciferase reporter containing mutant (MT) FLT1-3'UTR, or an empty reporter (Control), along with miR-181a-5p for 48 h. Relative luciferase activity for each reporter was measured by a dual-luciferase reporter assay and normalized as percentage against control ($^*P < 0.05$; $\Delta > 0.05$). C. After HG treatment, gene expressions of miR-181a-5p in hRECs were measured by qRT-PCR ($^*P < 0.05$).

formed with u6 sRNA as control probe. All PCR reactions were carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, USA) with manufacturer's recommended conditions.

Viability assay

HRECs were suspended from 6-well plates and re-cultured in 96-well plate (3.5×10^3 cells/well). After HG treatment, culture medium was replenished and hRECs were maintained for another 72 hours. The viability of hRECs was examined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen, USA) according to the manufacturer's recommendations. Optical density (O.D.) at 570 nm of each well was measured through a SpectraMax M5 fluorescence microplate reader (Molecular Devices, USA) and normalized to the O.D. under control condition.

Lentiviral transfection of miR-181a-5p

Lentivirus of mature hsa-miR-181a-5p mimics (Lenti-miR181-mimics), and a scrambled miRNA lentivirus (Lenti-C) were purchased from SunBio (SunBio Medical Biotechnology, China). For lentiviral transduction, hRECs were incubated with Lenti-miR181-mimics or Lenti-C (MOI =20 ~ 30) and 8 μg/ml polybrene for 48 h. After replenished with fresh culture medium, hRECs were cultured for another 3 or 5 days and transduction efficiency was evaluated by qRT-PCR.

Dual-luciferase reporter assay

The 3'-untranslated region (3'-UTR) of human VEGF-R1 gene (FLT1), which contains the putative miR-181a-5p binding sequences, was inserted between SpeI/HindIII restriction sites of a pmiR-REPORT luciferase plasmid (Applied Biosystems, USA) to generate a wild type FLT1 luciferase vector (FLT1-3'UTR). The miR-181a-5p at FLT1 3' UTR was point-mutated by a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, USA), and then inserted into pmiR-REPORT plasmid to generate a mutant (MT) FLT1 luciferase vector ((MT) FLT1-3'UTR). Human HEK293T cells were maintained at 37°C and co-transfected with FLT1-3'UTR, (MT)

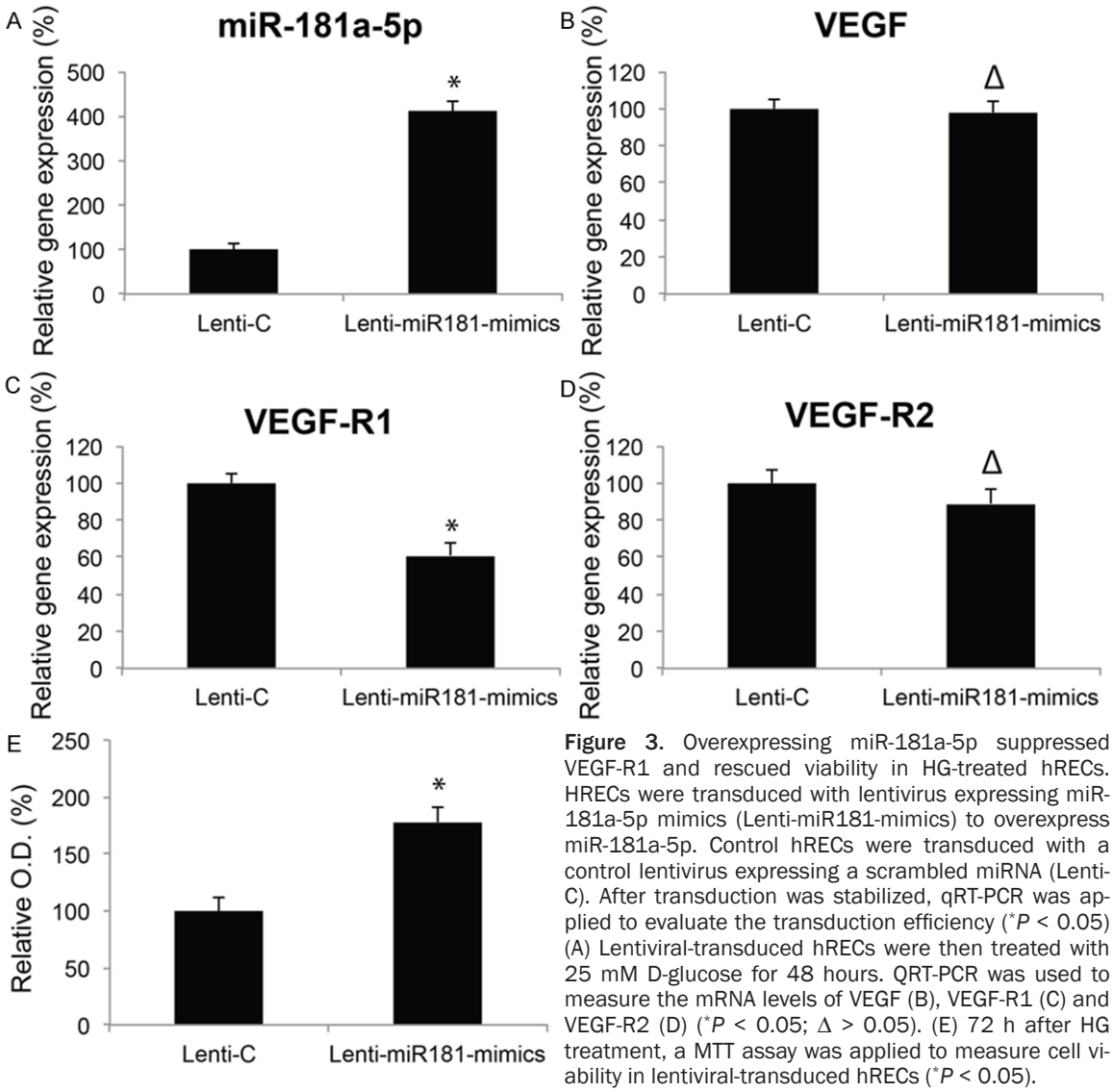

Figure 3. Overexpressing miR-181a-5p suppressed VEGF-R1 and rescued viability in HG-treated hRECs. HRECs were transduced with lentivirus expressing miR-181a-5p mimics (Lenti-miR181-mimics) to overexpress miR-181a-5p. Control hRECs were transduced with a control lentivirus expressing a scrambled miRNA (Lenti-C). After transduction was stabilized, qRT-PCR was applied to evaluate the transduction efficiency ($^{*}P < 0.05$) (A) Lentiviral-transduced hRECs were then treated with 25 mM D-glucose for 48 hours. QRT-PCR was used to measure the mRNA levels of VEGF (B), VEGF-R1 (C) and VEGF-R2 (D) ($^{*}P < 0.05$; $\Delta > 0.05$). (E) 72 h after HG treatment, a MTT assay was applied to measure cell viability in lentiviral-transduced hRECs ($^{*}P < 0.05$).

FLT1-3'UTR or an empty pmiR-REPORT luciferase plasmid (Control), along with miR-181a-5p mimics oligonucleotide (GenePharma, Shanghai, China) by Lipofectamine 2000 (Sigma-Aldrich, USA). 48 h after transfections, hRECs were lysed and relative firefly luciferase activities were measured with a dual-luciferase reporter assay (Promega, USA) according to the manufacturer's recommendations.

Statistical analysis

All experiments were repeated at least three times. Data were presented as mean ± S.E.M. SPSS 13.0. software (SPSS, Chicago, IL, USA) was used for statistical analysis. Comparisons were made by student's t-test. $P<0.05$ was determined to be statistically significant.

Results

High glucose upregulated VEGF signaling pathways and reduced viability in hRECs

We firstly evaluated the diabetic effect of high glucose (HG) on hRECs. HRECs were cultured *in vitro* and treated with 25 mM D-glucose for 48 h. The results of qRT-PCR showed that gene expression level of VEGF and its receptors, VEGF-R1 and VEGF-R2, were all significantly upregulated in hRECs (**Figure 1A-C**, $^{*}P < 0.05$). It also demonstrated that HG dramatically reduced viability in hRECs (**Figure 1D**, $^{*}P < 0.05$).

MiR-181a-5p targets VEGF-R1 and was downregulated by HG in hRECs

We then wondered what the molecular pathways were associated with HG induced VEGF

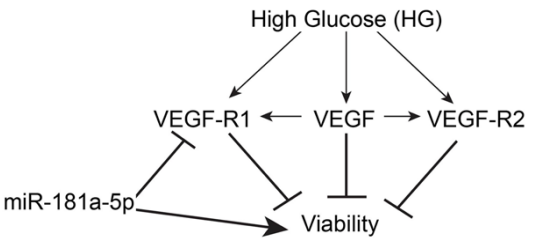

Figure 4. A summary showing the mechanisms of miR-1815a-5p in regulating VEGF signaling pathway under HG condition in hRECs. Arrowheads represent stimulation whereas Ts represent inhibition.

upregulation in hRECs. Using some of the online bioinformatics tools, such as TargetScan (www.targetscan.org), we identified miR-181a-5p was very likely the upstream regulator of VEGF-R1, as it may bind the 3'UTR of FLT1 (VEGF-R1) gene (**Figure 2A**). Thus, we constructed two luciferase plasmids. One is to include the wild type FLT-1 3'UTR. The other is to include a mutated FLT-1 3'UTR with abolished binding site of miR-181a-5p. Then we performed a dual-luciferase reporter assay and demonstrated that FLT1 gens was indeed the downstream target of miR-181a-5p (**Figure 2B**, $^{*}P < 0.05$). Furthermore, we measured the gene expression level of miR-181a-5p under the influence of HG in hRECs. The result of qRT-PCR showed that miR-181a-5p expression was significantly downregulated by HG treatment (**Figure 2C**, $^{*}P < 0.05$).

MiR-181a-5p upregulation inhibited VEGF-R1 and restored viability in HG-induced hRECs

We then asked whether miR-181a-5p had a functional role in regulating VEGF signaling pathways in HG treated hRECS. We constructed lentivirus containing miR-181a-5p mimics (Lenti-miR181-mimics), and transduced it into hRECs to ectopically overexpress miR-181a-5p. The control hRECs were transduced with a lentivirus containing a scrambled miRNA (Lenti-C). After lentiviral transduction was stabled, the transduction efficacy was verified by qRT-PCR, which shows miR-181a-5p expression was considerably upregulated by Lenti-miR181-mimics in hRECs (**Figure 3A**, $^{*}P < 0.05$).

We also examined effect of miR-181a-5p upregulation on VEGF signaling pathways in hRECs. The results of qRT-PCR showed that gene expression levels of VEGF and VEGF-R2 were not affected by miR-181a-5p upregulation (**Figure 3B**, **3D**, $^{*}\Delta < 0.05$). However, expression of VEGF-R1, the downstream target of miR-181a-5p, was significantly downregulated by miR-181a-5p upregulation (**Figure 3C**, $^{*}P < 0.05$).

Finally, we examined effect of miR-181a-5p upregulation on HG-induced growth inhibition in hRECs. We treated the lentiviral-transduced hRECs with 25 mM D-glucose for 24 h, followed by a MTT assay in another 72 h. The result demonstrated that, more viably cells were observed in miR-181a-5p upregulated hRECs than in control hRECs (**Figure 3E**, $^{*}P < 0.05$).

Therefore, our results strongly suggest that miR-181a-5p upregulation protected hRECs from HG-induced reduced viability through the inhibition on VEGF-R1 receptor.

Discussions

In diabetic retinopathy, activation of VEGF signaling pathways is always associated with glucose elevation [4, 7, 17]. In the present study, we used qRT-PCR to show that in an *in vitro* explant of human retinal endothelial cells, HG treatment elevated the mRNA levels VEGF as well as its receptors VEGF-R1 and VEGF-R2. Also, we showed that cell viability was significantly reduced in hRECs under HG condition. These results are in line with previous studies showing that VEGF pathways were upregulated and cell apoptosis was induced by high concentrations of D-glucose in hRECs [13, 18, 19], thus proving that hRECs explant with HG treatment is a very reliable *in vitro* model to study the cellular and molecular mechanisms in diabetic retinopathy.

The mechanism of VEGF activation under HG condition is extreme complex. Studies have suggested that multiple signaling pathways were involved, such as PKC/ERK pathway [20], Angiotensin converting enzyme pathway [21, 22], or hypoxia-inducible factor-1α/p300 pathway [23]. Interestingly, recent studies also demonstrated that, besides the conventional molecular pathways, epigenetic regulation of miRNAs played important role in VEGF regulation in DR. In a genomic study, McArthur and colleagues demonstrated that upregulating miR-200b effectively reduced VEGF production *both in vitro* and *in vivo*, and protected

HG-induced diabetic angionenesis [12]. In another study, Haque and colleagues showed that miR-152 inhibited VEGF/VEGF-R2 elevation through the suppression on (pro)renin receptor [13]. In the present study, we identified a novel miRNA candidate, miR-181a-5p to be the mechanistic regulator in VEGF regulation. Using dual-luciferase assay, we showed that miR-181a-5p was the upstream regulator of VEGF-R1. Using qRT-PCR, we also demonstrated that miR-181a-5p was substantially downregulated in hRECs under HG condition. Most importantly, functional assays confirmed that miR-181a-5p upregulation could suppress the production of VEGF-R1 mRNA, thus subsequently protected hRECs from HG-induced cell death. Therefore, our data presented strong evidence revealing a critical role of miR-181a-5p in DR through the regulation on VEGF signaling pathways.

Another interesting finding of our study is that, while miR-181a-5p had significantly inhibitory effect on VEGF-R1 mRNA production in hRECs, VEGF and VEGF-R2 productions were little affected. It is worth noting that, no collateral inhibition or compensatory elevation of VEGF-R2 was observed while VEGF-R1 was downregulated by miR-181a-5p, thus suggesting that the inhibitory effect of miR-181a-5p on VEGF-R1 is specific and selective. It is also worth noting that, inhibition on VEGF-R1 alone by miR-181a-5p was able to rescue hREC viability. This suggests that, VEGF activating VEGF-R1 may be the predominant pathologic pathway in hRECs under HG condition.

In summary, our study described the effects of a novel miRNA, miR-181a-5p in regulating VEGF signaling pathways in human retinal endothelial cells in diabetic retinopathy (**Figure 4**). The finding of our work may help identify novel genetic biomarker or develop new treatment strategy to prevent vision loss for patients suffered from diabetic retinopathy.

Disclosure of conflict of interest

None.

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