Nutrition, Metabolism & Cardiovascular Diseases (2019) **29**, 621–632

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

journal homepage: www.elsevier.com/locate/nmcd

MicroRNA-24 attenuates vascular remodeling in diabetic rats through PI3K/Akt signaling pathway

W. Cai [a,b,c,1], J. Zhang [a,b,1], J. Yang [a], Z. Fan [a], X. Liu [a,b], W. Gao [a,b], P. Zeng [a,b], M. Xiong [a,b], C. Ma [a,b], J. Yang [a,b,*]

[a] *Department of Cardiology, The First College of Clinical Medical Science, China Three Gorges University & Yichang Central People's Hospital, Yichang 443003, China*

[b] *Department of Central Experimental Laboratory, The First College of Clinical Medical Science, China Three Gorges University & Yichang Central People's Hospital, Yichang 443003, China*

[c] *Yichang Key Laboratory of Ischemic Cardiovascular and Cerebrovascular Disease Translational Medicine, China*

Received 3 August 2018; received in revised form 2 March 2019; accepted 4 March 2019
Handling Editor: L. Laviola
Available online 13 March 2019

KEYWORDS
Vascular remodeling;
Diabetes mellitus;
MicroRNA-24;
PI3K

Abstract *Background and aims:* The vascular remodeling plays a crucial role in pathogenesis of diabetic cardiovascular complications. In this study, we intended to explore the effects and potential mechanisms of microRNA-24 (miR-24) on vascular remodeling under diabetic conditions.
Methods and Results: MiR-24 recombinant adenovirus (Ad-miR-24-GFP) was used to induce miR-24 overexpression either in carotid arteries or high glucose (HG)-induced vascular smooth muscle cells (VSMCs). Cell proliferation was analyzed using CCK-8 method. Cell migration was examined using wound-healing and transwell assay. mRNA and protein expressions of critical factors were, respectively, measured by real-time PCR and western blot as follows: qRT-PCR for the levels of miR-24, PIK3R1; western blot for the protein levels of PI3K (p85α), Akt, p-Akt, mTOR, p-mTOR, 4E-BP1, p-4E-BP1, p70s6k, p-p70s6k, MMP 2, MMP 9, collagen I, as well as collagen III. Carotid arteries in diabetic rats suffered balloon injury were harvested and examined by HE, immunohistochemical and Masson trichrome staining.

The expression of miR-24 was decreased in HG-stimulated VSMCs and balloon-injured carotid arteries of diabetic rats, accompanied by increased mRNA expression of PIK3R1. The up-regulation of miR-24 suppressed VSMCs proliferation, migration, collagen deposition not only induced by HG *in vitro*, but also in balloon-injured diabetic rats, which were related to inactivation of PI3K/Akt signaling pathway.
Conclusion: The up-regulation of miR-24 significantly attenuated vascular remodeling both in balloon-injured diabetic rats and HG-stimulated VSMCs via suppression of proliferation, migration and collagen deposition by acting on PIK3R1 gene that modulated the PI3K/Akt/mTOR axes.

Introduction

Diabetes mellitus (DM) is a primary risk factor for coronary artery disease (CAD) [1,2], patients with both CAD and diabetes suffer from increased cross-sectional luminal narrowing and heavy plaque burdens [3,4]. Compared with nondiabetic patients, patients with diabetes often

* Corresponding author. Department of Cardiology, The First College of Clinical Medical Sciences, Institute of Cardiovascular Diseases, China Three Gorges University, Yichang 443000, Hubei Province, China.
E-mail address: yangjian@ctgu.edu.cn (J. Yang).

[1] W. Cai and J. Zhang contribute equally to this study.

https://doi.org/10.1016/j.numecd.2019.03.002
0939-4753/

exhibit severe neointimal hyperplasia following coronary interventions [5,6]. Even the implantation of drug-eluting stents is unsatisfying in remitting the restenosis and unfavorable clinical outcomes [7]. As the common pathological basis of diabetic vascular complications, vascular remodeling is the main cause of its disability and death [8]. A variety of internal environmental disorders of diabetes can lead to impaired vascular endothelial barrier function and promote vascular remodeling [9–13]. Diabetic vascular remodeling is a complex pathological process, whose pathogenesis is related to inflammation, endothelial dysfunction and neointimal proliferation [9,14]. In the neointimal, the vascular smooth muscle cells (VSMCs) in contractile type are transformed into synthetic type, producing and secreting a large number of extracellular matrix (ECM) and obtaining the ability to migrate and proliferate. This is the most critical link in vascular remodeling [15,16]. While, the existing treatments are still too simple to improve the VSMCs function, such as inhibiting the proliferation of VSMCs, but it does not play a role in the inflammation, migration and the formation of ECM of VSMCs. Therefore, penetrates the mechanism of diabetes intimal hyperplasia after vascular injury, in order to overcome the single problem of previous research, it is of great theoretical and clinical significance to find the “multiple intervention” target that can inhibit the VSMCs phenotype conversion, inflammation, migration, excessive proliferation and ECM formation at the same time.

MicroRNAs (miRNAs) are composed of ~22 nucleotides that bind to complementary sites located on the 3′ untranslated region (UTR) of their target mRNAs [17–19]. Binding of miRNAs to a target leads to down-regulation of protein levels, either through mRNA cleavage or repression of translation [16]. miRNAs are estimated to target ~60% of all human protein-coding genes and are involved in regulating key physiological processes and intracellular signaling pathways [20]. Functionally, an individual miRNA can regulate the expression of multiple target genes, which is the basis of their multipoint intervention [21–24]. The studies of miRNAs are rapidly growing and recent studies have revealed a significant role of miRNAs in vascular biology and disease [23,25]. Many miRNAs are highly expressed in the vasculature, and their expression is dysregulated in diseased vessels, including miR-143, miR-145 and so on [26]. Alberto Polimeni et al. found that miR-24 is highly expressed in the vascular wall [27], our previous study confirmed this and the unpublished data has proposed that miR-24 was low expression in endothelial cells. The previous studies have confirmed that the level of miR-24 in plasma of diabetes is significantly lower than that in healthy controls, and miR-24 level is decreased 2–3 fold in STZ-induced mice [28]. Moreover, it has been reported that miR-24 expression is downregulated in atherosclerotic plaques, and miR-24 expression is significantly lower in patients with atherosclerosis, as compared with healthy subjects. MiR-24 could inhibit the development of atherosclerosis [29]. However, the role and potential mechanism of miR-24 in the vascular remodeling remains to be fully elucidated. Pathway analysis for predicted target genes revealed that miR-24 was associated with numerous pathways, and PI3K/Akt was one of them [30]. Our previous data showed that PI3K signaling pathway was involved in the migration of VSMCs [31]. However, the relationship between miR-24 and PIK3R1 gene is unclear.

In this study, we intended to explore the possible protective effects and underlying mechanisms of miR-24 on VSMCs proliferation, migration, collagen deposition *in vitro* in response to high glucose stimuli as well as neointimal formation *in vivo* following arterial injury in diabetic rats.

Methods

Animals and ethics statement

All animals used in our study were purchased from the Animal Center of China Three Gorges University (CTGU), Yi Chang, China. The procedures for experiments and animal care were approved by the Animal Care and Use Committee of CTGU and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 80-23). All animals were housed in a room with a 12-h light/12-h dark cycle and an ambient temperature of 21°C–26 °C.

MiR-24 expression adenoviral vector in vitro and in vivo

The synthesis of rno-miR-24 precursor DNA (MI0000854), and recombinant adenoviruses expressing miR-24 or Scramble as a control were provided by Genechem (Shanghai, China). Plaque analysis was used to adjust the concentration of virus titer to 1×10^{10} pfu/ml. For the transfection *in vitro*, VSMCs seeded in 100 mm petri dish at 70% confluence were transfected with the adenovirus at multiplicities of infection (MOI) of 25 for 3 h before medium change, following with another 3 d to obtain approximately 95% efficiency. For the transfection *in vivo*, adenovirus was delivered to the rat carotid arteries immediately after injury, and locally incubated for 20 min before blood perfusion restoring.

Cell culture and high glucose treatment

Primary VSMCs were isolated from the thoracic aorta of male Sprague–Dawley rats (130–150 g) as previously described [32], and then cultured in Dulbecco’s Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. All VSMCs were used for experiments between the 3rd and 5th passages. For HG stimulation experiments, serum-starved VSMCs were stimulated with fresh medium containing 30 mM glucose (HG). All cells were divided into four groups according to different treatment ($n = 3$ for each group): non-glucose group (NG); 30 mM of glucose stimulation group (HG); the adenovirus expressing miR-24-GFP was transfected and then stimulated by 30 mM of glucose (Ad-miR-24 + HG); the adenovirus expressing scramble-GFP was transfected and then stimulated by 30 mM of glucose (Ad-Scramble + HG).

740Y-P peptide treatment

After adenovirus transfection, HG treatment and serum starvation, VSMCs were cultured in the presence of the 740Y-P peptide (binding and activating p85 subunit of PI3K) at the given concentrations (1–200 μg/ml) for 48 h. All cells were divided into four groups according to different treatment (n = 3 for each group): the adenovirus expressing scramble-GFP was transfected and then stimulated by 30 mM of glucose (Ad-Scramble + HG); the adenovirus expressing scramble-GFP was transfected and then stimulated by 30 mM of glucose and 740Y-P peptide (Ad-Scramble + HG + 740Y-P); the adenovirus expressing miR-24-GFP was transfected and then stimulated by 30 mM of glucose (Ad-miR-24 + HG); the adenovirus expressing miR-24-GFP was transfected and then stimulated by 30 mM of glucose and 740Y-P peptide (Ad-miR-24 + HG + 740Y-P).

Cell proliferation

Cell proliferation was assessed using a cell counting kit-8 assay. For CCK-8, VSMCs were seeded in 96-well plates (Corning) at 8×10^3 cells per well in 200 μl culture medium after transfection of adenovirus. Following synchronization by DMEM/F12 for 24 h, VSMCs were stimulated with HG. After 24 h of stimulation (For 740Y-P peptide stimulation test, 740Y-P peptide was added after HG treatment and stimulated for 48 h), 20 μl of CCK-8 was added, and the absorbance was measured at 450 nm.

Cell migration

VSMCs migration was assessed using a transwell chamber (8 μm, Corning) and wound healing assay. For transwell chamber assay, 1×10^5 serum starved cells were seeded into the upper chamber in 200 μl serum-free medium with or without Ad-miR-24. 600 μl DMEM/F12 containing NG or HG was added to the lower chamber. 24 h later, the non-migrating cells were subsequently removed with a cotton swab and the migrated cells were fixed in methanol and stained with 0.1% crystal violet. A microscope (Nikon, Tokyo, Japan) was used to determine the number of migratory cells by counting the cells in five randomly selected fields of view.

For wound healing assay, the confluent monolayer VSMCs treated as mentioned above were wounded with a yellow tip and washed with phosphate buffered saline (PBS) to remove debris. After stimulation with HG for 24 h (For 740Y-P peptide stimulation test, 740Y-P peptide was added after HG treatment and stimulated for 48 h), the images of migration were photographed by light microscope under a magnification of 100× and quantified as percentage of wound closure.

Diabetic rat model and carotid artery balloon injury

Male Sprague–Dawley rats, 8 weeks of age and weighing 170 g, were used for all studies. Each rat was assigned to one of the following four groups randomly and equally (n = 10 for each group): balloon injury of carotid artery in normal rats (Normal rats group); balloon injury of carotid artery in diabetic rats (Diabetic rats group); Carotid artery balloon injury with Scramble-GFP adenovirus transfection in diabetic rats (Ad-Scramble group); Carotid artery balloon injury with miR-24-GFP adenovirus transfection in diabetic rats (Ad-miR-24 group). Diabetic rat model was constructed using a combination of high-fat diet (HFD, 60% fat) and low-dose streptozotocin (STZ, Sigma) injection as previously described [33]. Male SD rats (170 g) were given HFD for 4 weeks and received a single intraperitoneal injection of STZ (30 mg/kg). Two days later, their blood glucose levels were measured and rats with non-fasting blood glucose level of >15 mM were used for later experiments. Normal rats were fed by general diet for 4 weeks. Two weeks later, we implemented the rat carotid artery balloon-injury model as described previously [1]. The rats were anesthetized via an intraperitoneal injection of 10% chloral hydrate (2.8 ml/kg). Following the blunt dissection of the left carotid artery, an arterial balloon catheter (balloon diameter 1.25 mm, balloon length 20 mm) was inserted through the external carotid arteriotomy in the case of systematic heparinization (100 U/kg, intravenous injection). The catheter was inflated solely until moderate resistance was achieved and pulled down the entire length of the common carotid artery from the incision to the aortic arch 3 times. The catheter was subsequently deflated and withdrawn, and the external carotid branch was ligated. All animals were fed with conventional diet until sacrificed.

Western Blotting

Total protein was extracted from cells and carotid arteries using the protein extraction kit containing 1 mM PMSF and 10 mM phosphatase inhibitor (Beyotime, Shanghai, China). Total protein was quantified using a BCA protein assay kit (Beyotime, Shanghai, China). Equal amount (150 μg) was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were subsequently blocked and incubated with antibodies against PI3K (p85α, San Ying Biotechnology, China), Akt (San Ying Biotechnology, China), p-Akt (San Ying Biotechnology, China), mTOR (San Ying Biotechnology, China), p-mTOR (Abcam, US), 4E-BP1 (CST, US), p-4E-BP1 (CST, US), p70s6k (CST), p-p70s6k (CST, US), MMP 2 (San Ying Biotechnology, China), MMP 9 (San Ying Biotechnology, China), collagen I (San Ying Biotechnology, China) and collagen III (San Ying Biotechnology, China) overnight at 4 °C. After being washed, the membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 2 h at room temperature and subsequently analyzed using enhanced chemiluminescence dependent detection system. Meanwhile, we tested the cell-permeable p85 binding peptide (740Y-P peptide) for its ability to activate p85 and its downstream proteins, thus stimulating cell proliferation and migration. After adenovirus transfection and HG stimulation, VSMCs were grown for 2 days in the presence of the 740Y-P peptide (1–200 μg/ml) in media containing 10% serum, western blot was used to determine the optimum concentration of 740Y-P peptide for Akt activation in VSMCs.

Quantitative RT-PCR

The mRNA expression of miR-24 and PIK3R1 both in the cells and the vessels were assessed using quantitative real-time PCR. Total RNA was extracted using the Trizol Reagent (Invitrogen) and reverse transcribed to cDNA. The expression level of the two genes was measured using ABI Prism 7500 Sequence Detection System (PE Applied Biosystems). U6 and GAPDH were used as internal control. $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression levels. The primer designs were as follows.

miR-24, Forward: 5′-CGCAGTGGCTCAGTTCAGCA-3′,
Reverse: 5′-AGTGCGTGTCGTGGAGTCG-3′;
PIK3R1, Forward: 5′-CGAAAACACAGAAGACCAATACTCA-3′,
Reverse: 5′-TCCCTCGCAATAGGTTCTCG-3′;
U6, Forward: 5′-CGATACAGAGAAGATTAGCATGGC-3′,
Reverse: 5′-AACGCTTCACGAATTTGCGT-3′;
GAPDH, Forward: 5-TGGAGAAACCTGCCAAGTATGAT-3′,
Reverse: 5′-TCAAAGGTGGAAGAATGGGAGT-3′.

Histologic examination

Four weeks after operation, the arteries were fixed in 4% paraformaldehyde for one week and then embedded in paraffin. Three round cross-sections (approximately 4 μm thickness) were cut from the paraffin and stained with hematoxylin–eosin (HE). For morphologic analysis of neointimal formation, Image-Pro Plus 6.0 professional image analysis software was used. The medial and intimal cross-sectional areas were measured and the intimal/medial ratios were calculated.

Immunohistochemical detection

To quantify the proliferative activity of VSMCs *in vivo*, immunohistochemical staining of PCNA was performed. Briefly, the carotid sections were incubated with anti-PCNA antibody overnight at 4 °C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. PBS substituted for the primary antibodies were used as negative controls. Lastly, color development was achieved with diaminobenzidine, and hematoxylin was applied as a counterstain prior to cover-slipping. Data was represented as its percentage of total cells which was positive for PCNA.

Masson trichrome staining

Carotid samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. With standard histological techniques, samples were stained with Masson trichrome to detect collagen deposition in carotid sections. Light microscope images were captured with a color video camera and analyzed with image analysis software.

Statistical analyses

All data were presented as mean ± SEM and the statistical significance between groups were determined by SPSS 18.0. Independent T-tests for comparison between two groups, One-way analysis of variance (ANOVA) was used for comparisons among three or more groups. Statistical significance was inferred at $P < 0.05$.

Results

Adenovirus was transfected into the VSMCs and carotid artery balloon-injured diabetic rats successfully

Our previous study indicated that transfection of VSMCs with adenovirus did not exhibit effects on cell viability at MOI of 25 and the average transfection efficiency exceeded 95%. As shown in Supplementary material 1A, extensive green fluorescence was observed in the cytoplasm of VSMCs after treatment. *In vivo*, at four weeks post-transfection of adenovirus into the balloon-injured carotid artery in diabetic rats, successful Adenovirus transfection was evidenced by green fluorescence (Supplementary material 1B).

MiR-24 inhibits proliferation and migration of VSMCs mainly through PI3K/Akt signaling pathway

740Y-P peptide was known as its ability to activate PI3Kp85, thus activating the downstream signaling pathway. In this experiment, western blot was used to determine the optimum activation concentration of 740Y-P peptide for downstream PI3K signaling molecule, Akt. While, a maximal response observed at 50 μg/ml (Fig. 1A). CCK-8, wound healing assay and western blot were used to determine whether the effect of miR-24 on VSMCs proliferation and migration were achieved by inhibiting the activation of PI3K downstream proteins. The results showed that the activation of PI3K (p85α) and its downstream molecule Akt was inhibited by miR-24 under HG. After adding PI3Kp85 agonist 740Y-P peptide, the activation levels of PI3K (p85α) and Akt increased significantly, which indicated that miR-24 could inhibit the proliferation and migration of VSMCs by acting on PI3K-related signaling pathway (Fig. 1B–G).

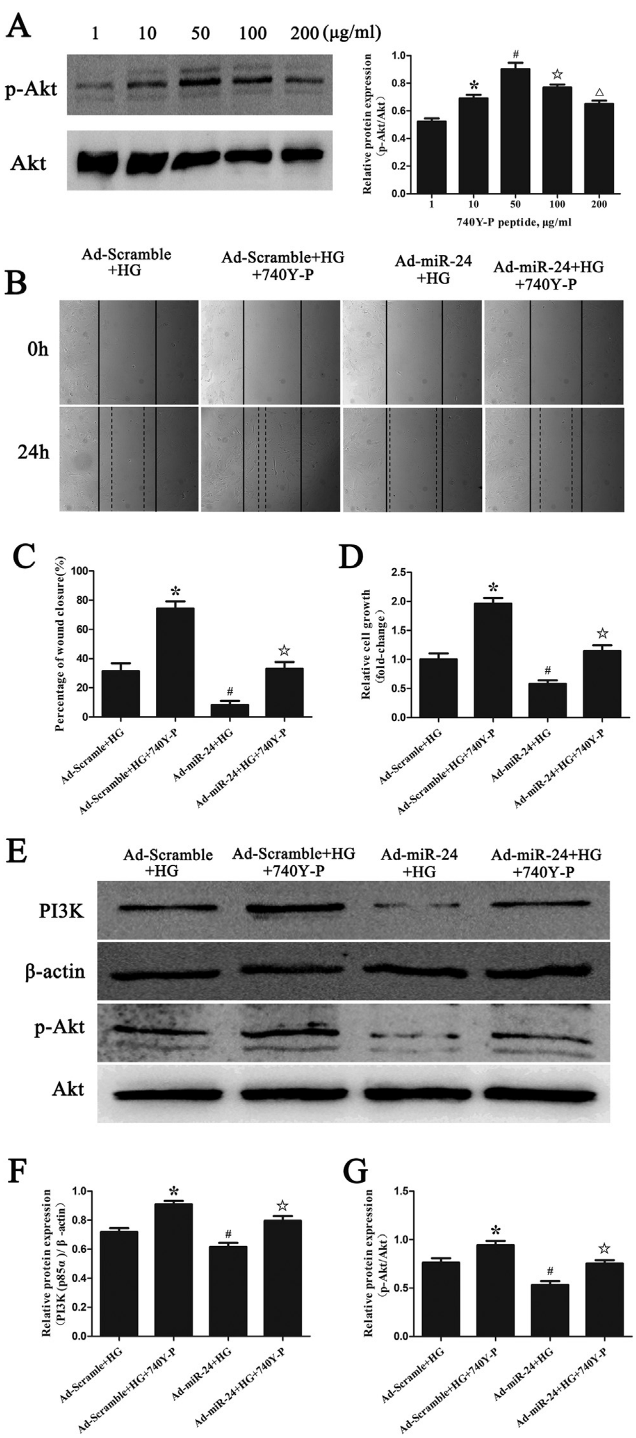

Figure 1 (A) The optimum concentration of 740Y-P peptide for phosphorylation of Akt is 50 μg/ml ($^{*}P < 0.05$ vs. 1 μg/ml group, $^{\#}P < 0.05$ vs. the previous two groups, $^{☆}P < 0.05$ and $^{\triangle}P < 0.05$ vs. the nearest front group). (B–D) MiR-24 inhibited the proliferation and migration of VSMCs. While, 740Y-P peptide could resist the decrease of proliferation and migration ability of VSMCs induced by miR-24. (E–G) MiR-24 inhibited PI3K (p85α) expression and Akt phosphorylation in VSMCs. Nevertheless, 740Y-P peptide could resist PI3K-related signaling pathway inhibition induced by miR-24. $^{*}P < 0.05$ and $^{\#}P < 0.05$ vs. Ad-scramble + HG, $^{☆}P < 0.05$ vs. Ad-miR-24 + HG.

MiR-24 inhibited VSMCs proliferation induced by HG

In subsequent experiments, we identified the role of miR-24 in VSMCs proliferation using CCK-8 kit. VSMCs proliferation was significantly enhanced due to HG stimulation for 16 h when compared to the NG group. After transfection, the VSMCs treated with Ad-miR-24 markedly inhibited VSMCs proliferation compared with either HG or Ad-Scramble + HG group. However, there was no significant difference between the HG and Ad-Scramble + HG group (Fig. 2A).

MiR-24 suppressed HG-induced VSMCs migration

Transwell chambers and wound healing assays were performed to investigate the effect of miR-24 on VSMCs migration induced by HG. In response to HG stimulation, VSMCs acquired strong capacity of migration when compared to the non-stimulated cells. Strikingly, when VSMCs were transfected with Ad-miR-24, the number of cells on the bottom side of the transwell chamber was dramatically decreased under HG conditions. While there was no effect of Ad-Scramble transfection (Fig. 2B). In addition, VSMCs treated with HG moved faster than the control, miR-24 reduced this increase, while Ad-Scramble had no effect on it (Fig. 2C).

The MMPs play a vital role in VSMC migration, especially MMP 2 and MMP 9, which were closely related to changes in cell migration ability. Consisted with the results of cell migration assays, miR-24 up-regulation caused significant decrease of protein expressions of MMP 2 and MMP 9 in HG-stimulated VSMCs (Fig. 2D).

Relative expression of miR-24 and PIK3R1 in VSMCs and injured arteries

We detected the expression of miR-24 and PIK3R1 in balloon injured carotid arteries of normal and diabetic rats. The results showed that the expression level of miR-24 in the former was significantly higher than that in the latter, while, the opposite results were found in the expression of PIK3R1 (Fig. 3A–B). In addition, as results of real-time PCR shown, miR-24 expression was significantly down-regulated after 16 h HG incubation, while obviously increased by transfection with Ad-miR-24 for 3 d in VSMCs. However, Ad-Scramble had no obvious effect on it (Fig. 3C). Moreover, HG induced a high expression level of PIK3R1 in VSMCs (Fig. 3D). Compared with Ad-Scramble + HG group, Ad-miR-24 transfection greatly reduced PIK3R1 gene levels (Fig. 3E–F).

MiR-24 up-regulation inhibited the activation of the PI3K/Akt signaling pathway under HG stimulation

In vitro, to determine whether miR-24 could modulate the activity of PI3K/Akt signaling pathway-related molecules through the regulation of PIK3R1, western blot was used to show the protein levels of PI3K (p85α), Akt, p-Akt, mTOR, p-mTOR, 4E-BP1, p-4E-BP1, p70s6k, p-p70s6k. PI3K (p85α), Akt, mTOR, 4E-BP1, p70s6k could be sensitized once

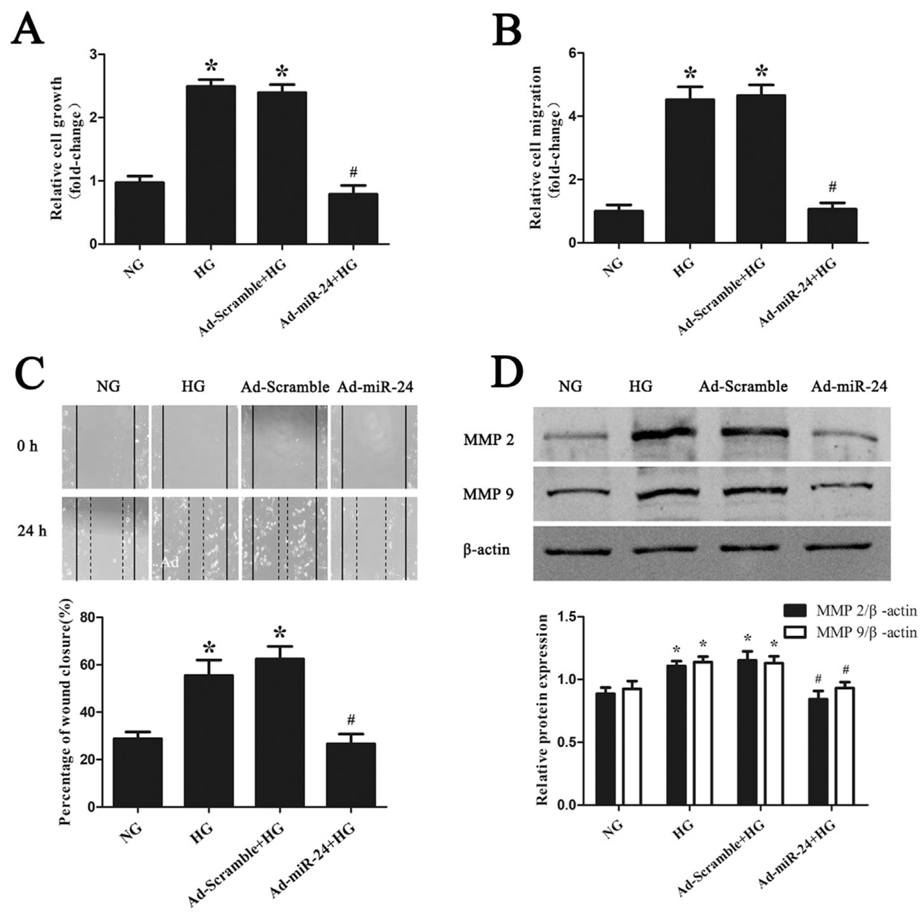

Figure 2 MiR-24 inhibits VSMCs proliferation and migration. (A) VSMCs were transfected with Ad-scramble, Ad-miR-24 or nothing for 72 h, and then stimulated with or without HG for another 16 h, VSMCs proliferation was detected using CCK-8. (B) transwell migration assay. VSMCs on transwell inserts were stained with 0.1% crystal violet. The number of migratory VSMCs was significantly higher in HG-treated group compared with the NG group, while up-regulation of miR-24 inhibited this increase. (C) wound healing assay demonstrated that treatment of Ad-miR-24 decreased the migratory ability of VSMCs. The migration distance of VSMCs treated with Ad-miR-24 was significantly shorter compared with HG or Ad-Scramble + HG cells. (D) HG increased the expression level of MMP 2 and MMP 9, overexpression of miR-24 inhibits the result. Results were presented as mean ± SEM, $^{*}P < 0.05$ compared with cells treated with NG. $^{\#}P < 0.05$ compared with cells treated with HG or Ad-Scramble + HG.

PIK3R1 was activated. As shown in Fig. 4A–F, the expression level of PI3K (p85α) and phosphorylation levels Akt, mTOR, 4E-BP1 and p70s6k were significantly decreased in the Ad-miR-24 + HG group.

MiR-24 up-regulation inhibited the expression of collagen fibers

Collagen fibers play an important role in vascular remodeling. The effect of miR-24 on the collagen fibers production induced by HG was investigated by western blot. Compared with the NG group, HG significantly increased Collagen I and Collagen III generation in VSMCs. However, miR-24 up-regulation could obviously reverse this increase when compared to the Ad-scramble + HG group (Fig. 4G).

Up-regulation of miR-24 inhibited the activation of PI3K/Akt signaling pathway in the balloon-injured carotid arteries

It is well known that phosphorylation and subsequent activation of PI3K/Akt is the major signal involved in serum-stimulated VSMC proliferation and migration. To determine whether miR-24 affect the PI3K/Akt pathway, we detected phosphorylation levels of Akt, mTOR, 4E-BP1, p70s6k in VSMCs treated with Ad-miR-24, which were closely related to the proliferation of VSMCs. We observed there was a rapid decrease in phosphorylation levels of Akt, mTOR, 4E-BP1 and p70s6k in VSMCs transfected with Ad-miR-24 (Fig. 5A–F). Furthermore, the migration of VSMCs is the initiating factor of neointimal hyperplasia, the expression of MMP 2 and MMP 9 could reflect the condition of VSMCs migration. Consisted with the proliferation related proteins expression, the protein levels of MMP 2 and MMP 9 were both significantly inhibited by miR-24 overexpression (Fig. 5G), which played a crucial role in attenuating neointimal hyperplasia after injury.

MiR-24 up-regulation inhibited intimal hyperplasia

Neointimal hyperplasia is an important pathogenesis of vascular remodeling. Four weeks after the balloon injury and adenovirus transfection, the degree of neointimal hyperplasia was evaluated morphologically and

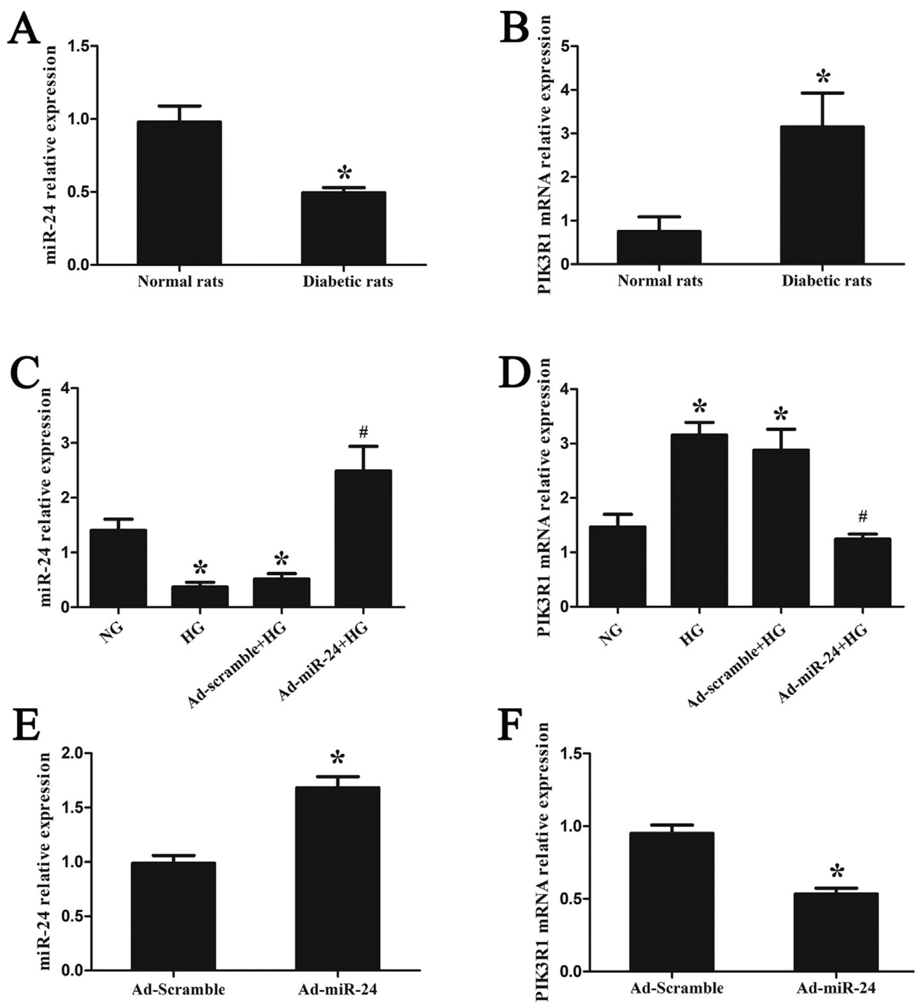

Figure 3 Real-time PCR was carried out to detect the gene expression of miR-24 and PIK3R1 in VSMCs and carotid artery balloon-injured rats. (A–B) Real-time PCR was carried out to compare the gene expression level of miR-24 and PIK3R1 after balloon injury of the carotid artery between normal and diabetic rats. (C, D) After transfection, VSMCs were cultured in 30 mM glucose (HG), miR-24 and PIK3R1 gene levels were measured by real-time PCR. (E, F) Seven days post-transfection of adenovirus into the balloon-injured carotid artery in diabetic rats, miR-24 and PIK3R1 gene levels were identified by real-time PCR. Data from our independent experiments were shown and expressed as mean ± SEM. $^{*}P < 0.05$ vs. normal rats group (A–B). $^{*}P < 0.05$ compared with cells treated with NG, $^{\#}P < 0.05$ compared with cells treated with HG and Ad-Scramble + HG (C–D). $^{*}P < 0.05$ vs. Ad-Scramble (E–F).

quantitatively. Ad-miR-24 treatment significantly reduced the neointimal area compared with Ad-Scramble, the extent of its area reduction was up to 73%. The intimal/medial ratio was also markedly less in Ad-miR-24 transfected arteries than in Ad-Scramble transfected arteries. However, the lumen area was contrary to the previous ones (Fig. 6A). In addition, there was no significant difference in medial area and external elastic lamina area. Immunostaining results showed that PCNA expression markedly increased and was distributed in the neointimal induced by balloon-injury, but miR-24 up-regulation repressed this increase, the degree of reduction reached by 61% (Fig. 6B).

MiR-24 up-regulation decreased collagen deposition

For Masson trichrome staining, vascular damage increased the percentage of collagen expressing area within the carotid artery, while treatment with miR-24 prevented these increase (Fig. 6C). Meanwhile, the levels of collagen I and collagen III were both reduced by 58% and 64% in Ad-miR-24 group, respectively, when compared to the Ad-Scramble group (Fig. 6D).

Discussion

In this study, we found that the expression level of miR-24 after balloon injury of the carotid artery in normal rats was higher than that in diabetic rats, while the opposite result was observed in the expression of PIK3R1. Therefore, we considered the interaction between the two, and then we did a study on the role of miR-24 in carotid artery balloon injury in diabetic rats. In further experiments, we demonstrated that miR-24 up-regulation exerted protective effect against neointimal formation in diabetic rats following balloon injury through interfering with the HG-induced proliferation, migration and excessive collagen generation in VSMCs. MiR-24 up-regulation, by adenovirus

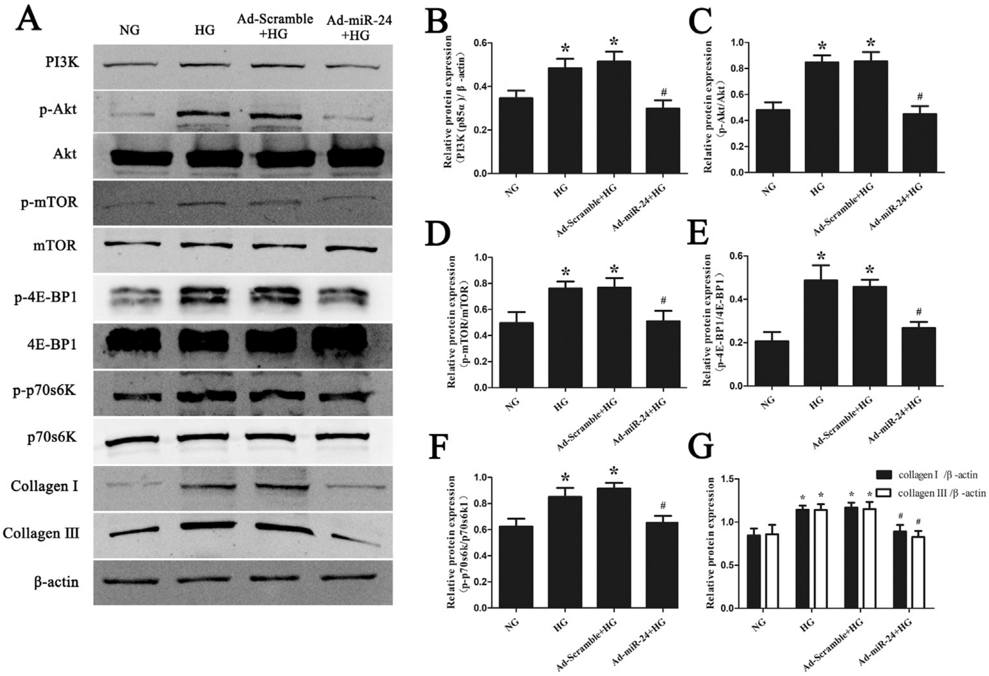

Figure 4 Cell proliferation and collagen deposition associated proteins were inhibited by miR-24. Cells were treated with miR-24 for 3 d to, respectively, detect phosphorylated and total levels of proteins of interest. (A–F) PI3K (p85α) and the phosphorylation levels of Akt, mTOR, 4E-BP1, p70s6k in the Ad-miR-24 + HG group were markedly lower than Ad-scramble + HG group. (G) Collagen I and Collagen III expression levels. $^{*}P < 0.05$ vs. NG, $^{\#}P < 0.05$ vs. HG and Ad-Scramble + HG.

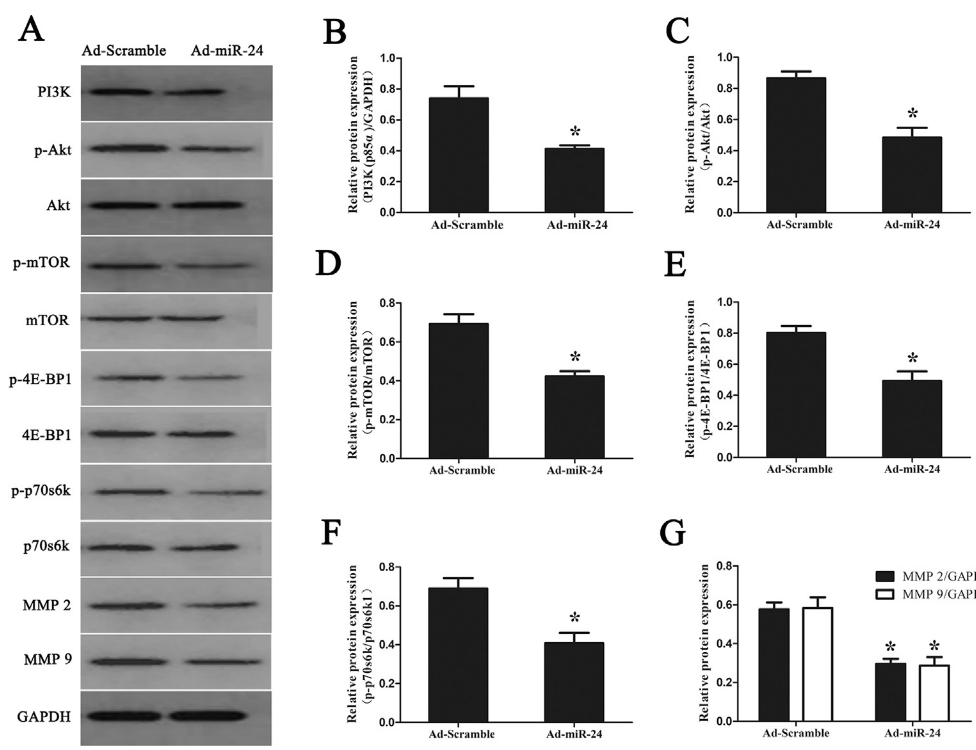

Figure 5 Western blot was carried out to detect the levels of associated molecules in PI3K/Akt signaling pathway. (A–F) PI3K (p85α) and the phosphorylation levels of Akt, mTOR, 4E-BP1, p70s6k in the miR-24 group were markedly lower than Ad-scramble group ($^{*}P < 0.05$ vs. Ad-Scramble). At the same time, as the downstream migration related molecules of PI3K/Akt signaling pathway, the expression level of MMP 2 and MMP 9 were also decreased after miR-24 up-regulated (Fig. G $^{*}P < 0.05$ vs. Ad-Scramble).

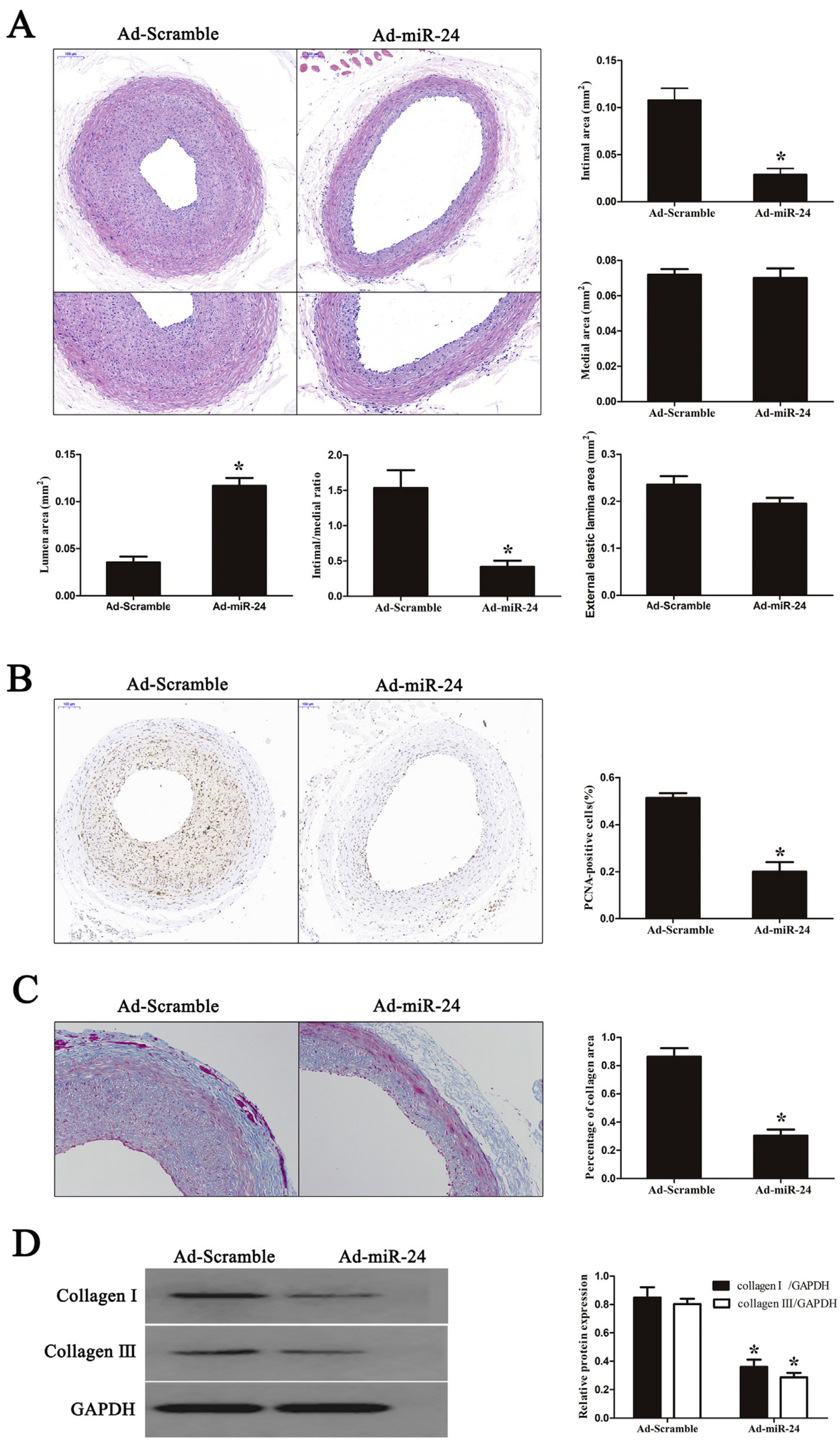

Figure 6 Effects of miR-24 on neointimal formation at four weeks after vascular injury and gene delivery. (A) Representative cross-sections of HE in different groups and Quantitative analysis of the intimal area, medial area, external elastic lamina area, lumen area as well as Ratio of intimal to medial. (B) Immunohistochemistry and bar graph were used to detect the expression of PCNA positive cells in neointimal. Scale bar represents 100 μm. (C) Representative Masson trichrome stained vessel sections. Collagen displayed as the blue performance and calculated as the percentage of collagen area to intimal and medial area. Scare bar represents 50 μm. (D) Western blot was used to detect the level of collagen I and collagen III. Values were presented as mean ± SEM, compared to Ad-scramble group, *P both <0.05.

expressing miR-24, suppressed HG-induced proliferation, migration and excessive collagen generation *in vitro* and attenuated neointimal formation *in vivo*. The underlying mechanism may be related to the decrease of PIK3R1 gene expression after miR-24 overexpression, which inactivates the PI3K/Akt signaling pathway. These results indicate that miR-24 may be a therapeutic option for proliferative vascular diseases.

Diabetes is an independent risk factor for CVD [34]. Diabetic individuals have lower long-term survival rates and higher rates of repeat revascularization [35,36]. However, existing diabetes drugs can only reduce vascular complications to a certain extent by strictly controlling blood glucose [37]. Even with the application of drug eluting stents (DES), the adjusted risk of restenosis was higher in patients with DM than in patients without DM [3,5,38]. Thus, optimal therapies against neointimal hyperplasia in diabetics are limited. At present, cell transplantation and gene therapy are mainly used in the prevention and treatment of diabetic vascular remodeling at home and abroad [39–41]. Hence, gene therapy, an applied form of biotechnology, has gradually become feasible. In our study, the adenovirus vector mediated gene delivery system can efficiently deliver miR-24 into injured vessels and isolated VSMCs, and the effects are considerable. At 4 weeks after adenovirus transfection, the strong green fluorescence was still detectable in all the arterial layers. Therefore, adenovirus vector would be an optimal candidate for efficient cardiovascular gene therapy.

In addition to an effective gene delivery system, appropriate target gene is the second important element for vascular gene therapy. Numerous studies have identified a lot of therapeutic genes, including tissue inhibitors of matrix metalloproteinases (TIMP-3), nitric oxide synthase (NOS) and p53, these genes could only regulate one factor contribute to restenosis following balloon dilation [42]. miRNA is a highly conserved endogenous non-coding small molecular RNA [17], multiple studies have shown that different vascular specific miRNAs, such as miR-17, miR-18a, miR-19a, miR-19b-1, miR-205, miR-92a, miR-145 participate in the pathophysiological process of regulating vascular remodeling or play a protective role by regulating their respective target mRNA. Studies have shown that miR-24 inhibits tumor cell growth [43], while other studies have suggested that miR-24 may be involved in the regulation of pathological angiogenesis [27,44]. We previously found that miR-24 inhibits proliferation and inflammation of VSMCs by inhibiting Wnt4, HMGB1 and PDGF-BB signaling pathway [31,45,46]. However, single action on a signaling pathway cannot play a complete role in anti-intimal hyperplasia. As is well-known that one miRNA can regulate hundreds of genes at the same time, and one gene can be regulated by hundreds of miRNAs [21]. Apparently, miR-24 has multiple intervention targets, Wnt4, HMGB1and PDGF-BB are only three of them, PIK3R1 is another important target for miR-24. In this study, our experiments confirmed that the expression of miR-24 and PI3K/Akt related molecules were inversely correlated both *in vitro* and *in vivo*.

PI3K/Akt/mTOR pathway plays important roles in regulating cell survival, proliferation, migration, differentiation and apoptosis [47–49]. The phosphorylated PI3K and Akt, as well as its downstream effector mTOR became activated during the progression of vascular injury [50]. As downstream molecules, the activation of 4E-BP1 and p70s6k are regulated by mTOR, which are certainly involved in cell proliferation [47,51]. Recently, PI3K/Akt/mTOR pathway is reported to be involved in the VSMCs proliferation during the processes of atherogenesis [50]. Previous studies have shown that PI3K/Akt is an important signaling pathway for growth factors or cytokines induced VSMCs proliferation and migration [47,52]. The absence of Akt decreased VSMCs survival and migration, which implied an essential role of PI3K/Akt signaling in regulating physiological functions of VSMCs [2,53,54]. In the present study, an intervention with miR-24 was proven to reduce PI3K (p85α) level and activation levels of Akt, mTOR, 4E-BP1, p70s6k proteins in HG-stimulated VSMCs and balloon-injured diabetic rats. Therefore, miR-24 mediated inhibitory mechanisms on VSMCs proliferation and migration as well as balloon injury-induced neointimal hyperplasia in rat carotid artery may be attributed to suppression of PI3K/Akt cascades.

In addition to VSMCs proliferation, increased VSMCs migration is another key process in promoting neointimal formation [48]. Experimental data have shown that MMPs are important regulators of cell migration and play significant roles in the degradation of the ECM, facilitating VSMCs migration [48,55,56]. MMP 2 and MMP 9 degrade the basement membrane and promote intimal hyperplasia in various animal models [57]. Decreased MMP 2 and MMP 9 contribute to inhibition of matrix degradation and suppression of migration [48]. Our previous study have confirmed that PI3K/Akt signaling pathway was linked to the migration of VSMCs [31]. Lu et al. found that PI3K/Akt played an important role in the secretion of MMPs [58]. Thus, we imagine that miR-24 promotes the migration of VSMCs by activating the PI3K/Akt signaling pathway and promoting the production of MMPs. In our study, the miR-24-mediated reduced MMP 2 and MMP 9 expression levels could have contributed to the reduction in VSMCs migration observed in the intimal hyperplasia.

It is well known that VSMCs exist in a quiescent and differentiated state in the normal mature arterial wall, and control the vascular tone through their contractile machinery [16,52]. In pathological vascular injury, VSMCs become activated and switch to a synthetic phenotype with a high rate of proliferation and migration, that together contribute to impaired vascular remodeling and neointimal formation [59]. The transformed cells acquire a marked capacity for the synthesis and secretion of ECM components, becoming the main source of ECM [15]. Collagen, as part of the ECM, plays an indispensable role in vascular remodeling [60]. Previous study demonstrated that miR-24 was decreased in myocardial tissues of infarction rats, it may inhibit myocardial fibrosis in many ways [61]. What's more, Qu et al. found that miR-24 down-regulation is believed to cause cardiac fibrosis, up-regulation of miR-24 could abrogated the fibrogenesis [62]. Our study has confirmed miR-24 up-regulation could effectively inhibited collagen deposition after vascular injury. Daniel A. Duprez have indicated that Type I and III collagens are the major fibrillar components in both normal and diseased tissue. Moreover, these two collagen fibers play an important role in vascular remodeling [60]. Xing Zhou revealed that PI3K/Akt/m-TOR signaling pathway was critically involved in the activation of systemic sclerosis (SSC) fibroblasts [63]. Our *in vitro* and *in vivo* experiments confirmed that up-regulation of miR-24 reduced collagen I and collagen III production, which was accompanied by inhibition of PI3K/Akt/mTOR signaling pathway. The results suggest that inhibition of balloon injury-induced collagen deposition in the neointimal by miR-24 may be partly attributed to suppression of PI3K/Akt signaling pathway activation in VSMCs.

In summary, results from this study provide the evidence that miR-24 attenuates the proliferation, migration and collagen deposition of VSMCs *in vitro* and inhibits balloon-injury induced neointimal formation *in vivo*, which may be ascribed to downregulation of PI3K/Akt/mTOR signaling pathway. We believe that miR-24 represents a powerful, new multifunction gene that provides a potential target for the treatment of occlusive vascular diseases.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by National Natural Science Foundation of China, China (Grant Nos. 81770360, 81470387, 81500230, 81800258).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.numecd.2019.03.002.

References

[1] Uemura S, Matsushita H, Li W, Glassford AJ, Asagami T, Lee KH, et al. Diabetes mellitus enhances vascular matrix metalloproteinase activity: role of oxidative stress. Circ Res 2001;88(12):1291–8.
[2] Shi L, Ji Y, Jiang X, Zhou L, Xu Y, Li Y, et al. Liraglutide attenuates high glucose-induced abnormal cell migration, proliferation, and apoptosis of vascular smooth muscle cells by activating the GLP-1 receptor, and inhibiting ERK1/2 and PI3K/Akt signaling pathways. Cardiovasc Diabetol 2015;14(1):18.
[3] Wang K, Wen L, Peng W, Li H, Zhuang J, Lu Y, et al. Vinpocetine attenuates neointimal hyperplasia in diabetic rat carotid arteries after balloon injury. PLoS One 2014;9(5):e96894.
[4] Kennedy MW, Fabris E, Ijsselmuiden AJ, Nef H, Reith S, Escaned J, et al. Combined optical coherence tomography morphologic and fractional flow reserve hemodynamic assessment of non- culprit lesions to better predict adverse event outcomes in diabetes mellitus patients: COMBINE (OCT–FFR) prospective study. Rationale and design. Cardiovasc Diabetol 2016;15(1):144.
[5] Minha S, Bental T, Assali A, Vaknin-Assa H, Lev EI, Rechavia E, et al. A comparative analysis of major clinical outcomes using drug-eluting stents versus bare metal stents in diabetic versus nondiabetic patients. Cathet Cardiovasc Interv 2011;78(5):710–7.
[6] Jansen F, Zietzer A, Stumpf T, Flender A, Schmitz T, Nickenig G, et al. Endothelial microparticle-promoted inhibition of vascular remodeling is abrogated under hyperglycaemic conditions. J Mol Cell Cardiol 2017;112:91–4.
[7] Jensen LO, Thayssen P, Junker A, Maeng M, Tilsted HH, Kaltoft A, et al. Comparison of outcomes in patients with versus without diabetes mellitus after revascularization with everolimus- and sirolimus-eluting stents (from the SORT OUT IV trial). Am J Cardiol 2012;110(11):1585–91.
[8] Sun H, Zhong M, Miao Y, Ma X, Gong HP, Tan HW, et al. Impaired elastic properties of the aorta in fat-fed, streptozotocin-treated rats. Cardiology 2009;114(2):107–13.
[9] Pant R, Marok R, Klein LW. Pathophysiology of coronary vascular remodeling: relationship with traditional risk factors for coronary artery disease. Cardiol Rev 2014;22(1):13–6.
[10] Spinetti G, Kraenkel N, Emanueli C, Madeddu P. Diabetes and vessel wall remodeling: from mechanistic insights to regenerative therapies. Cardiovasc Res 2008;78(2):265–73.
[11] Patel TP, Rawal K, Bagchi AK, Akolkar G, Bernardes N, Dias DDS, et al. Insulin resistance: an additional risk factor in the pathogenesis of cardiovascular disease in type 2 diabetes. Heart Fail Rev 2016;21(1):11–23.
[12] Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. Int J Vasc Med 2012;(5):918267.
[13] Spinetti G, Kraenkel N, Emanueli C. Diabetes and vessel wall remodelling: from mechanistic insights to regenerative therapies. Cardiovasc Res 2008;78(2):265–73.
[14] Suga T, Iso T, Shimizu T, Tanaka T, Yamagishi S, Takeuchi M, et al. Activation of receptor for advanced glycation end products induces osteogenic differentiation of vascular smooth muscle cells. J Atheroscler Thromb 2011;18(8):670–83.
[15] Zhang J, Chen J, Xu C, Yang J, Guo Q, Hu Q, et al. Resveratrol inhibits phenotypic switching of neointimal vascular smooth muscle cells after balloon injury through blockade of Notch pathway. J Cardiovasc Pharmacol 2014;63(3):233–9.
[16] Quintavalle M, Condorelli G, Elia L. Arterial remodeling and atherosclerosis: miRNAs involvement. Vasc Pharmacol 2011;55(4):106–10.
[17] Kataoka M, Wang DZ. Non-coding RNAs including miRNAs and lncRNAs in cardiovascular biology and disease. Cells 2014;3(3):883–98.
[18] Hynes CJ, Clancy JL, Preiss T. miRNAs in cardiac disease: sitting duck or moving target? IUBMB Life 2012;64(11):872–8.
[19] Egea V, Schober A, Weber C. Circulating miRNAs: messengers on the move in cardiovascular disease. Thromb Haemost 2012;108(4):590–1.
[20] Rivera-Barahona A, Pérez B, Richard E, Desviat LR. Role of miRNAs in human disease and inborn errors of metabolism. J Inherit Metab Dis 2017;40(5):1–10.
[21] Paul P, Chakraborty A, Sarkar D, Langthasa M, Rahman M, Bari M, et al. Interplay between miRNAs and human diseases: a review. J Cell Physiol 2017.
[22] Jones Buie JN, Goodwin AJ, Cook JA, Halushka PV, Fan H. The role of miRNAs in cardiovascular disease risk factors. Atherosclerosis 2016;254:271–81.
[23] Jamaluddin MS, Weakley SM, Zhang L, Kougias P, Lin PH, Yao Q, et al. miRNAs: roles and clinical applications in vascular disease. Expert Rev Mol Diagn 2011;11(1):79–89.
[24] Yang Y, Yu T, Jiang S, Zhang Y, Li M, Tang N, et al. miRNAs as potential therapeutic targets and diagnostic biomarkers for cardiovascular disease with a particular focus on WO2010091204. Expert Opin Ther Pat 2017;27(9):1021.
[25] Small EM, Frost RJ, Olson EN. MicroRNAs add a new dimension to cardiovascular disease. Circulation 2010;121(8):1022–32.
[26] Ultimo S, Zauli G, Martelli AM, Vitale M, McCubrey JA, Capitani S, et al. Cardiovascular disease-related miRNAs expression: potential role as biomarkers and effects of training exercise. Oncotarget 2018;9(24):17238.
[27] Polimeni A, Rosa SD, Indolfi C. Vascular miRNAs after balloon angioplasty. Trends Cardiovasc Med 2013;23(1):9–14.

[28] Xiang Y, Cheng J, Wang D, Hu X, Xie Y, Stitham J, et al. Hyperglycemia repression of miR-24 coordinately upregulates endothelial cell expression and secretion of von Willebrand factor. Blood 2015;125(22):3377–87.
[29] Zheng Y, Li Y, Liu G, Qi X, Cao X. MicroRNA-24 inhibits the proliferation and migration of endothelial cells in patients with atherosclerosis by targeting importin-α3 and regulating inflammatory responses. Exp Ther Med 2018;15(1):338.
[30] Li D, Li J. Association of miR-34a-3p/5p, miR-141-3p/5p, and miR-24 in decidual natural killer cells with unexplained recurrent spontaneous abortion. Med Sci Mon Int Med J Exp Clin Res 2016; 22:922–9.
[31] Yang J, Chen L, Ding J, Fan Z, Li S, Wu H, et al. MicroRNA-24 inhibits high glucose-induced vascular smooth muscle cell proliferation and migration by targeting HMGB1. Gene 2016;586(2):268–73.
[32] Tokunou T, Shibata R, Kai H, Ichiki T, Morisaki T, Fukuyama K, et al. Apoptosis induced by inhibition of cyclic AMP response element-binding protein in vascular smooth muscle cells. Circulation 2003; 108(10):1246–52.
[33] Yang B, Yang J, Bai J, Pu P, Liu J, Wang F, et al. Suv39h1 protects from myocardial ischemia-reperfusion injury in diabetic rats. Cell Physiol Biochem 2014;33(4):1176.
[34] Arnold M. Prevention and management of coronary artery disease in patients with diabetes mellitus. Acta Med Indones 2009;41(1):41.
[35] Kovacic JC, Castellano JM, Farkouh ME, Fuster V. The relationships between cardiovascular disease and diabetes: focus on pathogenesis. Endocrinol Metab Clin N Am 2014;43(1):41–57.
[36] Beckman JA, Paneni F, Cosentino F. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part II. Eur Heart J 2013;34(31):2444–52.
[37] Low Wang CC, Hess CN, Hiatt WR, Goldfine AB. Clinical update: cardiovascular disease in diabetes mellitus: atherosclerotic cardiovascular disease and heart failure in type 2 diabetes mellitus – mechanisms, management, and clinical considerations. Circulation 2016;133(24):2459–502.
[38] Akin I, Bufe A, Eckardt L, Reinecke H, Senges J, Richardt G, et al. Comparison of outcomes in patients with insulin-dependent versus non-insulin dependent diabetes mellitus receiving drug-eluting stents (from the first phase of the prospective multicenter German DES. DE registry). Am J Cardiol 2010;106(9): 1201–7.
[39] Li Y, Liu S, Zhang Z, Xu Q, Xie F, Wang J, et al. RAGE mediates accelerated diabetic vein graft atherosclerosis induced by combined mechanical stress and AGEs via synergistic ERK activation. PLoS One 2012;7(4):e35016.
[40] Porter KE, Kirsten R. The vascular smooth muscle cell: a therapeutic target in type 2 diabetes? Clin Sci 2013;125(3–4):167–82.
[41] Conte MS, Nugent HM, Gaccione P, Roy-Chaudhury P, Lawson JH. Influence of diabetes and perivascular allogeneic endothelial cell implants on arteriovenous fistula remodeling. J Vasc Surg 2011; 54(5):1383–9.
[42] Yang J, Jiang H, Chen SS, Chen J, Li WQ, Xu SK, et al. Lentivirus-mediated RNAi targeting CREB binding protein attenuates neointimal formation and promotes re-endothelialization in balloon injured rat carotid artery. Cell Physiol Biochem 2010;26(3):441.
[43] Reduce the apoptosis threshold in cancer cells. Oncogene 2013; 32(19):2442.
[44] Zhou Q, Anderson C, Zhang H, Li X, Inglis F, Jayagopal A, et al. Repression of choroidal neovascularization through actin cytoskeleton pathways by MicroRNA-24. Mol Ther 2014;22(2): 378–89.
[45] Yang J, Fan Z, Yang J, Ding J, Yang C, Chen L. MicroRNA-24 attenuates neointimal hyperplasia in the diabetic rat carotid artery injury model by inhibiting Wnt4 signaling pathway. Int J Mol Sci 2016;17(6).
[46] Yang J, Zeng P, Yang J, Liu X, Ding J, Wang H, et al. MicroRNA-24 regulates vascular remodeling via inhibiting PDGF-BB pathway in diabetic rat model. Gene 2018;659:67–76.
[47] Cui X, Wang S, Cai H, Lin Y, Zheng X, Zhang B, et al. Overexpression of microRNA-634 suppresses survival and matrix synthesis of human osteoarthritis chondrocytes by targeting PIK3R1. Sci Rep 2016;6:23117.
[48] Zhang QZ, Guo YD, Li HM, Wang RZ, Guo SG, Du YF. Protection against cerebral infarction by Withaferin A involves inhibition of neuronal apoptosis, activation of PI3K/Akt signaling pathway, and reduced intimal hyperplasia via inhibition of VSMC migration and matrix metalloproteinases. Adv Med Sci 2017;62(1):186–92.
[49] Chan S. Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer. Br J Cancer 2004;91(8):1420–4.
[50] Morello F, Perino A, Hirsch E. Phosphoinositide 3-kinase signalling in the vascular system. Cardiovasc Res 2009;82(2):261–71.
[51] Marx SO, Totary-Jain H, Marks AR. Vascular smooth muscle cell proliferation in restenosis. Circ Cardiovasc Interv 2011;4(1): 104–11.
[52] Fang H, Yang S, Luo Y, Zhang C, Rao Y, Liu R, et al. Notoginsenoside R1 inhibits vascular smooth muscle cell proliferation, migration and neointimal hyperplasia through PI3K/Akt signaling. Sci Rep 2018;8(1):7595.
[53] Stabile E, Zhou YF, Saji M, Castagna M, Shou M, Kinnaird TD, et al. Akt controls vascular smooth muscle cell proliferation in vitro and in vivo by delaying G1/S exit. Circ Res 2003;93(11):1059–65.
[54] Chien YC, Sheu MJ, Wu CH, Lin WH, Chen YY, Cheng PL, et al. A Chinese herbal formula "Gan-Lu-Yin" suppresses vascular smooth muscle cell migration by inhibiting matrix metalloproteinase-2/9 through the PI3K/AKT and ERK signaling pathways. BMC Complement Altern Med 2012;12(1):1–12.
[55] Lee EJ, Kim DI, Kim WJ, Moon SK. Naringin inhibits matrix metalloproteinase-9 expression and AKT phosphorylation in tumor necrosis factor-α-induced vascular smooth muscle cells. Mol Nutr Food Res 2009;53(12):1582–91.
[56] Zhang Y, Fu J, Zhang Z, Qin H. miR-486-5p regulates the migration and invasion of colorectal cancer cells through targeting PIK3R1. Oncol Lett 2018;15(5):7243–8.
[57] Pan CH, Li PC, Chien YC, Yeh WT, Liaw CC, Sheu MJ, et al. Suppressive activities and mechanisms of ugonin J on vascular smooth muscle cells and balloon angioplasty-induced neointimal hyperplasia. Phytother Res 2018;32(2):312–20.
[58] Ma L, Zhang L, Wang B, Wei J, Liu J, Zhang L. Berberine inhibits Chlamydia pneumoniae infection-induced vascular smooth muscle cell migration through downregulating MMP3 and MMP9 via PI3K. Eur J Pharmacol 2015;755:102–9.
[59] Chen J, Zhang J, Xu L, Xu C, Chen S, Yang J, et al. Inhibition of neointimal hyperplasia in the rat carotid artery injury model by a HMGB1 inhibitor. Atherosclerosis 2012;224(2):332–9.
[60] Duprez DA, Gross MD, Ix JH, Kizer JR, Tracy RP, Shea S, et al. Collagen biomarkers predict new onset of hypertension in normotensive participants: the Multi-Ethnic Study of Atherosclerosis. J Hypertens 2018.
[61] Chen Z, Lu S, Xu M, Liu P, Ren R, Ma W. Role of miR-24, furin, and transforming growth factor-β1 signal pathway in fibrosis after cardiac infarction. Med Sci Monit 2017;23:65–70.
[62] Qu X, Du Y, Shu Y, Gao M, Sun F, Luo S, et al. MIAT is a pro-fibrotic long non-coding RNA governing cardiac fibrosis in post-infarct myocardium. Sci Rep 2017;7:42657.
[63] Zhou X, Liu C, Lu J, Zhu L, Li M. 2-Methoxyestradiol inhibits hypoxia-induced scleroderma fibroblast collagen synthesis by phosphatidylinositol 3-kinase/Akt/mTOR signaling. Rheumatology 2018;8(43):74791–805.