

miR-548j-5p Regulates Angiogenesis in Peripheral Artery Disease

Chiu-Yang Lee

Division of Cardiovascular Surgery, Department of Surgery, Taipei Veterans General Hospital

Shing-Jong Lin

Division of Cardiology, Taipei Veterans General Hospital

Tao-Cheng Wu (✉ dcwu@vghtpe.gov.tw)

Taipei Veterans General Hospital

Original investigation

Keywords: microRNAs, angiogenesis, peripheral artery disease

DOI: <https://doi.org/10.21203/rs.3.rs-56069/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Peripheral artery disease (PAD) is a vascular disease involving diffuse atherosclerosis, which is associated with increased cardiovascular mortality and morbidity. Critical limb ischemia is the most severe complication of PAD. In addition to medical and interventional treatment, therapeutic angiogenesis is a novel therapy for PAD. Circulating microRNAs (miRNAs) are considered to be key regulators of gene expression, but their role in ischemic-induced angiogenesis is poorly characterized. There is little knowledge of specific miRNAs associated with PAD.

Methods: To determine the regulation of miRNAs, we obtained miRNA profiles using RNA isolated from patients with PAD and a control group. The effect of the specific miRNA in angiogenesis were evaluated by *in vitro* angiogenic function of endothelial progenitor cells (EPCs), *in vivo* angiogenesis assay and a hind-limb ischemic model.

Results: Circulating miR-548j-5p was significantly reduced in patients with PAD compared with the controls. miR-548j-5p promoted EPC angiogenesis by enhancing migration and tube formation. The endothelial nitric oxide synthase and stromal cell-derived factor (SDF)-1 signaling pathways appeared to be potential targets for miR-548j-5p. Furthermore, a directed *in vivo* angiogenesis assay of EPCs and a hind-limb ischemia mouse model demonstrated that miR-548j-5p enhanced capillary density and blood flow recovery in hind-limb ischemia.

Conclusion: Taken together, our data indicates that up-regulation of miR-548j-5p promotes angiogenesis in ischemic tissue and might be a novel therapeutic approach for PAD.

Background

Peripheral artery disease (PAD) is an atherosclerotic disease, which increases the risk of cardiovascular mortality and morbidity [1]. The prevalence of PAD is greater than 20% in individuals over 60 years of age and incidence rates have been predicted to increase with aging populations [2]. Critical limb ischemia (CLI) is the most severe clinical presentation of PAD, and causes intermittent claudication, ulceration and gangrene of the foot; it is associated with amputation and cardiovascular death [3]. The pathophysiology of PAD is a complex process involving endothelial dysfunction, oxidative stress, platelet activation and inflammation [4]. Despite pharmacological control of risk factors, the clinical outcomes for patients with PAD are poor. Thus, the development of new therapeutic targets for patients with PAD is an important issue.

Recently, microRNAs (miRNAs) have emerged as novel regulators of vascular biology. miRNAs are small, non-coding RNAs that regulate gene expression at the post-transcriptional level to repress expression [5]. miRNAs play key roles in inflammation, angiogenesis, endothelial function and restenosis associate with PAD [6]. Previous studies have shown that specific miRNA expression profiles in patients with PAD may serve as prognostic predictors [7]. In addition, some miRNAs, such as miR-93 [8] and miR let-7g [9], have

been shown to mediate angiogenesis through various molecular pathways. However, the association between miRNAs and PAD has not yet been fully characterized.

The present study aimed to explore specific miRNAs relevant to PAD and investigate their angiogenic effects and mechanisms on endothelial progenitor cell (EPC) migration, tube formation and angiogenesis in PAD, in the hope of providing a novel therapeutic strategy for patients with PAD.

Methods

Study population

A total of 40 subjects were included in the current study, including 25 patients with PAD who were diagnosed via the ankle-brachial index or angiography. Patients with acute coronary syndrome, acute ischemic limb or malignancy were excluded. A total of 15 healthy individuals without any evidence of PAD served as the control group. Blood sampling was performed and samples were subjected to RNA isolation (FocusGenomics Biotech Co., Ltd, Taiwan). Following miRNA array analysis, we selected specific miRNAs for comparison between the patients with PAD and the control group. This study was approved by the Taipei Veterans General Hospital's Institutional Review Board for Research (approval no. 2013-08-020B#3), and written informed consent was provided by all the participants prior to their inclusion within the study.

MiRNA library construction and next-generation sequencing

RNA was isolated according to the manufacturer's protocol. Total RNA from samples was further constructed into libraries using an Illumina TruSeq Small RNA Library Prep kit (Cat. RS-200-0012; Illumina, San Diego, CA, USA). In brief, 1 µg of high-quality total RNA from each sample was ligated with adapters using T4 RNA ligase. The adapter-ligated RNA samples were reverse-transcribed to cDNA, amplified by primers containing a specific sequence index, and then size-validated using an Agilent 2100 bioanalyzer (Cat. G2943, Agilent, Santa Clara, CA, USA) loaded with a DNA 1000 kit (Agilent, cat. 5067-1504). The size-checked libraries were loaded onto Novex TBE gels (Cat. EC6265BOX, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and then size-selected and gel-eluted to obtain proper fragments. The eluted libraries were qualified using an Agilent 2100 bioanalyzer loaded with a DNA 1000 kit, and quantified using Qubit (Thermo Fisher Scientific, Inc., Cat. Q33216) and real-time PCR (Q-PCR). Each library was diluted in equal concentrations and the same volumes were taken for pooling. Pooled libraries were sequenced for 10 M reads/samples with a high-throughput, 50-bp single-end sequencing reagent on an Illumina MiSeq sequencing system.

Scratch injury model and EPCs

Human EPC isolation, cultivation and characterization were performed as previously described [10]. EPC migration was evaluated using a scratch injury model. EPCs were transfected with miR-548j-5p mimic or

antagomir, or a control. After serum-starvation of EPCs overnight, a scratch injury was applied with a scalpel, and EPC sprouting was examined before and 12/24 h after scratching.

Tube formation assay

An EPC tube formation assay was performed using an *in vitro* angiogenesis assay kit (Chemicon, Temecula, CA, USA). EPCs transfected with miR-548j-5p mimic or antagomir were placed onto a matrix with medium for 16 h. Tubule formation was inspected by inverted light microscopy. Six random fields were used to calculate the average number of complete tubes formed by cells using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Western blot analysis

EPCs were lysed in a lysis buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 10% glycerol, 0.5 mM phenylmethanesulfonyl fluoride, 2 µg/mL aprotinin, pepstatin, and leupeptin). Proteins in the cell lysates were separated using sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis, followed by transfer onto poly (vinylidene fluoride) membranes. The membranes were then probed with monoclonal antibodies against phosphorylated eNOS (Upstate Biotechnology, Lake Placid, NY, USA), SDF-1 and actin. Protein band densitometry was performed using ImageQuant software (Promega, Madison, WI, USA).

Directed *in vivo* angiogenesis assay (DIVAA)

Angiogenesis *in vivo* was evaluated using a DIVAA kit (Trevigen, Gaithersburg, MD, USA). Briefly, angioreactors were filled with EPCs transfected with 50,000 miR-548j-5p mimic, negative control, miR-548j-5p antagomir or scramble control and VEGF/FGF1 embedded in 20 µl basement membrane extract [11].

Angioreactors were incubated at 37°C for 1 h. For positive controls, angioreactors were filled with BME supplemented with VEGF (12.5 ng/ml) plus FGF1 (37.5 ng/ml). Two angioreactors were implanted in each immunocompromised nude mouse (eight-week-old male nude mice) subcutaneously in the dorsal region. The angioreactors were removed 14 days after implantation and photographed. The presence of blood vessels was assessed using FITC-Lectin detection, and fluorescence was determined using a plate reader in mean relative fluorescence units.

Ischemic hind-limb model and EPC transplantation

Eight-week-old male nude mice were purchased from the BioLASCO Taiwan Co., Ltd. After 2 weeks observation, unilateral hind-limb ischemia was induced by left femoral artery ligation. EPCs transfected with miR-548j-5p mimic (n=6) or antagomir (n=6) or a control (n=6) were injected intramuscularly at six different sites on the ischemic limb distal to the arterial occlusion site. The blood flow of the hind limb was measured using a Laser Doppler perfusion imaging system (Moor Instruments Limited, Devon, UK) before and after surgery, and then weekly. The results were expressed as the ratio of perfusion in the ischemic vs. the non-ischemic limb.

The mice were sacrificed 3 weeks after surgery and the limbs were fixed overnight in methanol. The ischemic muscles were embedded in paraffin, and then deparaffinized to incubate them with rat monoclonal antibodies against murine CD31 (BD PharMingen, San Diego, CA, USA). New capillaries were identified based on morphology and positive staining for CD31 using the avidin-biotin-complex technique and Vector Red Chromogenic substrate (Vector Laboratories, Burlingame, CA, USA) after counterstaining with hematoxylin. The visible capillaries were counted under 10 random fields, and the capillary density was expressed as the number of capillaries/mm.

All animals were housed and handled in accordance with the criteria outlined in the National Institutes of Health "Guide for Care and Use of Laboratory Animals". The study protocol was approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital, Taipei, Taiwan (approval number: IACUC_2013-076).

Statistical analysis

Data are expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using the unpaired Student's *t*-test or one-way analysis of variance, followed by Scheffé's multiple comparison *post hoc* test using Statistical Package of the Social Sciences software (version 14; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Detection of circulating miRNAs

The baseline clinical characteristics of the PAD group and control group are shown in Table 1. In total, 40 subjects were studied; 25 patients with documented PAD and 15 individuals without evidence of PAD (control group) from Taipei Veterans General Hospital, Taipei, Taiwan, R.O.C. The mean age of participants was 74 years. A total of 17 patients were male, and the median body mass index was 23.19 ± 2.82 . There were 19 patients with hypertension and 15 with diabetes mellitus. Coronary artery disease and chronic kidney disease were present in 5 and 10 patients, respectively.

Table 1. Baseline characteristics of the study population.

	PAD group (<i>n</i> = 25)	Control group (<i>n</i> = 15)
Demographics		
Age (years)	74	44
Male sex	17 (8)	13 (2)
BMI	23.19 ± 2.82	25.5 ± 0.64
Risk factors		
Hypercholesterolemia	3 (22)	0
Hypertension	19 (6)	1 (14)
Diabetic mellitus	15 (10)	1 (14)
Coronary artery disease	5 (20)	0
Chronic kidney disease	10 (15)	0

The concentration of several circulating miRNAs was measured. The PAD group had a significantly decreased expression of miR-548j-5p (AAAAGUAAUUGCGGUCUUUGGU) compared with the control group (Fig. 1).

miR-548j-5p enhances migration and tube formation in EPCs

Mobilization and tube formation in EPCs in the ischemic area are important for neovascularization. Compared with the control group, transfection of miR-548j-5p mimics into EPCs significantly increased their migration, as shown in Fig. 2; however, transfection of miR-548j-5p antagomir into EPCs decreased their migration.

The effect of miR-548j-5p on tube formation in EPCs was also investigated, as shown in Fig. 3. Transfection of miR-548j-5p mimics into EPCs significantly increased their tube formation compared with the control group; thus, miR-548j-5p could modulate the angiogenic activities of EPCs.

miR-548j-5p promotes the angiogenic potential of EPCs according to DIVAA

The angiogenic effects of miR-548j-5p were further analyzed by DIVVA, as shown in Fig. 4. Transfection of miR-548j-5p mimics into EPCs significantly increased the invasion of vessels in the angioreactors compared with the negative control. However, transfection of miR-548j-5p antagomir into EPCs decreased the invasion of vessels in the angioreactors compared with the scramble control. These findings indicate that miR-548j-5p could promote the angiogenic capacity of EPCs.

miR-548j-5p regulates EPC function via targeting of eNOS and SDF-1

EPCs with miR-548j-5p mimic transfection exhibited significantly upregulated eNOS expression, as shown in Fig. 5. In addition, miR-548j-5p mimic transfection of EPCs upregulated SDF-1 expression; however, miR-548j-5p antagomir transfection of EPCs downregulated eNOS and SDF-1 expression. These results suggest that miR-548j-5p could regulate EPC function via the eNOS and SDF-1 signaling pathways.

miR-548j-5p promotes blood flow recovery in hind-limb ischemia in a mouse model

To evaluate the angiogenic effect of miR-548j-5p, a hind-limb ischemia model was developed. Compared with the control group, the mice with miR-548j-5p mimic transfection of EPCs exhibited significantly enhanced flow recovery. However, the mice with miR-548j-5p antagomir transfection showed delayed blood flow recovery after surgery (Fig. 6A and B). Anti-CD31 immunostaining showed a decreased capillary density in mice with miR-548j-5p antagomir transfection of EPCs compared with the control mice, but the miR-548j-5p mimic mice exhibited significantly increased capillary density in muscles (Fig. 6C). Therefore, miR-548j-5p could enhance the angiogenic activities of EPCs.

Discussion

To the best of our knowledge, this is the first study to report down-regulation of miR-548j-5p in PAD. Transfection of EPCs with miR-548j-5p could enhance the activity of eNOS and SDF-1 and improve EPC functions, such as migration and tube formation. Whereas, miR-548j-5p antagomir transfection impaired the angiogenic activities of EPCs. Transfection of EPCs with miR-548j-5p also improved blood flow recovery in a hind-limb ischemic mouse model. These results suggest that miR-548j-5p could induce angiogenesis.

Recently, circulating miRNAs have served as novel biomarkers for cardiovascular disease [12]. In patients with PAD, individual miRNAs have been found to be differentially-expressed in circulation. Bogucka-Kocka *et al.* [13] used dysregulation of 26 miRNAs to propose novel biomarkers for lower-extremity arterial disease. In patients with CLI, higher levels of circulating miR15a and miR16 predicted a poorer prognosis compared with diabetic individuals [14]. Hazarika *et al.* [15] found that miR-93 was differentially-expressed in the ischemic hind-limb muscle of mice. Overexpression of miR-93 enhances cell proliferation and endothelial cell tube formation, which suggests that miR-93 modulated ischemic-induced angiogenesis independent of HIF-1 α regulated angiogenic genes [15].

miR-146a was found to be a regulator of vascular remodeling. Heuslein *et al.* [16] found that suppression of miR-146a enhanced arteriogenesis in muscular collateral circulation via upregulation of pro-inflammatory endothelial activation. miR29a inhibited the expression of endothelial cell metalloproteinase domain-containing protein 12 in ischemia in hyperglycemia. Modulation of miR29a improved impaired post-ischemic angiogenesis in diabetes [17]. In the present study, miR-548j-5p was observed to facilitate migration and tube formation in EPCs, indicating that miR-548j-5p may contribute to angiogenesis in PAD by regulating the angiogenic activities of EPCs.

Nitric oxide (NO) is a key role for angiogenesis in ischemic disease and eNOS synthetization of NO could promote migration of EPCs [18,19]. SDF-1 is a chemokine that has a strong chemotactic effect on EPCs. After vascular injury, SDF-1 induces migration of EPCs to the injured area and promotes endothelialization [20,21]. Under hyperglycemic conditions miR-133a was found to be up-regulated and induced endothelial dysfunction through inhibition of eNOS [22]. Chen *et al.* [23] reported that the diabetes-induced expression of miR-133a, impaired angiogenesis in PAD via a reduction in NO synthesis. miR-133a antagonism improved post-ischemic angiogenesis. In the current study, we found that up-regulation of miR-548j-5p induced the expression of eNOS and SDF-1 in EPCs. These results revealed that miR-548j-5p enhanced migration and tube formation in EPCs by targeting the eNOS signaling pathway.

There are some limitations in our studies such as in vitro and in vivo experiments not conducted with control miRNAs. We also need more cases to confirm the importance of miR-548j-5p in the future.

Conclusion

The present study demonstrated that miR-548j-5p contributes to angiogenesis by promoting migration and tube formation in EPCs, which are associated with eNOS and SDF-1 expression. Up-regulation of miR-548j-5p improved the neovascularization in hind-limb ischemic mice. Thus, miR-548j-5p may have therapeutic potential for PAD. Further studies are needed to evaluate the therapeutic effects and to examine the clinical implications of these findings.

Abbreviations

CLI, critical limb ischemia

eNOS, endothelial nitric oxide synthase

EPC, endothelial progenitor cell

FGF1, fibroblast growth factor-1

miRNA, microRNA

NO, nitric oxide

PAD, peripheral artery disease

SDF-1, stromal cell-derived factor-1

SEM, standard error of the mean

VEGF, vascular endothelial growth factor

Declarations

Authors' contributions

CYL and TCW contributed to the study conception and design, literature review and preparation of the manuscript. SHL and TCW contributed to the study conception and design, drafted the manuscript, revised it critically for important intellectual content, and gave final approval. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

This work was assisted in part by the Division of Experimental Surgery of the Department of Surgery, Taipei Veterans General Hospital.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical approval

This study was approved by the Taipei Veterans General Hospital's Institutional Review Board for Research (approval number 2013-08-020B#3), and the animal study protocol was approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital, Taipei, Taiwan (approval number: IACUC_2013-076)

Funding

This study was partially supported by grants V105C-108 and V107C-172 (to Tao-Cheng Wu) from Taipei Veterans General Hospital, Taipei, Taiwan, R.O.C.

Consent to participate

Written informed consent was provided by all the participants prior to their inclusion within the study.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Code availability

Not applicable

References

1. Criqui MH, Aboyans V. Epidemiology of peripheral artery disease. *Circ Res.* 2015; doi:10.1161/CIRCRESAHA.116.303849.
2. Stock JK. The challenge of peripheral arterial disease: How do we improve outcome? *Atherosclerosis.* 2018; doi:10.1016/j.atherosclerosis.2017.12.031.
3. Abu Dabrh AM, Steffen MW, Undavalli C, Asi N, Wang Z, Elamin MB, et al. The natural history of untreated severe or critical limb ischemia. *J Vasc Surg.* 2015; doi:10.1016/j.jvs.2015.07.065.
4. Morisaki K, Yamaoka T, Iwasa K. Risk factors for wound complications and 30-day mortality after major lower limb amputations in patients with peripheral arterial disease. *Vascular.* 2018; doi:10.1177/1708538117714197.
5. Quiat D, Olson EN. MicroRNAs in cardiovascular disease: from pathogenesis to prevention and treatment. *J Clin Invest.* 2013; doi:10.1172/JCI62876.
6. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov.* 2012; doi:10.1038/nrd3864.
7. Stather PW, Sylvius N, Wild JB, Choke E, Sayers RD, Bown MJ. Differential microRNA expression profiles in peripheral arterial disease. *Circ Cardiovasc Genet.* 2013; doi:10.1161/CIRCGENETICS.111.000053.
8. Shu X, Mao Y, Li Z, Wang W, Chang Y, Liu S, et al. MicroRNA93 regulates angiogenesis in peripheral arterial disease by targeting CDKN1A. *Mol Med Rep.* 2019; doi:10.3892/mmr.2019.10196.
9. Hsu PY, Hsi E, Wang TM, Lin RT, Liao YC, Juo SH. MicroRNA let-7g possesses a therapeutic potential for peripheral artery disease. *J Cell Mol Med.* 2017; doi:10.1111/jcmm.12997.
10. Wu TC, Chan JS, Lee CY, Leu HB, Huang PH, Chen JS, et al. Rivaroxaban, a factor Xa inhibitor, improves neovascularization in the ischemic hindlimb of streptozotocin-induced diabetic mice. *Cardiovasc Diabetol.* 2015; doi:10.1186/s12933-015-0243-y.
11. Flores-Perez A, Marchat LA, Rodriguez-Cuevas S, Bautista-Pina V, Hidalgo-Miranda A, Ocampo EA, et al. Dual targeting of ANGPT1 and TGFBR2 genes by miR-204 controls angiogenesis in breast cancer. *Sci Rep.* 2016; doi:10.1038/srep34504.
12. Fichtlscherer S, Zeiher AM, Dimmeler S. Circulating microRNAs: biomarkers or mediators of cardiovascular diseases? *Arterioscler Thromb Vasc Biol.* 2011; doi:10.1161/ATVBAHA.111.226696.
13. Bogucka-Kocka A, Zalewski DP, Ruszel KP, Stepniowski A, Galkowski D, Bogucki J, et al. Dysregulation of MicroRNA Regulatory Network in Lower Extremities Arterial Disease. *Front Genet.* 2019; doi:10.3389/fgene.2019.01200.
14. Spinetti G, Fortunato O, Caporali A, Shantikumar S, Marchetti M, Meloni M, et al. MicroRNA-15a and microRNA-16 impair human circulating proangiogenic cell functions and are increased in the proangiogenic cells and serum of patients with critical limb ischemia. *Circ Res.* 2013; doi:10.1161/CIRCRESAHA.111.300418.

15. Hazarika S, Farber CR, Dokun AO, Pitsillides AN, Wang T, Lye RJ, et al. MicroRNA-93 controls perfusion recovery after hindlimb ischemia by modulating expression of multiple genes in the cell cycle pathway. *Circulation*. 2013; doi:10.1161/CIRCULATIONAHA.112.000860.
16. Heuslein JL, McDonnell SP, Song J, Annex BH, Price RJ. MicroRNA-146a Regulates Perfusion Recovery in Response to Arterial Occlusion via Arteriogenesis. *Front Bioeng Biotechnol*. 2018; doi:10.3389/fbioe.2018.00001.
17. Chen L, Okeke E, Ayalew D, Wang D, Shahid L, Dokun AO. Modulation of miR29a improves impaired post-ischemic angiogenesis in hyperglycemia. *Exp Biol Med (Maywood)*. 2017; doi:10.1177/1535370217716424.
18. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med*. 2003; doi:10.1038/nm948.
19. Yang CY, Chen C, Lin CY, Chen YH, Lin CY, Chi CW, et al. Garcimultiflorone K inhibits angiogenesis through Akt/eNOS- and mTOR-dependent pathways in human endothelial progenitor cells. *Phytomedicine*. 2019; doi:10.1016/j.phymed.2019.152911.
20. Piao JM, Wu W, Yang ZX, Li YZ, Luo Q, Yu JL. MicroRNA-381 Favors Repair of Nerve Injury Through Regulation of the SDF-1/CXCR4 Signaling Pathway via LRRC4 in Acute Cerebral Ischemia after Cerebral Lymphatic Blockage. *Cell Physiol Biochem*. 2018; doi:10.1159/000488821.
21. Yu G, Liu P, Shi Y, Li S, Liu Y, Zhu W. Sitagliptin Stimulates Endothelial Progenitor Cells to Induce Endothelialization in Aneurysm Necks Through the SDF-1/CXCR4/NRF2 Signaling Pathway. *Front Endocrinol (Lausanne)*. 2019; doi:10.3389/fendo.2019.00823.
22. Li P, Yin YL, Guo T, Sun XY, Ma H, Zhu ML, et al. Inhibition of Aberrant MicroRNA-133a Expression in Endothelial Cells by Statin Prevents Endothelial Dysfunction by Targeting GTP Cyclohydrolase 1 in Vivo. *Circulation*. 2016; doi:10.1161/CIRCULATIONAHA.116.017949.
23. Chen L, Liu C, Sun D, Wang T, Zhao L, Chen W, et al. MicroRNA-133a impairs perfusion recovery after hindlimb ischemia in diabetic mice. *Biosci Rep*. 2018; doi:10.1042/BSR20180346.

Figures

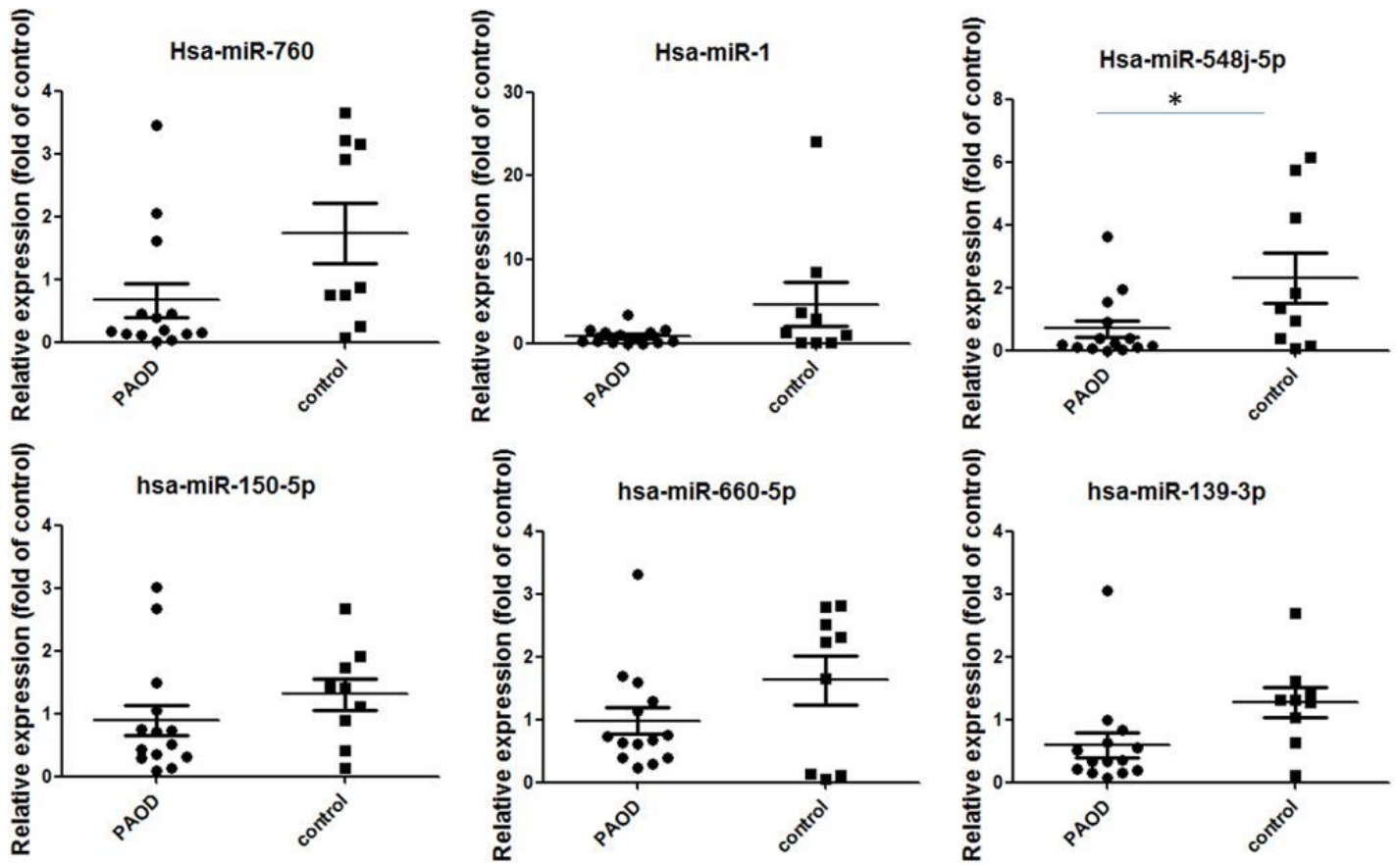
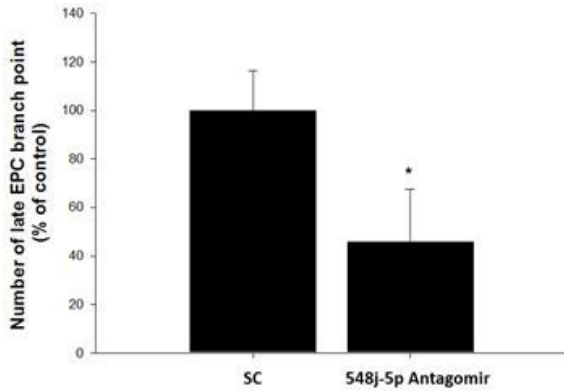
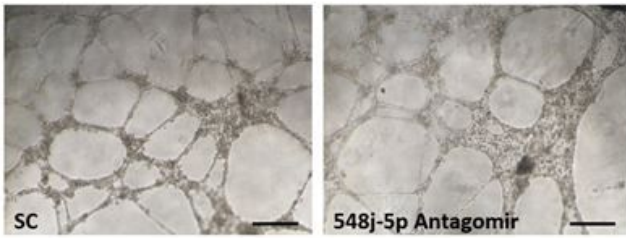


Figure 1

Expression of microRNAs in patients with PAD compared with the control group. Candidate miRNAs in the PAD and the control group were analyzed. miR-548j-5p was significantly decreased in patients with PAD group compared to controls. (*P<0.05 vs. the control group).

A.



B.

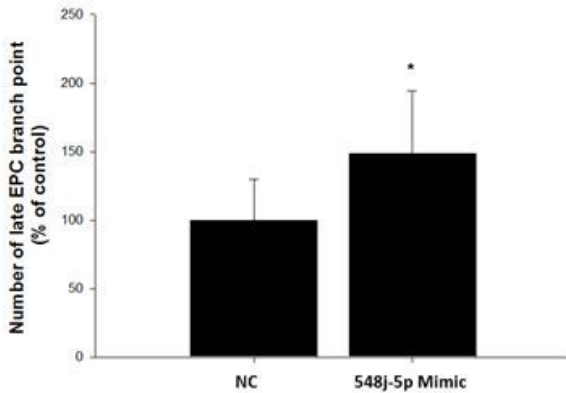
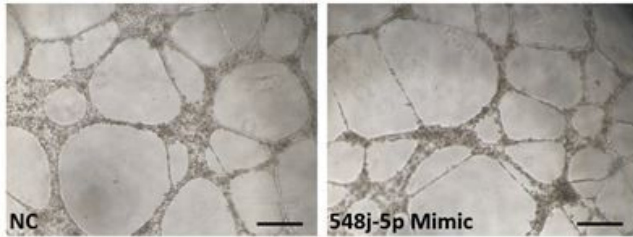
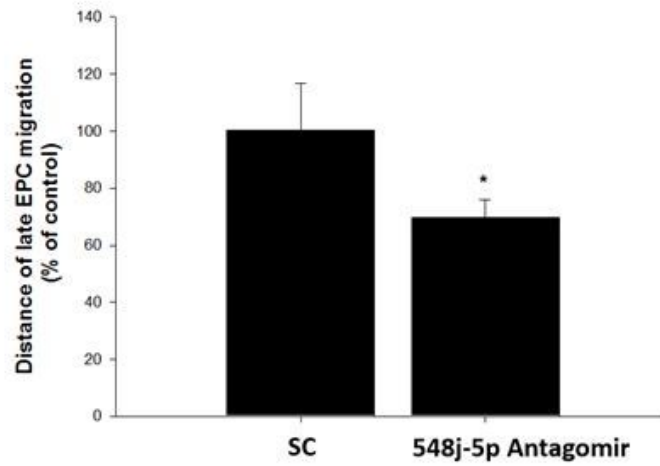
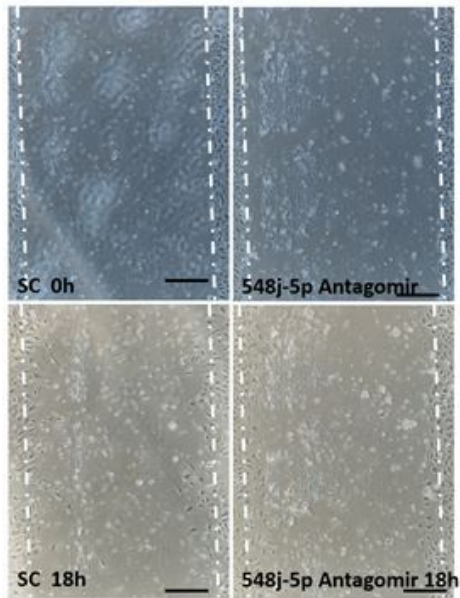


Figure 2

Effect of miR-548j-5p on human EPC tube formation. An in vitro angiogenesis assay for EPCs was performed using ECMatrix gel. (A) Human EPCs transfected with miR-548j-5p antagomir exhibited impaired tube formation compared with the negative control in vitro. (B) Human EPCs transfected with miR-548j-5p mimic exhibited enhanced tube formation compared with the negative control in vitro. Data are presented as the mean \pm SEM. (* $P < 0.05$ vs. the control, $n = 6$ for each experiment).

A.



B.

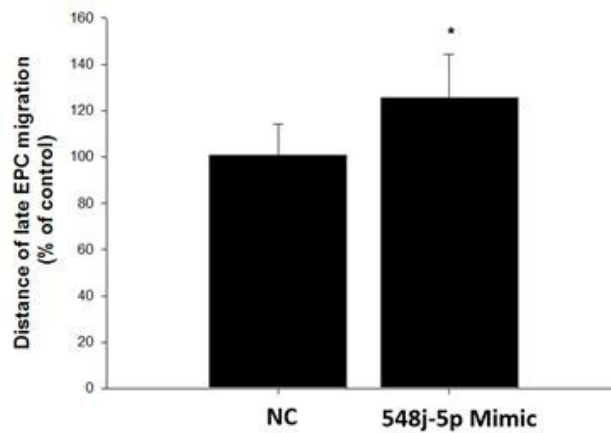
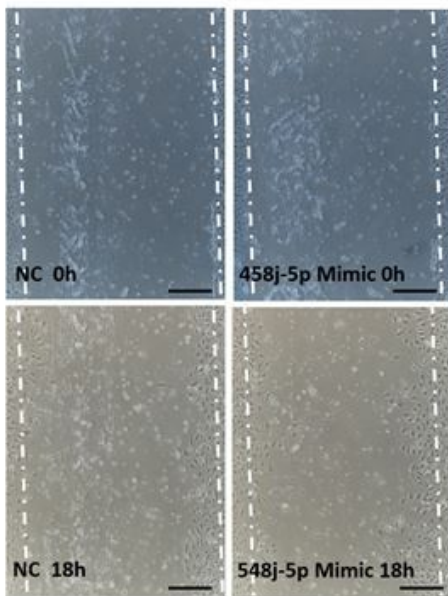


Figure 3

Effect of miR-548j-5p on human EPC migration. The migratory function of EPCs was evaluated using a scratch injury model. (A) Human EPCs transfected with miR-548j-5p antagomir exhibited suppressed EPC mobilization compared with the negative control in vitro. (B) Human EPCs transfected with miR-548j-5p mimic exhibited enhanced EPC mobilization compared with the negative control in vitro. Data are presented as the mean \pm SEM. (*P<0.05 vs. the control, n=6 for each experiment).

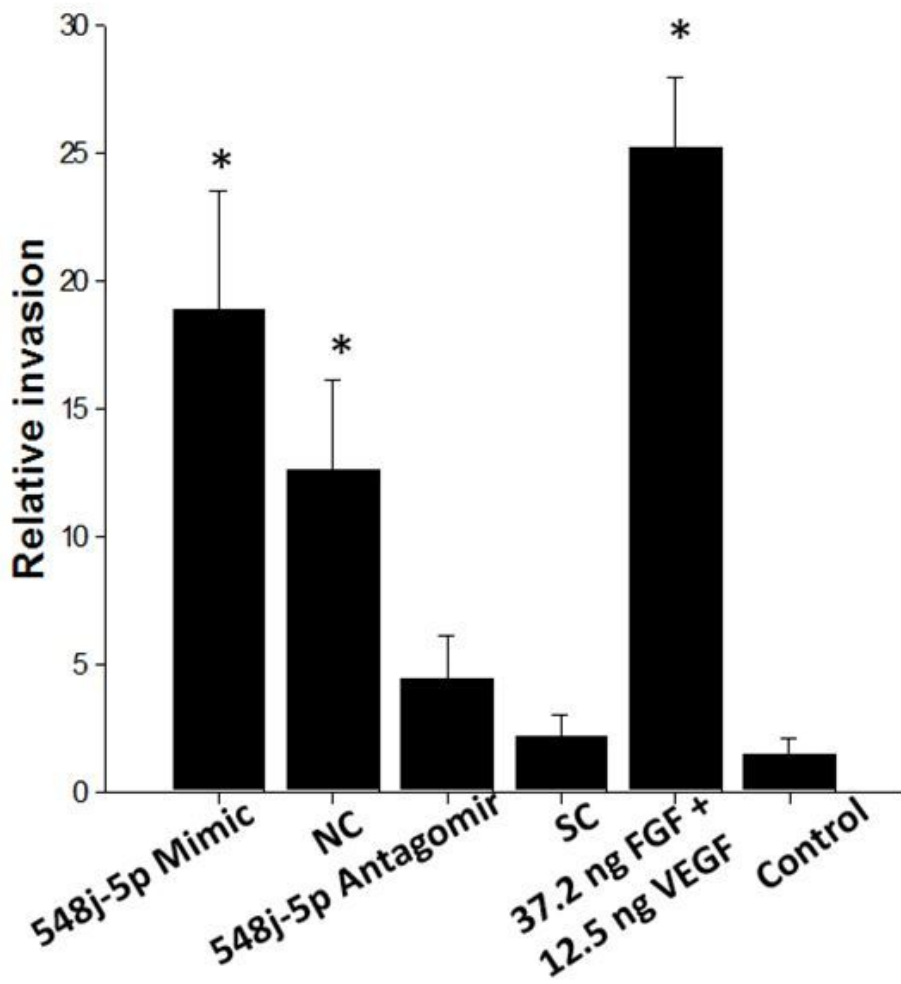
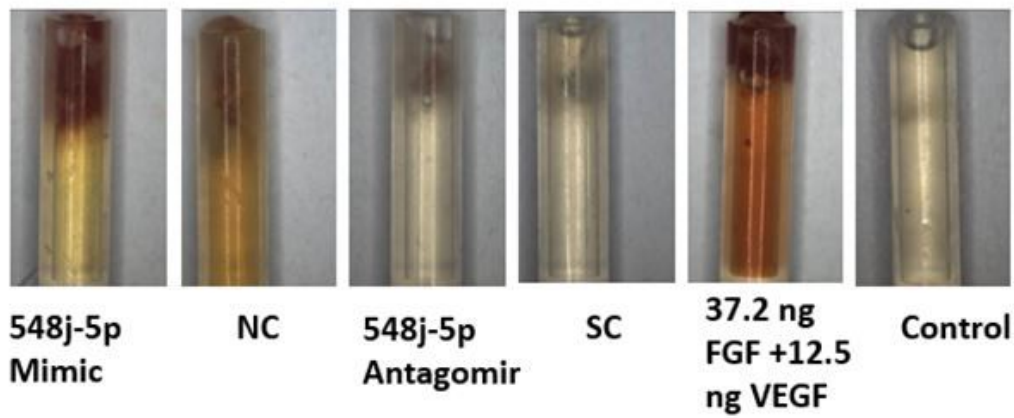
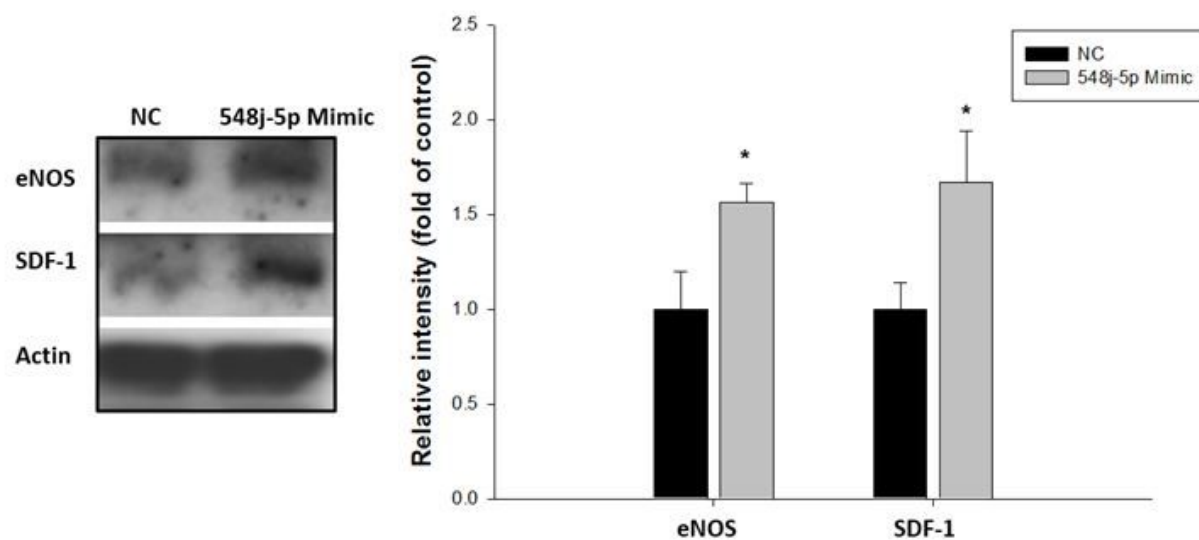


Figure 4

Effect of miR-548j-5p on blood vessel formation in vivo, assessed using a directed in vivo angiogenesis assay. Representative images of angioreactors extirpated from nude mice 9 days after implantation. miR-548j-5p enhanced blood vessel formation. Data are presented as the mean \pm SEM. (* $P < 0.05$ vs. the control, $n = 6$ for each experiment).

A.



B.

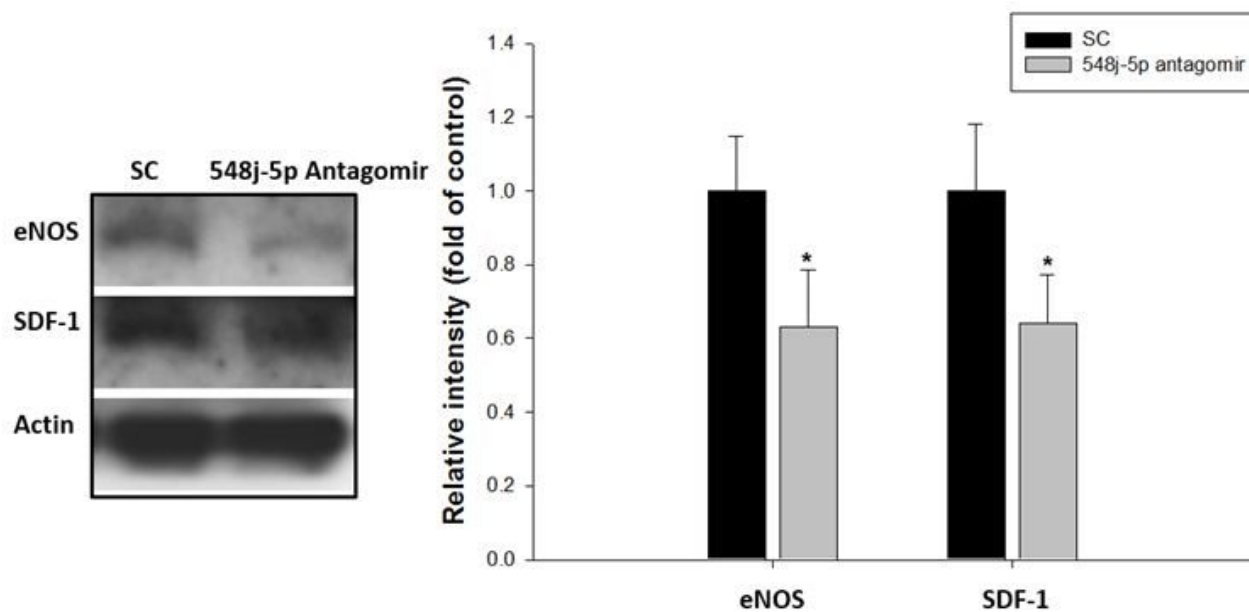


Figure 5

Effect of miR-548j-5p on eNOS and SDF-1 production in EPCs. (A) Impaired expression of eNOS and SDF-1 in EPCs was observed in EPCs transfected with miR-548j-5p antagomir compared with the scramble control. (B) Enhanced expression of eNOS and SDF-1 was observed in EPCs transfected with miR-548j-5p mimic compared with the negative control. Data are presented as the mean \pm SEM. (* $P < 0.05$ vs. the control, $n = 6$ for each experiment).

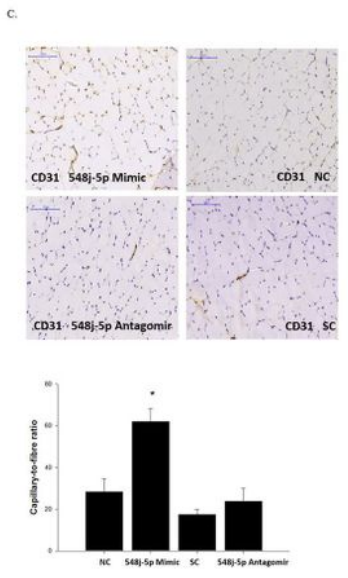
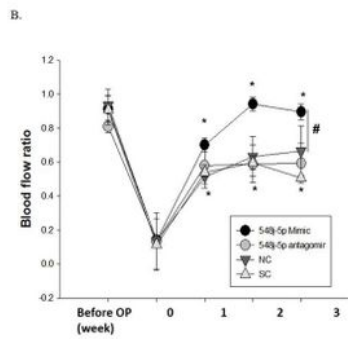
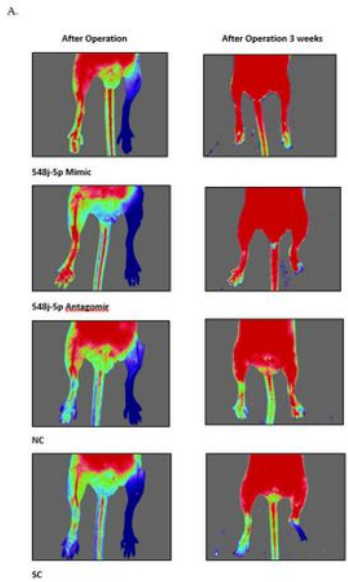


Figure 6

Effect of miR-548j-5p on hind-limb perfusion. (A) Serial Doppler analysis of hind-limb perfusion before and 3 weeks after hind-limb ischemia surgery in nude mice that received transplants of EPCs transfected with miR-548j-5p mimic, miR-548j-5p antagomir, negative control or scramble control. The color scale illustrates blood flow variation from minimal (dark blue) to maximal (red) values. Arrows indicate the ischemic limb after hind-limb ischemia surgery. (B) Quantification analysis of perfusion recovery using

the laser Doppler perfusion imaging ratio (ischemia/normal hind-limb) in the different groups. (C) Mice were sacrificed 3 weeks after surgery and capillaries in the ischemic muscles were visualized using anti-CD31 immunostaining. Data are presented as the mean \pm SEM. (*P<0.05 vs. 0 weeks, #P<0.05 vs. miR-548j-5p antagomir, n=6 for each experiment).