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Upregulation of MicroRNA-126 Contributes to Endothelial Progenitor Cell Function in Deep Vein Thrombosis via Its Target PIK3R2

Qingyou Meng,[1] Wenbin Wang,[2] Xiaobin Yu,[1] Wendong Li,[1] Lingshang Kong,[1] Aimin Qian,[1] Chenglong Li,[1]* and Xiaoqiang Li[1]*

[1]*Department of Vascular Surgery, the Second Affiliated Hospital of Soochow University, Suzhou 215000, China*
[2]*Department of General Surgery, the Fourth Affiliated Hospital of Anhui Medical University, Hefei 230022, China*

ABSTRACT

Deep vein thrombosis (DVT) is a common complication of surgery. Endothelial progenitor cells (EPCs) are recruited into resolving venous thrombi. In this report, we investigated the effects of miR-126 on EPCs function and venous thrombus resolution. We demonstrated that overexpression of miR-126 enhanced EPCs′ migration and tubulogenic activity in vitro, and promoted EPCs′ homing and thrombus resolving in vivo. Moreover, we identified that miR-126 directly targeted PIK3R2 and affected PI3K/Akt signaling axis. Overall, our findings demonstrated that miR-126 promoted EPCs function through suppressing PIK3R2 expression and modulation of miR-126 may represent a potential therapeutic intervention for treating DVT. J. Cell. Biochem. 116: 1613–1623, 2015.

KEY WORDS: miR-126; EPCs; MIGRATION; HOMING; THROMBUS RESOLUTION

Deep vein thrombosis (DVT), which may lead to pulmonary hypertension, recurrent thrombosis, or even the fetal pulmonary embolism (PE), is one of the common complications of surgery. More than one-third of patients with DVT will develop post-thrombotic syndrome (PTS), and 5–10% of patients develop severe PTS, which can manifest as venous ulcers [Kahn et al., 2008]. Currently, the major preventive agents for DVT are prophylactic anticoagulants, such as low molecular weight heparin (LMWH) and direct Xa inhibitor (rivaroxaban), which could reduce the incidence of venous thromboembolism to 3.7% and 1.1% [Eriksson et al., 2008]. However, they would also increase the risk of bleeding and wound complications.

Stem cell therapy is a promising novel therapy for patients with ischemic diseases. The discovery of marrow-derived circulating endothelial progenitor cells (EPCs) cause people′s great interest because of their plasticity to differentiate into endothelial cells (ECs) and as a source of paracrine proangiogenic factors [Asahara and Isner, 2002]. It has been proven that EPCs are recruited into resolving venous thrombi [Modarai et al., 2005]. In our previous studies, the results also showed exogenous EPCs transplantation could significantly improve the microenvironment in venous thrombus and promoted the resolution of thrombus [Li et al., 2007; Meng et al., 2010; Lei et al., 2012]. However, studies demonstrated that EPCs recruitment to sites of neovascularition was limited due to the weak viability, migration and homing ability [Tongers et al., 2011]. Therefore, looking for a method to improve the function of EPCs has gained abroad attention.

MicroRNAs (miRs) are an emerging class of highly conserved, noncoding small RNAs that regulate gene expression at the posttranscriptional level by inhibiting protein translation or by

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*Correspondence to: Chenglong Li and Xiaoqiang Li, Department of Vascular Surgery, the Second Affiliated Hospital of Soochow University, No. 1055, Sanxiang Rd., Suzhou 215000, Jiangsu, China. E-mail: nihao229@163.com (XL) or lcljm11@163.com (CL)

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promoting mRNA degradation [Bartel, 2004]. Recently, evidence supporting a role for endothelial miRs in the control of neovascularization has been provided [Urbich et al., 2008; Mriouah et al., 2012]. miR-126, a newly discovered miRNA, is one of the most frequent miRNA isolated in vascular ECs and plays an important role in maintaining endothelial cell proliferation, migration, and tubulogenic activity [Landskroner-Eiger et al., 2013]. Knock-down of miR-126 in zebra fish caused the blood vessels collapse and hemorrhage [Fish et al., 2008]. Likewise, miR-126-deficient mice exhibited the defects in the integrity of vessel, hemorrhages and partial embryos lethality [Wang et al., 2008]. These findings suggested that EPCs and miR-126 both have proangiogenic properties. However, the biological function and correlation between miR-126 and EPCs in recanalization of venous thrombus remained to be further elucidated.

In this study, we discovered that miR-126 enhanced EPCs migration and tubulogenic activity, by targeting PIK3R2 directly. The mechanism of miR-126 effecting on EPCs is through PI3K/Akt signal pathway. In addition, we firstly demonstrated the proangiogenic role of miR-126 in the process of venous thrombus resolution in rat DVT models, by regulating EPCs. Based on the results in this research, modulation of miR-126 may represent a potential therapeutic intervention in EPC-mediated angiogenesis in DVT.

MATERIALS AND METHODS

ANIMALS

Male Spraque-Dawley rats were purchased from the Experiment Animal Center of Soochow University (Suzhou, China). The animals were maintained under a conventional state (12:12 h light–dark cycle, temperature 25°C) and provided with standard laboratory food and water. The University of Soochow Institutional Animal Care and Use Committee approved all the procedures.

ISOLATION AND IN VITRO CULTURE OF EPCs

Isolation, ex vivo expansion and culture of EPCs were performed as previously described [Li et al., 2007; Meng et al., 2010; Lei et al., 2012]. Briefly, bone marrow was harvested from both femurs and tibias of 3–4 weeks male SD rats. Mononuclear cells (MNCs) were fractionated by density gradient centrifugation with Ficoll-paque (GE Healthcare, Piscataway, NJ). After two washing steps, cells were cultured in endothelial basal medium-2 (Lonza) supplemented with EGM-2MV single aliquots (Lonza) containing vascular endothelial growth factor (VEGF), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor, ascorbic acid, hydrocortisone, gentamycin, amphotericin-B, and fetal bovine serum. On the fourth day, nonadherent cells were removed by washing with PBS, and fresh medium was added. The adherent cells cultured in EGM-2MV showed a spindle shape on Day 7. The adherent cells were confluent on Day 14, and passaged continuously. The second and third generations of cultured EPCs were used for our research.

IN VITRO TRANSFECTION

miR-126 mimics (miR10000832-1-5), inhibitor (miR20000832-1-5), PIK3R2siRNA (Q0000029741-1-A), and their negative control (NC) were purchased from Ribobio Corporation (Guangzhou, China). Transfection was performed by electroporation system according to the instruction of manufacture (Bio-Rad, Hercules, CA). Briefly, EPCs in EGM-2-MV medium without FBS and growth factors were resuspended at a density of 5×10^7 cells/ml. 100 μl EPCs mixed with 120 pmol miR-126 mimics or 300 pmol inhibitors or 300 pmol siRNAs transferred into cuvettes and the loaded cuvettes were placed on ice. Voltage on the Gene-Pulser II was set at 180 V and 25 ms. EPCs were electroporated two times, and then transferred to culture dish in complete EGM-2-MV medium. The electroporated cells were incubated at 37°C in 5% CO_2. After 48 h of transfection, cells were harvested for further analysis. Transfection efficiency was monitored by the transfection of GMR-miRTM microRNA FAM labeled microRNA control (Ribobio, Fig. S1).

SCRATCH WOUND ASSAY

Cells transfected with miR-126, siRNA, or NC oligonucleotides as aforementioned were harvested and loaded into 24-well plate at a density of 4×10^6 cells/well. Following incubation for 24 h, cells reached 100% confluence and formed a monolayer. Then, a linear scratch was made using a 200 μl pipette-tip on cell monolayer. Wash the plate once with PBS and replace with the serum-free medium. Several images were taken at 0, 24, 48, and 72 h at 40× magnification and the wounding size was measured in three wells of each group.

MIGRATION CHAMBER ASSAY

To further validate EPC migration ability, migration chamber assay was performed by using transwells chamber with 8.0 μm pore size (Corning Costar, Cambridge, MA). Briefly, EPCs subjected to electroporation with NC, miR-126, or siRNA oligonucleotides were harvested 48 h after transfection and resuspended in serum-free EBM medium. A total of 3×10^5 EPCs were loaded into the upper chambers. The lower chamber was filled with the medium containing 20% FBS. After 24 h of incubation, the non-migrated cells were wiped away from upper side of the membrane. The migrated cells were fixed with crystal violet stain and photographed for counting at 200× magnification. Migration activity was evaluated as the mean number of migrated cells in three random microscopic fields (200×) per chamber. All groups were performed in triplicate.

TUBULE FORMATION ASSAY

The in vitro angiogenic activity of EPCs was determined by matrigel tube formation assay. Briefly, EPCs transfected with miR-126, siRNA, or NC oligonucleotides were seeded onto matrigel- (BD Bioscience) coated 48-well plate in EBM medium at a density of 5×10^4 cells per well. After 15 h of incubation, tube formation was observed under light microscope. Images of tube morphology were taken in five random microscopic fields per sample at 100× magnification. The cumulative mean of the tube lengths and tube numbers were measured by the JEDA801D pathology microscope image analysis system.

VECTOR CONSTRUCTION

To stably express miR-126 in EPCs, the lentiviral expression vector pLVX-IRES-ZsGreen-miR-126 (Clontech Laboratories) was constructed. The 403 bp segment including the mature miR-126 sequence

and its 142 bp 5′ and 188 bp 3′-flanking regions were PCR-amplified from genomic DNA and subcloned into *Eco*R1 and *Bam*H1 sites of pLVX-IRES-ZsGreen vector.

LENTIVIRUS PRODUCTION AND CELLS TRANSDUCTION

293T cells were plated in the dish and reached a confluence of ~70% after 20 h. The pLVX-IRES-ZsGreen vector or pLVX-IRES-ZsGreen-rno-miR-126 plasmid was then transfected into 293T cells along with pCMV Δ8.91 and pMD.G using Lipofectamine 2000 (Invitrogen, Paisley, Scotland, UK). The supernatant containing the lentivirus was harvested at 72 h and filtered through a 0.45 μm low protein binding polysulfonic filter (Millipore, Bedford, MA). EPCs, inoculated in advance in 75 cm^2 flask and presenting with ~10% confluence after 24 h, were infected with 1 ml lentivirus suspension at a multiplicity of infection (MOI) of 10 in the presence of 8 μg/ml polybrene (Chemicon, Temecula, CA). Green fluorescence was observed to indicate the transduction efficiency at 48 h post-transduction. Positive cells were selected for GFP expression by FACS-sorting and named EPCs/pLVX-IRES-ZsGreen-miR-126 and EPCs/pLVX-IRES-ZsGreen Vector respectively. miR-126 expression was confirmed by qRT-PCR.

RAT MODEL OF VENOUS THROMBOSIS AND CELLS THERAPY

This model has been widely used by our group for the study of venous thrombosis as described previously with minor modifications [Li et al., 2007; Meng et al., 2010]. In brief, male SD rats (250–300 g) purchased from the Experiment Animal Center of Soochow University were anesthetized and a midline laparotomy was performed. The inferior vena cava (IVC) below the renal veins was exposed and all side branches are ligated with nonreactive 7-0 Prolene suture. Caudal to the left renal vein, a 7-0 Prolene suture is tied down on the IVC. This model provided a total stasis environment leading to thrombosis and proven valuable in the study of vein thrombus [Diaz et al., 2012]. Based on our group's observations, the survival rate for this model is around 95%. On the third day after IVC ligation, the alive rats were divided as three groups for cells transplantation via tail intravenous injection: A (n = 12), blank control group (blank control), which received 1 ml cell culture medium; B (n = 12), EPCs/pLVX-IRES-ZsGreen Vector group (EPCs/vector), which received 1.0×10^6 EPCs transfected with lentivirus particle of pLVX-IRES-ZsGreen vector; C (n = 12), EPCs/pLVX-IRES-ZsGreen-miR-126 group (EPCs/miR-126), which received 1.0×10^6 EPCs transfected with lentivirus particle of pLVX-IRES-ZsGreen-miR-126.

TISSUE HARVESTING AND HISTOLOGY

On the 7th and 14th day post-operation, the rats were sacrificed with overdose of 10% chloral hydrate. Firstly, the IVC with thrombus was carefully removed, and absorbed excess blood with filter paper. The thrombi were weighed at once. Then, the samples were placed into 4% paraformaldehyde overnight. Next day, a graded ethanol series were used to dehydrate the samples. Whereafter, all of the samples were treated by dimethylbenzene and embedded in paraffin. In order to observe thrombus organization and recanalization, hematoxylin and eosin (HE) staining and immunohistochemical staining for CD34 and MMP-2 were performed.

CONSTRUCTION OF PLASMIDS AND LUCIFERASE ACTIVITY ASSAY

A 501-bp full-length fragment of the wild-type (wt) PIK3R2-3′UTR or mutant PIK3R2-3′UTR (mut) containing the putative miR-126 binding site were synthesized (Sangon, Shanghai, China). After digestion by *Spe*I and *Hin*dIII, the fragments of wt and mutant PIK3R2-3′UTR were cloned into the *Spe*I and *Hin*dIII sites of pMIR-Report luciferase vector (Applied Biosystems) and named pMIR/PIK3R2/wt and pMIR/PIK3R2/mut, respectively. HEK 293T cells were seeded in 24-well plates 24 h prior to transfection. In each well, cells were transfected with 50 ng of either pMIR/PIK3R2/wt or pMIR/PIK3R2/mut, together with 5 ng of the pRL-TK vector (Promega, Madison, WI) containing Renilla luciferase and 100 nM of the miRNA oligonucleotides. Transfection was performed by Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured by using dual-luciferase reporter assay (Promega) after 48 h post-transfection. Firefly luciferase activity was normalized to Renilla luciferase activity.

WESTERN BLOT ANALYSIS

EPCs were lysed using M-PER reagents and Halt Protease Inhibitor Cocktail kits (Pierce, Appleton, WI) after 48 h post-transfection. The protein concentrations of the lysates were measured by BCA Protein Assay Kit (Pierce). Then the lysates were fractionated by 10% SDS–PAGE and transferred to PVDF membranes. The membranes were blocked with TBST buffer containing 5% non-fat milk and then hybridized with following specific antibodies: PIK3R2 (1:1,000, sc-131324, Santa Cruz Biotechnology), PI3K (1:1,000, sc-7177, Santa Cruz Biotechnology), total AKT (1:1,000, cs-9272, Cell Signaling), phospho-AKT (1:1,000, cs-9271, Cell Signaling) or β-actin (1:5,000, Kangchen). After rewarming and washing, the membranes were incubated with appropriate secondary antibodies. Labeled bands were detected using the ECL chemiluminescent kit (Pierce). β-Actin was used for loading control.

STATISTICAL ANALYSIS

The values were presented as mean ± SD. The differences between groups were assessed by one-way ANOVA when there were more than two groups, or analyzed using Student's *t*-test, when there were only two groups. All statistical analyses were performed using SPSS 15.0 software. A two-tailed value of $P < 0.05$ was considered statistically significant.

RESULTS

miR-126 HAS EFFECT ON IN VITRO MIGRATION OF EPCs

In order to detect the effect of miR-126 on the motility of EPCs, wound healing assay and migration chamber assay were performed. As shown in Figure 1, the relative wound size of EPCs transfected with NC or miR-126 oligonucleotides was analyzed at 0, 24, 48, and 72 h post-wounding. Compared with NC mimics, miR-126 mimics significantly enhanced the migration of EPCs across the wound space at 24, 48, and 72 h (64.2 ± 4.5% vs. 76.3 ± 6.5%, 75.1 ± 5.7% at 24 h, $P = 0.039$; 29.9 ± 5.3% vs. 41.8 ± 4.0%, 44.5 ± 6.7% at 48 h, $P = 0.036$; 8.1 ± 1.8% vs. 20.4 ± 3.4%, 19.1 ± 3.8% at 24 h, $P = 0.003$, Fig. 1A,B). In contrast, miR-126 inhibitor significantly

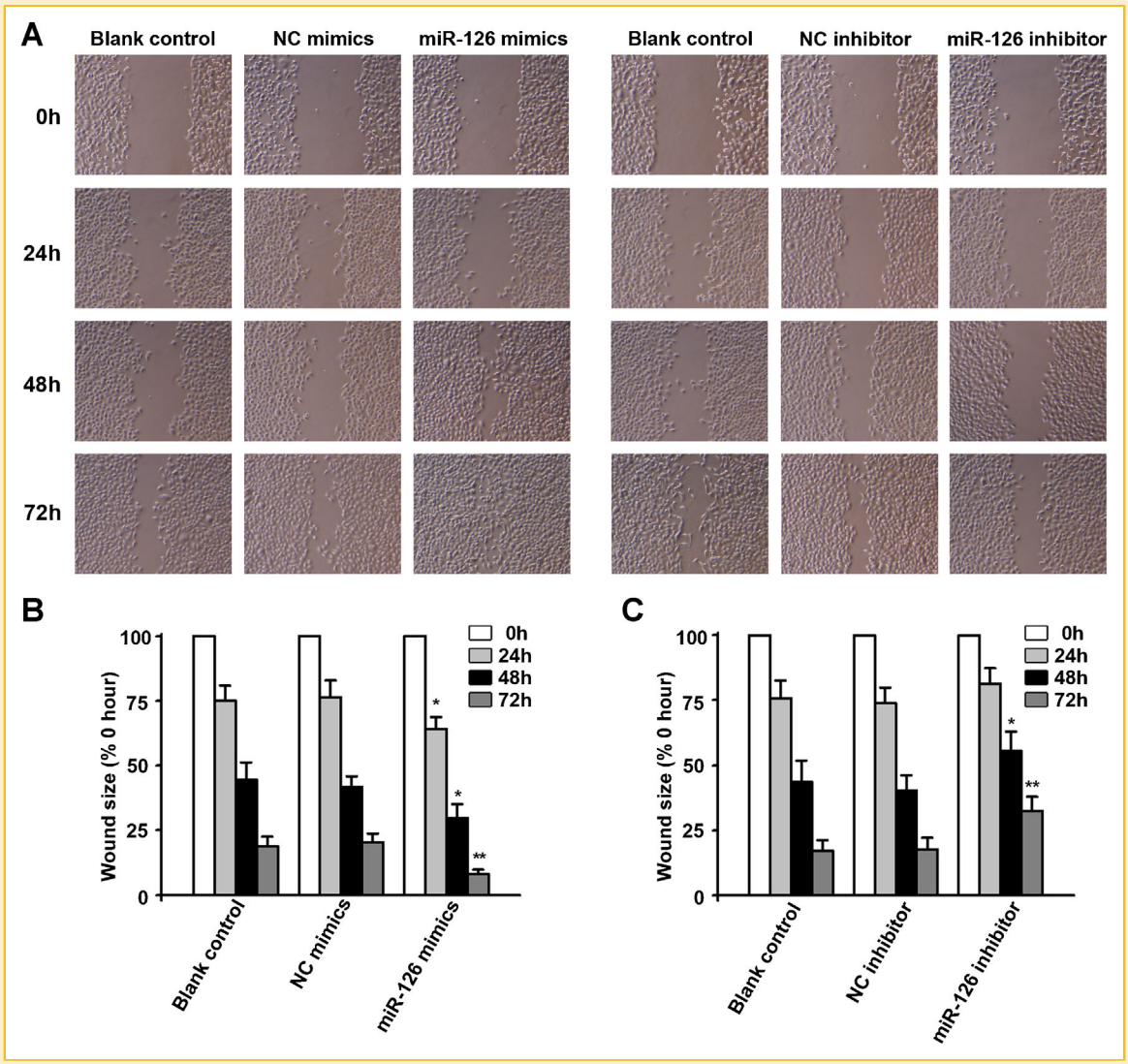

Fig. 1. Effect of miR-126 on wound healing assay of EPCs. A: Representative micrographs were shown in the up panel (magnification 40×). Wound closure was monitored by microscopy at the indicated times. B–C: Statistical analysis of relative wound size is shown in the below panel. The symbols * and ** denotes significant statistical difference of $P < 0.05$ and $P < 0.01$ relative to NC.

suppressed the migration of EPCs at the same time ($81.3 \pm 5.9\%$ vs. $73.9 \pm 6.0\%$, $75.8 \pm 6.8\%$ at 24 h, $P = 0.195$; $55.5 \pm 7.5\%$ vs. $40.4 \pm 5.8\%$, $43.8 \pm 8.1\%$ at 48 h, $P = 0.042$; $32.5 \pm 5.5\%$ vs. $17.7 \pm 4.5\%$, $17.3 \pm 3.9\%$ at 24 h, $P = 0.009$, Fig. 1A,C). As shown in Figure 2, consistent with the wound healing assay, migration chamber assay also showed that miR-126 mimics significantly promoted EPCs migration into the chamber membrane compared with control group (55.5 ± 12.8 vs. 32.1 ± 9.9, 34.7 ± 9.3 $P = 0.037$, Fig. 2B), and vice versa (15.8 ± 4.3 vs. 37.5 ± 11.5, 38.7 ± 12.6 $P = 0.04$, Fig. 2C).

miR-126 HAS EFFECT ON IN VITRO ANGIOGENESIS OF EPCs

In order to explore whether miR-126 affected the angiogenic activity of EPCs in vitro, matrigel tube formation assay was employed. After 15 h of incubation, EPCs transfected with miR-126 mimics showed a significant improvement of capillary tube formation compared with NC ($P < 0.05$). In contrast, miR-126 inhibitor-infected cells showed a significant impairment of capillary tube formation compared with NC ($P < 0.05$) (Fig. 3). The above results suggested that miR-126 played an important role in the angiogenic process.

miR-126 IMPROVES IN VIVO HOMING OF EPCs

Studies have shown that EPCs can be mobilized from bone marrow, migrate to ischemic tissue, and contribute to ischemia-induced neovascularization [Wassmann et al., 2006; Meng et al., 2013]. It is also proven that EPCs are recruited into resolving venous thrombi [Moldovan and Asahara, 2003; Modarai et al., 2005]. Our preceding observations demonstrated that miR-126 affected migration and tubule formation of EPCs in vitro. Furthermore, we would test whether overexpression of miR-126 could promote EPCs homing and

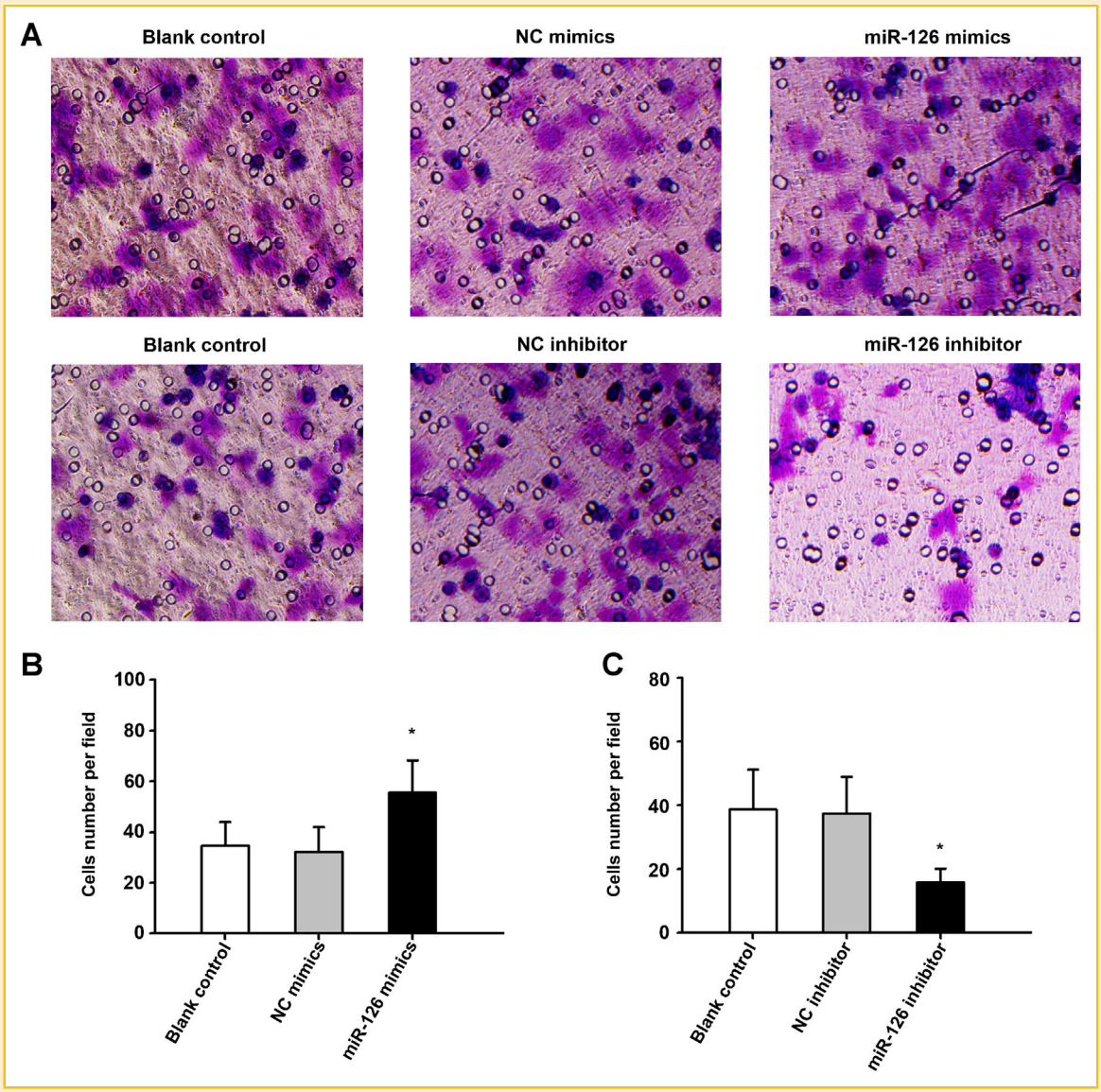

Fig. 2. Effect of miR-126 on migration chamber assay of EPCs. A: Representative pictures of migrated EPCs in four groups (magnification 200×). Cell migration was analyzed at 24 h following the cell inoculation in the chamber. B: The number of migrated EPCs was increased in miR-126 mimic group. C: The number of migrated EPCs was decreased in miR-126 inhibitor group. The symbol * are regarded as significant difference with $P < 0.05$ relative to NC.

thrombus resolution in vivo. We successfully constructed the model of rat DVT by ligaturing the infrarenal IVC. Lentiviral vector expressing miR-126 or empty vector was respectively transfected into EPCs and the EPCs were injected to rats′ models of DVT. On the day of 7 and 14 post-operations, the rats were sacrificed and the histological study was performed. Because GFP was expressed on the transplanting EPCs, we could observe EPCs homing in section by using fluorescence microscope. As shown in Figure 4A, transplanted EPCs were recruited into the thrombus in both EPCs/vector and EPCs/miR-126 group. In contrast, no GFP was observed in control group. We can also find that the larger number of GFP positive cells was appeared in EPCs/miR-126 group at Day 7 and 14 compared with EPCs/vector group (154.2 ± 37.7 vs. 243.6 ± 59.3, $P = 0.042$ at Day 7; 429.4 ± 63.7 vs. 582.5 ± 81.9, $P = 0.009$ at Day 14, Fig. 4A,B). The results indicated that miR-126 enhanced the homing of EPCs in rat DVT.

miR-126 INHIBITS THROMBUS FORMATION AND PROMOTES THROMBUS ORGANIZATION, RECANALIZATION AND RESOLUTION

Firstly, we observed whether miR-126 had effect on thrombus formation. As shown in Figure 4D, miR-126 significant decreased the weight of venous thrombus (IVC + thrombus at harvest). Compared with control group, the weight of thrombus decreased in EPCs/vector and EPCs/miR-126 groups at Day 7 and 14. Furthermore, the thrombus weight of EPCs/miR-126 group was even lower than that of EPCs/vector group (34.6 ± 8.9 mg vs. 48.7 ± 13.5 mg, 67.3 ± 15.3 mg

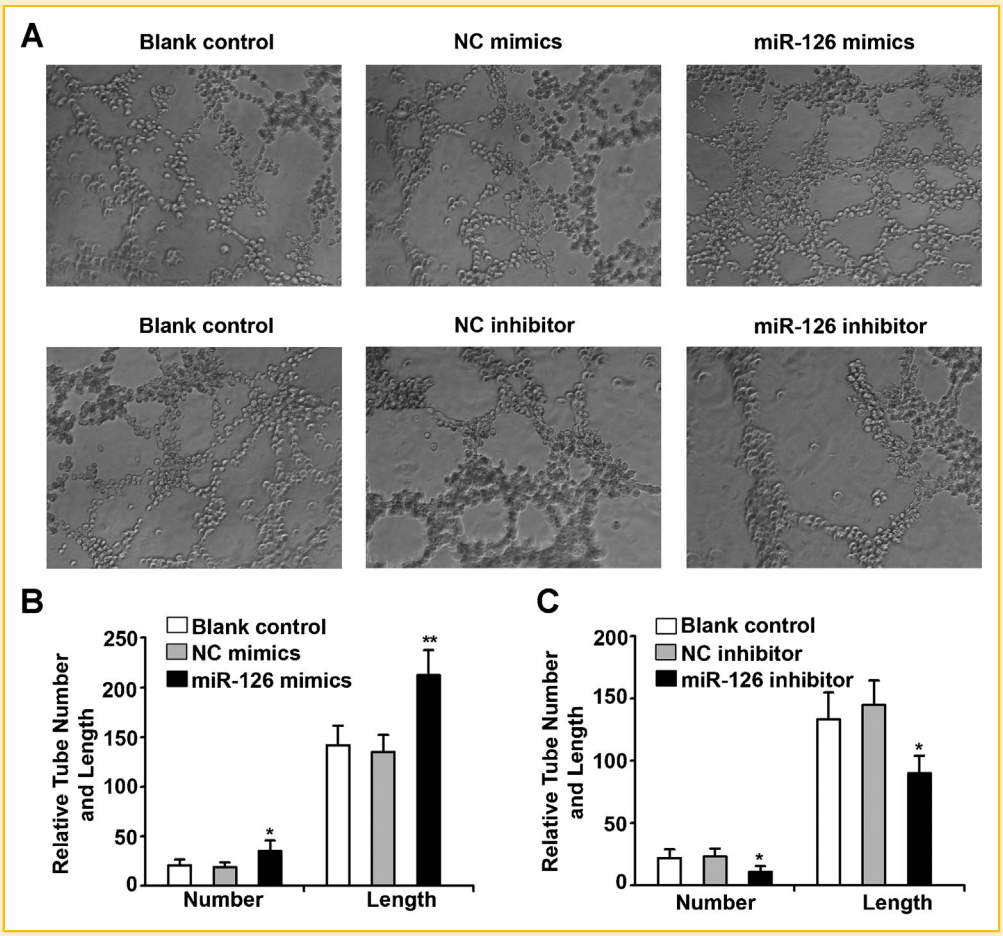

Fig. 3. Effect of miR-126 on Matrigel tube formation assay of EPCs. A: Representative micrographs for in vitro angiogenesis are shown (magnification 100×). The effects of miR-126 in EPCs to form capillary tubes in Matrigel assay were determined 15 h after seeding. B: The relative tube number and length were increased in miR-126 mimic group. C: The relative tube number and length were decreased in miR-126 inhibitor group. The symbols * and ** are regarded as significant difference with $P < 0.05$ and $P < 0.01$ relative to NC.

at Day 7, $P = 0.041$; 18.9 ± 6.4 mg vs. 29.6 ± 8.5 mg, 52.3 ± 14.1 mg at Day 14, $P = 0.045$). Therefore, the data implied that the thrombus formation was inhibited by miR-126 in vivo.

It has been proven that venous thrombi resolution by a process that is similar to the formation of granulation tissue in wound healing [Wakefield et al., 1999]. Both of thrombus organization and recanalization are involved in the dynamic and complex process. In order to detect the extent of venous thrombus organization, the sections were subjected to HE staining. As shown in Figure 4C, nucleated cell, including ECs, monocytes, and neutrophil granulocytes entering into the perimeter of the thrombus at Day 7, indicated the early organization occurred. Compared with the hyperchromatic cells dried blue, the red blood cells, platelets, and fibrin were dried red in the center of thrombus. No matter at Day 7 or 14, there were more nucleated cells and channels in EPCs/vector and EPCs/miR-126 group in contrast with control group. Meanwhile, EPCs/miR-126 group showed a larger scale of organization in comparison with EPCs/vector group, which displayed more channels and nucleated cells appearing.

Furthermore, the newborn capillaries in venous thrombus demonstrate the recanalization of thrombus. To validate this phenomenon, CD34, a common cell-surface marker for capillary ECs, was detected using immunohistochemical staining. As shown in Figure 5A, ECs was specifically dried brown, and distinguished from other nucleated cells. Consistent with HE staining, the newborn capillaries were most located at the perimeter of the thrombus at Day 7. Compared with control group, more small vessels were formed in EPCs/vector and EPCs/miR-126 group at Day 7. By day of 14, a large number of blood vessels appeared and some of vessels converged into larger blood vessels in both EPCs transplanted groups. In addition, no matter at Day 7 or at Day 14, there were more and larger vessels formed in EPCs/miR-126 group compared with EPCs/vector group. As shown in Figure 5B, we calculated the number of newborn capillary in thrombus. The results showed exogenous EPCs contributed to capillary vessels formed and miR-126 improved the proangiogenic properties of EPCs.

It has been proven that DVT resolution involves profibrotic growth factors, collagen deposition, matrix metalloproteinase (MMPs) expression, and activation [Wakefield et al., 2008]. To investigate the molecular mechanisms underlying the increased thrombus resolution ability of EPCs overexpressing miR-126, we immunohistochemically

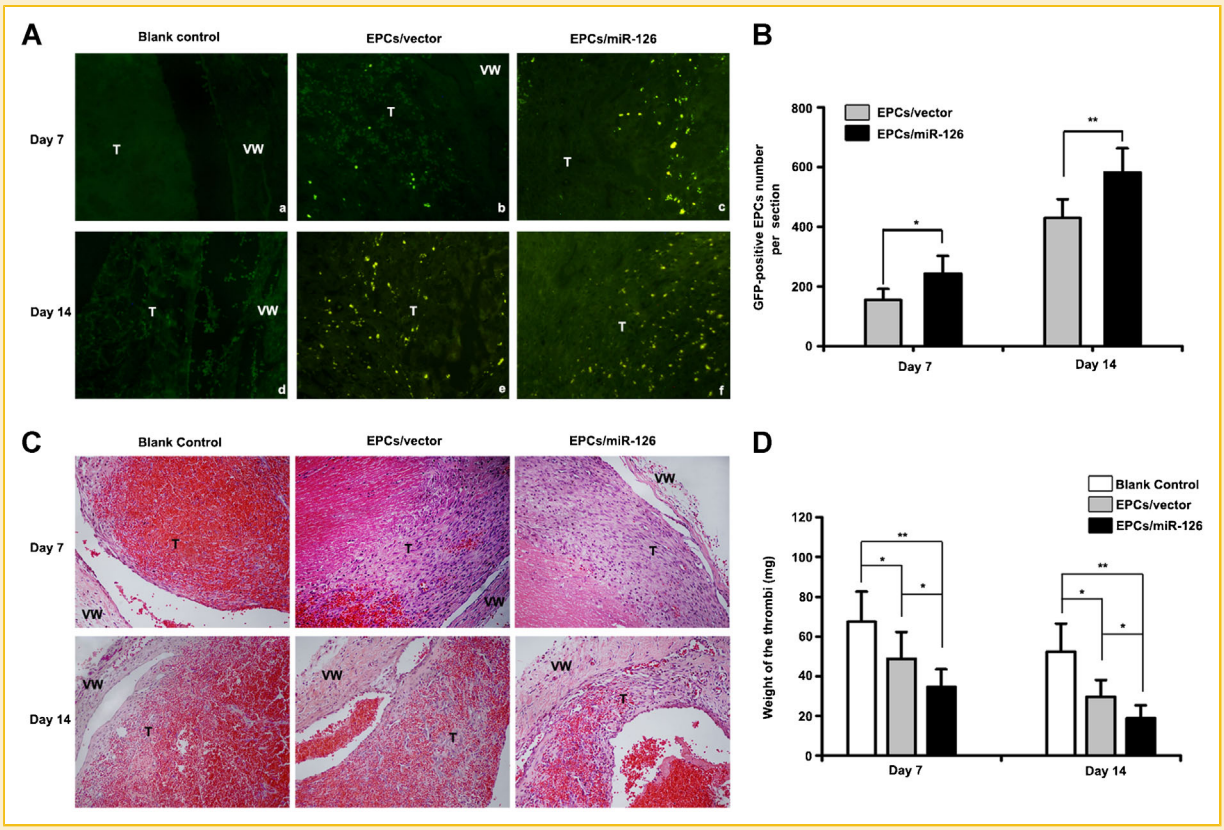

Fig. 4. miR-126 promotes EPCs homing and thrombus organization, and inhibits thrombus formation in vivo. A: Representative images of GFP-positive EPCs recruitment in the deep venous thrombus. T indicates thrombus and VW means venous wall. The pictures are with magnification of 200×. B: Quantification of GFP-positive EPCs in thrombus sections. C: Representative images of HE staining for the thrombus sections are presented with original magnification of 200×. T means thrombus and VW indicates venous wall. D: Weight of the venous thrombi 7 days and Day 14 post-operation. Data are expressed as mean ± SD. The symbols * and ** are regarded as significant and great significant difference with $P < 0.05$ and $P < 0.01$, respectively.

examined the expression of MMP-2 in venous thrombus. As shown in Figure 5C,D, compared with control group, more MMP-2-positive cells could be observed in EPCs/vector and EPCs/miR-126 group at Day 7 and 14. Additionally, compared with EPCs/vector group, the larger number of MMP-2-positive cells were appeared in EPCs/miR-126 group at Day 7 ($P = 0.048$) and Day 14 ($P = 0.021$).

PIK3R2 IS A TARGET GENE OF miR-126

After an extensive review of online microRNA database (that is, TargetScan, Microrna.org and miRanda), we selected PIK3R2 as the candidate target gene of rno-miR-126 in EPCs. To validate this hypothesis experimentally, we first performed luciferase reporter assays to verify a direct interaction between miR-126 and the 3′UTR of PIK3R2. Luciferase reporters were constructed containing either a wt PIK3R2 3′UTR sequence (pMIR/PIK3R2/wt), or a mutated PIK3R2 3′UTR (pMIR/PIK3R2/mut) (Fig. 6A,B). We assessed luciferase activity by co-transfecting the luciferase reporter vectors with the miR-126 mimics, inhibitor or NC. Luciferase activity of pMIR/PIK3R2/wt was markedly decreased in cells transfected with miR-126 mimics, compared to luciferase activity of pMIR/PIK3R2/mut ($P = 0.006$, Fig. 6C). Conversely, the luciferase activity of reporter plasmid was not interfered after transfection with miR-126 inhibitor. This result strongly indicated that 3′UTR of PIK3R2 carries the direct binding sites of rno-miR-126.

Furthermore, to determine miR-126 down-regulated PIK3R2, we detected PIK3R2 expression by Western blot assays. As shown in Figure 6D, miR-126 mimics decreased the expression of PIK3R2 compared to NC mimics ($P = 0.008$); while miR-126 inhibitor increased PIK3R2 protein levels ($P = 0.035$). Taken together, these data implied that miR-126 may attenuate the expression of PIK3R2 by directly targeting the 3′UTR of PIK3R2.

PI3K and Akt are also two major proteins in PI3K/Akt signaling pathway, which regulates multiple critical steps in angiogenesis and vessel homeostasis [Shiota et al., 2010]. Because PIK3R2 was direct targeted by miR-126, we investigated whether miR-126 was able to interfere with these pathways using Western blot. As shown in Figure 6C, PI3K and phospho-AKT (p-AKT) levels were significantly enhanced in the EPCs transfected with miR-126 mimics compared to NC mimics ($P < 0.05$). A reverse result was observed when miR-126 was knocked down in EPCs transfected with miR-126 inhibitor compared to NC inhibitor ($P < 0.05$).

To further elucidate the effect of miR-126 on rat EPCs was modulated by downregulation of PIK3R2, we performed the loss-of-function study. Briefly, we silenced PIK3R2 to investigate whether the

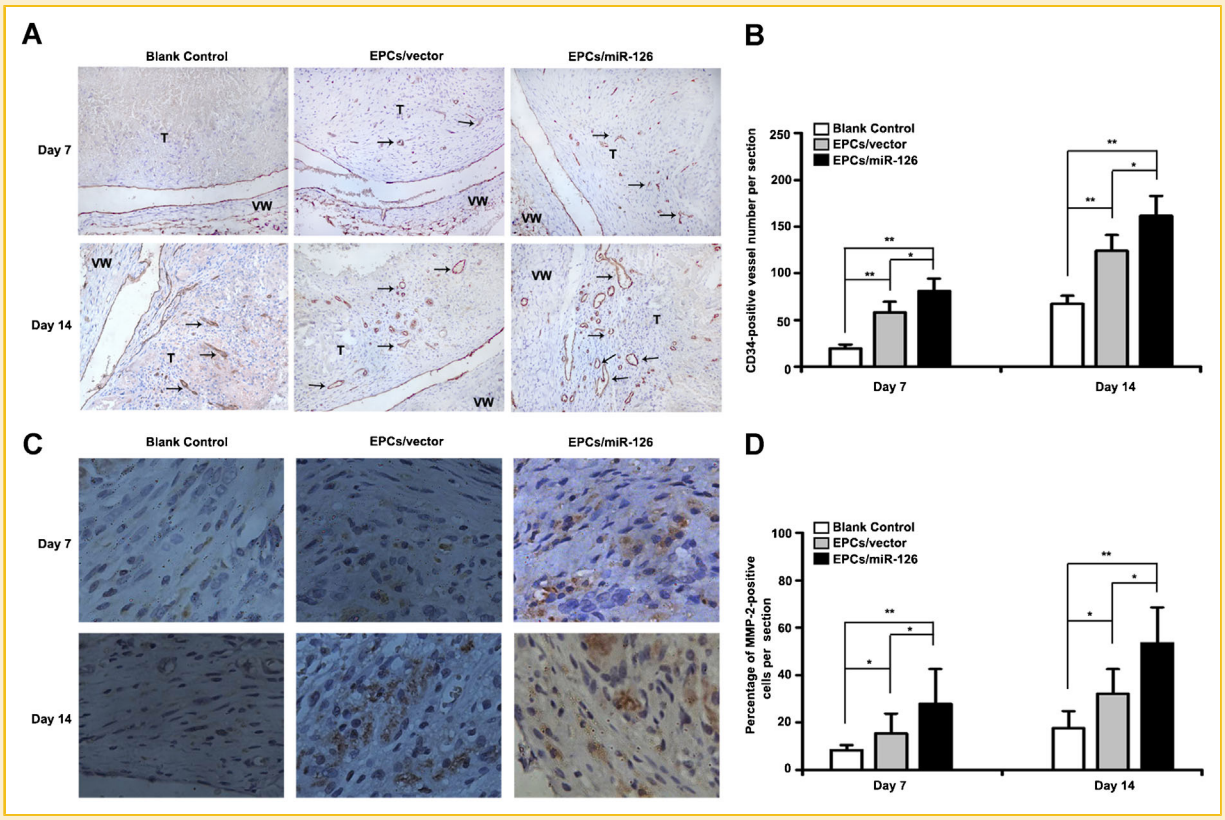

Fig. 5. miR-126 promotes thrombus recanalization and resolution. A: Representative images of CD34 staining for the thrombus sections are presented with original magnification of 200×. T and VW mean thrombus and venous wall, respectively. Arrows indicate the new-formed vessels with CD34 expression. B: CD34-positive vessels were quantified and presented as mean ± SD. C: Representative images of MMP-2 staining for the thrombus sections are presented with original magnification of 400×. D: The percentage of MMP-2-positive cells were quantified per section and presented as mean ± SD. $^{**}P < 0.01$, $^{*}P < 0.05$.

reduced expression of PIK3R2 could mimic the promoting effect of miR-126. EPCs were infected with siPIK3R2 and then cell motility and tubule formation were examined in vitro. As shown in Supplementary Figure S2, knockdown of PIK3R2 significantly promoted cell migration and tubule formation ability, which was similar to those induced by miR-126 mimics. These results showed that miR-126 directly targeted PIK3R2 and involved in the regulation of PI3K/Akt signaling pathways.

DISCUSSION

In this report, we investigated the effects of miR-126 on rat EPCs function and venous thrombus resolution by employing up-regulation and down-regulation strategies. We demonstrated that the ectopic expression of miR-126 could enhance EPCs migration and tubulogenic activity in vitro. PIK3R2 is a target of miR-126 predicted by bioinformatics. We confirmed this relationship using Western blot and luciferase reporter assay. In addition, to further explore the role of miR-126 in EPCs under venous thrombus, EPCs stably expressing miR-126 were achieved by lentiviral vectors and delivered into rats with DVT. The results showed that miR-126 promoted the homing of EPCs and neovascularization in venous thrombus.

As we know, mature ECs expressing both hemostatic and fibrinolytic factors, play a vital role in the process of thrombus formation and resolution [Kwaan and Samama, 2010]. Many of ECs functions have been explored and demonstrated also in EPCs. For example, ECs express the protease activated receptors (PAR)-1, serving as thrombin receptors [Chi et al., 2001]. Smadja and colleagues evidenced that functional PAR-1 were also present on EPCs. PAR-1 activation promoted cells proliferation and CXCR4-dependent migration and differentiation, leading to a proangiogenic effect [Smadja et al., 2006]. In further studies, their data showed that EPCs, in addition to their angiogenic potential, have both anticoagulant and antifibrinolytic properties. Thrombin may modulate these properties and contribute to thrombus recanalization by EPCs [Smadja et al., 2008]. In agreement with these observations, Modarai et al. [2005] demonstrated that EPCs are recruited into the thrombus during resolution and can orchestrate thrombus recanalization. In our previous reports [Li et al., 2007; Meng et al., 2010], we found that EPCs significantly improved the microenvironment, and promoted the resolution of acute venous thrombus and the recanalization of chronic thrombus.

Recently, 28 miRNAs commonly expressed in all profiling studies have been identified in ECs [Heusschen et al., 2010]. Data suggested that several miRNAs regulated aspects of vascular development and

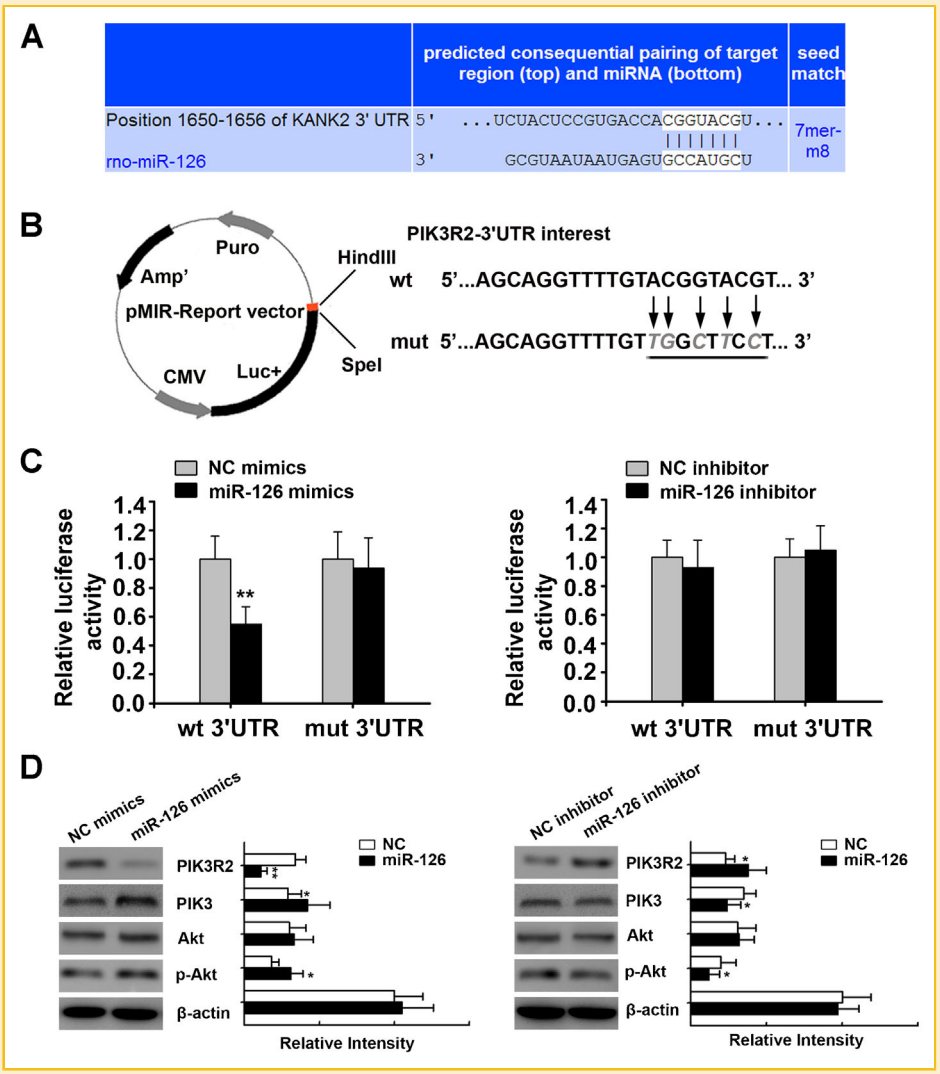

Fig. 6. miR-126 targets 3′UTR of PIK3R2 gene and attenuates PIK3R2 expression. A: Putative binding sites of rno-miR-126 in the rat PIK3R2 3′UTR (white sequences) predicted by TargetScan. B: Schematic graph of the constructed reporter plasmid containing the putative binding sites of rno-miR-126 in the PIK3R2 3′UTR predicted. PIK3R2-3′UTR mut indicates the PIK3R2-3′UTR with mutation in miR-126-binding site. The mutated nucleotides in PIK3R2-3′UTR fragments are underlined. C: Dual luciferase report assays were performed on HEK 293T cells. Left: miR-126 mimics down-regulated luciferase activities controlled by wild-type PIK3R2-3′UTR ($^{**}P < 0.01$), but did not affect luciferase activity controlled by mutant PIK3R2-3′UTR. Right: miR-126 inhibitor did not interfere the luciferase activity of reporter plasmids. D: Western blot showed PIK3R2, PI3K, Akt, and p-Akt protein expression. Left: effect of miR-126 mimics on protein levels. Right: effect of miR-126 inhibitor. β-Actin expression is shown for loading normalization (left panels), analysis with relevant quantification (right panel). Columns, mean; bars, ±SD, $^{**}P < 0.01$, $^{*}P < 0.05$.

angiogenesis, including pro-angiogenic miRs (miR-27b, miR-130a, miR-126, and miR-210) and anti-angiogenic miRs (miR-221/222, miR-34, and miR-217). miR-126 is one of the highly expressed miRNAs, plays an important role in endothelial functions. Fish et al. showed that miR-126 positively regulate endothelial cell migration, proliferation, and the stability of capillary tubes [Fish et al., 2008]. ECs lacking miR-126 exhibit a diminished angiogenic response to growth factors such as VEGF and bFGF [Kuhnert et al., 2008; Wang et al., 2008].

Exploring the correlation between miRs and stem cells to improve the function of stem cells has become a research hotspot. Some studies investigated whether miR-126 has an impact on EPCs. For example, Zhang showed that miR-126 inhibited the transition of EPCs to mesenchymal cells [Zhang et al., 2013]. Meng et al. provided evidence that miR-126 was downregulated in EPCs from diabetic patients, and impaired EPCs-mediated function via its target Spred-1, and through Ras/ERK/VEGF and PI3K/Akt/eNOS signal pathway [Meng et al., 2012]. Yan et al. analyzed the EPCs number and expression of miR-126 in patients with preeclampsia, then proved that miR-126 is essential for angiogenic properties of EPCs in vitro and placental vasculogenesis in vivo [Yan et al., 2013]. In the present study, we verified the role of miR-126 in rat EPCs, and firstly tested the role of miR-126 in venous thrombus resolution in rat DVT models.

First of all, we transfected EPCs with miR-126 mimics and inhibitor to upregulate and downregulate miR-126 expression. We found that overexpression of miR-126 could improve rat EPCs functions in vitro, which are crucial in the angiogenic process. In contrast, inhibition of miR-126 impaired EPCs functions. The results were in accordance with previous studies [Meng et al., 2012; Yan et al., 2013].

Secondly, we tried to investigate the role of miR-126 in the process of venous thrombus resolution. Although upregulation of miR-126 promoted vasculogenesis in the models of myocardial infarction and ischemia hindlimb [van Solingen et al., 2009; Chen and Zhou, 2011], little is known about its effect on venous thrombus. We constructed rat models of DVT by ligating IVC, which provided reproducible thrombus beginning at 3** h and extending to 21 days. Day 7 and 14 as two key time points were chose for subacute and chronic DVT studies. Consistent with our previous reports [Li et al., 2007; Meng et al., 2010], our in vivo data showed that compared with control group, transplanted-EPCs could be recruited into thrombus, significantly inhibited thrombus formation and improved thrombus organization and recanalization. In our present research, the results demonstrated that miR-126 promoted EPCs homing into venous thrombus and accelerated thrombus organization and recanalization, which was consistent with the proangiogenic role of miR-126 in other ischemia models.

MMPs, a family of zinc-dependent endopeptidases, play an important role in the degradation of extracellular matrix and basement membrane components [Verma and Hansch, 2007]. To data, 24 different vertebrate MMPs have been identified, of which MMP-2 and MMP-9 has been proven to be indispensable for the process of thrombus resolution [Wakefield et al., 2008]. Nosaka et al. found that both MMP-2- and MMP-9-positive cells could be detected in the whole course of thrombus formation after IVC ligation in mice [Nosaka et al., 2010]. Meanwhile, several studies have suggested that early EPCs may contribute to neovascularization by secretion of cytokines and MMP-9, whereas late EPCs participated by providing building blocks and secreting MMP-2 [Yoon et al., 2005; Renault and Losordo, 2007]. In the present study, the second and third generations of cultured EPCs were used for our research. The EPC population is mainly composed of late EPCs. Therefore, the expression of MMP-2 in venous thrombus was detected using immunohistochemical staining. The data showed that overexpression of miR-126 resulted in a larger number of MMP-2-positive cells appearing at the indicated time points. Collectively, all of results indicated miR-126 improved the functions of EPCs in vivo.

Finally, to explore the underlying mechanism by which miR-126 modulates EPCs biological function, we investigated the potential mRNA targets of miR-126 by online database. Phosphatidylinositol-3 kinase (PI3K) regulatory subunit p85 beta (PIK3R2) is a target of miR-126 predicted by bioinformatics. We confirmed this relationship using Western blot and luciferase reporter assay. Furthermore, we tested if PIK3R2 knockdown could mimic the promoting effect of miR-126 in EPCs by siRNA. Consistent with overexpression of miR-126 in EPCs, we found that PIK3R2 knockdown had similar effect to enhance the migration and tube formation ability of EPCs in vitro. It has been proven that PIK3R2 acted as a suppressor of the PI3K/Akt signaling pathway activation [Ueki et al., 2003]. Recent studies have demonstrated that the PI3K/Akt signaling pathway played a critical role in EPC proliferation migration and tubulogenic activity [Li et al., 2012; Yan et al., 2013]. In the present research, we detected whether the expression of miR-126 could affected the PI3K/Akt signaling axis in rat EPCs. Western analysis indicated a decrease in PIK3R2 protein and an increase in PI3K and phosphor-Akt protein by overexpression of miR-126. Conversely, downregulation of miR-126 increased the expression of PIK3R2 and decreased the expression of PI3K and phosphor-Akt. Taken together, these findings implied that miR-126 promoted EPCs function via targeting PIK3R2 to enhance PI3K/Akt activation.

In conclusion, our results demonstrated that miR-126 upregulation inhibited PIK3R2 expression, leading to enhanced EPCs-mediated vasculogenesis in vitro and in DVT. Thus, we may reasonably speculate that miR-126 may be a promising potential therapeutic target for DVT therapy.

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