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MicroRNA-24 inhibits the oxidative stress induced by vascular injury by activating the Nrf2/Ho-1 signaling pathway

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HIGHLIGHTS

- *MiR-24* up-regulation inhibits HG-stimulated oxidative stress of VSMCs.
- *MiR-24* up-regulation promotes reendothelialization in balloon-injured diabetic rats.
- *MiR-24* up-regulation inhibits Ogt and keap1 expression, and promotes Nrf2 and Ho-1 expression.

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ABSTRACT

Background and aims: The process of endothelial repair in diabetic patients after stent implantation was significantly delayed compared with that in non-diabetic patients, and oxidative stress is increasingly considered to be relevant to the pathogenesis of diabetic endothelial repair. However, the mechanisms linking diabetes and reendothelialization after vascular injury have not been fully elucidated. The aim of this study was to evaluate the effect of microRNA-24 (*miR-24*) up-regulation in delayed endothelial repair caused by oxidative stress after balloon injury in diabetic rats.

Methods: In vitro, vascular smooth muscle cells (VSMCs) isolated from the thoracic aorta were stimulated with high glucose (HG) after *miR-24* recombinant adenovirus (Ad-*miR-24*-GFP) transfection for 3 days. *In vivo*, diabetic rats induced using high-fat diet (HFD) and low-dose streptozotocin (30 mg/kg) underwent carotid artery balloon injury followed by Ad-*miR-24*-GFP transfection for 20 min.

Results: The expression of *miR-24* was decreased in HG-stimulated VSMCs and balloon-injured carotid arteries of diabetic rats, which was accompanied by increased expression of *Ogt* and *Keap1* and decreased expression of *Nrf2* and *Ho-1*. Up-regulation of *miR-24* suppressed VSMC oxidative stress induced by HG *in vitro*, and *miR-24* up-regulation promoted reendothelialization in balloon-injured diabetic rats. The underlying mechanism was related to the activation of the Nrf2/Ho-1 signaling pathway, which subsequently suppressed intracellular reactive oxidative species (ROS) production and malondialdehyde (MDA) and NADPH oxidase (Nox) activity, and to the restoration of Sod and Gsh-px activation.

Conclusions: The up-regulation of *miR-24* significantly promoted endothelial repair after balloon injury through inhibition of oxidative stress by activating the Nrf2/Ho-1 signaling pathway.

1. Introduction

Diabetes is considered to be a risk equivalents of coronary heart disease (CHD) [1]. It has reached an epidemic level worldwide and patients suffering from this disease are at significantly higher risk for cardiovascular disease (CVD) [2]. The mortality rate of CVD is 2–4 times that of type 2 diabetes mellitus (T2DM) compared with people without diabetes. Moreover, CVD is the most common cause of

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mortality among people with T2DM [3]. As the latest technology in the treatment of CHD, percutaneous coronary intervention (PCI) has been widely accepted given its most significant therapeutic effect. However, the incidence of restenosis and delayed endothelialization after stent implantation in diabetic patients is significantly higher than that in non-diabetic patients [4,5]. Drug-eluting stents (DES) significantly reduce the restenosis rate of bare metal stents (BMS); however, drug-coated stents can inhibit the migration and proliferation of VSMCs but also affect the function of vascular endothelial cells (ECs), leading to delayed vascular endothelial repair [6,7]. In the context of DM, the process of vascular endothelialization repair is severely blocked due to the synergistic effects of inflammation, oxidative stress, abnormal glucose metabolism and other factors, and the incidence of late thrombosis is significantly higher than that of non-DM patients [8]. Therefore, promoting the endothelialization process after diabetic vascular injury can provide a new scientific basis for late thrombosis after PCI.

Previous studies have found that oxidative stress is the key node of delayed development of vascular endothelialization, and oxidative stress can damage vascular ECs and cause loss of barrier function [9]. After losing endothelial protection, VSMCs in the mesangium are exposed to multiple stimuli and actively proliferate, migrate and synthesize copious amounts of extracellular matrix (ECM) deposited in the vascular wall [10]. Meanwhile, VSMCs could release a large number of oxidative products and inactivate the vasoprotective molecule nitric oxide (NO), thus contributing to the onset of endothelial dysfunction [11]. With delayed endothelialization coverage, diabetic patients are more likely to develop stent thrombosis than non-diabetic patients [12]. Thus, inhibiting VSMCs oxidative stress to accelerate the re-endothelialization of injured blood vessels and form intact intima is of great significance to the recovery of vascular function and even inhibition of the occurrence and development of stent thrombosis in diabetes mellitus.

MicroRNAs (miRNAs) are short, noncoding RNAs that regulate gene expression by base pairing with target messenger RNAs (mRNAs) [13]. MiRNAs can lead to the same biological effects through multiple targets. In addition, multiple miRNAs can target the same gene, thus forming a huge information network between miRNAs and mRNAs [14]. MiRNAs play important roles in most biological processes, including cell development, proliferation, differentiation, immune reaction, and apoptosis [15]. Encouragingly, numerous studies have revealed that miRNAs play crucial role in the occurrence and progression of CVD. Some miRNAs promote the development of CVD, while others have the opposite effect [16,17]. Lots of results clearly indicate that miRNAs are involved in the process of reendothelialization after vascular injury. Santulli and colleagues found that delivery of Ad-p27-126TS to balloon-injured arteries in rats not only induced faster and more complete reendothelialization, but also improved neointimal hyperplasia [18,19]. In addition, studies have shown that miR-483–3p could impair endothelial cell survival by inhibiting transcription factor VEZF1 in T2DM, thereby limiting vascular repair capacity upon injury [20]. There was also a study demonstrated that inhibition of miR-221/222 was able to enhance reendothelialization. However, the opposite effect was observed on neointimal formation [21]. It has been reported that *microRNA-24* (*miR-24*) participates in many pathophysiological processes, including tumor formation and ischemic reperfusion injury [22–25]. However, the role of *miR-24* in vascular oxidative stress and reendothelialization has rarely been reported. *MiR-24* is highly expressed in the vessel wall, and dysregulation of *miR-24* is associated with dysfunction or even damage of vascular ECs; all of these effects lead to the development of CVD [26]. Previous studies demonstrated that Nrf2/Ho-1 signaling acts as a sensor for oxidative stress involved in CVD [27]. However, the relationship between *miR-24* and the Nrf2/Ho-1 signaling pathway has not been fully elucidated.

Accordingly, the aim of this study was to explore the possible protective effects and underlying mechanisms of *miR-24* on oxidative stress *in vitro* in response to high glucose (HG) and vascular injury *in vivo* following arterial endothelial denudation in diabetic rats.

2. Materials and methods

2.1. Animals and ethics statement

All animals used in this experiment were purchased from the Animal Center of China Three Gorges University (CTGU). 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).

2.2. Construction and production of adenoviral vectors

The rno-*miR-24* precursor DNA was designed and synthesized by Genechem (Shanghai, China), and a scramble miRNA was used as a negative control. 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 dishes at 70% confluence were transfected with the adenovirus at multiplicities of infection (MOI) of 25 for 3 h before medium change, and cells were incubated for an additional 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.

2.3. Cell culture and HG treatment

Primary VSMCs were isolated from the thoracic aorta of male Sprague–Dawley rats (130–150 g). Briefly, rat thoracic aortas were harvested following euthanasia. After scraping off the adventitia and endothelium, thoracic aortas were cut into sections of 2 × 2 mm. The primary VSMCs were obtained using the tissue explants adherent method. When the cells reached 80–90% confluence, primary cells were passaged and grown 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. Cells at passages 3–5 were used for the following experiments. Serum-starved VSMCs were stimulated with fresh medium containing 30 mM glucose (HG). All cells were divided into four groups according to different treatments (n = 3 for each group): non-glucose group (NG); 30 mM glucose stimulation group (HG); transfection of *miR-24*-GFP adenovirus followed by 30 mM glucose stimulation group (Ad-*miR-24* + HG group); transfection of scramble-GFP adenovirus followed by 30 mM glucose stimulation group (Ad-Scramble + HG group).

2.4. miR-24 target prediction and luciferase assays

The potential targets of *miR-24* were identified using TargetScan. Luciferase assays were performed using 293T cells with the firefly luciferase report vector (0.1 μg), miRNA expression plasmid (0.4 μg) and the control vector containing renilla luciferase (0.02 μg) in a 24-well plate according to the protocol of the luciferase assay kit (E2910, Promega). The luciferase activity was assayed 48 h later with the Dual-Luciferase® Reporter Assay System (Promega) and was normalized to renilla luciferase activity.

2.5. ROS detection

ROS generation was measured with an ROS assay kit (Beyotime Biotechnology, China) according to the manufacturer's instruction. In brief, after adenovirus transfection for 3 d, VSMCs were seeded onto 8-well plate at a density of 3×10^5 (each well). Cells were synchronized for 24 h and incubated with 30 mM glucose for 16 h. The fluorescent probe DCFH-DA was added at a final concentration of 10 mM and

incubated at 37 °C for 20 min. Pictures were obtained using a fluorescence microscope, and the fluorescence mean density was measured from three random fields for each well. For flow cytometry, VSMCs were treated with 30 mM glucose for 16 h. Cells were harvested, centrifuged, and washed with phosphate buffered saline (PBS). Following incubation with DCFH-DA (10 μM), the supernatant was removed, and cells were suspended in sheath fluid. DCF fluorescence intensity was analyzed using a flow cytometer (BD verse, US).

2.6. MDA, Sod and Gsh-px determination

The malondialdehyde (MDA) content was measured to evaluate the degree of lipid peroxidation. In addition, the activities of intracellular antioxidants superoxide dismutase (Sod) and glutathione peroxidase (Gsh-px) were determined to assess the body's antioxidant levels. After adenovirus transfection and HG stimulation, the suspensions of VSMCs were harvested and homogenized by cell lysis buffer (Beyotime Biotechnology, China). The MDA content was measured by the thiobarbituric acid (TBA) method. Sod and Gsh-px activity was detected using WST-8 and colorimetric methods, respectively, according to the manufacturer's instructions. All of the above experimental assay kits were purchased from Nanjing Jiancheng Institute of Bioengineering, and the experimental steps were performed according to manufacturer's instructions.

2.7. Diabetic rat and carotid artery balloon injury model

Forty male Sprague–Dawley rats weighing 170 g were randomly divided into the following four groups: 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). A diabetic rat model was constructed using a combination of HFD (60% fat) and low-dose streptozotocin (STZ, Sigma) injection. Male SD rats were given HFD for 4 weeks and received a single intraperitoneal injection of STZ (30 mg/kg). The blood glucose levels were measured 2 d after injection. Rats with non-fasting blood glucose levels greater than 15 mM were used for later experiments. Normal rats were fed a general diet for 4 weeks. Two weeks later, the rat carotid artery balloon-injury model was generated. The rats were anesthetized via an intraperitoneal injection of 10% chloral hydrate (2.8 ml/kg). The common, internal and external carotid arteries were exposed sequentially. A balloon catheter (balloon diameter 1.25 mm, balloon length 20 mm) was introduced into the common carotid in the case of systematic heparinization (100 U/kg, intravenous injection). The inflated catheter was dragged back and forth through entire length of the common carotid artery thrice. The catheter was subsequently deflated and withdrawn, and the external carotid branch was ligated. All animals were fed a conventional diet until sacrificed.

2.8. Immunofluorescence staining

The spatial expression of Ogt, Keap1, Nrf2, Ho-1, Nox 2 and Nox 4 were evaluated with immunofluorescence. All labeled sections were incubated with antibodies against Ogt, Keap1, Nrf2, Ho-1, Nox 2 and Nox 4 in a humidified container at 4 °C overnight. All antibodies were purchased from Beyotime Biotechnology. The sections were incubated with tetramethyl rhodamine isothiocyanate-conjugated second antibodies. DAPI (Beyotime Biotechnology, China) solution was added to stain the cell nucleus for 3 min. Sections were then washed in PBS and sealed with a coverslip. The slides were analyzed with laser confocal microscopy (NIKON, ECLIPSE C1). ImageJ software was used for quantitative analysis.

2.9. Western blotting

For total, nuclear and cytosolic protein extractions, cells or vessels were lysed in lysis buffer according to the manufacturer's protocol (Beyotime Biotechnology, China). Extracted protein was quantified using a BCA assay kit (Beyotime Biotechnology, China). Equal amounts of protein (150 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were subsequently blocked and incubated with antibodies against Ogt (Proteintech Group, Inc., catalog number: 11576-2-AP, Rabbit Polyclonal, 110 KD), Keap1 (Proteintech Group, Inc., catalog number: 10503-2-AP, Rabbit Polyclonal, 70 KD), Nrf2 (Proteintech Group, Inc., catalog number: 16396-1-AP, Rabbit Polyclonal, 68 KD), Ho-1 (Proteintech Group, Inc., catalog number: 27282-1-AP, Rabbit Polyclonal, 33 KD), Nox 2 (Proteintech Group, Inc., catalog number: 19013-1-AP, Rabbit Polyclonal, 55 KD), Nox 4 (Proteintech Group, Inc., catalog number: 14347-1-AP, Rabbit Polyclonal, 62 KD), endothelial nitric oxide synthase (Enos, Abcam, catalog number: ab5589, Rabbit Polyclonal, 133 KD), inducible nitric oxide synthase (Inos, Affinity, catalog number: AF6270, Rabbit Polyclonal, 131 KD), Gapdh (Hangzhou Xianzhi Biology Co., catalog number: AB-P-R 001, Rabbit Polyclonal, 37 KD), β-actin (Servicebio, catalog number: GB11001, Rabbit Polyclonal, 45 KD) overnight at 4 °C. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature and analyzed by the ECL detection system.

2.10. Quantitative RT-PCR

The mRNA expression of *miR-24*, *Ogt*, *Keap1*, *Nrf2*, *Ho-1*, *Nox 2*, and *Nox 4* was assessed using quantitative real-time PCR. Total RNA was extracted using the Trizol Reagent (Invitrogen) and reverse transcribed to cDNA. The expression levels of these genes were measured using ABI Prism 7500 Sequence Detection System (PE Applied Biosystems). *U6* and *Gapdh* were used as internal control. Each sample was collected and analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression levels. The primers are listed in Table 1.

2.11. Immunohistochemical detection

Briefly, the carotid sections were incubated with anti-CD31, anti-Enos and anti-Inos 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 was used

Table 1
Primers used in this study.

Name	Primer	Sequence	Size
U6	Forward	5' - CGATACAGAGAAGATTAGCATGGC - 3′	61 bp
	Reverse	5' - AACGCTTCACGAATTTGCGT - 3′	
miR-24	Forward	5' - CGCAGTGGCTCAGTTCAGCA - 3′	66 bp
	Reverse	5' - AGTGCGTGTCGTGGAGTCG - 3′	
Gapdh	Forward	5' - TGGAGAAACCTGCCAAGTATGAT - 3′	142 bp
	Reverse	5' - TCAAAGGTGGAAGAATGGGAGT - 3′	
Ogt	Forward	5' - TGGAAGAAGCCAAGGCATGTT - 3′	113 bp
	Reverse	5' - TGCCAGCCAAATCTCCCCT - 3′	
Keap1	Forward	5' - GTGGAGAGATATGAGCCAGATCG - 3′	180 bp
	Reverse	5' - CATCCGCCACTCATTCCTCT - 3′	
Nrf2	Forward	5' - TACTCCCAGGTTGCCCACA - 3′	208 bp
	Reverse	5' - TCTCCAGAGAGCTATCGAGTGACT - 3′	
Ho-1	Forward	5' - GGAAGGCTTTAAGCTGGTGATG - 3′	93 bp
	Reverse	5' - GCATAGACTGGGTTCTGCTTGTTT - 3′	
Nox 2	Forward	5' - CGAAAACTTCTTGGGTCAGCAC - 3′	136 bp
	Reverse	5' - TTGAGCAACACGCACTGGAA - 3′	
Nox 4	Forward	5' - AACACTCTACTGGATGACTGGAAAC - 3′	88 bp
	Reverse	5' - ACCACTGGAATGATTGGATGTCT - 3′	

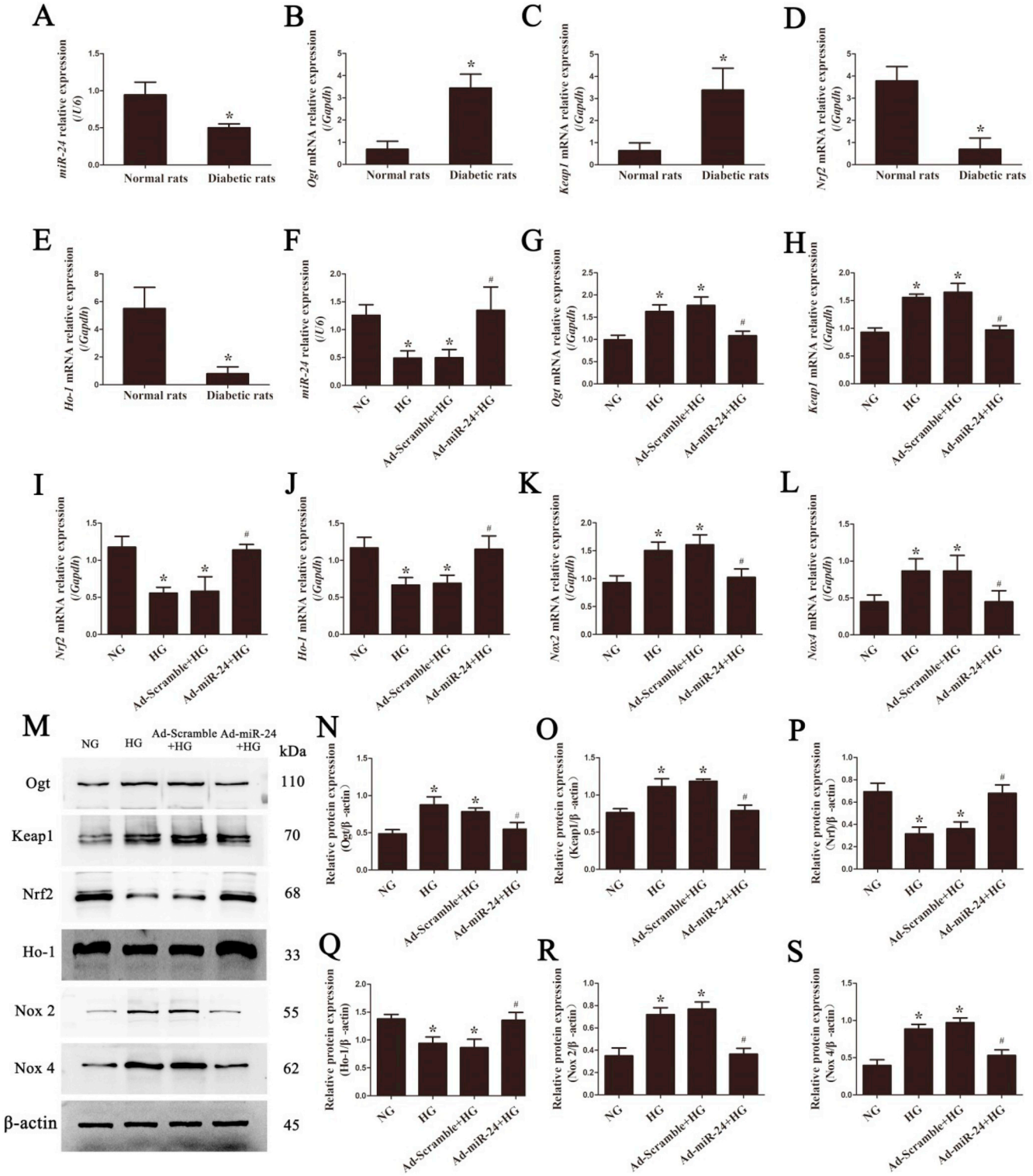

Fig. 1. Real-time PCR and Western blot were performed to detect gene and protein expressions of the following molecules in VSMCs and carotid artery balloon-injured rats.

(A–E) Real-time PCR was performed to compare the gene expression level of *miR-24*, *Ogt*, *Keap1*, *Nrf2*, and *Ho-1* after balloon injury of the carotid artery between normal and diabetic rats. *U6* and *Gapdh* were used as internal parameters of the above genes, respectively. (F) Relative expression of *miR-24* in VSMCs using *U6* as an internal control. (G–L) The mRNA levels of *Ogt*, *Keap1*, *Nrf2*, *Ho-1*, and *Nox 2* and *Nox 4* in VSMCs were analyzed by real-time PCR and normalized to the internal control gene *Gapdh*. (M − S) Original representative Western blots (left panel) and relative levels of Ogt, Keap1, Nrf2, Ho-1, Nox 2 and Nox 4 proteins (right panels). β-actin served as a loading control. Data from our independent experiments expressed as the mean ± SD. $^{*}p < 0.05$ *vs.* NG, $^{\#}p < 0.05$ *vs.* HG and Ad-Scramble + HG, $n = 4$.

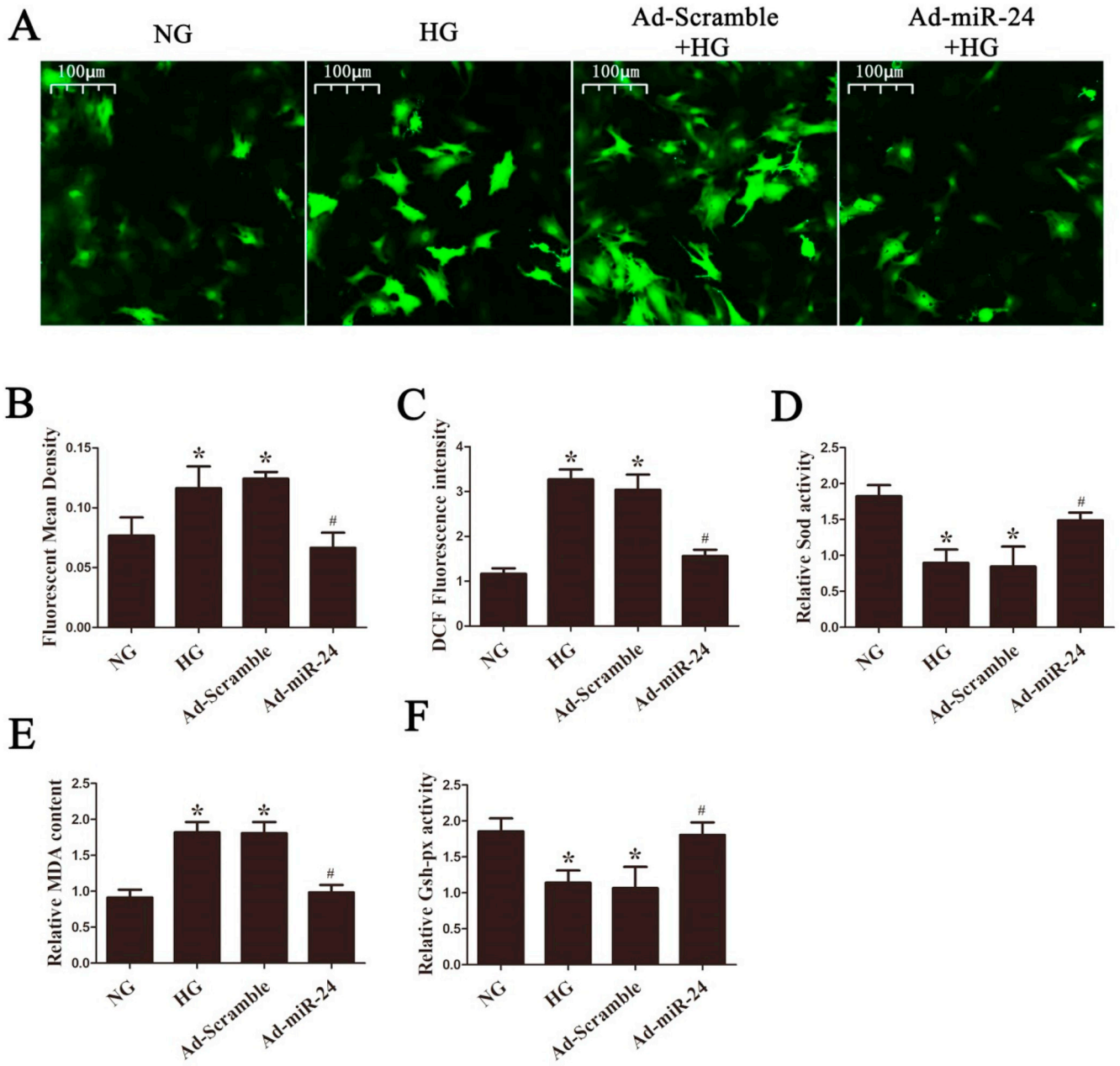

Fig. 2. *miR-24* reduced ROS and oxidative damage in VSMCs.
(A–B) Intracellular ROS generation was detected using the DCFH-DA probe. Fluorescence expression was observed by confocal laser microscopy. (C) Flow cytometry analysis of ROS using a DCFH-DA kit. (D–E) Changes in MDA content and Sod and Gsh-px activities in VSMCs. $^{*}p < 0.05$ compared with cells treated with NG; $^{\#}p < 0.05$ compared with cells treated with HG and Ad-Scramble + HG. Data are reported as the mean of four independent experiments.

as a negative control. Color development was achieved with diaminobenzidine, and hematoxylin was applied as a counterstain prior to cover-slipping.

2.12. Serum biochemistry detection

To assess the oxidative stress *in vivo*, the levels of Sod, MDA, and Gsh-px were investigated. Blood samples from the external jugular vein were collected when rats were anesthetized. After centrifugation at 1500 rpm for 10 min, the serum was isolated, and Sod, MDA, and Gsh-px levels were determined using a biochemical detection kit (Nanjing Jiancheng Institute of Bioengineering) according to the manufacturer's instruction.

2.13. Statistical analyses

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

3. Results

3.1. Ogt was a direct target of miR-24

Through a bioinformatics search, *Ogt* and *Keap1* were identified as potential targets of *miR-24* in a 3′-UTR-dependent manner. Luciferase

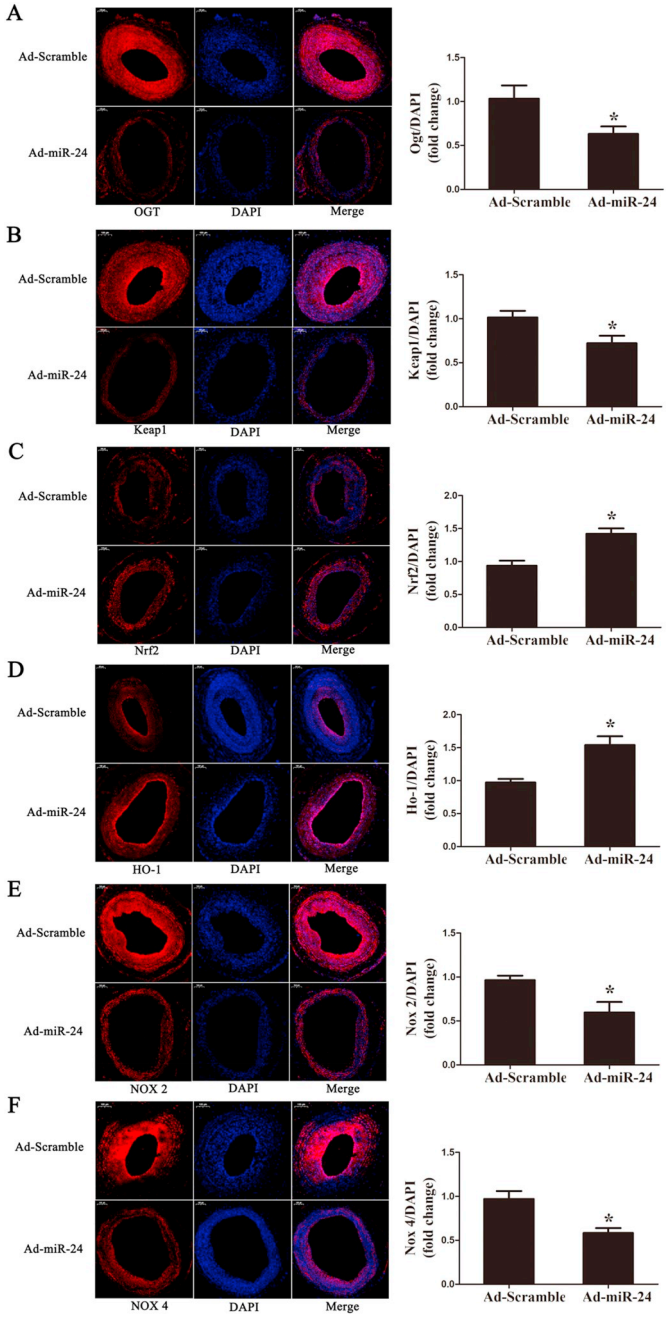

Fig. 3. *miR-24* facilitated activation of Nrf-2/Ho-1 signal transduction after vascular injury.
(A–F) Ogt, Keap1, Nrf2, Ho-1, Nox 2, and Nox 4 localization and levels in balloon-injured carotid arteries assessed by immunofluorescence assay. Image-Pro Plus 6.0 was used to calculate the fluorescence intensity of these proteins in injured vascular tissues. All data are expressed by the fluorescence intensity ratio of the above proteins to DAPI. Values are presented as the mean ± SD compared to Ad-scramble group, $*p < 0.05$, $n = 4$.

assays were performed to confirm this hypothesis. Unfortunately, luciferase activity of the *Keap1* 3′-UTR was not significantly reduced by *miR-24*, indicating that *miR*-24 has no targeted binding effect on the 3′-UTR of the *Keap1* gene, while *miR-24* suppressed more than 34% activity of the *Ogt*-3′-UTR compared with the *Ogt*-3′-UTR-NC group. Moreover, this activity was only slightly reduced in the *Ogt*-3′-UTR-M group. Overall, these results strongly supported direct suppression of *Ogt* by *miR-24* (Supplementary Figs. 1A–B).

3.2. *miR-24 up-regulation inhibited Ogt and Keap1 expression but promoted that of Nrf2 and Ho-1 in balloon-injured carotid arteries*

We detected the expression of *miR-24*, *Ogt*, *Keap1*, *Nrf2* and *Ho-1* in balloon-injured carotid arteries of normal and diabetic rats. The results showed that *miR-24*, *Nrf2*, and *Ho-1* expression levels were significantly higher in the former compared with the latter. Opposite results were observed for *Ogt* and *Keap1* expression (Fig. 1A–E).

3.3. *miR-24 up-regulation inhibited Ogt, Keap1, Nox 2 and Nox 4 expression but promoted Nrf2 and Ho-1 expression in VSMCs*

Real-time PCR and Western blot were used to detect the expression levels of Ogt, Keap1, Nrf2, Ho-1, Nox 2 and Nox 4 in VSMCs. The results showed that *miR-24* expression was significantly down-regulated after 16 h HG stimulation but increased by transfection with Ad-*miR-24* for 3 d in VSMCs. HG induced high expression levels of Ogt, Keap1, Nox 2 and Nox 4 but low expression levels of Nrf2 and Ho-1. However, *miR-24* up-regulation reversed these changes (Fig. 1F–S).

3.4. *MiR-24 up-regulation decreased ROS generation in VSMCs*

Compared with the NG group, HG significantly increased ROS levels in VSMCs. However, *miR-24* could inhibit the increased intracellular ROS generation induced by HG (Fig. 2A–C).

3.5. *Effect of miR-24 on MDA content and Sod and Gsh-px activities*

Under HG stimulation, MDA levels increased by 96%, while Sod and Gsh-px activities decreased by 50% and 58%, respectively. However, *miR-24* over-expression not only suppressed MDA generation but also promoted the activation of Sod and Gsh-px compared to the Ad-Scramble group (Fig. 2D–F).

3.6. *miR-24 up-regulation activated the Nrf2/Ho-1 signaling pathway in balloon-injured carotid arteries*

An adenoviral vector carrying *miR-24* induced a significant increase in local *miR-24* expression in balloon-injured carotid arteries (Supplementary Fig. 1C). Consistent with the results *in vitro*, *miR-24* up-regulation inhibited Ogt, Keap1, Nox 2 and Nox 4 expression levels but increased Nrf2 and Ho-1 expression levels (Fig. 3).

3.7. *miR-24 up-regulation inhibited oxidative stress in vivo*

As downstream molecules of the Nrf2/Ho-1 signaling pathway, the protein levels of Nox 2 and Nox 4 were attenuated by *miR-24 in vivo* (Fig. 4A–C). After 7 d of balloon injury, serum concentrations of Sod and Gsh-px were markedly increased, and MDA content was decreased in the Ad-*miR-24* group compared to the Ad-Scramble group (Fig. 4F–H). These results indicated that *miR*-24 might be involved in oxidative stress after vascular endothelial denudation.

3.8. *miR-24 up-regulation promoted vascular endothelium repair*

Enos, which could produce nitric oxide (NO), is an important indicator of EC function. However, Inos, another source of NO, is induced by inflammation and oxidative stress, which can induce excessive NO and thus cause vascular dysfunction. Up-regulation of *miR-24* significantly increased the expression of Enos but had an opposite effect on Inos in diabetic injured arteries. Moreover, CD31 expression was also increased in the Ad-*miR-24* group compared to the Ad-Scramble group (Fig. 4D and E and Fig. 5). These results indicated that *miR-24* over-expression might be related to endothelial repair after vascular injury.

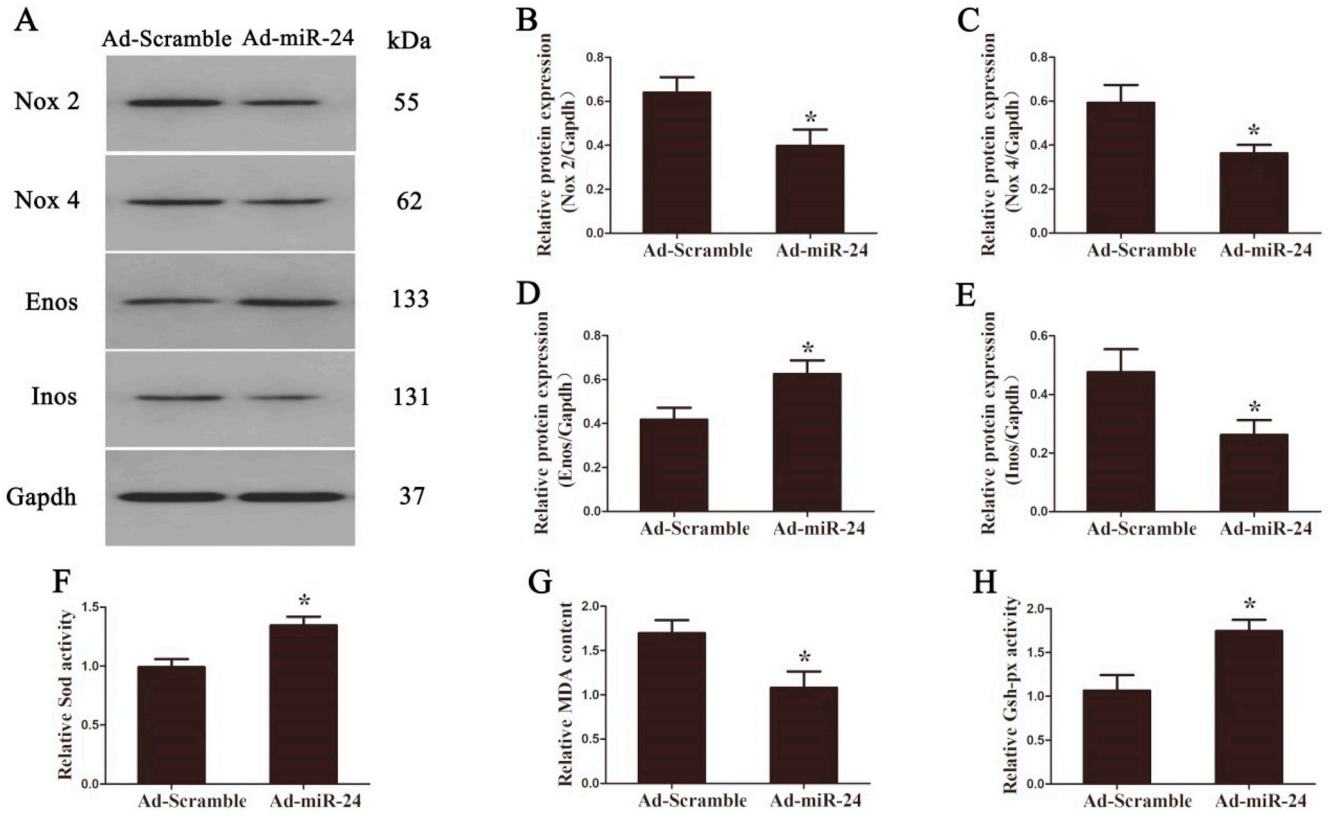

Fig. 4. *miR-24* inhibited oxidative stress induced by balloon injury in diabetic rats. Protein expression was measured in balloon-injured carotid arteries were treated with Ad-*miR-24* or Ad-Scramble for 7 d.
(A–E) Western blot analysis showing Nox 2, Nox 4, Enos, and Inos protein expression in Ad-*miR-24* and Ad-Scramble treated arteries. Gapdh was used as internal control. (F–H) *miR-24* up-regulation increased Sod and Gsh-px levels but reduced MDA levels in serum induced by arterial injury. $n = 4$ for each group. Data are presented as the mean ± SD, $^*p < 0.05$ *vs.* Ad-scramble group.

4. Discussion

The present study clearly demonstrated that *miR-24* up-regulation enhanced vascular endothelium repair by attenuating oxidative damage induced by vascular endothelial injury. The conclusion was established based on the observations below. Primarily, we found that *miR-24*, *Nrf2*, and *Ho-1* expression levels after balloon injury of the carotid artery in normal rats were higher than in diabetic rats, while the opposite result was observed for the expression of *Ogt* and *Keap1*. Therefore, we considered the interaction between them and studied the role of *miR-24* in carotid artery balloon injury in diabetic rats. In further experiments, we demonstrated that *miR-24* was decreased in HG-treated VSMCs, accompanied by the elevated production of oxidants and the reduced production of anti-oxidants. Moreover, *miR-24* up-regulation via adenovirus-mediated expression of *miR-24* suppressed HG-induced oxidative stress *in vitro* and promoted endothelial repair *in vivo*. The underlying mechanism may be related to Nrf2/Ho-1 signaling pathway activation after *miR-24* over-expression. These results indicated that *miR*-24 might be a therapeutic option for the prevention of vascular disease.

Diabetes is a major risk factor for CVD, and cardiovascular complications are the leading cause of mortality among diabetic patients [28]. Given the synergistic effect of inflammation, oxidative stress, abnormal glucose metabolism, lipid metabolism and other factors in the diabetic environment, the process of vascular endothelial repair is severely blocked, and the incidence of late thrombosis in diabetic patients is significantly increased compared with non-diabetic patients [29]. Oxidative stress plays an important role in diabetic vascular injury, and excessive production of oxidative stress inhibits reendothelialization [30]. Incomplete reendothelialization is a major clinical problem limiting the long-term efficacy of percutaneous coronary angioplasty (PTCA) [31]. Although EC implantation, endothelial progenitor cell mobilization, cytokines, exosomes, and statins play a role to some degree, there is no effective way to promote vascular reendothelialization without affecting vascular wall function [32–35]. Therefore, novel strategies to prevent stent thrombosis and other CVDs that could selectively suppress oxidative stress and promote reendothelialization are urgently needed.

Gene therapy, an applied form of biotechnology, has gradually become feasible. Choosing the correct vectors and suitable genes is probably the most critical step in gene therapy applications [36]. In our study, the adenovirus vector could efficiently deliver *miR-24* into injured vessels and isolated VSMCs, and the effects were appreciable [37,38]. The option of a target gene is just as important as vector selection. MiRNAs are considered promising clinical tools both as disease biomarkers and therapeutic targets [39–41]. Targeting miRNAs that have the ability to regulate hundreds of genes could be the future of gene therapy [42]. A portion of miRNAs, including *miR-143*, *miR-145*, *miR-221*, and *miR-222*, are highly expressed in the vascular wall and involved in vascular biology and diseases [16,43,44]. Recent studies have revealed that *miR-24* was aberrantly expressed and down-regulated in the plasma and vascular system under diabetic conditions [45,46]. In addition, numerous studies reported that *miR-24* plays an important role in VSMCs [47]. Our previous research also demonstrated that *miR-24* levels were decreased 2- to 3-fold in STZ-induced mice [38,48]. However, little is known about the effect of *miR-24* on VSMC biological function and its underlying mechanisms. Theoretically, *miR-24* replacement therapy for diabetes and diabetic complications will attract researchers' attention and interesting. An individual miRNA can regulate the expression of multiple target genes [44], e.g., *miR-24*. Our

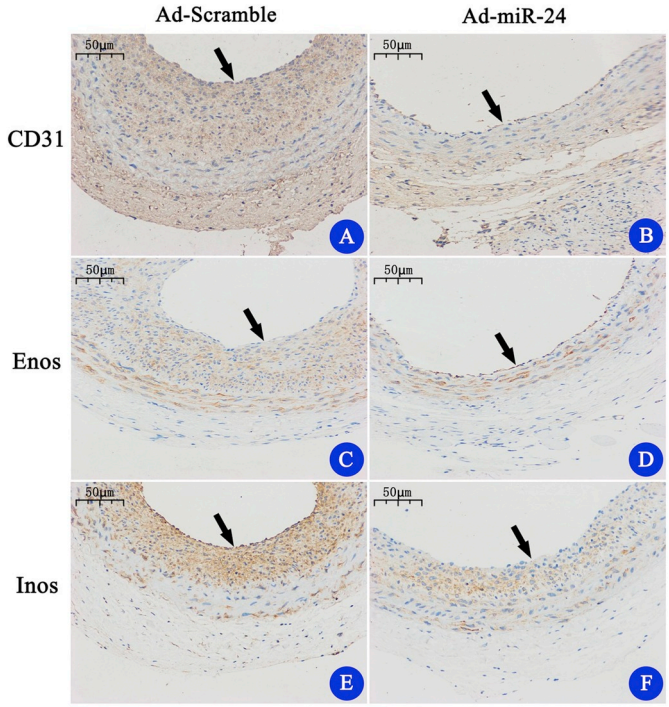

Fig. 5. *miR-24* up-regulation facilitated reendothelialization at 4 weeks after balloon injury.
Representative images of CD31 (A and B), Enos (C and D) and Inos (E and F) positivity in immunohistochemical staining. The black arrow indicates the representative region, $n = 4$.

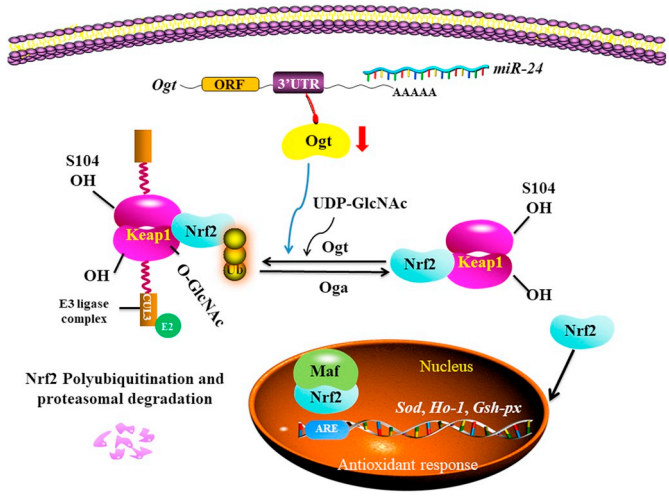

Fig. 6. The mechanism of stimulated oxidative stress in VSMCs through the Ogt/Keap1/Nrf2 signaling pathway.
Up-regulation of *miR-24* could inhibit the expression of Ogt. Ogt is required to restrain Nrf2 through the O-GlcNAcylation of Keap1 on S104, promoting its optimal interaction with CUL3 for efficient Nrf2 ubiquitination. Ogt inhibition results in a reduction in global O-GlcNAcylation; meanwhile, Keap1 is deglycosylated, which reduces Nrf2 ubiquitination and degradation. Subsequently, Nrf2 is translocated into the nucleus and bound to Maf protein, binding to the ARE region of the antioxidant genes and activating its expression. As a result, oxidative stress is reduced in VSMCs.

previous study showed that *miR-24* could inhibit vascular remodeling of diabetic rats by regulating Pi3k/Akt, Hmgb1, Wnt4 and Pdgf-bb signaling pathways [37,38,48,49]. In this study, we hypothesized that *miR-24* could promote vascular endothelial repair by reducing the release of reactive oxygen products via activating the Nrf2/Ho-1 signaling pathway.

Ogt can transfer O-GlcNAc to serine/threonine residues of numerous cellular proteins, and its substrate Keap1 can negatively regulate Nrf2, the main transcription factor of intracellular antioxidative stress [50]. In our experiments, *Ogt* and *Keap1* gene expression was simultaneously down-regulated. However, the target gene of *miR-24* remains to be explored. Previous studies have demonstrated that *miR-24* targets *Ogt* in a 3′-UTR-dependent manner [51]. Our experiments confirm this notion, but whether it has a targeting effect on *Keap1* remains unclear. Therefore, we performed a double luciferase reporter gene assay to assess the targeting relationship between *miR-24* and *Keap1* gene; however, the result was negative. Based on our data, we infer that *miR-24* regulates the Nrf2/Ho-1 signaling pathway by indirectly regulating Keap1 after targeting *Ogt*.

Nrf2 is a transcription factor that plays an important role in oxidative stress and inflammation in cells and is usually located in the cytoplasm. Under quiescent conditions, Keap1 promoted Nrf2 ubiquitination and inhibited Nrf2 activity; under oxidative stress, Nrf2 is released from Keap1 and transported to the nucleus [52]. In the nucleus, Nrf2 activates the transcription of many genes involved in oxidative stress responses, such as Ho-1 and Sod [50]. Ho-1 is a key enzyme for the degradation of heme, and its antioxidant products can maintain cell microenvironment homeostasis [53]. Previous studies demonstrated that Nrf2/Ho-1 signaling is a critical approach to reduce HG-induced apoptosis [54,55]. In addition, the Nrf2/Ho-1 axis plays a significant role in antioxidative stress [56]. In the present study, an intervention with *miR-24* was proven to decrease the gene and protein expression of Ogt and Keap1 but increased that of Nrf2 and Ho-1. Therefore, *miR-24*-mediated inhibitory mechanisms of VSMCs oxidative stress and balloon injury-induced endothelial injury in rat carotid arteries may be attributed to activation of the Nrf2/Ho-1 signaling pathway. Fig. 6 shows a summary of the mechanism of the Ogt/Keap1/Nrf2 signaling pathway in stimulated VSMCs. In addition, some limitations at this point also should be recognized. Our study only used the Ogt/Keap1/Nrf2 signaling pathway as the starting point, and whether other signaling pathways are involved remains to be further studied. Thus, further experiments, such as the use of the inhibitors or mimics of Ogt/Keap1/Nrf2 signaling-related molecules, should be performed to provide a deep understanding of the precise mechanism.

ROS, including hydrogen peroxide, superoxide and peroxynitrite, contribute significantly to endothelial dysfunction following coronary revascularization [57]. Nox 2 and 4 are two members of a family of enzymes consisting of seven isoforms responsible for producing various ROS [58]. They are both upregulated during vascular endothelial injury, thereby contributing to ROS production and consequent inhibition of vascular repair [59]. In this study, our data confirmed that HG induced a significant increase in ROS in cultured VSMCs, while *miR-24* up-regulation inhibited HG-induced ROS generation. The same result was observed in carotid artery balloon-injured diabetic rats. Sod is the only known enzyme to directly scavenge a free radical, thus protecting tissue from ROS-induced oxidative stress injury [60]. Our data clearly demonstrated that *miR-24* up-regulation greatly facilitated Sod expression under HG stimulation not only *in vitro* but also *in vivo*.

Membrane lipid peroxidation that results in a loss of integrity can lead to irreversible cell damage. Lipid peroxidation, as measured by MDA content, is recognized as an important criterion for assessing oxidative stress [61]. HG significantly increased MDA levels, while *miR-24* reversed this increase. The enhanced activity of the intracellular antioxidant Gsh-px may mediate the rapid removal of ROS and thereby protect cells from possible oxidative damage. Gsh-px activity can thus be used to evaluate the ability of cells to resist oxidative damage [62]. Our study confirmed that *miR-24* up-regulation restores Gsh-px activity and subsequently prevents cells from further oxidative damage.

CD31 is a marker typically used to evaluate vascular re-endothelialization [63]. It is found that *miR-24* could accelerate CD31-positive staining in the vessel endothelium. Moreover, Enos plays an

important role in regulating vascular homeostasis. Damage to the endothelium results in a loss of NO production from Enos, which plays a central role in maintaining vascular integrity and endothelial function [64]. Enos levels in the blood vessel wall were significantly elevated by *miR-24* in injured arteries. Inos is absent in blood vessels under physiological conditions, but its expression may be induced under inflammatory and oxidative stress conditions. Inos can induce the production of large amounts of NO, which may cause vascular dysfunction [65]. Moreover, uncontrolled Inos expression could inhibit Enos generation under oxidative stress. Fortunately, *miR-24* suppressed Inos expression, which exerts a synergistic effect with the increase of Enos, thus promoting endothelial regrowth and endothelial barrier restoration. Taken together, our present study suggests that *miR-24* reduces the release of HG-induced oxidation products and promotes vascular endothelial repair. Furthermore, the molecular mechanism is potentially linked to Nrf2/Ho-1 signaling pathway activation. Thus, we believe that *miR*-24 might be a novel therapeutic target for the treatment of patients with CAD and diabetes.

In recent years, *miR-24-3p* as a tumor suppressor has been studied in a variety of tumors, such as lung cancer, liver cancer, and nasopharyngeal carcinoma, suggesting that it plays a role in inhibiting tumor cell proliferation and promoting tumor cell apoptosis. One study indicates that *miR-24* inhibited cardiomyocyte apoptosis through the Keap1/Nrf2 pathway [66]. It seems that there is a contradiction between the observations, this may be due to the fact that *miR-24* acts on different signaling pathways in different cells. The object of our study was VSMCs. Our data indicated that *miR-24* inhibited oxidative stress of VSMCs through the Nrf2/Ho-1 signaling pathway, thereby promoting endothelial repair after vascular injury. These results indicated that *miR-24* played different roles in different cells. In our study, both *in vivo* and *in vitro* experiments were conducted under diabetic conditions, and the cell status and stimulation in the diabetic environment were significantly different compared with those in non-diabetic patients. Therefore, the role of miR-24 in the cardiovascular system needs to be further explored. Finally, we would like to stress that our study was conducted using cell and animal models; thus, the obtained data cannot be directly transferred to humans. Moreover, the sample size of our research is not large enough, which limits the persuasiveness of our research results. However, one of the highlights of this study is that we performed a more in-depth mechanistic study in cells, and the animal model produced consistent results. The common findings are explained by oxidative stress, so the idea is clear. In summary, more studies are needed to support the final conclusion and usefulness of *miR-24* in inhibiting oxidative stress after vascular injury.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Jing Zhang and Wanyin Cai designed the study, analyzed and interpreted the data and wrote the manuscript. Zhixing Fan, Chaojun Yang, Xiaowen Liu and Wenqi Gao contributed to data acquisition. Mengting Xiong and Cong Ma performed part of the experimental procedures. Jian Yang provided financial support and guided the completion of the project. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.08.023.

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