MicroRNA-93 regulates angiogenesis in peripheral arterial disease by targeting CDKN1A

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Abstract. MicroRNAs (miRNAs) are considered to be critical mediators of gene expression with respect to tumor progression, although their role in ischemia-induced angiogenesis is poorly characterized, including in peripheral arterial disease (PAD). Furthermore, the underlying mechanism of action of specific miRNAs in PAD remains unknown. Reverse transcription-quantitative polymerase chain reaction analysis revealed that microRNA-93 (miR-93) was significantly upregulated in patients with PAD and in the EA.hy926 endothelial cells in response to hypoxia. Additionally, miRNA (miR)-93 promoted angiogenesis by enhancing proliferation, migration and tube formation. Cyclin dependent kinase inhibitor 1A (CDKN1A), verified as a potential target gene of miR-93, was inhibited by overexpressed miR-93 at the protein and mRNA expression levels. Furthermore, a hind-limb ischemia model served to evaluate the role of miR-93 in angiogenesis *in vivo*, and the results demonstrated that miR-93 overexpression enhanced capillary density and perfusion recovery from hind-limb ischemia. Taken together, miR-93 was indicated to be a promising target for pharmacological regulation to promote angiogenesis, and the miR-93/CDKN1A pathway may function as a novel therapeutic approach in PAD.

Introduction

Peripheral arterial disease (PAD) is an atherosclerotic disease, which affects the arteries of the limbs. It occurs via a complex process including endothelial dysfunction in animals (1) and humans (2), lipid metabolic disturbance, thrombosis and inflammation (3). It has been reported that the incidence of PAD is as high as 15-20% in people >70 years old (4,5), with the prevalence of intermittent claudication (IC) rising to ~6% in people >60 years old (6). PAD is usually associated with atherosclerosis in the whole-body vasculature and is considered to be a principal cause of mortality worldwide. Although there have been multiple studies conducted regarding relevant risk factors in PAD, the underlying epigenetic mechanism is poorly characterized (7-9).

MicroRNAs (miRNAs) are short noncoding RNAs that are involved in the mediation of human gene expression by binding to mRNA, in addition to suppressing protein synthesis (7). There are no fewer than 1,500 human miRNAs in the miRBase database, which serve a critical role in the post-transcriptional modification of gene expression via targeting of the 3'-untranslated region (UTR) of specific mRNAs (8), thereby affecting various cellular processes in embryonic development and disease conditions (9-11). Previous studies have indicated that the determination of miRNA expression in patients with PAD may serve a prognostic and diagnostic role in the future (12,13).

Recently, regarding the study of tumor and hind limb ischemia, miRNA (miR)-93 has been considered to be able to mediate angiogenesis in various molecular pathways (14,15). miR-93 is a member of the miRNA-106b~25 cluster, and has been demonstrated to serve a key oncogenic role by regulating cell proliferation, migration, the cell cycle and tube formation (16-18). However, the association between miR-93 and PAD remains unknown and the target gene of miR-93 in PAD has not been fully characterized.

The present study determined the expression levels of miR-93 in the serum of patients with PAD and investigated the function of miR-93 in angiogenesis in a cell model and in an ischemic hind-limb mouse model. In addition, the study aimed to identify the underlying downstream targets involved in PAD.

Materials and methods

Clinical samples. A total of 146 patients with PAD (79 male and 67 female) with a mean age of 58.9±9.8 years were admitted to the 1st Hospital of Lanzhou University (Lanzhou, China) to receive drug therapy and other ancillary treatment. In addition,

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32 normal control subjects (18 male and 14 female) with a mean age of 57.6 ± 8.1 years were recruited. The ankle brachial index (ABI) was used to assess the severity of PAD (19). The blood samples from the PAD patients at the time of admission and from the controls were collected and centrifuged at 200 x g for 10 min at 4˚C to harvest the separated serum, and stored at -80˚C for further analysis. Written informed consent was obtained from all patients and the experiment was approved by the ethics committee of 1st Hospital of Lanzhou University (Lanzhou, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR analyses of miR-93 were performed on patient serum using specific TaqMan assays (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (20). In addition, RNA was isolated from EA.hy926 cells to analyze the cyclin dependent kinase inhibitor 1A (CDKN1A) mRNA expression and the miRNA-93 expression using TaqMan assays (Life Technologies; Thermo Fisher Scientific, Inc.) as previously described (21). PCR primer sequences were obtained from previously published studies (20,21).

Cell culture and transfection. EA.hy926 endothelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.), 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 1% glutamine, 1% nonessential amino acids, and penicillin (100 U/ml) at 37˚C and 5% CO_2 under humidified conditions. The cells were cultured in hypoxic conditions when the oxygen concentration of the incubator was adjusted to 1%. EA.hy926 cells ($2x10^6$/ml) cultured in the normal and hypoxic conditions were respectively transfected with miRNA-93 mimics using RNAiMAX Reagent (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Oligonucleotide transfection RNA oligos were chemically synthesized and purified by Shanghai Genepharma Co. Ltd. (Shanghai, China). The following rno-mir-93 agomir sequences were used: Sense, 5'-CAAAGUGCUGUUCGU GAGGUAG-3' and antisense, 5'-ACCUGCACGAACAGC ACUUUGUU-3'. The sequences of the rno-mir-93 agomir negative control used were as follows: Sense, 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense 5'-ACGUGACAC GUUCGGAGAATT-3'. The final concentration of miRNA mimics used for transfection was 100 nM, and the cells were harvested at 12 h for subsequent experimentation.

Cell proliferation, migration and tube formation. In order to assess the functions of transfected cells under hypoxic conditions, cell proliferation, migration and tube formation assays were performed. Cell proliferation was determined using the MTT method (12). Transfected cells were seeded into 96-well plates at 10,000 cells/well and viability was detected. In order to identify transfected cell migration ability, a wound healing assay was performed. Transfected cells were cultured in serum-free medium for 24 h, and an artificial wound was created using a 100-μl sterile pipette tip. A total of 24 h post-wound infliction, the width of the scratch gap was measured using an inverted microscope (magnification, x10). In the tube formation assay, 10 μl growth factor-reduced Matrigel was seeded

into each well and permitted to polymerize for 30 min at 37˚C. Subsequently, 10,000 cells were placed on the Matrigel for 24 h at 37˚C and images were viewed using an inverted microscope (magnification, x10). A total of three independent detections were performed for each assay, with analysis of five random visual fields for each chamber.

Luciferase reporter assays. The miRNA body map web tool, including EIMMO (http://www.mirz.unibas.ch/ElMMo3/) and miRanda-mirSVR (http://microRNA.org/), was employed to identify potential target genes of miRNA-93. EA.hy926 cells were co-transfected with the wild-type (WT) or mutant (Mut) CDKN1A 3'-UTR reporter genes or negative control miRNA mimics (pMIR-Control; Genscript Cor--p., Piscataway, NJ, USA). Following culture for 36 h, the luciferase activity was determined via comparison with *Renilla* luciferase activity when the cells had been lysed with a passive lysis buffer, using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

Western blotting. Western blot analysis using EA.hy926 cells was performed as previously described (21,22). The total proteins were incubated with primary antibodies against CDKN1A (1:1,000; cat. no. SAB4300419; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and subsequently incubated with a secondary antibody (1:2,000; cat. no. ab6721; Abcam, Cambridge, UK). GAPDH (1:2,000; cat. no. G5262; Sigma-Aldrich; Merck KGaA) was used as an internal control. The quantification of the western blotting results was performed using ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD, USA).

Hind-limb ischemic model. C57Bl/6J mice (n=12) weighing 230 ± 20 g were purchased from the Institute of Laboratory Animal Sciences, Peking Union Medical College (Beijing, China). All the procedures were approved by the Animal Experimental Ethics Committee, 1st Hospital of Lanzhou University. A critical hind limb ischemia model was created as previously described (23). Prior to surgery, the mice were anesthetized via injection of ketamine 90 mg/kg and xylazine 10 mg/kg. The ligation and division of the left femoral artery and vein were conducted to surgically create severe unilateral hind limb ischemia. At the time of surgery, mice were randomly divided into two groups (n=6 per group): The negative control group (NC intramuscular injection); and the miR-93 group (premiR-93 intramuscular injection). PremiR-93 (PM10951) or miR-mimic NC (Genscript Corp.) were dissolved in PBS and intramuscularly injected into the gastrocnemius muscle (100 μM in 25 μl), as previously described (24). Laser Doppler perfusion imaging (LDPI) was conducted 2 weeks post-surgery to detect the blood flow of the ischemic and normal limbs, as previously stated (25). The scores for muscle necrosis and ambulatory impairment were assessed respectively.

Histology. Alterations in muscle tissue morphology were examined with hematoxylin and eosin (H&E) and platelet endothelial cell adhesion molecule (CD31) staining. The hind limb tissues were fixed in 4% paraformaldehyde for 48 h at room temperature, dehydrated, paraffin-embedded and sliced into tissue sections (4 mm). All the slices were stained using

H&E and anti-CD31 for histological analysis, according to the manufacturer's specific instructions (26).

Statistical analysis. Descriptive statistics were calculated and are presented as the mean ± standard error of the mean in the figures. One-way analysis of variance was performed for multiple group comparisons followed by the Student-Newman-Keuls test for group-wise comparisons. The Student's t-test was used for comparison between two groups. P<0.05 was considered to indicate a statistically significant difference. The statistical analysis was performed using SPSS software (SPSS for Windows 17.0; SPSS, Inc., Chicago, IL, USA). A minimum of three repeats were performed per assay.

Results

Expression levels of miR-93 in patients with PAD. In order to detect the importance of miR-93 in PAD, the expression of miR-93 was analyzed using RT-qPCR. miR-93 expression in patients with PAD was significantly upregulated when compared with the controls (Fig. 1A). It has been demonstrated that PAD severity, as determined by the ABI, is also correlated with the degree of functional impairment (27,28). Among participants able to walk for 6 min without stopping at baseline, the diagnosis of PAD degree was made in accordance with baseline ABI categories (<0.50; 0.50<0.70; 0.70<0.90; and 0.90<1.10) (29). Furthermore, to validate the fold-change in miR-93 expression in patients with differing degrees of PAD, corresponding expression of miR-93 was determined, and the results demonstrated that there was a positive association between miR-93 expression and PAD severity (Fig. 1B). Collectively, these findings indicated that miR-93 was involved in the development and progression of PAD.

CDKN1A is a potential target of miR-93. To further identify the underlying mechanism by which miR-93 facilities proliferation, migration and tube formation of EA.hy 926 cells, we verified CDKN1A as a direct target of miR-93 using accessible databases (30,31). The luciferase reporter was constructed, which contained the wild-type or mutant miR-93 target sequences of CDKN1A 3'-UTR. The luciferase reporter assay was performed to demonstrate whether the CDKN1A 3'-UTR is a direct target of miR-93 (Fig. 2A). As indicated in Fig. 2B, compared with the mutant reporter gene, the luciferase activity of the wild-type 3'-UTR reporter gene was significantly downregulated, suggesting that miR-93 is able to bind to the CDKN1A 3'-UTR. It was additionally observed that miR-93 significantly inhibited CDKN1A expression at the mRNA and protein levels, using RT-qPCR and western blotting (Fig. 2C and D). Taken together, these findings demonstrated that miR-93 may directly suppress CDKN1A expression in EA.hy926 cells by targeting the CDKN1A 3'-UTR.

Effects of miR-93 in EA.hy926 cells in response to hypoxia. To examine the effect of miR-93 on endothelial cells under hypoxic conditions, the expression of miR-93 was analyzed using RT-qPCR, and the results demonstrated that miR-93 expression was upregulated in response to hypoxia when compared with normal conditions (Fig. 3A). Additionally, cells were transfected with miR-93, in which the miR-93 level was quantified by
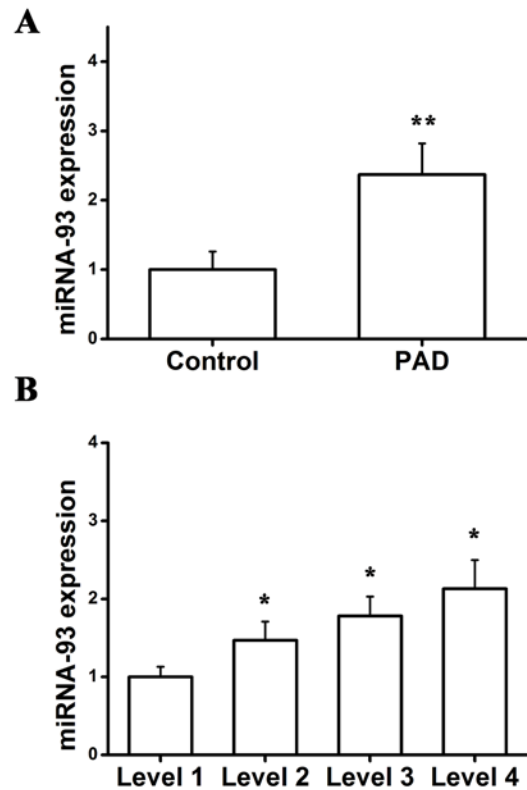

Figure 1. Expression levels of miR-93 in patients with PAD. (A) RT-qPCR analysis of miR-93 expression in patients with PAD (n=48) and controls (n=25). **P<0.01 vs. control group. (B) RT-qPCR analysis of miR-93 expression in patients with differing degrees of PAD. *P<0.05 vs. Level 1. The experiment was repeated three times. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; PAD, peripheral arterial disease.

RT-qPCR at 48 h post-transfection. miR-93 was successfully overexpressed in EA.hy926 cells (Fig. 3B), and a number of experiments were performed *in vitro*, including the MTT assay, wound healing assay and tube formation assay. The data demonstrated that the proliferation, migration and tube formation of cells that were transfected with miR-93 and cultured in hypoxic conditions were markedly enhanced (Fig. 3C-E). Thus, the aforementioned data suggested that miR-93 maintained endothelial cell activity by promoting proliferation, migration and tube formation in response to hypoxia.

Overexpression of miR-93 in the hind-limb ischemic model to improve perfusion recovery. It has been reported that BALB/cJ mice have decreased expression of miR-93 and exhibit little increase in miR-93 following hind-limb ischemia when compared with C57B1/6J mice (32). Thus, local intramuscular injections of premiR-93 and NC were administered to detect whether the overexpression of miR-93 improved perfusion in the hind-limb ischemic model. The LDPI experiment indicated that miR-93 increased the blood flow ratio and improved tissue necrosis and ambulatory impairment, as presented in Fig. 4A-C. Consistent with improved perfusion recovery, ischemic hind limb muscles from the mice injected with premiR-93 indicated decreased muscle necrosis and a higher capillary density when compared with NC-treated mice (Fig. 4D), suggesting that overexpression of miR-93 may be sufficient to promote angiogenesis following hind-limb ischemia.
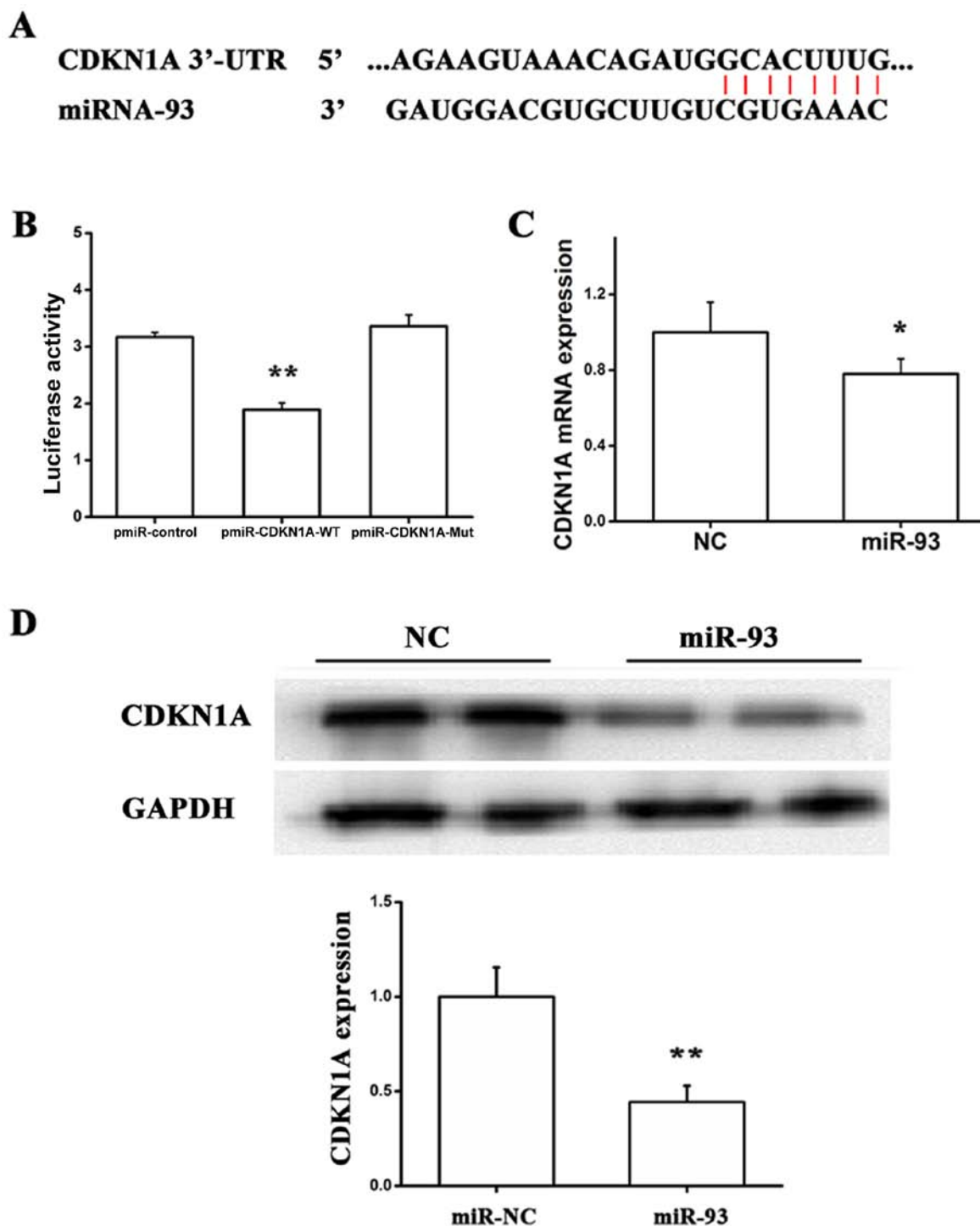
Figure 2. CDKN1A is a potential target of miR-93. (A) Schematic representation of the CDKN1A 3'-UTR as a direct target of miR-93. (B) Luciferase activity of EA.hy926 cells co-transfected with the WT or Mut CDKN1A 3'-UTR reporter genes or negative control miRNA mimics. **P<0.01 vs. pMIR-CDKN1A-Mut. (C) Quantification of CDKN1A mRNA expression levels by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. NC. (D) CDKN1A protein expression was determined by western blotting in EA.hy926 cells co-transfected with miR-93 or control, and the quantification was performed using Image J. n=3. **P<0.01 vs. miR-NC. The experiment was repeated three times. CDKN1A, cyclin dependent kinase inhibitor 1A; miR, microRNA; UTR, untranslated region; NC, negative control; WT, wild-type; Mut, mutant.

Discussion

The discovery of miRNAs was considered a milestone in molecular biology, and miRNAs are critical regulators involved in numerous cellular processes, including proliferation, migration, apoptosis and differentiation. miRNAs have been closely associated with the development and progression

of tumors (33,34). In the present study, miR-93 expression in the peripheral blood of patients with PAD was compared with the control group, and it was observed that miR-93 was upregulated in patients with PAD. To validate the molecular mechanisms underlying PAD, EA.hy926 endothelial cells were used to observe miR-93 expression in response to hypoxia. *In vitro* analyses demonstrated that miR-93 promoted
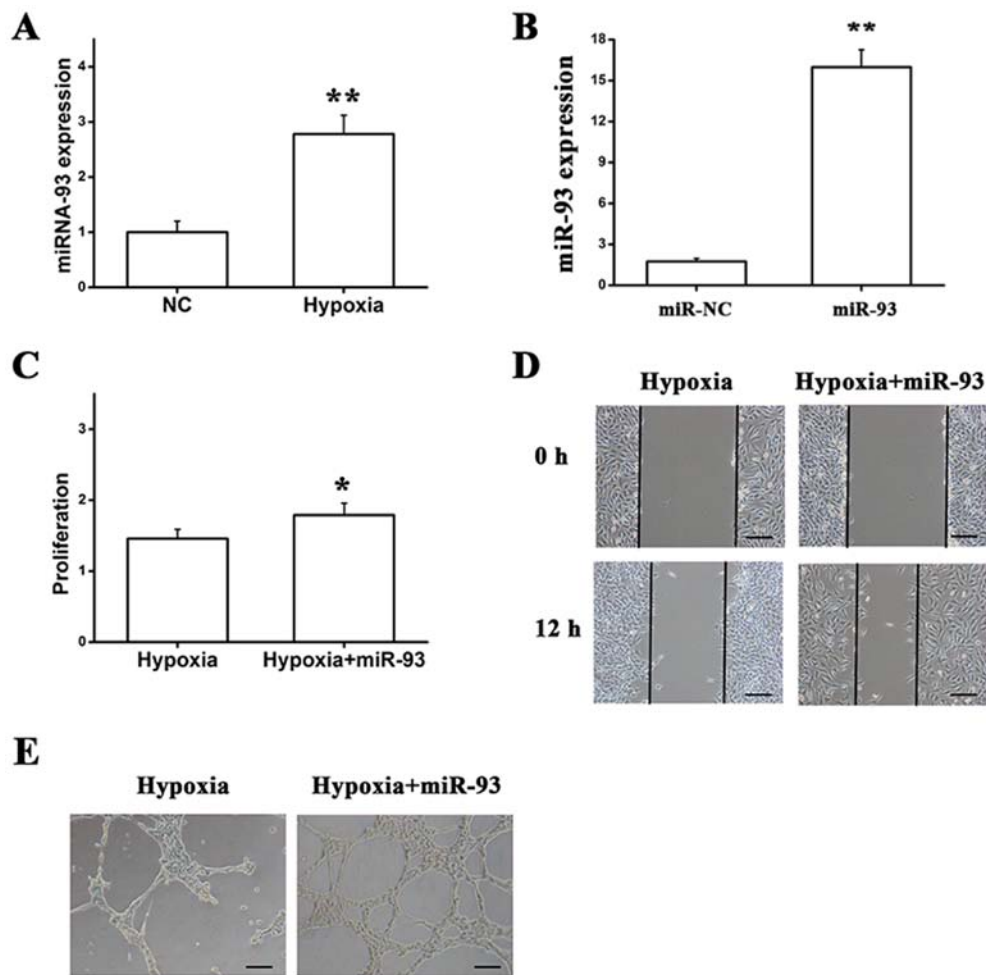
Figure 3. Effects of miR-93 in EA.hy926 cells in response to hypoxia. (A) RT-qPCR analysis of miR-93 expression in EA.hy926 cells when cultured in normal and hypoxic conditions. [**]P<0.01 vs. NC. (B) RT-qPCR analysis of miR-93 expression in EA.hy926 cells transfected with the miR-93 vector. [**]P<0.01 vs. NC. (C) The proliferation of cells transfected with miR-93 or the control when cultured in hypoxic conditions was determined using an MTT assay. [*]P<0.05 vs. hypoxia. (D) Representative images of cell migration following transfection with miR-93 or the control when cultured in hypoxic conditions at 0 and 12 h. Scale bar, 50 μm. (E) Representative images of tube formation of cells transfected with miR-93 or the control when cultured in hypoxic conditions for 12 h. Scale bar, 50 μm. n=3. The experiment was repeated three times. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; NC, negative control.

EA.hy926 cell proliferation, migration and tube formation by binding to CDKN1A, which was demonstrated to be a direct target. The aforementioned findings revealed that miR-93 enhanced the proliferation, migration and tube formation of EA.hy926 cells by directly targeting CDKN1A.

PAD induces tissue hypoperfusion and eventually results in critical limb ischemia (35). A number of miRNAs have been reported to be associated with the development of PAD and involved in vascular disorders. Liu *et al* (36) reported that miRNA-15b is associated with apoptosis and angiogenesis in myocardial infarction. Kuehbacher *et al* (37) demonstrated that miR-27b may be regarded as a pro-angiogenic miRNA by regulating angiogenesis through the angiogenic inhibitor, thrombospondin-1. Chamorro-Jorgane *et al* (38) indicated that miR-16 modulates angiogenic signaling and vascular integrity, serving a role in angiogenesis by suppressing the proliferation, migration and angiogenic capacity of endothelial cells. However, little information is available pertaining to the association between miRNA-93 expression and PAD. The results of the present study revealed that miRNA-93 was upregulated in EA.hy926 endothelial cells when cultured in

hypoxic conditions, and in freshly frozen muscle tissues. To further verify the angiogenic function of miRNA-93, the effect of miRNA-93 on endothelial cells was observed *in vitro* and the results suggested that miRNA-93 may facilitate proliferation, migration and tube formation under hypoxic conditions, indicating that miRNA-93 may contribute to the progression of PAD by regulating endothelial cell activities (32).

In previously established models of PAD, a series of studies (32,39,40) indicated that C57Bl/6 J mice recover well, while BALB/cJ mice exhibit poor perfusion recovery following hind-limb ischemia. *In vivo*, C57Bl/6 J mice were used as a model and it was observed that hind-limb blood perfusion was improved, and scores for muscle necrosis and ambulatory impairment were significantly decreased following injection of premiR-93, which indicated that overexpression of miR-93 promoted angiogenesis to improve recovery from hind-limb ischemia. Furthermore, extensive histological staining of the hind-limb tissue revealed a high density of CD31, which is considered to be a key marker of endothelial cells. Consistent with the results of the *in vitro* studies (32,41), these findings further confirmed the role of miR-93 in ischemia-induced angiogenesis.
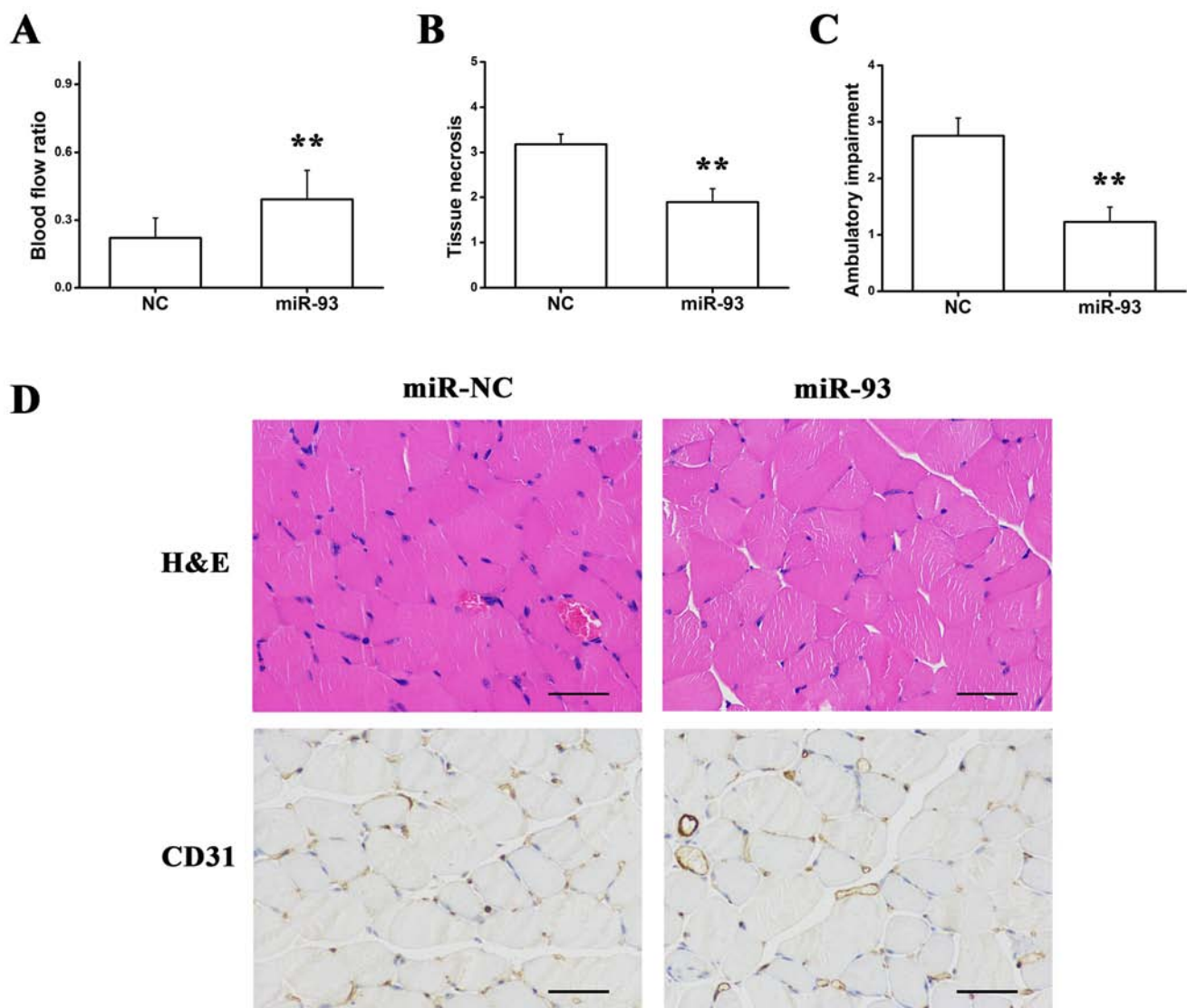
Figure 4. Overexpression of miR-93 in the hind-limb ischemic model to improve perfusion recovery. (A) miR-93 significantly increased the blood flow ratio, and improved (B) tissue necrosis and (C) ambulatory impairment. **P<0.01 vs. NC. (D) Representative images of H&E and CD31 staining. Scale bar, 100 μm. n=6. The experiment was repeated three times. miR, microRNA; H&E, hematoxylin and eosin; CD31, platelet endothelial cell adhesion molecule; NC, negative control.

CDKN1A is regarded as an important inhibitor of the cell cycle, mediator of DNA damage and effector of the tumor suppressor cellular tumor antigen p53, displaying a key role in the development and progression of various cancer types (42). CDKN1A, an endothelial dysfunction-associated gene, is associated with the decrease in human umbilical vein endothelial cell viability caused by sodium arsenite (43). Yamagata *et al* (44) reported that docosahexaenoic acid regulates the expression of numerous genes that involve CDKN1A, which is associated with senescence and dysfunction in endothelial cells.

Previous studies have emphasized the functional implications of CDKN1A during the development of PAD (45,46). As previously stated, PAD is an atherosclerotic disease, in which the pathological response contributes to a complex inflammatory reaction induced by endothelial functional loss (45). In the present study, using a luciferase reporter assay, CDKN1A was identified as a direct target of miR-93 in EA.hy926 cells, suggesting that miR-93 exerts an important effect in the regulation of angiogenesis during PAD via binding to CDKN1A.

In conclusion, miR-93 contributes to angiogenesis by enhancing the proliferation, migration and tube formation of EA.hy926 endothelial cells, which is associated with the reduced expression of CDKN1A. Overexpression of miR-93 was able to ameliorate ischemia in the mouse hind-limb, providing a novel opportunity to elucidate whether miR-93/CDKN1A may be a promising therapeutic target for PAD.

Availability of data and materials

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

Authors' contributions

XS designed the study, performed experiments, analyzed the data and wrote the manuscript. YM performed a number of *in vitro* and *in vivo* experiments. ZL performed the animal experiments. WW, YC and SL analyzed the data and drafted the manuscript. XL designed and supervised the study, and edited the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients and the experiment was approved by the ethics committee of 1st Hospital of Lanzhou University (Lanzhou, China). All the procedures were performed in accordance with national (D.L.n.26, March 4th, 2014) and international laws and policies (directive 2010/63/EU), and were approved by the Animal Experimental Ethics Committee, 1st Hospital of Lanzhou University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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