



Upregulated MiR-9-5p Protects Against Inflammatory Response in Rats with Deep Vein Thrombosis *via* Inhibition of NF- κ B p50

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Abstract— Recently, microRNAs (miRNAs) have been demonstrated to play important roles in the cardiovascular system, including heart, blood vessels, plasma, and vascular diseases. Deep vein thrombosis (DVT) refers to the formation of blood clot in the deep veins of the human body and is a common peripheral vascular disease. Herein, we explored the mechanism of miR-9-5p in DVT through nuclear factor- κ B (NF- κ B). The expression of miR-9-5p in DVT rats was measured through the establishment of DVT rat models, followed by the alteration of miR-9-5p and NF- κ B p50 in rats through the injection of constructed lentiviral vectors so as to explore the role of miR-9-5p and NF- κ B p50 expression in rats. Next, the expression of NF- κ B p50 and levels of inflammation-related factors plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6), tumor necrosis factor α (TNF- α), and interleukin-8 (IL-8) were measured after the injection with lentiviral vectors, followed by the assessment of platelet aggregation and TXB2 content. MiR-9-5p was found to be downregulated in DVT rats. Through dual luciferase reporter gene assay, NF- κ B p50 was verified as the target gene of miR-9-5p and miR-9-5p could negatively regulate NF- κ B p50. MiR-9-5p over-expression decreased the levels of PAI-1, TNF- α , IL-6, and IL-8 and platelet aggregation as well as TXB2 content, thus inhibiting thrombosis. Meanwhile, over-expressed NF- κ B p50 could reverse the anti-inflammatory or anti-thrombotic effect of miR-9-5p. In summary, miR-9-5p over-expression can suppress the NF- κ B signaling pathway through p50 downregulation, thus alleviating inflammation and thrombosis in DVT rats. MiR-9-5p could serve as a potential therapeutic target for DVT.

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INTRODUCTION

Thrombosis is defined as tissue infarction within the blood vessel due to formation of a clot, leading to decreased blood flow [1]. Venous thrombosis is a chronic disease which frequently occurs in the deep veins of the legs or arms [2, 3]. Deep vein thrombosis (DVT) and pulmonary embolism (PE) are collectively known as venous thromboembolism (VTE), representing a primary cause of morbidity and mortality across the globe [4]. Notably, a previous study revealed that inflammation accompanying thrombosis is a potential cause for venous outflow obstruction in DVT and is engaged in post-thrombotic syndrome [5]. DVT is easily misdiagnosed due to its hidden onset and symptoms at the beginning [6]. Moreover, it has been demonstrated that DVT could also be complicated by post-thrombotic syndrome and is correlated with various inflammatory disorders including cancers, with no effective methods for prophylaxis despite the presence of some therapies [7]. Therefore, it is imperative to explore the molecular mechanism underlying DVT in order to uncover therapeutic biomarkers for better diagnoses.

MicroRNAs (miRNAs) are crucial regulators of numerous biological processes such as cell differentiation, proliferation, apoptosis, and metabolism, and abnormal expressions of miRNAs are known to be implicated in diverse human diseases [8]. Particularly, a previous study evidenced the abnormal expression of miRNAs in venous thrombosis and suggested that miRNAs may be associated with the molecular mechanism of DVT [9]. In addition, miR-9-5p has been found to exert a suppressive effect in liver fibrosis [10]. Moreover, there is evidence showing that miR-9 functions critically in human hypertension [11]. Although miR-9-5p has been proposed to regulate nuclear factor- κ B (NF- κ B) by which miR-9-5p influences visceral blood flow and the production of pro-inflammatory cytokines during hemorrhagic shock [12], the role of miR-9-5p in DVT remains to be unknown. NF- κ B, a transcription factor involved in the modulation of multiple genes, has been previously identified to be close related to cell proliferation and inflammatory responses [13]. For instance, NF- κ B is engaged in the regulation of vascular inflammation which is critical for the development of atherosclerosis [14]. Also, NF- κ B p50 has been demonstrated to play an important role in the mediation of tissue factor in DVT [15]. Notably, NF- κ B has been found abnormally activated in multiple human malignancies and is associated with

angiogenesis [16]. Furthermore, another study suggested that miR-9 could inhibit cell migration and invasion in uveal melanoma partly by suppressing the NF- κ B1 signaling pathway [17]. These abovementioned findings lead to a presumption that miR-9-5p might interact with NF- κ B p50 in DVT. Therefore, the current study aims to explore the effect of miR-9-5p on inflammation of DVT rats and the potential mechanism in relation to NF- κ B p50.

MATERIALS AND METHODS

Study Subjects

The current study comprised of 110 male Sprague-Dawley (SD) rats raised under specific pathogen free (SPF) conditions (aged eight–12 weeks, weighing 200–300 g), which were obtained from Hunan SJA Laboratory Animal Co., Ltd. (SCXK (Xiang) 2009-0004, Changsha, Hunan, China). A total of 10 rats were categorized as normal controls, while 10 rats were used for extraction of rat vein endothelial cells and the remaining 90 rats were employed for model establishment. The rats were fed in 12-h light/12-h dark conditions with free access to food and water.

Construction of NF- κ B p50 Over-expression Lentiviral Vector

The coding sequence (CDS) of NF- κ B p50 was ligated into the lentiviral vector pMDLg/pRRE (Addgene plasmid no. 12251; <http://n2t.net/addgene:12251>; RRID: Addgene_12251) [18] as shown in Supplementary Fig. 1A, according to the lentiviral packaging vector kit (TaKaRa, Otsu, Shiga, Japan). Subsequently, the 293T cells were co-transfected with pMDLg/pRRE vector and the lacZ cosmid vector to obtain the 1st-generation recombinant lentivirus. After centrifugation, the lentiviruses were filtered and stored at -80°C . The virus titer was determined, followed by screening using the 293T cell differential infection method. The wild-type (Wt) virus strain was discarded. Next, the virus was cultured and amplified with Dulbecco's modified Eagle's medium (DMEM) culture medium to obtain higher titer of the 4th-generation lacZ lentivirus.

Establishment and Grouping of DVT Rat Models

After 1 week of adaptive feeding, DVT rat models were established using the “quantitative hitting + external plaster fixation” method. Briefly, the rats were not anesthetized and fixed in the prone position. A wound quantitative hitting device was used with the instantaneous striking energy of 5 J to strike the proximal side of bilateral thighs once respectively, which caused femoral fracture. After that, hip herringbone plaster fixation was adopted. After fixation, the rats were housed at room temperature with free access to food and water. A total of 4 rats died during model establishment (1 during operation, 2 the following day after operation, and 1 after 3 days of operation). The survival rate was calculated to be 95.56% (86/90). The 10 modeled rats were selected as the DVT group without any treatment.

The rats were injected with 100 μ L of the following different viral vectors through the tail vein: including agomir-negative control (NC), agomir-miR-9-5p, antagomir-NC, antagomir-miR-9-5p, agomir-NC plus overexpression (oe)-NC, agomir-miR-9-5p plus oe-NC, and agomir-miR-9-5p plus oe-NF- κ B p50, 10 rats per kind of injections. After 24 h of injection, the rats were euthanized, and the abdominal cavity was dissected to observe thrombosis of the inferior vena cava. The vessel wall and the embolus were carefully separated. One portion of separated venous wall tissue was used for reverse transcription quantitative polymerase chain reaction (RT-qPCR). The other portion tissue was fixed with 4% paraformaldehyde solution, subjected to ethanol gradient dehydration, cleared with xylene, embedded with paraffin, and sliced into 5- μ m sections which were then baked at 60 $^{\circ}$ C for 6 h and stored at room temperature. The remaining tissues were stored in liquid nitrogen for further experimentation.

ELISA

ELISA was performed according to the instructions of the ELISA kits (69-20224, 69-40133, 69-25328, 69-22085, Wuhan Moshake Biotechnology Co., Ltd., Wuhan, Hubei, China) to measure the levels of plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6), tumor necrosis factor α (TNF- α), and interleukin-8 (IL-8). Venous blood samples were obtained, allowed to stand for 1 h, and centrifuged at 3000 r/min for 10 min. Then, the supernatant was stored at -20 $^{\circ}$ C.

Cells were inoculated into a 96-well plate. The supernatant was collected when the cells reached 70% confluence. In addition, the levels of IL-6, IL-8, and TNF- α in cells were assayed. OD values were measured using an

excitation wavelength of 450 nm, and the concentrations of IL-6, IL-8, and TNF- α in cell supernatant were calculated according to the standard curve.

Platelet Aggregation and TXB2 Determination

Five rats in each group were selected, and blood samples were obtained from the abdominal aorta. Then, platelet suspension or platelet-rich plasma (PRP) was prepared according to the routine method. The platelet concentration in suspension or PRP was adjusted to 3×10^{11} /L, and modified Born turbidimetry was used to assess the platelet aggregation. Next, 200 μ L of platelet suspension or PRP was incubated for 5 min at 37 $^{\circ}$ C, then added with 200 μ mol/L ADP (3 μ L) or 10 U/mL thrombin (1 μ L) or 10 g/L AA (2 μ L), followed by plotting the 5-min platelet aggregation curve. The inhibition rate of aggregation (%) = (maximum aggregation rate of the control group - maximum aggregation rate of the administration group / maximum aggregation rate of the control group) \times 100%. Indomethacin was added to the thrombin-induced platelet aggregation tube to terminate reaction, and another 200 μ L of blank platelet suspension without induction agent was obtained for centrifugation. The obtained supernatant was used to determine the TXB2 (thromboxane B2) content released by platelets using radioimmunoassay.

Identification of Thrombosis

The proximal and distal end of femoral vein markers were excised at the obvious thrombus and fixed in 10% formalin for 48 h. After washing, dehydration, and clearing, the tissues were embedded with paraffin using a ZMN-803 automatic tissue embedding machine and then sliced using SHAN DON automatic slicer. After that, HE staining was performed using ZMN-2 8 02 full computer automatic tissue staining machine to observe thrombus and blood vessels wall changes under an optical microscope (XP-330, Shanghai Bingyu Optical Instrument Co., Ltd., Shanghai, China). The thrombosis was assessed and recorded by pathological professionals according to the histological examination of thrombus grading evaluation criteria. If the thrombosis was uncertain, the proximal, middle, and distal points were obtained for slicing and the points with obvious thrombus were included in the statistics. According to the degree of vascular obstruction by thrombus, 0 represented no thrombus, 1 represented vascular obstruction < 50%, 2 represented vascular obstruction > 50% (not completely blocked), and 3 represented complete vascular obstruction.

Dual Luciferase Reporter Gene Assay

The target gene of miR-9-5p was predicted by biological prediction website TargetScan (http://www.targetscan.org/vert_71/). The promoter region of NF- κ B p50 was constructed into pGL3-Basic vector (E1751; Promega, Madison, WI, USA) (Supplementary Fig. 1B) to obtain recombinant vector pGL3-NF- κ B p50-Wt. The mutation (Mut) sequence containing mutated binding site was designed based on pGL3-NF- κ B p50-Wt and was then inserted into the pMIR-reporter reporter plasmid (Ambion™, AM5795) (Supplementary Fig. 1C) using T4 DNA ligase after restriction endonuclease digestion. Subsequently, a recombinant vector pNF- κ B p50-Mut was obtained. The HEK293T cells were seeded in a 24-well plate at a concentration of 3×10^4 cells/well. Next, pGL3-NF- κ B p50-Wt and pNF- κ B p50-Mut were co-transfected with miR-9-5p mimic respectively with Rellina plasmid serving as internal control. After 48 h, a dual luciferase reporter gene analysis system (Promega, Madison, WI, USA) was applied to measure relative luciferase units (RLU). The activation of the target reporter gene was calculated using the ratio of the firefly RLU to the Renilla RLU.

In vitro Culture and Treatment of Vascular Endothelial Cells

The vein endothelial cells of normal rats were treated with 0.25% ethylenediaminetetraacetic acid (EDTA)-trypsin for 1 min at 37 °C and then made into cell suspension. The cell suspension was then inoculated into new culture bottle supplemented with medium (a final concentration of 4 mL/bottle) and stored in 5% CO₂ at 37 °C for further use.

Subsequently, the cells were introduced with agomir-NC, agomir-miR-9-5p, antagomir-NC, antagomir-miR-9-5p, agomir-NC plus oe-NC, agomir-miR-9-5p plus oe-NC, or agomir-miR-9-5p plus oe-NF- κ B p50 using Lipofectamine 2000 kit (11668–019, Invitrogen, Carlsbad, CA) as described in the specifications.

RNA Isolation and Quantitation

Total RNA was extracted from rat tissues and cells according to the instructions of Trizol. Primers (Table 1) were designed and synthesized by TaKaRa (Otsu, Shiga, Japan). The obtained RNA was reversely transcribed into cDNA using a PrimeScript RT kit (RR036A, TaKaRa, Otsu, Shiga, Japan). The cDNA was subjected to real-time fluorescence quantitative PCR using ABI7500 quantitative PCR instrument

(7500, ABI, Oyster Bay, NY, USA) in accordance with the instructions of the SYBR® Premix Ex Taq™ II Kit (RR820A, TaKaRa, Otsu, Shiga, Japan). The relative level of miR-9-5p was calculated with 2 μ g of total RNA serving as a template and U6 as internal control by relative quantification method ($2^{-\Delta\Delta CT}$ method).

Western Blot Analysis

Total protein in the tissues and cells was extracted with radio-immunoprecipitation assay (RIPA) lysate containing phenylmethanesulfonyl fluoride (PMSF) (R0010, Solarbio, Beijing, China) and incubated on ice for 30 min. The supernatant was obtained by centrifugation at 12000 \times g and 4 °C for 10 min for protein quantitative detection. Then, 50 μ g of protein was dissolved in 2 \times sodium dodecyl sulfate (SDS) loading buffer and boiled at 100 °C for 5 min, followed by separation with 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the protein was transferred onto a polyvinylidene fluoride membrane which was blocked with 5% skim milk powder at room temperature for 1 h. After that, the membrane was incubated overnight at 4 °C with the diluted rabbit polyclonal antibody NF- κ B p50 (ab32360, dilution ratio of 1:5000, Abcam, Cambridge, UK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab181602, dilution ratio of 1:10000, Abcam, Cambridge, UK). After being washed with Tris-buffered saline with Tween 20 (TBST) 3 times (5 min each time), the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (ab205718, dilution ratio of 1:10000, Abcam, Cambridge, UK) for 1 h, reacted with electrochemical luminescence (ECL) solution (ECL 808-25, Biomiga, San Diego, CA, USA) for 1 min at room temperature, and developed. GAPDH was used as internal control, and the ratio of the gray value of the target band to the internal control band was used as the relative expression of the protein.

CCK-8 Assay

The treated cells were suspended with concentration adjusted to 1×10^5 cells/mL and then inoculated in a 96-well plate overnight. Cell viability was measured using a CCK-8 kit (Beyotime Biotech, Shanghai, China) at 0th, 24th, 48th, and 72nd hours with the addition of 10 μ L CCK-8 solution after inoculation. The OD value at 450 nm was measured using a microplate reader, and the growth curve was plotted.

Table 1. Primer Sequence for RT-qPCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
miR-9-5p	GCCGCTCTTTGGTTATCTAGCT	GTGCAGGGTCCGAGGTAT
U6	ATGACGTCTGCCTTGGAGAAC	TCAGTGTGCTACGGAGTTCAG

RT-qPCR reverse transcription quantitative polymerase chain reaction, miR-9-5p microRNA-9-5p

Flow Cytometry

An Annexin-V-FITC/PI apoptosis detection kit (CA1020, Beijing Solarbio Technology Co., Ltd., Beijing, China) was used to detect cell apoptosis. After 48 h of transfection, the cells were trypsinized using EDTA-free trypsin. After centrifugation, the cells were stained with a mixture of Annexin-V-FITC and Binding buffer (ratio of 1:40) for 30 min. Next, the cells were stained with a mixture of PI and binding buffer (ratio of 1:40) for 15 min. Subsequently, cell apoptosis was detected by flow cytometer.

Statistical Analysis

The experimental data were analyzed using SPSS 21.0 software (IBM Corp. Armonk, NY, USA). Measurement data were expressed as mean \pm standard deviation. Comparisons between two groups were conducted using *t* test, and comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Low Expression of miR-9-5p in Venous Wall Tissues of DVT Rats

A rat DVT model was established to mimic *in vivo* pathological conditions. The tail vein injection process went smooth. After the injection, vital signs of the rats were stable and they could drink water and eat normally. Observations were entailed to ensure no obvious swelling, ischemia, and necrosis in the soft tissues of the tail. In addition, HE staining revealed the presence of thrombosis in the inferior vena cava. The blood vessels were observed to be significantly thickened, and some of the branches were also thickened and deeply stained (Fig. 1a). Additionally, the expression of miR-9-5p in the venous vascular wall of normal rats and DVT rats was measured by RT-qPCR. The results (Fig. 1b) showed that DVT rats presented with lower expression of miR-9-5p compared to the normal rats ($p < 0.05$).

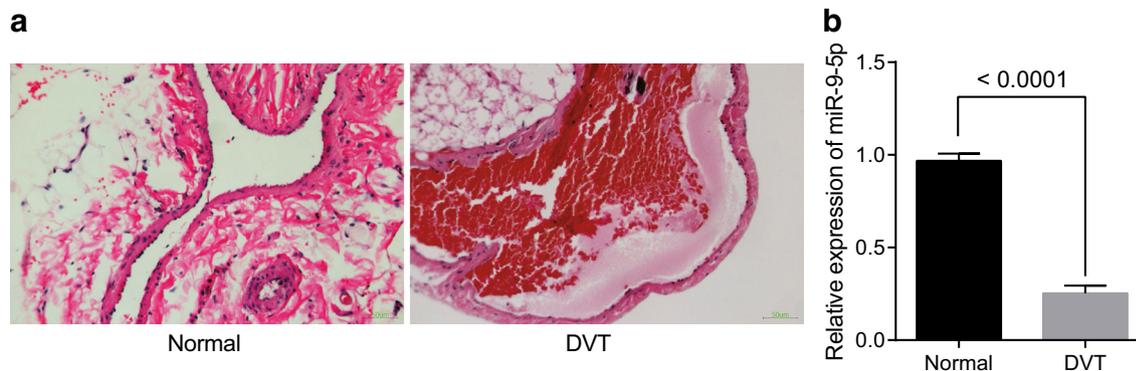


Fig. 1. miR-9-5p is poorly expressed in venous wall tissues of DVT rats. **a** The thrombosis in the rats after DVT model establishment as observed by HE staining ($\times 200$). **b** The expression of miR-9-5p in the venous wall of normal rats and DVT rats determined by RT-qPCR. The abovementioned results were measurement data and expressed as mean \pm standard deviation. The independent sample *t* test was used for comparisons between two groups. $n = 5$. miR-9-5p microRNA-9-5p, RT-qPCR reverse transcription quantitative polymerase chain reaction, DVT deep vein thrombosis, HE hematoxylin-eosin, ANOVA analysis of variance.

Over-expression of miR-9-5p Inhibits Thrombosis and Inflammation in DVT Rats

Following the results indicating downregulation of miR-9-5p in DVT rats, we further investigated whether miR-9-5p affects thrombosis in DVT rats. RT-qPCR (Fig. 2a) demonstrated that rats injected with agomir-

miR-9-5p presented with significantly higher expression of miR-9-5p compared with rats injected with agomir-NC ($p < 0.05$). Results of ELISA assay (Fig. 2b) revealed that the levels of PAI-1, TNF- α , IL-6, and IL-8 were reduced in rats injected with agomir-miR-9-5p compared with those in rats injected with agomir-NC ($p < 0.05$). Meanwhile,

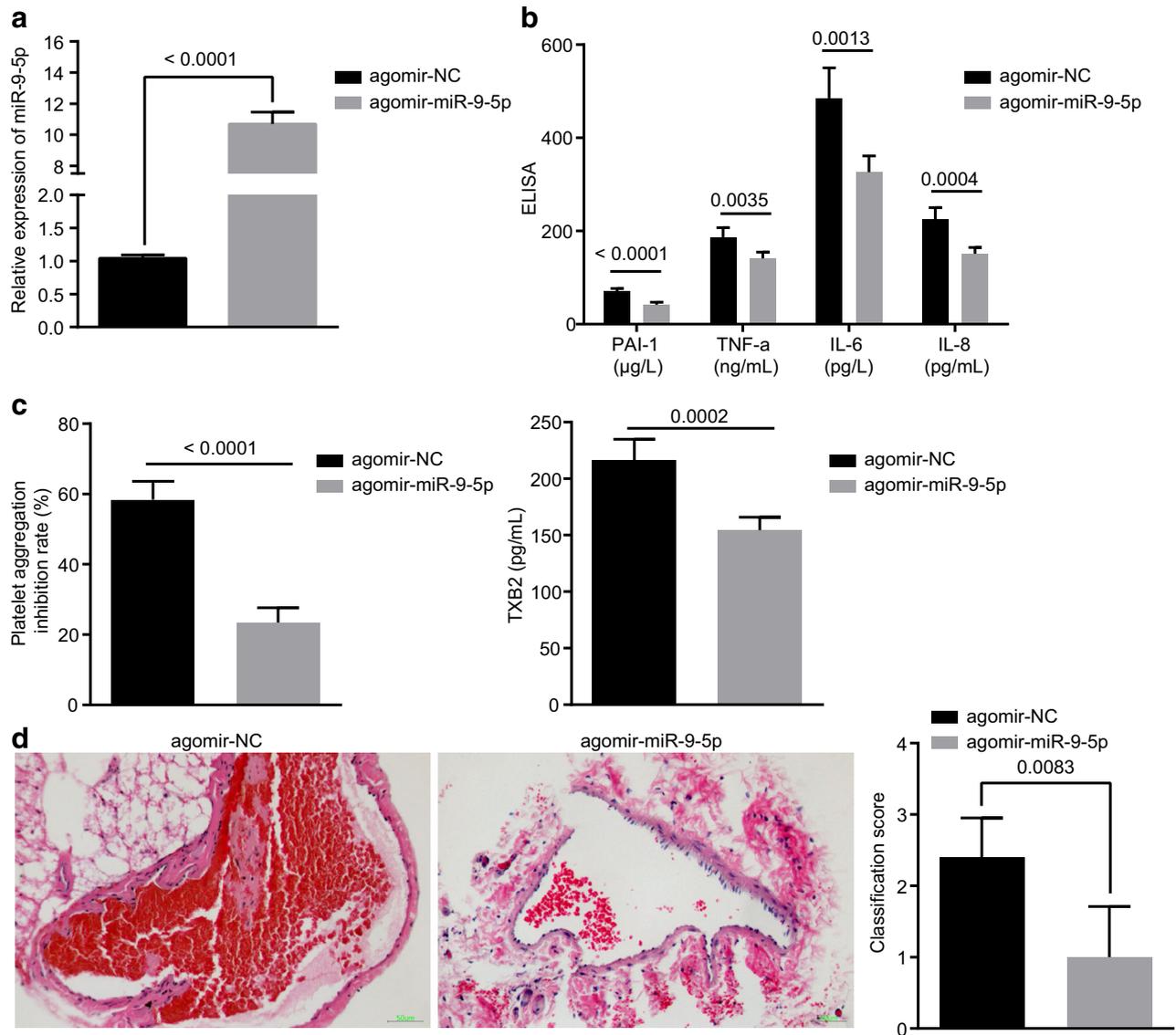


Fig. 2. Over-expressed miR-9-5p suppresses thrombosis and inflammation in DVT rats. **a** The delivery efficiency of agomir-miR-9-5p measured by RT-qPCR. **b** The levels of PAI-1, TNF- α , IL-6, and IL-8 after alteration of miR-9-5p determined by ELISA. **c** Platelet aggregation and TXB2 content after alteration of miR-9-5p examined by turbidimetry and radioimmunoassay. **d** Thrombosis after alteration of miR-9-5p observed by HE staining ($\times 200$). The above-mentioned results were measurement data and expressed as mean \pm standard deviation. The independent sample t test was used for comparisons between two groups. $n = 5$. miR-9-5p microRNA-9-5p, RT-qPCR reverse transcription quantitative polymerase chain reaction, NC negative control, PAI-1 plasminogen activator inhibitor-1, TNF- α tumor necrosis factor α , IL-6 interleukin-6, IL-8 interleukin-8, ELISA enzyme-linked immunosorbent assay, TXB2 thromboxane B2, DVT deep vein thrombosis.

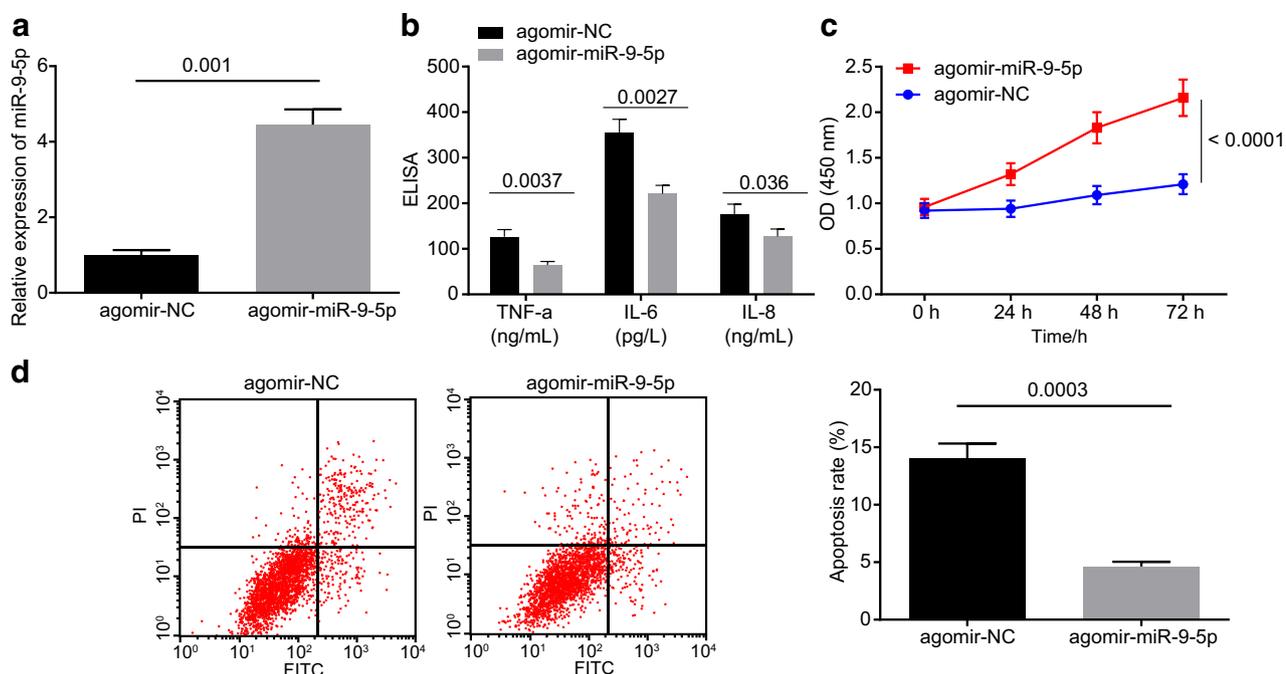


Fig. 3. Over-expression of miR-9-5p inhibits inflammatory response and apoptosis and promotes proliferation of vascular endothelial cells. **a** The transfection efficiency of agomir-miR-9-5p determined by RT-qPCR. **b** The contents of TNF-α, IL-6, and IL-8 in vascular endothelial cells after upregulation of miR-9-5p detected by ELISA. **c** The proliferation of vascular endothelial cells after upregulation of miR-9-5p assayed by CCK-8. **d** The apoptosis of vascular endothelial cells after upregulation of miR-9-5p determined by flow cytometry. The abovementioned results were measurement data and expressed as mean ± standard deviation. One-way ANOVA was used for comparison among multiple groups. The experiment was repeated three times. miR-9-5p microRNA-9-5p, RT-qPCR reverse transcription quantitative polymerase chain reaction, NC negative control, TNF-α tumor necrosis factor α, IL-6 interleukin-6, IL-8 interleukin-8, ELISA enzyme-linked immunosorbent assay, CCK-8 cell counting kit 8, ANOVA analysis of variance.

platelet aggregation inhibition rate and TXB2 content were also found to be inhibited as a result of agomir-miR-9-5p administration (Fig. 2c). As depicted by HE staining (Fig. 2d), rats injected with agomir-miR-9-5p showed darker coloration on a small part of the left femoral vein, while the proximal end of the vein was noted to be relatively less bloody, in addition to lower suspicion of thrombosis and lower thrombosis grading score compared to rats injected with agomir-NC ($p < 0.05$).

Restoration of miR-9-5p Inhibits Inflammatory Response and Apoptosis of Rat Vascular Endothelial Cells

To further explore the *in vitro* effects of miR-9-5p on inflammation, vascular endothelial cells were isolated from the veins of rats and transduced with agomir-miR-9-5p. The results of RT-qPCR (Fig. 3a) showed that expression of miR-9-5p in vascular endothelial cells transduced with agomir-miR-9-5p was upregulated ($p < 0.05$). Additionally, the contents of TNF-α, IL-6, and IL-8 in vascular

endothelial cells were determined by ELISA, which showed that TNF-α, IL-6, and IL-8 contents in vascular endothelial cells over-expressing miR-9-5p were downregulated ($p < 0.05$) (Fig. 3b). Next, the proliferation and apoptosis of vascular endothelial cells were tested by CCK-8 and flow cytometry. The proliferation ability of vascular endothelial cells transduced with agomir-miR-9-5p was found to be increased while the apoptosis rate was decreased when compared to that transduced with agomir-NC (Fig. 3c, d) ($p < 0.05$).

NF-κB p50 Is a Target Gene of miR-9-5p

Subsequently, the focus of the experiment shifted to elucidating the mechanism of miR-9-5p in DVT. Firstly, website prediction results (Fig. 4a) revealed that NF-κB p50 was a target gene of miR-9-5p. According to the results of the dual luciferase reporter gene assay (Fig. 4b), the luciferase activity of NF-κB p50-Wt was found to be downregulated by miR-9-5p mimic compared to the NC

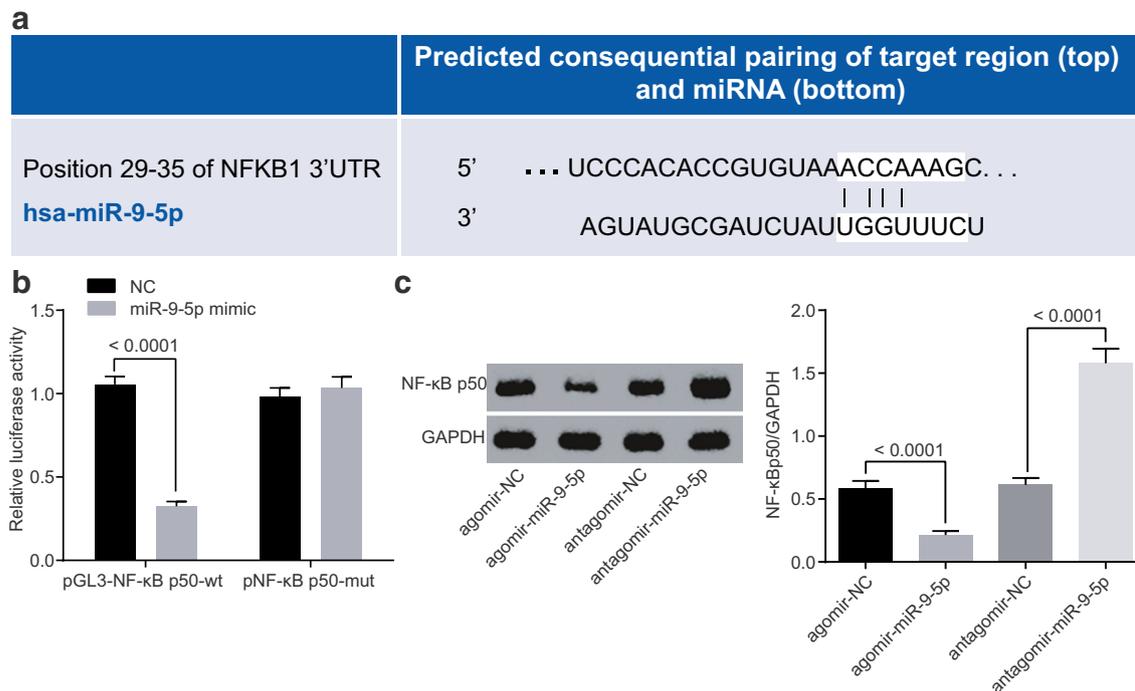


Fig. 4. miR-9-5p negatively regulates NF- κ B p50. **a** Binding site between miR-9-5p and NF- κ B p50 predicted by bioinformatics website. **b** The binding of NF- κ B p50 to miR-9-5p detected by dual luciferase reporter gene assay. **c** The expression of NF- κ B p50 after alteration of miR-9-5p assessed by Western blot analysis. The abovementioned results were measurement data and expressed as mean \pm standard deviation. The independent sample *t* test was used for comparisons between two groups, and one-way ANOVA was used for comparisons among multiple groups. The experiment was repeated three times. miR-9-5p microRNA-9-5p, NC negative control, ANOVA analysis of variance, NF- κ B nuclear factor- κ B.

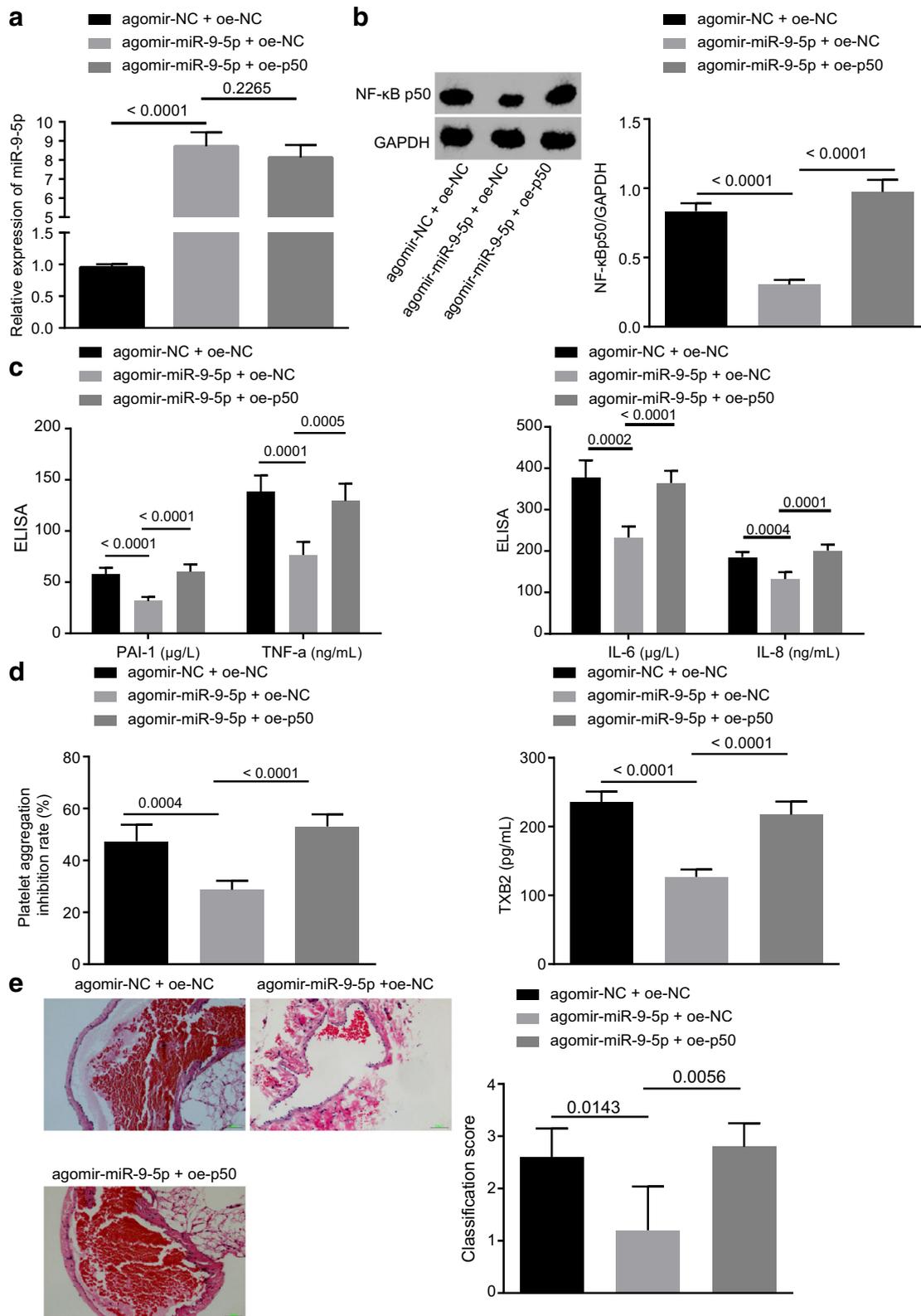
($p < 0.05$) but that of NF- κ B p50-Mut was not significantly changed ($p > 0.05$). Western blot analysis showed that the expression of NF- κ B p50 was reduced in rats injected with agomir-miR-9-5p while elevated after the injection of antagomir-miR-9-5p (Fig. 4c). Taken together, the aforementioned findings evidenced that NF- κ B p50 was a target gene of miR-9-5p and miR-9-5p could negatively regulate NF- κ B p50.

MiR-9-5p Inhibits Thrombosis and Inflammation in DVT Rats by Downregulating NF- κ B p50

Upon determining the targeting relationship between miR-9-5p and NF- κ B p50, we further aimed to uncover whether NF- κ B p50 is involved in the inhibitory role of miR-9-5p in thrombosis and inflammation of DVT rats. The miR-9-5p over-expression efficiency in rat venous wall tissue was detected by RT-qPCR, which showed that the expression of miR-9-5p was upregulated in rats injected with both agomir-miR-9-5p and oe-NC or both agomir-miR-9-5p and oe-NF- κ B p50 in contrast to that in rats injected with both agomir-NC and oe-NC ($p < 0.05$;

Fig. 5a). In addition, Western blot analysis demonstrated that the expression of NF- κ B p50 was upregulated upon being injected by both agomir-miR-9-5p and oe-NF- κ B p50 (Fig. 5b). The results of ELISA (Fig. 5c) suggested that the injection of both agomir-miR-9-5p and oe-NF- κ B p50 led to the upregulation of PAI-1, TNF- α , IL-6, and IL-8 levels relative to the injection of both agomir-miR-9-5p and oe-NC ($p < 0.05$). The results of serum platelet

Fig. 5. miR-9-5p negatively regulates NF- κ B p50 to alleviate thrombosis and inflammation in DVT rats. **a** The efficiency of miR-9-5p over-expression assessed by RT-qPCR. **b** The efficiency of NF- κ B p50 over-expression determined by Western blot analysis. **c** The levels of PAI-1, TNF- α , IL-6, and IL-8 measured by ELISA. **d** The platelet aggregation and TXB2 content examined by turbidity and radioimmunoassay. **e** Thrombosis after alteration of miR-9-5p and NF- κ B p50 observed by HE staining ($\times 200$). The abovementioned results were measurement data and expressed as mean \pm standard deviation. The enumeration data between two groups were analyzed by chi-square test, and one-way ANOVA was used for comparisons among multiple groups. The experiment was repeated three times. $N = 5$. miR-9-5p microRNA-9-5p, NF- κ B nuclear factor- κ B, RT-qPCR reverse transcription quantitative polymerase chain reaction, NC negative control, ELISA enzyme-linked immunosorbent assay, TXB2 thromboxane B2, ANOVA analysis of variance, PAI-1 plasminogen activator inhibitor type-1, HE hematoxylin-eosin.



aggregation and TXB2 content showed that the injection with both agomir-miR-9-5p and oe-NF- κ B p50 resulted in higher platelet aggregation and TXB2 content than the injections of both agomir-miR-9-5p and oe-NC. ($p < 0.05$; Fig. 5d). Moreover, HE staining (Fig. 5e) demonstrated that in rats injected with both agomir-miR-9-5p

and oe-NF- κ B p50, most of the left vascular veins darkened, the proximal venous blood slowed down, more suspected thrombosis appeared, and the thrombus grading score was significantly increased, when compared with rats injected with both agomir-miR-9-5p and oe-NC ($p < 0.05$).

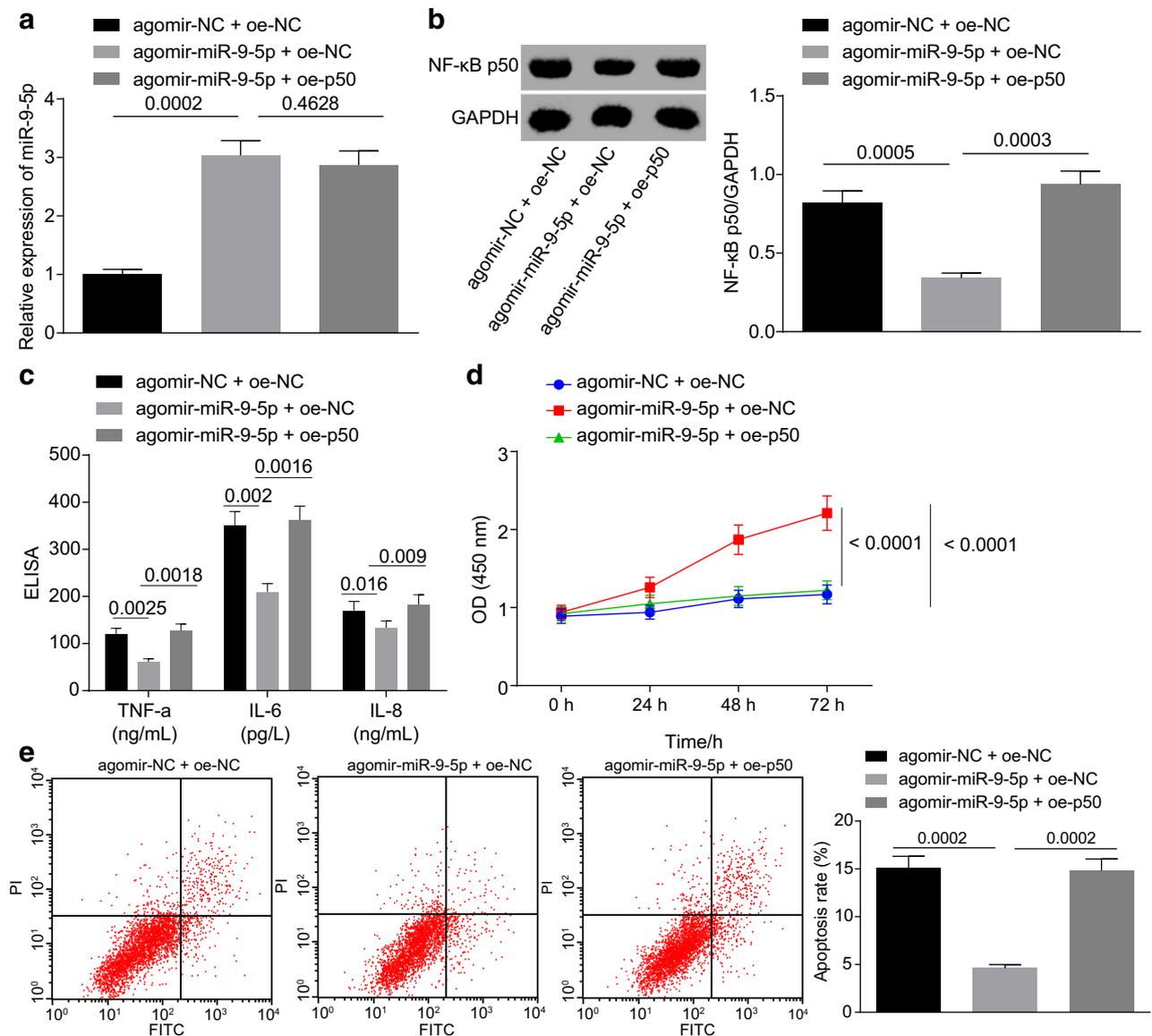


Fig. 6. miR-9-5p reduces inflammatory response and apoptosis of rat vascular endothelial cells by targeting NF- κ B p50. **a** The efficiency of transfection with agomir-miR-9-5p and NF- κ B p50 over-expression plasmids. **b** The expression of NF- κ B p50 in rat vascular endothelial cells measured by Western blot analysis. **c** The content of TNF- α , IL-6 and IL-8 in rat vascular endothelial cells assayed by ELISA. **d** Proliferation of vascular endothelial cells detected by CCK-8. **e** Apoptosis of vascular endothelial cells detected by flow cytometry. The abovementioned results were measurement data and expressed as mean \pm standard deviation. One-way ANOVA was used for comparisons among multiple groups. The experiment was repeated three times. miR-9-5p microRNA-9-5p, NF- κ B nuclear factor- κ B, RT-qPCR reverse transcription quantitative polymerase chain reaction, NC negative control, TNF- α tumor necrosis factor α , IL-6 interleukin-6, IL-8 interleukin-8, ELISA enzyme-linked immunosorbent assay, CCK-8 cell counting kit 8, ANOVA analysis of variance.

miR-9-5p Inhibits Inflammatory Response and Apoptosis of Rat Vascular Endothelial Cells by Negatively Regulating NF- κ B p50

Subsequently, we further assessed the *in vitro* roles of miR-9-5p in inflammatory response and apoptosis of vascular endothelial cells. The results of RT-qPCR (Fig. 6a) showed that the expression of miR-9-5p was increased in cells transduced with agomir-miR-9-5p and oe-NC or agomir-miR-9-5p and oe-NF- κ B p50 in comparison with cells transduced with agomir-NC and oe-NC ($p < 0.05$). Western blot analysis (Fig. 6b) revealed that the expression of NF- κ B p50 in the cells introduced with agomir-miR-9-5p and oe-NF- κ B p50 was upregulated compared with that in cells transduced with agomir-miR-9-5p and oe-NC ($p < 0.05$). Similarly, ELISA (Fig. 6c) demonstrated that upregulation of both miR-9-5p and NF- κ B p50 led to higher levels of TNF- α , IL-6, and IL-8 than that with upregulation miR-9-5p alone ($p < 0.05$).

Additionally, we detected the cell viability and apoptosis of vascular endothelial cells by means of CCK-8 and flow cytometry. The results showed that the cell viability of vascular endothelial cells transduced with agomir-miR-9-5p and oe-NF- κ B p50 was decreased while the apoptosis rate was increased compared to vascular endothelial cells transduced with agomir-miR-9-5p and oe-NC ($p < 0.05$) (Fig. 6d, e).

DISCUSSION

DVT is one of the most prevalent peripheral vascular diseases affecting the human venous system accompanied by high morbidity and mortality rates [19]. In addition, DVT can often lead to worsening disorders such as PE, recurrent thrombosis, and pulmonary hypertension [20]. Although major strides have been made in the treatment of DVT such as anti-coagulation and surgical interventions, the existence of side effects including bleeding and wound complications still remains to be problematic [21]. Interestingly, recent studies have evidenced the implication of miRNAs in the cardiovascular system [22]. For example, miR-96 was demonstrated to be upregulated in DVT and suggested to be an important predictor for DVT [23]. Thus, aiming to provide better treatment modalities for DVT patients, the current study investigated the effect of miR-9-5p on the DVT, and the findings revealed that over-expressed miR-9-5p could inhibit DVT through suppression of NF- κ B p50.

Initially, we found that miR-9-5p was poorly expressed in venous wall tissues of DVT rats. Similarly, the down-regulation of miR-9-5p has been reported in numerous diseases. For example, miR-9-5p was poorly expressed in liver fibrosis and restoration of miR-9-5p acted as suppressor in liver fibrosis by inhibiting TGF- β 1-induced hepatic stellate cell activation [10]. In addition, miR-9 was previously found to be expressed at low levels in cardiac fibroblast (CF) and its over-expression wielded a suppressive effect on CFs proliferation and collagen production [24]. miRNAs play important roles in various biological processes during development and tissue homeostasis by regulating around 90% of human genes, and numerous miRNAs have been detected in the extracellular space like blood and other body fluids [25]. In this study, NF- κ B p50 is a putative target gene of miR-9-5p as determined by dual luciferase reporter gene assay. Furthermore, over-expressed miR-9-5p was found to negatively regulate NF- κ B p50 in rats with DVT. NF- κ B1 is the mature form of p50 and its precursor p105 [26]. NF- κ B transcription factor p50 (presumably the p50/p65 heterodimer) transcriptionally regulates the expression of tissue factor (TF) in stimulated human and murine monocytes/macrophages and vascular endothelial cells *in vitro* and in the murine model of experimental DVT *in vivo*. Supplementing the importance of our findings, inhibition of p50 was previously found to be conducive to partial suppression of DVT [15]. Similar to our study, Guo *et al.* also reported the relationship between NF- κ B1 and miR-9 in gastric adenocarcinoma and that miR-9 over-expression could suppress cell proliferation and tumor growth by inhibiting NF- κ B1 [27]. Also, over-expressed miR-9 plays an inhibitory role in melanoma cell proliferation and metastasis with decreased F-actin polymerization and downregulation of multiple GTPases involved in cytoskeleton remodeling through the suppression of NF- κ B1 [28].

Additionally, we demonstrated that over-expressed miR-9-5p could inhibit NF- κ B p50, thus suppressing inflammatory response and thrombosis in DVT rats, corresponding to decreased levels of PAI-1, TNF- α , IL-6, IL-8, platelet aggregation, and TXB2 content. Both IL-6 and IL-8 are pro-inflammatory cytokines associated with activation of the immune system [29]. Meanwhile, TNF- α is also a potent pro-inflammatory mediator which could induce IL-6 and IL-8 through NF- κ B [30]. PAI-1 has been found to be involved in inflammation in various diseases [31]. In addition, the regulation of platelet function is crucial for the prevention for thrombosis, which could be beneficial in the treatment of DVT [32]. Furthermore, platelets are vital for thrombosis and hemostasis and contain numerous miRNAs [33]. Simultaneously, platelets have been reported to potentially contribute to inflammation responses by interacting with monocytes and

neutrophils in the peripheral bloodstream [34]. A previous study further demonstrated that several miRNAs are engaged in the regulation of PAI-1 molecule and could serve as potential biomarkers in inflammatory and thrombotic disorders [35]. Also, PAI-1 is known to be involved in formation of blood clots and venous thrombosis, and miR-335-5p has been found to repress the development of lower extremity deep venous thrombosis (LEDVT) in rats by suppressing PAI-1 [36]. Importantly, another study highlighted the role of miR-9 in monocytes and/or macrophages during inflammation and further evidenced the involvement of miR-9 in immune response through the regulation of NF- κ B family in monocytes and polymorphonuclear neutrophils [37]. The aforementioned NF- κ B family is considered to be the fundamental mediator of the inflammatory process [38]. Notably, NF- κ B1 served as a target of miR-9 experimentally *in silico* and the promotion of miR-9 could prevent from being negatively regulated by p50 homodimers in monocytes in systemic anti-inflammatory response syndrome and in cancer [39].

Taken together, the findings of the current study demonstrated that upregulation of miR-9-5p can inhibit thrombosis and inflammatory response through suppression of NF- κ B p50. Thus, it can be hypothesized that miR-9-5p overexpression can serve as a potential therapeutic target for DVT in the future. Our *in vitro* and *in vivo* experiments only demonstrate that miR-9-5p downregulates p50 expression and inhibits the NF- κ B signaling pathway, by which reduces inflammation to prevent thrombus formation. However, the detailed mechanism remains to be perfected in the aspect of specific molecules, reducing the inflammatory response and vascular endothelial cell proliferation. Further researches are needed to provide more comprehensive and specific findings. In addition, bioinformatics analyses can be used to predict more miR-9-5p-related functions so as to discuss the function of miR-9-5p in DVT in a more in-depth manner.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical Approval. All animal experiments were carried out in accordance with the principles and procedures of the National Institute of Animal Health Care Guidelines and approved by the Animal Ethics Committee of Qingdao Municipal Hospital.

Conflict of Interest. The authors declare that they have no conflict of interest.

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