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Resveratrol Induces Endothelial Progenitor Cells Angiogenesis via MiR-542-3p by Targeting Angiopoietin-2 and Involves in Recanalization of Venous Thrombosis

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Background: Endothelial progenitor cells (EPCs) play an important role in therapeutic angiogenesis. Besides, resveratrol (RSV) exerts many pharmacological functions in regulation of cell function. Furthermore, microRNAs (miRNAs) have been considered to be of great significance in biological process. In this study, we aimed to investigate the effect of RSV on EPCs and its potential mechanism that involved in recanalization of venous thrombosis.





Material/Methods: EPCs were treated with RSV, and angiogenic functions was evaluated by tube formation and migration assays. miR-542-3p expression level in EPCs was assessed and exogenously modified. Bioinformatic analysis was applied to detect the potential target of miR-542-3p. Effects of RSV treatment *in vivo* venous thrombosis rat model were evaluated.

Results: RSV enhanced angiogenic function of EPCs and decreased expression of miR-542-3p. Dual luciferase reporter gene and western blot results confirmed angiopoietin-2 (ANGPT2) was a direct target of miR-542-3p. It was found that inhibition of miR-542-3p contributed to angiogenesis of EPCs and elevated ANGPT2 protein level. Finally, in a rat model of venous thrombosis, RSV-treated EPCs promoted recanalization of thrombi.

Conclusions: We demonstrated that RSV can contribute to progenitor cells angiogenesis via miR-542-3p by targeting ANGPT2, subsequently enhanced recanalization of thrombi.

MeSH Keywords: **MicroRNAs • Neovascularization, Pathologic • Stem Cells • Venous Thrombosis**

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Background

Venous thrombosis (VT) constitutes a major global burden of disease with about 10 million cases occurring every year [1]. Although the recent application of endovascular treatment techniques provided new therapeutic solutions for VT patients, it is known to be inadequate modality for restoration and preservation of damaged vessel and dysfunctional endothelium. Endothelial progenitor cells (EPCs), as a set of multipotent cells with the potential of differentiating into mature endothelial cells, is involved in formation of new vessels and repair of injured damaged vasculature [2]. Recently, mounting evidence shows that EPCs fulfill critical roles as appropriate candidate for various vascular diseases [3,4].

Resveratrol (3,5,4'-trihydroxystilbene) is a pleiotropic phytochemical which originally derived from the skin of grapes, blueberries, raspberries, mulberries, and peanuts. It is also used as a dietary supplement and naturally occurs in red wine and some medicinal herbs. Many previous studies have revealed that resveratrol (RSV) plays an important role on cell biological process such as anti-inflammation and apoptosis and protect against the symptoms of metabolic diseases associated with aging [5–7]. Recent studies have revealed that RSV exerted protective effect on EPCs [8,9], but the mechanism is still unclear.

MicroRNAs (miRNAs) refer to a family of small non-coding RNA of 22–24 nucleotides in length and they are implicated in a wide variety of cellular process including proliferation, differentiation, apoptosis and development [10]. The discovery of miRNAs revealed a further layer of regulators, also playing a key role in the fine regulation of vascular pathophysiologic processes. Aberrant expression of miR-542-3p has been reported in a variety of human diseases including neuroblastoma [11], breast cancer [12], and colorectal cancer [13]. Several studies also demonstrated that miR-542-3p participated in angiogenic activity of endothelial cells [14,15]. However, the role of miR-542-3p in endothelial progenitor cells is unexplored.

In the present study, we investigated the effect of RSV on angiogenic function of EPCs and the potential role of miR-542-3p involved in this mechanism. Furthermore, our results established that the therapeutic effect of RSV-treated EPCs in treating venous thrombosis.

Material and Methods

Cell culture

Eighty milliliters of peripheral blood were collected from healthy adult volunteers under informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood

by density gradient centrifugation with 15 mL of density gradient solution. Then the collected PBMCs were washed with phosphate-buffered saline (PBS) and resuspended with EGM-2 medium (Lonza, MD, USA), followed by incubation of CD34 antibody and dextran-coated magnetic beads. After incubation, the cells were transferred to FACS tubes and then seeded on fibronectin-coated T75 flask (Millipore, MO, USA) with EGM-2 medium. This medium was changed after 4 days culture and early EPCs developed an elongated spindle-shaped morphology after 7 days culture. Then medium was changed every 2 days. After 2 to 3 weeks, late EPCs were identified by their formation of a cobblestone-like morphology, their expression of surface markers including CD34, CD31, VEGFR-2, and vWF. Thereafter, late EPCs colonies were trypsinized and cultured on fibronectin pre-coated wells or plates ($2 \times 10^4/\text{cm}^2$) for further experiments. EPCs was pretreated with RSV (Sigma-Aldrich, MO, USA) before subsequent experiments. The protocols were approved by the Institutional Review Board of our institution.

Tube formation assay

Before the experiment, Matrigel (BD Biosciences, NJ, USA) basement membrane matrix was added to 24-well plate. 5×10^4 EPCs was seeded into the plate with EGM-2MV medium for 24 hours. Formed tube was monitored and measured by ImageJ software (Media Cybernetics, MD, USA).

Migration assay

Migration of EPCs was evaluated using a Transwell system. 1×10^5 EPCs were placed on the top compartment pretreated with Matrigel. EBM-2MV medium supplemented with 20% fetal bovine serum (FBS) was added into the lower compartment. After 24 hours in 5% CO₂ incubator, the membranes were fixed and stained with hematoxylin. EPCs penetrated the membrane were measured and calculated under a light microscope.

Quantitative RT-PCR analysis

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA was converted to cDNA with the PrimeScript RT reagent Kit (Takara, Dalian, China). Real-time polymerase chain reaction (RT-PCR) was performed using SYBR[®] Green Q-PCR Mix (Thermo Scientific, MBI, USA). miRNA expression was measured with miRNA qPCR Quantitation Kit (GenePharma, Shanghai, China). Expression of U6 and GAPDH were monitored as internal controls. Primers were as follows: angiotensin-2 (ANGPT2), forward 5'-CCGCTCGAGAACTTGCATCACTTAACGGACCA-3' and reverse 5'-ATTTGCGGCCGCTAGTCCCGAGTATAAAGCTGT-3'; miR-542-3p, forward 5'-UGUGACAGAUUGAUA ACU GAAA-3' and reverse 5'-GTG CAG GGT CCG AGG T-3'; U6, forward 5'-GCTTCGGCAGCACATATACTAAAAT-3' and

reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3';
GAPDH, forward 5'-CATGAGAAGTATGACAACAGCCT-3' and
reverse 5'-AGTCCTTCACGATACCAAAGT'.

Cell transfection

MiR-542-3p mimics or inhibitor was transfected into cells to change the expression level of miR-542-3p in EPCs. After incubation for 48 hours, EPCs were harvested for the further experiments.

Western blot analysis

Total proteins were extracted from EPCs using radioimmuno-precipitation assay (RIPA) buffer (Sigma-Aldrich, MO, USA) and quantified by bicinchoninic acid (BCA) method. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred into polyvinylidene difluoride (PVDF) membranes. After that, the PVDF membranes were incubated with antibodies for ANGPT2 (Abcam, MA, USA) and β -actin (Sigma, MO, USA). After reaction with appropriate horseradish peroxidase conjugated secondary antibodies, the protein bands were examined using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) on x-ray films (Kodak, Tokyo, Japan).

Luciferase assay

The 3'-UTR of ANGPT2 containing the putative miRNA target site(s) was cloned into the SpeI and HindIII sites of the pMIR-REPORT Luciferase vector (Ambion, TX, USA). We transfected 293T cells with firefly luciferase reporter vector, miRNA, and Renilla luciferase control vector, respectively. The ratio between firefly and Renilla luciferase activities was analyzed to determine the interaction between miR-542-3p and potential target gene.

Venous thrombosis model construction

The 24 immuno-deficient male nude rats (n=8 for each group) aged 8–12 weeks old (Charles River Laboratories Supplier in China, Beijing, China) were anesthetized and the inferior vena cava (IVC) was exposed and ligated with 6-0 Prolene sutures. IVC segment thrombus was induced by blocking the blood flow at the confluence of iliac veins for 15 minutes. Three days after this procedure, EPCs mixed with EGM-2MV was injected via tail vein. The study protocol was approved by the Institutional Animal Care and Use Committee of our institution.

Histology analysis

The rats were sacrificed and IVC segments with thrombus were harvested 7 days after cell transplantation. Collected thrombi

were weighed before fixation. Then the thrombi were treated with 4% paraformaldehyde overnight and dehydrated using a graded ethanol series, treated by dimethylbenzene and embedded in paraffin. The slices were cryosectioned at 8- μ m intervals and performed with hematoxylin and eosin (H&E) staining.

Digital subtraction angiography (DSA)

IVC venography was acquired with digital subtract angiography (DSA) by injecting contrast media into rat caudal vein 5 days after cell transplantation. All the acquired images were analyzed using ImageJ software.

Statistical analysis

All values are expressed as mean \pm standard deviation. Student's *t*-test was applied when there 2 groups while one-way analysis of variance (ANOVA) for more than 2 groups comparison. All statistical analyses were performed using SPSS version 21. A 2-tailed value of $P < 0.05$ was considered as significant difference.

Results

RSV promoted EPCs angiogenic function *in vitro*

Isolated EPCs were identified with Dil and UEA-1 staining and flow cytometry analysis before application for *in vitro* experiment (Figure 1). To determine the optimal concentration of RSV, EPCs were incubated with different concentration of RSV for 2 days. Dose-dependent EPCs viability indicated that no significant change was observed at concentration of 25 μ mol/L to 75 μ mol/L (Figure 2). Thus, we chose the concentration of 25 μ mol/L for the subsequent experiments. Transwell assay revealed that RSV-treated EPCs showed enhanced migration compared with that in control group (Figure 3A). Furthermore, the angiogenic function of EPCs also increased in the presence of RSV (Figure 3B).

RSV repressed miR-542-3p expression in EPCs

MiR-542 is considered to play an important role in regulating the angiogenesis in various types of cells including endothelial cells [14], but the effect of miR-542-3p on EPCs is still unclear. Besides, emerging evidence showed that RSV could influence the expression of multiple miRNAs [16,17]. RT-PCR was performed to determine the expression of miR-542-3p in RSV-treated EPCs and the results showed that RSV could reduce miR-542-3p expression in EPCs (Figure 4A). Next, we altered the expression of miR-542-3p by transfecting miR-542-3p mimics into RSV-treated EPCs, respectively. Our data established that overexpression of miR-542-3p could reverse the effect of RSV on EPCs (Figure 4B–4D).

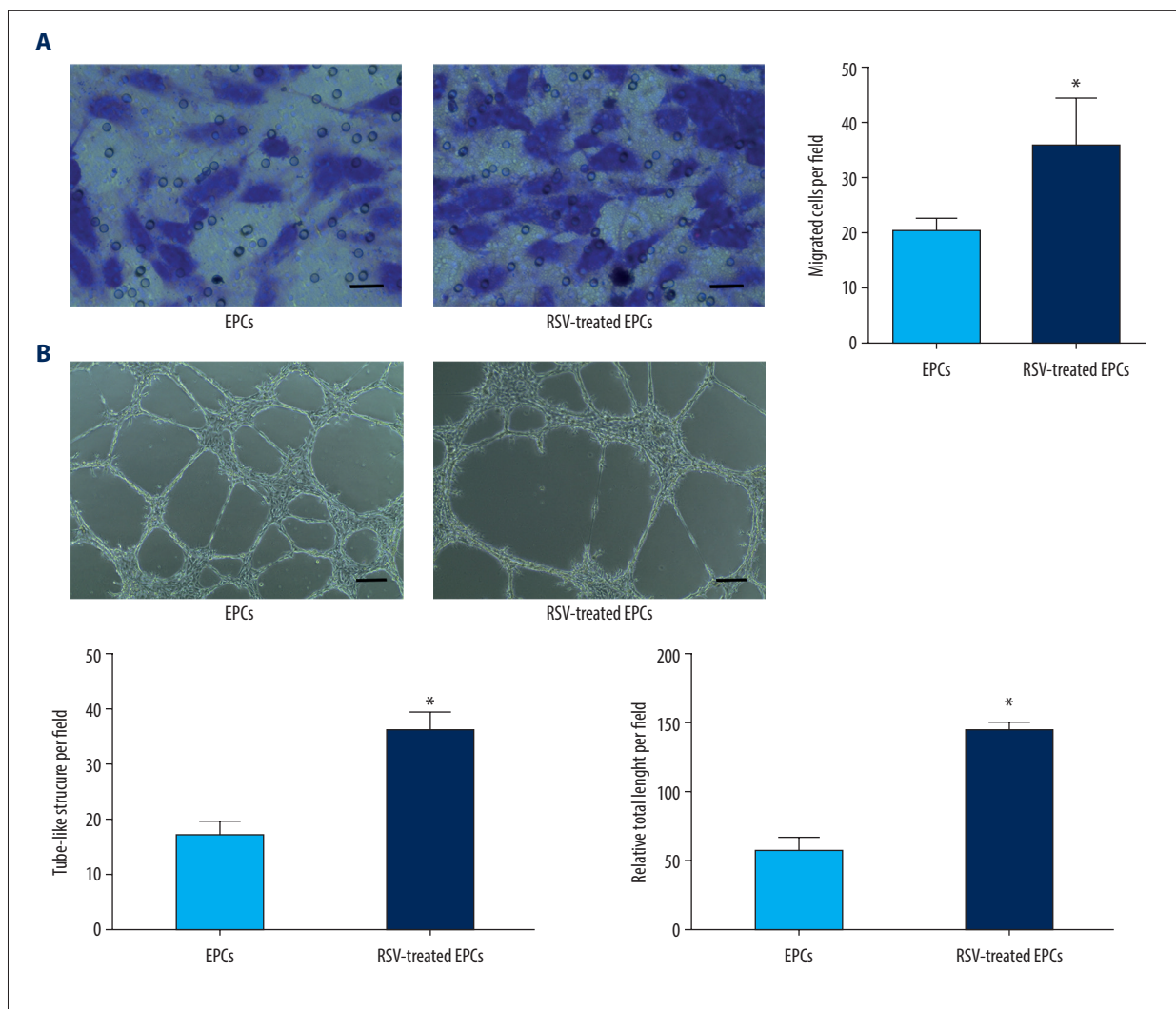


Figure 1. Identification of endothelial progenitor cells (EPCs). **(A)** Dil and UEA-1 staining of EPCs. **(B)** Flow cytometry analysis of CD34, CD31, VEGFR2 and vWF expression of EPCs.

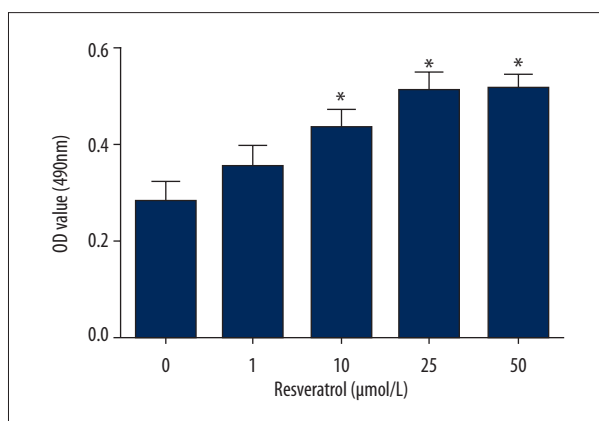


Figure 2. Endothelial progenitor cells viability at different concentration of resveratrol.

RSV regulated angiogenesis of EPCs via miR-542-3p/ANGPT2

It is known that miRNAs regulate the stability and translation of protein-coding mRNAs through binding to the 3'-untranslated region (3'UTR) of target mRNAs. Bioinformatic analysis was conducted to predict the potential target of miR-542-3p. Among the potential target genes, ANGPT2 was chosen for subsequent verification using luciferase assays for its widely function in tumor cells (Figure 5A). The results showed that miR-542-3p led to suppressed luciferase activity (Figure 5B). The data from western blot and RT-PCR also revealed that miR-542-3p regulated the expression of ANGPT2 on post-transcriptional level (Figure 5C, 5D). What's more, we found that decreased expression of ANGPT2 could also counteract the effect of RSV on EPCs (Figure 5E-5G). Above all, we concluded that RSV could regulate angiogenesis of EPCs via miR-542-3p/ANGPT2.

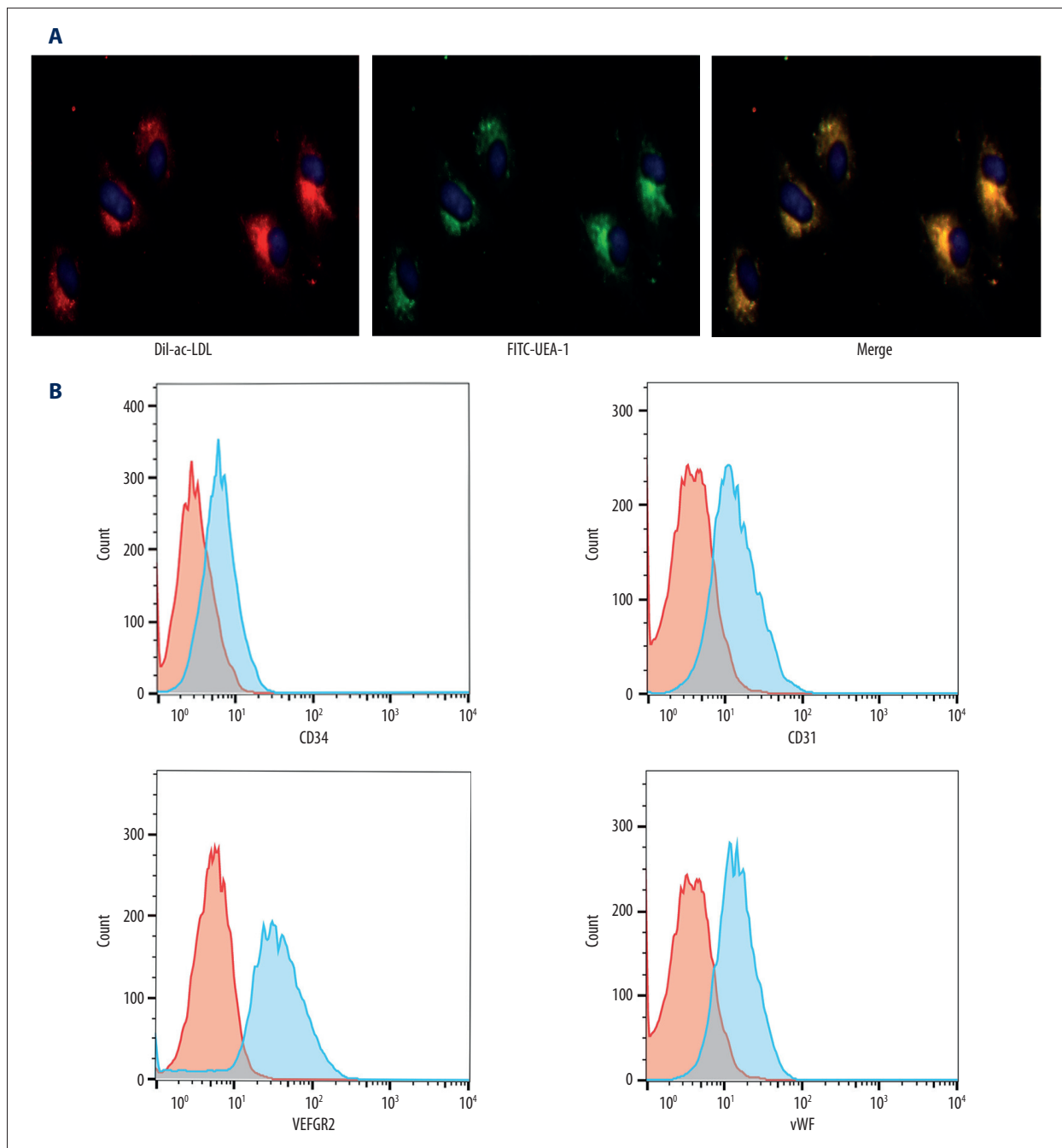


Figure 3. Resveratrol (RSV) regulates migration and tube formation of endothelial progenitor cells (EPCs). **(A)** Migrated cell counting (200 \times). * $P < 0.05$ versus RSV group ($n = 3$). **(B)** Relative structure-like tube number and length (200 \times). * $P < 0.05$ versus RSV group ($n = 3$).

RSV-treated EPCs promoted recanalization of venous thrombosis

Rat model of venous thrombosis was established and underwent RSV-treated EPCs, EPCs or saline injection via tail-vein, respectively. Before sacrifice, we performed venography to detect the recanalization of venous thrombosis in the inferior

vena cava. The more contrast medium was found in rats received RSV-treated EPCs compared to that in other groups (Figure 6A, 6B). Then the venous thrombi segments were harvested 7 days after cell transplantation. The weight of thrombi received RSV-treated EPCs was significantly lower than that in other groups (Figure 6C). Also, histological analysis revealed that more nucleated cells and channels in the RSV-treated

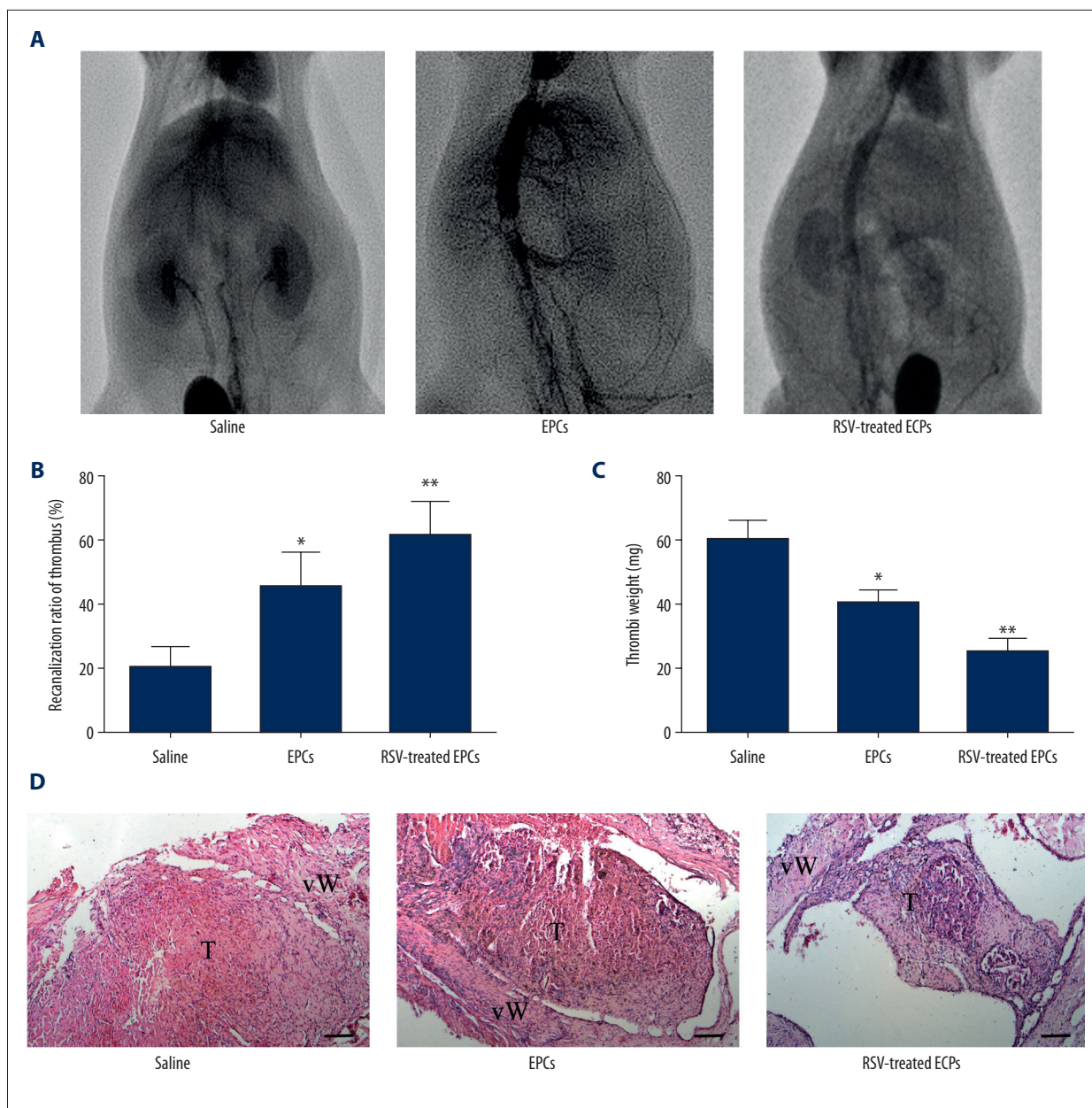


Figure 4. MiR-542-3p is involved in resveratrol-induced regulation on endothelial progenitor cells. **(A)** MiR-542-3p expression detected by quantitative real-time polymerase chain reaction. * $P < 0.05$ versus control group. **(B)** Migrated cell counting among different groups. * $P < 0.05$ versus control group. **(C)** Relative structure-like tube number. * $P < 0.05$ versus control group. **(D)** Relative structure-like tube length. * $P < 0.05$ versus control group.

EPCs groups (Figure 6D). Taken together, we established that RSV-treated EPCs promoted recanalization of venous thrombosis *in vivo*.

Discussion

The results from these experiments together demonstrate that resveratrol (RSV) induces endothelial progenitor cells

angiogenesis *in vitro*. Specifically, in line with data from *ex vivo* assays of tube formation and migration, RSV-treated EPCs contributed to recanalization of venous thrombosis *in vivo* rat models. Besides, we found that RSV could exert its influence via regulating miR-542-3p expression. Bioinformatic analysis further revealed that ANGPT2 was potential target of miR-542-3p and inhibition of ANGPT2 protein could reverse the effect of RSV on EPCs function. Epidemiological evidence has showed a significant lower incidence of cardiovascular

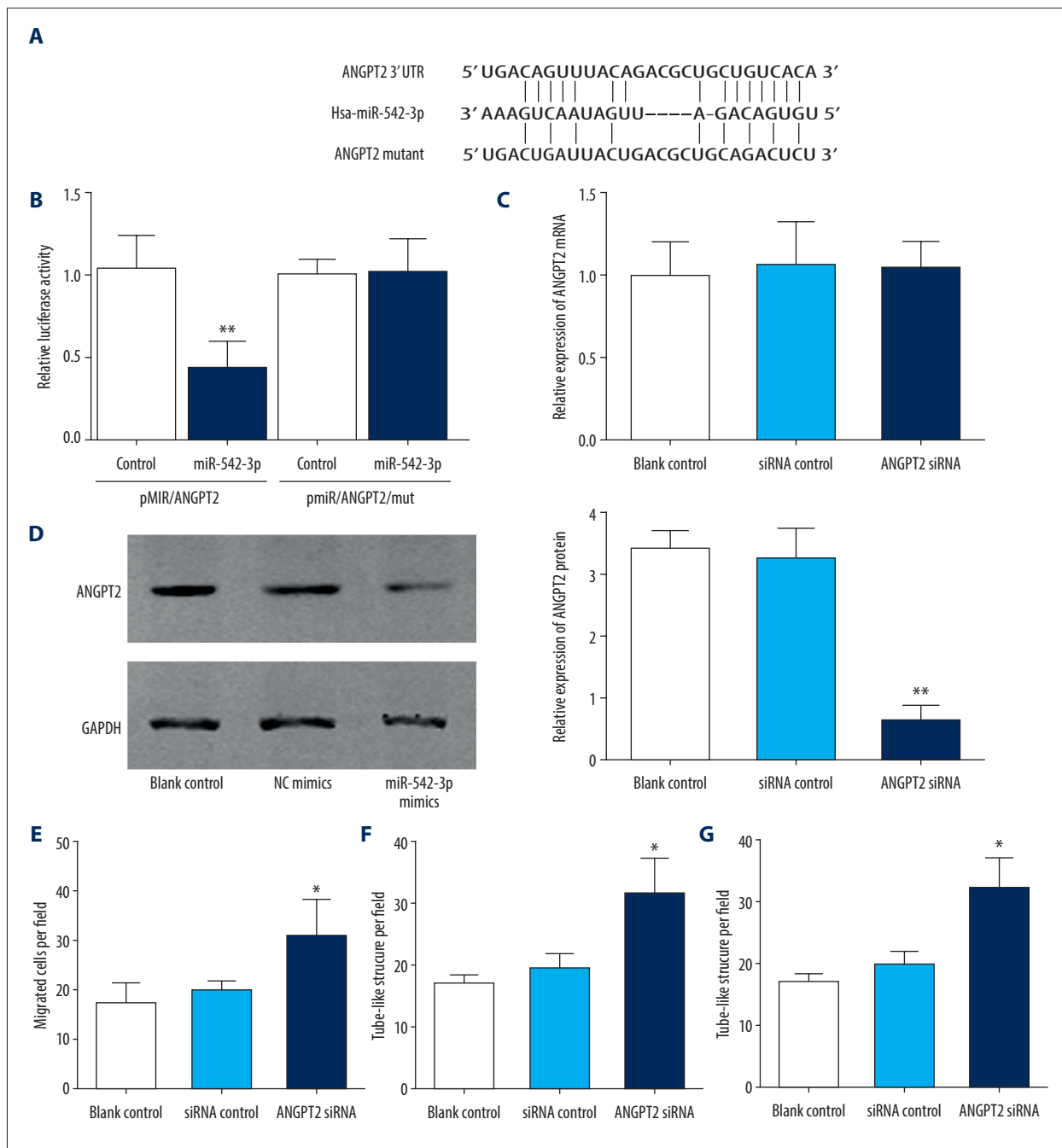


Figure 5. Resveratrol (RSV) regulated angiogenesis of endothelial progenitor cells (EPCs) via miR-542-3p/angiopoietin-2 (ANGPT2). **(A)** The potential binding site of miR-542-3p on ANGPT2 3'UTR region. ANGPT2 mutant indicates the ANGPT2 3'UTR with mutation in miR-542-3p binding site. **(B)** Luciferase activity in 293T cells cotransfected with either control or miR-542-3p together with a luciferase reporter plasmid carrying the wildtype or a mutated ANGPT2 3'UTR. Each bar represents mean values \pm standard error of the mean (SEM) (n=3), ** $P < 0.01$ versus control group. **(C)** ANGPT2 mRNA expression detected by quantitative real-time polymerase chain reaction. **(D)** ANGPT2 protein expression detected by western blot in EPCs transfected with NC mimics or miR-542-3p mimics, respectively. Each bar represents mean values \pm SEM (n=3), ** $P < 0.01$ versus control group. **(E)** Migrated cell counting among different groups. * $P < 0.05$ versus control group. **(F)** Relative structure-like tube number. * $P < 0.05$ versus control group. **(G)** Relative structure-like tube length. * $P < 0.05$ versus control group. All assays were performed in triplicate and repeated 3 times.

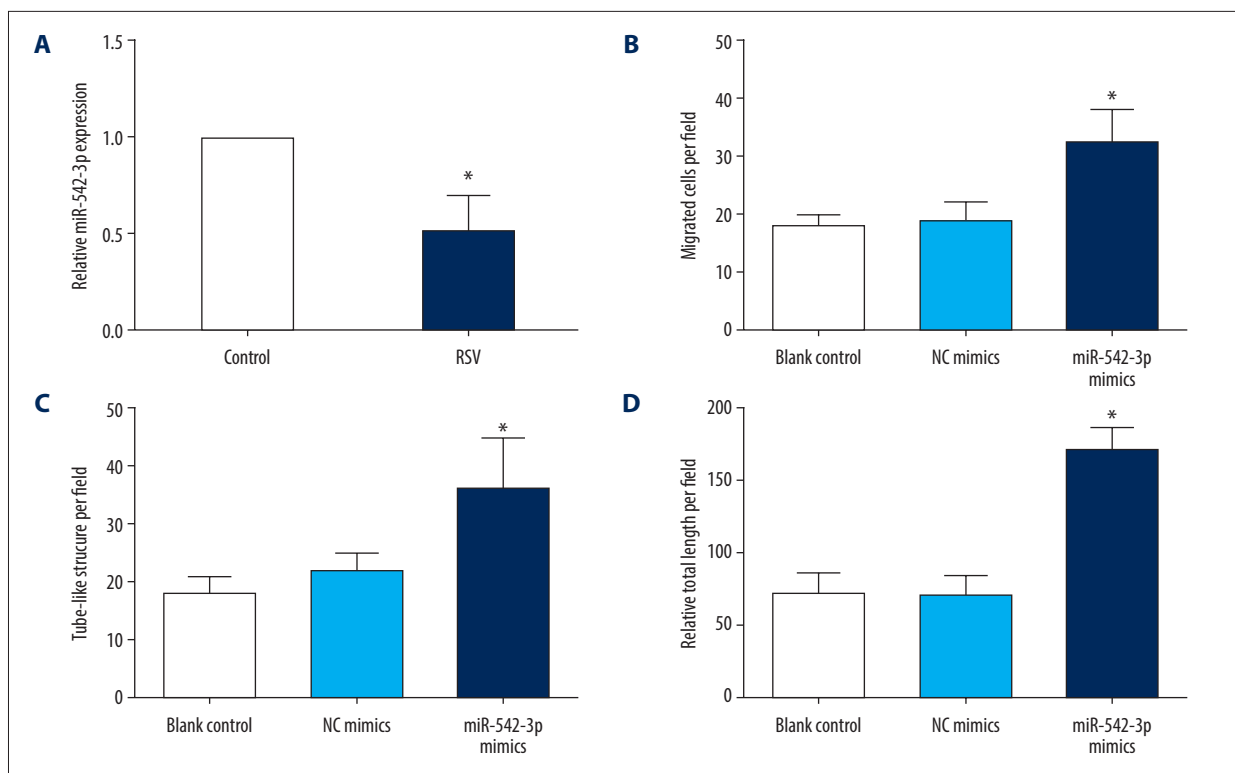


Figure 6. Resveratrol (RSV)-treated endothelial progenitor cells (EPCs) promoted recanalization of venous thrombosis. (A) Digital subtract angiography (DSA) images in rats transplanted with EPCs, RSV-treated EPCs or saline, respectively. (B) The recanalization of thrombus was quantified by the area ratio of contrast agent in vascular with thrombosis. Each bar represents mean values \pm standard error of the mean (SEM) (n=8), * $P < 0.05$ versus saline group; ** $P < 0.01$ versus saline group. (C) The thrombi weight at day 8 post model construction. Each bar represents mean values \pm SEM (n=8), * $P < 0.05$ versus saline group; ** $P < 0.01$ versus saline group. (D) Hematoxylin and eosin staining of thrombus section. (200 \times). T indicates thrombus and vW indicates venous wall.

diseases in individuals regularly drinking red wine [18], which referred to “French paradox”. It is partially due to the presence of RSV in the red wine. Previous studies have demonstrated RSV was able to prevent damage to endothelial cells and reduce neointimal formation after endothelial injury [6,7]. In addition, the results published by Gu et al. support our findings on the role of RSV in EPCs and confirmed its beneficial effect in the intima-injured rat models [19]. Endothelial progenitor cells were first reported by Asahara and coworkers in 1997 [20]. It was found that EPCs have the ability of migration towards injury site and incorporate into damaged vasculature *in vivo* [21]. Previous studies have also explored the potential role of EPCs in the context of various vascular-related disease animal models such as hind limb ischemia [22], myocardial infarction [23], and carotid artery injury [2]. Following these observations, we propose a novel mechanism of RSV on EPCs and subsequent therapeutic effect on venous thrombosis. The role of RSV in angiogenesis has been widely studied in tumor cells. Recent paper also revealed the effect of RSV on embryonic stem cells [24]. Xia et al. [25] reported that RSV reduced EPCs senescence through augmentation of telomerase

activity by Akt-dependent mechanism. Another study showed that RSV-treated EPCs contributed to reendothelialization in intima-injured rats [19]. In line with previous studies, we found the treatment of EPCs with RSV increased their angiogenic function.

MicroRNAs, as a class of ~22-nt non-coding RNAs, have been shown to participate in various biological events including cell proliferation, differentiation and aging. In our study, we found a novel role of miRNAs underlying the RSV-dependent regulation of EPCs. Our data showed that RSV repressed miR-542-3p expression in EPCs, leading to increased ANGPT2 expression. Furthermore, both inhibition of ANGPT2 protein level and overexpression of miR-542-3p could reverse the effect of RSV. This finding is consistent with the previously published papers showing aberrant expression of miR-542-3p in human cancers. Althoff et al. reported miR-542-3p exerted tumor suppressive functions in neuroblastoma [11]. Another study revealed that miR-542-3p suppressed osteoblast cell proliferation and differentiation [26]. In the present study, we found the downregulation of miR-542-3p contributed to angiogenesis

of EPCs. We also identified RSV regulated EPCs angiogenesis via miR-542-3p/ANGPT2. ANGPT2 is considered as a key regulator of tumor angiogenesis and progression. Previous study showed that inhibition of ANGPT2 inhibited tumor growth and metastasis [27]. In our study, ANGPT2 was identified as target of miR-542-3p with bioinformatic analysis and luciferase assay, which involved the regulation of RSV-treated EPCs function. In addition, our results suggest that RSV-treated EPCs contributed to recanalization of venous thrombosis in rat models. Venous thrombosis is a major global burden of disease.

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Conclusions

The resolution of venous thrombus refers to a complex process of organization of neovascular channels within the thrombus, which requires the orchestration of different cells [28]. Although previous evidence demonstrated the naturally ability of EPCs in resolving thrombi [29], dysfunction of EPCs in thrombi context limited its application in clinical. In our system, we established treatment with RSV promotes angiogenic ability of EPCs by inhibiting miR-542-3p and therefore increasing ANGPT2 expression, leading to better recanalization of venous thrombosis *in vivo*, which provide suggestions on RSV-treated EPCs as a therapeutic choice for thrombus resolution.