microRNA-646 inhibits angiogenesis of endothelial progenitor cells in pre-eclamptic pregnancy by targeting the VEGF-A/HIF-1α axis

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Abstract. Pre-eclampsia is a complication that occurs during pregnancy, the pathological feature of which is a change in vascular endothelial homeostasis. microRNA (miR)-646 is an anti-angiogenic miRNA that has been indicated to exhibit potential anti-angiogenic effects in endothelial cells cultured *in vitro* and in ischemia-induced angiogenesis. However, whether miR-646 has therapeutic potential in placental angiogenesis in pre-eclampsia remains to be determined. In the current study, human peripheral blood-derived endothelial progenitor cells (EPCs) were isolated to study the coordination between miR-646, vascular endothelial growth factor (VEGF)-A and hypoxia-inducible factor (HIF)-1α expression in preeclampsia EPCs. EPCs were isolated from human peripheral blood to demonstrate a potential interaction between miR-646 and targets (VEGF-A) *in vitro*. The number of EPCs and the expression of miR-646 in patients with preeclampsia was detected, and the effects of miR-646 on EPC function and preeclampsia angiogenesis was assessed. Clinical specimens demonstrated that miR-646 expression was enhanced in pregnancy with preeclampsia. The results indicated that miR-646 suppressed EPCs multiplication, differentiation and migration. miR-646 was observed to exert an anti-angiogenic function by suppressing the expression of angiogenic cytokines VEGF-A and HIF-1α. Additionally, luciferase results displayed that miR-646 downregulated VEGF-A expression by directly binding to a specific sequence in its 3'-untranslated region. The results of the current study demonstrated that the miR-646/VEGF-A/HIF-1α axis is significant for angiogenic properties of EPCs *in vitro* and *in vivo* placental vasculogenesis. The results of the present study provide a new insight into microRNA regulation of vessel homeostasis and angiogenesis, and a basis for alternative treatments for patients with pre-eclampsia.

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Introduction

The pathological basis of pre-eclampsia is generally considered to be an abnormal placental vasculature that is caused by endothelial dysfunction (1). Pre-eclampsia and fetal growth restriction are late pregnancy complications that are associated with insufficient uterine vascular density and suboptimal placental perfusion (2). Furthermore, vessel formation is driven by progenitor cells and leads to vascularization and vasculogenesis, which is distinguished by the presence or absence of pre-existent vessels (3,4). Damaged endothelial cells cannot be repaired by already differentiated endothelial cells, but can be repaired by endothelial progenitor cells (EPCs) (5).

EPCs are precursors of vascular endothelial cells and originate from bone marrow, similar to angioblast and umbilical vein endothelial cells, which together belong to a subgroup of hematopoietic stem cells (6). There are two sources of EPCs that can be detected *in vitro*, immature and mature EPCs. While early immature EPCs display a linear growth structure and are spindle-shaped, late mature EPCs form cobblestone-like, oval shaped structures (7). EPCs take part in vascularization during embryonic development, and also participate in postnatal vascularization and reparative processes post-trauma (8). Therefore, EPCs hold extensive prospects for vascular tissue engineering and potential clinical application in coronary artery disease and wound healing (9). Some studies have indicated that the number of EPCs in patients with pre-eclampsia is reduced, and the function of EPCs is diminished (10,11). Available evidence demonstrates that neovascularization may be due to impaired availability or function of EPCs (12,13). Therefore, EPCs may be a diagnostic tool and a direct target for medical intervention during pre-eclampsia.

microRNAs (miRNAs or miRs) are conserved non-coding RNAs that are 19-26 nucleotides in length and that regulate gene expression by binding to specific sites in the 3'-untranslated region (UTR) of mRNAs. miRNAs are associated with a variety of *in vivo* physiological processes, including angiogenesis and embryonic development (14,15). miR-646 is a newly discovered miRNA, and is a common miRNA isolated from vascular endothelial cells (16). miR-646 has been demonstrated to serve a key role in many aspects of angiogenesis, including in vascularization and wound healing (16). Li *et al* (17) revealed that miR-646 is not only expressed in normal cells, but also has low expression in renal cell carcinoma, and serves an important role in renal cell angiogenesis,

which is associated with the NOB1/MAPK pathway (17). A growing number of studies have demonstrated that miRNAs are abundantly expressed in pre-eclampsia during pregnancy, and the dysregulation of miRNAs is associated with the pathogenesis of pre-eclampsia (18).

Although a few studies have identified a functional relationship between miR-646, angiogenesis and endothelial cells, the biological functions and mechanisms of miR-646 and EPCs in pregnancy-associated vascular complications remain to be determined. In the current study, the number of EPCs, the expression of miR-646 and its relationship to blood in patients with and without pre-eclampsia, was assessed. Additionally, the effects of miR-646 on proliferation, angiogenesis and migration of EPCs were investigated. The current study aimed to reveal the regulatory mechanisms of miR-646 function in EPCs, and to provide molecular evidence for miR-646 regulation of vascular endothelial growth factor (VEGF)-A and hypoxia inducible factor-1α (HIF1α) expression, and reveal the role of this regulation in the pathogenesis of pre-eclampsia.

Materials and methods

Patients and blood samples. This research was conducted at the department of Obstetrics and Gynecology, Zhongnan Hospital of Wuhan University (Wuhan, China) from August 2017 to July 2018. The study group included 20 women without pre-eclampsia (control group) and 20 women with pre-eclampsia. The inclusion criteria were as follows: i) Women with single pregnancy; ii) women aged <35 years old and >18 years old; iii) The diagnostic criteria for pre-eclampsia were a systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg after 20 weeks of gestation, whereas women with previous normal blood pressure had proteinuria ≥1+ (300 mg/24 h) after pregnancy (19). 4) Patients did not receive any previous or ongoing treatment. Exclusion criteria: Patients with chromosomal abnormalities, anatomical variations, hormonal disorders and infectious diseases were excluded. The formal medical history was obtained for all of the women. The patients underwent obstetric examination, ultrasound examination of the abdomen, hematology and urine protein analysis. Peripheral blood was collected during regular review (every 4 weeks) of the pregnant women. To compare the expression of miR-646 in the placenta of the control group to the patients with pre-eclampsia, umbilical cord blood was also collected at birth. Baseline characteristics were recorded for all pregnant women enrolled (Table I). The ethical approval of the current study's protocol was gained from the Ethics Committee of the Zhongnan Hospital of Wuhan University, and detailed written consent was obtained from all enrolled subjects.

miRNA and target gene expression assay and reverse transcription-quantitative (RT-q) PCR. A TRIzol kit (Qiagen GmbH) was used to extract total RNA from peripheral blood and umbilical cord blood of pregnant women. cDNA was subsequently synthesized from total mRNA (8 μg) using miScript II RT kit (Qiagen GmbH). The reverse transcription procedure includes incubation at 37˚C for 1 h, followed by inactivation by briefly incubating at 95˚C. The expression of miRNAs was quantified using the miRNA-specific TaqMan miRNA assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR

was performed using a FastStart Univepreeclampsial SYBR Green Master (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression of miR-646 was normalizes to the relative expression of U6, and the expression of the target genes VEGF-A and HIF-1α were normalized to β-actin for quantification. The RT-PCR primers used were as follows: VEGF-A forward, 5'-GAGGAGCAGTTACGGTCTGTG-3' and reverse, 5'-TCCTTTCCTTAGCTGACACTTGT-3'; HIF-1α forward, 5'-ATGCGGTCAGCAAGAGCATC-3' and reverse, 5'-AGA CGATACTCTCCGACTGGG-3'; Akt forward, 5'-CTACCC ACACAGCAGTACGC-3' and reverse, 5'-AAGTCGCTGGTG TTAAGCCG-3'; β-actin forward, 5'-CGGAGTGAGCGA TCTTACAGG-3' and reverse, 5'-TCATCAGCGACTCTG ACCACA-3'. SYBR Premix Ex TaqTM was then used on an Applied Biosystem 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), and each reaction was repeated three times in succession. Immediately after the initial denaturation step (95˚C; 2 min), the program was set up to consist of 40 cycles (95˚C, 15 sec; 62˚C, 15 sec; 72˚C, 45 sec). Finally, the resulting PCR was subjected to melting curve analysis. The relative gene expression of miR-646 and the target gene were analyzed using the $2^{-\Delta\Delta Ct}$ method (20).

EPCs cell culture and characterization. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using the methods described by Schildberger *et al* (21). PBMCs were isolated using Ficoll-Paque density gradient centrifugation (300 x g; 25 min; 15˚C) in a lymphocyte separation solution (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cells were then washed 3 times with PBS then resuspended in endothelium-based medium-II (Applied Biosystems; Thermo Fisher Scientific, Inc.) containing 5 EGM-2-MV-SingleQuots (Lonza Group, Ltd.) at a concentration of $5x10^5$ cells/ml. Medium contained 10% FBS, 40 ng/ml human VEGF (Sigma-Aldrich; Merck; KGaA; cat. no. 127464-60-2), 40 ng/ml human insulin-like growth factor-1 (IGF-1; Sigma-Aldrich; Merck; KGaA; cat. no. 67763-96-6), 50 ng/ml human epidermal growth factor (Sigma-Aldrich; Merck; KGaA; cat. no. 62253-63-8), 120 μg/ml penicillin and 120 μg/ml streptomycin. PBMCs ($5x10^6$) were seeded on a fibronectin-coated six-well culture dish. After 4 days of cell-induced culture at 37˚C, adherent cells were observed to form small round EPC clusters under Olympus BX50 (Olympus Corporation; Magnification, x400). After 6-8 days of cell-induced culture, the spindle cells gradually turned into a cluster of round cells in which a plurality of spindle-shaped cells were observed to germinate from the central core and designated as colony forming units (CFU). The method of identification of EPCs was as follows: The attached cells were then incubated with DiI-Ac-LDL (2.4 mg/ml) for 4 h at 37˚C, then fixed with 2% paraformaldehyde at room temperature for 10 min, and finally incubated with FITC-UEA-1 (10 mg/ml) at room temperature for 1.5 h. Adherent cells were observed by a microscope (magnification, x400; Olympus IX81; Olympus Corporation), and the cells co-stained with DiI-Ac-LDL and FITC-UEA-1 were identified as differentiated mature EPCs.

Transfection in vitro. The miR-646 mimics and miR-646 inhibitors oligonucleotides (Applied Biosystems; Thermo

Table I. Demographic of all pregnant women enrolled in the study.

Characteristics	Control (n=20)	Preeclampsia (n=20)	P-value
Age (years)	29.7±2.3	31.7±3.2	NS
Gestational age (weeks)	11.5±2.1	12.3±2.8	NS
Pregnancy time (weeks)	10.6±1.7	11.6±1.5	NS
Median maternal weight (kg)	53.9±4.3	55.2±3.9	NS
BMI (kg/m^2)	23.4±3.1	28.4±3.8	NS
Proteinuria	20% (4/20)	100% (20/20)	<0.05
S/D ratio of umbilical artery	2.7±0.8	3.6±0.7	<0.05
Birth weight (g)	2316±632	3034±517	<0.05
Apgar score	9.0±0.87	8.3±0.76	NS

Data are presented as the mean ± standard deviation or as a percentage; P>0.05; NS, not significant; S/D ratio, systole/diastole (S/D) ratio.

Fisher Scientific, Inc.) were diluted in PBS to a concentration of 30 μM. Cells were transfected with Superfect (Invitrogen; Thermo Fisher Scientific, Inc.) using miR-646 mimics (2.5 nM), miR-646 inhibitor (2.5 nM) or a VEGF-A shRNA plasmid (Applied Biosystems; Thermo Fisher Scientific, Inc.). The control (siR-RibTM; 2.5 nM) was then transfected with the Cy3-small interfering (si)RNA kit (Tiangen Biotech Co., Ltd.) to determine miRNA transfection efficiency. Efficiency was determined by comparison to the miRNA mimic negative control (miR-NC; NC) siRNA, which was provided by Guangzhou RiboBio Co., Ltd. A period of 48 h after transfection, harvested cells were used for subsequent analysis.

Protein extraction and western blot analysis. Proteins were extracted from EPCs with different transfection conditions with RIPA lysis buffer (2 mM Tris HCl; pH 7.5; 15 mM NaCl; 0.1 mM Na2EDTA; 0.1 mM EGTA; 0.1% Triton; 0.25 mM sodium pyrophosphate; 0.1 mM β-glycerophosphate; 0.1 mM Na3VO4; 0.1 μg/ml leupeptin; Tiangen Biotech Co., Ltd.). Protein concentration was detected using the BCA method. Proteins (10 μg) were resolved in 15% SDS-PAGE. They were then transferred onto an Immobilon-P membrane (EMD Millipore). The membrane was blocked with 5% non-fat milk in TBST for 1 h at room temperature. The membrane was subsequently probed with primary antibodies against VEGF-A (1:2,000; cat. no. ab46160; Abcam) or β-actin (1:5,000; cat. no. sc-58673; Santa Cruz Biotechnology, Inc.), and incubated overnight at 4˚C. After extensive washing with PBST, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (1:10,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. An Odyssey Infrared Imaging System (LI-COR Biosciences) was used to visualize protein bands. The relative intensity of protein bands compared with β-actin was quantified using Image J software version 1.47 (National Institutes of Health).

Proliferation and colony formation. MTT assay was used to determine the cell proliferation rate. Cells were inoculated into 96-well plates (1x10^3 cells per well) and MTT reagent was added (25 μl; 5 mg ml) to each well. Supernatant was removed from the medium and DMSO reagent was added to each well.

The absorbance was measured at 520 nm using a microplate reader (595 nm). For colony formation experiments, cells were resuspended at 1x10^3 cells/well and cultured at 37˚C for 5-6 days until macroscopic colonies appeared. Finally, the colonies were stained with crystal violet at room temperature for 30 min.

Angiogenesis experiment. EPCs formed capillary structures as previously described. Pre-registration 96-well plates were coated with Matrigel at 37˚C 30 min prior to the experiment. EPCs (1x10^3 cells per well) were then cultured at 37˚C in 250 ml of Ham's F12K medium (Applied Biosystems; Thermo Fisher Scientific, Inc.). A total of 1x10^6 tumor cells were then transfected with miR-646 mimics or inhibitors, and cultured for 48 h under the same conditions. An image capturing capillary-like structure formation was obtained at 12 h using an inverted microscope (Olympus Corporation; magnification, x50). Finally, the tubular structure was evaluated.

EPCs cell migration assay. Transwell assay was used to examine EPC migration. Transfected cells were harvested at 24 h after transfection and resuspended in cell culture medium (Eagle's Minimum Essential Medium; Thermo Fisher Scientific, Inc.) supplemented with 5% FBS. A total of 7x10^3 cells were added in the upper-chamber of the transwells, and the lower-chambers were filled with 600 μl of cell culture medium (Eagle's Minimum Essential Medium; Thermo Fisher Scientific, Inc.). Cells were then stored in a 37˚C, 5% CO_2 atmosphere for 48 h. After 48 h, the unmigrated cells were gently removed from the upper side of the transwell membrane using a cotton swab. The migrated cells attached to the lower side of the membrane were then fixed in methanol and stained with crystal violet at room temperature for 30 min. Finally, the average number of cells was counted manually. Images of the samples were then captured using an inverted microscope (magnification, x400; Olympus IX81; Olympus Corporation).

Luciferase reporter assay. To ascertain whether miR-646 inhibited VEGF-A expression, TargetScan was used, which is an online publicly available algorithm, to analyze miR-646 sequence 3'-UTR (NM_001025369) of VEGF-A. Sequence-combined
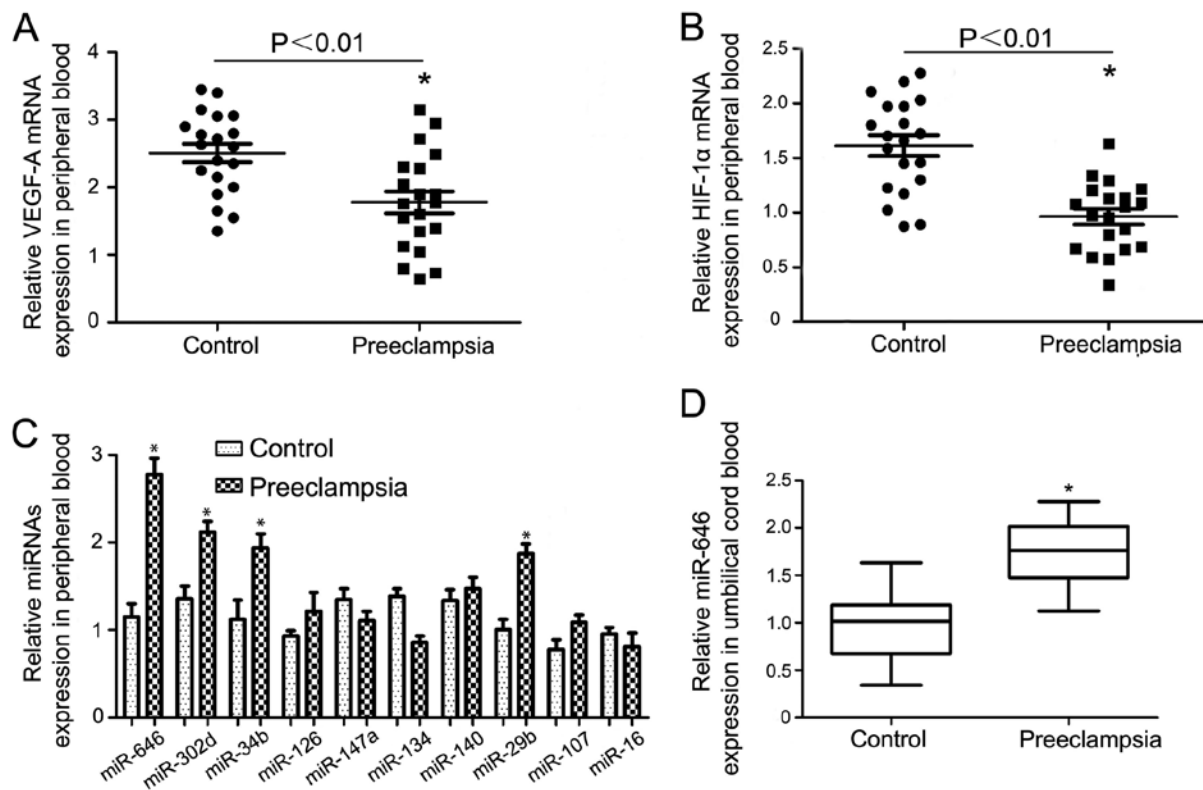
Figure 1. Abnormal increased expression of miR-646 in patients with preeclampsia. (A) VEGF-A mRNA expression was significantly lower in the peripheral blood of patients with preeclampsia compared with control pregnant women. (B) A similar reduction in expression was observed for HIF-1α. (C) TaqMan qPCR results indicated that the difference in expression level of mir-646 was most significant in peripheral blood. (D) miR-646 expression was determined in umbilical cord blood of preeclampsia cases, miR-646 expression was determined and normalized to U6 using RT-qPCR, and analysis was performed using the Ct method. Data are expressed as the mean ± standard deviation. *P<0.01 vs. control group; n=20; miR, microRNA; VEGF, vascular endothelial growth factor; HIF, hypoxia inducible factor; RT-q, reverse transcription-quantitative.

index comprehensive ranking was performed to confirm that the combined prediction score for the miR-646 binding site in the 3'UTR was the highest scoring site. Once it was established that miR-646 could target VEGF-A, subsequent experiments were performed. EPCs were transfected with either wild type or mutant VEGF-A constructs without the miR-646 binding site. This part of the experiment allowed EPC cells to grow to 70-80% confluence in 24-well plates and Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used with a luciferase reporter vector (200 ng) (Promega Corporation), and appropriate miRNA (50 nM, mimics and inhibitors) were co-transfected. Following the experimental protocol, after 72 h, the cells were washed and lysed with passive lysis buffer (Sigma-Aldrich; Merck KGaA). The relative activity of luciferase was then assessed using a dual luciferase vector assay (Thermo Fisher Scientific, Inc.). Finally, relative reporter gene activity was determined by normalizing to *Renilla* luciferase activity.

Statistical analysis. All experiments were performed in triplicate, and the results are presented as the mean ± standard deviation of 3 independent experiments. Statistical comparisons were performed using a one-way ANOVA. Statistical analyses were performed using R software (R version 3.3.2), GraphPad Prism Software (7.0; GraphPad Software, Inc.), and the SPSS 17.0 statistical software package (IBM Corp). P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical data of enrolled patients. The clinical data of the patients enrolled in the current study are presented in Table I. Patients with pre-eclampsia and control subjects were matched by their age, body mass index (BMI) and gestational age, respectively. The umbilical cord blood vessels and placenta indicators of the two groups of pregnant women were observed using ultrasound examination, and some data had significant gaps. In patients with clinical pre-eclampsia, lower placental microvessel density (MVD) indicated abnormal vasculogenesis in the placenta (Table I). The data indicated that the fetuses of patients with pre-eclampsia have an elevated risk of preterm birth (Table I).

Increased expression of miR-646 in patients with preeclampsia. A previous clinical study demonstrated that VEGF-A and HIF-1α expression were lower in the peripheral blood of patients with preeclampsia compared with the controls (Fig. 1A and B). To investigate whether specific miRNAs regulate VEGF-A or HIF-1α expression in preeclampsia, the total of 20 potential miRNAs that targeted the VEGF-A gene were determined. The expressions of these 20 miRNAs were determined using RT-qPCR. The expression of the first 10 of these miRNAs is presented in Fig. 1C. The expression of miR-646 in the preeclampsia group was significantly higher compared with control pregnant women (Fig. 1C). The higher expression of miR-646 was confirmed in umbilical
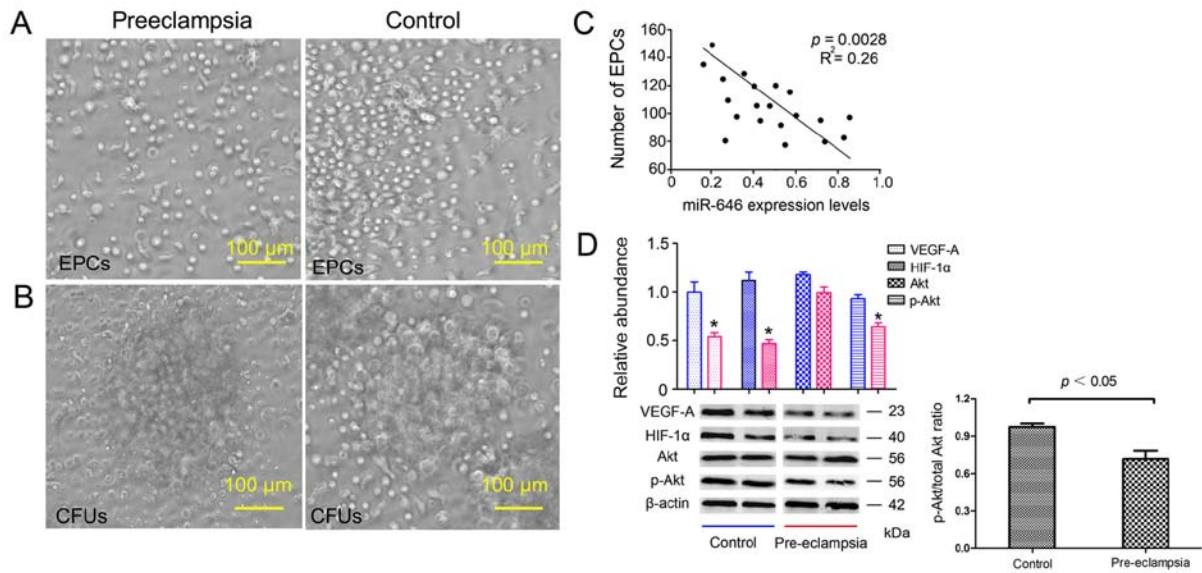
Figure 2. Morphology of EPCs and CFUs representative of one patient with preeclampsia and one with a normal pregnancy following 7 days of cell culture. (A) The number of EPCs decreased in the patient with preeclampsia compared with the control (magnification, x200). (B) The numbers of CFUs were lower in the patient with preeclampsia, and the diameter was significantly reduced. Only typical CFUs with spindle cells gradually turning into a cluster of round cells and germinating from the central core were counted using light microscopy (magnification, x200). (C) Pearson's correlation analysis indicated a correlation between EPC number and miR-646 expression in patients with preeclampsia; significant negative correlation, R^2=0.26, P=0.0028. (D) Protein levels of VEGF-A, HIF-1α, Akt and p-Akt in patients with preeclampsia and healthy controls, VEGF-A, HIF-1α and p-Akt level in the preeclampsia group decreased, and the difference was statistically significant. Blue and red represent control and pre-eclampsia group respectively. *P<0.05 vs. control group. EPC, endothelial progenitor cells; CFU, colony forming unit; miR, microRNA; VEGF, vascular endothelial growth factor; HIF, hypoxia inducible factor; p, phosphorylated.
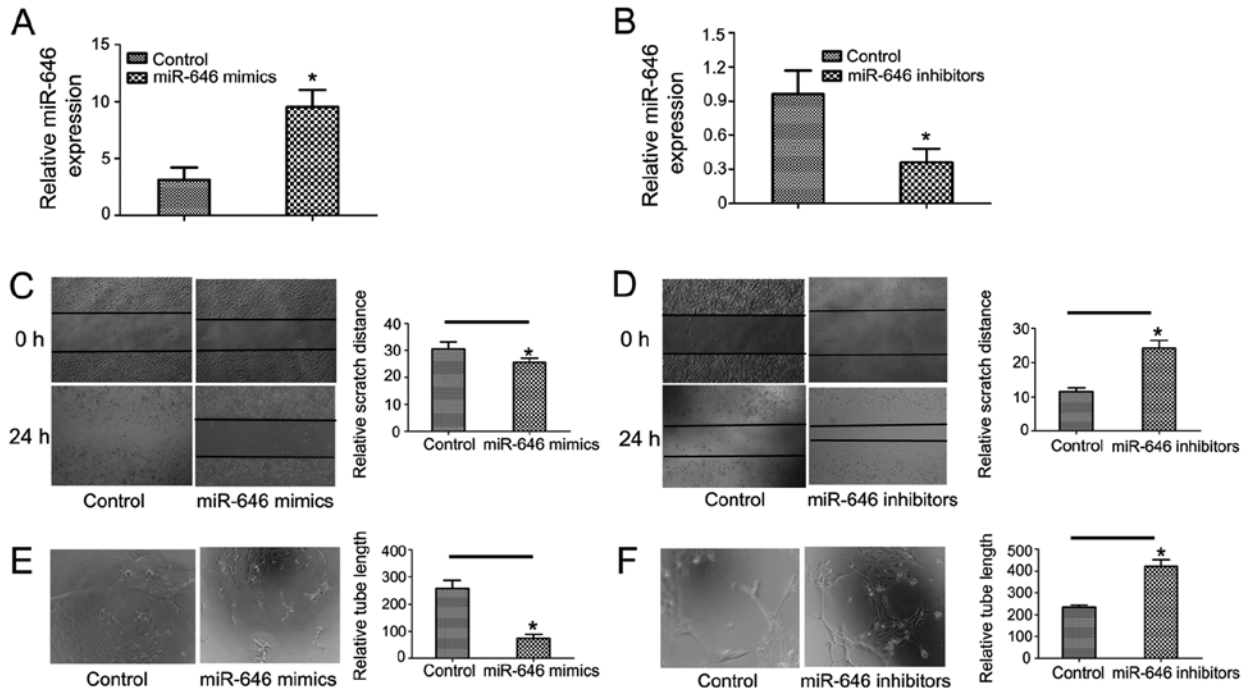

Figure 3. The effect of miR-646 on EPCs proliferation, migration and tube formation. EPCs were transfected with control or miR-646 mimics or inhibitors. At 48 h after transfection, cells were harvested. RT-qPCR analysis was performed to examine miR-646 levels after transfection with miR-646 mimics (A) and with miR-646 inhibitor (B) Representative images after transfection with miR-646 mimics (C) and with miR-646 inhibitor (D) were captured at 0 and 24 h to assess the cell migration into the open space. Quantification of the percentage of the distance migrated was achieved by measuring these distances in 3 high-magnification. Representative images of tube formation after transfection with miR-646 mimics (E) and with miR-646 inhibitor (F) Magnification, x1,000. Quantification of the vascular tube structure was conducted by measuring the total tubule length in 3 high-magnification. Data are expressed as the mean ± standard deviation. *P<0.05 vs. control. miR, microRNA; EPC, endothelial progenitor cells; qPCR, quantitative PCR.

cord blood of preeclampsia cases, suggesting that the upregulation of miR-646 may be associated with preeclampsia (Fig. 1D).

Therefore, the data indicated that miR-646 was upregulated in patients with pre-eclampsia.

Table II. Bioinformatics prediction differential expression miRNAs (top 20).

ID	Species (miRNA)	Species (Target)	miRNA	Target	Sum
MIRT061535	Homo sapiens	Homo sapiens	hsa-miR-646	VEGFA	2
MIRT000722	Homo sapiens	Homo sapiens	hsa-miR-302d-3p	VEGFA	2
MIRT002466	Homo sapiens	Homo sapiens	hsa-miR-34b-5p	VEGFA	3
MIRT003428	Homo sapiens	Homo sapiens	hsa-miR-126-3p	VEGFA	4
MIRT003810	Homo sapiens	Homo sapiens	hsa-miR-147a	VEGFA	2
MIRT003811	Homo sapiens	Homo sapiens	hsa-miR-134-5p	VEGFA	5
MIRT003812	Homo sapiens	Homo sapiens	hsa-miR-140-5p	VEGFA	2
MIRT003813	Homo sapiens	Homo sapiens	hsa-miR-29b-3p	VEGFA	3
MIRT003814	Homo sapiens	Homo sapiens	hsa-miR-107	VEGFA	2
MIRT003890	Homo sapiens	Homo sapiens	hsa-miR-16-5p	VEGFA	4
MIRT004055	Homo sapiens	Homo sapiens	hsa-miR-93-5p	VEGFA	2
MIRT004271	Homo sapiens	Homo sapiens	hsa-miR-17-5p	VEGFA	3
MIRT004272	Homo sapiens	Homo sapiens	hsa-miR-150-5p	VEGFA	2
MIRT004273	Homo sapiens	Homo sapiens	hsa-miR-195-5p	VEGFA	2
MIRT004274	Homo sapiens	Homo sapiens	hsa-miR-15b-5p	VEGFA	4
MIRT004275	Homo sapiens	Homo sapiens	hsa-miR-15a-5p	VEGFA	5
MIRT004276	Homo sapiens	Homo sapiens	hsa-miR-520g-3p	VEGFA	6
MIRT004277	Homo sapiens	Homo sapiens	hsa-miR-378a-3p	VEGFA	2
MIRT004278	Homo sapiens	Homo sapiens	hsa-miR-330-3p	VEGFA	3
MIRT004443	Homo sapiens	Homo sapiens	hsa-miR-383-5p	VEGFA	3

miR, microRNA; VEGF, vascular endothelial growth factor.

The number of EPCs was significantly lower in patients with preeclampsia, accompanied by morphological differences in EPCs. Microscopic images of EPCs from patients with preeclampsia and control groups are presented in Fig. 2. Compared with the control group, the number of EPCs in patients with pre-eclampsia decreased significantly, and the CFUs in patients with pre-eclampsia were ~1.5 times larger. Fig. 2A and B demonstrated that pre-eclampsia significantly reduced EPC and CFU counts. EPC number: 132±47 vs. 186±51; P<0.05; CFU count: 3.3±3.7 vs. 11.43±10.6, P<0.01 (data not shown). As presented in Fig. 2C, the number of EPCs was negatively correlated with miR-646 levels in the pre-eclampsia group (R^2=0.26; P =0.0028), suggesting that miR-646 levels may be important for the regulation of the amount of EPCs in pre-eclampsia. Protein levels of VEGF-A, HIF-1α and p-Akt (Akt phosphorylation level), which are three important regulators of angiogenesis, also decreased in pre-eclampsia, relative to β-actin (Fig. 2D). The ratio of phosphorylated AKT/total AKT ratio was significantly decreased in the pre-eclampsia group. Specifically, this supports the hypothesis that miR-646 is involved in the pathogenesis and regulation of pre-eclampsia by regulating EPCs.

miR-646 inhibition and overexpression in primary cultured EPC cells. To investigate the mechanism of action by which miR-646 regulates EPCs, miR-646 overexpression or suppression was performed in normal non-preeclampsia EPCs. A period of 48 h after transfection, the expression efficiency was determined using a Cy3-siRNA transfection control. The cells exhibited intense and extensive cytosolic delivery by Cy3-siRNA (Fig. 3A and B). The expression of miR-646 was confirmed in different transfection groups using RT-qPCR. The results confirmed that miR-646 was expressed in these groups, and the increase in miR-646 mimic transfected cells was higher (1.3±0.9 vs. 7.2±1.4; P<0.01; Fig. 3A), while miR-646 expression was suppressed by miR-646 inhibitor (0.9±0.37 vs. 0.46±0.32; P<0.01; Fig. 3B).

The function of miR-646 on EPCs proliferation, migration, colony formation and tube formation. Based on the correlation between miR-646 and VEGF-A expression, and the important role of VEGF-A in angiogenesis, nutritional maintenance and homeostasis of the placenta, the antiangiogenic effect of miR-646 was assessed by transfecting EPCs with a miR-646 mimic or inhibitor *in vitro*. Important processes for angiogenesis include cell proliferation and migration. Therefore, the effect of miR-646 on VEGF-A induced EPC proliferation and migration was assessed. The data demonstrated that the overexpression of miR-646 in EPCs significantly inhibited their proliferation and migration. The inhibition of miR-646 expression significantly promoted the proliferation and migration of EPCs (Fig. 3B and C). By calculating the length of the formed tubules using inverted phase contrast microscopy, the ability of EPCs to form tubular structures was revealed. Additionally, miR-646 overexpression inhibited EPC tube formation, while miR-646 inhibitor transfection increased tube formation, clonal formation experiments indicated a similar role for miR-646 (Fig. 3D-F).
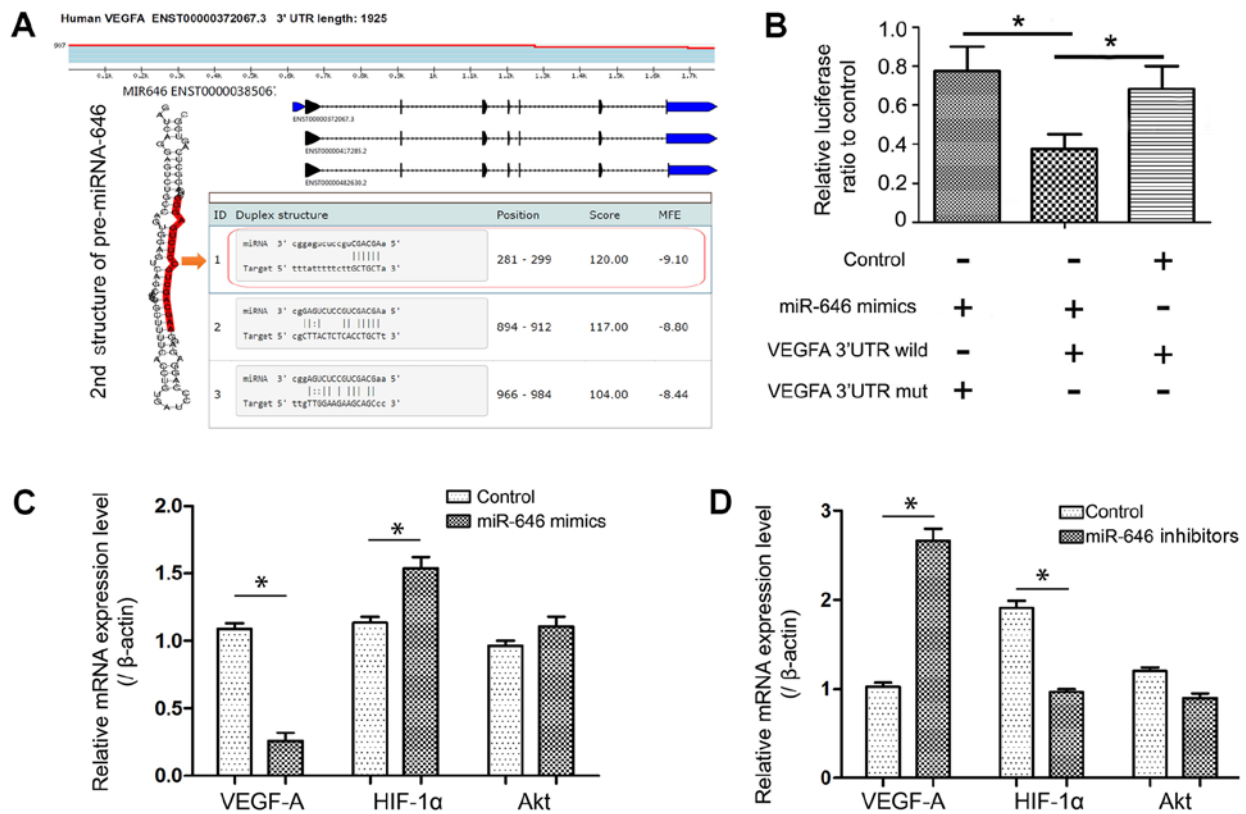
Figure 4. miR-646 directly targets the 3'UTR of VEGF-A and downregulates its expression. (A) Bioinformatics analysis combined with information from databases predicted miR-646 binding sites in the 3'UTR of VEGF-A. The full-length genomic transcript of VEGF and its miR-646 binding site are shown along with the secondary structure of miR-646. (B) EPCs were transfected with the luciferase reporter plasmid carrying the wild type or mutant binding sequences of VEGF-A and the miRNA mimics or inhibitors. (C) miR-646 regulates VEGF-A gene expression in EPCs. VEGF-A mRNA level was decreased in the miR-646 mimic group. In contrast, HIF-1α mRNA was increased compared with the control group. (D) VEGF-A mRNA was increased in the miR-646 inhibitor group, but HIF-1α mRNA was decreased compared with the control group. The mRNA level of AKT corresponds to the previous protein levels indicated in Fig. 2, demonstrating that AKT mRNA levels were not significantly different between the transfection group and control group; *P<0.05 vs. control. miR, microRNA; UTR, untranslated region; VEGF, vascular endothelial growth factor; EPC, endothelial progenitor cells; HIF, hypoxia inducible factor; mut, mutant.

miR-646 directly targeted VEGF-A 3'-UTR and downregulated its expression, regulating the VEGF-HIFα-Akt signaling axis. VEGF has been identified as a major cellular molecule that regulates angiogenesis (22). Using online microRNA target databases (miRBase, http://www.mirbase.org; miRWalk, http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/; and TargetScan, http://www.targetscan.org/), it was demonstrated that VEGF-A is a potential target of miR-646 (Table II). To ascertain whether miR-646 inhibited VEGF-A expression, the online publicly available algorithm TargetScan was used to analyze miR-646 target sequences in the 3'-UTR of VEGF-A. With reference to sequence information, the wild type or mutant sequence was cloned, which contained the full-length genomic transcript of VEGF and its miR-646 binding target site, visually indicating the secondary structure of miR-646 (Fig. 4A). These fragments containing the binding sequence were used for luciferase reporter assays (Fig. 4B). Additionally, RT-qPCR was used to measure VEGF-A expression in these different groups with and without the miR-646 binding site. The results demonstrated that the lack of a binding site in the 3'UTR attenuated the miR-646 mediated degradation of VEGF-A mRNA (Fig. 4B). HIF-1α and AKT are two important factors in VEGF/AKT signaling, which regulates multiple key forms of angiogenesis and vascular homeostasis (23).

The results indicated that HIF-1α mRNA was increased after transfection with miR-646 mimics (Fig. 4C). Although the mRNA level of AKT did not change significantly, it was indicated that the p-AKT protein level was significantly decreased in combination with the previous western blotting results, demonstrating that the phosphorylation level of AKT changed under the action of miR-646. It was hypothesized that this phenomenon may be due to exogenously increasing the expression of miR-646, which can directly target the expression of VEGF-A, resulting in decreased VEGF-A expression. The consequence of decreased VEGF-A expression is inhibition of placental angiogenesis. The hypoxic-ischemic internal environment eventually leads to an increase in the expression of hypoxia-inducible factor-HIF-1α. Therefore, in the miR-646 inhibitor group, HIF-1α and AKT expression levels were significantly lower compared with the control group (P<0.05; Fig. 4D).

To clarify whether the inhibitory effect of miR-646 on EPC proliferation is regulated by the negative regulation of VEGF-A, a study of gene function loss was performed. Specifically, whether the knockdown of VEGF-A mimics the effect of miR-646 via silencing VEGF-A was assessed by performing an MTT analysis, counting the number of EPCs and their spindle-like morphology. EPCs were transfected
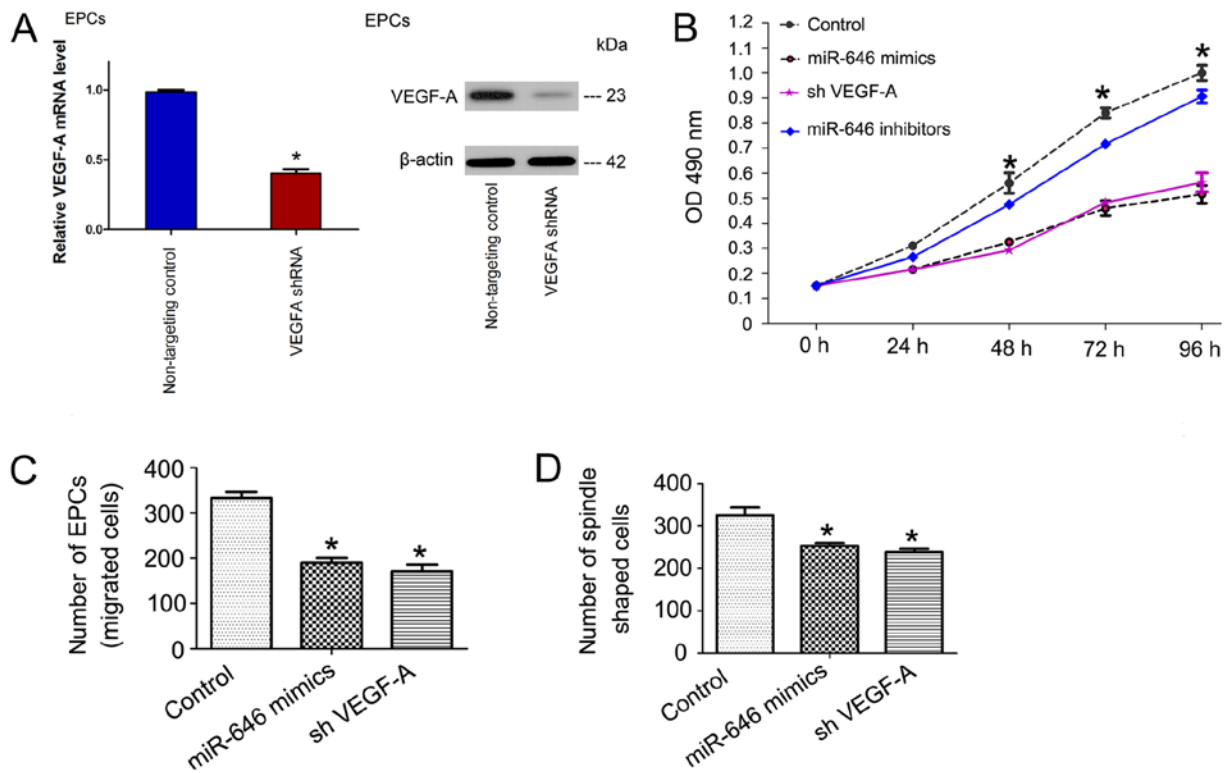
Figure 5. VEGF-A is associated with miR-646 functional suppression in EPCs. (A) Relative VEGF-A mRNA and protein levels in EPCs after sh-VEGF-A transfection. (B) MTT demonstrated that sh-VEGF-A and miR-646 mimics suppressed the proliferation of EPCs 24 h after incubation. (C) The number of migrating cells was decreased in the sh-VEGF-A and miR-646 mimic groups compared with the control. (D) Statistical analysis showed that the average number of spindle-shaped cells in sh-VEGF-A and miR-646 mimic groups was decreased. Data were normalized to control EPCs and expressed as means ± standard deviation; *P<0.05 compared with the control group. VEGF, vascular endothelial growth factor; miR, microRNA; EPC, endothelial progenitor cells; sh, short-hairpin.

with shVEGF-A and EPCs proliferation, differentiation and migration capabilities were detected. As indicated in Fig. 5, silencing of VEGF-A significantly inhibited cell growth, differentiation and migration, similar to that induced by the miR-646 mimetic. The data demonstrated that miR-646 directly targeted the angiogenic gene VEGF-A, and is associated with the regulation of the VEGF-Akt signaling pathway.

Discussion

To the best of our knowledge, the current study revealed, for the first time, that miR-646 is expressed in EPCs in patients with pre-eclampsia and an important mechanism of miR-646 is associated with regulating the angiogenesis characteristics of early EPCs. The present study revealed that the expression of miR-646 in the peripheral or cord blood of the patients with pre-eclampsia is higher compared with control patients, the change in the number of EPCs and the level of miR-646 in the pre-eclampsia group demonstrated a negative correlation and miR-646 inhibited proliferation, differentiation and migration of EPCs by directly targeting the angiogenesis-related gene VEGF-A. The mechanism of action of miR-646 on EPCs is via the VEGF-Akt signaling pathway.

miRNAs have been considered to serve important regulatory roles in a variety of gynaecological and obstetric diseases, including leiomyoma, endometriosis and preterm birth (24). Additionally, studies have demonstrated the differential expression of miRNAs in peripheral or cord blood of patients with pre-eclampsia. This significantly different expression miRNAs

can affect pregnancy via regulating vascular events, including angiogenesis (25). Additionally, a number of miRNAs can cause premature birth via regulating genes involved in angiogenesis (26). One study revealed that miR-646 inhibits angiogenesis, trophoblast proliferation and migration (17). The current study indicated that miR-646 may serve a key role in placental angiogenesis. However, these novel findings require further investigation.

In the current study, patients with pre-eclampsia and control patients were indicated to exhibit significant differences in the expression of miR-646 in peripheral blood, which is a point not previously assessed. The standardization of data indicated that miR-646 in the cord blood of patients with pre-eclampsia was significantly higher. *In vitro* data indicated that miR-646 overexpression in EPC inhibited cell proliferation, migration and angiogenesis, suggesting that miR-646 may be a novel inhibitor of angiogenesis. Consistent with these findings, a recent study demonstrated that miR-646 inhibited the proliferation, migration and angiogenesis potential of mesenchymal stem cells (27). The further clinical value of the present study is that miRNAs and their targets may be used as biomarkers for pre-eclampsia in early pregnancy. These biomarkers may aid in the prediction of pre-eclampsia to avoid adverse outcomes in pregnant women and perinatal children. The current study also indicated that in patients with pre-eclampsia, the serum levels of miR-646 and VEGF-A continued to be abnormal and may be associated with changes in uterine artery Doppler energy. The genetic basis of pre-eclampsia, and the determination of biomarkers with a predictive value for this condition requires study in the future. Further research will allow the identification

of the best combination of biomarkers for clinical use. In the current study, miR-646 was identified as a biomarker that serves as an indicator of preeclampsia and may be a good target for drug intervention. It was demonstrated that the protein levels of VEGF-A and HIF were decreased in the pre-eclampsia group, and the phosphorylated AKT (p-AKT) level was also significantly decreased (Fig. 2). Furthermore, the level of VEGF-A mRNA was decreased following the overexpression of miR-646, but the HIF-1α mRNA expression was increased (Fig. 4). It was hypothesize that this phenomenon may be due to exogenously increasing the expression of miR-646, which can directly target the expression of VEGF-A, resulting in decreased VEGF-A expression. The consequence of decreased VEGF-A expression is the inhibition of placental angiogenesis. A hypoxic-ischemic internal environment eventually leads to an increase in the expression of hypoxia-inducible factor HIF-1α. Although the mRNA level of Akt did not change significantly in the transfection group, it was indicated that the p-Akt level was significantly decreased in combination with previous western blotting analysis (Fig. 2), indicating that the phosphorylation level of AKT (p-AKT) changed under the action of miR-646, suggesting that miR-646 may serve a role in the regulation of VEGF/AKT pathway in patients with pre-eclampsia.

VEGF-A is an important regulator for new vessel development and establishment, and serves an important role in an angiogenic switch initiating new vessel formation against ischemia (28). Through bioinformatics analysis, it was revealed that miRNAs may regulate VEGF-A, and the comprehensive score of miR-646 was identified to be the highest scoring binding site (Table II). Endothelial cells are able to adapt to the pathological environment and produce pro-angiogenic and hypoxic regulators, including VEGF-A, IGF-1 and HIF-1α (29-31). A HIF-1α dependent pathway serves an important role in new vessel development against hypoxic environment, including tissue hypoxia (23). HIF-1 is a heterodimeric transcription factor complex that binds to DNA. It is composed of two basic helix-loop-helix domains. The α (1α or 2α) subunits are regulated by oxygen and are the hypoxia inducible domains. In contrast, 1β domain is a non-oxygen responsive subunit and is expressed constitutively (32-34). Previous studies have demonstrated that VEGF-A was first discovered in decidual cells in early pregnancy, and is capable of regulating angiogenesis in embryonic development and physiology of placenta in early pregnancy (35). The dysregulation of VEGF-A expression is important in the development of placental lesions, including pre-eclampsia, preterm birth and intrauterine growth restriction, and is closely associated with multiple steps in the development and progression of pre-eclampsia (36-38). Previous studies have demonstrated that preterm birth may be associated with low levels of VEGF-A expression, and changes in VEGF-A expression may help uncover the specific cause of pre-eclampsia (39,40).

In the current study, it was demonstrated that the miR-646-mediated reduction of VEGF-A is likely to be a key event in placental angiogenesis. Endovascular disorders can be attributed in part to the overexpression of miR-646 in peripheral or cord blood of patients with pre-eclampsia. Here, it is demonstrated that miR-646 is an endogenous inhibitor of VEGF-A thereby modulating EPC-mediated angiogenesis *in vitro*. mir-646 may be a therapeutic target for cytokine regulation that interferes with placental vascular growth in pre-eclampsia.

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Availability of data and materials

The data and materials are available from the corresponding author upon reasonable request.

Authors' contributions

DD conceived the idea, designed the study, performed all the experiments, analyzed the data and wrote the manuscript. YKh and YKo performed RT-qPCR, MTT and transwell assay. YZ carried out the angiogenesis assay and the luciferase reporter assay and helped in revising the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The ethical approval of the current study's protocol was gained from the Ethics Committee of the Zhongnan Hospital of Wuhan University, and detailed written consent was obtained from all enrolled subjects.

Patients consent for publication

All patients provided written consent for publication.

Competing interests

The authors declare that they have no competing interests.

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