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MicroRNA-126 Regulates EPCs Function: Implications for a Role of miR-126 in Preeclampsia

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ABSTRACT

Preeclampsia is a specific vascular complication in pregnancy, which has a pathophysiology of altered endothelial homeostasis. There is extensive evidence that endothelial progenitor cells (EPCs) dysfunction underlies the endothelial cells loss that occurs during preeclampsia. MicroRNA-126 (miR-126), an angiogenesis-related miRNA, has been shown to have potential angiogenic effects both in cultured endothelial cells in vitro and ischemia-induced angiogenesis in vivo. However, whether miR-126 has therapeutic potential in placental vasculogenesis of preeclampsia remains unclear. In this report, we analyzed the EPCs number and expression of miR-126 in patients with preeclampsia, then investigated the effects of miR-126 on EPCs function and rat placenta by employing up-regulation and down-regulation strategies. We confirmed that miR-126 enhanced EPCs proliferation, differentiation and migration. However, a strong reduction in EPCs function was observed in vitro after miR-126 inhibitor transfection. MiR-126 exerts pro-angiogenic functions by suppressing the synthetize of antiangiogenic factors PIK3R2. Similar to miR-126 overexpression, PIK3R2 downregulation promoted EPCs function. In pregnant rats, we also found that miR-126 increased vascular sprouting, placenta and fetus weights. These findings suggest that miR-126 is essential for angiogenic properties of EPCs in vitro and placental vasculogenesis in vivo, providing basis for an alternative therapeutic approach in patients with preeclampsia. J. Cell. Biochem. 114: 2148–2159, 2013.

KEY WORDS: MICRORNA-126; ENDOTHELIAL PROGENITOR CELLS; VASCULOGENESIS; PREECLAMPSIA

Preeclampsia is a pregnancy-specific syndrome with clinical manifestations of high blood pressure and proteinuria appearing after the 20 weeks′ gestation, and is the second most common cause of fetal and maternal morbidity and mortality worldwide [Roberts and Cooper, 2001; 2002; Redman and Sargent, 2005]. The generally accepted pathological basis of preeclampsia is aberrant placental vasculature that caused by endothelial cell dysfunction [Roberts, 1998; Sipos et al., 2010]. However, the damaged endothelial cells cannot be repaired by terminally differentiated endothelial cells, but by the endothelial progenitor cells (EPCs). EPCs, a heterogeneous group of endothelial cell precursors that derived from bone marrow as well as nonmarrow sites, play an important role in vascular formation and angiogenesis [Asahara, 1997; Hill et al., 2003]. They possess the capacity to migrate to sites of neovascularition, differentiate into endothelial cells and release a source of paracrine factors for angiogenesis. Nowadays, the main stream of EPC-related research has been conducted in adult cardiovascular field, despite the fact that EPCs are more abundant and more active in the cord blood than adult peripheral blood [Ingram, 2004]. Studies have demonstrated that preeclampsia reduces EPCs number and impairs EPCs function [Lin et al., 2009; Luppi et al., 2010]. The existing evidence suggests that aberrant vessel formation may be contributed by the impaired availability or function of the EPCs forming them. Therefore, EPCs could be both diagnostic tools and direct targets of medical interventions for preeclampsia.

MicroRNAs, a class of small RNAs (~20–25 nt) that negatively modulate target gene expression at the posttranscriptional level by either degradation or translational repression of a target mRNA [Makeyev and Maniatis, 2008]. MiRNAs have been mainly associated with a wide range of physiological processes, including vasculogenesis and embryonic development [Bartel, 2004]. Furthermore, miRNAs have been implicated in diseases such as vascular disease, which make them attractive new targets for therapy [Fish and Srivastava, 2009]. To date, several human miRNAs have been shown to be invovled in vasculogenesis, such as miR-27b, let-7, and miR-221/222, which regulate a range of processes that includes cell development, differentiation, proliferation, and apoptosis [Zhang, 2010]. These findings suggest that miRNAs are critical modulators of

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vasculogenesis. MiR-126, a newly discovered miRNA, is one of the most frequent miRNA isolated in vascular endothelial cells and plays important roles in many aspects of vasculogenesis, such as tube formation and wound healing in vitro [Wang et al., 2008]. Knockout of miR-126 in mouse embryos and zebrafish resulted abnormal vasculogenesis, hemorrhage, and loss of vascular integrity, demonstrated that miR-126 is required for the vasculogenesis and maintenance of vascular structure [Fish et al., 2008].

These findings suggest that EPCs and miR-126 may have pro-angiogenic properties. However, the biological function and mechanisms of miR-126 and EPCs in vessel-related complications in pregnancy, such as preeclampsia, remain to be further elucidated. In this study, we examined EPCs number, miR-126 expression and the correlation between them in preeclampsia patients. In addition, we tested the role of miR-126 on EPCs proliferation, differentiation, and migration. Next, we explored the underlying mechanism of miR-126 functions in EPCs. Finally, we tested our hypothesis in pregnant rats based on the results in vitro. Our study will provide a better understanding of placenta vasculogenesis.

MATERIALS AND METHODS

STUDY POPULATION AND BLOOD COLLECTION

The study was conducted at the Department of Obstetrics and Gynecology, Union Hospital, Huazhong University of Science and Technology (HUST) from November 2011 to March 2012. The study groups consisted of 12 women with uncomplicated pregnancy (controls) and 12 women with preeclampsia. All subjects were in the third trimester of pregnancy. Pre-eclampsia was diagnosed as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mm Hg arising after 20 weeks′ gestation and proteinuria of ≥1+ (300 mg/24 h) in a previously normotensive woman [Chesley, 1980]. Exclusion criteria included rupture of membranes, tobacco use, history of cardiovascular disease, diabetes mellitus, or other significant pre-existing metabolic disorders, prenatal maternal infection, fetal anomaly, and multifetal gestation. Controls remained normotensive and nonproteinuric during pregnancy and were delivered of healthy infants of appropriate weight. All women received detailed history-taking, a general systemic and obstetric examination, ultrasonographic abdominal evaluation, hematology testing, and urine analysis. All the subjects underwent cesarean section (controls undergoing cesarean section due to their own demands). After the delivery of placenta, cord blood (40–60 ml) was collected into sterile tubes containing heparin immediately and processed within 2 h of collection for cell culture. To compare the basal expression of miR-126 in placenta from controls and pre-eclampsia patients, placenta were also collected. Table I lists clinical characteristics of the two groups. Ethical approval for the study protocol was obtained from the ethics committee of Union hospital, Tongji Medical College, HUST, China and written informed consent was obtained from all subjects.

TABLE I. Baseline Characteristics of Study Population. Data is Listed as Mean ± SD or Percentage (Number/Total)

Parameters	Pre-eclampsia (n = 12)	Normal (n = 12)	*P*-value
Maternal age (years)	31.4 ± 4.03	30.3 ± 3.67	>0.05
BMI	27.4 ± 3.64	25.8 ± 4.22	>0.05
Gestational weeks at delivery	35.54 ± 3.77	37.5 ± 1.76	>0.05
SBP at delivery	121 ± 9	156 ± 13	<0.05
DBP at delivery	73 ± 11	98 ± 8	<0.05
Proteinuria	0% (0/12)	100% (12/12)	<0.05
S/D ratio of umbilical artery	3.08 ± 0.95	2.37 ± 0.63	<0.05
Birth weight (g)	3,612 ± 628	2,508 ± 854	<0.05
Placental weight (g)	523.2 ± 98.7	552 ± 103.3	<0.05
Apgar score	8.5 ± 0.89	9.2 ± 0.76	>0.05
Placental MVD	54.8 ± 5.72	77.6 ± 7.92	<0.05

BMI, body mass index in pregnancy (kg/m^2); DBP, diastolic blood pressure; SBP, systolic blood pressure; S/D ratio, systole/diastole (S/D) ratio; MVD, microvessel density. Proteinuria is defined in Methods Section.

CELL CULTURE AND EPCs CHARACTERIZATION

Heparinized cord blood was used to isolate peripheral blood mononuclear cells (PBMCs) by employing the method described previously [Asahara, 1997]. Briefly, PBMCs were isolated using Ficoll-Paque density gradient centrifugation (400*g*, 30 min, 20°C) in a lymphocyte separation solution (TBD Sciences, Inc., Tianjin, China). Then washed twice with phosphate buffer solution (PBS), and resuspended at a final concentration of 1×10^6 cells/ml in endothelial basal medium-2 (EBM-2, Lonza, USA) supplemented with EGM-2-MV-SingleQuots (Lonza) containing 5% foetal bovine serum, 50 ng/ml human vascular endothelial growth factor (VEGF), 50 ng/ml human insulin-like growth factor 1, 50 ng/ml human epidermal growth factor, 100 μg/ml penicillin, and 100 μg/ml streptomycin. PBMCs (1×10^7) were seeded on fibronectin-coated (Sigma–Aldrich) six-well culture dishes (Corning). After 2 days of culture, non-adherent cells were removed and the medium was replaced every 2 days. After 3 days, attached cells appeared and formed small round EPC clusters (Fig. 1B), then the round cells started elongating and had a spindle shape similar to that of the EPC Asahara first reported. After 7 days of culture, they gradually turn to clusters consisting of round cells centrally with multiple spindle-shaped cells sprouting from the central core, which we called colony-forming units (CFU) [Hur et al., 2007] (Fig. 1C). All experiments were performed with EPCs at day 7 (Fig. 1). Identification of EPCs was performed by staining with DiI-acetylated-low-density lipoprotein (DiI-Ac-LDL, Molecular Probes, Carlsbad, CA) and FITC-labeled Ulex europaeus agglutinin-I (FITC-UEA-I, Sigma–Aldrich, St. Louis, MO). In brief, attached cells were incubated with DiI-Ac-LDL (2.4 mg/ml) at 37°C for 4 h, washed three times with PBS, fixed with 2% paraformaldehyde, and then incubated with FITC-UEA-1 (10 mg/ml) for 1 h. Adherent cells were visualized with a laser scanning confocal microscope (LSCM, Leica) and those stained for both DiI-Ac-LDL and FITC-UEA-1 were identified as differentiating EPCs [Asahara, 1997; Hill et al., 2003] (Fig. 1D). Dual-staining cells in 10 randomly selected high power fields were counted by two independent investigators.

IN VITRO TRANSFECTION

The miR-126 mimic and miR-126 inhibitor oligonucleotides (Ribobio, Guangzhou, China) were diluted in the PBS at a final concentration of 20 μM. According to the manufacturer′s instructions, cells were seeded into 24-well plates and transfected respectively with miR-126 mimic, miR-126 inhibitor, or PIK3R2 shRNA plasmid (GeneSil, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Efficiency of transfection was determined by Cy3-siRNA transfection control (siR-Ribo™, China). The overexpression and inhibition efficiency were

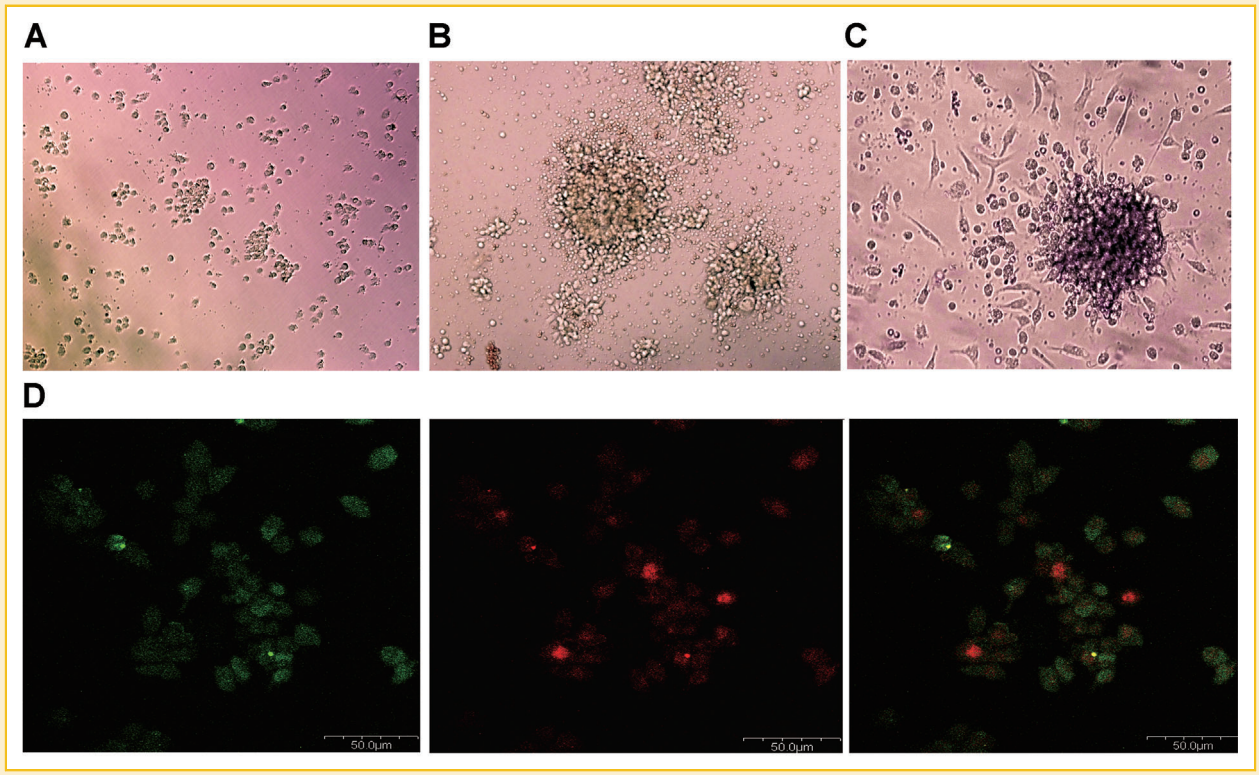

Fig. 1. Characteristics of EPCs and colony-forming units (CFUs) in culture derived from cord blood samples. A: Brightfield image of freshly isolated PBMCs (original magnification 100×). B: Small round EPC clusters at 3 days of culture (magnification 200×). C: CFUs were characterized by round cells centrally with multiple spindle-shaped cells (length at least three times greater than width) radiating from the central core (200×). D: EPCs showed both DiI-acLDL (red) and FITC-UEA-lectin (green) staining in confocal microscopy (400×).

determined by comparing it against the negative control (NC) siRNA provided by Ribobio. After 24 h of transfection, cells were harvested for further analysis.

QUANTITATIVE RT-PCR FOR miR-126 AND TARGET GENES

Total RNA was extracted from tissues or cultured cells using TRIZOL reagent (Invitrogen, USA). RNA was quantified and reverse transcribed into complementary DNA (cDNA) using Bulge-Loop miRNA qRT-PCR Primer set (Ribobio, China) and PrimeScript RT reagent Kit (Takara, Japan) [Chen et al., 2005]. The U6 small nucleolar RNA and GAPDH were used as internal controls. The expression level of miR-126 was normalized to U6, and the expression levels of target genes were normalized to GAPDH. The RT-PCR primer sequences were as follows: PIK3R2, forward primer: 5′-gCAAgATCCAggg-CgAgTACA-3′, reverse primer: 5′-TgAggTCCACAACggAgCAg-3′. PI3K, forward primer: 5′-CACACACTACATCAgTggCTCAAAg-3′, reverse primer: 5′-TCCAgCACATgAACgTgTAAACAg-3′. Akt, forward primer: 5′-CTTgCTTTCAgggCTgCTCA-3′, reverse primer: 5′-TACACgTgCTgCCACACgATAC-3′. GAPDH, forward primer: 5′-ACCACAGTCCATGCCATCAC-3′, reverse primer: 5′-TCCACCA-CCCTGTTG CTGTA-3′. Each reaction was performed in triplicate by employing SYBR Premix Ex Taq™ (TaKaRa, Japan) on an Applied Biosystem 7300 Real-time PCR system (Applied Biosystems, Foster City, CA). The PCR reaction consisted of 40 cycles (95°C for 15 s, 62°C for 15 s, 72°C for 45 s) after an initial denaturation step (95°C for 2 min). The results were subjected to melting curve analysis. The relative gene expressions of miR-126 and target genes were analyzed using $2^{-\triangle\triangle Ct}$ method.

WESTERN IMMUNOBLOTTING

EPCs transfected with miR-126 mimic and inhibitor were washed in ice-cold PBS and extracted following the manufacturer's protocol. The protein concentrations were determined with BCA protein assay kit (Beyotime, Shanghai, China). Forty micrograms protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto polyvinylidene fluoride (PVDF) membrane. The bands were incubated with 5% nonfat milk in phosphate-buffered saline with 0.1% Tween-20 to block the nonspecific binding sites, and then incubated with a mouse monoclonal anti-human PIK3R2 (diluted at 1:3,000; Abcam, USA) and a rabbit monoclonal anti-human Akt, PI3K (diluted at 1:2,000; Cell Signal, USA) overnight at 4°C. β-actin (1:3,000; Santa Cruz) was used as an internal control. After rewarming and washing, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature and visualized by ECL-PLUS (Amersham Biosciences).

CELL PROLIFERATION, COLONY FORMING AND CELL DIFFERENTIATION ASSAY

Cell proliferation was determined at 24 h by MTT. Cells were seeded into 96-well plates at 1.0×10^3 cells per well and MTT (20 μl, 5 mg/ml, Sigma, USA) was added to each well. Then the plates were

incubated for 4 h at 37°C. The medium was removed and dimethyl sulfoxide (DMSO) was added to each well. Absorbance was measured at 490 nm using a microplate reader (Model 550, Bio-Rad, USA). For colony formation assay, cells were resuspended at 1,000 cells/well and incubated in medium for 1 week until visible colonies appeared. Then, colonies were fixed in methanol and stained with crystal violet for 20 min. Colonies were counted in each well. The cell differentiation assay was conducted as previously described [Wang et al., 2011]. Briefly, EPCs transfected respectively with miRNA-126 mimic, inhibitor, and mimic/inhibitor control siRNA were resuspended and replated onto fibronectin-coated culture dishes at a density of 1×10^6 cells/well. Then cells were incubated for 24 h at 37°C in 5% CO_2 incubator. The elongated and spindle-shaped cells represented differentiated cells according to Asahara's first report, and the number of cells was counted in three random fields under a phase-contrast microscope.

MIGRATION ASSAY

The ability of EPCs to migrate was detected using Transwells (8-mm pore size, Corning, USA). Briefly, cells transfected as aforementioned were harvested 24 h after transfection and resuspended in medium containing 1% fetal bovine serum. 5×10^4 cells were added to the upper chamber and the lower chamber was filled with 500 μl of medium containing 10% fetal bovine serum (FBS) and 50 ng/ml VEGF as a chemoattractant. Cells were incubated for 36 h at 37°C in 5% CO_2 atmosphere. After 36 h, non-migrated cells were removed from upper side of the membrane with a cotton-tipped swab. The cells attached on the lower side of the membrane were fixed in methanol and stained with crystal violet. Then the average number of cells were counted manually (three randomly selected 200× fields per transwell) and imaged under an inverted microscope. Each experiment was performed in triplicate.

IN VIVO VASCULOGENESIS IN PREGNANT RATS

All procedures were approved by the Animal Care and Use Committee of Tongji Medical College, Wuhan, China. Adult female Wistar rats ($n = 30$) weighing 250–270 g (12 weeks of age) were obtained from the Hubei Experimental Animal Center, China and maintained in a 12-h light–dark cycle in a pathogen-free animal facility at Tongji Medical College. To test our hypothesis that miR-126 promotes vasculogenesis in pregnant rats, we performed an experiment in which a chemically modified oligonucleotide specific for miR-126 (agomir-126 and antagomir-126, Ribobio, China) was injected into placenta. Transfection compounds were prepared according to the manufacturer's instructions at the optimal concentration (20 μl transfection compounds containing 400 pmol agomir-126 or antagomir-126). All animals were made pregnant and divided randomly into three groups (10 rats in each group), at day 15 of gestation, all rats received placental injection via laparotomy. In control group, rats were injected with 20 μl saline. In agomir-126 and antagomir-126 group, rats were injected 20 μl transfection compound with 400 pmol agomir-126 or 400 pmol antagomir-126, respectively. They were sacrificed 3 days after the injection, the placenta and fetuses were harvested. The in vivo transfection efficiency of agomir-126 and antagomir-126 was determined by detecting the expression of miR-126 in placenta tissues.

IMMUNOHISTOCHEMICAL ANALYSIS

Formalin-fixed and paraffin-embedded placental tissues were used for immunohistochemical staining. The 6 μm thick sections were deparaffinizd and antigens retrieval, then blocked by 10% normal goat serum for 1 h and incubated with rabbit polyclonal anti-rats CD34 (1:100; Santa Cruz) or PBS serving as control at 4°C overnight, then the sections were incubated with secondary antibody (1:100; Boster, China). Afterward, peroxidase reactivity was detected using a DAB substrate Kit. Finally, the sections were counterstained with hematoxylin. Microvessel density was assessed by determining the average of the number of vessels in five random high-power fields in each section.

STATISTICAL ANALYSIS

Each cell experiment was performed three times independently. Results from each independent experiment were presented as mean ± SD. The relationship was calculated using Spearman's correlation coefficient. Student *t*-test and ANOVA were employed to analyze the in vitro and in vivo data using SPSS 13.0 software. A *P*-value of <0.05 was interpreted to be statistically significant.

RESULTS

PATIENT CLINICAL CHARACTERISTICS

Clinical characteristics of the patient are summarized in Table I. Preeclampsia patients and control subjects were matched by age, body mass index (BMI) and gestational weeks. In preeclampsia patients, the lower placental MVD indicated abnormal vasculogenesis in placenta, and the increased S/D ratio of umbilical artery revealed vasospasm and inadequate blood supply of the fetus.

DIFFERENCE BETWEEN PREECLAMPSIA GROUP AND CONTROL GROUP

Examples of microscopic images of EPCs and CFUs from two groups are shown in Figure 2. The number of EPCs and CFUs were significantly lower in preeclampsia patients compared with healthy controls, and the diameter of CFUs was approximately twofold shorter in preeclampsia. Figure 3A illustrates that preeclampsia markedly decreased the number of EPCs and CFUs counts (EPCs number: 78.3 ± 37.5 vs. 118.7 ± 30.1, $P < 0.05$; CFUs counts: 2.9 ± 4.8 vs. 9.75 ± 12.2, $P < 0.05$). Microvessel density in placenta was also detected using immunohistochemistry, Figure 2C shows two sections from the preeclampsia group and control group. There were more capillaries with thin walls in healthy placenta (Fig. 3A). To further understand the role of miR-126 in preeclampsia, we compared its expression in EPCs and placenta from preeclampsia group and control group. Results showed that miR-126 was expressed in both EPCs and placenta of two groups. MiR-126 level decreased notably in preeclampsia patients (Fig. 3B), suggesting that the downregulation of miR-126 may be involved in preeclampsia. As shown in Fig. 3C, EPCs numbers were moderately positively correlated with miR-126 levels in preeclampsia group ($r = 0.655$, $P < 0.05$), indicating that miR-126 level may be the regulatory factor for EPCs number in preeclampsia. The mRNA and protein levels of PI3K and Akt, two important regulators in angiogenesis, were also decreased in

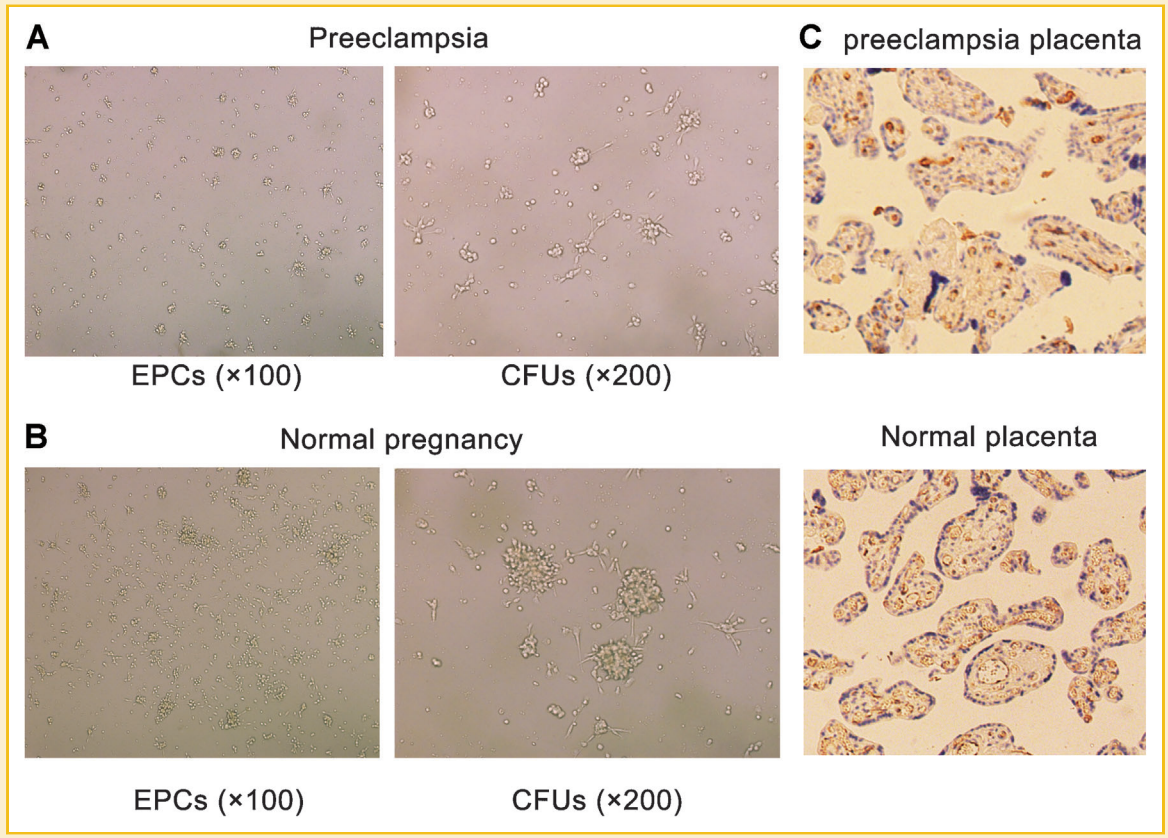

Fig. 2. Example of microscopic images of EPCs and CFUs representative of one patient with preeclampsia (A) and one with normal pregnancy (B) after 7 days of culture. EPCs number decreased in the preeclampsia patient compare to control (100×). The numbers of CFUs were lower in preeclampsia patient (200×). Only typical CFUs were counted by light microscopy. C: CD34 immunohistochemical staining was used for assessing microvessel density and vessels area in preeclampsia and normal placenta. Vessels appear as brown areas.

preeclampsia (Fig. 3D). In summary, we hypothesized that miR-126 involved in the pathogenesis of preeclampsia by regulating EPCs.

MiR-126 OVEREXPRESSION AND INHIBITION IN TRANSFECTED EPCs

To study the role of miR-126 in EPCs, we established a miR-126 overexpression and inhibition model in EPCs. After 24 h of transfection, transfection efficiency was determined by Cy3-siRNA transfection control (siR-Ribo™, China). All cells presented strong and extensive cytosolic delivery of Cy3-siRNA (Fig. 4A). The expressions of miR-126 in different groups were confirmed by qRT-PCR. MiR-126 was expressed in all the groups and showed greater increases in miR-126 mimic transfected cells (1.0 ± 1.2 vs. 8.1 ± 1.7, $P < 0.05$), whereas miR-126 expression was substantially decreased by miR-126 inhibitor (1.0 ± 0.26 vs. 0.32 ± 0.21, $P < 0.05$) (Fig. 4A).

EFFECTS OF miR-126 ON CELL PROLIFERATION AND COLONY FORMATION

Using MTT assays, we observed the cell proliferation in miR-126 mimic, miR-126 inhibitor, and control groups. There was a significant increase in cell proliferation 24 h after transfection with miR-126 mimics (Fig. 4C). Similarly, miR-126 overexpression resulted in increased colony formation compared to control group 1 week after incubation (Fig. 4B). In contrast, cell proliferation and colony formation were restrained in miR-126 inhibitor group versus control group (Fig. 4). These results indicate proliferative and colony formation effects of miR-126 in EPCs.

EFFECTS OF miR-126 ON CELL DIFFERENTIATION AND MIGRATION

We investigated the effects of miR-126 on EPC differentiation. The miR-126 mimic group showed a marked increase in the number of spindle-shaped attached cells in comparison to that observed in mimic control group ($P < 0.05$), however, miR-126 inhibitor group showed a notably decrease in differentiated cells compared with inhibitor control group ($P < 0.05$) (Fig. 5A). Next, we used transwell migration technique to examine the effect of miR-126 on EPC migration. Migration assay showed that miR-126 significantly increased the migration of EPCs as compared with control group (Fig. 5B). These results indicate a differentiating and migrating promoter role for miR-126 in EPCs.

MiR-126 REGULATES PI3K–AKT SIGNALING AXIS BY DIRECTLY TARGETING PIK3R2

PIK3R2 has been reported to be an important molecule that regulates angiogenesis negatively. Using an online microRNA target database we found PIK3R2 as the putative target of miR-126. To confirm the target gene of miR-126, we used RT-PCR and Western blotting to detect the PIK3R2 expression in four groups. Results showed that miR-126 mimic attenuated the expression of PIK3R2 compared to the

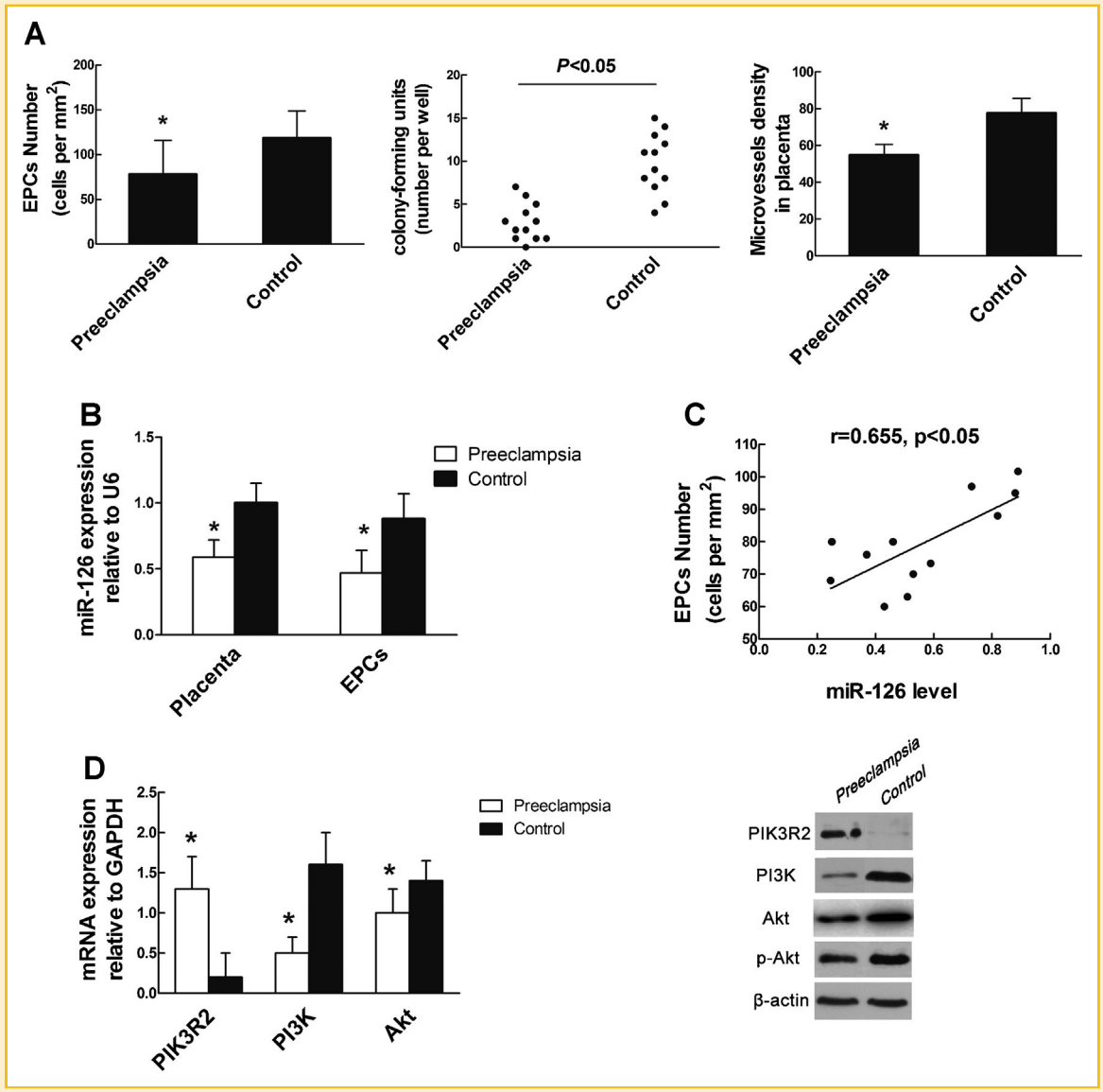

Fig. 3. A: Statistical analysis of EPCs numbers (left), CFUs counts (middle) and microvessle density (right) between preeclampsia patients and normal healthy controls. Each dot equals to average CEUs per culture well from a different individual. B: miR-126 is expressed in EPCs and placenta from preeclampsia patients and controls. MiR-126 expression was determined normalized to U6 by real-time PCR and analysis was performed by the Ct method. C: Correlation between miR-126 levels and EPCs numbers in patients with preeclampsia. D: mRNA and protein levels of PIK3R2, PI3K, and Akt in preeclampsia and healthy controls.* $P < 0.05$ versus control group.

mimic control. We also found an increase in the mRNA and protein levels of PI3K and Akt, which are down streams of PIK3R2 (Fig. 6A,D). PI3K and Akt are also two major proteins in PI3K–Akt signaling axis which regulates multiple critical steps in angiogenesis and vessel homeostasis. However, in the miR-126 inhibitor group the expression of PIK3R2 increased due to less degradation by miR-126, PI3K, and Akt expressions were significantly lower than that in the inhibitor control group ($P < 0.01$) (Fig. 6B,E).

To elucidate whether the function-promoting effect of miR-126 was modulated by down-regulation of PIK3R2 in EPCs, we performed the loss-of-function study. Briefly, we silenced PIK3R2 to investigate whether the reduced expression of PIK3R2 could mimic the function-promoting effect of miR-126. EPCs were infected with shPIK3R2 and then we examined cell proliferation, differentiation, and migration. As shown in Figure 7, knockdown of PIK3R2 significantly promoted cell growth, differentiation and migration, which is similar to those induced by miR-126 mimic. These results suggest that miR-126 targets antiangiogenic gene PIK3R2 directly and involved in the regulation of PI3K–Akt signaling pathways.

ROLES OF miR-126 IN PLACENTAL ANGIOGENESIS

To further confirm the above findings, agomir-126, antagomir-126, or saline were inoculated into the placenta of each rat respectively ($n = 10$) via laparotomy. The miR-126 expressions in three groups are presented in Figure 8B. Though no abortion took place, the size and weight of placenta and fetus were significantly reduced in

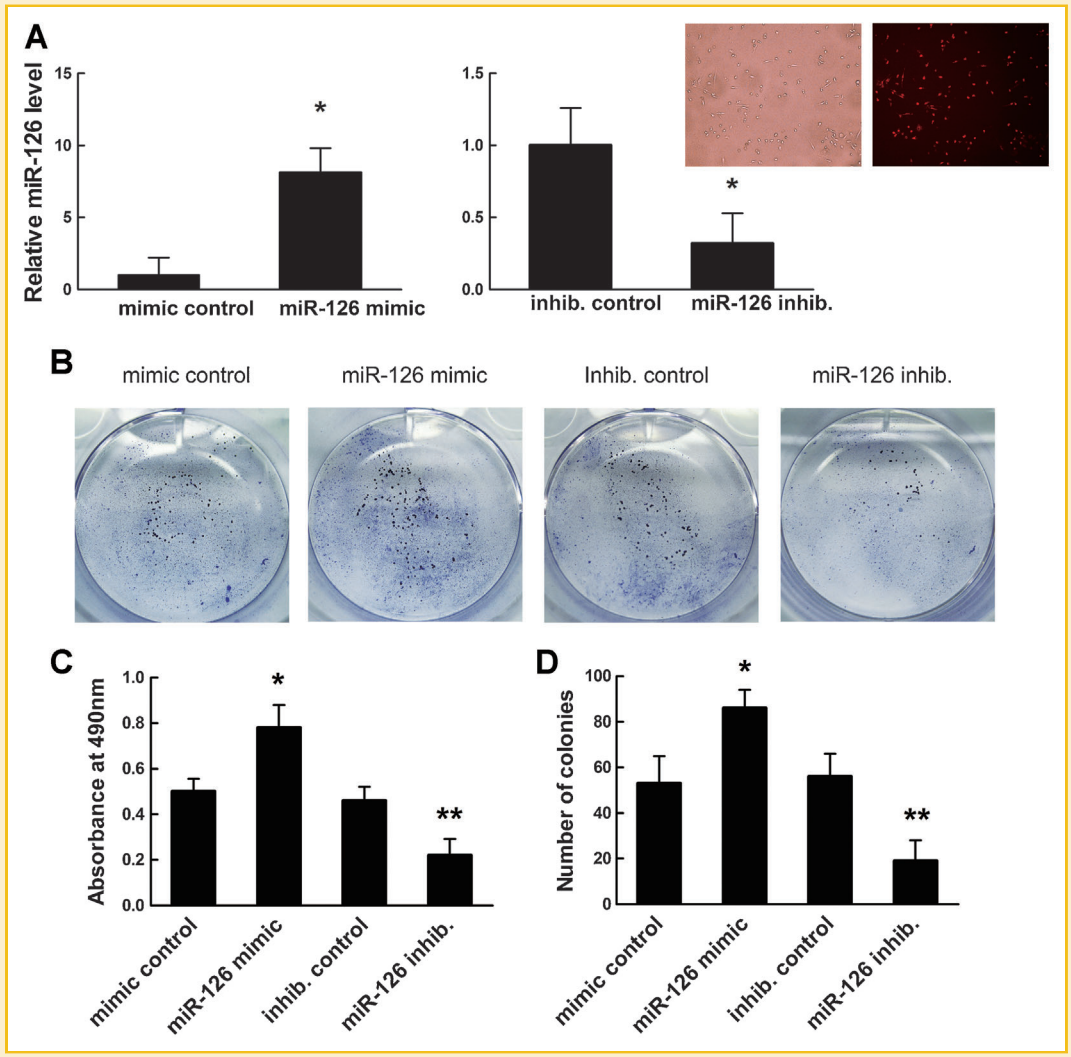

Fig. 4. Overexpression and inhibition of miR-126 influence cell proliferation and colony formation of EPCs in vitro. A: Transient transfection of miR-126 mimic significantly increased expression of miR-126 in EPCs, whereas miR-126 expression dropped notably in miR-126 inhibitor group confirmed by qRT-PCR. Transfected EPCs showed a strong and extensive expression of Cy3 under inverted fluorescence microscope. B: Colony formation of EPCs in six-well plates was assayed in four groups. C and D: Histogram shows that cell proliferation and colony forming ability of EPCs after miR-126 mimic transfection was significantly increased compared to control EPCs. Proliferation and colony efficiency of EPCs was decreased by miR-126 inhibitor. $^{*}P < 0.05$ versus mimic control group. $^{**}P < 0.05$ versus inhib. control group.

antagomir-126 group (Fig. 8A). The placenta from antagomir-126 rat looked paler than control and agomir-126 group, indicating that there might be abnormal angiogenesis in placenta. To confirm the hypothesis that antagomir-126 inhibits placental angiogenesis in vivo, we detected the microvessel density of placenta from three groups (Fig. 8C). The results showed that placental microvessel density was also increased significantly in agomir-126 group as compared with control group. However, in the miR-126 antagomir group, the size and weight of placenta and fetus, microvessel density were decreased compared with control groups ($P < 0.05$).

DISCUSSION

Our study is the first to investigate miR-126 expression in EPCs from preeclampsia patients and effects of miR-126 on important angiogenic characteristics of early EPCs. The main findings of the present study are: (i) both numbers of EPCs and levels of miR-126 in EPCs were lower in preeclampsia group than in the normal group; Changes in EPCs numbers were positively correlated with changes in miR-126 levels in the preeclampsia group. (ii) miR-126 enhanced EPCs proliferation, differentiation and migration, by targeting antiangiogenic gene PIK3R2 directly. The mechanism of miR-126 effecting on EPCs is through PI3K–Akt signal pathway. (iii) miR-126 had pro-angiogenic properties in vivo, which increased vascular sprouting, placenta and fetus weights.

In the first part of our research, we confirmed that the EPCs number, CFUs counts, placental MVD, and miR-126 levels in EPCs were lower in preeclampsia, which are consistent with researches before [Lin et al., 2009; Luppi et al., 2010]. The reduction of above-mentioned indicators in preeclampsia is consistent with increased systole/diastole (S/D) ratio in the umbilical arteries, a feature typical

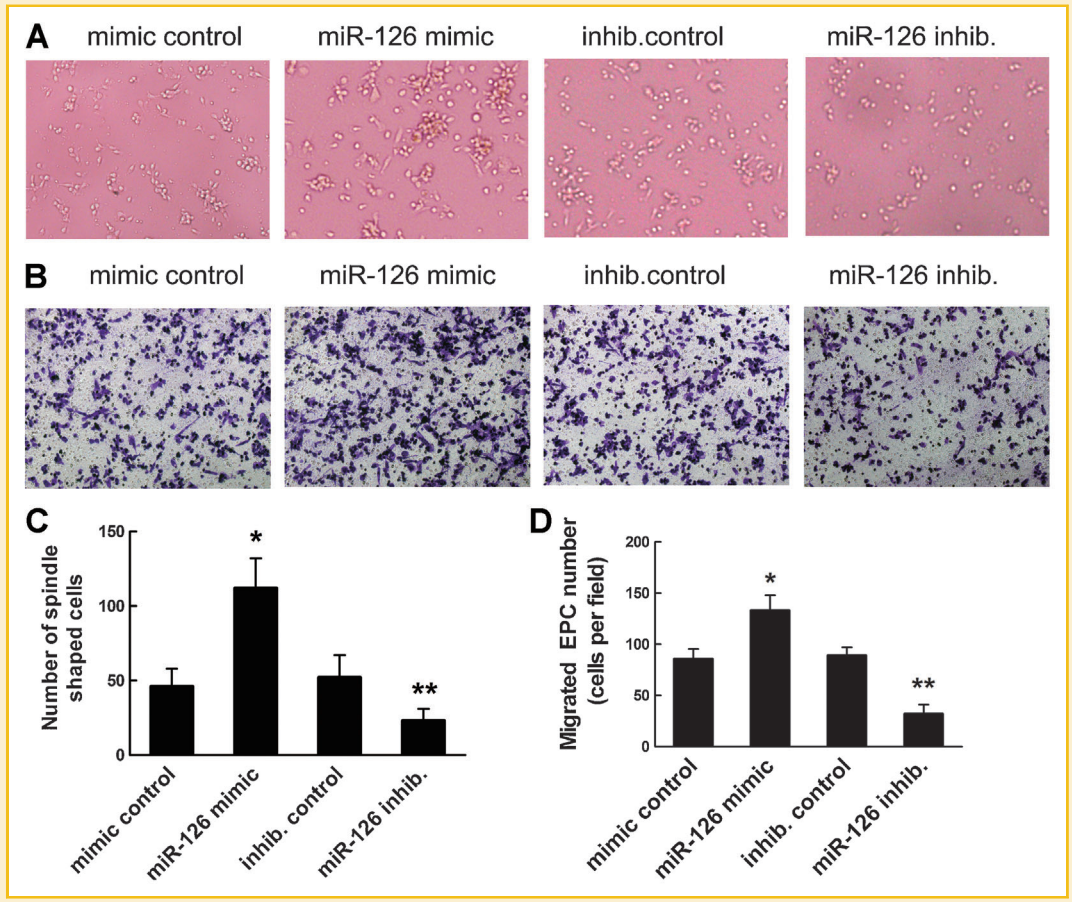

Fig. 5. MiR-126 influences differentiation and migration of EPCs. A: Cell differentiation assay. Spindle-shaped cells of four groups were observed under a phase-contrast microscope. B: Representative pictures of migrated EPCs in four groups. C: Histogram showed that miR-126 mimic increased average number of spindle-shaped cells. Cell differentiation was decreased by miR-126 inhibitor transfection. D: The number of migrated EPCs was increased in miR-126 mimic group and decreased in miR-126 inhibitor group. Results are presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ versus mimic control group. $^{**}P < 0.05$ versus inhib. control group.

for preeclampsia, which means an increased peripheral resistance of the placental vessels due to the abnormal placental vasculogenesis [Sibley et al., 2002]. In addition, levels of miR-126 were positively related to the number of EPCs in preeclampsia patients, indicating that miR-126 level may be the regulatory factor for EPCs number in preeclampsia. Through a combination of clinical data and laboratory parameters of preeclampsia patients, we hypothesized that miR-126 may involve in the placental vasculogenesis by regulating EPCs.

To further investigate the role of miR-126 in EPCs, we transfected miR-126 mimic and inhibitor into EPCs to up-regulate and down-regulate its expressions and then examined the changes of EPCs functions. Our results clearly showed that enhancement of miR-126 in human EPCs by miR-126 mimics promoted EPCs proliferation, migration, and differentiation in vitro. In contrast, inhibition of miR-126 impaired EPCs functions. These findings suggest that miR-126 enhances EPCs functions, which are crucial for vasculogenesis. To better understand the mechanism by which miR-126 modulates EPCs biological function, we investigated the potential mRNA targets of miR-126 and possible signal transduction pathway. Research before have demonstrated that phosphatidylinositol-3 kinase (PI3K)–Akt signaling axis regulates multiple critical steps in angiogenesis and vessel homeostasis, including endothelial cell survival, migration, and capillary-like structure formation [Li et al., 2011; Shiojima, 2002]. The PI3K–Akt axis is activated by many angiogenic growth factors such as VEGF, which are also the stimulants and chemo-attractants of EPCs [Tanimoto, 2002; Everaert et al., 2010]. Besides, our group has proved that ILK, a downstream substrate of PI3K and an important upstream kinase for the regulation of protein kinase B (PKB/Akt), could improve the angiogenic properties of EPCs [Wang et al., 2011]. The protein level of PI3K and Akt were also expressed differently between preeclaimpsia patients and control subjects in our research. Accordingly, the mobilization of EPCs from bone marrow and forming new blood vessels through proliferation and differentiation depend on a PI3K–Akt manner.

PIK3R2, a negative regulator of PI3 kinase, restrains the VEGF signaling pathway by inhibiting the activity of PI3 kinase [Ueki et al., 2003]. Fisher et al. have proved that transfection of miR-126 caused a significant decrease in luciferase activity derived from PIK3R2 RNAs, and miR-126 exerts pro-angiogenic functions by suppressing the synthetize of antiangiogenic factors PIK3R2 in endothelial cells. However, the mechanism by which miR-126 modulates EPCs remains unclear. So in our research, we detected

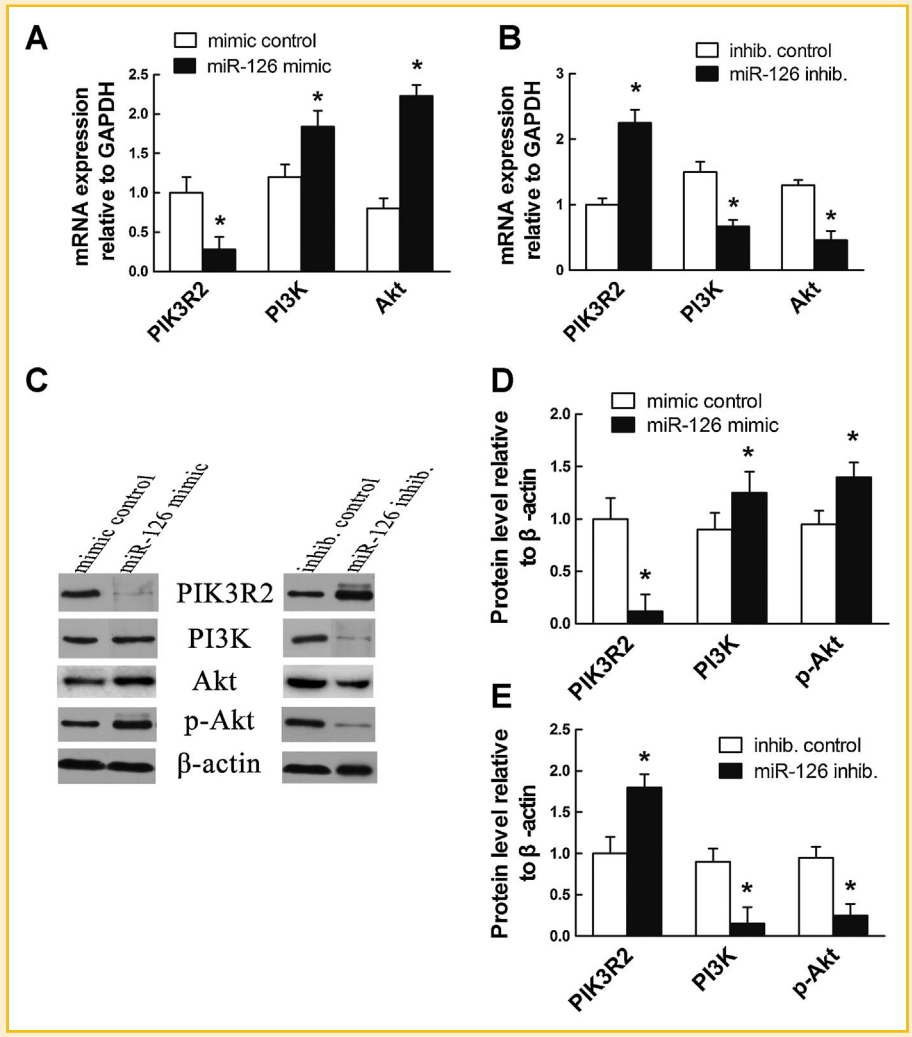

Fig. 6. MiR-126 regulates PIK3R2 and downstream genes expressions in EPCs. A: The expression of PIK3R2 and downstream genes were analyzed by qRT-PCR. PIK3R2 mRNA level was decreased in miR-126 mimic group, in contrast, PI3K and Akt mRNA levels were increased compared to control group. B: In miR-126 inhibitor group, PIK3R2 mRNA level was increased, but PI3K and Akt mRNA levels were decreased compared to inhibitor control group. $^{*}P < 0.05$ versus control groups. C: Western blotting showed PIK3R2, PI3K, Akt, and p-Akt protein expression in four groups. β-actin has been used as internal control. D and E: Effect of miR- 126 mimic and inhibitor on PIK3R2, PI3K, and p-Akt protein levels. The expression level of PIK3R2, PI3K, and Akt were analyzed using semi-quantitative analysis. $^{*}P < 0.05$ versus control groups.

the expression of PIK3R2, PI3k, and Akt in order to assess whether the PI3K–Akt signaling axis was affected by upregulate or downregulate the expression of miR-126 in EPCs. Using RT-PCR and Western blots, we confirmed that overexpression of miR-126 resulted in a declining expression of PIK3R2, an increasing expression of PI3K and Akt in human EPCs. Conversely, downregulation of miR-126 by miR-126 inhibitor increased the expression of PIK3R2 and decreased the expression of PI3K and Akt, which is consistent with the findings in endothelial cells. We also observed that silence of PIK3R2 by shRNA could mimic the promoting effect of miR-126 in EPCs. These results suggest that miR-126 promotes EPCs function by represses the expression of PIK3R2.

In the previous section, we have confirmed that overexpression of miR-126 by miR-126 mimic enhanced EPCs function in vitro. Therefore, in the last section we tried to confirm the role of miR-126 in placental angiogenesis in vivo models. Although upregulation of miR-126 promotes vasculogenesis in developing zebrafish and mouse embryos, little is known about its effect on placenta. To investigate the role of miR-126 in placental vasculogenesis, we used agomir-126 and antagomir-126, RNA-like oligonucleotides which have the property to enhance tissue and cellular uptake, locally injected into rat placenta at Day 15 of gestation. The size and weight of rat placenta and fetus were analyzed to assess the pregnancy outcome. CD34 staining of rat placental tissues was also performed to assess vessel number in rat placenta.

In most mammals, main growth of fetal weight and the placental capillary beds occurs during the last half of gestation. During this period, the branching growth of the placenta appears to keep pace with the increasing metabolic demands of fetal growth [Reynolds et al., 2005; Thornburg et al., 2010]. The placental capillary beds grow primarily by branching, resulting in a large increase in capillary number density [Kaufmann et al., 2004]. The angiogenic activity of

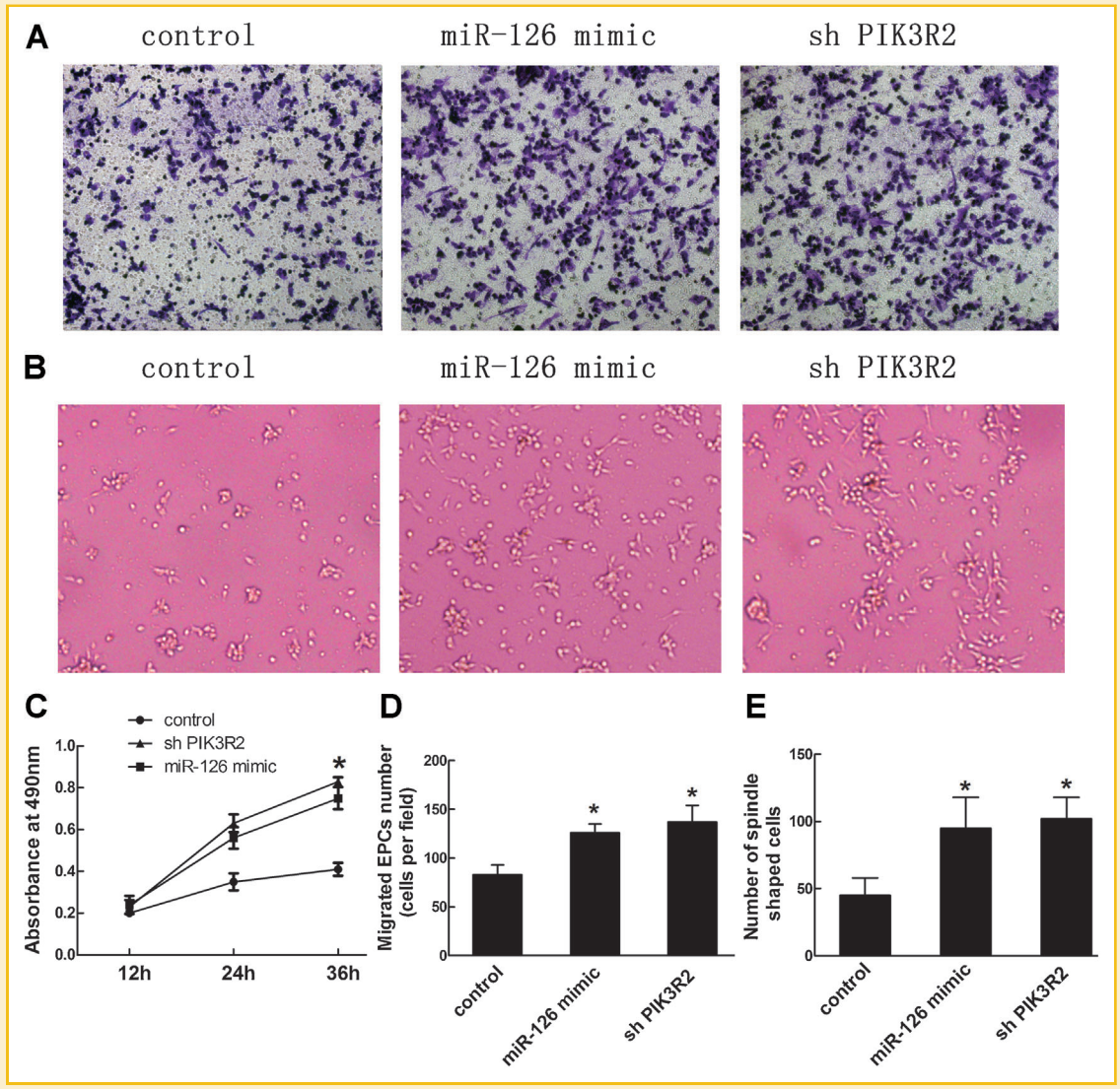

Fig. 7. PIK3R2 is involved in miR-126-induced functional enhancements in EPCs. A and B: EPCs were infected with sh-PIK3R2 or miR-126 mimic. Representative micrographs of cell migration and differentiation in three groups. C: MTT showed that sh-PIK3R2 and miR-126 mimic increased the proliferation of EPCs 24 h after incubation. D: The number of migrating cells was increased in sh-PIK3R2 and miR-126 mimic group compared to control EPCs. E: Statistical analysis showed that average number of spindle-shaped cells in sh-PIK3R2 and miR-126 mimic group was increased. Data were normalized to control EPCs and expressed as means ± SD; $^{*}P < 0.05$ compared to control group.

placental tissues throughout the last half of gestation could not proceed without migration and proliferation of EPCs in vivo. Therefore we investigated the effect of miR-126 on fetal growth and the development of placental capillary beds during the last half of pregnancy.

In this study, we locally injected agomir-126 and antagomir-126 in placenta to up- and down-regulating miR-126 expression in vivo, and examined its effect on microvessel density of placenta for the first time. In our study, the number of capillaries in placenta was increased in agomir-126-treated rats, indicating that agomir-126 improves perfusion and maternal–fetal exchanges. Likewise, fetal weights increased in agomir-126-treated rats due to improved vascularization in placenta, while the fetal weights decreased in antagamor-126 group due to placenta ischemia. So these results implied a link between miR-126 expression and development of placenta and fetus, placental locally application of agomir-126 could improve pregnancy outcome. The result in vivo was consistent with our hypothesis that miR-126 augments vasculogenesis, it was also consistent with the role of miR-126 in other animal models. However, since the level of miR-126 was not determined in fetal tissues in this study, we cannot tell whether the altered fetal size is caused by the change of miR-126 level or placental vessels number with certainty, which requires further study. In addition, we cannot formally exclude the possibility that miR-126 has effects on other cell types in the placenta, although placenta has been defined as a major hematopoietic stem cell pool in mid-gestation mouse and EPCs are produced in the placenta itself [Mikkola et al., 2005; Ottersbach and Dzierzak, 2009; Robin et al., 2009].

In conclusion, we have demonstrated that miR-126 acts as an endogenous suppressor of PIK3R2 to enhance EPC-mediated

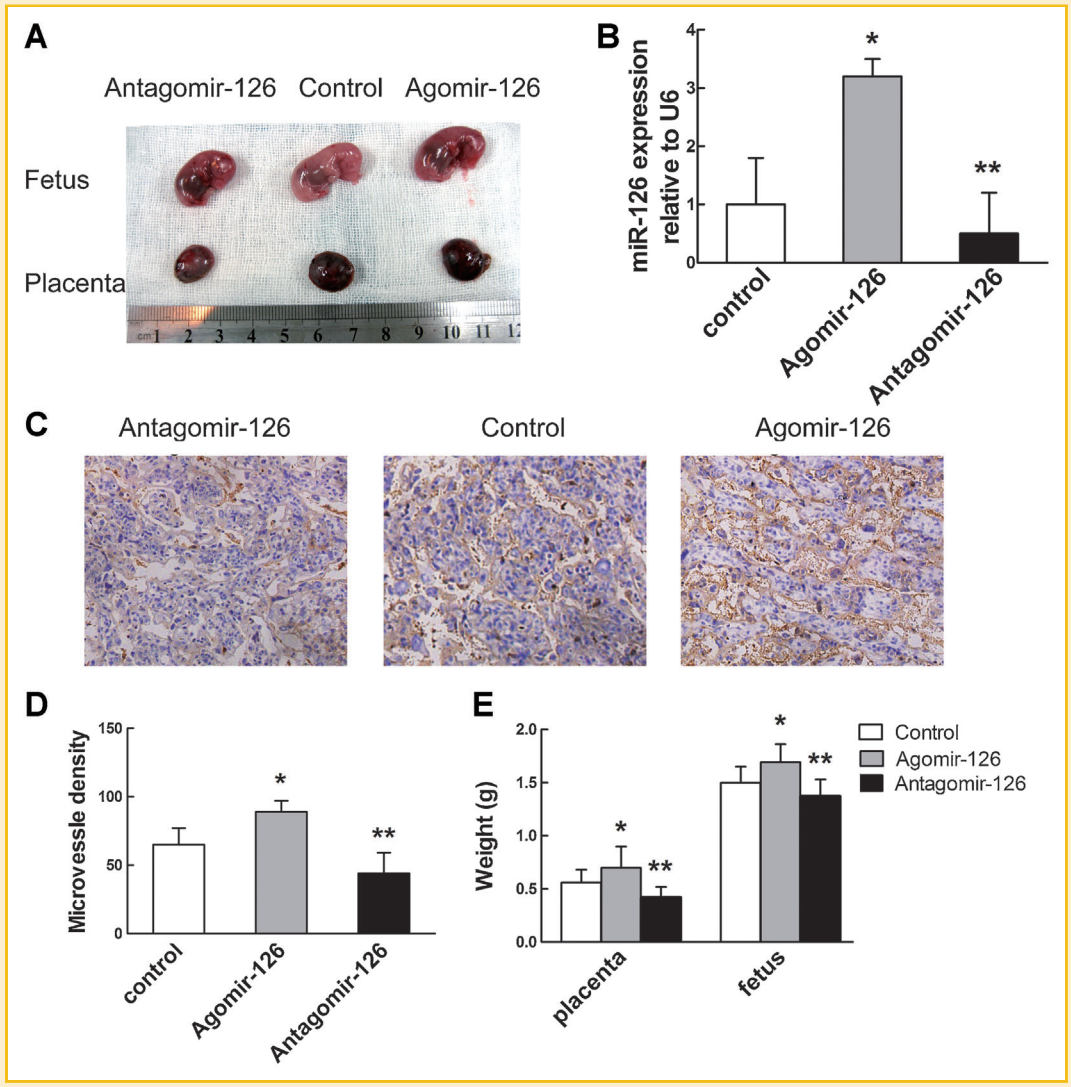

Fig. 8. Effect of miR-126 on rat placenta and fetus. A: Size of placenta and fetus of three representative rats transfected with agomir-126, antagomir-126, and negative control. B: miR-126 level was measured in placenta tissue in three groups. C: Microvessel density of rat placenta using CD34 immunohistochemical staining (paraffin section, SABC). D: Histogram showed that microvessel density was increased in agomir-126 group and it was decreased in antagomir-126 group compared with control group ($^{*}P < 0.05$). E: The weight of rat placenta and fetus was measured, it was increased in agomir-126 group compared with control group and it was decreased in antagomir-126 group compared with control group. ($^{*}P < 0.05$).

vasculogenesis in vitro and in vivo, which might offer a therapeutic advantage to interfere with the complex modulation of placental vessel growth in preeclampsia.

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