MicroRNA let-7g possesses a therapeutic potential for peripheral artery disease

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Abstract

Peripheral artery disease (PAD) is a manifestation of systemic atherosclerosis and conveys a significant health burden globally. Critical limb ischaemia encompasses the most severe consequence of PAD. Our previous studies indicate that microRNA let-7g prevents atherosclerosis and improves endothelial functions. This study aimed to investigate whether and how let-7g therapy may improve blood flow to ischaemic limbs. The present study shows that let-7g has multiple pro-angiogenic effects on mouse ischaemic limb model and could be a potential therapeutic agent for PAD. Mice receiving intramuscular injection of let-7g had more neovascularization, better local perfusion and increased recruitment of endothelial progenitor cells after hindlimb ischaemia. The therapeutic effects of let-7g’s on angiogenesis are mediated by multiple regulatory machinery. First, let-7g increased expression of vascular endothelial growth factor-A (VEGF-A) and VEGF receptor-2 (VEGFR-2) through targeting their upstream regulators HIF-3α and TP53. In addition, let-7g affected the splicing factor SC35 which subsequently enhanced the alternative splicing of VEGF-A from the anti-angiogenic isoform VEGF-A165b towards the pro-angiogenic isoform VEGF-A164a. The pleiotropic effects of let-7g on angiogenesis imply that let-7g may possess a therapeutic potential in ischaemic diseases.

Keywords: microRNA let-7g • vascular endothelial growth factor-A • endothelial progenitor cells • peripheral artery disease • angiogenesis

Introduction

Peripheral artery disease (PAD) is often associated with diabetes and coronary artery disease, leading to significant morbidity and mortality [1, 2]. The prevalence of asymptomatic PAD is estimated to be around 3–10%, increasing to 15–20% in persons over 70 years [3]. Critical limb ischaemia (CLI) is the most severe clinical manifestation of PAD, which causes intermittent claudication, gangrene and foot ulceration. Patients with CLI are at risk of devastating complications including amputation and mortality [4, 5].

MicroRNAs (miRNAs) are small (~22 nucleotide long) non-coding RNAs that regulate gene expression at the post-transcriptional level by degradation of mRNAs or inhibition of protein translation [6]. The miRNA let-7 family plays a pivotal role in cell proliferation, cancer and cardiovascular diseases [7, 8]. Our group previously reported that let-7g prevents atherosclerosis by inhibiting the uptake of oxidized-low density lipoprotein (ox-LDL) into endothelial cells (ECs) and vascular smooth muscle cells [9]. Our recent study further showed that reduced let-7g levels impairs endothelial functions through targeting transforming growth factor beta (TGF-β) and sirtuin-1 (SIRT-1) signalling pathways [10]. We demonstrated that low let-7g levels can increase thrombosis, inflammation, senescence and anti-angiogenesis, all of which contribute to vascular diseases. Another group showed that hypoxia could up-regulate the let-7 family (including let-7g) in ECs, which in turns targeted argonaute 1 (AGO1) leading to de-suppression of vascular endothelial growth factor-A (VEGF-A) [11]. Another recent study showed that PAD patients had elevated levels of anti-angiogenic VEGF-A isoform (VEGF-A165b) and decreased levels of
pro-angiogenic isofrm VEGF-A\textsubscript{165a} (the human orthologue of murine VEGF-A\textsubscript{164a}) [12]. Therefore, an increase in let-7g may be beneficial to PAD patients.

This study aimed to investigate whether let-7g therapy could improve blood flow to ischaemic tissue in the hindlimb ischaemic animal model. The effects of hypoxia on the let-7g and let-7g-regulated genes were investigated in muscle cells. Let-7g’s effects on VEGF-A, vascular endothelial growth factor receptor 2 (VEGFR-2), endothelial progenitor cells (EPCs) and the underlying mechanisms were explored.

**Materials and methods**

**Muscle cell culture**

C2C12, a mouse myoblast cell line, was used for the in vitro studies. C2C12 cells were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM; Thermo Scientific, Waltham, MA, USA) and supplemented with 10% foetal bovine serum (FBS). For the hypoxic experiment, the C2C12 cells were cultured under 1% O\textsubscript{2}, 5% CO\textsubscript{2} and 94% N\textsubscript{2} for 24 hrs. For the oxygen glucose deprivation (OGD) experiment, the C2C12 cells were cultured using an identical hypoxia protocol, but the cells were incubated in DMEM medium without glucose and serum (Thermo Scientific) at 37°C for 6 hrs. Following the OGD insult, fresh culture medium was added in the cultures. The cells were then allowed to re-oxygenate under normal conditions (37°C, 5% CO\textsubscript{2}, 95% air) for 18 hrs. For the siRNA study, C2C12 cells were incubated at 37°C in a humidified atmosphere for 24 hrs. C2C12 cells were transfected with different doses of CDK6 siRNA (5, 10, 50, 100 nM) or control siRNA for 24 hrs.

**Animal model of hindlimb ischaemia**

Ten-week-old male C57BL/6 mice (Charles River Technology; BioLASCO Taiwan Co., Ltd, Taipei, Taiwan), weighing 22–25 g were used for all experiments. To produce hindlimb ischaemia, mice were sedated with isoflurane (Abbott Laboratories Ltd., Quenborough, Kent, United Kingdom), and anaesthetized by intraperitoneal administration of pentobarbital sodium (40 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). The mice were placed in a supine position on a warming pad at 37°C with the left hindlimbs shaved. The left femoral artery and all attached side-branches were dissected free and then excised along its entire length. The veins were left intact during the procedure and the overlying skin was then closed [13].

The negative control-miRNA and let-7g mimic were from Thermo Scientific. Both types of miRNAs were formulated with a commercial PEI-based nanoparticle (called in vivo-jetPEI\textsuperscript{TM}) for animal studies. Mice were then divided into two groups (n = 6 for each group) for intramuscular injection (IM) of 5 nM negative control-miRNA (placebo group) or let-7g mimic (let-7g group). IM injection was given at three sites of the gastrocnemius muscle at the medial thigh on day 1, 8 and 15 after induced hindlimb ischaemia [14]. Noticelby, the day for induction of ischaemic limb was defined as day 0. All animal procedures were compliant with the standards and approved protocols by Kaohsiung medical university-institutional Animal Care and Use Committee (IACUC).

**Measurement of hindlimb perfusion**

The blood flow of ischaemic (left) limb and normal (right) limb was measured using the Laser Doppler Perfusion Image (LDPI, Moor-LD12-2x; Moor, Co., United Kingdom) on day 2, 7, 14 and 21 postoperatively. The LDPI system uses a near infrared laser beam (633 and 830 nm) and has an average measurement depth of approximately 1.0–2.0 mm. This imaging technique provides a non-invasive measurement of blood flow by determining the Doppler frequency shift of light reflected by the moving red blood cells [15]. During the scanning procedure, mice were placed in a supine position on a warming pad at 37°C and the normal and ischaemic regions were identified. The machine then focused on each region and consecutively measured the intensity of blood flow over the region of interest (leg and foot). Colour-coded images were recorded, and analyses were performed automatically by calculating the average perfusion of each leg (ischaemic and non-ischaemic legs separately). The perfusion ratio calculated as blood flow in left (ischaemic) versus right (normal) legs of each animal was used to assess the circulation to the ischaemic limb.

**Immunohistochemistry to measure capillary density**

Capillary density, as an index of angiogenesis, was determined by counting the total number of endothelial cells relative to the total surface area via light microscopy. Ischaemic (left) leg of each animal was harvested on day 21 after surgery and embedded in 10% formalin. The gastrocnemius muscle was excised and fixed overnight in 10% formalin in phosphate-buffered saline (PBS). Paraffin-embedded sections of 5 μm-thickness muscle sections were first treated with 0.3% H\textsubscript{2}O\textsubscript{2} for 30 min. and incubated with block reagent for 1 hr at room temperature. Sections were then incubated with the primary antibody specifically against CD31 (1:100; GenTex, Irvine, CA, USA) for 1 hr, followed by incubation with biotinylated secondary antibody (1:200). The capillary density was defined as: (capillary surface area)/(total surface area of each section) (×100%). The capillary density for each mouse was counted under five randomly selected 200× fields using Image J software.

**Definition of EPCs**

Bone marrow and peripheral blood were obtained from each mouse on day 21 after induction of hindlimb ischaemia. To obtain whole marrow cells, femoral and tibial bones were aseptically harvested and the bone marrow cavity was washed with DMEM. Marrow cells were then centrifuged at 300 × g for 10 min. and the supernatant was removed. Mononuclear cells were isolated from peripheral blood by gradient centrifugation using Histopaque 1083 (Sigma-Aldrich). To determine the proportion of EPCs in bone marrow and peripheral blood, whole marrow cells and circulating mononuclear cells were incubated with monoclonal antibodies against CD34 and VEGFR-2 for 1 hr. Secondary detection was performed using Alexa Fluor 488- and Texas Red 615-conjugated secondary antibodies (BioSmart, Houston, TX, USA). Isotype-identical antibodies (IgG) were served as controls. Flow cytometry analyses were performed by utilizing a fluorescence-activated cell sorter (FACS, Beckman Coulter FC500 flow cytometer). Cells carried both CD34 and VEGFR-2 (CD34+VEGFR-2+) were defined as EPCs.
mRNA and protein analysis

The gastrocnemius muscles of ischaemic and non-ischaemic limbs were harvested on day 21 after mice were killed. Total RNA and protein extracted from the gastrocnemius muscle or C2C12 cells were used to analyse the expression levels of VEGF-A, VEGF-A164a, VEGF-A165b, VEGFR-2, Hypoxia-inducible factor 3-alpha (HIF-3α), Tumour Protein 53 (TP53), Cyclin D-dependent kinases 6 (CDK6) and splicing factor SC35. Total RNA was extracted with TRizol® Reagent (Invitrogen), and cDNA was produced using 1 μg of starting mRNA (Applied Biosystems, Darmstadt, Germany) and random hexamers. The sequences of PCR primers are shown in Table 1, and primer sequences for VEGF-A164a and VEGF-A165b were from Kikuchi et al. [12]. Quantitative real-time PCR (qPCR) was performed with an ABI PRISM 7900 sequence detector and SYBR green reagents (Applied Biosystems, Waltham, MA, USA). All samples were run in triplicate. The relative amount of mRNA of interest was normalized to 18S rRNA. For let-7g level quantification, U6 was used as the internal control. Dissociation curves were performed to confirm the specificity of PCR products.

| Gene     | Primer                  |
|----------|-------------------------|
| VEGF-A F | 5′-AAC GAA AGC GCA AGA AAT CC-3′ |
| VEGF-A R | 5′-GCT CAC AGT GAA CGC TCG AG-3′ |
| VEGF-A164a F | 5′-C AGA AAA TCA CTG TGA GCC TTG TT-3′ |
| VEGF-A164a R | 5′-C TTG GCT TGT TCG TAC TCG AA-3′ |
| VEGF-A165b F | 5′-C AGA AAA TCA CTG TGA GCC TTG TT-3′ |
| VEGF-A165b R | 5′-C TTT CCG GTG AGA GTG CTG C-3′ |
| VEGFR-2 F | 5′-CCA CCC CAG AAA TGT ACC AAA C-3′ |
| VEGFR-2 R | 5′-AAA ACG CGC GTC TCT GGT T-3′ |
| 18S F    | 5′-TTG ATT AAG TCC CTG CCC TTG TT-3′ |
| 18S R    | 5′-CGA TCC GAG GGC CTC ACT A-3′ |
| HIF-3 F  | 5′-TGG GAA CTT CAT GTC GAC GC-3′ |
| HIF-3 R  | 5′-GCA ATG CCT GGT GCT TAT CT-3′ |
| TP53 F   | 5′-CAG GTA CTC TCC TCC CCT CAA T-3′ |
| TP53 R   | 5′-AAC TGC ACA GGG CAC GTC TT-3′ |
| CDK6 F   | 5′-TCT CAC AGA GTA GTG CAT GCT-3′ |
| CDK6 R   | 5′-CGA GTG AGG GCC CAT CTG AAG A-3′ |
| SC35 F   | 5′-TCC AAG TCC AAG TCC TCT TC-3′ |
| SC35 R   | 5′-ACT GCT CCC TCT TCT TCT GG-3′ |

Results

Let-7g increases perfusion in the rodent ischaemic hindlimb

The let-7g level was decreased in C2C12 cells under the hypoxia or OGD condition (Fig. 1A). In addition, our in vivo data showed that let-
7g level was significantly decreased in the ischaemic limb (Fig. 1B). Injection of let-7g to hindlimbs of normal mice significantly increased let-7g levels in gastrocnemius muscles at 24 hrs, then the level declined gradually at 48 hrs, and returned to the baseline at 72 hrs (Fig. 1C). The timeframe of the protocol for animal experiment of hindlimb ischaemia is shown in Fig. 1D.

The operation of ligation and excision of the left femoral artery effectively reduced the perfusion ratio to 0.20 at 48 hrs post-operation for mice in either let-7g-treated or placebo group (Fig. 2A and B). The perfusion ratio was significantly increased in let-7g-treated mice compared with placebo-treated mice on day 7 (0.346 ± 0.015 versus 0.256 ± 0.009, t-test \( P = 0.0004 \)). Representative images of the hindlimb perfusion on day 2 and day 21 are shown in Fig. 2A. In the let-7g-treated group, the perfusion ratio improved promptly and reached to 0.63 on day 21 post-operation (Fig. 2B).

### Capillary density analysis

To confirm that the improved perfusion ratio is attributed to neo-vascularization, immunohistochemistry staining with anti-CD31 antibody was performed in the gastrocnemius muscle of ischaemic legs. Representative photographs of histological sections are shown in Fig. 2C. The capillary density in the gastrocnemius muscle was significantly higher in the let-7g-treated group than that in the placebo group on day 21 post-operation (3.50 ± 0.39% versus 1.00 ± 0.14%, \( P = 0.0001 \)) (Fig. 2D).

### Let-7g enhances EPC recruitment

Mobilization and recruitment of EPCs (defined as CD34+VEGFR-2+ cells) to the ischaemic region are important for neo-vascularization. Flow cytometry analysis was performed to detect EPCs in the bone marrow and in peripheral mononuclear cells. On day 21, EPC percentage among the bone marrow cells was highest in the ischaemic leg of let-7g-treated group and lowest in the non-ischaemic leg of placebo group (0.258 ± 0.018% versus 0.168 ± 0.013%, \( P = 0.0272 \)) (Fig. 3A), while the data were similar between non-ischaemic legs of let-7g-treated group (0.207 ± 0.015%) and ischaemic legs of placebo group (0.198 ± 0.015%). Additionally, the percentage of EPCs in the circulating mononuclear cells was significantly higher in the let-7g treated group than in the placebo group (0.055 ± 0.009% versus 0.027 ± 0.005%, \( P = 0.019 \)) (Fig. 3B).

### Let-7g effects on VEGF-A and VEGFR-2

To assess the role of let-7g in the VEGF-A signal pathway, we analysed the expression ratio of VEGF-A and VEGFR-2 in gastrocnemius...
muscle of the ischaemic/non-ischaemic hindlimb. Twenty-one days after the induction of hindlimb ischaemia, mRNA ratios of VEGF-A and VEGFR-2 were significantly higher in let-7g-treated group than those in the placebo group (Fig. 3C and D, $P = 0.0017$ and 0.0017 for VEGF-A and VEGFR-2, respectively). Similarly, the ratios of VEGF-A and VEGFR-2 protein levels of ischaemic/non-ischaemic hindlimb are higher in the let-7g-treated than placebo-treated animals (Fig. 3E). Using the ratio from placebo-treated animals as the reference, the relative ratios for let-7g-treated animals were 1.45 for VEGF-A protein and 1.20 for VEGFR-2 protein.

**HIF-3α and TP53 as let-7g target genes**

Let-7g is unlikely to directly bind to VEGF-A since let-7g increased VEGF-A expression. We used the IPA software to indicate that let-7g may directly bind to HIF-3α and TP53, both of which are up-stream regulators of VEGF-A [16–18]. The luciferase reporter assay confirmed that HIF-3α harbours two let-7g binding sites, and let-7g could dose-dependently reduce luciferase activity (Fig. 4A and B). Destruction either of the let-7g binding sites in HIF-3α 3’UTR aborted let-7g’s effect on luciferase activity. The luciferase assay also confirmed that let-7g could bind to TP53 3’UTR leading to TP53 down-regulation (Fig. 4C).

**Let-7g enhances VEGF-A alternative splicing through modulating CDK6 and SC35**

The splicing factor SC35 and the transcription factor E2F1 collaboratively control the alternative processing at exon 8 of VEGF-A, which leads to VEGF-A₁₆₄₄ (i.e. the rodent ortholog of human...
VEGF-A165a and VEGF-A165b isoforms [19]. SC35 was found to be a transcription target of E2F1, and E2F1 increased the expression of SC35 in cellular models [19, 20]. Using IPA, let-7g was predicted to directly suppress CDK6, which inhibited the release of E2F1 and subsequently down-regulated SC35 expression [21, 22]. Luciferase reporter assay confirmed that let-7g could directly knock down CDK6 (Fig. 4D). The in vivo data showed that the mRNA ratio of HIF-3\textalpha, TP53, CDK6 and SC35 in the gastrocnemius muscle of ischaemic/non-ischaemic hindlimb were lower in the let-7g treated group than placebo group (\(P < 0.005\)) (Fig. 5A). Let-7g treatment increased the ratio of pro-angiogenic VEGF-A164a in ischaemic/non-ischaemic hindlimb, but decreased the ratio of anti-angiogenic VEGF-A165b levels (Fig. 5A). Western blot analysis showed similar findings as mRNA data. The ratios of protein amounts of VEGF-A165b, HIF-3\textalpha, TP53, CDK6 and SC35 were lowered by let-7g when compared to the ratios in the placebo-treated animals (Fig. 5B). Using the ratio from placebo-treated animals as the reference, the relative ratios for let-7g-treated animals were 0.34 for VEGF-A165b, 0.70 for HIF-3\textalpha, 0.68 for TP53, 0.59 for CDK6 and 0.20 for SC35. Because no commercially available antibody for VEGF-A164a, there was no VEGF-A164a protein data in Fig. 5B.

**Down-regulation of CDK6 affects VEGF-A alternative splicing machinery**

Our luciferase reporter assay and in vivo protein/mRNA expressions suggested that let-7g could directly knock down CDK6 that is an upstream regulator of splicing factor SC35 [21, 22]. To confirm that down-regulation of CDK6 can promote pro-angiogenic isoform VEGF-A164a and inhibit anti-angiogenic isoform VEGF-A165b, C2C12 cells were transfected with different doses of CDK6 siRNA. As shown in Fig. 6A, the expressions of CDK6 and SC35 were dose-dependently inhibited by CDK6 siRNA. Although the mRNA levels of total VEGF-A were not changed, the pro-angiogenic isoform VEGF-A164a increased dose-dependently by CDK6 knockdown while an opposite pattern was found for anti-angiogenic isoform VEGF-A165b.
Hypoxic effect on muscle cells

When C2C12 cells were subjected to either hypoxia or OGD, the expressions of all tested molecules (VEGF-A, VEGFR-2, VEGF-A164a, VEGF-A165b, TP53, HIF-3α, CDK6 and SC35) were increased (Fig. S1).

Discussion

Therapeutic angiogenesis has been pursued as a potential treatment for ischaemic disorders like PAD. Using cellular studies, we and others have demonstrated that let-7g can induce angiogenesis [10, 11]. The present study showed that let-7g increased VEGF-A expression by regulating HIF-3α and TP53. MiRNA let-7g also enhanced the alternative splicing of VEGF-A towards the pro-angiogenic isoform VEGF-A164a via targeting in the 3′UTR of CDK6 that further affects splicing factor SC35. Furthermore, let-7g treatment increased bone marrow derived and circulating EPCs. These effects are summarized as a hypothetic schema in Fig. 6B.

Several regulatory pathways can contribute to the pro-angiogenic effects of let-7g. Improved local perfusion through neo-vasculization as indicated by the increase in capillary density could be attributed to up-regulation of pro-angiogenic genes (VEGF-A and VEGFR-2). The present study revealed dual regulatory mechanisms for let-7g's
Fig. 5 Let-7g influenced the expression levels of VEGF-A upstream genes and VEGF-A splice isoforms. (A) The ratios of mRNA levels (ischaemic versus non-ischaemic legs) of VEGF-A upstream genes (HIF-3α and TP53), VEGF-A isoform determinants (CDK6 and SC35) and VEGF-A two isoforms on day 21 after induced hindlimb ischaemia. (n = 6 for each group). (B) Western blot data show that the protein ratios are lower in the let-7g-treated than the miRNA control-treated animals. Compared with the control group, the ratio in the let-7g group is 0.34× for VEGF-A165b, 0.70× for HIF-3α, 0.68× for TP53, 0.59× for CDK6 and 0.20× for SC35 (n = 2 for each group). Data in the bar figures are presented as mean ± S.E.M., **P < 0.01, ***P < 0.001.

Fig. 6 Down-regulation of CDK6 affects VEGF-A alternative splicing machinery. (A) Knockdown of CDK6 by siRNA suppresses CDK6 and SC35. Although the mRNA levels of total VEGF-A were not changed, the pro-angiogenic isoform VEGF-A164a increased dose-dependently by CDK6 knockdown while an opposite pattern was found for anti-angiogenic isoform VEGF-A165b. Data are presented as mean ± S.E.M., **P < 0.01, ***P < 0.001. (B) Hypothetic schema shows multiple regulatory pathways of let-7g on angiogenesis in ischaemic hindlimb model.
modulation on the VEGF-A signalling. First, let-7g increased the expression of total VEGF-A by direct repression of HIF-3α and TP53. Secondly, leg-7g suppressed the expression of alternative splicing factor SC35 by inhibiting its up-stream regulator CDK6. Down-regulation of CDK6 and SC35 further resulted in an increase in pro-angiogenic isoform VEGF-A164a and a decrease in anti-angiogenic isoform VEGF-A165b. On the other hand, both the bone marrow derived and circulating EPCs were significantly increased by let-7g treatment. VEGF-A can release EPCs from the bone marrow into peripheral circulation [23, 24], and circulating EPCs can incorporate into capillaries and interstitial arteries for neo-vasculization [25]. The multiple mechanisms indicate the potential of let-7g in treating ischaemic diseases.

VEGF-A is the major factor for angiogenesis and neo-vasculization and it is up-regulated as an adaptive response to ischaemic and hypoxic injury. Several hypoxia regulated/responsive elements located in the 5′ and 3′ UTR of the VEGF-A gene are involved in the fine-tuning of VEGF-A expression [26]. HIF-1α and HIF-2α are transcription factors that activate VEGF-A expression, whereas HIF-3α exerts a negative regulation of the angiogenic response by competing with HIF-1α and HIF-2α binding sites [16]. In the present study, we demonstrated that let-7g could bind to two binding sites in the HIF-3α 3′UTR to silence HIF-3α expression. It might explain why let-7g treatment led to increase VEGF-A. In addition, we found let-7g could also repress TP53 expression via annealing to its 3′UTR. TP53 has been shown to inhibit VEGF-A promoter activity to down-regulate VEGF-A expression [17, 18]. Furthermore, TP53 could also up-regulate thrombospondin-1 (also known as THBS1) that is an angiogenesis inhibitor [27]. Our previous study has also shown that let-7g could directly bind to THBS1 3′UTR and knock down its expression [10]. A recent study showed that several let-7 family members have hypoxia regulated/responsive elements in their promoters, and HIF-1α-induced let-7 could directly suppress AGO1 [11]. AGO1 is involved in miRNA-mediated silencing complex (miRISC) that causes gene silencing. VEGF-A is translationally suppressed by AGO1-miRISC under normoxia and let-7 can increase VEGF-A by repressing AGO1-miRISC leading to VEGF-A RNA release [11]. Accordingly, let-7g has multiple routes to increase VEGF-A.

By alternative splicing of eight exons within the VEGF-A gene, at least 14 different isoforms have been reported and they can be grouped into two families: the proangiogenic (denoted as VEGF-Axxxa family) and the antiangiogenic (denoted as VEGF-Axxxb family), xxx where refers to the number of amino acids [28]. The opposite effects of VEGF-A splice isoforms on angiogenesis might be an important factor for VEGF-A therapy. Because of the critical role of VEGF-A in ischaemic diseases, there were several clinical trials of VEGF-A gene therapy for PAD. However, limited success was achieved in clinical outcome [29]. Interestingly, it has been known that PAD patients had elevated VEGF-A levels but poor neo-vasculization in legs [12]. Further studies showed that PAD patients had elevated serum levels of the anti-angiogenic isoform VEGF-A165b but reduced serum levels of the pro-angiogenic isoform VEGF-A165a (the human ortholog of murine VEGF-A164a) [12]. VEGF-A165b competes with VEGF-A165a/VEGF-A164a for the binding to VEGFR-2 [28]. In the present study, let-7g treatment increased VEGF-A164a levels but decreased VEGF-A165b levels in murine ischaemic limb model. The ratio of VEGF-A165b and VEGF-A164a can be determined by SC35, which is a pre-mRNA splicing factor for alternative processing at VEGF-A exon 8 [19]. The SC35 protein was reported to increase the VEGF-A165b/VEGF-A ratio and to decrease tumour neovascularization in immune-deficient nude mice [19]. Our data demonstrated that let-7g down-regulated the CDK6/SC35/VEGF-A165b cascade and up-regulated the expression of total VEGF-A as well as the VEGF-A164a isoform. These regulatory mechanisms enforce the potential application of let-7g in treating PAD.

To sum up, the present study showed that let-7g intramuscular injection might be a potential therapeutic strategy in PAD. The proangiogenic effects of let-7g via multiple mechanisms imply that let-7g may possess a therapeutic potential for neovascularization in ischaemic diseases. Further studies are warranted to test the therapeutic effects for its clinical utility.

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Conflicts of interest
The authors confirm that there are no conflicts of interest.

Supporting information
Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Hypoxic effect on gene expression levels in muscle cells.

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