Metformin Inhibits the Phenotypic Transformation of Aortic Vascular Smooth Muscle Cells Induced by Hyperglycemia Through Regulation of the Mir-21-mediated PTEN/pAKT/Egr-1 Pathway

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Abstract

Background The phenotypic transformation of arterial vascular smooth muscle cells (VSMC) is one of the key mechanisms in the formation of atherosclerotic plaque. It is unclear whether metformin can inhibit the phenotypic transformation of VSMC. In this study, we observed the effect of metformin on the phenotypic transformation of VSMC in vivo and in vitro and its mechanism.

Methods Patients who underwent simple coronary artery bypass grafting (CABG) were divided into non-diabetic(non-DM) group and diabetes (DM) group according to whether diabetes was combined. The miR-21 and VSMC contractile marker protein, smooth muscle contractile protein (αSMA) and synthetic marker protein osteopontin (OPN) were isolated from the internal mammary artery.30 male, clean, and 6 week old wild type C57/BL6J mice were randomly divided into 3 groups:the control group (NC), the diabetes mellitus group (DM) and the metformin intervention group(DM+MET).Diabetic mice model was established by high-fat diet combined with low-dose streptozotocin. Metformin was given by gavage for 8 weeks.The aortic tissue from aortic root to renal artery was retained, miR-21 was determined by real-time PCR, and SMC and OPN were determined by Western Blotting (WB). In vitro, human aortic smooth muscle cells (HA-SMC) were divided into three groups: control group, HG group and HG+ group. The content of miR-21/PTEN/pAKT/Egr-1 protein was determined by WB, the migration ability was determined by MTT and scratch method.

Results 1.In vivo, the expression of miR-21 and OPN in the aortic VSMC of DM petients and diabetic mice increased and the intima media thickness increased. Metformin treatment reduced the expression of miR-21 and OPN in the aorta and decreased the thickness of intima media. 2.In vitro, glucose concentration dependently upregulates the expression of miR-21 and osteopontin in HA-SMC.

Conclusion Metformin can inhibit the phenotype transformation of VSMC induced by HG, which may inhibit the migration of VSMC through miR-21/PTEN/pAkt/Egr-1.

Background

Phenotypic transformation of vascular smooth muscle cells (VSMCs) from the contractile to the synthetic type is one of the key mechanisms of atherosclerotic plaque formation. Synthetic VSMCs can cross the basement membrane under the influence of inflammatory factors and proliferate in the endothelium [1]. Metformin, as an insulin sensitizer, inhibits hepatic gluconeogenesis. TheUnited Kingdom Prospective Diabetes Study(UKPDS)results in 1998 showed that metformin could reduce the risk of diabetes-related myocardial infarction by 39%. Basic research investigations have also confirmed that metformin has anti-arteriosclerotic effects [2]; however, the mechanisms underlying these effects remain unclear.

Micro-deoxyribonucleic acids (miRNAs or miRs) are small non-coding RNA molecules that inhibit messenger RNA by base pairing, making the target RNA unstable or inhibiting translation processes. They inhibit the expression of proteins involved in critical cellular processes such as the cell cycle, proliferation, differentiation, and apoptosis [3]. The expression of MIR-21 in the kidney [4–7], retinal [8–10], and

cardiovascular tissue [11–12] is increased in diabetic patients. Among the target molecules of MIR-21, the phosphatase and tensin homologous gene (*PTEN*) on human chromosome 10 and phosphorylated serine/threonine kinase (pAkt) play important roles in cell proliferation and migration [13–15]. Previous studies have shown that metformin can inhibit the MIR-21/PTEN/pAkt pathway in tumor cells and inhibit cell proliferation and migration [16]. However, the effects of metformin on MIR-21 and related signaling pathways under HG and those on the phenotypic transformation of VSMCs have not yet been studied.

In this study, we first measured the levels of miR-21, smooth muscle actin (αSMA), which is a contractile phenotype marker, and osteopontin (OPN), a synthetic phenotype marker protein, in the internal thoracic artery of diabetic patients undergoing coronary artery bypass grafting (CABG) and in the aortic root of diabetic mice treated with metformin. Secondly, we used human aortic smooth muscle cells (HA-SMCs) to observe the effects of HG on MIR-21 and its downstream signal pathway *in vitro*. We also studied whether metformin inhibits MIR-21 and OPN expression in VSMCs induced by HG through the PTEN/pAkt/early growth response-1 (Egr-1) pathway and its effects on cell proliferation and migration.

Methods

Patients

Consecutive patients hospitalized in the Department of Cardiology of TEDA International Cardiovascular Disease Hospital from October 2018 to December 2018 and who planned to undergo simple CABG were selected for this study. All patients had signed informed consent forms. The inclusion criteria were as follows: 1) elective CABG for the first time; 2) internal mammary artery to be used as a bridging vessel; 3) internal mammary artery was reserved for about 0.5 cm. Exclusion criteria were as follows: 1) history of other cardiac surgery; 2) previous PCI implantation and CABG; 3) episode of acute myocardial infarction one month before enrollment; 4) presence of severe liver and kidney dysfunction, chronic obstructive pulmonary disease, or severe infection.

Data regarding the gender, age, history of diabetes mellitus (DM is defined as diabetes mellitus or diabetic patients taking drugs), smoking history (past and current smokers), and hypertension (140 mmHg/90 mmHg or use of antihypertensive drugs) were collected. Height and weight were measured, and the body mass index (BMI) was calculated.

Venous blood (3 ml) from patients fasting for more than 8 h was collected in the coagulation promoting tube and centrifuged immediately (3000 rpm, 5 min). High-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (TC), triglyceride (TG), creatinine (Cr), and uric acid (UA) levels were measured using 1 ml of supernatant collected using a biochemical analyzer. The supernatant remaining in the tube was placed in a 2 ml EDTA anticoagulant tube and used to determine the levels of glycosylated hemoglobin (HbA_{1c}, high performance liquid chromatography). All subjects were operated on by the same surgical and anesthesia team. Midazolam injection, etomidate fat emulsion injection, sufentanil citrate, and rocuronium injection were used to induce anesthesia. Midazolam injection,

propofol injection, and cisatracurium benzenesulfonate were used to maintain the anesthesia assisted with endotracheal intubation and ventilator-assisted breathing, right internal jugular vein puncture, deep venous tube, and right radial artery manometric tube. The patient was in the supine position, disinfected, and covered with a sterile towel. Using median thoracotomy, 0.5 cm of the left internal mammary artery was collected. The tissue was washed with PBS 3 times. The outer membrane was peeled off, the inner membrane was scraped off with a fine toothpick, the intact middle membrane was placed in the cryopreservation tube, and the tube was stored in a liquid nitrogen tank for further analysis.

Animals

Thirty 6-week-old wild-type, male C57/BL6J mice, weighing 20.3 ± 2.0 g were purchased from the Beijing HuaFuKang Biotechnology Co., Ltd. The animal experiments were approved by the medical ethics committee of Chu Hsien-I Memorial Hospital of Tianjin Medical University and operated strictly according to the ethical requirements. The mice were randomly divided into three groups: the normal control group (NC group, n = 10), diabetes group (DM group, n = 10), and metformin treatment group (DM + MET group, n = 10). The NC group was fed with a normal diet, and the DM and DM + MET groups were fed with a high-fat diet. After 10 weeks, 20 μl of streptozotocin solution (30 mg/kg) was injected into the caudal vein of mice in DMand DM + MET group every alternate day five times. The mice were given ad libitum access to food and water. The NC group was injected with sodium citrate solution by the same method. After this injection regimen, random blood glucose levels were measured using the blood collected from the tail vein. Mice with blood glucose levels exceeding 16.7 mmol/l were considered a successful model for type 2 DM. The DM + MET group was administered 227.5 mg/kg/d metformin once a day for ten weeks after successful diabetes modeling. The NC group and DM group mice were administered the same volume of normal saline by gavage.

After 10 weeks of treatment, the mice were sacrificed by cervical spondylectomy, and the abdominal aorta was severed from the aortic root to the proximal end of the renal artery. The tissue around the blood vessels was removed and perfused with normal saline, and the residual blood was washed and dried using filter paper. The aorta, approximately 1 cm in length, was excised from the aortic root to the aortic arch and fixed in 4% paraformaldehyde for subsequent paraffin embedding. The remaining aortic tissues were collected in sterile cryopreservation tubes and immediately stored in liquid nitrogen.

Cell culture

HA-SMCs were purchased from American Type Culture Collection (ATCC) (lot:60628482). ATCC-SMC special medium f-12k (ATCC 30-2004) supplemented with 0.05 mg/ml ascorbic acid (Sigma- Aldrich, Mo, USA, A-4544), 0.01 mg/ml, bovine insulin (Sigma- Aldrich, I-1882), 0.01 mg/ml human transferrin (Sigma-Aldrich, T-5391), 10 ng/ml sodium selenite (Sigma- Aldrich, S-9133), 0.03 mg/ml endothelial growth factor (Sigma-Aldrich, E-2759), 10 mm HEPES (Sigma- Aldrich, H-0887), 10 mm TES (Sigma-Aldrich, T-5691), and fetal bovine serum (10%, GIBOCO, MA, USA) was used. HA-SMCs were cultured in 6-well plates containing the medium and incubated at 37 ℃, with 5% CO_2. Cells grown in the plates were used when cell growth reached 30% confluency.

The expression of MIR-21 was determined by real-time fluorescence quantitative polymerase chain reaction (RT-PCR). Total RNA was extracted from animal tissues or cells, and the concentration was determined. cDNA was synthesized using the reverse transcription kit (Thermo Fischer, MA, USA), and U6 was used as an internal reference. The cDNA was diluted eight times. Primer sequence of target gene fragment:

U6	forward	5'- CGCTTCGGCAGCACATATAC-3'
	Reverse	5'- AAATATGGAACGCTTCACGA-3'
miRNA-21	forward	5'- TGCGCTAGCTTATCAGACTGA-3'
	Loop	5'-gtcgtatccagtgcagggtccgaggtattcgactggatacgac tcaacatc-3'
MicroRNA antisense strand universal primer:R primer		5'- ccagtgcagggtccgaggtatt- 3'

Reaction conditions: 94 ℃ for 3 min, 94 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 45 s, 35 cycles. The dissolution curve was constructed, and the final data were analyzed using $2^{-\triangle\triangle Ct}$.

Transfection of mir-21mimics and inhibitor in VSMC

Add 5 to each hole of the 6-hole plate μ L Lipofectamine 2000, 245 for the latter μ The medium was diluted with low serum of L opti mem and incubated at room temperature for 5 min.According to the product instructions, miR-21 inhibitor control, miR-21 inhibitor, miR-21 mimics control and miR-21 mimics were added into DEPC water to prepare 20 μMol / L solution, stored at − 20 ℃. Mimics / inhibitor (NC) 5 when used μL and 245 μL opti MEM was mixed and diluted, and incubated at room temperature for 5 min.Mir-21mimics and inhibitor were purchased from Shanghai genepharma Co., Ltd.Diluted spare mimics/inhibitor (NC) and diluted spare Lipofectamine 2000 were mixed, gently mixed and set for 20min at room temperature to form the mimics/inhibitor (NC) -Lipo2000 complex.Suck out the original culture medium from the 6-well plate and rinse it twice with PBS. The above mixture was added dropwise to the wells to be transfected, and 1500 was added μ L opti MEM medium. 2ml opti MEM medium was added to the non transfection wells.The treated cells were incubated in a 37℃ heated CO_2 incubator for 6h.0.5% serum was starved for 24 h and finally high glucose / plus glucose treatment for 24 h.Cells were processed for the next assay.The cells were randomly divided into five groups: normal glucose group (glucose concentration 5.5mmol/l), high glucose + mir-21inhibitor NC group (glucose concentration 25mmol / L + mir-21inhibitor NC), high glucose + mir-21inhibitor group (glucose concentration 25mmol / L + mir-21inhibitor NC), High glucose + mir-21mimics NC group (glucose concentration 25mmol / L + mir-21mimics NC) and high glucose + mir-21mimics group (glucose concentration 25mmol / L + mir-21mimics NC)

Western blotting

Western blotting (WB) was used to determine the expression of signaling pathway proteins. The tissue lysates were prepared using a buffer containing 10 μl PMSF (100 mM) and 10 μl phosphatase inhibitor. Total protein was extracted, and the protein concentration was determined using the BCA method. After protein denaturation, electrophoresis, and film transfer and sealing, the tanon film system (Shanghai Beiyi Bioequip Information Co., Ltd) and ECL developer were used to develop the images. The images were analyzed to quantify the protein expression.

Cell proliferation analysis.

The MTT assay was used to determine cell proliferation. The density of the VSMC cells was adjusted to 5×10^4 cells/ml in a complete medium on a 96-well plate, and 100 μL of cell suspension was used from each well. The cells were cultured overnight at 37 ℃. The cells were treated as per the abovementioned treatment strategies for the different groups, with 3 multiples of each treatment strategy, and cultured at 37 ℃ in a 5% CO_2 saturation humidity incubator for 24 h. MTT (10 μl) was added per well and incubated at 37 ℃ for 4 h. After he incubation period, the medium was removed, and 150 μL DMSO was added to the well and the plates were shaken for 10 min. The absorbance was measured at 568 nm using an enzyme labeling instrument.

Cell migration was analyzed using the scratch method. HA-SMCs, in the logarithmic growth stage and in good growth conditions, were inoculated in 6-well plates at 2×10^5 cells/well and cultured overnight in a 5% CO_2 incubator at 37 ℃. The cells were treated as per the abovementioned treatment strategies for the different groups for 6 h respectively. The intervention solution was sucked out, and the complete medium was added. The cells were further grown for 48 h. After achieving 90% confluency, horizontal line scratches on the bottom of the plate were made using the gun head. The cells were washed with PBS 3 times, the scratched cells were removed, and serum-free medium was added. Images were taken at 0 h, and the cultures were further observed for 24 h.

Hematoxylin & eosin and immunohistochemical staining of mouse aorta.

The paraffin sections of the mouse aorta were dehydrated, waxed, embedded, sliced and baked, dewaxed, and stained (Mayer's hematoxylin dye). Sections were sealed with neutral gum after air drying and examined using a microscope. After dewaxing, the sections were blocked with endogenous peroxidase and serum. The primary antibody and enzyme-labeled secondary antibody of α-SMA (1:200) and OPN (1:200) were added. Finally, the sections were counter-stained, and observed under the microscope.

Statistical methods

SPSS 19.0 was used for data analysis. The data are expressed as mean ± standard deviation. The data of multiple groups were compared using one-way ANOVA; LSD-*t* test was used for pairwise comparisons between groups. $P < 0.05$ indicated statistical significance. Graphpad 7.0 was used for the graphical representation of the data.

Results

MIR-21 expression in the internal mammary artery increased in diabetic patients undergoing CABG, and the contractile VSMCs were converted to the synthetic type.

There were no significant differences in gender, age, BMI, hypertension, carotid stenosis, smoking history, preoperative left ventricular ejection fraction (LVEF), end-diastolic left ventricular posterior wall thickness (LVPWTd), end-diastolic intraventricular septal thickness (IVSTd), TC, TG, LDL, HDL, Cr, and UA between the two groups ($P > 0.05$). Compared with the non-DM group, the level of HbA_{1c} in the DM group increased significantly ($P < 0.05$), and the SYNTEX score was higher ($P = 0.053$) (Table 1).

Compared with the non-DM group, the level of MIR-21 in the internal mammary artery tissue of the DM group increased significantly ($P < 0.05$) (Table 1, Fig. 1). The expression of αSMA decreased (Fig. 1B) and that of OPN increased ($P < 0.05$) (Fig. 1A). Overall, in patients with diabetes, the results indicate changes in the VSMC phenotype in the internal mammary artery, and increase in the expression levels of MIR-21 (Table 1).

Table 1
Comparison of baseline data between the two groups

	Non-DM group	DM group	P
N	11	18	-
Sex(male,%)	6(54.5)	15(83.3)	0.127
Age(year)	64.4 ± 9.5	62.8 ± 6.0	0.680
BMI(kg/m2)	25.0 ± 1.9	24.5 ± 2.5	0.572
Hypertension(n,%)	6(54.5)	7(38.9)	0.463
Carotid artery stenosis (n, %)	2(18.2)	7(38.9)	0.345
Smoking (n, %)	7(63.6)	16(88.9)	0.243
LVEF (%)	58 ± 13.3	60.3 ± 7.0	0.669
IVSTd (mm)	11.1 ± 2.3	11.3 ± 1.2	0.778
LVPWTd (mm)	10.2 ± 1.7	10.0 ± 0.5	0.862
SYNTEX (min)	28.5 ± 9.3	38.3 ± 6.1	0.053*
HDL (mmol/l)	1.2 ± 0.3	1.1 ± 0.2	0.302
LDL (mmol/l)	2.5 ± 0.7	3.4 ± 1.2	0.077
TC (mmol/l)	4.1 ± 0.9	5.1 ± 1.3	0.065
TG (mmol/l)	1.9 ± 0.7	1.8 ± 0.9	0.687
HbA_{1c} (%)	5.9 ± 0.4	7.5 ± 1.0	0.000*
Cr (umol/l)	62.8 ± 12.9	71.6 ± 19.5	0.252
UA (umol/l)	325.3 ± 92.5	348.3 ± 112.8	0.632
miR-21	1.5 ± 0.8	3.0 ± 1.2	0.005*
OPN	0.5 ± 0.2	0.7 ± 0.1	0.019*
α-SMA	0.8 ± 0.1	0.6 ± 0.1	0.002*

*P < 0.05.BMI: body mass index, TC: total cholesterol, TG: triglyceride, HDL-C: high-density lipoprotein cholesterol, LDL-C:

low-density lipoprotein cholesterol, LVEF: left ventricular ejection fraction, LVSTd : end diastolic ventricular septal thickness, LVPWTd : end diastolic left ventricular posterior wall thickness, Cr : creatinine, UA : uric acid, OPN : osteopontin, α-SMA□Smooth muscle contractile protein.

H&E and immunohistochemical staining of OPN and α-SMA in internal mammary artery between the two groups

H&E staining showed that VSMCs in the non-DM group were neatly aligned, had uniform nuclear size, showed cytoplasmic staining, and had a clear structure. Compared with the non-DM group, in the DM group, the thickness of media increased, the arrangement of cell growth was irregular, and nuclear size and cytoplasmic staining results were normal (Fig. 1C).

The results showed that αSMA and OPN proteins were mainly expressed in the whole layer of the artery (Fig. 1D). We primarily measured the middle layer of the artery dominated by VSMCs. Compared with the non-DM group, the content of OPN and percentage of positively stained cells in the DM group increased (12.0 ± 3.4 vs. 33.7 ± 6.8, P = 0.000), and the percentage of cells staining positive for αSMA decreased (28.2 ± 8.6 vs. 17.5 ± 6.2, P = 0.001).

Effect of metformin on α-SMA, OPN, and miR-21 expression in diabetic mice

Comparison of general data among mice in each group at the end of the experiment showed no significant differences in bodyweight and Cr among the three groups. Compared with the NC group, fasting blood glucose (FPG), TG, and TC increased in the DM and DM + MET groups (P < 0.05). Compared with the DM group, FPG, TG, and TC levels and *Mir*-21 expression decreased in the DM + MET group (P < 0.05; Table 2).

Table 2
Comparison of general biochemical indexes of mice among groups

	NC Group	DM Group	DM + MET Group	P
N	10	10	10	
weight(g)	33.1 ± 6.7	34.8 ± 1.3	29.2 ± 3.1*#	0.011
FPG(mmol/l)	6.9 ± 1.4	18.5 ± 3.2*	13.5 ± 3.0	0.000
TG(mmol/l)	1.4 ± 0.2	4.6 ± 1.0*	3.3 ± 0.2#	0.000
TC(mmol/l)	3.1 ± 0.1	6.5 ± 0.1*	4.6 ± 0.0#	0.000
HDL(mmol/l)	1.9 ± 0.1	1.1 ± 0.1*	1.9 ± 0.0#	0.000
Cr(umol/l)	59.8 ± 9.0	68.7 ± 14.6	64.5 ± 14.6	0.567
miR-21	0.8 ± 0.1	1.5 ± 0.2*	1.1 ± 0.2#	0.001

Note: * compared with NC group, P < 0.05, # compared with DM group, P < 0.05,TC: total cholesterol, TG: triglyceride, HDL-C: high-density lipoprotein cholesterol.

Comparison of aortic αSMA and OPN expression among the three groups.

Compared with the NC group, αSMA expression decreased (Fig. 2A) and OPN expression increased in the DM group (Fig. 2B). Compared with the DM group, αSMA expression in the aorta of the DM + MET group increased, OPN expression decreased, and there was no significant difference of αSMA and OPN between DM + MET group with the NC group.

Immunohistochemical results of αSMA and OPN in mouse aorta between the three groups

Compared with the NC group, αSMA optical density in the DM group decreased and that of OPN increased significantly. Compared with the DM group, αSMA optical density in the DM + MET group increased slightly, and the OPN expression decreased significantly. The results showed that metformin could inhibit the conversion of VSMCs from the contractile to the synthetic type (Fig. 2C).

Effect of metformin on the Mir-21 in the aorta of diabetic mice

Compared with the NC group, the expression of *Mir-21* in the aorta of mice in the DM and DM + MET groups was significantly higher. Compared with the DM group, the expression of *Mir-21* in the aorta of mice in the DM + MET group decreased (Fig. 2D).

Effects of metformin on the expression of MIR-21 induced by HG and its downstream signaling pathway proteins in HA-SMCs

Effect of HG on the expression of MIR-21 and its downstream signaling proteins in HA-SMCs

HA-SMCs were inoculated in 24-well plates and randomly divided into 15 groups with 3 wells corresponding to each group. Groups 1–5 were supplied with the medium containing 5.5 mmol/l glucose, groups 6–10 were supplied with medium containing 25 mmol/l glucose, and groups 11–15 were supplied with mannitol at the same concentration as that supplied to the control group. All groups were treated for 0, 6, 12, 24, and 48 h. Expression of MIR-21 in HA-SMCs was measured. We found that the expression of miR-21 increased significantly after 12 h in HA-SMCs supplemented with 25 mmol/l glucose, and that of MIR-21 increased gradually with time (Fig. 3B). Expression of MIR-21 in the 5.5 mmol/l glucose concentration group and mannitol group did not change significantly with the time. Therefore, the glucose concentration of 25 mmol/l was selected for 24 h treatment in further investigations (Fig. 3B).

Effect of HG on the expression of PTEN, pAKT, Egr-1, and OPN proteins in HA-SMC

The cells were inoculated in 6-well plates and randomly divided into 2 groups with 3 wells in each group: normal sugar group (5.5 mol/l, n = 3) and a high glucose group (25 mmol/l, n = 3). The intervention lasted for 24 h. The expression levels of PTEN, pAkt, Egr-1, and OPN were measured using western blotting (Fig.

4A). Compared with the normal glucose group, the expression of PTEN decreased significantly and that of pAkt, Egr-1 and OPN increased significantly in the HG group ($P < 0.05$). Compared with the HG group, the expression of miR-21 in the HG + MIR-21 inhibitor group decreased significantly, and that of MIR-21 in the HG + miR-21 mimics group increased significantly (Fig. 4B). Compared with the HG + miR-21 mimics NC group, the expression of PTEN protein decreased and that of pAkt, Egr-1, and OPN proteins increased in the HG + miR-21 mimics NC group, but the difference was not significant (Fig. 4C). Compared with the HG + miR-21 inhibitor NC group, the expression of PTEN protein decreased, the expression of Egr-1 and OPN protein increased significantly, and there was no difference in pAKT expression in the HG + miR-21 inhibitor NC group (Fig. 4D). The results showed that the inhibitor of miR-21 could inhibit OPN expression in HA-SMCs induced by HG and inhibit the transformation of HA-SMCs.

Effect of metformin on the expression of MIR-21 and its downstream signaling proteins in HA-SMCs under HG conditions

HA-SMCs were treated with metformin at different concentrations (0 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, and 40 μM) for 6 h, 12 h, 24 h, and 48 h, respectively. The cell proliferation rate was measured using the MTT method. The concentration of 40 μM metformin was selected for the final experiment, and the 24 h proliferation rate was found to be 61%, which was statistically different from that observed for the control group. VSMCs were randomly divided into HG group, HG group + miR-21 mimics group, HG + MET group, and HG + MET + miR-21 mimics group. Among them, HG + miR-21 mimics NC group and HG + miR-21 mimics group were transfected with NC and mimics 48 h after intervention, starved with 0.5% serum for 24 h, and treated with HG and 40 μM/l metformin for 24 h. Compared with the HG group, the expression of miR-21 in VSMCs in the HG + MET group decreased significantly ($P < 0.05$); Compared with the HG group, the expression of MIR-21 in VSMCs increased significantly in the HG + MIR-21 mimics group. After metformin intervention, the expression of MIR-21 promoted by MIR-21 mimics decreased, and the expression of MIR-21 decreased (Fig. 5A). The proliferation of VSMCs was measured using the MTT assay. Compared with the HG group, there was no significant difference in the VSMC proliferation rate in the HG + MET group. The VSMC proliferation rate in HG + miR-21 mimics group and HG + miR-21 mimics + MET group increased significantly ($P < 0.05$), but there was no significant difference in VSMC proliferation rate between HG + miR-21 mimics group and HG + miR-21 mimics + MET group (Fig. 5D).

WB was used to determine the changes in PTEN, pAkt, and Egr-1 protein expression in each VSMC group. Three independent experiments were performed in each group to calculate the mean value (Fig. 5B). Compared with the HG group, the expression of pAkt, Egr-1, and OPN protein in the HG + MET group decreased significantly ($P < 0.05$). Compared with the HG + MIR-21 mimics group, the expression of pAkt, Egr-1, and OPN protein in the HG + MIR-21 mimics + MET group decreased significantly ($P < 0.05$). The results showed that metformin could block the activation of pAkt/Egr-1/OPN pathway induced by MIR-21 upregulation, produce an effect similar to that of miR-21 inhibitor, promote the expression of the synthetic phenotypic protein of VSMCs, and further convert them to the synthetic phenotype.

The migration ability of VSMCs was evaluated using the scratch test. Scratch closure rate = Percentage of blank area treated 0h-percentage of blank area area treated at 24h. Three independent experiments were conducted for each group intervention, and the mean was calculated. Figure 5C shows that compared with the HG group, the migration ability of VSMCs in the HG + MET group decreased, but the difference was not statistically significant. The migration ability of VSMCs in the HG + miR-21 mimics group increased significantly. Compared with the HG + MIR-21 mimics group, the migration ability of VSMCs in the HG + MIR-21 mimics + MET group decreased significantly. The results showed that metformin could inhibit VSMC migration induced by MIR-21 mimics.

Figure 5 Effects of metformin on HG induced expression of mir-21 and its signal pathway protein, proliferation and migration of HA-SMC.(A)RT-PCR of miR-21 of HG,HG + miR-21 mimics,HG + miR-21 mimics + MET group.(B)Western blotting of proteins PTEN\pAKT\Egr-1\OPN and cell marker GAPDH. (C)Metformin inhibitored VSMCs migration measured by scratch wound assay. Magnification ×100. (D)Metformin had no effect on the proliferation of HASMC induced by HG by MTT. *Compared with NC group, P < 0.05.All the data are presented as mean ± SD (One-way NOVA).

Discussion

In this study, we found that metformin inhibits the phenotypic transformation of aortic smooth muscle cells induced by HG. MIR-21 plays an important role in HG-induced expression of OPN in aortic VSMCs of diabetic mice or in the internal mammary artery of patients with diabetes. Moreover, metformin can reduce the expression of MIR-21 in the aorta. At the same time, metformin was found to affect the expression of OPN through the PTEN/pAkt/Egr-1/OPN pathway downstream of miR-21, and inhibit the migration ability of HA-SMCs.

In previous studies, MIR-21 has been shown to play an important role in hyperglycemia-induced transformation of HA-SMCs. In patients with diabetes, the expression of MIR-21 increased in the heart tissue; however, no change in MIR-21 expression in peripheral blood was observed. The expression of MIR-21 is regulated by inflammation [11]. HG could also promote the proliferation and collagen synthesis of rat cardiac fibroblasts by upregulating Mir-21 [12]. There are few studies on MIR-21 expression in VSMCs of patients with diabetes. *In vivo* and *in vitro* studies [17] have found that hyperglycemia can alter the interaction between the vascular endothelial cells and VSMCs by upregulating the expression of MIR-21 in VSMCs, which might be achieved by inhibiting the expression of Notch receptor Jag1. The restoration of EC-VSMC interaction is believed to be a possible target for the treatment of macrovascular complications associated with diabetes. Similar results were obtained in this study. The expression of MIR-21 and OPN increased and that of αSMA decreased in the aortic tissue of diabetic mice. Therefore, there is a possibility of a causal relationship between the changes in blood glucose, Mir-21, and OPN expression.

We found that glucose increased the expression of MIR-21 in HA-SMC in a concentration- and time-dependent manner, and inhibited the expression of downstream target molecules, PTEN, pAkt, Egr-1, and

OPN, of miR-21. Downregulating the expression of MIR-21 can enhance PTEN expression, and finally inhibit the expression of OPN protein. Yang et al. [18] observed that MIR-21 has a high homology with the 3'UTR of PTEN mRNA, and PTEN is the target gene of MIR-21. Numaga-Tomita T et al. found that Akt could be activated and VSMCs could be transitioned to the contractile phenotype by inhibiting the typical TRPC6 channels and PTEN using plasma membrane hyperpolarization [19]. Egr-1 is a transcription factor required for OPN expression. It is directly connected with the *OPN* gene promoter and positively regulates its expression [20]. In VSMCs, Egr-1 is directly regulated by Akt. A variety of cardiovascular risk factors, including ROS, endothelin, and insulin-like growth factor, can activate Akt and upregulate Egr-1 protein expression [21–23]. This study confirmed, for the first time, that VSMCs can upregulate the expression of Egr-1/OPN protein by upregulating the level of MIR-21 and activating Akt.

OPN is a marker protein for the synthetic phenotype of VSMCs, indicating higher proliferation and migration ability. This study found that the migration and proliferation of VSMCs was enhanced by HG administration. The administration of MIR-21 mimics or inhibitor can enhance or inhibit the migration and proliferation of VSMCs caused by HG. MIR-21 is the first miRNA found to promote the proliferation of aortic smooth muscle cells [24]. Under the action of different factors, MIR-21 acts on different pathways to produce various outcomes [25–28]. Qiu F et al. [29] found that inhibition of MIR-21 can inhibit hyperglycemia and increase the proliferation and angiogenesis of human retinal microvascular endothelial cells, which may be achieved by regulating the Akt/ERK pathway through *Maspin.* Our study provides new mechanistic evidence for the proliferation and migration of smooth muscle cells induced by HG.

As a hypoglycemic drug, metformin is considered effective for reducing the risk of atherosclerosis [30]. Some studies have found that metformin significantly reduces atherosclerosis/calcification in ApoE$^{-/-}$ mice and effectively inhibits the phenotypic transformation of VSMCs [31], which is independent of the hypoglycemic effect. This study found that the expression of the VSMC contractile marker protein αSMA was significantly lower in the DM mouse model. Although the formation of typical atherosclerotic plaques was not observed in the aorta of the DM mice, the aortic middle layer thickness in DM + MET group decreased significantly. Multivariate analysis showed that the effect of metformin was more potent than that of blood glucose. Therefore, metformin may play a cardiovascular protective role in addition to its hypoglycemic function; however, the exact mechanism is not clear.

This study found that metformin could inhibit the upregulation of MIR-21 expression in VSMCs induced by 25 mmol/l glucose, and then cause the corresponding changes in the downstream PTEN/pAkt/Egr-1/OPN pathway. After transfection of miR-21 mimics, metformin could reduce the expression levels of miR-21 and change the expression of downstream signaling proteins. The inhibitory effect of metformin on miR-21 has been verified in various tumors and endothelium [32–36].

The effect of metformin on MIR-21 in HA-SMCs was evaluated for the first time in this study. This study also found that metformin significantly reduced the migration ability of VSMCs induced by HG but had no significant effect on the proliferation of VSMCs. Dong M Zhou et al. [37] also found similar results

using metformin that inhibited HG-induced VSMC proliferation and migration by promoting the mir-142-3p mediated HMGB1 expression. The mechanism of metformin-mediated inhibition of migration still needs to be further studied.

This study has some limitations. This study did not analyze the mechanisms behind metformin-mediated miR-21 inhibition. The signaling pathway of metformin inhibiting HG-induced VSMC migration still needs to be further studied.

In conclusion, this study provides new evidence that MIR-21 mediates the HG-induced phenotypic conversion of VSMCs and targets the PTEN/pAkt/Egr-1/OPN signaling pathway. Metformin inhibits the HG-induced VSMCs phenotypic conversion by downregulating the expression of MIR-21.and migration of VSMCs Therefore, the results of this study provide information for the design of novel anti-arteriosclerosis treatment strategies based on MIR-21 expression.

NOTE

Hui Zheng and Ying Liu contributed equally to this work. They are co-first authors.

Abbreviations

ascular smooth muscle cells (VSMCs)

coronary artery bypass grafting (CABG)

smooth muscle actin (SMA)

low-density lipoprotein (LDL)

high-density lipoprotein (HDL)

percutaneous coronary intervention (PCI)

body mass index (BMI)

American Type Culture Collection (ATCC)

left ventricular posterior wall thickness (LVPWTd)

left ventricular ejection fraction (LVEF)

Interventricular septum thickness end-diastole (IVSTd)

Declarations

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Metabolic disease hospital of Tianjin Medical University（DXBYYhMEC2017-26).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZH ,LY and LL participated in the study design, data collection, data analysis, and drafted the article. SXY was responsible for cell experiments and molecular biology experiments.LXC and MS are in charge of the animal experiment. LYM and ZSJ are responsible for data sorting and statistics.YJH and CBC are in charge of the experimental design.All authors approved the final version of the manuscript for submission.

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References

1. Sartore S, Chiavegato A, Faggin E,et al. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. Circ Res. 2001;89(12):1111–21. doi:10.1161/hh2401.100844.
2. Akira A, Yuki S, Mototsugu N,et al. Metformin Attenuates Early-Stage Atherosclerosis in Mildly Hyperglycemic Oikawa-Nagao Mice. J Atheroscler Thromb. 2019;26(12):1075–83. doi:10.5551/jat.48223.
3. Wang M, Li W, Chang GQ,et al. MicroRNA-21 regulates vascular smooth muscle cell function via targeting tropomyosin 1 in arteriosclerosis obliterans of lower extremities. Arterioscler Thromb Vasc Biol. 2011;31(9):2044–53. doi:10.1161/ATVBAHA.111.229559.

4. Pezzolesi MG, Satake E, McDonnell KP, et al.Circulating TGF-β1-Regulated miRNAs and the Risk of Rapid Progression to ESRD in Type 1 Diabetes.Diabetes. 2015,64(9):3285–3293. doi: 10.2337/db15-0116.
5. Dey N, Das F, Mariappan MM,et al. MicroRNA-21 orchestrates HG-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes.J Biol Chem. 2011,22;286(29):25586–25603.doi: 10.1074/jbc.M110.208066.
6. Fiorentino L, Cavalera M, Mavilio M,et al. Regulation of TIMP3 in diabetic nephropathy: a role for microRNAs. Acta Diabetol. 2013;50(6):965–9. doi:10.1007/s00592-013-0492-8.
7. Wang Y,LiuL, Peng W,et al. Ski-related novel protein suppresses the development of diabetic nephropathy by modulating transforming growth factor-β signaling and microRNA-21 expression.J Cell Physiol. 2019,7. doi: 10.1002/jcp.28425. [Epub ahead of print].
8. Jiang Q, Lyu XM, Yuan Y,et al.Plasma miR-21 expression: an indicator for the severity of Type 2 diabetes with diabetic retinopathy.2017,37(2):BSR20160589. doi: 10.1042/BSR20160589.
9. Haque R, Iuvone PM, He L,et al. The MicroRNA-21 signaling pathway is involved in prorenin receptor (PRR) -induced VEGF expression in ARPE-19 cells under a hyperglycemic condition.Mol Vis. 2017,23:251–262.
10. Usui-Ouchi A, Ouchi Y, Kiyokawa M,et al. Upregulation of Mir-21 Levels in the Vitreous Humor Is Associated with Development of Proliferative Vitreoretinal Disease.PLoS One. 2016,11(6):e0158043. doi: 10.1371/journal.pone.0158043.
11. Liang YZ, Li JJ, Xiao HB,et al. Identification of stress-related microRNA biomarkers in type 2 diabetes mellitus: A systematic review and meta-analysis.J Diabetes. 2018,17. doi:10.1111/1753-0407.12643. [Epub ahead of print].
12. Liu S, Li W, Xu M,et al. Micro-RNA 21Targets dual specific phosphatase 8 to promote collagen synthesis in HG-treated primary cardiac fibroblasts. Can J Cardiol. 2014;30(12):1689–99. doi:10.1016/j.cjca.2014.07.747.
13. Dai L, Chen F, Zheng Y, et al.miR-21 regulates growth and EMT in lung cancer cells via PTEN/Akt/GSK3βsignaling.Front Biosci (Landmark Ed). 2019, 24:1426–1439.
14. Liu HY, Zhang YY, Zhu BL,et al.miR-21 regulates the proliferation and apoptosis of ovarian cancer cells through PTEN/PI3K/AKT.Eur Rev Med Pharmacol Sci. 2019,23(10):4149–4155. doi: 10.26355/eurrev_201905_17917.
15. Chen WJ, Pang JH, Lin KH,et al. Propylthiouracil, independent of its antithyroid effect, promotes vascular smooth muscle cells differentiation via. PTEN inductionBasic Res Cardiol. 2010;105(1):19–28. doi:10.1007/s00395-009-0045-z.
16. Luo M, Tan X, Mu L,et al.MiRNA-21 mediates the antiangiogenic activity of metformin through targeting PTEN and SMAD7 expression and PI3K/AKT pathway.Sci Rep. 2017,7:43427. doi: 10.1038/srep43427.
17. Lee S, Yu LH,Lim LR,et al. Down regulation of Jag-1 in VSMCs contributes to impaired angiogenesis under HG condition: Experimental study using aortic rings of rats. Clin Hemorheol Microcirc.

2015;61(3):497–511. doi:10.3233/CH-141915.

18. Fan Y, Yao W,Jun X,et al. Effect of Corilagin on the miR-21/smad7/ERK signaling pathway in a schistosomiasis-induced hepatic fibrosis mouse model. Parasitol Int. 2016;65(4):308–15. doi:10.1016/j.parint.2016.03.001.
19. Numaga-Tomita T, Shimauchi T, Oda S,et al.TRPC6 regulates phenotypic switching of vascular smooth muscle cells through plasma membrane potential-dependent coupling with PTEN. FASEB J. 2019,4:fj201802811R. doi: 10.1096/fj.201802811R. [Epub ahead of print].
20. Liu QF, Yu HW, Liu GN.Egr-1 upregulates OPN through direct binding to its promoter and OPN upregulates Egr-1 via the ERK pathway.Mol Cell Biochem. 2009,332(1–2):77–84. doi:10.1007/s11010-009-0176-4.
21. Rondeau V, Jain A, Truong V, et al. Involvement of Akt-dependent CREB signaling pathway in hydrogen peroxide (H2O2)-induced early growth response protein-1 (Egr-1) expression in vascular smooth muscle cells. Can J Physiol Pharmacol. 2019;97(9):885–92. doi:10.1139/cjpp-2019-0061.
22. Bouallegue A, Simo Cheyou ER, Anand-Srivastava MB,et al.ET-1-induced growth promoting responses involving ERK1/2 and PKB signaling and Egr-1 expression are mediated by Ca^{2+}/CaM-dependent protein kinase-II in vascular smooth muscle cells.Cell Calcium. 2013,54(6):428–435. doi: 10.1016/j.ceca.2013.10.002.
23. Youreva V, Kapakos G,Srivastava AK.Insulin-like growth-factor-1-induced PKB signaling and Egr-1 expression is inhibited by curcumin in A-10 vascular smooth muscle cells.Can J Physiol Pharmacol.2013,91(3):241–247. doi: 10.1139/cjpp-2012-0267.
24. Albinsson S, Sessa WC.Can microRNAs control vascular smooth muscle phenotypic modulation and the response to injury?Physiol Genomics. 2011,43(10):529–533.doi: 10.1152/physiolgenomics.00146.2010.
25. Sun P, Tang LN, Li GZ,et al. Effects of MiR-21 on the proliferation and migration of vascular smooth muscle cells in rats with atherosclerosis via the Akt/ERK signaling pathway. Eur Rev Med Pharmacol Sci. 2019;23(5):2216–22. doi:10.26355/eurrev_201903_17269.
26. Stein JJ, Iwuchukwu C, Maier KG,et al. Thrombospondin-1-induced vascular smooth muscle cell migration and proliferation are functionally dependent on microRNA-21.Surgery. 2014,155(2):228–233. doi: 10.1016/j.surg.2013.08.003.
27. Li Y, Yan L, Zhang W,et al. MicroRNA-21 inhibits platelet-derived growth factor-induced human aortic vascular smooth muscle cell proliferation and migration through targeting activator protein-1. Am J Transl Res. 2014;6(5):507–16.
28. Sarkar J, Gou D, Turaka P, et al.MicroRNA-21 plays a role in hypoxia-mediated pulmonary artery smooth muscle cell proliferation and migration.Am J Physiol Lung Cell Mol Physiol.2010,299(6):L861-871.doi: 10.1152/ajplung.00201.2010.
29. Qiu F, Tong H, Wang Y,et al. Inhibition of miR-21-5p suppresses HG-induced proliferation and angiogenesis of human retinal microvascular endothelial cells by the regulation of AKT and ERK

pathways via maspin. Biosci Biotechnol Biochem. 2018;82(8):1366–76. doi:10.1080/09168451.2018.1459179.

30. Hao B, Xiao Y, Song F,et al. Metformin-induced activation of AMPK inhibits the proliferation and migration of human aortic smooth muscle cells through upregulation of p53 and IFI16.Int. J Mol Med. 2018;41(3):1365–76. doi:10.3892/ijmm.2017.3346.
31. Cai Z, Ding Y,Zhang M. et al,Ablation of Adenosine Monophosphate-Activated Protein Kinase α1 in Vascular Smooth Muscle Cells Promotes Diet-Induced Atherosclerotic Calcification In Vivo. Circ Res. 2016;119(3):422–33. doi:10.1161/CIRCRESAHA.116.308301.
32. Wang J, Gao Y, Duan L,et al. Metformin ameliorates skeletal muscle insulin resistance by inhibiting miR-21 expression in a high-fat dietary rat model.Oncotarget. 2017,8(58):98029–98039. doi: 10.18632/oncotarget.20442.
33. Kalogirou C, Schäfer D, Krebs M,et al.Metformin-Derived Growth Inhibition in Renal Cell Carcinoma Depends on miR-21-Mediated PTEN Expression.Urol Int. 2016,96(1):106–115. doi: 10.1159/000441011.
34. Deng Y. Ma W.Metformin inhibits HaCaT cell viability via the miR-21/PTEN/Akt signaling pathway. Mol Med Rep. 2018;17(3):4062–6. doi:10.3892/mmr.2017.8364.
35. Luo M, Tan X, Mu L,et al. MiRNA-21 mediates the antiangiogenic activity of metformin through targeting PTEN and SMAD7 expression and PI3K/AKT pathway.Sci Rep. 2017, 7:43427. doi: 10.1038/srep43427.
36. Agnieszka S, Katarzyna K, Jolanta SK,et al.Metformin Increases Proliferative Activity and Viability of Multipotent Stromal Stem Cells Isolated from Adipose Tissue Derived from Horses with Equine Metabolic Syndrome.Cells 2019, 8, 80: doi:10.3390/cells8020080.
37. Dong-Ming Z, Feng R, Hai-Zhen N,et al. Metformin inhibits HG-induced smooth muscle cell proliferation and migration.Aging (Albany NY). 2020,24;12(6):5352–5361. doi:10.18632/aging.102955.

Figures

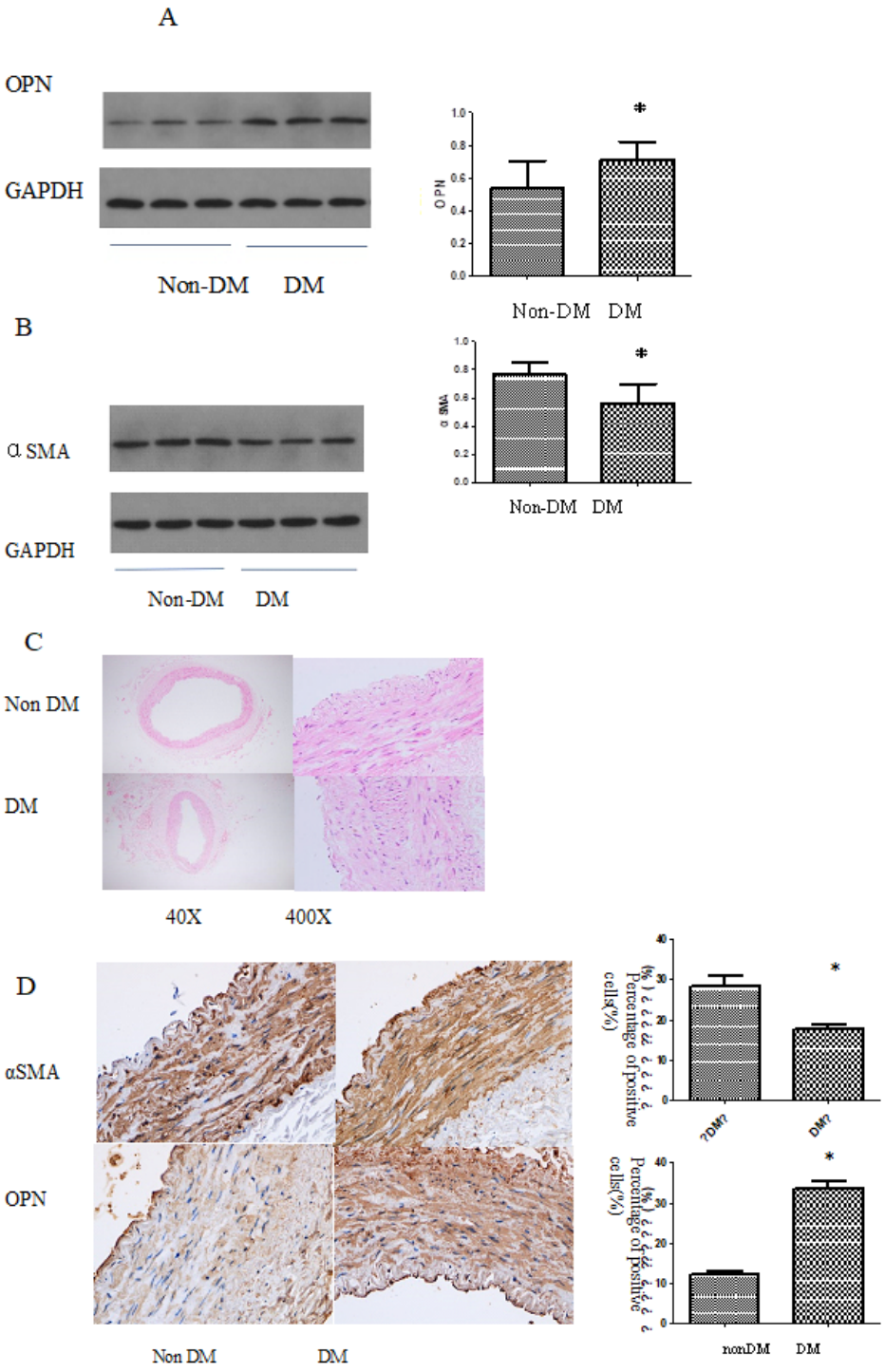

Figure 1

Expression of phenotypic proteins in vascular smooth muscle cells of patients with diabetes. (A) Western blotting of phenotypic proteins marker OPN and cell marker GAPDH. (B) Western blotting of phenotypic proteins marker αSMA and cell marker GAPDH. (C) The Aortic root of non-DM and DM groups were stained with hematoxylin and eosin (HE). Magnification ×40 and ×400. (D) Immunohistochemical images

stained for aSMA and OPN of the aortic root. Magnification ×400. *compared with non-DM group, P < 0.05. All the data are presented as mean ± SD (Student's t-test).

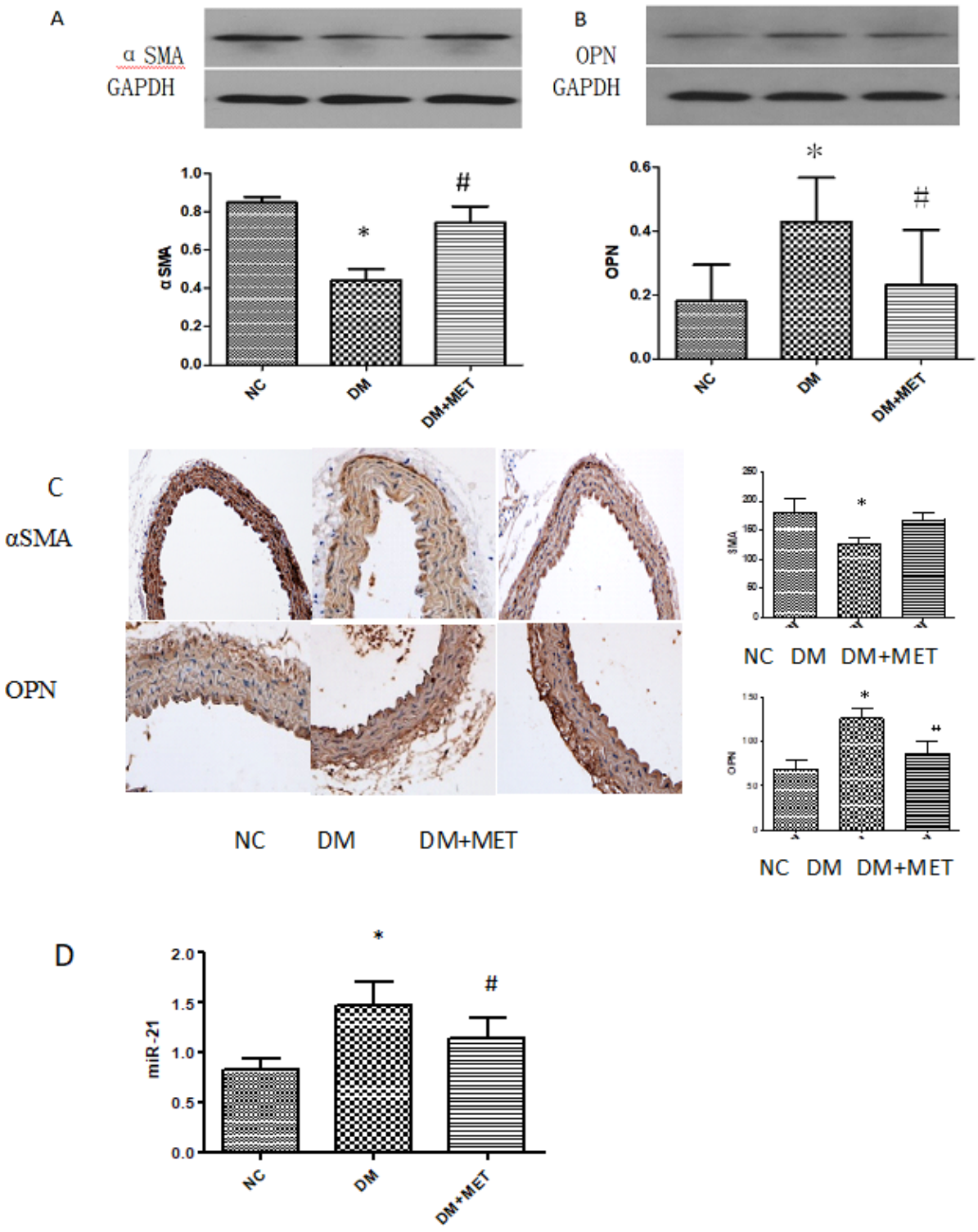

Figure 2

Metformin can influence the expression of marker proteins of aortic smooth muscle phenotype in diabetic mice. (A) Western blotting of smooth phenotypic marker αSMA and cell marker GAPDH. (B) Western blotting of contractile phenotype marker OPN and cell marker GAPDH. (C) Immunohistochemical images

of α-SMA and OPN staining of the aortic root. Magnification ×400. (D) RT-PCR of miR-21 of the NC, DM, and DM+MET group.*<0.05. All the data are presented as mean ± SD (One-way NOVA).

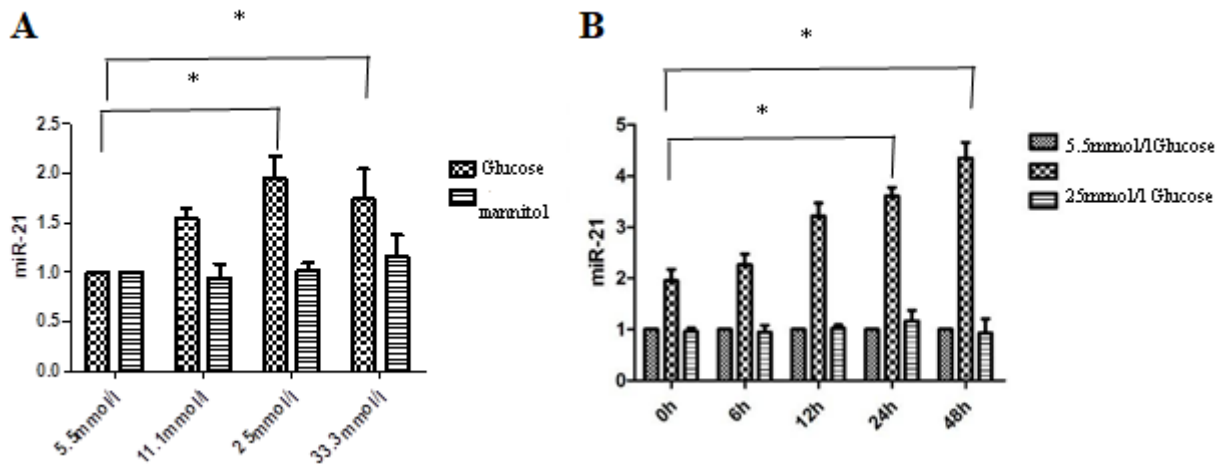

Figure 3

(A) effect of different glucose concentrations on the expression of MIR-21 in VSMC after 48 h, B: effect of 25 mmol/L glucose on the expression of MIR-21 in VSMCs after different time intervals. Note: *compared with 0 h, P < 0.05

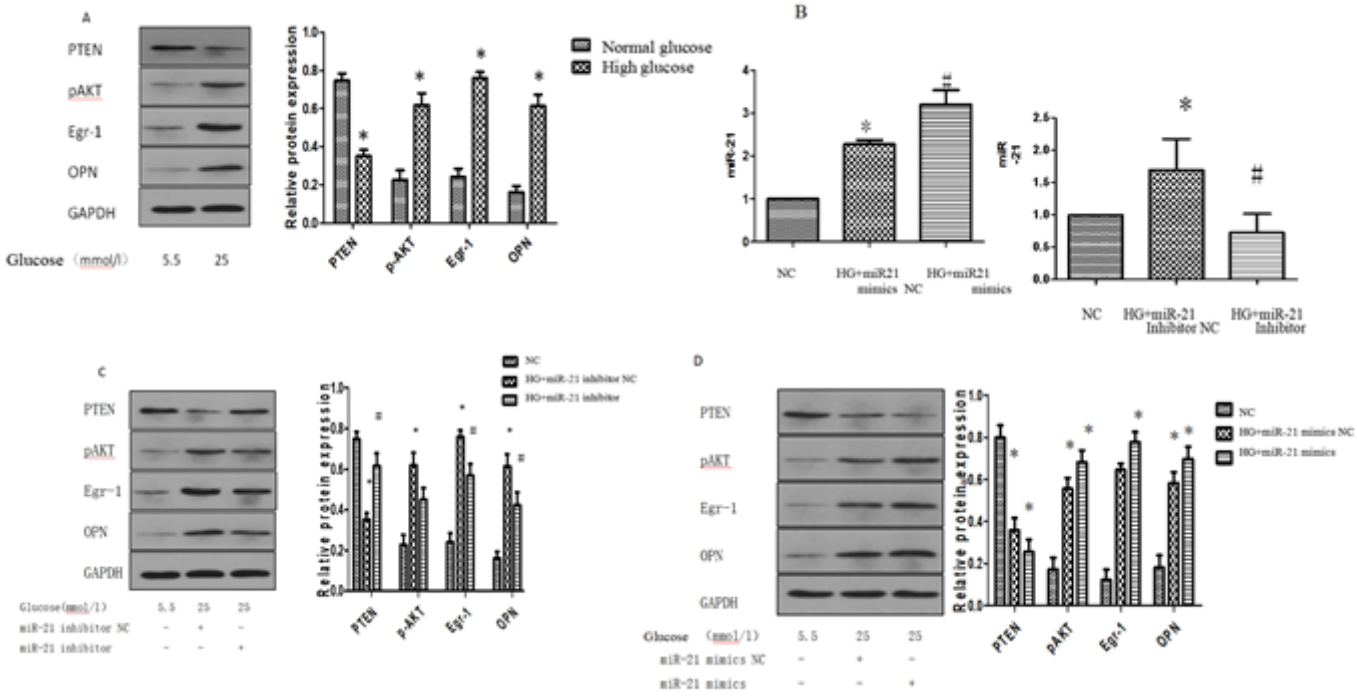

Figure 4

Effects of HG on the expression of hasmcmir-21 and its downstream signal pathway proteins.(A)Western blotting of proteins PTEN\pAKT\Egr-1\OPN and cell marker GAPDH of NC group and HG group.(B)RT-PCR of miR-21 of NC,HG+miR21 mimics/inhibitor NC,HG+miR21 mimics/inhibitor group.(C)Western blotting of proteins PTEN\pAKT\Egr-1\OPN and cell marker GAPDH of NC,HG+miR-21inhibitor NC and HG+miR-

21 inhibitor.(D)Western blotting of proteins PTEN\pAKT\Egr-1\OPN and cell marker GAPDH of NC,HG+miR-21mimics NC and HG+miR-21 mimcs. Compared with NC group, P<0.05,#compared with miR-21mimics/inhibitor NC. All the data are presented as mean±SD (One-way NOVA).

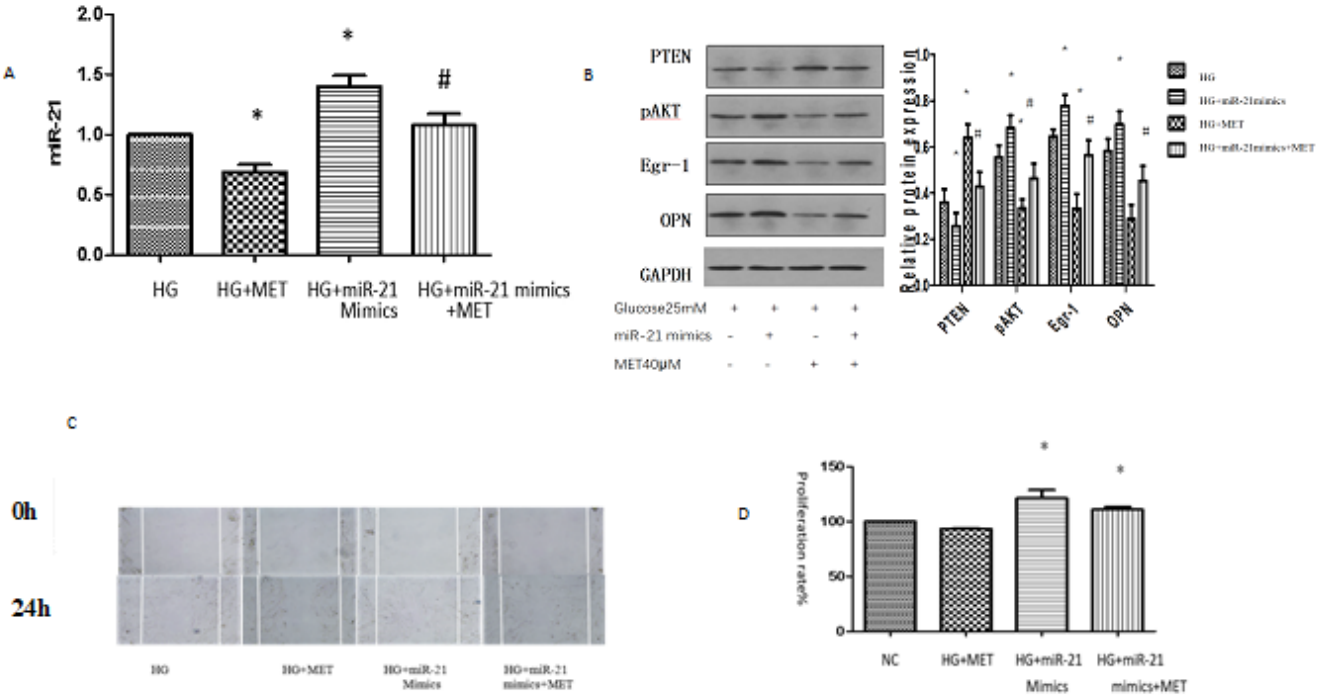

Figure 5

Effects of metformin on HG induced expression of mir-21 and its signal pathway protein, proliferation and migration of HA-SMC.(A)RT-PCR of miR-21 of HG,HG+miR-21 mimics,HG+miR-21 mimics+MET group.(B)Western blotting of proteins PTEN\pAKT\Egr-1\OPN and cell marker GAPDH.(C)Metformin inhibitored VSMCs migration measured by scratch wound assay. Magnification ×100. (D)Metformin had no effect on the proliferation of HASMC induced by HG by MTT. *Compared with NC group, P<0.05.All the data are presented as mean±SD (One-way NOVA).