Clarification of the Role of miR-9 in the Angiogenesis, Migration, and Autophagy of Endothelial Progenitor Cells Through RNA Sequence Analysis

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Abstract

We have previously reported that miR-9 promotes the homing, proliferation, and angiogenesis of endothelial progenitor cells (EPCs) by targeting transient receptor potential melastatin 7 via the AKT autophagy pathway. In this way, miR-9 promotes thrombolysis and recanalization following deep vein thrombosis (DVT). However, the influence of miR-9 on messenger RNA (mRNA) expression profiles of EPCs remains unclear. The current study comprises a comprehensive exploration of the mechanisms underlying the miR-9-regulated angiogenesis of EPCs and highlights potential treatment strategies for DVT. We performed RNA sequence analysis, which revealed that 4068 mRNAs were differentially expressed between EPCs overexpressing miR-9 and the negative control group, of which 1894 were upregulated and 2174 were downregulated. Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses indicated that these mRNAs were mainly involved in regulating cell proliferation/migration processes/pathways and the autophagy pathway, both of which represent potential EPC-based treatment strategies for DVT. Reverse transcriptase quantitative polymerase chain reaction confirmed the changes in mRNA expression related to EPC angiogenesis, migration, and autophagy. We also demonstrate that miR-9 promotes EPC migration and angiogenesis by regulating FGF5 directly or indirectly. In summary, miR-9 enhances the expression of VEGFA, FGF5, FGF12, MMP2, MMP7, MMP10, MMP11, MMP24, and ATG7, which influences EPC migration, angiogenesis, and autophagy. We provide a comprehensive evaluation of the miR-9-regulated mRNA expression in EPCs and highlight potential targets for the development of new therapeutic interventions for DVT.

Keywords

deep vein thrombosis, miR-9, endothelial progenitor cell, angiogenesis, autophagy

Introduction

Endothelial progenitor cells (EPCs) are pluripotent cells that express specific markers of endothelial cells and differentiate into mature endothelial cells. These cells were first isolated from adult peripheral blood by Asahara et al. in 1997[1–3] and have been shown to secrete angiogenic growth factors and cytokines[4]. In the field of regenerative medicine, EPCs have potential therapeutic applications in vascular tissue engineering and cell-based therapy, especially for cardiovascular and vascular-related diseases[5]. Bone-marrow-derived EPCs are initially released into venous microcirculation; therefore, the concentration of EPCs in the veins may be higher than in the arteries[6]. Previous studies have demonstrated that EPCs are mobilized from the bone marrow into the lesion created when the intima is damaged[7]. Circulating EPCs can accelerate thrombus recanalization by restoring damaged or lost endothelium, which enhances

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neovascularization and prompts recanalization of deep vein thrombosis (DVT)[3,8–10]. Angiogenesis and thrombolysis are crucial for recanalization following DVT[4,11,12], and numerous studies have shown that EPCs are recruited to the thrombus to accelerate thrombus resolution, where they play a vital role in physiological and pathological neovascularization in adults[8,9,12,13]. Moreover, EPCs can differentiate into endothelial cells to participate in angiogenesis and thus represent a promising therapeutic approach for thrombus resolution, for which there are currently limited treatment options with insufficient success rates[12–14]. However, the function of EPCs can be impaired by cardiovascular risk factors, ischemic disease, and graft vasculopathy[15–18]; therefore, exploring methods to improve EPC homing and angiogenesis is important.

MicroRNAs (MiRNAs, MiRs) are small noncoding RNAs comprising approximately 22 nucleotides that influence post-transcriptional gene regulation by targeting the 3′-untranslated regions of messenger RNAs (mRNAs)[19–22]. These small RNAs play a major role in the regulation of cell migration, proliferation, apoptosis, and angiogenesis, which is essential for the development and progression of vascular disease[23]. Our previous studies have suggested that miR-9 plays a crucial role in regulating angiogenesis and migration of EPCs by targeting transient receptor potential melastatin 7 (TRPM7) through the AKT autophagy pathway, thereby promoting thrombolysis and recanalization following DVT[24]. However, the detailed mechanisms underlying regulation of miR-9 in EPCs are poorly understood. The present study aimed to examine the mRNA expression of miR-9 in EPCs using RNA sequence (RNAseq) analysis and to analyze the effects on EPC angiogenesis and migration.

Materials and Methods

Isolation and Culture of Human EPCs

We isolated and characterized human EPCs as previously described[18,25]. Peripheral blood was obtained from the veins of recruited subjects, then carried out density gradient centrifugation using Ficoll-Isopaque Plus (Histopaque-1077, Sigma, St. Louis, MO, USA) to isolate mononuclear cells. After washing with phosphate-buffered saline (PBS), isolated mononuclear cells were seeded in fibronectin-coated cell culture flasks, then incubated in complete Endothelial Basal Medium-2 (EBM-2, Lonza, Walkersville, MD, USA) at 37°C and 5% CO_2. The culture medium was changed every 4 days. After 14 days of culture, EPCs were detached by the addition of 0.025% trypsin containing 0.02% ethylenediaminetetraacetic acid and harvested for further analysis or transplantation.

Characterization of EPCs

Isolated EPCs were identified using both confocal microscopy and flow cytometry as previously described[18]. Cells that were positive for DiI-conjugated acetylated low-density lipoproteins and Ulex europaeus agglutinin 1 staining on

confocal microscopy and those with antibodies against CD31, CD34, CD45, CD133, and CD309 (all antibodies were purchased from Becton-Dickinson, Franklin Lakes, NJ, USA) on flow cytometry were determined to be EPCs.

Cell Treatment

To detect mRNAs that are regulated by miR-9 in EPCs, recombinant lentiviral particles carrying miR-9 mimics or vehicle controls provided by GenePharma Co. Ltd (Shanghai, China) were used to infect EPCs according to the manufacturer's instructions. Polybrene (5 µg/ml) was used to enhance infection efficiency. Infected cells were then selected by incubating with 3 µg/ml puromycin (Invitrogen, San Diego, CA, USA) for 1 week to induce stable miR-9 overexpression in EPCs and negative controls (NCs; EPCs are infected with the lentivirus carrying the empty vector).

mRNA Sample Preparation and RNA Sequencing Analysis

Total RNA was extracted from 1×10^7 cells to construct sequencing libraries using RNAiso Plus (TaKaRa Bio, Inc., Ostu, Shiga, Japan). The RNA Integrity Number was measured using an Agilent Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) to assess RNA quality prior to sequencing.

We performed RNAseq analysis as previously described[10]. Briefly, the NEBNext® Poly(A) mRNA Magnetic Isolation Module was used to isolate intact poly(A)+ RNA from previously isolated total RNA, and the NEBNext® Ultra RNA Library Prep Kit for Illumina was used to construct the library. Library determination and quantification were performed using the Agilent Bioanalyzer 2100 and Qubit. Sequencing was performed on an Illumina HiSeq 2000 paired-end module (Illumina, San Diego, CA, USA).

Gene Ontological and Pathway Analysis

Gene ontology (GO) analysis was performed to determine processes or functional categories of differential mRNA expression, as described previously[26]. Genes were considered differentially expressed if |log2 fold change| value was ≥ 1 between the two groups with a q value of <0.05. Hierarchical clustering with average linkage was performed to identify distinguishable mRNA expression patterns. Differentially expressed mRNAs were classified into three domains: molecular function, cellular component, and biological process. To investigate whether genes share a similar biological function, we searched for overrepresentation in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Enriched genes were identified by hypergeometric distribution, with q value <0.05 used as the selection criterion for enrichment.

Table 1. Primers Used for RT-qPCR.

Has-VEGFA	Forward (5'–3')	AGGGCAGAATCATCACGAAGT
	Reverse (5'–3')	AGGGTCTCGATTGGATGGCA
Has-FGF5	Forward (5'–3')	TGTACCGCTATGGTTACACTCG
	Reverse (5'–3')	GGCAGGGACAGTTGCTTCT
Has-FGF12	Forward (5'–3')	GGGACCAAGGACGAAAACAG
	Reverse (5'–3')	TTGCTGGCGGTACAGTGTG
Has-MMP2	Forward (5'–3')	TGACTTTCTTGGATCGGGTCG
	Reverse (5'–3')	AAGCACCACATCAGATGACTG
Has-MMP7	Forward (5'–3')	GAGTGAGCTACAGTGGGAACA
	Reverse (5'–3')	CTATGACGCGGGAGTTTAACAT
Has-MMP10	Forward (5'–3')	TGCTCTGCCTATCCTCTGAGT
	Reverse (5'–3')	TCACATCCTTTTCGAGGTTGTAG
Has-MMP11	Forward (5'–3')	CCGCAACCGACAGAAGAGG
	Reverse (5'–3')	ATCGCTCCATACCTTTAGGGC
Has-MMP24	Forward (5'–3')	GCCGGGCAGAACTGGTTAAA
	Reverse (5'–3')	CCCGTAAAACTGCTGCATAGT
Has-ATG7	Forward (5'–3')	CAGTTTGCCCCTTTTAGTAGTGC
	Reverse (5'–3')	CCAGCCGATACTCGTTCAGC
Has-GAPDH	Forward (5'–3')	CATGAGAAGTATGACAACAGCCT
	Reverse (5'–3')	AGTCCTTCCACGATACCAAAGT

RT-qPCR: reverse transcription quantitative polymerase chain reaction.

Reverse Transcription Quantitative Polymerase Chain Reaction

We selected mRNA transcripts that are closely related to angiogenesis, migration, and autophagy (VEGFA, FGF5, FGF12, MMP2, MMP7, MMP10, MMP11, MMP24, and ATG7) and validated their expression levels by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The RNA remaining after RNAseq was reverse transcribed into complementary DNAs (cDNAs) using the PrimeScript RT reagent kit (TaKaRa Bio, Inc.). Next, RT-qPCR was performed using the SYBR Green quantitative PCR (qPCR) Master Mix (Bio-Rad, CA, USA) according to the manufacturer's instructions. We determined miRNA levels using the Hairpin-it™ miRNA RT-PCR Quantitation Kit (GenePharma Co. Ltd) according to the manufacturer's instructions and analyzed mRNA expression levels using mRNA primers (GENEWIZ, Jiangsu, China). Glyceraldehyde 3-phosphate dehydrogenase was used as the endogenous control for mRNAs. Reactions were processed using a High Throughput Quantitative PCR LightCycler480 II (Roche, Basel, Switzerland) and relative expression levels of each candidate gene calculated using the $2^{\Delta\Delta CT}$ method[10]. Each experiment was performed independently and in triplicate. The primers used for RT-qPCR are shown in Table 1.

Immunofluorescence

The experiment of immunofluorescence was performed as previously described[10]. In short, transfected EPCs were grown on cover slides for 24 h, washed with PBS, and then fixed with 4% paraformaldehyde for 10 min at room temperature. After washing three times with PBS, EPCs were blocked with Immunol Staining Blocking Buffer (Beyotime, P0102, Shanghai, China) for 1 h at room temperature.

Immunostaining was performed using FGF5 antibody (Abcam, ab168743, Cambridge, UK) to detect the protein expression levels of FGF5 in EPCs. Cell nuclei were stained with 4',6-diamidino-2-phenylindole. The coverslips were washed with PBS and inverted onto cover slips that had been treated with Fluoromount-GTM seal. For each experiment, images were acquired by confocal microscopy under the same conditions, and no digital image processing was performed.

Tube Formation Assay

Tube formation assay was performed as previously described[10]. Briefly, NC EPCs and FGF5-overexpressing EPCs (2×10^4 cells) were seeded in Matrigel-coated wells of a 24-well plate to induce tube formation. After 12 h of incubation, tubular structures of EPCs in the Matrigel were examined through an Image Navigator®, and the extent of tube formation assessed by measuring the number and length of tubes using ImageJ software. We examined at least five random fields per well to determine cell-tube formation ability.

Migration Assay

Cell migration was evaluated using 24-well chambers (8-mm pore size; BD, Franklin Lakes, NJ, USA). Medium containing 10% fetal bovine serum (300 µl) was added to the lower wells and EPCs (5×10^3 cells in 200 µl serum-free EBM-2 medium) were added to the upper transwell chambers. The chambers were incubated for 12 h at 37°C in a 5% CO_2 incubator. Migration of cells was evaluated using an inverted microscope and the number of cells in each chamber was quantified using Image J. Three wells were analyzed per group.
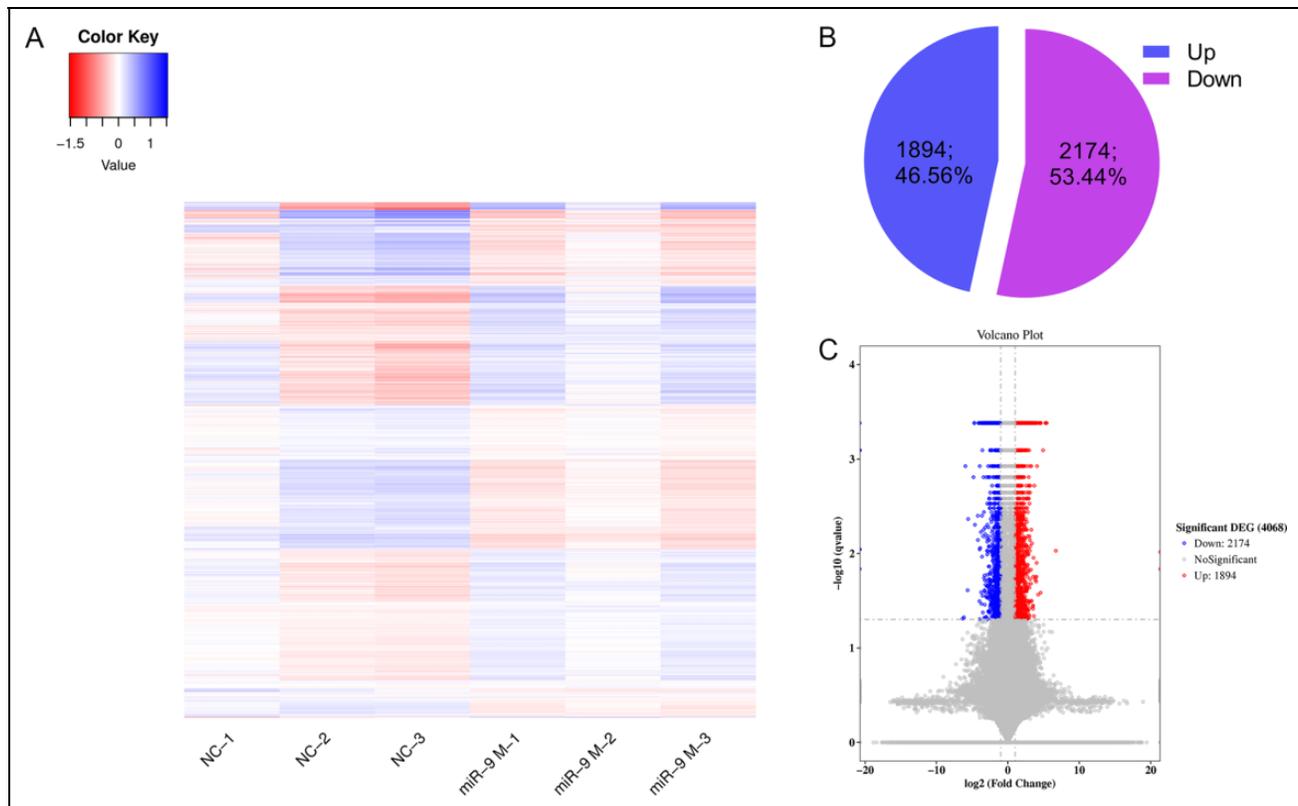
Figure 1. Differentially expressed mRNAs identified using RNASeq analysis. (A) Heat maps and hierarchical clustering of mRNAs in the miR-9 mimics groups and the NC groups. Blue and red indicate upregulation and downregulation, respectively. NC indicate NC group and 9M indicate miR-9 mimics group. (B) A total of 4086 mRNAs were differentially expressed in the miR-9 mimics groups ($n = 3$) compared with the NC groups ($n = 3$), including 1894 (46.56%) upregulated and 2174 (53.44%) downregulated mRNAs (|log2 fold change| ≥ 1, q value <0.05). (C) mRNA volcano plots of miR-9-overexpressing EPCs versus NC EPCs (red: upregulated, blue: downregulated, and gray: no significant mRNAs). EPCs: endothelial progenitor cells; mRNA: messenger RNA; NC: negative control; RNAseq: RNA sequence.

Table 2. RNAseq Analysis Results of the Main Factors Involved in Angiogenesis and Migration Regulated by miR-9 Overexpression.

Gene symbol	Log2 fold change	test_stat	*P* value	*P* adj	Significant
VEGFA	1.80434	2.17582	0.00675	0.037264	Ups
FGF5	3.91977	7.7593	5.00E-05	0.000415	Ups
FGF12	2.06785	3.46969	5.00E-05	0.000415	Ups
MMP2	2.23677	2.10103	0.0001	0.004512	Ups
MMP7	1.15593	1.89401	0.005	0.028731	Ups
MMP10	1.8382	2.62685	5.00E-05	0.000415	Ups
MMP11	1.42101	3.22442	5.00E-05	0.000415	Ups
MMP24	2.96222	5.2389	5.00E-05	0.000415	Ups
ATG7	−1.2249	-2.30742	0.002	0.012854	Down

RNAseq analysis was performed after EPC infection with LV3-NC and miR-9 mimics lentivirus, and three groups of LV3-NC and three groups of miR-9 mimics were detected; gene symbol means differentially expressed genes; *P* value was calculated by *t* test, and *P* adj was obtained by *P* value normalized by Benjamini–Hochberg; significant means the up- or downregulation of the gene expressed in the miR-9 overexpression group compared with the LV3-NC.
EPCs: endothelial progenitor cells; NC: negative control; RNAseq: RNA sequence.

Statistical Analysis

All data are presented as mean $\pm$ standard error of the mean. Student's *t* test was used to analyze differences between groups. GraphPad Prism software was used for statistical analysis and a *P* value of <0.05 was considered statistically significant.

Results

Differential Expression Profiles of mRNAs

After processing sequencing data to reduce noise, remove outliers, and normalize, hierarchical clustering revealed systematic variations in mRNA expression between EPCs that
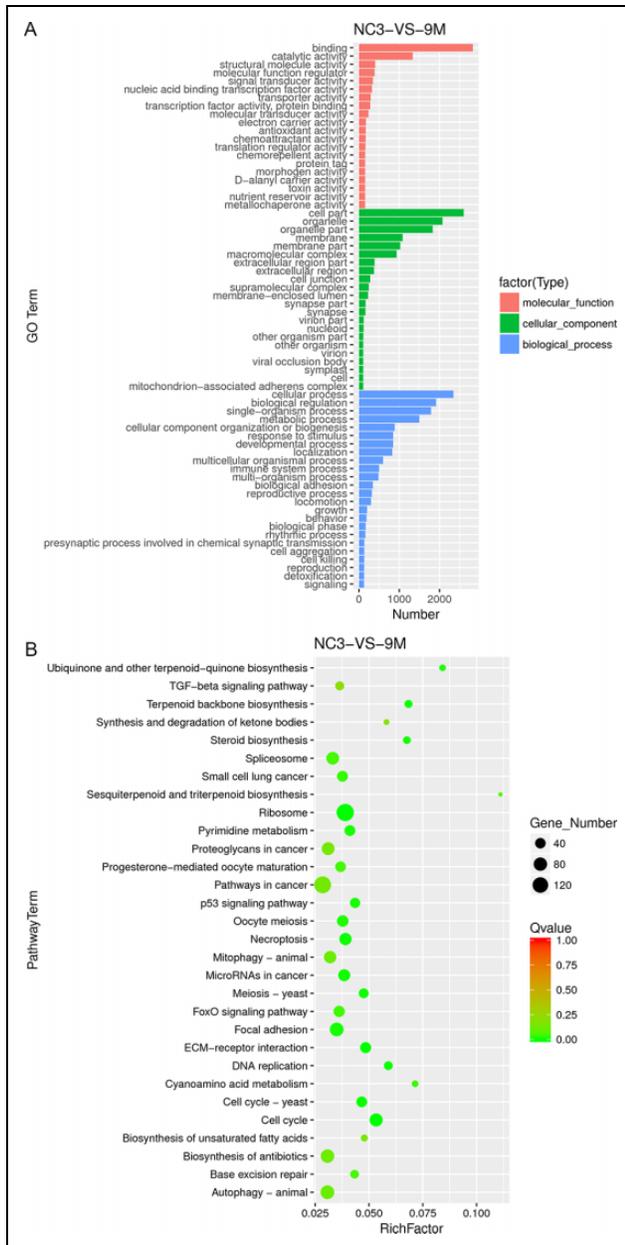
Figure 2. GO and KEGG pathway analysis. (A) GO analysis assigns high-level terms from each of the following three major GO terms: biological processes, cellular components, and molecular functions. (B) KEGG analysis of the differential genes. The top 30 enriched pathways were shown. The vertical axis represents the name of the pathway, the horizontal axis represents the rich factor, the size of the dots indicates the number of differentially expressed genes in the pathway, and the color of the dots corresponds to different *q* value ranges. GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.
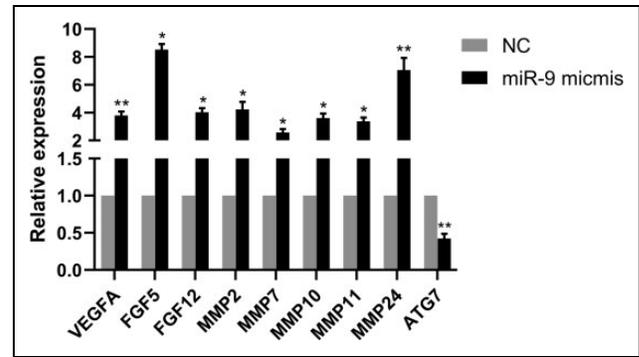
Figure 3. RT-qPCR validation of selected mRNAs in EPCs. *$P < 0.05$, **$P < 0.01$ compared to the NC group. Data are represented as mean $\pm$ standard error of the mean. EPCs: endothelial progenitor cells; mRNA: messenger RNA; NC: negative control; RNAseq: RNA sequence; RT-qPCR: reverse transcription quantitative polymerase chain reaction.

volcano plot analysis are shown in Fig. 1C. Because of the high number of DE genes, we sorted the results into mRNAs related to cell migration, angiogenesis, and autophagy to expose meaningful sets of relevant genes, including VEGFA, FGF5, FGF12, MMP2, MMP7, MMP10, MMP11, MMP24, and ATG7 (Table 2).

GO and KEGG Pathway Analysis

In the molecular function domain, GO terms included binding, catalytic activity, structural molecular activity, molecular function regulator, signal transducer activity, transcription factor activity, and protein binding. In the cellular component domain, the top five GO terms were cell part, organelle, organelle part, membrane, macromolecular complex, extracellular region part, extracellular region, cell junction, supramolecular complex, and membrane-enclosed lumen. In the biological process domain, the GO terms for differentially expressed mRNAs included structural cellular process, biological regulation, single-organism process, metabolic process, cellular component organization or biogenesis, cell adhesion, cell growth, and so on (Fig. 2A). From KEGG pathway analysis, we identified 30 signaling pathways that were enriched in EPCs regulated by miR-9. These pathways related to cell proliferation, migration, and angiogenesis including the transforming growth factor-β, p53, and FoxO signaling pathways; focal adhesion; cell cycle; and autophagy (Fig. 2B).

Validation of the Microarray Results by RT-qPCR

We selected nine mRNAs with high DES scores and analyzed further with RT-qPCR to evaluate the consistency of the results of mRNA DE from sequencing. Expression trends of these transcripts confirmed the results of RNAseq (Fig. 3). Our data show that mRNA expression of VEGFA, FGF5, FGF12, MMP2, MMP7, MMP10, MMP11, and MMP24 was upregulated, while ATG7 was downregulated in

overexpressed miR-9 mimics and NC EPCs (Fig. 1A). Differential expression (DE) analysis identified 4068 mRNAs that were significantly altered in miR-9-overexpressing EPCs compared with NC EPCs: 1894 mRNAs were upregulated and 2174 were downregulated (Fig. 1B). The results of
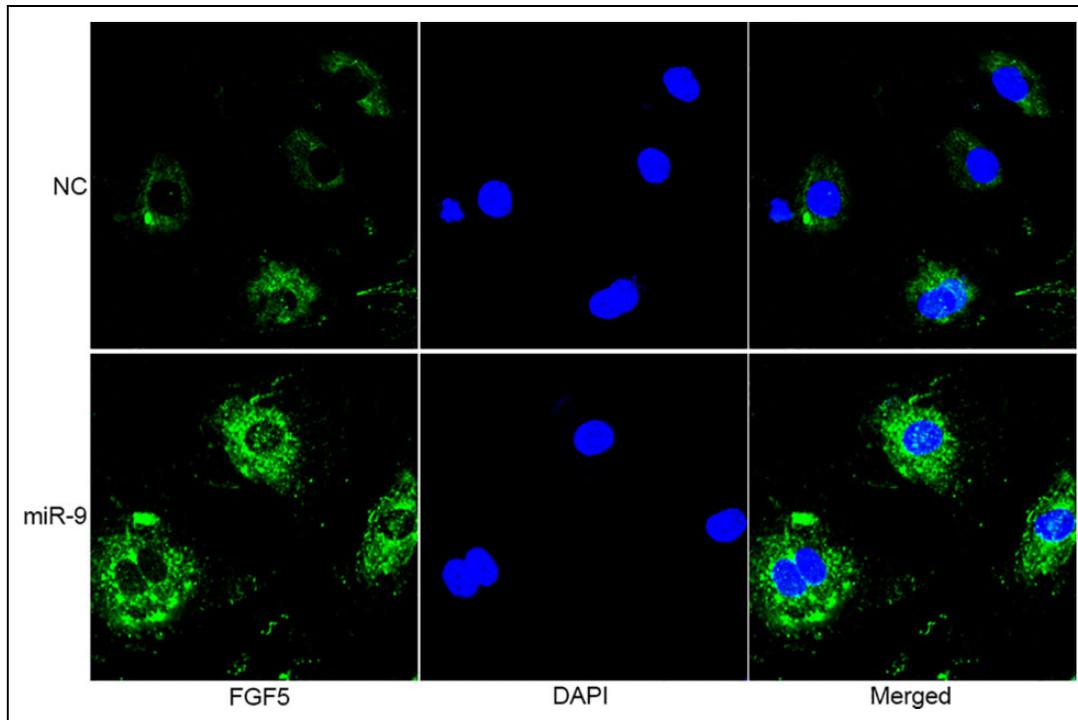
Figure 4. The expression levels of FGF5 protein in EPCs. Immunofluorescence results showed that miR-9 significantly upregulated FGF5 protein expression. *P < 0.05. EPCs were immunostained with an antibody against FGF5 (green). Nuclei were stained with DAPI (blue). Results are representative of ≥3 independent experiments. DAPI: 4',6-diamidino-2-phenylindole; EPCs: endothelial progenitor cells.

miR-9-mimic-expressing EPCs compared with NC EPCs. All DE was in the expected direction.

MiR-9 Enhances the Protein Expression of FGF in EPCs

To examine the effect of miR-9 on the protein expression of FGF5 in EPCs, immunofluorescence technology was performed. As shown in Fig. 4, the results of immunofluorescence showed that miR-9 significantly upregulates FGF5 protein expression.

Effect of FGF5 on Migration and Angiogenesis of EPCs

Tube formation and transwell analyses revealed that FGF5 significantly enhances EPC angiogenesis and migration (Fig. 5).

Discussion

DVT is a common and difficult clinical problem, and complications associated with the condition—such as pulmonary embolism and pulmonary vein thrombosis—can be life-threatening[10]. The current treatment for venous thromboembolism is primary anticoagulation; however, this causes impairment of hemostasis, and many patients who present with DVT have already progressed past the point where anticoagulants are effective[27]. Thus, identifying new biomolecular treatment therapies for DVT could have great

clinical value[10]. There is increasing evidence of the critical role of EPCs in the angiogenesis and resolution of DVT. However, the limited capacities of homing and angiogenesis of EPCs impair their therapeutic effects. Intriguingly, our previous study demonstrated that miR-9 prevents apoptosis but promotes proliferation, migration, and angiogenesis of EPCs by targeting TRPM7, which enhances recanalization following venous thrombosis[24]. Although we identified the PI3K/Akt/autophagy pathway to be involved in the process, the effects of miR-9 on mRNA expression profiles of EPCs remain unclear.

The present study involved a comprehensive exploration of the mechanisms underlying the influence of miR-9 on the migration and angiogenesis of EPCs to highlight potential treatment strategies based on miR-9 targets. We determined the mRNA expression profiles of miR-9-overexpressing EPCs and NC EPCs and demonstrate for the first time, to the best of our knowledge, that a total of 4068 mRNAs are differentially expressed between these two cell lines. The results of GO and KEGG analyses indicated the downregulated and upregulated mRNAs to be mainly involved in cell adhesion, angiogenesis, and cell differentiation, which suggests that differentially expressed mRNAs might regulate EPC migration, angiogenesis, and differentiation by influencing the expression of related genes.

The results of RT-qPCR supported RNAseq analysis and confirm that miR-9 significantly increased the expression
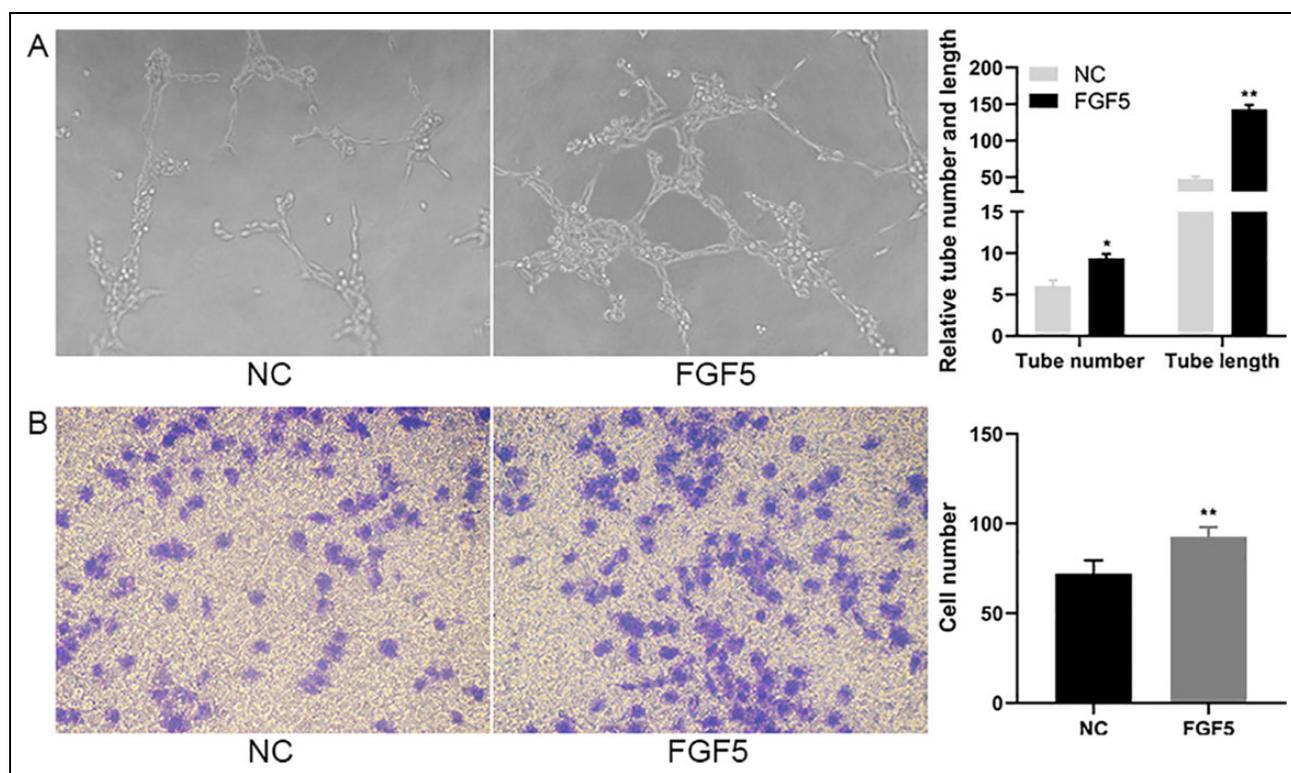
Figure 5. Effect of FGF5 on angiogenesis and migration of EPCs. (A) FGF5 significantly improves the angiogenic capacity of EPCs. (B) FGF5 enhances the EPC migration. *P < 0.05, **P < 0.01 compared to the NC group. Data are represented as mean $\pm$ standard error of the mean. EPCs: endothelial progenitor cells; NC: negative control.

levels of eight miR-9 mimics in EPCs but decreased the mRNA expression of ATG7. It has been reported that VEGFA regulates multiple functions and induces angiogenesis in vascular endothelial cells; previous studies have shown it to be a prominent proangiogenic factor that enhances angiogenesis *in vitro* and *in vivo* by inducing EPC proliferation, migration, and tube formation[28,29]. Additionally, VEGF promotes the recruitment of EPCs to ischemic sites and contributes to the growth of new blood vessels[30]. The matrix metalloproteinase (MMP) superfamily comprising metalloproteases that degrade the extracellular matrix to facilitate cell migration, invasion, and angiogenesis[10,31]. Among the members of this superfamily, MMP2 plays an essential role in vascular disease and promotes EPC migration and angiogenesis[3,32]. Autophagy has attracted attention as a potential therapeutic target for vascular disease, and the process plays an important role in vascular biology and homeostasis and is implicated in many physiological and pathological processes involving cell angiogenesis and migration[33]. Our previous studies have shown miR-9 to promote EPC migration, proliferation, and angiogenesis by regulating autophagy and MMP2 protein expression, thereby promoting thrombolysis and vascularization in DVT[24]. However, tissue inhibitor of matrix metalloproteinases (TIMPs), specific inhibitor of MMPs, had no significant differences in our RNAseq results. Therefore, miR-9 may upregulate the expression

levels of MMPs in EPCs through other targets and pathways. In fact, MMPs can be upregulated through a variety of pathways, such as the PI3K-AKT and autophagy pathway[24,33]. Our previous studies also showed that miR-9 can upregulate the PI3K-AKT pathway[24], which is consistent with this result. There may be other detailed mechanisms and pathways that need further study. Additionally, previous studies have shown that FGF5 promotes angiogenesis of human aortic endothelial cells and increases blood flow and function[34,35]. However, the role of FGF5 in regulating the migration and angiogenesis of EPCs has remained unclear until now. The present study demonstrates that FGF5 significantly enhances EPC migration and angiogenesis and identifies the major mRNAs that promote angiogenesis and migration, thus highlighting new targets for DVT therapy.

In summary, we have determined comprehensive mRNA profiles of EPCs regulated by miR-9. DE of VEGFA, FGF5, FGF12, MMP2, MMP7, MMP10, MMP11, MMP24, and ATG7 may participate in the migration, angiogenesis, and autophagy of EPCs, and may contribute to the use of EPCs for the treatment of DVT.

Ethical Approval

The present study was approved by the Ethics Committee of Soochow University, Suzhou, Jiangsu Province, China. All research involving the human was approved by the institutional review committee of Soochow University.

Statement of Human and Animal Rights

All experiments were performed with relevant laws and guidelines, and following institutional guidelines of Soochow University. Human blood samples were obtained using protocols approved by the Ethics Committee of Soochow University. This article does not contain any studies with animals performed by any of the authors.

Statement of Informed Consent

Human EPCs were extracted and isolated from the venous blood of recruited subjects.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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