

MicroRNA-126/stromal cell-derived factor 1/C-X-C chemokine receptor type 7 signaling pathway promotes post-stroke angiogenesis of endothelial progenitor cell transplantation

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Abstract. Stroke is the most common cause of mortality worldwide. Post-stroke angiogenesis is of great significance to the treatment of strokes. The aim of the present study was to investigate the mechanism underlying the angiogenesis-promoting effect of microRNA-126 (miR-126)-associated signaling pathways using a stroke model *in vivo* and a cell migration model *in vitro*. Bone marrow-derived endothelial progenitor cells (EPCs) were extracted and identified using a density gradient method. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to examine the expression levels of miR-126 and C-X-C chemokine receptor type 7 (CXCR7). Target genes of miR-126 were analyzed using TargetScan software version 7.1 (www.targetscan.org/). In addition, a reporter gene assay and RT-qPCR were performed to determine the target genes of miR-126. The effect of miR-126 on cell migration was examined using a cell migration model *in vitro* and a middle cerebral artery occlusion model of mice was established *in vivo*. The miR-126 antagomir-treated EPCs were infused into stroke mice. Microvessel density, nerve function score and infarction volume were assessed. Flow cytometric analysis indicated that cluster of differentiation (CD)34, CD133 and vascular endothelial growth factor receptor 2 were partly expressed on the cell surface of bone marrow-derived EPCs. In addition, the expression levels of Di-acetylated-low density lipoprotein and *Ulex europaeus* agglutinin 1 were positive. Stromal cell-derived factor 1 (SDF-1) was identified as a target gene of miR-126, which was confirmed by a reporter gene assay and RT-qPCR. Cell migration examination demonstrated that the neutralizing antibody of CXCR7 blocked miR-126 antagomir-induced migration of EPCs. Microvessel density increased, while nerve function score and infarction volume

decreased following infusion of miR-126 antagomir-treated EPCs. Furthermore, miR-126 antagomir improved the efficacy of EPC treatment. Thus, miR-126 improved the migration of EPCs via the miR-126/SDF-1/CXCR7 signaling pathway.

Introduction

Stroke is the leading cause of mortality and permanent disabilities worldwide (1). Patients with ischemic brain injury suffer from strokes and the prognosis is poor. Thus, the current treatment of strokes requires further improvement.

Previous studies have demonstrated that decreased quantities of microvessels, reduced collateral circulation and damaged neovascularization were associated with the poor prognosis of stroke (2). As a type of adult stem cell, endothelial progenitor cells (EPCs) are pivotal in keeping the endothelium intact and for vascular homeostasis during the process of angiogenesis (3,4). These functions allow EPCs to engage in vascular repair. Increasing evidence indicates that the quantity of EPCs decreased in stroke patients (5-7). Furthermore, studies demonstrate that infusion of EPCs attenuated tissue injury, promoted angiogenesis and improved functional recovery of ischemia organs, such as the heart and brain (8,9). These findings provide the theoretical basis for treatment of EPCs.

microRNAs (miRNA) are a type of non-coding RNA (length, 20-25 bp) and >30% of human genes are under post-transcriptional regulation of miRNAs (10). The post-transcription regulatory effect of miRNAs provides a novel strategy for gene expression regulation and influences cellular events (11). A previous study indicated that miRNA is involved in angiogenesis and miR-126 modification enhances the function of EPCs, so as to improve therapeutic efficacy (12).

Thus, the present study evaluated the expression level and function of miR-126 in EPCs, and demonstrated the miR-126-associated mechanism using a cell migration model *in vitro* and a middle cerebral artery occlusion model *in vivo*.

Materials and methods

Extraction, culture and identification of EPCs. A total of 15 healthy male mice, aged 8-10 weeks and weighing 20-25 g were purchased from Shanghai SLAC laboratory Animal Co., Ltd., Shanghai, China. The mice were housed in a sterilized and

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specific pathogen free environment with a temperature $\sim 25^{\circ}\text{C}$, 12-h light/dark cycle and free access to sterilized food and water. All procedures involving the mice were approved by the Animal Ethics Committee of the Weifang People's Hospital.

As reported previously (13), EPCs were extracted from mouse bone marrow. Thighbone and shinbone were harvested from the leg of mice after removal of muscle from entire leg for the extraction of mononuclear cells using the density gradient method. Extracted cells (1×10^6) were cultured in 24-well plates with Endothelial Basal Medium-2 (EBM-2; Clonetics Corporation, San Diego, CA, USA) at 37°C under 5% CO_2 for 3 days. Non-adherent cells were discarded after 3 days of culture. Dil-acetylated-low density lipoprotein (Dil-Ac-LDL) and *Ulex europaeus* agglutinin 1 (UEA-1) immunofluorescent staining (at 37°C for 1 h) were performed under a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). Meanwhile, cluster of differentiation (CD)34, CD133 and vascular endothelial growth factor receptor 2 (VEGFR2) were also measured by flow cytometry (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) using FITC-conjugated anti-CD34 antibody, PE-conjugated anti-CD133 antibody and APC-conjugated anti-VEGFR2 antibody.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from bone marrow-derived EPCs using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Reverse transcription was performed using M-MLV First-Strand Synthesis reagent (Thermo Fisher Scientific, Inc.), and mRNA expression levels of stromal cell-derived factor 1 (SDF-1) and miR-126 were examined using the SYBR method (SYBR-Green Quantitative RT-qPCR kit; QR0100; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). qPCR was performed using an iCycler IQ[®] RT-PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR reaction was performed under the condition of 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 15 sec, and finally 72°C for 10 sec. The expression level of SDF-1 was assessed with GAPDH serving as the internal control, and miR-126 with U6 RNA as the internal control. The data was quantified using $2^{-\Delta\Delta\text{Ct}}$ method (14). The primer sequences are presented in Table I.

Cell transfection. Bone marrow-derived EPCs or bEnd3 cells (mouse brain endothelial cell line; American Type Culture Collection, Manassas, VA, USA) (5×10^4 cells) were cultured in 6-well plates. Scramble and angomir-126 were transfected into the cells using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. A 48-h culture was maintained at 37°C and then transfected cells collected by centrifugation at $125 \times g$ for 5 min at room temperature.

Cell migration experiment. The cell migration experiment was performed via Transwell assay. Transfected cells (2×10^4) were cultured in the upper compartment of a Transwell plate. SDF-1 (100 ng/ml) or C-X-C chemokine receptor type 7 (CXCR7; 100 ng/ml) neutralizing antibody (MAB7167; R&D Systems, Inc., Minneapolis, MN, USA) was added to the lower compartment of the Transwell plate. Cell abundance was assessed using a fluorescence microscope (magnification, $\times 40$;

5 randomly selected visual fields). Untransfected EPCs served as the control.

Western blot analysis. Western blotting was performed to examine the expression level of CXCR-7 in the cells. Cells were lysed with RIPA Lysis buffer (Thermo Fisher Scientific, Inc.) and protein was determined by Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Then, 10% SDS-PAGE gel electrophoresis was performed with $20 \mu\text{g}$ protein being loaded per lane, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes at a constant voltage setting of 25 V for 2 h. The PVDF membranes were blocked with skimmed milk (5%) and then incubated with CXCR7 antibody (PA3-069; 1:3,000; Thermo Fisher Scientific, Inc.) at 25°C for 2 h or GAPDH antibody (ab37168; 1:3,000; Abcam) followed by addition of HRP-conjugated goat anti-rabbit secondary antibody (65-6120; 1:5,000; Thermo Fisher Scientific, Inc.). GAPDH served as the internal control. Specific protein bands were examined by chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc.).

Reporter gene assay. EPCs were cultured in 96-well plates at 37°C for 3 days. Co-transfection was performed with 20 ng angomir-126, 5 pmol negative control and 100 ng pLUC SDF-1 3'-untranslated region (UTR) wild type (WT) or mutant after 24 h of culture. Transfection was performed with Lipofectamine 2000. The reporter gene assay was performed using a Luciferase assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Animal experiments. The animal experiments were approved by the Animal Ethics Committee of the Weifang People's Hospital (Shandong, China). The mice were randomly assigned to three groups (5 mice per group), including a control group, EPC group and an EPC + miR-126 group. Middle cerebral artery occlusion was performed according to a previously reported protocol (15). In the EPC and EPC + miR-126 groups, EPCs (2×10^5) were infused via intravenous injection into the tail after 2 h of occlusion. Isovolumetric phosphate-buffered saline (PBS) was infused in the control group mice.

Neurological scoring. Then, 5-mark scoring was performed according to previously reported protocol (16). Scoring was as follows: 0, normal neurological function; 1, bending of contralateral trunk and foreleg as the leg was lifted; 2, torsion of contralateral trunk as leg was lifted, while normal at resting-state; 3, torsion of contralateral trunk at rest; 4, loss of autonomic movement.

Examination of microvessel density. Mice were sacrificed by cervical dislocation on day 2 and 7 after transfection. As reported previously (5), Fluoro-Jade and CD31 staining were performed on cerebral sections. Microvessel density was assessed using ImageJ software version 1.44 (National Institutes of Health- Bethesda, MD, USA).

Statistical analysis. SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA) was used for data processing. Measurement data are presented as normal distribution to mean \pm standard deviation. Student's t-test and analysis of variance (one-way for

Table I. Primer sequences of the three genes.

Gene	Primer (5' to 3')	
	Forward	Reverse
Stromal cell-derived factor 1	GTGGTCGTGCTGGTCCTC	CACACTTGTCTGTTGTTGTTCTTC
GAPDH	ACTCCCACTCTTCCACCTTC	CACCACCCTGTTGCTGTAG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

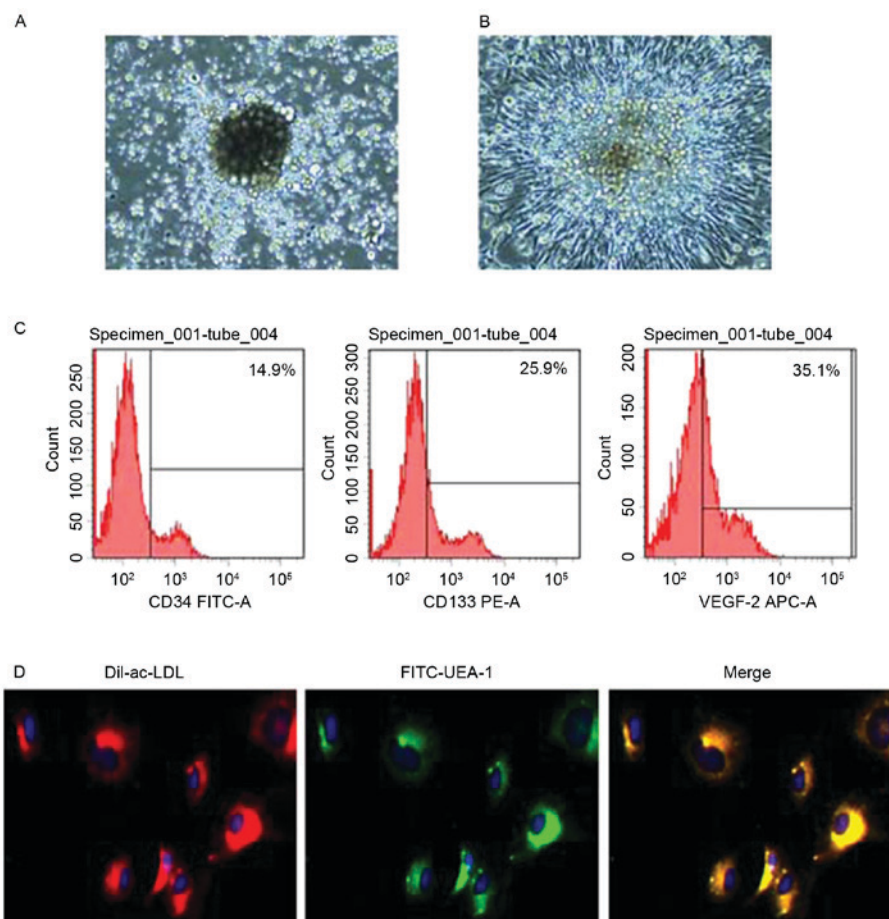


Figure 1. Identification of endothelial progenitor cells. Cellular morphology on days (A) 3 and (B) 7 of culturing under a phase contrast microscope (magnification, x40). EPCs were roughly circular and formed a cell colony at day 3 culture. EPCs were fusiform at day 7 culture. (C) Expression levels of CD34, CD133 and VEGFR2. (D) Expression levels of Dil-ac-LDL and UEA-1 (magnification, x40). Positive expressions of Dil-ac-LDL and UEA-1 in cultured EPCs, indicating the cultured cells were EPCs. CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; VEGFR2, vascular endothelial growth factor receptor 2; APC, allophycocyanin; Ac-LDL, acetylated-low density lipoprotein; UEA-1, *Ulex europaeus* agglutinin 1.

comparison among different groups, two-way for comparison among groups at different time points) were performed to establish the statistical significance and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of EPCs. As presented in Fig. 1A and B, EPCs were roughly circular and formed a cell colony following 3 days of culture. EPCs were fusiform after 7 days of culture. Flow cytometry indicated that CD34, CD133 and VEGFR2 were expressed in EPCs (Fig. 1C). Immunofluorescent staining

indicated that Dil-ac-LDL and UEA-1 were expressed in EPCs (Fig. 1D).

miR-126 and CXCR7 are expressed in EPCs. Western blot analysis demonstrated CXCR7 protein expression in EPCs. In addition, miR-126 and CXCR7 mRNA expression was observed in EPCs, as verified by qPCR (Fig. 2).

miR-126 regulates SDF-1 in EPCs. TargetScan software demonstrated that the 3'UTR region of SDF-1 was complementary base paired with the sequence of miR-126, indicating that SDF-1 is a target gene of miR-126 (Fig. 3A). The association between

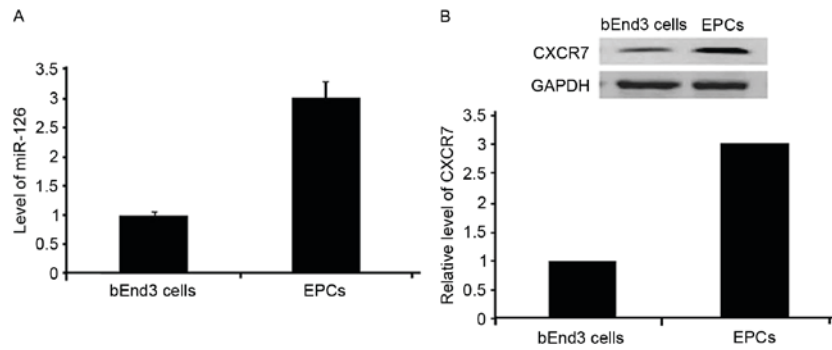


Figure 2. (A) miR-126 and (B) CXCR7 expression levels in EPCs. EPCs, endothelial progenitor cell; miR, microRNA; CXCR7, C-X-C chemokine receptor type 7. bEnd3, mouse brain endothelial cell line.

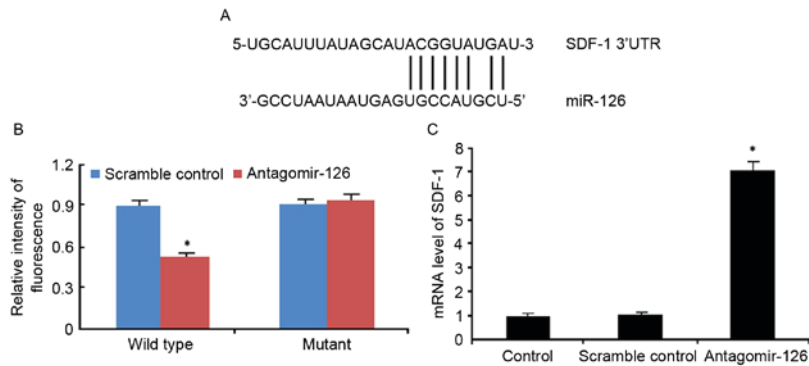


Figure 3. miR-126 regulated SDF-1 expression levels in endothelial progenitor cells. (A) Data from TargetScan software. (B) Fluorescence intensity in different groups. (C) Reverse transcription-quantitative polymerase chain reaction in the different groups. * $P < 0.05$ vs. scramble control. miR, microRNA; SDF-1, stromal cell-derived factor 1; UTR, untranslated region.

SDF-1 and miR-126 was determined using a reporter gene assay and RT-qPCR. The fluorescence intensity was decreased by 45% following transfection of antagomir-126 into the WT, while no difference was observed in the mutant (Fig. 3B). The miR-126 angomir significantly reduced the expression level of SDF-1, which was verified by RT-qPCR (Fig. 3C).

CXCR-7 inhibition abrogates miR-126 angomir-induced migration of EPCs. The Transwell assay demonstrated that inhibition of CXCR7 repressed migration of the miR-126 angomir-transfected cells, indicating that CXCR7 was involved in miR-126-induced migration of EPCs (Fig. 4).

miR-126 angomir improves the efficacy of EPC treatment in mice with middle cerebral artery occlusion. In the animal experiments, miR-126 angomir-transfected EPCs demonstrated significantly increased microvessel densities at 2 and 7 days after middle cerebral artery occlusion (Fig. 5A and B). In addition, miR-126 angomir-transfected EPCs improved the prognosis of neurological function, as verified by increased neurological scores and decreased infarct volumes (Fig. 6).

Discussion

Antagomir, as a group of oligonucleotides, is a type of chemically engineered single-strand mRNA inhibitor that efficiently blocks miRNA regulation of target gene expression. The antisense strand consists of two phosphorothioates at the 5' end,

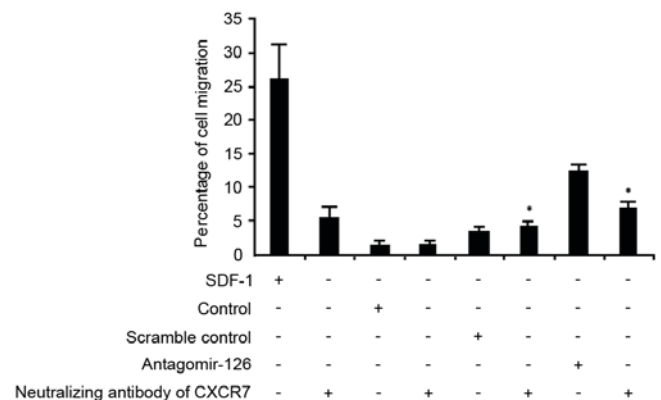


Figure 4. CXCR-7 is involved in miR-126-induced migration of endothelial progenitor cells. * $P < 0.05$ vs. control. CXCR7, C-X-C chemokine receptor type 7; miR, microRNA; SDF-1, stromal cell-derived factor 1.

four phosphorothioates, four cholesterol groups at the 3' end, with 2'-methoxy modification (17). Previous studies identified that miR-126 influenced angiogenesis in gene knock-out mice models or angomir treatment models, indicating that miR-126 may have attenuated structural damage to vessels (18-20). The present study demonstrated that miR-126 angomir contributed to EPC treatment.

SDF-1, also termed CXCL12, has been implicated in various types of disease, including numerous cancer types (21) and coronary artery disease (22). SDF-1 exerts a significant

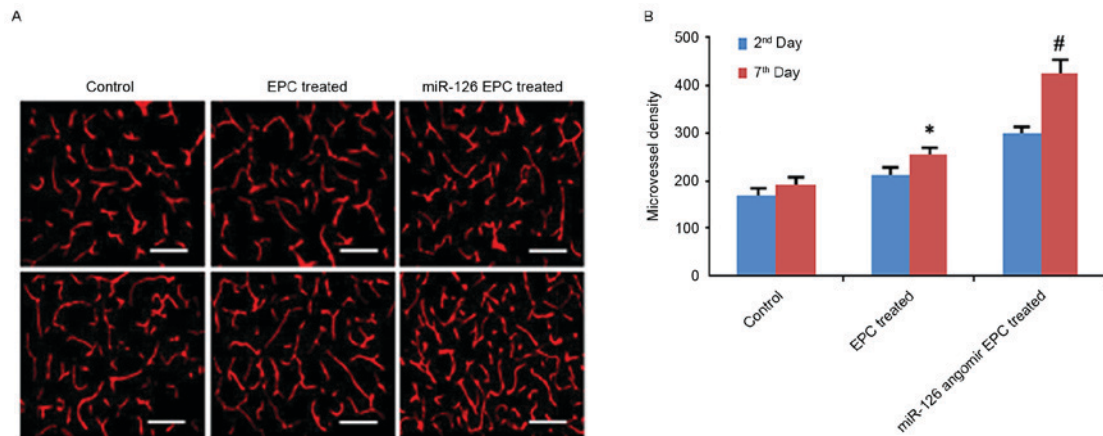


Figure 5. miR-126 transfected EPCs significantly increased microvessel density. (A) Immunofluorescence examination of microvessel density. Scale bar=50 μ m. (B) Analysis of microvessel density in the different groups. *P<0.05 vs. control. #P<0.01 vs. EPC treated. miR, microRNA; EPCs, endothelial progenitor cells.

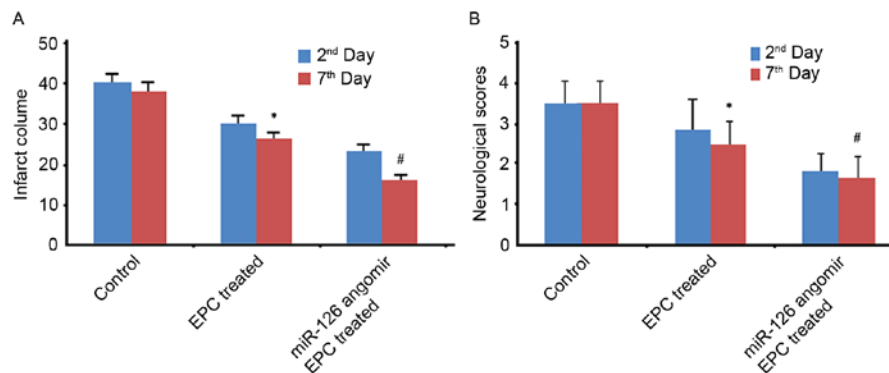


Figure 6. Analysis of (A) decreased infarct volume and (B) neurological score in the different groups. *P<0.05 vs. control. #P<0.01 vs. EPC treated. EPCs, endothelial progenitor cells; miR, microRNA.

regulatory role in various processes, such as morphogenesis, angiogenesis and immune responses, and is considered to be a potential target for drug development (23). Furthermore, CXCR7, also termed atypical chemokine receptor 3 or G-protein coupled receptor 159, is a high affinity receptor of CXCL12, and has recently been identified to be involved in the growth, migration, chemotaxis, adhesion and spreading of tumors (24). The current study elucidated that miR-126 exerted a protective effect of EPCs during angiogenesis via the SDF-1/CXCR7 signaling pathway. In addition, the neutralizing antibody of CXCR7 blocked the migration-induced effect of the miR-126 angomir on EPCs *in vitro*. Furthermore, infusion of the miR-126 angomir-treated EPCs was more effective than routine EPC treatment, which improved neurological scores, increased microvessel density and decreased infarct volume.

Interactions between miR-126 and SDF-1 has been demonstrated to increase elimination of endothelial apoptosis bodies induced by miR-126 (25). A potential mechanism is that miR-126 enhances the expression level of SDF-1 via inhibiting regulators of G protein signalling (25). The present study confirmed that silencing of miR-126 was associated with changes of SDF-1 expression levels, indicating miR-126 as a potential biomarker for clinical treatment. miRNAs have been associated with the occurrence and progression

of diseases (26). Furthermore, circulating miRNAs influence tumorigenesis (27) and cardiovascular diseases (28,29). Endothelial injury is a significant risk factor for patients with cardiovascular diseases. miRNAs secreted by EPCs have been demonstrated as cardiovascular disease markers in the clinical setting (30). Clinical trials have indicated that miR-126 expression levels decreased in acute coronary syndrome patients and diabetes patients (31,32). Thus, miR-126-associated signaling pathways have specific signals for EPCs, and selective activation of miR-126 was significant during EPC treatment.

In addition, miR-126 inhibits the expression of SDF-1 and stimulates angiogenesis when vessels are injured. Furthermore, downregulation of miR-126 inhibits angiogenesis in aging EPCs or EPCs with function disorders. The current study indicated that miR-126 improved the function of EPCs and angiogenesis via the downregulation of SDF-1.

In conclusion, miR-126 indeed improved migration of EPCs via the miR-126/SDF-1/CXCR7 signaling pathway. Furthermore, the miR-126 angomir improved efficacy of EPC treatment, and may present as a potential therapeutic target for cardiovascular diseases. The main study limitation is that the EPC used in the present study was from mouse, not human, whether this effect also exists in human EPCs remains unclear and requires further investigation.

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