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miR-200c serves an important role in H5V endothelial cells in high glucose by targeting Notch1

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Abstract. Diabetic vasculopathy is the leading cause of impairment and death in diabetic patients, a variety of factors are involved in its underlying pathological process, however, endothelial cell (EC) dysfunction serves a significant role in the process. MicroRNAs (miRNAs) have emerged as potential therapeutic candidates, due to their ability to regulate multiple targets involved in ECs. The aim of the present study was to investigate the role of miR-200c in regulating ECs in high glucose condition. To investigate the role of miR-200c in regulating hyperglycemia induced ECs by targeting Notch1, ECs H5V cells were cultured in high sugar conditions to initiate the inhibition of Notch1, the same cells in normal medium as the control. H5V cells were transfected with miR-200c mimics, miR-200c inhibitors or scrambled oligonucleotide controls, respectively. Notch1 and Hes1 mRNA and protein expression level were detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis. The present study reported that miR-200c was highly expressed by high glucose stimulation in H5V cells, however Notch1 was inhibited by high glucose, and it was also depressed by miR-200c in high glucose conditions. Notch1 was identified as the target gene of miR-200c, luciferase reporter assays confirmed the biochemical relationship for miR-200c decreasing Notch1. The current findings revealed that miR-200c may inhibit Notch1 expression in high glucose conditions, which suggested that miR-200c mediating Notch1/Hes1 may involve in the process of vascular damage caused by hyperglycemia.

Introduction

Microvascular related diseases are the most frequent complications of diabetes (1); especially diabetic foot, which afflicts ~10% of people with diabetes (2). The vascular endothelium is considered to serve an essential role in diabetes-associated microvascular dysfunction, especially diabetic foot. The endothelium serve a significant role in the regulation of microvascular function and development of physiological and pathophysiological inflammation. Endothelial cell (EC) injury is a critical element of diabetic foot (2). Previous studies have reported that high blood glucose induces EC apoptosis, and causes cellular dysfunction and cell death (1). However, the vascular function pathogenesis is complicated and there are a number of signaling pathways involved, including the Notch pathway.

The Notch1 signaling pathway is significant in cell differentiation, primarily determining and regulating cell survival (3). In mammals, four receptors (Notch1-Notch4) and five ligands, Jagged1, Jagged2, Delta-like (Dl)1, Dll3 and Dll4, have been discovered (4,5). Dysregulation or loss of Notch signaling underlies a wide range of human disorders (6). A previous study indicates that Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of Hes1 (7), Mutations in Notch1 cause aortic valve disease (8).

The Notch/Hes1 pathway has been identified to play a key role in mediating ECs function (8,9), participating in regulating angiogenesis, development and vascular inflammation reaction related pathophysiology process. DLL4-Notch signaling has a key role in regulation of tumor angiogenesis (4,10), and serve an important role in placental angiogenesis (11). Previous studies demonstrated that vaccarin attenuates the human EA.hy926 ECs oxidative stress injury through inhibition of Notch signaling (12). Another report indicated that Dll4 signaling through Notch1 regulates formation of tip cells during angiogenesis (4).

Hyperglycemia can cause inflammation and oxidative stress, leading to EC damage. It has also been demonstrated to mediate NF-κB activation, increase Nox4 expression, and increase inflammatory cytokine activation in vascular smooth muscle (13,14). Furthermore, hyperglycemia also causes the Notch signal pathway to be aberrantly activated (15).

MicroRNAs (miRNAs) are endogenous conservative noncoding small RNAs of 21-25 nucleotides and through binding to 3'-untranslated regions (3'UTRs) of the target mRNA (16), usually resulting in translational repression or target mRNA degradation and gene silencing. miRNAs

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regulate many cellular biological activities ranging from cell growth and differentiation, apoptosis, metabolism and angiogenesis. Studies demonstrated that hyperglycemia may cause abnormal high expression of clusters of miRNAs affecting a series of pathophysiology process (17). In addition, series of miRNAs serve an important role in the hyperglycemia-related pathophysiology process (18).

miR-200c is upregulated by oxidative stress and induces EC apoptosis and senescence via zinc finger E-box-binding homeobox (ZEB)1 inhibition (19). miR-200c may regulate vascular endothelial growth factor A, ZEBs, fms related tyrosine kinase 1, I kappa B kinase β, Krüppel-like factor 9, fibulin-5 (8) or ZEB1 (20) to participate in regulating EC function. The present study predicted that miR-200c and Notch1 had complementary pairing in promoter regions by using the computer software TargetScan version 7.1. Hyperglycemia-induced vascular lesions are the leading cause of diabetic lower limb ischemia. Previous studies have identified that miR-200c was significantly increased expression in the serum of type 2 diabetes patients with critical limb ischemia, which may suggest that miR-200c is possibly related to regulating mechanism of diabetic vascular endothelial injury. However, how miR-200c is involved in the diabetic vascular injury has never been reported.

The miR-200 family includes miR-200a, miR-200b, miR-200c, miR-429 and miR-141; they have emerged as noticeable markers for predicting cancer prognosis and tumors progression. miR-200c is a tumor suppressor in various cancers, such as pancreatic, gastric and breast cancer through the inhibition of Kirsten rat sarcoma viral oncogene homolog (KRAS) (21,22), and the mediation of Leydig tumor cells and murine adrenocortical tumor cells by vimentin. Another study using luciferase reporter plasmids observed that miR-200c inhibited the AKT and ERK pathways by directly targeting KRAS. It is suggested that miR-200c may be related to normal cells survival and function, rather than malignant cells. A previous study indicated that miR-200c served an important role in EC differentiation and vasculogenesis by targeting the transcription repressor ZEB1 (18).

The purpose of the present study was to investigate how high glucose mediated miR-200c and Notch1, and whether miR-200c can regulate EC differentiation and function via the Notch1/Hes1 signal pathway *in vitro*, in addition to the role of miR-200c in H5V apoptosis under high glucose conditions.

Materials and methods

Cell lines and cell culture. H5V cells, which are murine heart endothelial immortalized cells (23) (kindly provided by Nanjing Medical University (Nanjing, China), were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing fetal calf serum 10% (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator at 37˚C with 5% CO_2.

miRNA overexpression. An miRNA mimic (a synthetic RNA oligonucleotide duplex mimicking miRNA precursor) purchased from Suzhou GenePharma Co., Ltd, (Suzhou, China) was used for overexpression of miRNA. The synthetic RNA molecules were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) including pre-miR-200c and pre-miR-control (scrambled negative control RNA). H5V cells were seeded in six-well plates ($2x10^5$) in complete medium without antibiotics at least 24 h prior to transfection, when cell confluence was ~70%, a final concentration of 50 nM miRNA-200c-mimics, miRNA-200c-inhibitor and NC RNA were transfected into the cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. A total of 6 h later, the culture medium was replaced with DMEM supplemented with 2% fetal bovine serum, the cells continued to be cultured for 24 or 48 h; they were then harvested and processed for further analysis.

Western blot analysis. H5V cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), total protein was separated by 10% SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The membrane was blocked with 5% skimmed milk and incubated with primary antibodies (anti-Notch1; ab52627; 1:2,000; anti-Hes1; ab108937; 1:1,000, anti-GAPDH; ab181602; 1:5,000; all from Abcam, Cambridge, MA, USA) overnight at 4˚C. It was then incubated with horseradish peroxidase-conjugated anti-rabbit or anti mouse secondary immunoglobulins (B0014K052600; 1:2,000; BioSharp, Anhui, China) for 2 h at room temperature. The signal was then detected by enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The data were quantified by ImageJ version 1.43 (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, cDNA was synthesized using a miR-200c specific primer (Sangon Biotech Co., Ltd., Shanghai, China) in a reverse transcription system. The reaction conditions were as follows: 16˚C for 30 min, 42˚C for 42 min, 85˚C for 5 min. Quantitative detection of reverse transcription products was performed using specific sense and antisense primers of miR-200c and SYBR Green I dye (Molecular Probes; Thermo Fisher Scientific, Inc.). The PCR reaction condition was as follows: 95˚C for 5 min, 94˚C for 20 sec, 55˚C for 20 sec, and 72˚C for 20 sec, for 40 cycles, to obtain fluorescence intensity. U6 was used as an internal control. The sequence of specific primer for miR-200c was: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATGCACTGGATACGACTCCATC-3'; miR-200c sense primer, 5'-TAATACTGCCGGGTAAT-3'; miR-200c antisense primer, 5'-GTGCAGGGTCCGAGGT-3'. The Cq value was analyzed using the RFQ-PCR 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) analysis program version 2.0.6). Relative mRNA expression levels were determined by the $2^{-\Delta\Delta Cq}$ method (24) in comparison with control cells.

Cell survival assay. The cell suspension was inoculated in 96-well plates (100 μl/well). The culture plate was maintained

Table I. The algorithm predicted that Notch1 was a target gene of miR-200c.

Name of target region and miRNA	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 736-743 of Notch1 3'UTR	5' …UCUUUGUUUCAGGUUCAGUAUUA…
hsa-miR-200c-3p	3' AGGUAGUAAUGGGCCGUCAUAAU

The algorithm predicted that Notch1 was a target gene of miR-200c, the complementary base pairing between the 3'UTR of Notch1 and miR-200c. miR, microRNA; UTR, untranslated region; miRNA, microRNA.

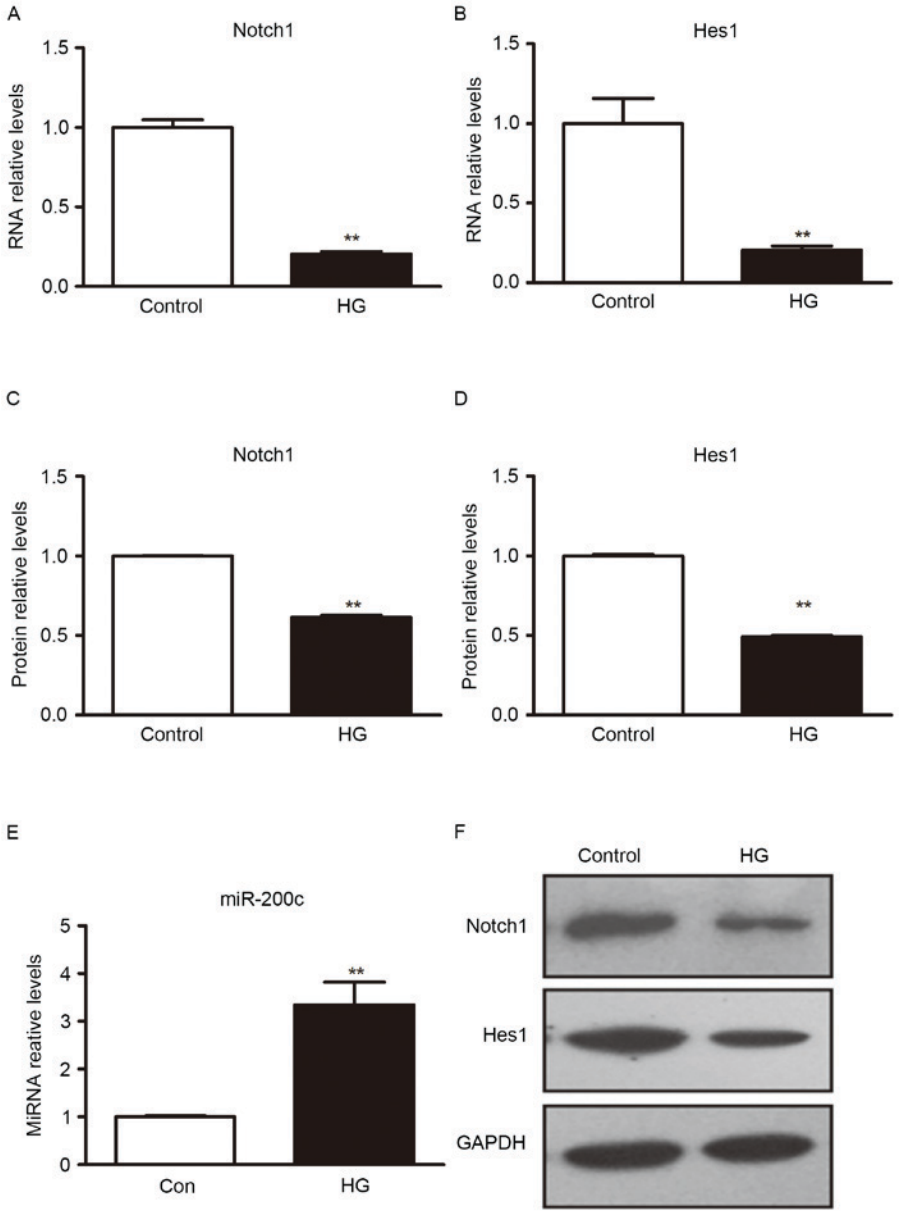

Figure 1. (A) Notch1 and (B) Hes1 were repressed by high glucose, while (E) miR-200c was induced by high glucose. (F) Protein levels of (C) Notch 1 and (D) Hes1 were decreased under high glucose conditions. Data are presented as mean ± standard deviation. **P<0.01 vs. control. Con, control; HG, high glucose (25 mM).

in the incubator (37˚C and 5% CO_2). A total of 10 μl CCK (Shanghai Qcbio Science & Technologies Co., Ltd., Shanghai, China) was added to each well, and the culture plate was incubated in incubator for 1-4 h. Absorbance was measured at 450 nm with a microplate reader (BioTex, Houston, TX, USA).

Immunofluorescence staining for Notch1 and Hes1. Cells were fixed with PBS containing 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 20 min, permeabilized and blocked with blocking buffer (Abcam) for 30 min. Cells were then incubated with rat anti-mouse Notch1 primary antibody (ab52627; 1:2,000; Abcam) at 4˚C overnight, followed by

incubation with Alexa fluoro 555 conjugated secondary antibodies (A32727; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min. Nuclei were stained with DAPI (Sigma-Aldrich; Merck KGaA). Images were acquired using a 50i Nikon fluorescence microscope (Nikon Corporation, Tokyo, Japan). Images were processed with Adobe Photoshop CS4 software version 11.0 (Adobe Systems, Inc., San Jose, CA, USA). The proliferation rate referred to the number of Notch/Hes1-positive cells divided by the number of DAPI-stained cells.

Luciferase reporter assay. Using NCBI GeneBank database (https://www.ncbi.nlm.nih.gov/gene/), the authors synthesized 3' untranslated region (3'-UTR) sequences of target genes at 100-120 nt in length containing the seed sequence. The full-length of 3'-UTR of the Notch1 gene was amplified by PCR from H5V genomic DNA and inserted into pGL3 control vector (Promega Corporation, Madison, WI, USA). Using the Qiagen XL-site directed Mutagenesis Kit (Qiagen, Valencia, CA, USA), the authors constructed several inserts by deletions of 4 bp from the complementarity site of the Notch1 gene. Lipofectamine 2000 was used to transfect H5V cells when cell confluence reached 80% in a 24-well plate with a 0.5 μg firefly luciferase reporter vector (Sangon Biotech Co., Ltd.) and 0.5 μg control vector containing Renilla luciferase (Sangon Biotech Co., Ltd.), phRL-TK (Promega Corporation) by Amaxa Nucleofector (Lonza Group, Basel, Switzerland). Each Nucleofector used 50 nM of the miR-200c or a scrambled oligonucleotide. At 48 h, the relative luciferase activities of Firefly/Renilla were consecutively measured through the dual luciferase assay (Promega Corporation). All data are presented as mean ± standard deviation.

Statistical analysis. All experiments were performed at least three times. Comparisons between two observations were analyzed by Student's paired t-test, and multiple comparisons with a one- or two-way analysis of variance. Multiple testing bias was assessed with the Bonferroni test. Data are expressed as mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

High glucose induced miR-200c expression. Exposing ECs to high glucose is thought to mimic the hyperglycemia of diabetic patients. miR-200c is upregulated by oxidative stress and induces EC apoptosis and senescence via Notch1 inhibition (19), hyperglycemia is the principal cause of induced oxidative stress (13). To investigate whether high glucose was able to induce expression of miR-200c in H5V cells, the H5V cells were cultured in different glucose concentration for comparison. Cells were cultured under normal glucose (5 mM) or high glucose (25 mM). Taking normal glucose as a control, the H5V cells were cultured for 8 h and miRNAs expression level was determined through RT-qPCR. When compared with cells in normal culture medium, miR-200c was upregulated under high glucose conditions (Fig. 1).

Notch1 and Hes1 gene was repressed by high glucose. The Notch1/Hes1 signal pathway serves an important role in EC development and its function (6,8). To determine how hyperglycemic stress affects Notch1 and Hes1, H5V cells were exposed to high glucose conditions, with normal culture medium as the control. Western blot analysis was used to detect Notch1 and Hes1 protein expression. RT-qPCR was used to detect Notch1 and Hes1 mRNA expression. The result indicated that, under the condition of high sugar medium, Notch1 and Hes1 protein and mRNA level expression were significantly decreased, compared with normal culture medium (P<0.01; Fig. 1).

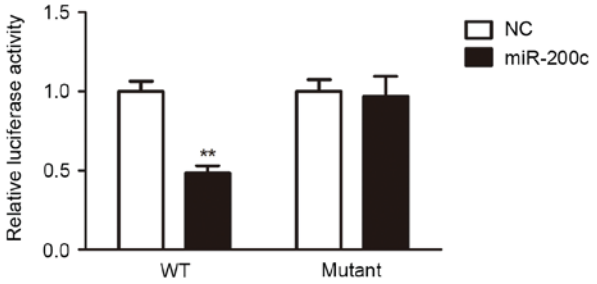

Figure 2. The luciferase activity of NC and miR-200c. The luciferase expression was decreased in H5V cells co-transfected with miR-200c and the wild-type Notch 3'-untranslated region compared to the control. Data are presented as mean ± standard deviation. **P<0.01 vs. the NC. NC, negative control; miR, microRNA.

miR-200c inhibited the Notch1/Hes pathway by targeting Notch1. To explore the function of miR-200c, computational algorithms (TargetScan and miRanda) were used to identify the potential target gene of miR-200c. It demonstrated that Notch1 was a target gene of miR-200c (Table I). Luciferase reporter assays were performed to clarify this finding. The full-length Notch1 was cloned downstream of the firefly luciferase gene and co-transfected with miR-200c mimics or scrambled oligonucleotides controls. Luciferase activity was measured 48 h following transfection. Luciferase expression was decreased in H5V cells that were co-transfected with miR-200c and the wild-type Notch 3'-UTR, compared to the control. However, no decrease can be observed in cells co-transfected with miR-200c and the mutant Notch1 3'-UTR in comparison with controls (Fig. 2). These results suggested that miR-200c can directly target Notch1.

To determine the level at which miR-200c influences Notch1 expression, the expression of Notch1 mRNA was analyzed following transfection with miR-200c mimics or scramble oligonucleotides controls in H5V cells. Following transfection with miR-200c mimics, transfection efficiency could be observed in Fig. 3E; the expression of Notch1/Hes1 mRNA in the H5V cell line was lower than in the controls (Figs. 3A and B). The effect of miR-200c on Notch1/Hes1 expression was also verified by western blotting analyses (Figs. 3C, D and F). The overexpression of miR-200c reduced Notch1/Hes1 protein levels significantly. These results provide evidence that miR-200c directly recognizes the 3'-UTR of Notch 1 mRNA and inhibits Notch 1 translation.

Although Notch 1 serves a wide-ranging role in controlling cell fate, differentiation and development (25), in ECs, the activation of Notch1 can trigger the Notch1/Hes1 signal pathway (7), which serves an important role in ECs' development and function. Therefore, it was presumed that

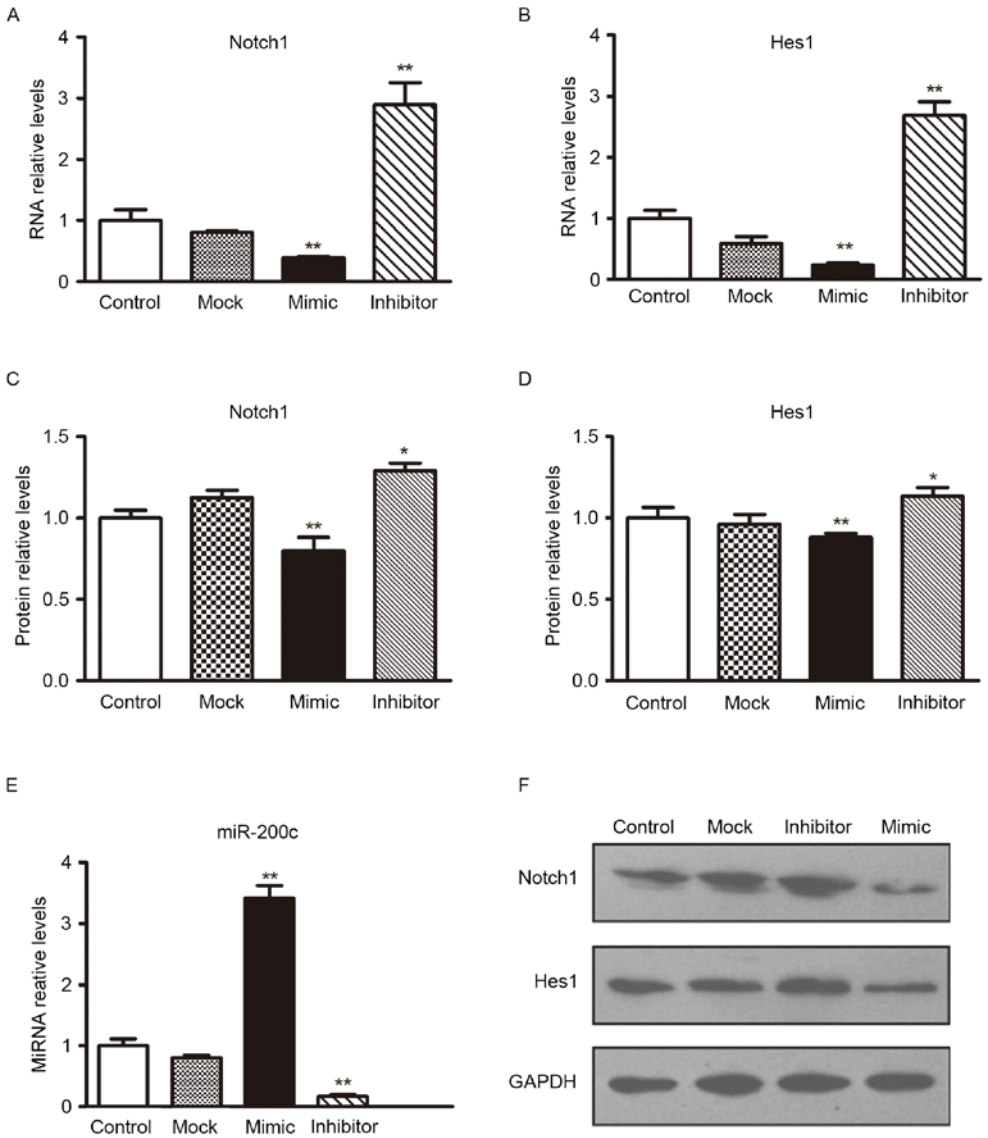

Figure 3. The relative levels of mRNA and protein expression of (A) Notch1 and (B) Hes1 in H5V cells transfected with miR-200c. Both (A and B) mRNA and (C, D and F) protein levels were lower in H5V cells that were transfected with the miR-200c mimic compared to the control. (E) Transfection efficiency of miR-200c in H5V cells. Data are presented as mean ± standard deviation. *P<0.05, **P<0.01 vs. mock group. The groups were control group, mock vector group, knock down group and miR-200c mimic (overexpression) group, respectively. miR, microRNA.

miR-200c may regulate that pathway by targeting Notch1. Therefore, the authors upregulated miR-200c levels via miR-200c mimics in H5V cells. As verified by similar results from protein quantification and an immunocytochemistry assay (Fig. 4), the western blotting results demonstrated that miR-200c decreased the expression levels of Notch 1 and Hes1 (Figs. 5C, D and F).

Subsequently, H5V cells were treated with miR-200c or a scramble control. At 24 h, they were transfected with Notch1-encoding vector (without an endogenous 3'-UTR) or mock vector (Fig. 3E presented that the transfection was successful in H5V cells). The miR-200c mimic inhibited the expression of Notch1, a similar alteration was observed in the expression levels of Hes1. However, the expression level of Notch1 and Hes1 was increased by enhancing miR-200c inhibition in H5V cells (Fig. 3). These results suggested that miR-200c inhibits the Notch1 and Hes1 pathway by targeting Notch1.

miR-200c inhibited the proliferation of VEC H5V by targeting Notch1. To investigate the biological consequences of the miR-200c-driven repression of Notch1 in H5V cells, the authors investigated whether increasing or decreasing the expression of miR-200c would have an impact on cell proliferation in H5V cells. H5V cells were transfected with miR-200c mimics, miR-200c inhibitors or their scrambled oligonucleotides controls, respectively, then were counted after 24 h by CCK8 assay. The result showed that the increased expression of miR-200c in H5V cells inhibited the cell proliferation compared with the control cells, while the decreased expression of miR-200c promoted the proliferation (Fig. 5).

The above results prompted us to validate that miR-200c could inhibit the proliferation of H5V cell by repressing Notch1 expression, it was involved in the development of vasculopathy induced by hyperglycemia. For this purpose, H5V cells were transfected with mock vector, scrambled oligonucleotides controls, Notch1-encoding vector + miR-200c or mock

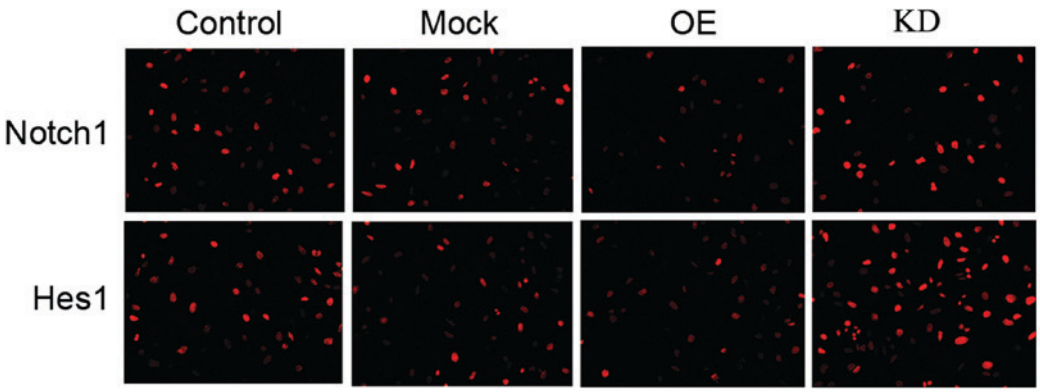

Figure 4. Activated miR-200c inhibited Notch1 and Hes1 demonstrated by immunocytochemistry assay. The fluorescence activity of Notch1 and Hes1 were decreased in H5V cells that were transfected with miR-200c. The groups were control group, mock vector group, knock down group and miR-200c mimic (overexpression) group, respectively. miR, microRNA; OE, overexpression; KD, knock down.

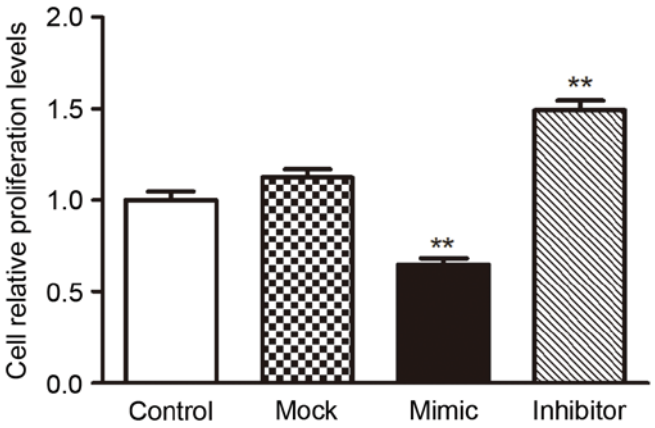

Figure 5. miR-200c inhibited the proliferation of VEC H5V by targeting Notch1. The cell relative proliferation level was lower in H5V cells that were transfected with miR-200c than the mock group. **P<0.01 vs. mock group. The groups were control group, mock vector group, knock down group and miR-200c mimic (overexpression) group, respectively. miR, microRNA.

vector + scrambled oligonucleotides controls, respectively. The results indicated that increased expression of miR-200c inhibited proliferation by repressing Notch1 in high glucose condition (Fig. 5).

Discussion

Exposure of the vascular endothelial tissue to high blood sugar causes vascular lesions of diabetic lower limbs, thereby causing ischemia, which serves a critical role in the pathogenesis of diabetic vascular complications. Previous studies indicated that high blood sugar dysregulated multiple miRNAs, including miR-200c (1,17,18), which results in cellular dysfunction, cell apoptosis and cell death through targeting their downstream proteins. Notch signaling has been widely connected in epithelial-mesenchymal transition, EC proliferation and apoptosis regulation (15), it is not surprising that aberrant gain or loss of Notch signaling components has been directly linked to multiple human disorders (6).

The present study has indicated that miR-200c served a key role in mice EC H5V apoptosis induced by a high glucose environment, the results demonstrated that miR-200c can downregulate Notch1 and Hes1 by directly binding 3'UTR of Notch1's promoter. This is similar to previous reports, which indicated that the Notch1/Hes1 signal pathway is vital to EC function.

Diabetic foot results in limited joint mobility of the ankle and foot, impairs muscular performance and reduces gait speed, increasing risk factors for ulceration (26). Diabetic feet are difficult to heal and are a significant risk factor for non-traumatic foot amputation. At present, popular diabetic foot treatments clinically include low-level light therapy and hyperbaric oxygen therapy, as well as various drugs and therapies such as antibiotics, neuropathic drugs, wound dressings, skin substitutes, growth factors and inflammatory modulators. The majority of these therapies target the treatment of diabetic foot ulcer to address the altered biochemical composition of the diabetic wound (2). However, no single treatment can be definitively recommended for the treatment of diabetic foot ulcers. These are clinical treatments according to disease symptoms, which cannot solve the key problem. In pathology, diabetic foot is caused by vascular lesions caused by insufficient blood supply of ischemic necrosis of lower limbs. Obviously, ECs are a key regulator of blood vessel function. This study aimed to explore the pathophysiology of diabetic foot, and offer some possible basis for molecular therapy.

From the current experiments, it was demonstrated that miR-200c was significantly upregulated in H5V cells stimulated by high glucose conditions. Overexpression of miR-200c may reduce H5V cells proliferation by targeting the Notch1/Hes1 pathway. These findings suggested that miR-200c mediating Notch1/Hes1 may involve in the process of vascular damage caused by hyperglycemia, the method of inhibiting miR-200c may provide a novel therapeutic approach for patients with diabetic foot.

The deficiencies of the present study are that it is only a cell study, the vasculogenesis process could not be presented, the process of vascular impairment in hyperglycemia could not be observed, and the regulation of miR-200c on the morphology and function of damaged vascular in high sugar could not be confirmed; therefore, more animal studies are required to further verify the function of miR-200c in ECs of diabetics.

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