Deep vein thrombosis (DVT) is a major health problem worldwide provoking abnormal coagulation of blood in deep veins, which is associated with high mortality and disability. Risk factors for DVT mainly include general anesthesia, caesarean section, estrogen therapy, pregnancy or puerperium, leg injuries, active cancer, inflammatory bowel disease, as well as systemic lupus erythematosus. A combination of blood stasis, plasma hypercoagulability, and endothelial dysfunction is also an important cause of thrombosis. The diagnosis of DVT is extremely challenging, requiring a high degree of vigilance, and anticoagulant therapy remains the current first choice treatment. Surgical thrombolysis is another option for DVT treatment, although it remains controversial. MicroRNAs (miRNAs) are endogenous small noncoding RNAs that play important regulatory roles in cells, with well-established functional roles in vascular disease shown in the previous literature. The miRNA-based therapy is a promising therapeutic strategy for many vascular diseases. For instance, miR-145 has been reported to prevent thrombosis by binding to 3’ untranslated region (UTR) of tissue factor involved in venous thrombosis, indicating a possible
therapeutic target of miR-145 in thrombosis with involvement of its target gene tissue factor. Furthermore, miRNA-296-5p (miR-296-5p) plays established roles in inhibition of vascular smooth muscle cell apoptosis in diabetes.\textsuperscript{10} Of note, miR-296 expression is implicated by the activities of primary human brain microvascular endothelial cells and the function of angiogenic growth factors.\textsuperscript{11} Interestingly, exosomes can transport and deliver large amounts of proteins, lipids, and nucleic acids.\textsuperscript{12} A previous study by Beltrami \textit{et al.} demonstrated that pericardial fluid exosomes could coordinate vascular repair through miRNA transfer.\textsuperscript{13} Therefore, we hypothesized that exosomal miR-296-5p might be involved in the behaviors of other endothelial cells and vascular diseases.

Serum response factor (SRF) is a transcription factor activated by a variety of extracellular stimuli that inhibits cell type-specific genes and promotes cell reprogramming to pluripotency.\textsuperscript{14} A previous study has revealed that the overexpression of miR-9 and miR-200 in cultured oligodendrocyte precursor cells contributes to decreased SRF expression and inhibited oligodendrocyte precursor cells differentiation.\textsuperscript{15} Besides, it has been reported that SRF could be targeted by miR-200c.\textsuperscript{16} Endothelial progenitor cells (EPCs), the precursor cells of vascular endothelial cells, also play important roles in various vascular diseases.\textsuperscript{17} More specifically, SRF has been reported to be involved in migration and angiogenesis of endothelial cells.\textsuperscript{18}

The present study explored potential functional roles of exosomal miR-296-5p in deep venous thrombosis by targeting SRF. We also substantiated the effects of exosomal miR-296-5p derived from EPCs in promoting thrombosis and recanalization of deep venous thrombosis, thus providing evidence that exosomal miR-296-5p presents a potential therapeutic target for deep venous thrombosis treatment.

**MATERIALS AND METHODS**

**Ethics statement**

All experimental procedures involving animals were made to minimize suffering and were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Chongqing Medical University.

**Isolation and identification of EPCs**

EPCs were isolated from male C57/BL6 mice aged 4–6 weeks. Bone marrow mononuclear cells were collected from the hips, vertebrae, and sternum, then separated and comminuted. The bone marrow mononuclear cells were further subjected to gradient centrifugation, and cultured with Endothelial Cell Basal Medium-2 (Lonza Group, Basel, Switzerland) supplemented with Endothelial Cell Basal Medium-2 MV Single Quots (Lonza Group), including vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin growth factor, ascorbic acid, hydrocortisone, gentamicin, amphotericin-B, and 10% fetal bovine serum (FBS) in a 5% CO\textsubscript{2} incubator. The adherent cells were removed after the first medium exchange for 48 hours, and the adherent cells were further cultured with fresh EBM-2 MV. The cells were serially passaged.

Next, 5 \mu g/mL of Dil-Ac-LDL solution (Biomedical Technologies, Stoughton, MA) was added and cultured in the dark at 37°C for 4 hours. The cells were fixed using 4% paraformaldehyde and stained with 1 mg/mL 4’,6-diamidino-2-phenylindole (Invitrogen, Waltham, MA) for 15 minutes. The endocytosis of Dil-Ac-LDL was observed using a fluorescence microscopy (Olympus, Tokyo, Japan), and cells were identified by flow cytometry analysis. Cells were incubated with antibodies against CD34, CD31, VE-cadherin, FLK-1, and CD11b. After washing with 3% FBS/phosphate-buffered saline (PBS), cell surface marker expression was quantitatively determined by fluorescence-activated cell sorting using the Guava easyCyte 8 system (EMD Millipore, Burlington, MA).

**β-galactosidase assay**

The aging of EPCs was determined by counting the proportion of senescence-associated β-galactosidase (SA-β-gal) positive cells. SA-β-gal was stained using a β-galactosidase staining kit (Beyotime Biotechnology, Shanghai, China). The first and third-generation EPCs were used to perform this assay, and number of SA-β-gal-positive cells was determined by counting blue cells among a total of at least 500 cells per field of view.

**Cell treatment**

The above-mentioned EPCs in logarithmic growth phase were seeded into the 6-well plate 24 hours before transfection. EPCs were then transfected with negative control (NC) inhibitor, miR-296-5p inhibitor, NC mimic, miR-296-5p mimic, pcDNA3.1, pcDNA3.1-MRTF-A (MRTF-A overexpression plasmid), or pcDNA3.1-p300 (p300 overexpression plasmid) using Lipofectamine 2000 (11668019; Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s instructions.

**Isolation and identification of exosomes**

EPCs were incubated with EGM-2MV medium without FBS, but supplemented with 1 × serum replacement solution (PeproTech, Rocky Hill, NJ) for 24 hours. The supernatant was filtered through 0.22 \mu m Steritop filter (Millipore, Billerica, MA) to remove residual dead cells and debris.

Then, the supernatant was centrifuged at 4,000 g to 200 \mu L at 4°C, followed by ultrafiltration using 15 mL Amicon Ultra-15 centrifugal filter device (Millipore). The ultrafiltrate was then washed twice with PBS and ultra-filtered again to a volume of 200 \mu L. To purify the exosomes, the liquid was placed in a sterile ultra-clear tube (Beckman Coulter, Miami, FL) in 30% sucrose-D\textsubscript{2}O buffer, followed by centrifugation at 100,000 g at 4°C for 1 hour to induce pelleting of the exosomes. Finally, the collected EPCs-Exo was stored at \textasciitilde –80°C for later use.

EPC-derived exosomes were characterized by Western blot analysis, transmission electron microscopy, and nanoparticle tracking analysis. Western blot analysis was performed using antibodies against CD63, CD9, TSG101, and Calnexin (Abcam, Cambridge, UK) to determine the specific markers for exosomes. The purified exosomes were fixed with 3% glutaraldehyde solution for 0.5 hours, followed by addition of 20 mL of exosomes to the copper
grid, and stained with 1% phosphotungstic acid for 5 minutes to conduct transmission electron microscopy. Finally, the exosomes were observed using a Zeiss Libra 120 (Zeiss, Oberkochen, Germany) electron microscope. Nanoparticle tracking analysis was performed using ZetaView PMX 120 (Particle Metrix, Dusseldorf, Germany) and its corresponding software (ZetaView).

Exosomes that were isolated from the transfected EPCs with miR-296-5p mimic, miR-296-5p inhibitor, and miR-296-5p inhibitor + sh-SRF, namely, Exo-NC mimic, Exo-miR-296-5p mimic, Exo-NC inhibitor, Exo-miR-296-5p inhibitor, and Exo-miR-296-5p inhibitor + sh-SRF, were resuspended with exosome-free PBS. Quantitative polymerase chain reaction (qPCR) was performed to measure miR-296-5p expression.

Dual-luciferase reporter gene assay
The target site sequence (WT) of the 3'-UTR region of miR-296-5p and SRF mRNA, as well as the sequence (MUT) after site-directed mutagenesis of the WT target site were synthesized and inserted into the pmiR-RB-REPORT vector (all commissioned by Guangzhou RiboBio, Guangzhou, China), whereas the empty plasmid was transfected as a control. The correct luciferase reporter plasmids WT and MUT were co-transfected into HEK293T cells with NC mimic or miR-296-5p mimic, respectively. After 48 hours of transfection, the cells were collected and lysed, followed by centrifugation for 3–5 minutes with the supernatant collected. The relative light unit was determined using the Renilla luciferase assay kit (YDJ2714, Shanghai Yuduo Biotechnology, Shanghai, China), with firefly luciferase serving as internal reference in the dual-luciferase reporter assay system (Promega, Madison, WI). The relative fluorescence values were analyzed by the Renilla luciferase kit and the R relative light unit value was obtained from the firefly luciferase measurement.

Chromatin immunoprecipitation
The binding of MRTF-A to the VE-cadherin promoter was investigated using the chromatin immunoprecipitation kit (Merck Millipore, Billerica, MA). EPCs were fixed with 1% formaldehyde for 10 minutes when cells reached about 70–80% confluence, and the DNA and protein were then cross-linked and randomly sheared by ultrasonic treatment. The cells were then centrifuged at 6540.3 g at 4°C. The supernatant was then collected and separated into three tubes, positive control antibody RNA polymerase II (INPUT), negative control antibody, normal human pre-immune serum IgG, and protein-specific antibody histone H3K9 (Abcam, Cambridge, UK, ab4441, anti-rabbit), H3K14 (Abcam, ab52946, anti-rabbit), and H4 (Abcam, ab9051, anti-rabbit) were added into the tubes, respectively, followed by incubation overnight at 4°C. The endogenous DNA-protein complexes were precipitated using protein agarose/sepharose, and then de-crosslinked at 65°C overnight. The DNA fragments were then purified by phenol/chloroform extraction, and the binding of SRF to VE-cadherin-pGL3 promoter region was assessed by using VE-cadherin promoter-specific primers.

Transwell assay
Cells (100 μL, 1 × 10^5 cells/mL) were seeded into the apical chamber of the Transwell chamber coated with Matrigel. Subsequently, 500 μL of 10% FBS medium was added to the basolateral chamber and then placed in a 37°C, 5% CO₂ cell incubator for 36 hours. The upper cells of the Transwell chamber were washed off using a cotton swab, and the remaining cells were fixed and stained with 1% crystal violet solution. Under the light microscope, four high power fields were randomly selected for cell counting.

Microtubule formation assay
EPCs in the logarithmic growth phase were digested, resuspended with EGM-2MV, and adjusted to a cell density of 4 × 10^4 cells/mL. Next, 500 μL of EPC suspension (2 × 10^4 cells/mL) were added to each well that had been coated with Matrigel (200 μL) and placed in a 37°C incubator. After 24 hours of incubation, the EPCs were observed under an inverted phase-contrast microscope, and the sum of the number of lumens was recorded in three randomly selected fields.

Mouse model of DVT
Totally, 30 male Kunming mice (aged 4–6 weeks, weighing 20–32 g) were used to establish a DVT model. The mice were anesthetized with 3% sodium pentobarbital (1 mL/kg), routinely sterilized, and a 2.0 cm incision was made along the longitudinal axis of the midline of the abdomen to separate the subcutaneous tissue and abdominal cavity. After exposing and separating the inferior vena cava, the visible side branches were ligated. A neurovascular clamp was clamped to the proximal and distal ends of about 1.0 cm for 30 seconds. A 5–0 polypropylene suture was placed along the longitudinal axis of the inferior vena cava, and the middle segment of the clamp segment was ligated with a 4-0 silk thread along with the venous wall and polypropylene suture. The polypropylene suture was then withdrawn to create a narrow lumen. No antibiotics were applied after surgery and the mice were given free access to food. A total of 25 mice survived and 5 died after the operation. The success rate of modeling was 83.3%. The modeled mice were classified into three groups (n = 8; with one backup). Exosomes isolated from EPCs transfected with NC antagonist or miR-296-5p antagonist, namely the Exo-NC antagonist and Exo-miR-296-5p antagonist, were injected into the mice. Eight mice in each group were euthanized by an overdose of anesthesia, and the vein wall and embolus were removed for analysis.

Hematoxylin-eosin staining
The thorbus tissue was fixed, dehydrated, embedded in paraffin using an embedding machine (SPCC-6D, Dongguan Spectrum Standard Experimental Equipment Technology, Dongguan, Guangdong, China), and cut into 2–4 μm-thick slices. After routine dewaxing and hydration, the slices were stained with hematoxylin for 5 minutes, differentiated using 1% hydrochloric acid alcohol, returned blue by 5% ammonia water, and stained with 0.5% eosin for 1 minute. Next, the slices were dehydrated, mounted, and the histopathological changes were observed under a light microscope.
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Immunofluorescence assay
Paraffin sections were routinely dehydrated, blocked with goat serum, and allowed to stand for 30 minutes. The cells were then incubated with primary anti-CD31 (1: 50, ab28364, Abcam) and allowed to stand overnight at 4°C. FITC-goat anti-rabbit secondary antibody (1: 50, Dako, Carpinteria, CA, USA) was added and incubated in the dark for 1 hour. The cells were then stained with 4',6-diamidino-2-phenylindole in the dark for 15 minutes. The cells were washed three times with PBS, followed by the addition of fluorescent, and sealed. The sample without primary antibody was used as negative control. Photographs were taken under a fluorescence microscope. The number of CD31-positive expressing cells was counted and the average value was taken. Red fluorescence indicates positive expression of CD31.

Real-Time-qPCR
Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). The miRNA was reversely transcribed using a reverse transcription kit (RR047A; Takara, Tokyo, Japan), whereas miRNA was reversely transcribed using a miRNA First Strand complementary DNA Synthesis (Tailing Reaction) kit (BS32451-0020; Shanghai Sangon Biotechnology, Shanghai, China). The sample was loaded using a SYBR Premix Ex TaqTM II (Perfect Real Time) kit (DRR081; Takara, Tokyo, Japan), and the sample was subjected to the real-time qPCR (RT-qPCR) reaction in a real-time PCR machine (ABI 7500; ABI, Foster City, CA). The primer of the U6 was provided by miRNA First Strand complementary DNA Synthesis (Shanghai Sangon Biotechnology, Shanghai, China; Table 1). The fold changes were calculated by means of quantitative (2-ΔΔCt method).

Western blot analysis
The total protein of the cells was isolated using a cell protein extraction kit (BC3640, Beijing Solarbio Technology, Beijing, China). The protein sample was mixed with 10% SDS gel loading buffer, boiled at 100°C for 10 minutes, ice-bathed, centrifuged, and loaded to each lane by micro-applicator electrophoresis. The proteins on the gel were transferred to a nitrocellulose membrane and blocked at 4°C overnight. The polyclonal primary antibody was used as negative control. Photographs were taken under a fluorescence microscope. The number of CD31-positive expressing cells was counted and the average value was taken. Red fluorescence indicates positive expression of CD31.

Table 1 Primer sequences for reverse transcription quantitative polymerase chain reaction

| Gene             | Sequence                  |
|------------------|---------------------------|
| VE-cadherin      | F: 5'-CAGCCTTTGGGAGGCTTCC-3' |
|                  | R: 5'-GGGGGACGCGAGTTTTCTT-3' |
| miR-296-5p       | F: 5'-TGCGTACTCAGGGTGG-3'  |
|                  | R: 5'-CTCACTCTCCGTGACAG-3'  |
| U6               | F: 5'-GGGGGACGCGAGTTTTCTT-3' |
|                  | R: 5'-GGGGGACGCGAGTTTTCTT-3' |
| GAPDH            | F: 5'-AATTTGGAAGGACTTCTGAT-3' |
|                  | R: 5'-CTCTCTCCTGGGCTG-3'    |

Statistical analysis
Statistical analysis was performed using SPSS 21.0 software (IBM, Armonk, NY). The measurement data were expressed as mean ± SD. Data comparison between two groups was analyzed by unpaired t-test, and data comparison among multiple groups were analyzed by one-way analysis of variance followed by a post hoc test for pairwise comparison. A value of P < 0.05 indicated statistical significance.

RESULTS
Identification of EPCs
The primary cultured EPCs were observed under the microscope and recorded. The cells on the third day after inoculation were observed, where the spindle-shaped adherent cells were visible at the bottom of the culture flasks. On the seventh day, the cells were fused and gradually changed from spindle shape into an oval or diamond shape. The cells were closely connected to form cell colonies, where the cells resembled paving stones (Figure 1a). On day 7, Dil-Ac-LDL uptake experiments were performed on the cells to determine the characteristics of EPCs, showing uptake of Dil-Ac-LDL specifically by EPCs (Figure 1b). Flow cytometry showed that CD34, CD31, and VE-cadherin were positive, but negative for FLK-1 and CD11b. Furthermore, most of the mononuclear cells cultured in vitro were EPCs (Figure 1c). Considering that the aging induced by in vitro culture may alter cell function and paracrine effects, senescence-associated β-galactosidase assay was performed to determine senescence of EPCs in different passages. The senescent cells were blue, and there was no significant difference in senescence ratio of EPCs in the first and third generations (Figure 1d,e).

Isolation and identification of EPCs exosomes
Electron microscopy analysis revealed that the exosomes showed typical cup-shaped morphology (Figure 2a) with a size of 80–150 nm and peaked at 98 nm (Figure 2b). The protein expression of exosome surface marker proteins CD63, CD9, TSG101, and calnexin measured by Western blot analysis showed CD9, CD63, and TSG101 was expressed in the EPCs-Exo, whereas calnexin showed no
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expression (Figure 2c). The miR-296-5p expression was increased in the exosomes (Figure 2d). The above data suggested that the exosomes were successfully isolated and miR-296-5p was highly expressed in the exosomes.

SRF was a target of miR-296-5p, whereas miR-296-5p negatively regulates SRF expression
Prediction results obtained from RAID version 2.0 (http://www.rna-society.org/raid/index.html) demonstrated that
there were binding sites between miR-296-5p and SRF, and that SRF was the target gene of miR-296-5p (Figure 3a). The results of luciferase reporter assay further verified that SRF was a target of miR-296-5p (Figure 3b). Besides, luciferase activity of SRF WT 3‘UTR was inhibited in cells transfected with miR-296-5p when compared with that in cells transfected with NC (P < 0.05), whereas the luciferase activity of MUT 3‘UTR had no significant difference (P > 0.05). The protein expression of SRF in EPCs was confirmed by Western blot analysis (Figure 3c,d). The results demonstrated that the protein expression of SRF in cells transfected with miR-296-5p mimic was significantly decreased (P < 0.05), whereas the protein expression of SRF in cells transfected with miR-296-5p inhibitor was increased (P > 0.05). Therefore, SRF was the target of miR-296-5p, whereas miR-296-5p could negatively regulate SRF expression.

SRF/MRTF-A activation is essential for maintenance of cerebral small vessel integrity. Indeed, MRTF-A is a key regulator of activation of VE-cadherin transcription in endothelial cells. The histone acetyltransferase p300 promotes MRTF-A-mediated transactivation of the VE-cadherin gene in endothelial cells. To test whether miR-296-5p had an effect on SRF/MRTF-A expression, MRTF-A and VE-cadherin expression in cells treated with Exo-NC mimic, Exo-miR-296-5p mimic, Exo-NC inhibitor, and Exo-miR-296-5p inhibitor was determined by Western blot analysis. Results showed that silencing of miR-296-5p resulted in elevated MRTF-A and VE-cadherin expression (Figure 3c,d). The binding of MRTF-A to VE-cadherin promoter assessed using the immunoprecipitation assay showed that, compared with the cells transfected with pcDNA3.1 plasmid, more VE-cadherin promoter was coprecipitated in cells transfected with MRTF-A (Figure 3e). These results suggested that silencing of miR-296-5p could promote the binding of MRTF-A to VE-cadherin promoter and elevate VE-cadherin expression (Figure 3f).

To determine whether p300 increases the protein transactivation of MRTF-A-dependent VE-cadherin, MRTF-A, p300, and VE-cadherin expression in cells transfected with pcDNA, MRTF-A, p300, and MRTF-A + p300 were assessed using Western blot analysis and co-immunoprecipitation assay. It was found that protein level of VE-cadherin in the cells individually transfected with MRTF-A or p300 was increased compared with the untransfected cells, whereas the elevation of VE-cadherin protein levels was more pronounced in the cells transfected with MRTF-A + p300 than in the cells individually transfected (Figure 3g,h). Compared with the pcDNA3.1 group, acetylation degree of histones H3K9, H3K14, and H4 was significantly elevated in MRTF-A and p300 groups. When MRTF-A and p300 were co-expressed, synergistic effects were observed. The results of co-immunoprecipitation showed that MRTF-A and p300 synergistically increased the transcription and expression of VE-cadherin by elevating the acetylation levels of the specific histones H3K9, H3K14, and H4 (Figure 3i). These results suggested that MRTF-A and p300 could synergistically increase the transcription and expression of VE-cadherin.

**Exosomes from EPCs transfected with miR-296-5p inhibitor increased migration and angiogenic capacity of EPCs in vitro**

Results from RT-qPCR showed that miR-296-5p expression was increased in Exo-miR-296-5p mimic, whereas SRF expression was decreased compared with NC mimic. The opposite trend was observed when compared with Exo-NC inhibitor. Furthermore, miR-296-5p expression was decreased in response to treatment with Exo-miR-296-5p inhibitor and Exo-miR-296-5p inhibitor + sh-SRF.

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**Figure 3** Silencing of miR-296-5p promotes the activation of serum response factor (SRF)/myocardin related transcription factor A (MRTF-A), thereby increasing VE-cadherin expression by acetylation. (a) The predicted binding site of miR-296-5p on the 3’ untranslated region of SRF. (b) HEK293 cell viability assessment using luciferase assay. (c, d) SRF, MRTF-A, and VE-cadherin expression in endothelial progenitor cells. (e, f) The results of chromatin immunoprecipitation analysis of MRTF-A and VE-cadherin promoter regions. (g, h) MRTF-A, p300, and VE-cadherin protein expression. (i) The level of histones H3K9, H3K14, and H4 measured using immunoprecipitation assay. The measurement data were expressed as mean ± SD. Data comparison between two groups was analyzed by unpaired t test. Data among multiple groups were analyzed by one-way analysis of variance followed by a post hoc test for pairwise comparison. *Comparison between groups, P < 0.05. The experiment was repeated three times. NC, negative control.
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Figure 4. Exosomes from cells transfected with silenced miR-296-5p resulted in increased migration in vitro and angiogenic capacity of endothelial progenitor cells. (a) Real time quantitative polymerase chain reaction was used to measure the expression of miR-296-5p and serum response factor (SRF). (b) The migration ability of endothelial progenitor cells was confirmed by Transwell assay (200×). (c) Histogram of cell migration ability. (d) Results of tube formation assay (200×). (e) Histogram results of tube formation assay. The measurement data were expressed as mean ± SD. Data among multiple groups were analyzed by one-way analysis of variance followed by a post hoc test for pairwise comparison. *Comparison between groups, \( P < 0.05 \). The experiment was repeated three times. NC, negative control.

and SRF expression was increased in response to Exo-miR-296-5p inhibitor treatment, whereas SRF expression in Exo-miR-296-5p inhibitor + sh-SRF-treated cells showed no significant difference (Figure 4a).

The migration ability of EPCs was determined by the Transwell assay. The results showed that the number of migrated cells treated with Exo-miR-296-5p mimic was reduced compared with Exo-NC mimic treatment. However, when compared with Exo-NC inhibitor-treated cells, the number of migrated cells treated with Exo-miR-296-5p inhibitor was increased, whereas there was no significant difference in the number of migrated cells treated with Exo-miR-296-5p inhibitor + sh-SRF (Figure 4b,c).

Angiogenesis ability was assessed by cell microtubule formation assay, which showed reduced tube formation ability of Exo-miR-296-5p mimic-treated cells compared with Exo-NC mimic-treated cells. In addition, when compared with Exo-NC inhibitor treatment, tube formation ability following Exo-miR-296-5p inhibitor treatment was notably increased, whereas there was no significant difference in Exo-miR-296-5p inhibitor + sh-SRF-treated cells (Figure 4d,e).

Suppression of exosomal miR-296-5p expression enhances thrombocyte tissue thrombolysis and recanalization
Hematoxylin-eosin staining revealed more nucleated cells and distinct channels in mice injected with Exo-miR-296-5p antagonim, with a reduced thrombus size and a large number of neovascularizations (Figure 5a). The miR-296-5p expression was increased in mice injected with Exo-NC antagonim compared with the control group, whereas miR-296-5p expression was reduced in the mice injected with Exo-miR-296-5p antagonim compared with the mice injected with Exo-NC antagonim (Figure 5b).

Immunofluorescence was used to determine CD31 expression in tissues. CD31 expression in mice injected with Exo-NC antagonim was lower than that without treatment, whereas mice injected with Exo-miR-296-5p antagonim exhibited higher expression than that in the mice injected with Exo-NC antagonim (Figure 5c). Western blot analysis showed that expression of SRF, MRTF-A, and VE-cadherin was reduced in the mice injected with Exo-NC antagonim compared with that without treatment, but it was increased in the mice injected with Exo-miR-296-5p antagonim (Figure 5d,e). The above data suggested that the suppression of exosomal miR-296-5p expression could enhance thrombolysis and recanalization.

DISCUSSION
DVT is a serious clinical condition with high mortality, calling for improved treatment modalities. Previous research has shown that exosomes derived from EPCs loaded with miR-126 could promote resolution and recanalization of DVT. Besides, it has also been revealed that miR-335-5p...
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The current study was designed to explore the regulatory role of exosomal miR-296-5p in the DVT. Collectively, the data of the study revealed that silencing of exosomal miR-296-5p could affect EPC function through the activation of SRF, thereby enhancing thrombolysis and recanalization in DVT.

Figure 5 Suppression of exosomal miR-296-5p expression enhances thrombolysis and recanalization. (a) Tissue hematoxylin-eosin staining results (200×). (b) The miR-296-5p expression in tissues. (c) Immunofluorescence map of CD31 in tissues (400×). (d, e) protein expression of serum response factor (SRF), myocardin related transcription factor A, and VE-cadherin in tissues. The measurement data were expressed as mean ± SD. Data among multiple groups were analyzed by one-way analysis of variance followed by a post hoc test for pairwise comparison. *Comparison between groups, \( P < 0.05 \). The experiment was repeated three times. NC, negative control.

Figure 6 The mechanism graph of the regulatory network and function of miR-296-5p. MiR-296-5p can target and inhibit serum response factor (SRF) gene expression. Inhibition of miR-296-5p expression increases SRF protein expression and promotes the binding between SRF and myocardin related transcription factor A (MRTF-A) on the VE-cadherin promoter. MRTF-A and p300 can synergistically promote the transcription and expression of VE-cadherin, thus improving the in vitro migration and angiogenesis ability of endothelial progenitor cells and enhancing thrombolysis and recanalization.
Initially, we found that miR-296-5p was highly expressed in exosomes, but that exosomes secreted from cells expressed by silencing miR-296-5p resulted in increased migration and angiogenic capacity of EPCs in vitro. These results are consistent with the findings reported by Li et al. that EPCs could accelerate the regression of DVT. Moreover, a recent study has revealed that miR-503 could inhibit hypoxia-induced proliferation, migration, and angiogenesis of EPCs by targeting Apelin. Besides, another study reported that the downregulation of miR-195 promotes cell proliferation, migration, and angiogenesis of human EPCs under hypoxic conditions. Furthermore, upregulation of miR-483-3p was observed in EPCs of patients with DVT, and its SRF targeting resulted in reduced migration and tube formation of EPCs, increased apoptosis in vitro, and reduced homing of EPCs and thrombosis in vivo. In addition, the exosomes secreted by mesenchymal stem cells could promote endothelial cell angiogenesis by transferring miR-125a. In this study, we also found that the downregulation of exosomal miR-296-5p in vivo could enhance the thrombocyte tissue thrombolysis and recanalization, as well as suppress DVT in our mouse model. Consistent with our result, a recent study reported by Kartal et al. demonstrated that ultrasonic accelerated catheter thrombolysis is a promising method for the treatment of DVT.

We also found that silencing of miR-296-5p could promote the binding of SRF and MRTF-A to the VE-cadherin promoter through SRF activation, thereby elevating VE-cadherin expression by histone acetylation. In accordance with the present results, a previous study has demonstrated that miR-22 may cause endosulfan-induced endothelial dysfunction by targeting SRF in human umbilical vascular endothelial cells. Xu et al. have revealed that miR-101-3p could inhibit HOTAIR-induced proliferation and invasion by directly targeting SRF in gastric cancer cells. Another study has reported that SRF/MRTF-A activation is essential for the maintenance of cerebral small vessel integrity, and that MRTF-A is a key regulator of VE-cadherin transcription in endothelial cells, whereas histone acetylation of p300 could promote MRTF-A-mediated transactivation of the VE-cadherin gene in human umbilical vein endothelial cells. A study reported by He et al. revealed that the transcription factors p300 and MRTF-A synergistically elevate migration-related gene expressions in MCF-7 breast cancer cells. A previous study reported by Franco et al. found that SRF plays an important role in mouse embryonic angiogenesis and small vessel integrity. Uppregulated miR-483-3p promotes endothelial progenitor cell dysfunction in patients with DVT through SRF. Accordingly, we hypothesized that silencing of miR-296-5p could activate SRF to promote resolution and recanalization of DVT. To verify the aforementioned hypothesis, a mouse model of DVT induced by inferior vena cava ligation was applied to substantiate the in vitro findings. The inferior vena cava ligation method we used is relatively simple, convenient, and economically commonly used, and it is commonly used to study the antithrombotic mechanism of drugs. The in vivo results further confirmed that silencing of exosomal miR-296-5p increased SRF, MRTF-A, and VE-cadherin expression.

Taken together, EPCs-exo carrying miR-296-5p uncovers a novel understanding of the pathogenesis of DVT, whereby we demonstrate therapeutic promotion of EPC resolution and recanalization in DVT (Figure 6). More in-depth preclinical investigations on EPCs-exo carrying miR-296-5p are required to yield a still better understanding of DVT treatment. However, the research is still at the preclinical stage, whereas the investigation on mechanism of miR-296-5p in DVT is still not yet well elucidated. Therefore, it is recommended that SRF interference experiment is needed in order to help to illuminate the underlying mechanisms.

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Ethical Approval. All experimental procedures involving animals were made to minimize suffering and were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Chongqing Medical University.

Data Availability Statement. The datasets generated/analyzed during the current study are available.

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