Downregulated miRNA-26a-5p induces the apoptosis of endothelial cells in coronary heart disease by inhibiting PI3K/AKT pathway

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Abstract. – OBJECTIVE: Multiple microRNAs (miRNAs) are abnormally expressed in endothelial cells during the occurrence of coronary artery disease (CAD). Previous researches have demonstrated that miRNA-26a-5p participates in regulating the proliferation of vascular smooth muscle cells and angiogenesis. The aim of this study was to clarify the role of miRNA-26a-5p in regulating cellular performances of endothelial cells in the progression of CAD.

PATIENTS AND METHODS: *In vivo* CAD model was successfully established by feeding high-fat diet in 8-week-old female ApoE/LDLR-/- mice. CAD mice were administered with miRNA-26a-5p NC or miRNA-26a-5p inhibitor, respectively. Meanwhile, coronary endothelial cells were isolated from CAD mice and normal controls. Relative levels of miRNA-26a-5p, the gene of phosphate and tension homology deleted on chromosome ten (PTEN) and vascular endothelial growth factor (VEGF) in CAD patients and coronary endothelial cells isolated from CAD mice were examined. The regulatory effect of miRNA-26a-5p on atherosclerosis-related genes in primary endothelial cells and HUVECs were detected as well. Moreover, the viability and apoptosis of primary endothelial cells with miRNA-26a-5p knockdown were assessed by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Dual-luciferase reporter gene assay was conducted to identify the relationship between miRNA-26a-5p and PTEN. Furthermore, the regulatory role of miRNA-26a-5p in phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway was examined in endothelial cells.

RESULTS: MiRNA-26a-5p and VEGF were significantly downregulated in CAD patients and primary endothelial cells isolated from CAD mice. However, PTEN was significantly upregulated. CAD mice administrated with miRNA-26a-5p inhibitor exhibited remarkably upregulated ET-1, TxA2, and ANG II, as well as downregulated eNOS and PGI2. Conversely, transfection of miRNA-26a-5p mimics in HUVECs obtained the opposite trends. PTEN was identified as the direct target gene of miRNA-26a-5p. Moreover, significantly reduced viability and enhanced apoptotic rate were observed in endothelial cells isolated from CAD mice administrated with miRNA-26a-5p inhibitor. In addition, the protein level of p-AKT in endothelial cells with miRNA-26a-5p knockdown was significantly down-regulated.

CONCLUSIONS: MiRNA-26a-5p influences the proliferative and apoptotic abilities of endothelial cells isolated from CAD mice by targeting PTEN to activate PI3K/AKT pathway.

Key Words

MiRNA-26a-5p, PI3K/AKT, CAD, Endothelial cells.

Introduction

Coronary artery disease (CAD) is caused by myocardial ischemia, hypoxia or necrosis due to structural or functional abnormalities in the coronary artery. The mortality of CAD ranks first all over the world, posing a serious threat to human health[1]. Currently, the pathological process of atherosclerosis (AS) is the main cause of CAD aggravation. Previous studies have shown that endothelial dysfunction is associated with every stage of AS, which is also believed to be the initiating factor of AS. Furthermore, most of the coronary events are closely related to arterial endothelial dysfunction[2].

MicroRNAs (miRNAs) are a kind of non-coding RNAs with 20-23 nucleotides in length, which can regulate gene expressions. MiRNAs are extensively expressed in eukaryotes and highly conserved[3]. As a vital mediator, miRNAs degrade target mRNAs or inhibit their translation by pairing to the 3'untranslated region (3'UTR) of mRNAs. This may eventually regulate the expressions of target genes at post-transcriptional level[4]. Functionally, miRNAs are capable of regulating various cellular phenotypes[5,6]. Abnormally expressed miRNAs have been identified in CAD. These miRNAs have been observed to influence

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cellular behaviors of endothelial cells, thus affecting the occurrence and progression of CAD[7,8].

MiR-26a is widely expressed in a variety of tissues, whose expression is not tissue-specific[9]. Whole-genome miRNA expression profiling has revealed abnormal expression of miR-26a in multiple diseases. Current studies[10-12] have indicated that miR-26 participates in the differentiation of vascular smooth muscle cells, hypertrophic cardiomyocytes and neurons by downregulating SMAD and GATA4. In liver cancer, miR-26a inhibits tumor angiogenesis by targeting vascular endothelial growth factor A (VEGFA) to inhibit the PIK3C2α/protein kinase B (AKT)/hypoxia inducible factor-1α (HIF-1α) pathway[13]. Meanwhile, another report has pointed out that miR-26a inhibits the angiogenesis of liver cancer by suppressing HGF and further inactivating phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (Mtor)/S6K and HIF-1α-VEGF pathway[14]. However, the potential role of miR-26a in the progression of CAD has rarely been elucidated. Therefore, the aim of this study was to clarify the role of miRNA-26a-5p in regulating cellular performances of endothelial cells in the progression of CAD.

Patients and Methods

Sample Collection

Coronary tissues were harvested from CAD patients (n=8) and controls (n=8) from July 2018 to February 2019. The investigation was approved by the Ethics Committee of Xiangya Hospital. Informed consent was obtained from each subject before the study.

Establishment of CAD Model in Mice

A total of 30 female ApoE/LDLR$^{-/-}$ mice aged 8 weeks old were provided by the Model Animal Research Center of Nanjing University. All mice were randomly assigned into two groups, including normal diet group (control group) and high-fat diet group (CAD group). Mice in CAD group were given a high-fat diet with 21% fat and 0.15% cholesterol for 15 weeks. After the successful establishment of the CAD model, CAD mice were administrated through the tail vein with 8 mg/kg miRNA-26a-5p inhibitor or negative control twice, with a one-week interval. All animal procedures were approved by relevant ethical requirements.

Isolation of Endothelial Cells and Cell Culture

Arterial tissues were thoroughly rinsed with Hank's Balanced Salt Solution (HBSS) and were clamped with a vessel clamp for one end. Subsequently, 10 mL of HBSS containing 0.2% collagenase was administrated into the lumen, followed by digestion at 37°C for 10 min. Subsequently, the mixture was centrifuged at 1600 rpm/min for 5 min. After that, the precipitant was suspended in 1 mL of Lonza and inoculated in a gelatin-coated flask. Human umbilical vein endothelial cells (HUVECs) were provided by Cell Bank (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA).

Target Gene Prediction

Target genes of miRNA-26a-5p were searched in miRBase, TargetScan, and miRDB. The intersection of predicted target genes from the three websites was selected for further functional and mechanistic verification.

Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was first extracted from tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The concentration of extracted RNA was quantified using NanoDrop 2000 spectrophotometer. Subsequently, qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Obtained cDNA was further amplified by real-time quantitative PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. Relative levels of genes were quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Primer sequences used in this study were as follows: PTEN F: CAAGATGATGTTTGAAACTATTCCAATG; R: CCTTTAGCTGGCAGACCACAA; VEGF F: AAAGGCTTCAGTGTGGTCTGAGAG; R: GGTTGGAACCGGCATCTTTATC; ET-1 F: GAGGCTGAGAATCAGACTGACA; R: TTACTGATAAACTTGCACATAACATTCT; TxA2 F: GGTGGCCCACTTGTTTGT; R: CATCCACGACCAGGAACA; ANG II F: GAGGCTGAGAATCAGACTGACA; R:

TTACTGATAAACTTGCACATAACATTCT; eNOS F: CGAGATATCTTCAGTCCCAAGC; R: GTGGATTTGCTGCTCTCTAGG; PGI2 F: TGGCTTCTGCTTTGGACGAC; R: TGCTC-CCTGCCTCTCACGAT; miRNA-26a-5p F: CCGCCGTTCAAGTAATCCAG; R: AGTG-CAGGGTCCGAGGTATT; R: GTCGTATC-CAGTGCAGGGTCCGAGGTATTCGCACTG-GATACGACAGCCTA.

Cell Transfection

Cells in the logarithmic growth phase were subjected to transfection at the confluence of 70-80%. Transfection vectors and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) were diluted in Opti-MEM, respectively. After incubation for 20 min, the mixtures were applied in each well. Complete fresh medium was replaced 6 h later. Transfection plasmids were provided by GenePharma (Shanghai, China).

Western Blot

Total protein was extracted from cells or tissues using the radioimmunoprecipitation assay (RIPA). The concentration of extracted total protein was quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After rinsing with Tris-Buffered Saline and Tween 20 (TBST), the membranes were incubated with the corresponding secondary antibody. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) and analyzed by the Image Software (NIH, Bethesda, MD, USA).

Cell Counting Kit-8 (CCK-8) Assay

Cells were first seeded into 96-well plates and cultured overnight. Absorbance (A) at 450 nm was recorded at the established time points according to the instructions of the Cell counting kit-8 (CCK-8 kit; Dojindo Laboratories, Kumamoto, Japan). Finally, viability curves were plotted.

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with miRNA-26a-5p NC/miRNA-26a-5p mimics and PTEN-WT/PTEN-MUT using Lipofectamine 2000. 24 h later, co-transfected cells were harvested. Luciferase activity was finally determined using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Cell Apoptosis

Cells were re-suspended in 500 μL of binding buffer at the dose of 1-5×10^5/mL. Next, the cells were incubated with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) in the dark for 30 min. Cell apoptosis was determined by flow cytometry (Partec AG, Arlesheim, Switzerland) within half an hour.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean ± standard deviation. Intergroup difference was analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

MiRNA-26a-5p was Downregulated in CAD Patients and Mouse Model

MiRNA-26a-5p expression in CAD patients and controls was determined. Results showed that the expression of miRNA-26a-5p was significantly downregulated in CAD patients (Figure 1A). Upregulated PTEN level and downregulated VEGF level were observed in CAD patients relative to normal controls (Figures 1B, 1C). *In vivo* CAD model was successfully established by feeding high-fat diet in 8-week-old female ApoE/LDLR$^{-/-}$ mice. Coronary endothelial cells were isolated from CAD mice and controls. It was found that miRNA-26a-5p expression was identically downregulated in endothelial cells isolated from CAD mice (Figure 1D). Similar trends of PTEN and VEGF levels were observed in the CAD mouse model (Figures 1E, 1F).

Silence of MiRNA-26a-5p Upregulated AS-Related Genes

After the establishment of the CAD model in mice, they were randomly administrated with miRNA-26a-5p NC or miRNA-26a-5p inhibitor through the tail vein, respectively. Subsequently, endothelial cells were isolated from these mice and cultured. QRT-PCR data showed the significantly lower expression level of miRNA-26a-5p in endothelial cells isolated from CAD mice administrated with miRNA-26a-5p inhibitor than con-

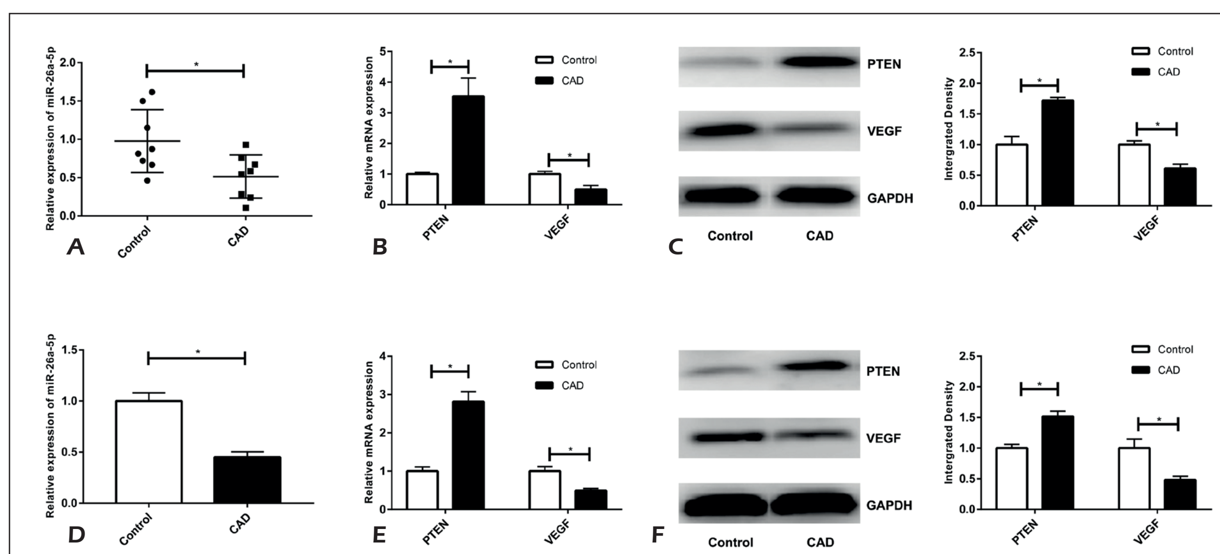

Figure 1. MiR-26a-5p was downregulated in CAD patients and mouse model. **A**, Relative level of miR-26a-5p in CAD patients (n = 8) and controls (n = 8). **B**, mRNA levels of PTEN and VEGF in CAD patients (n = 8) and controls (n = 8). **C**, Protein levels of PTEN and VEGF in CAD patients (n = 8) and controls (n = 8). **D**, Relative level of miR-26a-5p in endothelial cells isolated from CAD mice and controls. **E**, mRNA levels of PTEN and VEGF in endothelial cells isolated from CAD mice and controls. **F**, Protein levels of PTEN and VEGF in endothelial cells isolated from CAD mice and controls.

trols. This suggested the successful knockdown of miRNA-26a-5p *in vivo* (Figure 2A). Upregulated PTEN level and downregulated VEGF level were observed in endothelial cells isolated from CAD mice administrated with miRNA-26a-5p inhibitor (Figures 2B, C). Subsequently, the expression levels of AS-related genes were examined as well. In mice with miRNA-26a-5p knockdown, the expression levels of ET-1, TxA2, and ANG II were significantly elevated, while the expression levels of eNOS and PGI2 were reduced (Figures 2D, E).

Overexpression of MiRNA-26a-5p Downregulated AS-Related Genes

HUVECs were transfected with miRNA-26a-5p mimics to uncover the effect of miRNA-26a-5p on AS *in vitro*. Transfection efficacy of miRNA-26a-5p mimics in HUVECs was verified by qRT-PCR (Figure 3A). After transfection of miRNA-26a-5p mimics in HUVECs, the expression levels of ET-1, TxA2, and ANG II were downregulated, while the levels of eNOS and PGI2 were upregulated (Figures 3B, 3C).

Silence of MiRNA-26a-5p Suppressed Proliferation of Endothelial Cells Through PI3K/AKT Pathway

Bioinformatics predicted that potential binding sequences were identified between miRNA-26a-5p and PTEN (Figure 4A). Based on these binding sequences, PTEN-WT and PTEN-MUT vectors were constructed for dual-luciferase reporter gene assay. Remarkably declined luciferase activity was observed in endothelial cells co-transfected with PTEN-WT and miRNA-26a-5p mimics. The above results verified the binding relationship between PTEN and miRNA-26a-5p (Figure 4B). Meanwhile, significantly higher apoptotic rate and lower viability were discovered in endothelial cells isolated from CAD mice administrated with miRNA-26a-5p inhibitor than those of controls (Figures 4C, 4D). Furthermore, the protein level of p-AKT was significantly downregulated in endothelial cells with miRNA-26a-5p knockdown. All these findings suggested that miRNA-26a-5p positively regulated the PI3K/AKT pathway (Figure 4E).

Discussion

CAD is the most common pathological type of AS, which severely endangers human health. Multiple miRNAs have been discovered involved in the pathological process of AS, including endothelial cell damage, monocyte infiltration and vascular smooth muscle cell dysregulation. For example, miR-126 is a vascular-specific miRNA, which contributes to angiogenesis and endothelial cell repair[15]. Current studies[16] have highlighted that miR-126 is expressed in apoptosis body and maintains the sta-

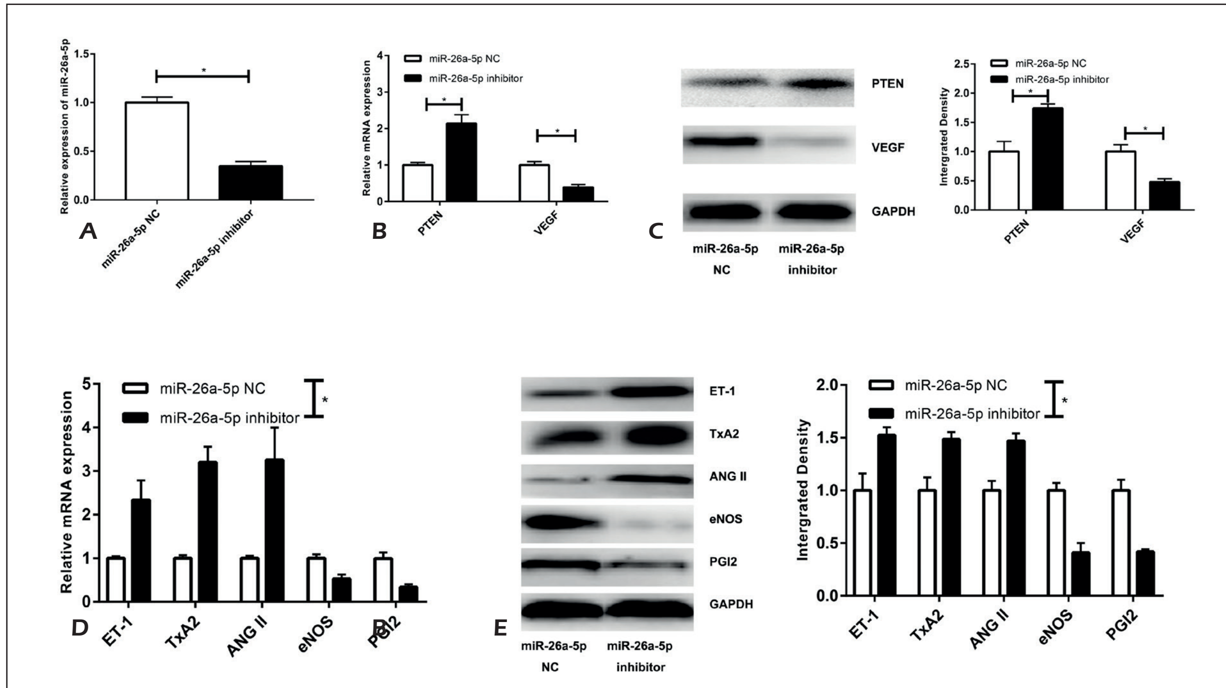

Figure 2. Silence of miR-26a-5p upregulated AS-related genes. **A**, Relative level of miR-26a-5p in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control. **B**, mRNA levels of PTEN and VEGF in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control. **C**, Protein levels of PTEN and VEGF in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control. **D**, mRNA levels of ET-1, TxA2, ANG II, eNOS, and PGI2 in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control. **E**, Protein levels of ET-1, TxA2, ANG II, eNOS, and PGI2 in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control.

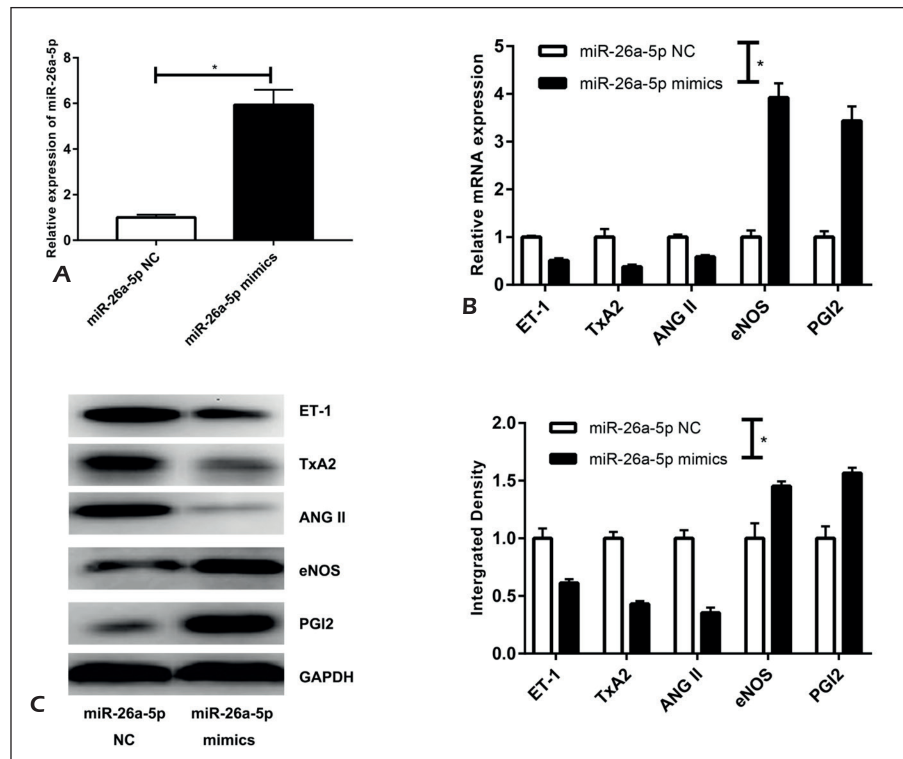

Figure 3. Overexpression of miR-26a-5p downregulated AS-related genes. **A**, Transfection efficacy of miR-26a-5p mimics in HUVECs. **B**, mRNA levels of ET-1, TxA2, ANG II, eNOS, and PGI2 in HUVECs transfected with miR-26a-5p NC or miR-26a-5p mimics. **C**, Protein levels of ET-1, TxA2, ANG II, eNOS, and PGI2 in HUVECs transfected with miR-26a-5p NC or miR-26a-5p mimics.

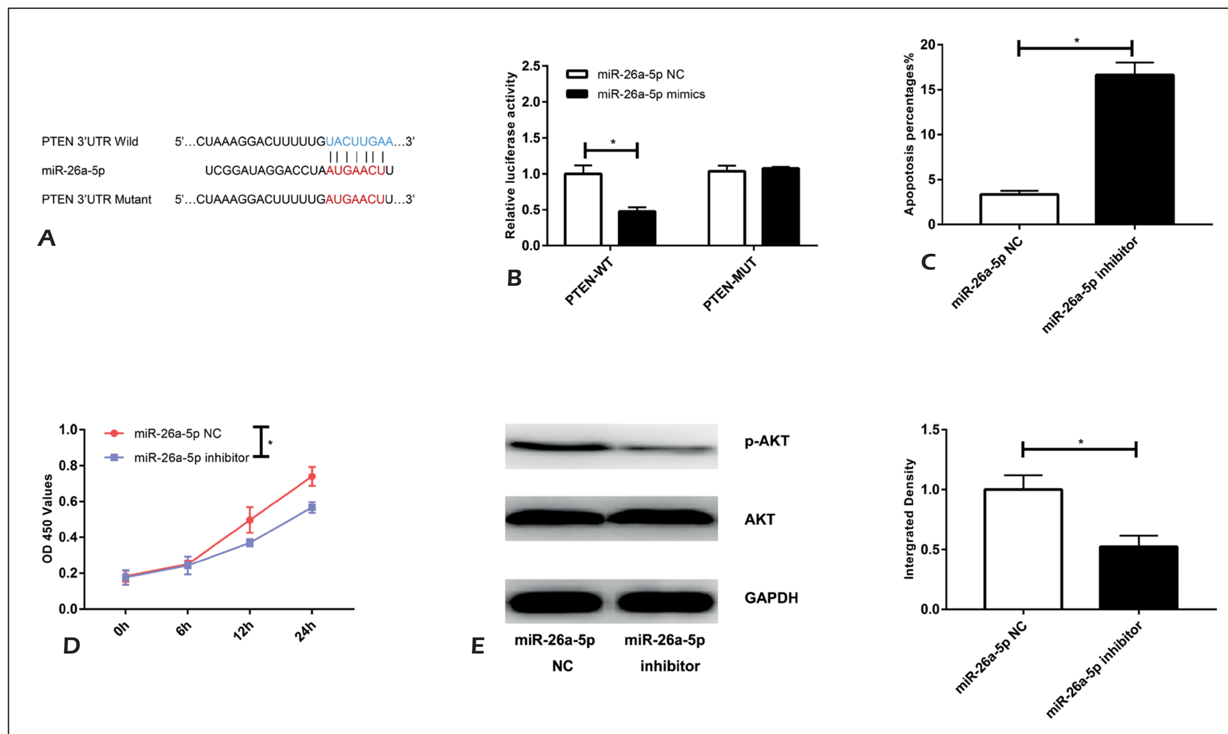

Figure 4. Silence of miR-26a-5p suppressed proliferation of endothelial cells through the PI3K/AKT pathway. **A**, Binding sequences between PTEN and miR-26a-5p. **B**, Luciferase activity in cells co-transfected with miR-26a-5p NC/miR-26a-5p mimics and PTEN-WT/PTEN-MUT. **C**, Apoptotic rate in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control. **D**, Viability in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control. **E**, Protein levels of p-AKT and AKT in endothelial cells isolated from CAD mice administrated with miR-26a-5p inhibitor or negative control.

bility of atherosclerotic plaques through targeting CXCL12. Silence of miR-10a stimulates endothelial inflammation by activating NF-κB[17]. MiR-34a has been verified to participate in the processes of cell damage and apoptosis, as well as dysregulation of endothelial cells. In endothelial progenitor cells isolated from coronary tissues of the elderly, miR-34a is remarkably upregulated[18]. SIRT1 is a histone deacetylase closely related to cellular behaviors. In endothelial cells, miR-34a maintains the normal function of cells, which also stimulates neovascularization after ischemia through targeting SIRT1[19]. Overexpression of miR-34a inhibits angiogenesis in vascular endothelial cells[20]. In this work, miRNA-26a-5p was significantly downregulated in CAD patients and endothelial cells isolated from CAD mice. AS-related genes were found significantly regulated by miRNA-26a-5p. Moreover, PTEN was verified as the downstream target of miRNA-26a-5p, whose expression level was negatively regulated by miRNA-26a-5p.

PTEN is a tumor-suppressor gene with dual phosphatase activities of lipid phosphatase and protein phosphatase. Located on chromosome 10q23.3, PTEN regulates the expressions of AKT and downstream genes by encoding phosphatases. PTEN exerts an anti-inflammatory effect through negatively mediating the PI3K/AKT pathway. In a hepatic ischemia-reperfusion model, silence of PDCD4 downregulates TLR4 and activates PI3K/AKT pathway, thus inhibiting NF-κB-induced inflammatory response[21]. Relevant studies have identified the exact role of PTEN in AS and cardiac hypertrophy. It alleviates inflammation by negatively mediating chemotaxis of neutrophils and reducing tissue damage[22]. In addition, PTEN maintains chromosomal integrity by targeting PI3K/AKT/GSK-3/GATA-6 pathway[23]. Previous investigations[24,25] have also pointed out that PTEN is capable of resisting AS through suppressing the migratory ability of vascular smooth muscle cells. As a vital gene in apoptosis pathway, p53 is directly regulated by PTEN[26]. MiRNA-21 regulates the proliferative and apoptotic rates of smooth muscle cells through PTEN and Bcl-2, and promotes intimal formation[27]. In this study, dual-lu-

ciferase reporter gene assay verified the binding relationship between miRNA-26a-5p and PTEN. Our findings suggested that miRNA-26a-5p positively regulated p-AKT level by targeting PTEN in endothelial cells.

VEGF is a classical activator of endothelial cells. It binds to the corresponding receptors on the membrane of endothelial cells, further enhancing cellular functions by targeting PI3K/AKT and MAPK/EKR1/2 pathways[28,29]. Meanwhile, VEGF enhances pro-angiogenic effect through mediating angiogenic factors, such as bFGF and TNF-α[30]. Our results proved that miRNA-26a-5p positively regulated VEGF expression in endothelial cells, eventually influencing cellular phenotypes of endothelial cells.

In this research, we only focused on the regulatory effects of miRNA-26a-5p on the proliferative and apoptotic abilities of endothelial cells. During the progression of CAD, inflammatory response and migratory ability of endothelial cells are of importance in maintaining the normal functions of endothelium, which are required for further explorations.

Conclusions

MiRNA-26a-5p influences the proliferative and apoptotic abilities of endothelial cells by targeting PTEN to activate PI3K/AKT pathway. Our findings suggest that miRNA-26a-5p may be utilized as a potential target for CAD treatment.

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Conflict of Interests

The Authors declared that they have no conflict of interests.

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