miR-132 inhibits high glucose-induced vascular smooth muscle cell proliferation and migration by targeting E2F5

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Abstract. The dysregulated behavior of vascular smooth muscle cells (VSMCs) serves an important role in the pathogenesis of cardiovascular diseases in diabetes. The present study aimed to investigate the effects of microRNA (miR)-132 on the proliferation and migration of VSMCs under high glucose conditions to mimic diabetes. We observed that the expression of miR-132 was significantly decreased and that of E2F transcription factor 5 (E2F5) was upregulated in high glucose (HG)-treated VSMCs or those obtained from diabetic rats. A dual luciferase reporter gene assay revealed that miR-132 could specifically bind to the 3'-untranslated region of E2F5 and significantly suppress the luciferase activity. The proliferation and migration of diabetic rat or HG-treated VSMCs were increased compared with non-diabetic rat VSMCs and those under normal glucose conditions. Upregulation of miR-132 significantly inhibited the proliferation and migration of diabetic rat VSMCs; similar effects were observed following E2F5 downregulation. The inhibitory effects of miR-132 on the proliferation and migration of HG-treated VSMCs could be reversed by E2F5 overexpression. In conclusion, miR-132 was proposed to inhibit the proliferation and migration of diabetic rat or high-glucose-treated VSMCs by targeting E2F5. The findings of the present study suggested that increasing the expression of miR-132 may serve as a novel therapeutic approach to inhibit the progression of cardiovascular disease in diabetes.

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Introduction

Diabetes mellitus (DM), as a form of chronic metabolic disease, is characterized by the lack of insulin and/or insulin resistance that results in hyperglycemia and abnormal metabolism (1,2). Long-term hyperglycemia can lead to various complications, including cardiovascular disease (CVD) that accounts for approximately a third of all mortalities worldwide (2,3). In addition, DM is considered as an independent risk factor for CVD, excluding other factors such as obesity, hypertension and age (4,5). Vascular smooth muscle cells (VSMCs) serve a key role in vascular remodeling (6). Accumulating evidence has indicated that the proliferation and migration of VSMCs were important features of numerous types of CVD, including atherosclerosis, hypertension and restenosis (7-9). It has been reported that high glucose (HG) can induce the excessive proliferation and migration of VSMCs, which promote the progression of diabetic vascular diseases (10,11). Thus, understanding the mechanism of HG-mediated VSMC proliferation and migration is important for improving current intervention strategies for the treatment of vascular diseases in diabetes.

MicroRNAs (miRNAs/miRs) are a type of small noncoding single-stranded RNAs (~21-23 nucleotides) that negatively regulate the expression of their target genes by complete or partial complementary binding with the 3'-untranslated region (3'UTR) of these target genes, to modulate various physiological and pathological processes, including cancer, DM, CVD and other diseases (12-14). Several studies have demonstrated that miRNAs are involved in regulating the dysfunction of VSMC behavior (15-17), including miR-145 (18), miR-155 (19), miR-146 (20), miR-504 (21), miR-24 (22) and miR-126 (23).

miR-132 originates from the miR-212/132 cluster that is located on chromosome 17 in humans (24). The majority of studies of miR-132 revealed its roles in cancer and the regulation of neurons (24-26). A recent study indicated that miR-132 regulated brain vascular integrity (27). Additionally, the expression levels of miR-132 are downregulated in VSMCs in diabetic rat models (21); however, the role and mechanism of miR-132 in diabetes-induced VSMC dysfunction are not clear. The present study aimed to investigate the effects of miR-132 on the proliferation and migration of VSMCs under HG conditions to mimic diabetes, which may provide insight

into the biological mechanism for the development of novel approaches to treat diabetic vascular complications.

Materials and methods

Animals. The diabetic animal model was generated as previously described (28). In total, 24 male Sprague-Dawley rats (age, 4-6 weeks; weight, 180-200 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). A single dose of streptozotocin (STZ; 65 mg/kg, citrate buffer) was injected into 12 rats. An equal volume of citrate buffer was used as the control treatment (n=12). When blood glucose levels were >250 mg/dl, the animals were defined as diabetic rats. After 2 days following treatment, the animals became hyperglycemic. All rats were sacrificed by cervical dislocation under ketamine administration. Thoracic aorta samples from non-diabetic and diabetic rats were collected and isolated; mRNA and protein were extracted for further analysis. All the procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee of Qianfoshan Hospital of Shandong (Jinan, China).

Cell culture and treatment. The isolation of primary VSMCs from the thoracic aorta of non-diabetic and diabetic rats was performed according to a previous study (29). Morphological and immunohistochemical analyses of VSMCs were performed as previously described (29). The 293 cells were purchased from the Shanghai Institute of Chinese Academy of Sciences (Shanghai, China). VSMCs and 293 cells were maintained in DMEM (HyClone; Thermo Fisher Scientific, Inc.) with 10% FBS (HyClone; Thermo Fisher Scientific, Inc.), as well as penicillin (100 U/ml; Bio-sciences Ltd., Dublin, Ireland) and streptomycin (100 U/ml; Bio-sciences, Ltd.) at 37˚C within 5% CO_2. Experiments were conducted on transduced and non-transduced VSMCs of passages 3-8. For HG treatment, VSMCs were cultured in normal media (5 mM glucose) for 24 h, and then incubated with HG (25 mM glucose) or 20 mM mannitol plus 5 mM glucose (normal glucose, NG) for 24 h at 37˚C with 5% CO_2.

Plasmid construction and infection. miR-132 precursor and the corresponding miR-control were designed and synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China), and were cloned into pCDH-CMV-MCS-EF1-coGFP (System Biosciences, LLC, Palo Alto, CA, USA). The gene encoding E2F transcription factor 5 (E2F5) lacking the 3'UTR was amplified from rat genomic DNA by PCR using the Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. PCR thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec, 72˚C for 1 min and 4˚C for 1 min. The following primers were used: E2F5 forward, AGA ATTCATGGCGGCGGCGGAGCCCA and reverse, ATG GATCCCTAATAATTTAGTATCTGAACATCAA. Then, the PCR product was cloned into pCDH-CMV-MCS-EF1-Puro (Addgene). The production and purification of lentivirus and cell infection (multiplicity of infection, 100) were performed as previously described (30,31). Empty lentiviral vector pCDH-CMV-MCS-EF1-Puro (vector) was used as the control for lenti-E2F5 transduction. VSMCs from non-diabetic rats were infected with lenti-E2F5 or vector. Small interfering (si)RNAs targeting two different sites (cat. nos. RSS350386 and RSS350386) of the rat E2F5 gene and the negative control (cat. no. 12935100; scramble) were purchased from Thermo Fisher Scientific Inc. Cells were transfected with E2F5 siRNAs (100 nM) or scramble (100 nM) using Lipofectamine® 2000 (Thermo Fisher Scientific Inc.) according to the manufacturer's protocols. miR-132 inhibitor (cat. no. 4464084) and corresponding negative control (inhibitor-NC; cat. no. 4464076) as well as miR-132 mimic (cat. no. 4464066) and corresponding negative control (miR-NC; cat. no. 4464058) were obtained from Thermo Fisher Scientific Inc. miR-132 mimic (100 nM) and miR-NC (100 nM) were used to transfect diabetic VSMCs. Normal VSMCs were transfected with miR-132 inhibitor (100 nM) and inhibitor-NC (100 nM) using Lipofectamine 2000 (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Cells were collected and used for analysis at 72 h after infection or transfection. All constructions were confirmed by plasmid DNA sequencing.

Dual luciferase assays. miR-132 mimic, miR-132 inhibitor and corresponding negative control (miR-NC and inhibitor-NC, respectively) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The wild-type E2F5 3'UTR and mutant-type E2F5 3'UTR were cloned into the reporter plasmid pGL3 (Promega Corporation, Madison, WI, USA) downstream of the luciferase reporter gene according to as previously described (26). For the dual luciferase assay, 293 cells were seeded into a 24-well plate at a density of $2x10^4$ cells per well. Cells were co-transfected with 1 μg of wild-type or mutant luciferase vector and miR-132mimics (100 nM), miR-132 inhibitor (100 nM) or their NCs. After co-transfection for 48 h, luciferase activity was detected using the dual luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

VSMC proliferation assay. A bromodeoxyuridine (BrdU) incorporation assay was employed to detect the proliferation of VSMCs (22). VSMCs were transfected with E2F5 siRNAs (100 nM) or scramble (100 nM) using Lipofectamine 2000 (Thermo Fisher Scientific Inc.) according to the manufacturer's protocols. miR-132 mimic was used to transfect diabetic VSMCs. Normal VSMCs were transfected with miR-132 inhibitor using Lipofectamine 2000 (Thermo Fisher Scientific Inc.) according to the manufacturer's protocols. Following transfection, stable infection or co-infection of miR-132, miR-NC, lenti-E2F5 and lenti-vector, cells were seeded into 96-well plates at $3x10^3$ cells/well. Cells were cultured with NG or HG media for 24 h, and were then starved in FBS-free DMEM containing the aforementioned concentrations of glucose (NG or HG) for 24 h at 37˚C with 5% CO_2. Subsequently, BrdU (10 μmol/l) was added into each well and incubated at 37˚C for 30 min. The cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and then detected using the cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Absorbance values were detected at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

VSMC migration assay. VSMC migration was detected by Transwell migration assay (22). VSMCs from non-diabetic rats were infected with lenti-E2F5 or vector. Following transfection, stable infection or co-infection of miR-132, miR-control, lenti-E2F5 and lenti-vector, cells were treated with NG or HG media for 48 h. Cells ($5x10^4$) were plated into the upper chamber with 8-mm pore size (BD Bioscience, Franklin Lakes, NJ, USA), while 800 μl DMEM containing 1% FBS was placed in the lower chamber. After incubation for 6 h at 37˚C, the cells in the upper membrane were removed with cotton swabs, and cells on the lower membrane were fixed in 4% paraformaldehyde for 15 min at room temperature and stained with crystal violet (1%) for 1 h at room temperature (Beyotime Institute of Biotechnology, Haimen, China). Cells were analyzed in 10 random fields per well (magnification, x100) using a phase contrast light microscope.

Protein extraction and western blotting. Thoracic aorta tissue or VSMC was lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors (Roche Diagnostics) according to the manufacturer's instructions. The protein concentrations were measured with a BCA Protein Assay kit (Applygen Technologies, Inc., Beijing, China) according to the manufacturer's protocols. Protein samples (20 μg for each sample) were fractionated by SDS-PAGE on 10% gels, and then transferred to a polyvinylidene fluoride membrane (EMD Millipore, Bedford, MA, USA). The PVDF membranes were incubated with anti-E2F5 antibody (1:1,500; cat. no. ab176017; Abcam, Cambridge, UK) or anti-β-actin antibody (1:6,000; cat. no. ab8226; Abcam) at 4˚C overnight. Then, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:4,000; cat. no. ab205718; Abcam) for 1 h at room temperature. Finally, the protein bands were detected using an enhanced chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc.) and a ChemoDoc XRS detection system (Bio-Rad Laboratories, Inc.). The results were analyzed with ImageJ software (version 1.34e; National Institutes of Health, Bethesda, MD, USA).

RNA extraction and reverse-transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from thoracic aorta tissue or VSMCs was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. miRNAs were reversely transcribed into cDNA using the miScript Reverse Transcription Kit (Qiagen GmbH; cat. no. 218060) according to the manufacturer's protocol. For mRNA detection, cDNA was generated using the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. miR-132 and E2F5 expression levels were quantified using the SYBR Green PCR Master Mix kit (Applied Biosystems; cat. no. 4309155). The sequences of the primers used are as follows: miR-132 forward, 5'-GGGTAACAGTCTACAGCCAT-3' and miR-132 reverse, 5'-GGCAATTGCACTGGATAC-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and U6 reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; rat E2F5 forward, 5'-GTACTTCCTTTGGCCTTAGTTT-3' and reverse, 5'-CTGGCACCATCACACCGACATA-3'; rat β-actin forward, 5'-TGTCACCAACTGGGACGATATG-3' and reverse, 5'-GGCTGGGGTGTTGAAGGTCTC-3'. The expression levels of miR-132 and E2F5 were quantified using the $2^{-\Delta\Delta Cq}$ method (32). RT-qPCR experiments were performed in triplicate.

Databases and bioinformatics analysis. The predicted target genes of miR-132 were identified using TargetScan (version 7.2; http://www.targetscan.org).

Statistical analyses. Data were expressed as the mean ± SD. One-way ANOVA followed by a Tukey's post-hoc test was conducted to assess significant differences. All statistical calculations were performed using SPSS 19.0 software (IBM Corp. Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-132 and E2F5 in thoracic aorta samples and VSMCs from non-diabetic and diabetic rats. To investigate the potential roles of miR-132 and E2F5 in VSMCs under diabetic conditions, the expression of miR-132 and E2F5 in thoracic aorta samples and VSMCs non-diabetic and diabetic rats was detected by RT-qPCR and western blotting. Compared with the non-diabetic control rats, the expression levels of miR-132 in thoracic aorta samples and VSMCs of diabetic rats were significantly decreased compared with the control ($P<0.001$; Fig. 1A and C). Conversely, the mRNA and protein expression levels of E2F5 in thoracic aorta samples and VSMCs of diabetic rats were upregulated than in non-diabetic control rats ($P<0.001$; Fig. 1A-D). Taken together, our results revealed the opposing expression profiles of miR-132 and E2F5, which suggested their conflicting roles in the abnormal behavior of VSMCs mediated by diabetic conditions.

Expression of miR-132 and E2F5 in HG-treated VSMCs. We investigated whether HG stimulation altered expression of miR-132 and E2F5. The results revealed that miR-132 was significantly downregulated ($P<0.001$), while the mRNA and protein expression levels of E2F5 were increased ($P<0.001$) in VSMCs incubated with HG media for 24 h compared with the control (Fig. 2). These findings further suggested the opposing expression profiles of miR-132 and E2F5 in HG-treated VSMCs.

E2F5 is a target of miR-132. Generally, miRNAs regulate gene expression by targeting the 3'UTR of these genes. Using TargetScan (http://www.targetscan.org), it was predicted that miR-132 binds to the 3'UTR of E2F5. As presented in Fig. 3A, the paired sequences between miR-132 and E2F5 3'UTR, and the mutant were determined. This prediction was confirmed by a dual luciferase reporter assay. The relative luciferase activity of the reporter containing the wild-type 3'UTR was significantly decreased by miR-132 overexpression compared with the control, but increased following transfection with miR-132 inhibitor ($P<0.001$; Fig. 3B). However, the luciferase activity of the mutant reporter was markedly affected by miR-132 overexpression and knockdown (Fig. 3B). These results suggested that miR-132 could directly bind to the 3'UTRs of E2F5 which were mutated in this study. We also overexpressed miR-132 in

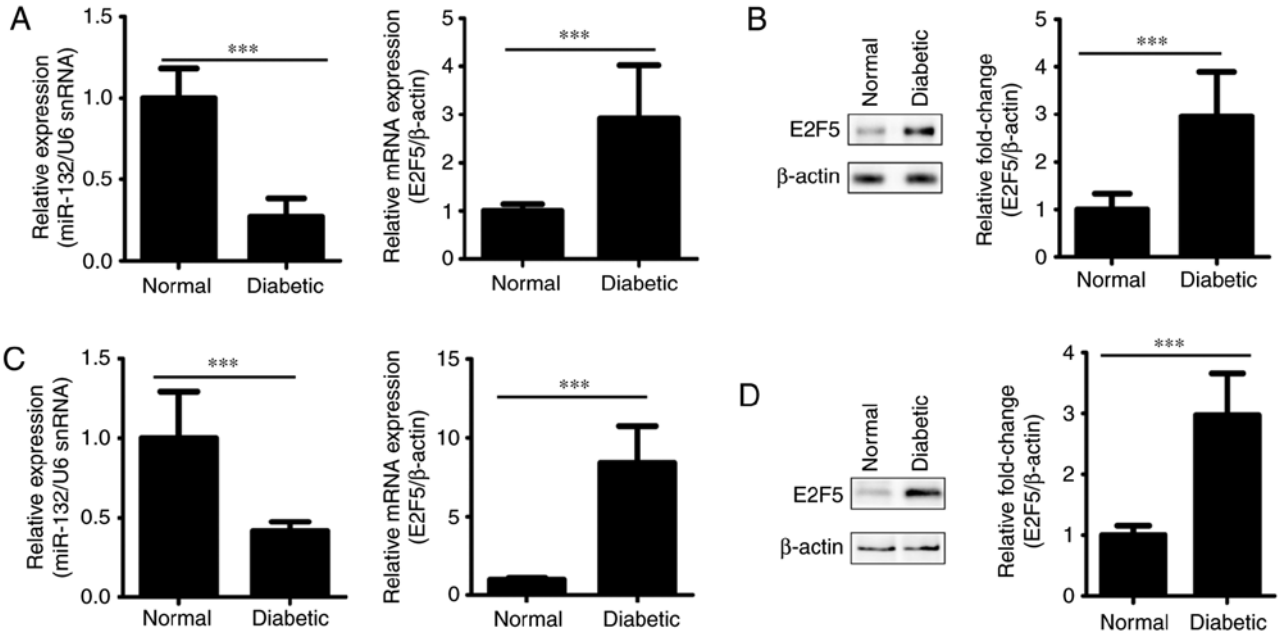

Figure 1. Expression levels of miR-132 and E2F5 in thoracic aorta samples and VSMCs of non-diabetic and diabetic rats. Thoracic aorta tissue and VSMCs were isolated from non-diabetic and diabetic rats (n=6). (A) The expression levels of miR-132 and E2F5 in thoracic aorta samples were detected by RT-qPCR. (B) The protein expression levels of E2F5 in thoracic aorta samples was determined by western blotting; left panel, representative results of 6 rats and right panel, the quantified data for western blotting. (C) The expression levels of miR-132 and E2F5 in VSMCs were detected by RT-qPCR. (D) The protein expression levels of E2F5 in VSMCs was determined by western blotting; left panel, representative results and right panel, the quantified data for western blotting. n=6. All data were presented as the mean ± standard deviation. ***P<0.001. The differences were tested by one-way ANOVA followed by a Tukey's post hoc test. E2F5, E2F transcription factor 5; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; snRNA, small nuclear RNA; VSMCs, vascular smooth muscle cells.

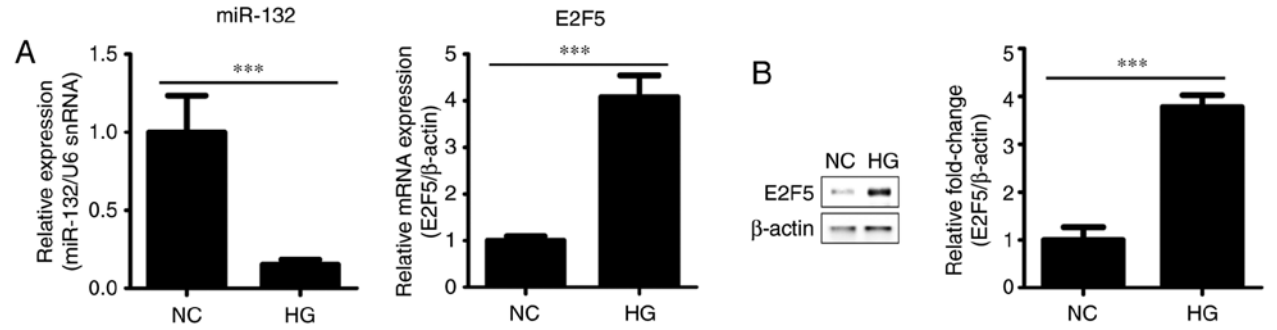

Figure 2. Expression levels of miR-132 and E2F5 in VSMCs treated with HG. VSMCs were incubated with HG for 48 h. (A) The expressions levels of miR-132 and E2F5 were detected by reverse transcription-quantitative polymerase chain reaction. (B) The protein expression levels of E2F5 were examined by western blotting; left panel, representative results and right panel, quantified data for western blotting. n=6. All data were presented as the mean ± standard deviation. ***P<0.001. The differences were tested by one-way ANOVA followed by a Tukey's posthoc test. E2F transcription factor 5; HG, high glucose conditions; miR, microRNA; NC, normal conditions; snRNA, small nuclear RNA; VSMCs, vascular smooth muscle cells.

VSCMs from diabetic rats. The expression levels of miR-132 and E2F5 were detected by RT-qPCR and western blotting, respectively. The results showed the successful transfection of cells with miR-132 mimics (Fig. 3C); the expression of E2F5 was significantly decreased by miR-132 overexpression in the VSCMs from diabetic rats compared with the control (Fig. 3D). Additionally, downregulation of miR-132 (Fig. 3E) significantly increased the expression of E2F5 in VSCMs compared with the control (Fig. 3F). Our findings suggested that E2F5 was a target of miR-132.

Effects of miR-132 and E2F5 on VSMC proliferation and migration. Excessive cell proliferation and migration of VSMCs are fundamental mechanisms underlying CVDs in diabetes (10). This prompted us to investigate the functions of miR-132 and E2F5 in the proliferation and migration of VSMCs from diabetic rats by the nuclear incorporation of BrdU (DNA synthesis) and Transwell migration assays. We reported that the proliferation and migration of VSMCs from diabetic rats were significantly increased than that of VSMCs of non-diabetic rats (P<0.001; Fig. 4A and B). Furthermore, miR-132 was overexpressed in VSMCs from diabetic rats (Fig. 3C and D), then the proliferation and migration of cells were analyzed. The results showed that diabetic rat-derived VSMCs transfected with miR-132 exhibited significantly reduced proliferation and migration compared with the control

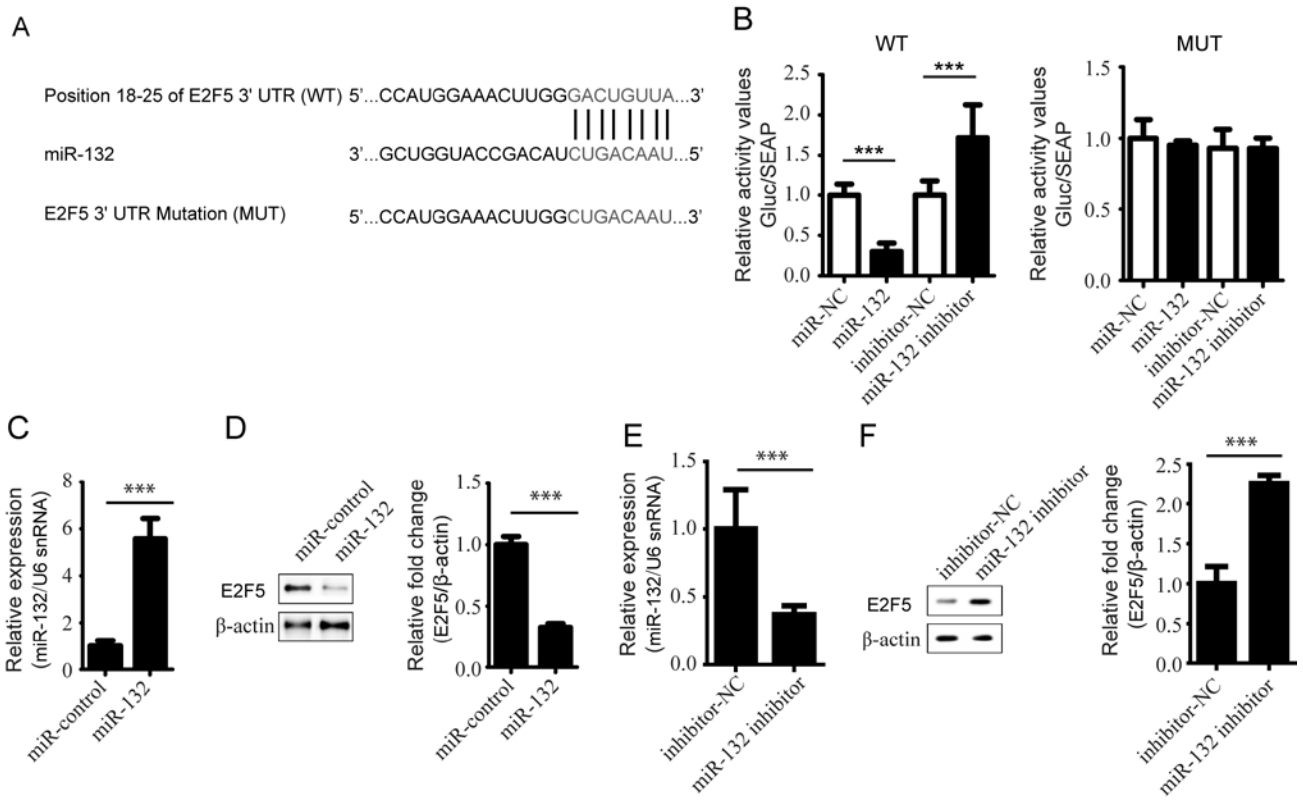

Figure 3. E2F5 is a target gene of miR-132. (A) Putative binding site of miR-132 in the 3'UTR of E2F5 mRNA. Mutation of E2F5 3'UTR was induced by mutating 7 nucleotides that were complementary to miR-132. WT or Mut3' UTR of E2F5 was subcloned into the dual-luciferase reporter vector, forming pGL3-E2F5WT and pGL3-E2F5MUT. (B) Luciferase activities were detected 48 h post-transfection. (C and D) VSMCs from diabetic rats were infected with lentivirus containing miR-132 or miR-control for 72 h. (C) The expression of miR-132 was measured by reverse transcription-quantitative polymerase chain reaction. (D) The protein expression levels of E2F5 were determined by western blotting; left panel, representative results and right panel, quantified data for western blotting. (E and F) VSMCs were transfected with miR-132 inhibitor or NC for 72 h. All data were presented as the mean ± standard deviation. ***P<0.001. The differences were tested by one-way ANOVA followed by a Tukey's post hoc test. 3'UTR, 3'-untranslated region; E2F transcription factor 5; miR, microRNA; mut, mutant; NC, negative control; VSMCs, vascular smooth muscle cells; WT, wild-type.

group (P<0.001; Fig. 4C and D). In addition, VSMCs from non-diabetic rats were infected with lenti-E2F5 or vector. The results demonstrated that the expression of E2F5 significantly increased 5.2-fold in the lenti-E2F5 infection group compared the control group (P<0.001; Fig. 4E). Overexpression of E2F5 significantly increased the proliferative and migration abilities of normal rat VSMCs compared with the control (P<0.001; Fig. 4F and G). Thus, these data suggested that miR-132 and E2F5 serve opposing roles in the proliferation and migration of VSMCs.

Downregulation of E2F5 inhibits diabetic rat VSMC proliferation and migration. To further confirm the function of E2F5 in VSMCs from diabetic rats, E2F5 expression was knocked down by delivery of E2F5 siRNAs, and the proliferation and migration of these VSMCs were analyzed. As determined by western blot analysis, the expression of E2F5 was significantly knocked down compared with the control (Fig. 5A). Of note, downregulation of E2F5 significantly inhibited the proliferation of diabetic rat VSMCs compared with those transfected with scramble siRNA (Fig. 5B). Additionally, compared with the control, downregulation of E2F5 significantly suppressed migration of diabetic VSMCs (Fig. 5C). Taken together, these results suggested that downregulation of E2F5 inhibited VSMC proliferation and migration similar to miR-132 overexpression.

E2F5 overexpression rescues the inhibitory effects of miR-132 on the proliferation and migration of VSMCs treated with HG. We further examined whether E2F5 was involved in the inhibitory effects of miR-132 on the HG-induced proliferation and migration of VSMCs, overexpression of E2F5 was conducted by infecting miR-132 overexpressed VSMCs; cell proliferation and migration were detected by the nuclear incorporation of BrdU and a Transwell migration assay, respectively. The results showed that miR-132 was successfully overexpressed under HG conditions compared with the corresponding control (Fig. 6A). In addition, miR-132 inhibited HG-induced upregulation of E2F5 in VSMCs (Fig. 6B). Furthermore, transduction with lenti-E2F5 exhibited non-significant effect on the expression of miR-132, but could rescue downregulated E2F5 expression induced by miR-132 (Fig. 6A and B). Furthermore, our results showed that E2F5 overexpression could significantly rescue the inhibitory effects of miR-132 on the proliferation and migration of VSMCs, compared with VSMCs that were co-infected with miR-132 and vector (P<0.001; Fig. 6C-E). Thus, our findings suggested that miR-132 suppressed the proliferation and migration of HG-treated VSMCs by targeting E2F5.

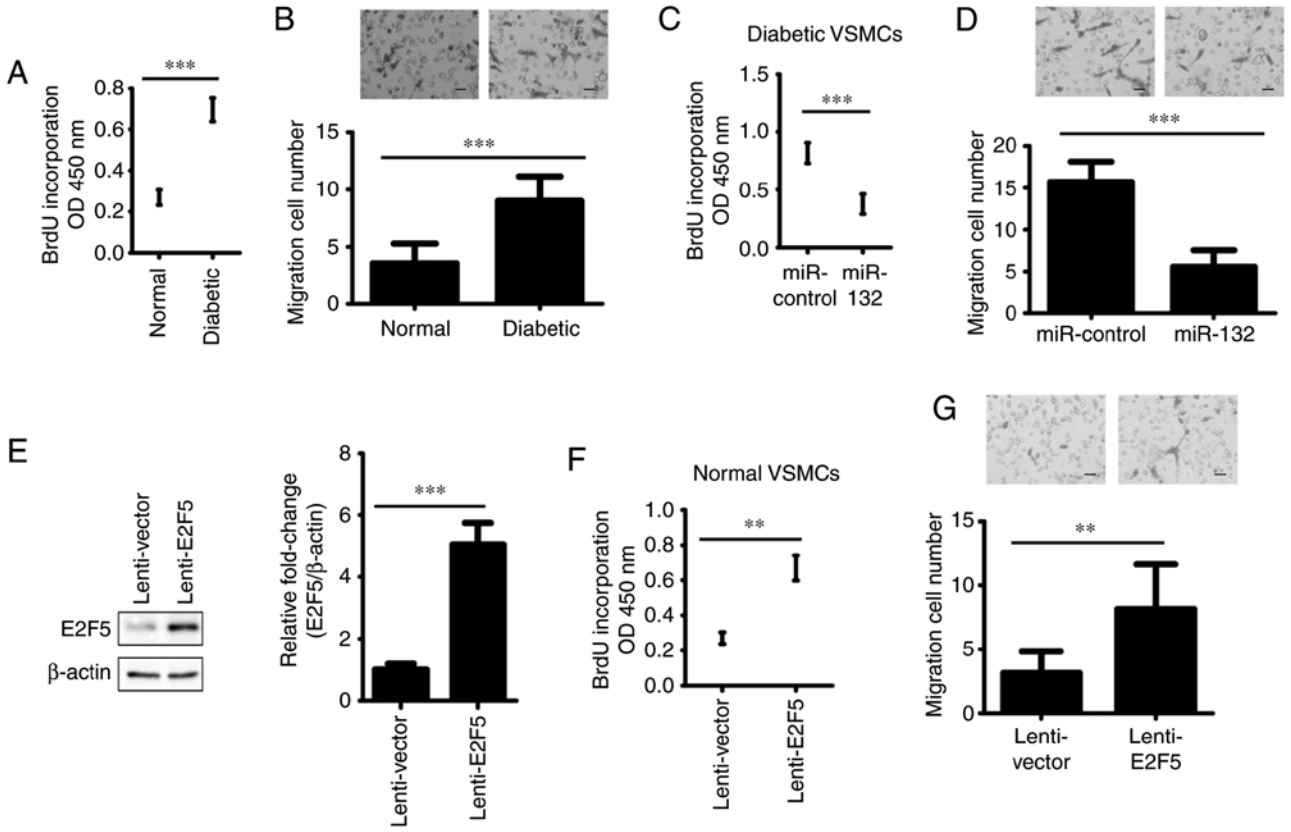

Figure 4. Effects of miR-132 and E2F5 on VSMC proliferation and migration. (A) The proliferation of VSMCs from diabetic and non-diabetic rats were detected by an BrdU incorporation assay. (B) The migration of VSMCs from diabetic and non-diabetic rats was analyzed by a Transwell migration assay. Magnification, x100. VSMCs from diabetic rats were infected with lentivirus containing miR-132 or miR-control, and non-diabetic rat VSMCs were infected with lenti-E2F5 or lenti-vector for 72 h. (C) The proliferation of VSMCs was detected by a BrdU incorporation assay. (D) A Transwell migration assay was conducted to analyze the migration of VSMCs. Magnification, x100. (E) The protein expression levels of E2F5 were determined by western blotting; left panel, representative results and right panel, the quantified data for western blotting. (F) After infection for 72 h, a BrdU incorporation assay was performed to examine the proliferation of VSMCs. (G) After infection for 72 h, a Transwell migration assay was conducted to analyze the migration of VSMCs. Magnification, x100. All data were presented as the mean ± standard deviation. **P<0.001, ***P<0.001. The differences were tested by one-way ANOVA followed by a Tukey's post hoc test. BrdU, bromodeoxyuridine; E2F transcription factor 5; miR, microRNA; OD, optical density; VSMCs, vascular smooth muscle cells.

Discussion

CVD-associated complications, such as restenosis, hypertension and atherosclerosis are the leading causes of mortality in patients with diabetes (2). The pathological development of diabetes-related CVDs is characterized by dysfunction of VSMCs (10). Previous studies have reported that miRNAs exhibit a pathophysiological role in VSMCs in diabetic CVD (33). It was demonstrated that miR-132 was downregulated in VSMCs from diabetic mice (21). Accordingly, our results showed that the expression levels of miR-132 were decreased in thoracic aorta tissue and VSMCs of diabetic rats. Furthermore, the expression of miR-132 was suppressed in HG-treated VSMCs. These findings suggested that miR-132 may serve a regulatory role in the dysfunction of VSMCs in diabetes.

It has been reported that miR-132 is involved in diabetic complications and CVDs, including diabetic cardiac microangiopathy, neointimal hyperplasia, cardiac hypertrophy endothelial cell function and angiogenesis (34-36). Rawal *et al* (36) have reported downregulation of proangiogenic miRNA-132 as an early modulator of diabetic cardiac microangiopathy. In addition, miR-132 improved the repair of infarcted heart tissue via angiogenic activation (37). A previous study (37) reported that miR-132 promotes the proliferation of endothelial cells and facilitates pathological angiogenesis. Additionally, miR-132 induced myofibroblast proliferation (38); however, accumulating evidence has indicated that miR-132 inhibited the proliferation of VSMC (34,39). These opposing effects of miR-132 on cell proliferation may be associated with the various function of miRNAs in different cell types and microenvironments. Hyperglycemia is harmful to VSMCs (40); the present study reported that HG-treatment induced the proliferation and migration of VSMCs, which is consistent with recent studies (22,28,41). VSMC proliferation and migration are fundamental processes of vascular dysfunction in diabetes. Therefore, CVD-associated VSMC dysfunction could be disrupted by regulating their proliferation and migration (11,42). In the present study, it was reported that miR-132 significantly inhibited the proliferation and migration of diabetic and HG-treated VSMCs.

miR-132 regulates biological functions by binding to the 3'UTR of target genes, inhibiting their expression. Previous studies have identified many downstream mRNA targets of miR-132, including p120 RasGAP in endothelial cells, RING finger protein 51 in cervical cancer cells and LRR binding FLII

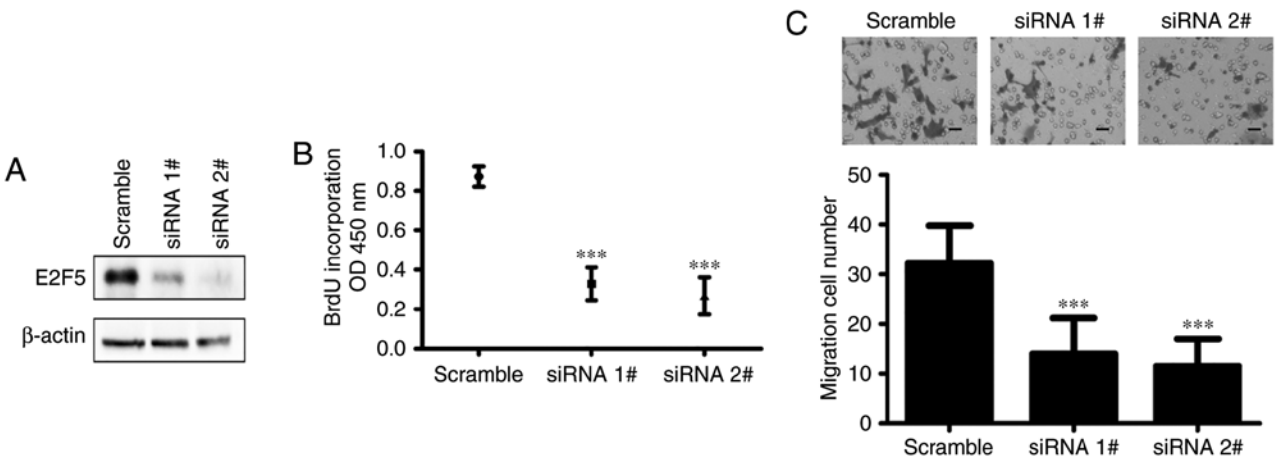

Figure 5. Knockdown of E2F5 inhibits diabetic VSMCs proliferation and migration. VSMCs were transfected with E2F5 siRNAs or scramble control for 72 h. (A) The protein expression levels of E2F5 was analyzed by western blotting. (B) The proliferation of VSMCs was detected by a BrdU incorporation assay. (C) The migration of VSMCs was measured by a Transwell migration assay. Magnification, x100. All data were presented as the mean ± standard deviation. ***P<0.001 vs. scramble. The differences were tested by one-way ANOVA followed by a Tukey's post hoc test. BrdU, bromodeoxyuridine; E2F transcription factor 5; siRNA, small interfering RNA; OD, optical density; VSMCs, vascular smooth muscle cells.

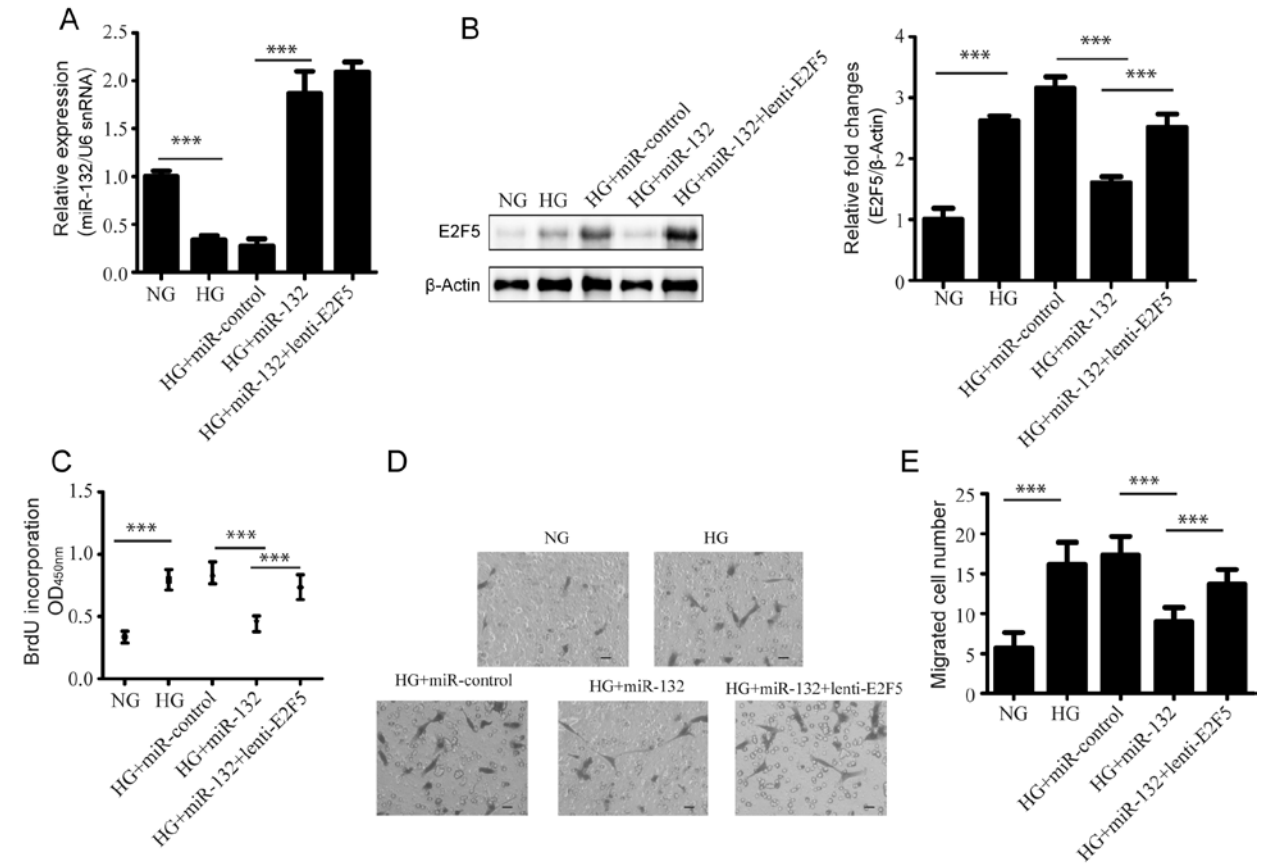

Figure 6. Effects of miR-132 and E2F5 on HG-induced VSMC proliferation and migration. VSMCs were co-infected with miR-132 and lenti-vector, or lenti-E2F5 for 72 h, and were then incubated under HG conditions for 48 h. (A) The expression of miR-132 was detected by reverse transcription-quantitative polymerase chain reaction. (B) The protein expression levels of E2F5 were determined by western blotting; left panel, representative results and right panel, quantified data for western blotting. (C) The proliferation of these VSMCs was detected by a BrdU incorporation assay. The migration of these VSMCs was measured by a Transwell migration assay; (D) representative images and (E) the quantified data. Magnification, x100. All data were presented as the mean ± standard deviation. ***P<0.001. The differences were tested by one-way ANOVA followed by a Tukey's post hoc test. BrdU, bromodeoxyuridine; E2F transcription factor 5; HG, high glucose; NG, normal glucose; miR, microRNA; snRNA, small nuclear RNA; VSMCs, vascular smooth muscle cells.

interacting protein 1 in VSMCs (34,43,44). TargetScan analysis revealed E2F5 as a potential target of miR-132. Furthermore, our results indicated that miR-132 interacted with the 3'UTR of E2F5, inhibiting its expression. In line with our observations, it has been reported that miR-132 suppresses ovarian cancer cell proliferation and migration by targeting E2F5 (26). E2F5 is a

key member of the E2F family that controls the transcription of proliferation-related regulatory genes (45,46). In the present study, diabetic rat-derived and HG-treated VSMCs exhibited high proliferative and migration potentials. In addition, E2F5 was observed to be upregulated in VSMCs from diabetic rats of and HG-treated VSMCs. VSMCs from non-diabetic rats exhibited increased proliferation and migration associated with E2F5 overexpression. In addition, the anti-proliferative and anti-migratory effects of miR-132 in HG-induced or diabetic rat VSMCs could be reversed by E2F5 overexpression. Numerous studies have proposed that E2F5 suppresses the migration of several cancer cells, including breast cancer, ovarian cancer and prostate cancer cells (26,47,48). A previous study (48) has reported that E2F5 binds to the promoter of heme oxygenase 1 (HMOX1) and inhibits its expression. Of note, it has been demonstrated that HMOX1 inhibits VSMC proliferation and migration by inhibiting the MAPK and AKT signaling pathways (49). Combined with the findings of these previous studies, we proposed that the anti-proliferative and anti-migratory effects of miR-132 may be associated with its inhibitory effects on E2F5 expression, which may activate the MAPK and AKT signaling pathways via the suppression of HMOX1; however, further investigation is required.

In summary, our results revealed that miR-132 was downregulated in diabetic rat-derived and HG-treated VSMCs. Furthermore, miR-132 inhibited the proliferation and migration of diabetic rat-derived and HG-treated VSMCs by targeting E2F5. The findings of the present study suggest miR-132 and E2F5 as potentially effective therapeutic targets for treating VSMC dysfunction and CVDs in diabetes.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QX, GY and ZG contributed to the design of the experiment. CZ, XJL, HL, JL, QX, YL and XQL performed the experiments, analyzed the data and wrote the manuscript. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

All the procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee of Qianfoshan Hospital of Shandong Province. All the mice were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (50).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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