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MicroRNA-126 inhibits cell viability and invasion in a diabetic retinopathy model via targeting IRS-1

SHIFENG FANG[1], XIANG MA[1,2], SUPING GUO[2] and JIANMIN LU[2]

[1]Dalian Medical University, Dalian, Liaoning 116044; [2]Department of Ophthalmology, The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011, P.R. China

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Abstract. Diabetic retinopathy (DR) is a sight-threatening complication of diabetes. IRS-1 was predicted to be the target gene of microRNA-126 (miR-126). The present study was designed to illustrate the involvement of miR-126 in the regulation of DR via targeting IRS-1. The present study revealed that the expression of miR-126 was significantly decreased while IRS-1 expression was increased in endothelial cells (ECs) and retinal pericytes (RPs) from a DR mouse model compared with healthy controls. Furthermore, a luciferase reporter assay confirmed the interaction between miR-126 and IRS-1. Following transfection with anmiR-126 mimic or miR-126 inhibitor, overexpression of miR-126 was demonstrated to suppress the invasion and viability of ECs and RPs and to inhibit the IRS-1 and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway protein expression levels, with inhibition of miR-126 leading to reverse results. Furthermore, transfection with small interfering RNA targeting IRS-1 altered the miR-126-induced effects observed in ECs, indicating that miR-126 may suppress angiogenesis in DR via inhibition ofIRS-1 expression. Taken together, the results of the present study suggested that miR-126 affected the expression of IRS-1, resulting in downregulated expression of PI3K/Akt pathway proteins, and also suppressed cell invasion and viability. These results may provide a potential therapeutic strategy for DR.

Introduction

Diabetic retinopathy (DR) is a leading cause of blindness and visual impairment among adults aged <40 years in the developed world (1). It is a sight-threatening complication of diabetes that is characterized by an early loss of capillary pericytes and thickening of the basement membrane, which may lead to uncontrolled endothelial proliferation and, subsequently, angiogenesis (2). Vascular endothelial growth factor (VEGF) is involved in the development of proliferative DR and diabetic macular edema (3). However, VEGF blocking is likely to produce systemic adverse effects. Thus, it is important to develop novel therapeutic strategies against DR by understanding the molecular mechanisms underlying its development and progression.

Insulin receptor substrate-1 (IRS-1) is a key molecule in insulin signaling, and is involved in signal transduction between the insulin receptor and phosphoinositide 3-kinase (PI3K) (4). IRS-1 is involved in the mediation of the metabolic and mitogenic effects of insulin in peripheral tissues, including skeletal muscle, liver and adipose tissue, by transmitting signals from the insulin receptor to downstream enzymes, which include PI3K, phosphoinositide-dependent kinase 1 and protein kinase B (Akt) (4). VEGF is an angiogenic factor is involved in the maintenance of vascular homeostasis (5) and in pathological angiogenesis in diabetic retinopathy (6). This regulation maybe clinically relevant, because intensive insulin treatment is associated with worsening of DR and is associated with VEGF expression levels. A previous study reported that activation of PI3K/Akt signaling pathway upregulates the expression of VEGF through a direct interaction with the VEGF promoter (7). Regulation ofPI3K/Akt activation is essential for the long term upregulation of VEGF (8). These previous studies suggested that the IRS-1/PI3K/Akt/VEGF pathway may be a promising molecular target for the prevention and treatment of DR.

Vascular abnormalities and pathologies are associated with diabetes (9). Loss of endothelial function and poor arterial collateral formation contribute to the morbidity and mortality of patients with diabetes (10). This is observed in multiple cell types, including endothelial cells (ECs) (11), vascular smooth muscle cells (12) and capillary pericytes (13). The dysfunction of the vasculature may be associated with the loss of the direct action of insulin on vascular cells, which has been demonstrated to be insulin-responsive (13). Insulin stimulates several signaling cascades in EC (8). There is selective inhibition of insulin-induced activation of the IRS-PI3K-Akt pathway in the micro- and macro-vessels of insulin resistant rodents (14).

Correspondence to: Dr Xiang Ma, Dalian Medical University, 9 South Lvshun Road, Dalian, Liaoning 116044, P.R. China
E-mail: maxiangg@sina.com

Abbreviations: DR, diabetic retinopathy; IRS-1, insulin receptor substrate 1; miRNA/miR, microRNA; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; 3'-UTR, 3'-untranslated region

Key words: diabetic retinopathy, insulin receptor substrate 1, microRNA-126, phosphoinositide 3-kinase/protein kinase B pathway

These reports suggested that the regulation of IRS-PI3K-Akt regulate affected angiogenesis in DR.

MicroRNAs (miRNAs/miRs) are an emerging class of small, highly conserved, noncoding RNAs that act as post-transcriptional regulators (15). miR-126 belongs to a highly conserved miRNA family and is located at intron 5 of EGF like domain multiple 7 (16). It may be involved in the control of vascular integrity and angiogenesis (17) as it modulates the release of angiogenic factors, including hypoxia-inducible factor 1-α, matrix metalloproteinases and VEGF, which is crucial in the development of proliferative DR (18).

The present study identified the function ofmiR-126 in the context of DR. Expression of miR-126 was significantly decreased in ECs and retinal pericytes (RPs) compared with healthy controls, resulting in overexpression of the target gene IRS-1, which regulates the PI3K-Akt-VEGF pathway. Taken together, the results suggested that miR-126 and its targeting of IRS-1 may provide novel insights into the treatment of DR.

Materials and methods

Establishment of a DR model and cell culture. Pericytes and their interactions with ECs in the vessel wall are involved in the regulation of vessel formation, stabilization and remodeling (2), so the present study isolated ECs and RPs from a murine DR model. The animals were obtained from the Animal Center of Dalian Medical University. All mice were kept in an environment maintained at 21˚C with 50% humidity under a 12/12 h light/dark cycle. All mice received food and water *ad libitum*. Diabetes mellitus was induced in 30 male C57/BL6 mice of 12 weeks old, weighing 18 to 22 g by a single intraperitoneal injection of streptozotocin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a dose of 100 mg/kg dissolved in 100 mM citrate buffer (pH 4.5). Ten control mice were treated with buffered saline alone. One week later, blood glucose levels were measured, and mice with a fasting-blood glucose of N12 mmol/l were considered diabetic and were used for further study. The DR model was established as previously described (19). The EC and RP cells were isolated according to the protocol as previously described (20,21). The present study was approved by the Animal Ethical and Welfare Committee of Dalian Medical University (Dalian, China).

The ECs and RPs were grown in minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA), 100 U/ml of penicillin and 100 mg/ml of streptomycin. All cells were incubated at 37˚C in a humidified 21% O_2, 5% CO_2 atmosphere.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed using a Roche Light Cycler 480 (Roche Diagnostics, Basel Switzerland), TaqMan MicroRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 200 ng total RNA extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) from 10^6 cells. RNA was reverse transcribed to cDNA using stem-loop primers and the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The obtained cDNA was amplified using a TaqMan miR-126 MicroRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). The67 bp cDNA product was amplified by PCR using the following primers: miR-126 forward, 5'-TATAAGATCTGAGGATAGGTGGGTTCCCGAGAACT-3' and reverse, 5'-ATATGAATTCTCTCAGGGCTATGCCGCCTAAGTAC-3' (22). The reaction mixtures were incubated at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min according to the Stratagene RT-qPCR instrument (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). miR-126 expression was normalized to U6 mRNA expression. Gene mRNA expression was normalized to β-actin. Primers used were as follows: U6forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; β-actin forward, 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and reverse, 5'-ATGGAGCCACCGATCCACA-3'. Relative gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (23). Three independent experiments were performed.

Western blot analysis. Cells (10^7) were lysed in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 μg/ml leupeptin). Protein was quantified using a Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total protein (30 mg) and pre-stained molecular weight markers were separated by 10% SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% Triton X-100) containing 5% nonfat milk at 4˚C overnight, and probed with primary antibodies against IRS-1 (cat. no. sc-8038; dilution, 1:400), VEGF (cat. no. sc-57496; dilution, 1:500), PI3K (cat. no. sc-365290; dilution, 1:400), Akt (cat. no. sc-81434; dilution, 1:500) and β-actin (cat. no. sc-130300; dilution, 1:500) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Then membranes were washed in TBST and incubated with a secondary horseradish peroxidase-conjugated antibody (cat.no. sc-516102; dilution, 1:5,000) for 2 h at room temperature. Following incubation with secondary antibodies, membranes were washed with TBST. An Odyssey CLx Western Blot Detection System (LI-COR Biosciences, Lincoln, NE, USA) was used to measure the band density. The band density of each gene was normalized to the corresponding density of β-actin using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). All experiments were performed in triplicate.

Transfection assay. Recombinant plasmids (2 mg) and 200 pmol miR-126 mimic, miR-126 mimic control, miR-126 inhibitor or miR-126 inhibitor control (cat. nos. 4464066 and 4464084; Ambion; Thermo Fisher Scientific, Inc.) were transfected into $3x10^6$ ECs and RPs for 48 h by electroporation, using a Nucleofector instrument (Lonza Cologne GmbH, Cologne, Germany) according to the manufacturer's protocol. For the IRS-1 interference experiment, HEK293 genomic DNA was used as the PCR template and the DNA fragment encoding mir-126 pre-miRNA (flanking upstream and downstream 30-50 nt) was amplified and inserted into the expressing vector pSilencer4.1CMV-puro (Ambion; Thermo Fisher Scientific, Inc.) as described previously (24). DNA fragments for anti-IRS-1 small interfering RNAs (siRNAs) were

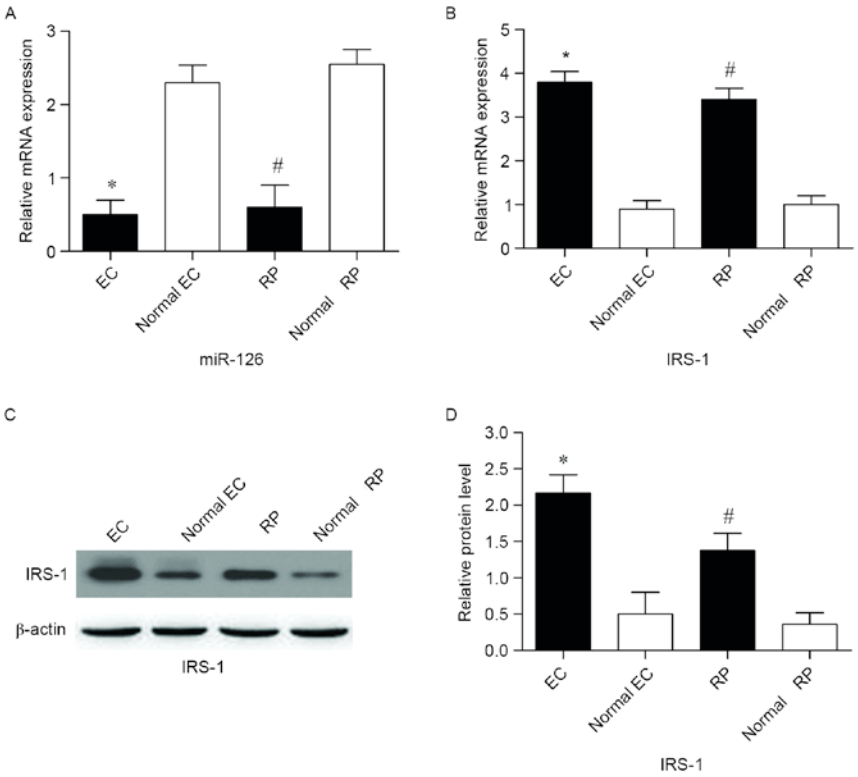

Figure 1. miR-126 and IRS-1 expression levels in ECs and RPs. (A) The expression levels of miR-126 in ECs and RPs compared with normal controls were measured using RT-qPCR. (B) IRS-1 mRNA in in ECs and RPs compared with normal controls was assessed using RT-qPCR. (C) Western blotting analysis was used to detect IRS-1 protein expression in ECs and RPs. (D) Relative protein expression was quantified and normalized to β-actin. Data are presented as the mean ± standard deviation of three experiments. *P<0.05 vs. normal EC group; #P<0.05 vs. normal RP group. miR-126, microRNA-126; IRS-1, insulin receptor substrate-1; EC, endothelial cell; RP, retinal pericyte; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

generated by annealing two complementary oligonucleotides and cloning into pSilencer4.1 CMV-puro vectors, as reported previously (25). Following transfection, the cells were allowed to recover by incubating for 4 h at 37˚C. The experiment was replicated three times.

Dual-luciferase reporter assay. The target gene was predicted using TargetScan Release 3.4 (http://www.targetscan.org/) (26). DNA fragments (414 nt) of the IRS-13'-untranslated region (3'-UTR) containing the predicted miR-126 binding site [IRS1-wild type (wt)] were cloned into the pGL3-promoter plasmid (Promega Corporation, Madison, WI, USA) and the miR-126 binding sites were replaced with 4 nt fragments to produce mutated 3'UTR pGL3-reporter plasmids (IRS1-mut) as previously described (16). The recombinant reporter vectors with normal and or mutated IRS-1 3'-UTRs were co-transfected with miR-126 mimic, miR-126 mimic control, miR-126 inhibitor or miR-126 inhibitor control into EC cells using the Trans Messenger transfection reagent (Qiagen GmbH, Hilden, Germany). The luciferase assay was performed using the Dual Luciferase Reporter Gene Assay kit (cat. no. RG027; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. All the experiments were performed in triplicate. The relative luciferase activities were normalized to that of the control cells.

Cell viability assay. Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were harvested and seeded into 96-well plates at $2x10^3$ cells per well and incubated in MEM medium for 24, 48, 72, 96 and 120 h, respectively. Following this, MEM was discarded and fresh medium containing MTT (5 mg/ml MTT in PBS; Sangon Biotech Co., Ltd., Shanghai, China) was added, and cells were incubated for additional 4 h. Dimethyl sulfoxide were used to dissolve the resultant formazan crystals and the absorbance at 490 nm was measured using an ELISA reader once every 24 h (BioTek Instruments, Inc., Winooski, VT, USA). All the experiments were repeated 5 times.

Invasion assay. A Transwell invasion chamber (Corning Incorporated, Corning, NY, USA) was washed with MEM, and 20 μl Matrigel (1 mg/ml) (BD Biosciences, Franklin Lakes, NJ, USA) was added to evenly cover the surface of the polycarbonate membrane (8 μm pore size) to create a Matrigel membrane. The chamber was divided into upper and lower chambers. For invasion assays, ECs and RPs ($4x10^5$) were serum-starved overnight and seeded into MEM medium containing 10% FBSon the top chamber. The bottom chamber contained 10% FBS in MEM, which acted as a chemoattractant. Following incubation for 48 h at 37˚C, cells from the top chamber were removed using a cotton swab and invading cells were fixed with 4% formaldehyde for 15 min at room temperature, then stained with a 0.1% crystal violet solution for 10 min. We randomly assessed 5 fields of view. The invading cells were photographed using an inverted microscope. We randomly assessed 5 fields of view, and total cell numbers were counted and quantified using ImageJ software (version1.48; National

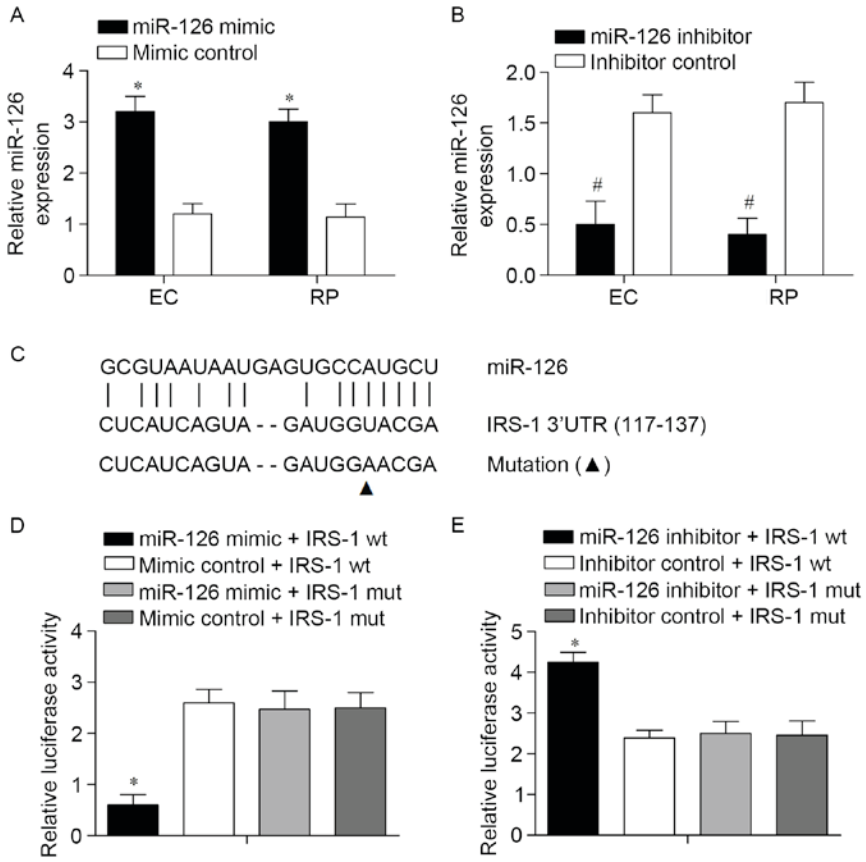

Figure 2. Target relationship between miR-126 and IRS-1. (A) The expression levels of miR-126 in ECs and RPs co-transfected with miR-126 mimic or mimic control, measured by RT-qPCR. (B) The expression levels of miR-126 in ECs and RPs co-transfected with miR-126 inhibitor or inhibitor control were assessed using RT-qPCR. (C) The wt and mut IRS-1 3'-UTR contained the target sequence formiR-126. (D) ECs were co-transfected with the miR-126 mimic or mimic control luciferase reporter vectors containing wt or mut IRS-1 3'-UTR. (E) A similar luciferase assay was performed in ECs transfected with the miR-126 inhibitor or inhibitor control. Luciferase activity were represented as firefly luciferase normalized to renilla luciferase. Data are presented as the mean ± standard deviation of three experiments. *P<0.05 vs. other groups. miR-126, microRNA-126; IRS-1, insulin receptor substrate-1; EC, endothelial cell; RP, retinal pericyte; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; wt, wild type; mut, mutant; 3'-UTR, 3'-untranslated region.

Institutes of Health, Bethesda, MD, USA). The results were presented as the mean ± standard deviation, and the experiment was repeated three times for each group.

Statistical analysis. Data were expressed as the mean ± standard deviation of at least three independent experiments. Multiple comparisons were conducted using a two-way analysis of variance (Tukey's test). Differences between two groups were tested for statistical significance using a paired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-126 and IRS-1 expression levels in ECs and RPs. The expression level of miR-126 was detected in ECs and RPs by RT-qPCR. The results indicated that miR-126 expression levels were significantly lower in ECs and RPs cultured from the DR model compared with normal controls (P<0.05; Fig. 1A). IRS-1 mRNA expression levels were increased in ECs and RPs compared with normal controls, as detected by RT-qPCR (P<0.05; Fig. 1B). IRS-1 protein expression levels were examined by Western blot analysis (Fig. 1C). The results revealed that IRS-1 protein levels were significantly elevated in ECs and RPs compared with normal controls (Fig. 1D).

IRS-1 is targeted by miR-126 in ECs and RPs obtained from a murine DR model. As miR-126levels were decreased in ECs and RPs from the DR model, its involvement in the biology of the two cell types was investigated. miR-126 mimics were used to amplify the expression of miR-126 and a synthetic inhibitor specific to miR-126 was used to suppress the expression of endogenous miR-126 in the ECs and RPs. The efficiency of this miR-126 mimic or inhibitor was confirmed using a qPCR assay (Fig. 2A and B). IRS-1 was predicted to be targeted by miR-126 by the bioinformatic software program TargetScan. According to the results, a potential binding target site of miR-126 was observed in the 3'-UTR of the IRS-1 gene (Fig. 2C). To experimentally confirm IRS-1 as an authentic target of miR-126 in ECs, IRS-1-wt or IRS-1-mut plasmids were transfected into ECs together with miR-126 mimics or mimic controls. Following transfection for 48 h, the results revealed that the luciferase activity in the IRS-1-wt with miR-126 mimic group was significantly reduced compared with the other three groups (Fig. 2D). The IRS-1-wt and IRS-1-mut luciferase reporter vectors were co-transfected with miR-126 inhibitors or inhibitor controls into ECs. The results revealed that transfection with the miR-126 inhibitor reversed the reduction in the expression level of luciferase with wild-type IRS-1 3'UTR in ECs

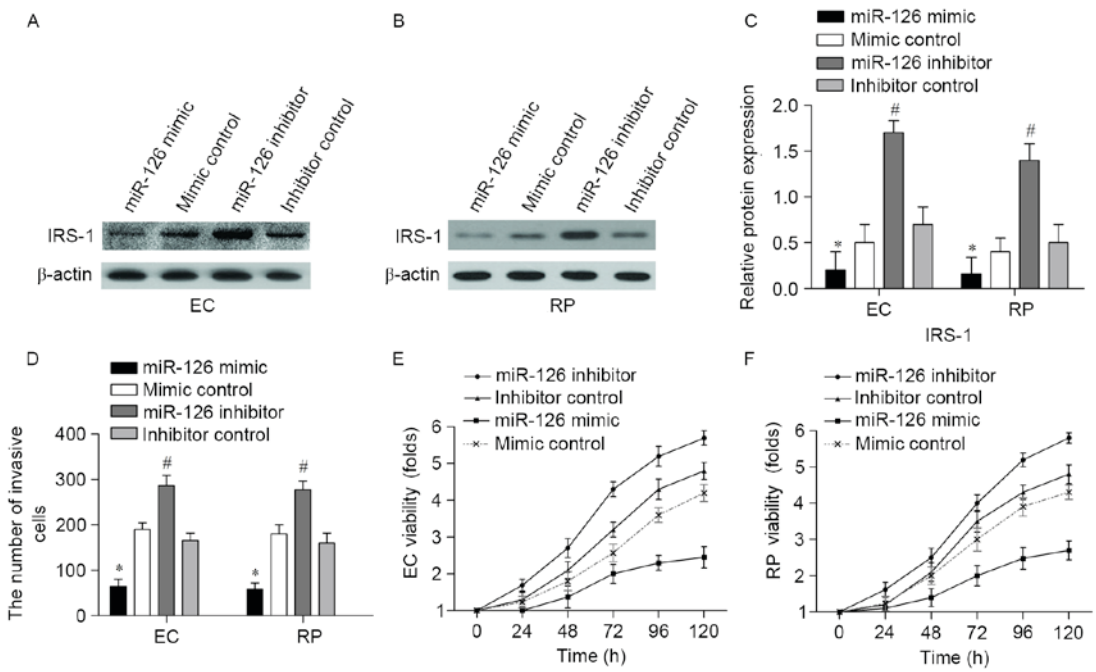

Figure 3. Effect of miR-126 on the invasion and viability of ECs and RPs. ECs and RPs were transfected with the miR-126 mimic, mimic control, miR-126 inhibitor or inhibitor control. Western blotting was performed to measure IRS-1expression levels of IRS-1 in (A) ECs and (B) RPs co-transfected with miR-126 mimic, mimic control, miR-126 inhibitor or inhibitor control. (C) Relative protein expressions in ECs and RPs were normalized to β-actin. (D) The number of invading ECs and RPs. The viability of (E) ECs and (F) RPs was determined at the indicated time points using MTT assays. All experiments were repeated three times with three replicates. *P<0.05 vs. mimic control group; #P<0.05 vs. inhibitor control group. miR-126, microRNA-126; EC, endothelial cell; RP, retinal pericyte; IRS-1, insulin receptor substrate-1.

(Fig. 2E). Taken together, these data demonstrated that IRS-1 is a target of miR-126.

miR-126 suppresses the invasion and viability of ECs and RPs. To further explore the impact of miR-126 in ECs and RPs, the present study examined whether overexpression or inhibition of miR-126 was capable of affecting cell viability. EC and RPs were transfected with miR-126 mimic, mimic control, miR-126 inhibitor or inhibitor control. The efficiency of the miR-126 mimic and inhibitor on the expression of IRS-1 was confirmed by western blotting in ECs and RPs (Fig. 3A and B, respectively). Transfection with the miR-126 mimic significantly decreased IRS-1 protein levels compared with the mimic control, and transfection with the miR-126 inhibitor significantly increased IRS-1 protein levels compared with the inhibitor control (Fig. 3C) Transfection with the miR-126 mimic significantly decreased the number of invasive ECs and RPs compared with the mimic control group (P<0.05; Fig. 3D), while transfection with the miR-126 inhibitor increased the number of invasive ECs and RPs compared with the inhibitor control group (P<0.05; Fig. 3D). In addition, overexpression of miR-126 decreased the viability of ECs compared with the mimic control group, and inhibition of miR-126 promoted EC viability compared with the inhibitor control group (Fig. 3E). Similar MTT results were obtained in RPs, with cell viability decreased by the overexpression of miR-126 compared with the mimic control (Fig. 3F). Furthermore, RP viability was increased following treatment with the miR-126 inhibitor (Fig. 3F). These results suggested that miR-126 suppressed the invasion and viability of ECs and RPs.

miR-126 inhibits the PI3K/Akt pathway by downregulating IRS-1. VEGF is an angiogenic factor that is involved in the maintenance of vascular homeostasis (27) and diabetic retinopathy (28). Insulin-induced VEGF expression has been reported to be mediated through the activation of PI3K/Akt, which is downstream to the insulin receptor (29).

Thus, the effect of miR-126 overexpression and suppression on the expression of IRS-1 and PI3K/Akt pathway was examined. The expression levels of PI3K/Akt pathway-associated genes, including PI3K, Akt and VEGF, were detected using western blotting analysis. The results revealed that transfection with the miR-126 mimic effectively decreased the expression of these proteins compared with cells transfected with the mimic control, while transfection with the miR-126 inhibitor increased the expression of these proteins in ECs compared with the inhibitor control (P<0.05; Fig. 4). Western blotting analysis was also performed in RPs, and decreased expression of these proteins was detected following transfection with the miR-126 mimic compared with the mimic control. Furthermore, transfection with the miR-126 inhibitor significantly increased the expression levels of these proteins compared with the inhibitor control (P<0.05; Fig. 4). These data suggested that the activation of the PI3K/Akt pathway was suppressed by miR-126 overexpression in ECs and RPs.

siRNA interference of IRS-1 nullified the effect of miR-126 inhibitor in ECs. To determine whether interference with IRS-1 expression counteracted the effects of miR-126 in ECs, the miR-126 inhibitor or inhibitor control were co-transfected with or without a siRNA IRS-1 vector into ECs. Western blotting was performed to measure the expression levels of

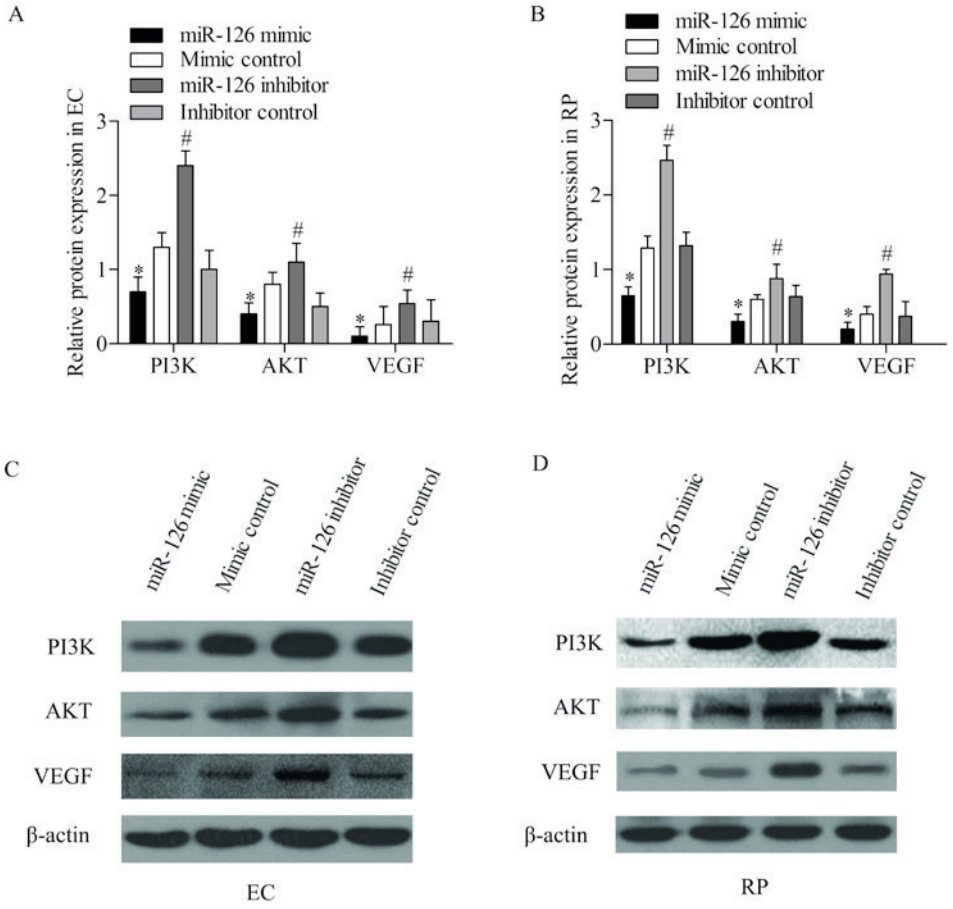

Figure 4. miR-126 regulates thePI3K/Akt pathway via targeting IRS-1. ECs and RPs were transfected with the miR-126 mimic, mimic control, miR-126 inhibitor or inhibitor control. The protein expression levels of IRS-1 and PI3K/Akt pathway proteins were measured by western blotting in (A) ECs and (B) RPs. (C) Relative protein expression levels were normalized to β-actin in (C) ECs (D) and RPs. Data are presented as the mean ± standard deviation of three experiments. *P<0.05 vs. mimic control; #P<0.05 vs. inhibitor control. miR-126, microRNA-126; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; IRS-1, insulin receptor substrate-1; EC, endothelial cell; RP, retinal pericyte; VEGF, vascular endothelial growth factor.

VEGF, PI3K and Akt (Fig. 5A). Compared with the inhibitor control, transfection with the miR-126 inhibitor increased the expression levels of these proteins (P<0.05; Fig. 5B). For the ECs transfected with the miR-126 inhibitor and siRNA IRS-1, the relative protein expression levels were significantly decreased compared with the miR-126 inhibitor transfection group (P<0.01; Fig. 5B) and the expression levels of these proteins were promoted in comparison with the siRNA IRS-1group (P<0.05; Fig. 5B). These results suggested that transfection with siRNA IRS-1 nullified themiR-126-induced inhibition of VEGF pathway protein expression levels in ECs. To further confirm the offset effect of IRS-1 silencing on miR-126 inhibition, we examined the number of invading ECs treated with the miR-126 inhibitor or inhibitor control, with or without the siRNA IRS-1 vector, was assessed. Interference withIRS-1 reversed the effect of miR-126 inhibitor on cell invasion in ECs (Fig. 5C). MTT assays were performed to examine the viability of ECs under similar treatments. Cell viability was increased when ECs were co-transfected with siRNA IRS-1 and miR-126 inhibitor compared with the siRNA IRS-1 transfection group (Fig. 5D). These results suggested that interference with IRS-1 restored the inhibitory effect of miR-126 in ECs.

Discussion

Diabetic retinopathy (DR) is characterized by the early dropout of capillary pericytes and the resultant angiogenesis (2). There is evidence to suggest that the IRS-1 protein is involved in the development of type 2 diabetes (30). Consistent with this, the present study demonstrated that the expression of IRS-1 was significantly increased in ECs and RPs from a DR mouse model compared with the healthy control. To examine the involvement of IRS-1 in DR, the noncoding 3'-UTR of IRS-1 was investigated in the present study. miRNAs are noncoding RNAs that suppress the expression of protein-coding genes by binding to a target sequence at the 3'-UTR of the target gene. In the present study, miR-126 was predicted to be the miRNA that targeted IRS-1, and thus may be involved in the pathogenesis of DR.

Expression of miR-126 is decreased in multiple types of cancer cell, and has previously been regarded as a cell growth suppressor that acts on IRS-1 (16) or as a metabolic regulator in hepatocytes (31). The loss of miR-126 has been reported in the plasma of patients with diabetes mellitus (DM). The target connection between IRS-1 and miR-126 was predicted using TargetScan, a bioinformatics software program. Thus, miR-126 was selected as the miRNA targeting IRS-1. The results

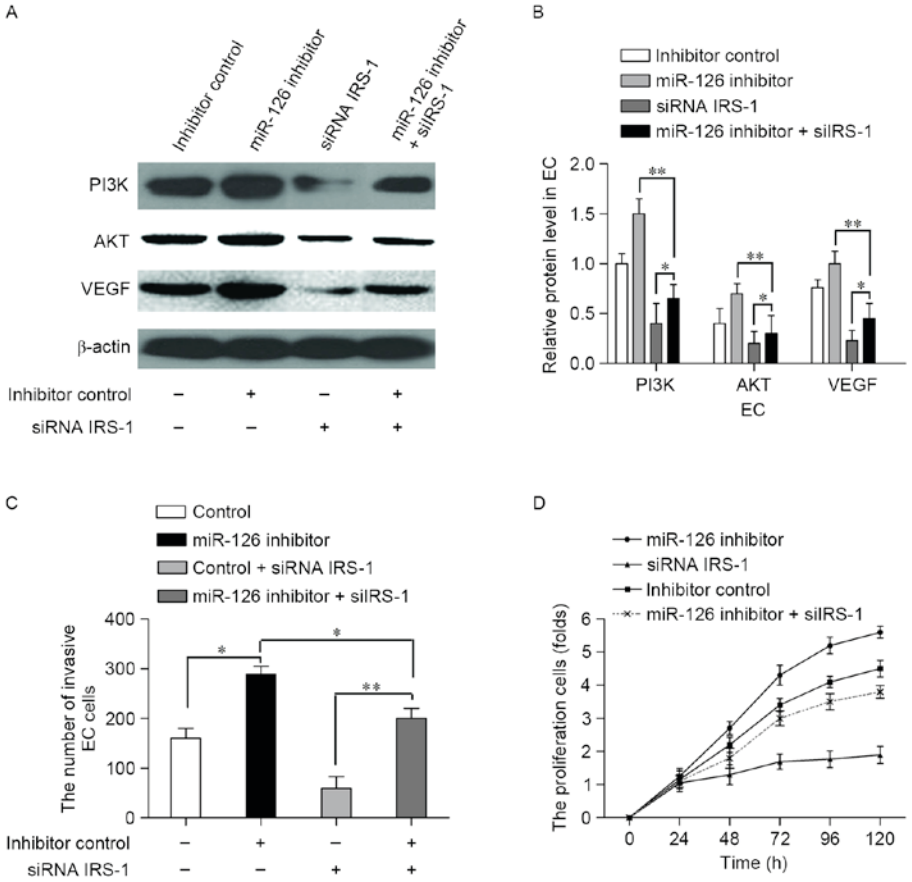

Figure 5. siRNA targeting IRS-1 offsets the suppression effect of miR-126 in ECs. Cells were transfected with the miR-126 inhibitor or inhibitor control with or without a siRNA IRS-1 vector. (A) VEGF, PI3K and Akt expression levels were detected using western blotting. (B) Relative protein expression levels in ECs were normalized to β-actin. (C) The number of invading ECs. (D) The viability of ECs, assessed by MTT assay. Data are presented as the mean ± standard deviation of three experiments. *P<0.05 and **P<0.01, with comparisons indicated by lines. siRNA, small interfering RNA; IRS-1, insulin receptor substrate-1; miR-126, microRNA-126; VEGF, vascular endothelial growth factor; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; EC, endothelial cell.

of the present study revealed thatmiR-126 expression was significantly decreased when IRS-1 expression was increased in ECs and RPs compared with healthy controls, which was consistent with the results of a former study in breast cancer cells (16). To verify the targeting reaction between miR-126 and IRS-1, IRS-1-wt and IRS-1-mutluciferase reporter vectors were constructed. The results revealed that overexpression of miR-126 inhibited luciferase expression when cells were transfected with the wt-IRS-1 luciferase reporter vector, but it was not inhibited in the mut-IRS-1 group. Furthermore, inhibition of miR-126 increased luciferase activity in the wt-IRS-1 transfection group compared with the mut-IRS-1 group. These results demonstrated that IRS-1 was a target gene for miR-126 in ECs and RPs from a DR mouse model.

miR-126 is enriched in endothelial cells and is involved in maintaining endothelial homeostasis and vascular integrity (32). Reduced levels of miR-126 were reported to be an underlying cause of endothelial progenitor cell (EPC) dysfunction in DM. As restoration of miR-126 expression in EPCs from patients with DM may promote EPC proliferation and migration and inhibit their apoptosis, miR-126 may restore the ability of EPCs to be incorporated into the damaged endothelium and work in concert with existing endothelial cells to form blood vessels (22). Thus, to investigate the involvement of miR-126 in EC and RP viability and invasion via targeting IRS-1, the effect of miR-126 inhibition and miR-126 overexpression on the viability and invasion of ECs and RPs was explored. The results demonstrated that transfection with the miR-126 mimic inhibited the expression of IRS-1, and inhibited cell viability and invasion. However, under inhibition of miR-126, IRS-1 expression was significantly increased and cell viability and invasion were promoted in ECs and RPs. As a cell growth suppressor, mir-126 has been reported to target IRS-1 and inhibit the cell cycle phase transition from G1/G0 to S (16). These results suggested that miR-126 negatively influenced viability and invasion in ECs and RPs obtained from DR mice model, via targeting IRS-1.

VEGF is a key regulatory factor associated with angiopoiesis, carcinogenesis and metastasis (33). Insulin upregulates VEGF expression, and this action is mediated by the insulin receptor (29). The effect of insulin on VEGF expression has been suggested to be a potential explanation for the worsening of diabetic retinopathy in DR (28). A previous report also indicated that insulin induces VEGF expression in vascular cells by activating the PI3K/Akt and Ras/MAP kinase pathways (8). As predicted by the Kyoto Encyclopedia of Genes and Genomes pathway database, VEGF is located downstream of IRS-1/PI3K/Akt (34). The present study revealed that expression of PI3K/Akt pathway proteins, PI3K, Akt and VEGF, were inhibited following transfection with the

miR-126 mimic compared with the mimic control group. The results indicated that the overexpression of miR-126 inhibited the expression of PI3K/Akt pathway proteins by decreasing IRS-1 expression, with the inhibition of miR-126 causing the reverse effect. Insulin has been reported to stimulate several signaling cascades in EC and vascular smooth muscle cells following activation of the insulin receptor via its tyrosine kinase subunit, by phosphorylating IRS-1 and IRS-2. Tyrosine-phosphorylated IRS-1 and IRS-2 interact with several downstream cellular proteins, including the p85 regulatory subunit of PI3K, resulting in the activation of Akt (14). In order to further confirm the inhibitory effect of miR-126 on the expression of PI3K/Akt pathway proteins and on the viability and invasion of ECs was via targeting IRS-1, interference with IRS-1 expression was performed in ECs using siRNA. The results revealed that transfection with siRNA targeting IRS-1 restored the inhibitory effect of miR-126 on the PI3K/Akt pathway protein expression in ECs. These results suggested that miR-126 suppresses the expression of IRS-1 in ECs and RPs. This result contributes to our understanding of the VEGF pathway regulatory network in DR. The present study demonstrated that IRS-1 is a novel DR-associated tumor promoting gene. Further studies are required to develop a therapeutic strategy targeting IRS-1 for DR treatment.

In conclusion, the results of the present study demonstrated that the overexpression of miR-126 affected the expression of IRS-1, resulting in the downregulation of VEGF pathway proteins, and suppressed the invasion and viability of ECs and RPs. The present study further elucidates the pathogenesis of DR and implicates miR-126 as a potential therapeutic target for DR.

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