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Down-regulated miR-149-5p contributes to preeclampsia via modulating endoglin expression

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ABSTRACT

Objective: Endoglin is expressed in human placenta and plays an important role in the pathogenesis of preeclampsia. Dysregulation of microRNAs in placental tissues has been recently suggested to be involved in the pathogenesis of preeclampsia. Until now, few studies have shed light on the correlation between endoglin and microRNAs, the latter of which may regulate the expression of *ENG*, a gene encoding endoglin, in placenta. In this study, we aim to investigate the regulation of *ENG* by microRNAs.
Study design: We located the microRNAs that might regulate the expression of *ENG*. Candidate microRNAs were tested if they had an impact on trophoblast function.
Main outcome measures: We compared endoglin expression between normotensive and preeclamptic placentas by using immunohistochemistry and real-time PCR. Downregulated microRNAs in preeclamptic placenta were revealed from a literature review. A bioinformatics assay was performed to predict those that might target *ENG*. Real-time PCR, Western blotting and dual luciferase assay were used to verify the targeting. The effects of the microRNAs on trophoblasts were evaluated by transwell invasion assay.
Results: The endoglin level was significantly higher in preeclamptic placenta than in normotensive placenta. *ENG* was validated as the direct target of miR-149-5p and was inversely correlated with it. MiR-149-5p promoted the invasion of trophoblast cells, and this promotion was abrogated by the overexpression of *ENG*.
Conclusions: Our findings highlight the importance of miR-149-5p in the pathogenesis of preeclampsia and provide new insight into the development of the disease.

1. Introduction

Preeclampsia (PE) is a major pregnancy complication, affecting 2–10% of all pregnancies and causing maternal and perinatal morbidity and mortality [1,2]. It is defined as onset of hypertension and proteinuria after 20 weeks of gestation [3] and is thought to be an implantation disorder [4] that elicits inadequate utero-placental blood perfusion and ischemia [5].

Endoglin, a *trans*-membrane glycoprotein, is a coreceptor for transforming growth factor (TGF)-β 1 and TGF-β 3 [6]. It is now well established by several studies that placental endoglin expression is elevated in PE [7–11] and that levels of circulating endoglin are elevated in PE [12,13]. Such observations raise the possibility that inappropriate expression of endoglin could contribute to PE.

MicroRNAs (miRNAs) are naturally occurring small noncoding RNAs that regulate gene expression, mRNA cleavage and/or translational repression [15,16] at the posttranscriptional levels through targeting the 3′ untranslated region (3′UTR) of mRNA. Their abnormal levels have been detected in placentas obtained from compromised pregnancies, including PE [17–19]. Few studies have shed light on the relevance between irregularly expressed miRNAs and *ENG* expression in preeclamptic placentas.

In this study, we initiated a demonstration of elevated endoglin expression in preeclamptic placenta. Second, we performed a literature review to identify differentially expressed, preferably downregulated, miRNAs in the preeclamptic placenta relative to normal pregnancies, searching for miRNAs that could potentially regulate endoglin expression. We discovered that miR-149-5p could inhibit *ENG* expression in the immortal extravillous trophoblast line HTR-8/SVneo. Moreover, miR-149-5p expression was decreased in placental tissues from patients with PE. As a result, third, we investigated the mechanism of how miR-149-5p is involved in *ENG* expression and, thus, regulates PE development. In this way, our findings have highlighted the important role of miR-149-5p in the pathogenesis of PE.

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2. Materials and methods

2.1. Patients and sample collection

A total of 30 pregnant women who underwent caesarean section at the Department of Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, PR China, from January 2016 to December 2016 were recruited for this study. We have excluded those that had twin pregnancy, chronic hypertension, renal disease and acute or chronic hepatitis. Fifteen were diagnosed with PE according to the diagnostic criteria of the American College of Obstetricians and Gynecologists (ACOG) Task Force on Hypertension in Pregnancy [3]. On the basis of whether the onset time of the clinical signs was earlier than the 34th week, PE was further classified as early-onset PE (E-PE) or late-onset PE (L-PE). The other 15 healthy and pregnant women comprised the control group. The clinical characteristics of the enrolled pregnant women are summarized in Supplementary Table 1. Two pieces of placental tissues were collected and stored at −80 °C immediately after delivery from the center area of the maternal placental surface for RNA extraction, and the remaining placenta samples were fixed in 4% formalin and embedded in paraffin for immunohistochemical analysis. This study was approved by the Shanghai First Maternity and Infant Hospital Ethics Board. All participants provided written consent.

2.2. Literature review and miRNA predictions of target genes

We scrutinized all studies on PubMed until December 2016 reporting the differential expression of miRNAs in PE using the keywords “microrna” and “preeclampsia”, from which a systematic review by Harapan Harapan and Mohd Andalas [20] was of importance. The review found 30 relevant articles between 2007 and 2015 from the MEDLINE database, from which it was known that 60 miRNAs were down-regulated in preeclamptic placentas. We used miRwalk2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html) to identify miRNAs that potentially could target endoglin, selecting database based on both of TargetScan (http://www.targetscan.org) and miRBase (http://www.mirbase.org). Finally we intersect between predicted miRNAs and published 60 miRNAs that were down-regulated in preeclamptic placentas. The output shown in Supplementary Fig. 1 was collected from TargetScan.

2.3. Sequences and constructs

The microRNA mimics miR-1207-5p, miR-149-5p, miR-328-5p, miR-377-3p and miR-133a-5p, together with the scramble negative control were designed and purchased from Shanghai GenePharma China. The sequences of the microRNA mimics above are shown in Supplementary Table 2. To construct pcDNA4-ENG, the *ENG*-expressing plasmid, the coding sequence of *ENG* (419-2395nt Genbank accession no. NM_001114753) was amplified and inserted into the pcDNA4.0 vector (Invitrogen, Carlsbad, CA) at the BamHI and Hind III restriction sites. To construct a pMIR-REPORT Luciferase plasmid (Ambion, Austin, Texas, USA) for *ENG*, 3′ UTR segments of human *ENG* mRNAs (2396-3077nt Genbank accession no. NM_001114753) containing the putative miR-149-5p binding sequences were amplified and cloned into pMIR-REPORT Luciferase plasmids at the Sac I and Hind III sites. The construct was called BD-WT. Mutated pMIR-REPORT plasmids, which carry site mutations in the 3′ UTR segments of human *ENG* mRNA that are complementary to the seed sequence of miR-149-5p, was generated based on the BD-WT plasmid using the Gibson Assembly Cloning Kit (NEB, Ipswich, MA, USA). The construct was called BD-MUT. The primers for vector construction are shown in Supplementary Table 3. All the constructs were confirmed by DNA sequencing.

2.4. Cell culture and transient transfection

HTR-8/SVneo cells were obtained from ATCC and cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 50 μg ml^{-1} streptomycin and 50 IU ml^{-1} penicillin. For transient transfection experiments, the cells were seeded in 6-well plates at 2×10^5 cells/well in complete medium. Twenty-four hours after seeding, cells were transfected respectively with 40 nM scrambled negative control, miR-1207-5p mimics, miR-149-5p mimics, miR-328-5p mimics, miR-377-3p mimics, and miR-133a-5p mimics. Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s instructions. Six hours after transfection, the cells were recovered in complete medium. RNA and protein detection assays were performed 48 h after transfection.

2.5. RNA extraction and quantitative polymerase chain reaction

Total RNA was isolated from cultured cells and placenta tissues using RNAiso Plus (Takara Biotechnology Co. Ltd. Dalian, China). For miR-149-5p detection, reverse transcription and quantitative real-time PCR were performed using the Mir-X MiRNA First-Strand Synthesis and SYBR qRT-PCR Kit (Clontech Laboratories, Inc. Dalian, China) following the manufacturer's instructions. The entire sequence of miR-149-5p was used as the 5′ primer. The kit supplied the 3′ primer for qPCR. For *ENG*, reverse transcription was performed using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Biotechnology Co. Ltd. Dalian, China), and qRT-PCR was performed using SYBR Premix Ex TaqII (Takara Biotechnology Co. Ltd. Dalian, China). All of the RT-qPCR experiments were run in triplicate, and a mean value was used to determine the mRNA or miRNA levels. Water without RT products was used as the negative control. Relative quantification of the mRNA or miRNA levels was calculated by the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ method, where ΔCT indicated the subtraction of the cycle threshold (CT) value of *GAPDH* or *U6* as the reference genes from that of the mRNA or miRNA of interest, and ΔΔCT indicated the ΔCT of the control group subtracted from that of the treatment group. The primer sequences for homo sapiens endoglin (*ENG*), transcript variant 1, mRNA (GenBank Accession: NM_001114753) were designed by Primerbank and are shown in Supplementary Table 3.

2.6. Immunohistochemistry

Paraffin sections were mounted on glass slides, dewaxed in xylene and rehydrated in a descending ethanol gradient, after which they were heated in 10 mmol of sodium citrate solution for antigen retrieval. Endogenous peroxidase was quenched with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min. After blocking with 5% BSA for 30 min, the slides were incubated overnight with 1:800 diluted anti-human endoglin antibody (Santa Cruz Biotechnology). After washing with PBS, the sections were incubated for 30 min with horseradish peroxidase-conjugated secondary antibodies at room temperature and were visualized with DAB (Boster Wuhan China). The slides were counterstained with Meyer’s hematoxylin. To acquire a negative control, the primary antibody was replaced with normal rabbit IgG at an appropriate dilution. Positive staining was scored by two independent researchers blinded to patient information according to the staining intensity and the percentage of stained syncytiotrophoblast cells. Staining intensity was graded as follows: 0 for no staining; 1, mild staining; 2, moderate staining; and 3, intense staining. The percentage of stained syncytiotrophoblast cells was scored as follows: 0, no staining of cells, 1, 1–25%; 2, 26–50%; and 3, > 50%. The sum of intensity and extension was designated as the staining score and was graded as follows: 0–1, negative expression (−); 2, weak positive expression (+); 3–4, moderate positive expression (++); and 5–6 strong positive expression (+++). Reference images were captured under an optical microscope (Nikon, Tokyo, Japan).

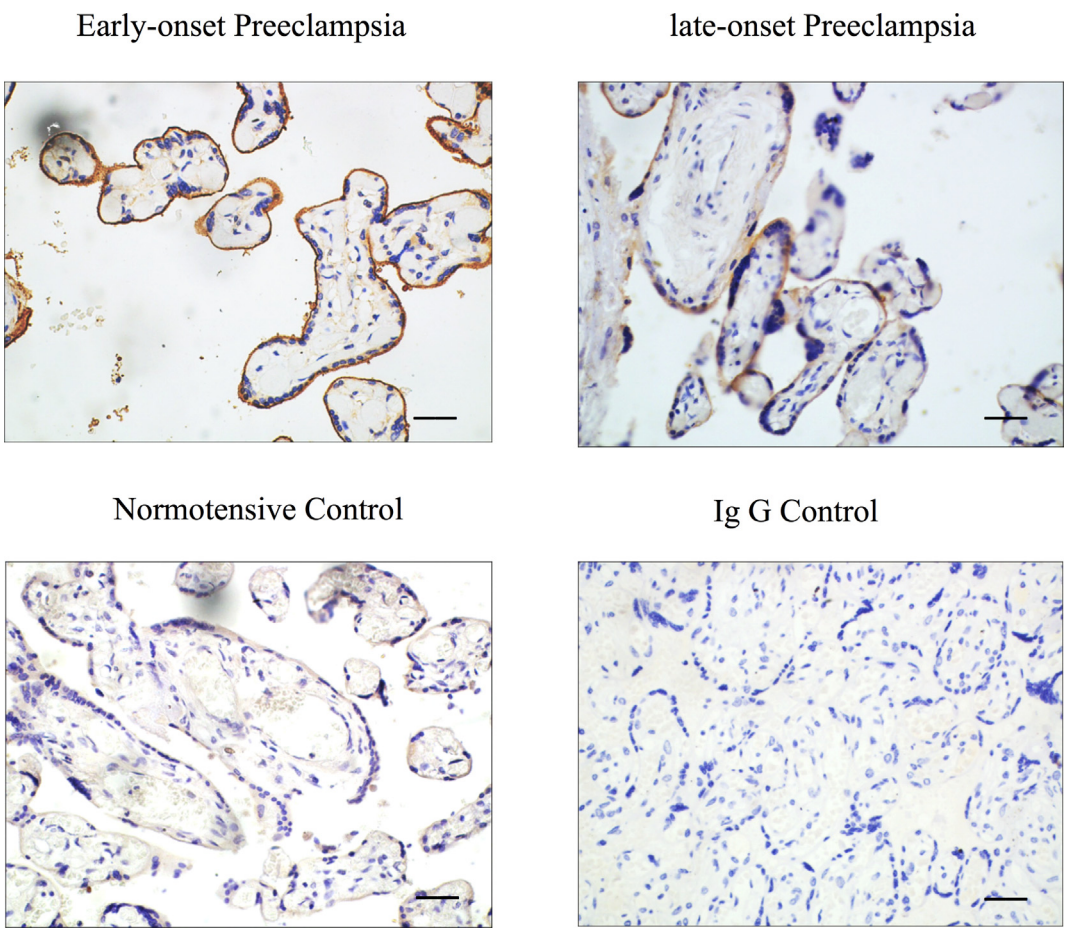

Fig. 1. Immunohistochemical staining of endoglin in preeclamptic and normotensive placentas. Expression of endoglin in early-onset preeclampsia, late-onset preeclampsia, normotensive control villous trophoblastic tissue and IgG control for endoglin. Magnification: ×400. Scale bars: 50 μm.

2.7. Western blot analysis

Protein samples were extracted from HTR-8/SVneo cells. A total of 40 μg of protein from each sample was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk solution, the membrane was incubated with rabbit anti-human endoglin (Santa Cruz Biotechnology) and mouse anti-human GAPDH in 5% milk in TBST at 4 °C. After overnight incubation, membranes were washed with TBST. Horseradish Peroxidase-Conjugated Secondary Antibody (Jackson, Mississippi, USA) was added to the membranes an incubated for 2 h at room temperature. After washing with TBST, the specific immunoreactive bands of proteins were photographed by using the Pierce ECL Western Blotting Substrate (Life Technologies) and the ChemiDoc MP System (Bio-Rad). The signal intensities from the bands were quantitated through ImageJ (National Institute of Health in USA). The relative densities of the detected molecules were measured by comparing their densitometry values with that of GAPDH in the same lane.

2.8. Luciferase reporter assay

HTR-8/SVneo cells were seeded into 24-well plates at the density of 5×10^4 cells/well 12 h before they were transfected with 80 ng of BD-WT or BD-MUT, 8 ng of pRL-TK vector encoding Renilla luciferase (Promega, WI, USA) and 40 nM miR-149-5p mimics according to the manufacturer's protocol. Luciferase activity was examined two days after transfection with a Dual-Luciferase Reporter Assay System (Promega, WI, USA). All transfections were carried out in triplicate.

2.9. Transwell invasion assay

HTR-8/SVneo cells were plated into 6-well plates and transfected with 40 nM of miR-149-5p mimics or scrambled negative control (NC), together with 2 μg of pENG or pcDNA4. Forty-eight hours later, transfected cells were trypsinized, and 1×10^5 cells were seeded in each transwell insert (8-μm pore size; Costar, Cambridge, MA) precoated with Matrigel (BD Biosciences) and were cultured with RPMI 1640 medium containing 1% FBS. Lower chambers were loaded with RPMI 1640 medium containing 10% FBS. The cells were cultured at 37 °C for 24 h to invade the Matrigel barrier. Cells that penetrated the membrane were fixed with cold methanol. The cell nuclei were stained with 0.5% crystal violet for 30 min and were subsequently washed thoroughly with tap water. Noninvaded cells on the upper surface of the membrane were removed using a cotton swab. In each insert, five microscopic fields were photographed under an optical microscope (Nikon, Tokyo, Japan). The cell invasion index was presented as the percentage of invaded cell number compared with the corresponding control. Each individual experiment was performed in triplicate.

2.10. Statistical analysis

Differences among several groups were determined by one-way analysis of variance, followed by the Student–Newman–Keul test. The Student *t* test or the Mann–Whitney *U* test (if normality test failed) was used for comparisons between two groups. For the immunochemical comparison of the PE group versus the normal group and the E-PE group versus the L-PE group, statistical analyses was performed using the Mann-Whitney *U* test. Spearman's correlation coefficient analysis

Table 1
Comparation of endoglin expression intensity in placenta among groups.

	n	Endoglin			
		−	+	++	+++
Normotensive	15	12	2	1	0
Preeclampsia[a]	15	0	2	5	8
Early –onset Preeclampsia[b]	10	0	0	2	8
Late-onset Preeclampsia	5	0	2	3	0

[a] $P < 0.01$compared with normotensive.
[b] $P < 0.01$compared with Late-onset Preeclampsia.

was performed to assess possible relationships between miR-149-5p and *ENG* mRNA expression levels in preeclamptic placentas. A P value $<$ 0.05 was considered to indicate statistical significance. SPSS Version 11 (SPSS Inc, Chicago, IL) was used for statistical analyses.

3. Results

3.1. Endoglin expression is increased in placentas of PE patients

Clinical characteristics in each group are shown in Supplementary Table 1. Among PE patients, 10 (67%) were diagnosed as E-PE and the other 5 (33%) as L-PE. Four small-for-gestation-age infants were delivered, which were all in the E-PE group. As expected, the immunohistochemical analysis (Fig. 1) showed that endoglin expression levels were significantly higher in placentas from PE patients than in those from normotensive pregnancies. In addition, we found increased expression of endoglin in the E-PE group compared with the L-PE group (Table 1).

3.2. MiR-149-5p suppresses ENG expression

Computational algorithms, including miRwalk2.0, TargetScan and miRBase, were used to locate the miRNAs that may regulate *ENG* expression. Among the 60 miRNAs that were downregulated in preeclamptic placentas (Supplementary Table 4), only miR-1207-5p [21], miR-149-5p [22], miR-328-5p [23], miR-377-3p [18] and miR-133a-5p [24] have the seed sequences that are complementary to the 3′UTR of *ENG* (Supplementary Fig. 1). As shown in Fig. 2A and B, only cells that were transfected with miR-149-5p mimics can suppress *ENG* expression. The levels of both *ENG* mRNA and protein in HTR-8/SVneo cells transfected with miR-149-5p mimics were, respectively, 53% and 48% lower than that in the scrambled negative control cells (NC).

3.3. ENG and miR-149-5p exhibit reverse expression patterns in placentas from PE patients

As shown in Fig. 3A and B, the relative expression of miR-149-5p in PE placentas was significantly down-regulated approximately 50%, while *ENG* mRNA expression was nearly 2.5-fold higher. Correlation analysis revealed an inverse correlation between miR-149-5p and *ENG* mRNA expression in PE patients' placentas (Fig. 3C).

3.4. ENG was validated to be the direct target of miR-149-5p in human trophoblast cells

According to bioinformatics analysis, the seed sequence of miR-149-5p was complementary to the 313–319 nt of the 3′ UTR in *ENG* mRNA. To obtain evidence as to whether miR-149-5p binds to the 3′-UTR of *ENG*, we generated wild type of luciferase reporter constructs BD-WT which contained the predicted binding sites for miR-149-5p in 3′-UTR of *ENG* and BD-MUT plasmids carrying the mutated seed sequence (Fig. 4A). MiR-149-5p mimics were transfected into HTR-8/SVneo cells together with one of the generated reporter plasmid, whereas pRL-TK control plasmid encoding a renilla luciferase was co-transfected as internal reference. Compared with scrambled control (negative control), miR-149-5p mimics could evidently reduce 49% of the relative luciferase activity of the BD-WT construct but could not affect the relative luciferase activity of the BD-MUT construct (Fig. 4B). The data strongly proved that *ENG* is the target gene of miR-149-5p in human trophoblast cells.

3.5. MiR-149-5p could regulate trophoblast cell invasion via targeting ENG

To demonstrate whether miR-149-5p can influence trophoblast cell behaviors via targeting *ENG*, we performed transwell invasion assay. MiR-149-5p mimics was transfected with or without pENG into HTR-8/SVneo cells. The transfection efficiency was determined by RT-PCR for miR-49-5p and *ENG* (Fig. 5A and B). As shown in Fig. 5C and D, the overexpression of miR-149-5p in HTR-8/SVneo cells significantly promoted cell invasiveness 1.7-fold. The overexpression of the *ENG*-expressing construct (pENG) alone appeared to have no effect on the invasive behavior of HTR-8/SVneo cells. However, it completely blocked the invasion-promoting effect caused by miR-149-5p. The data demonstrated that the involvement of miR-149-5p in trophoblast cell invasion was mediated, at least in part, by *ENG*.

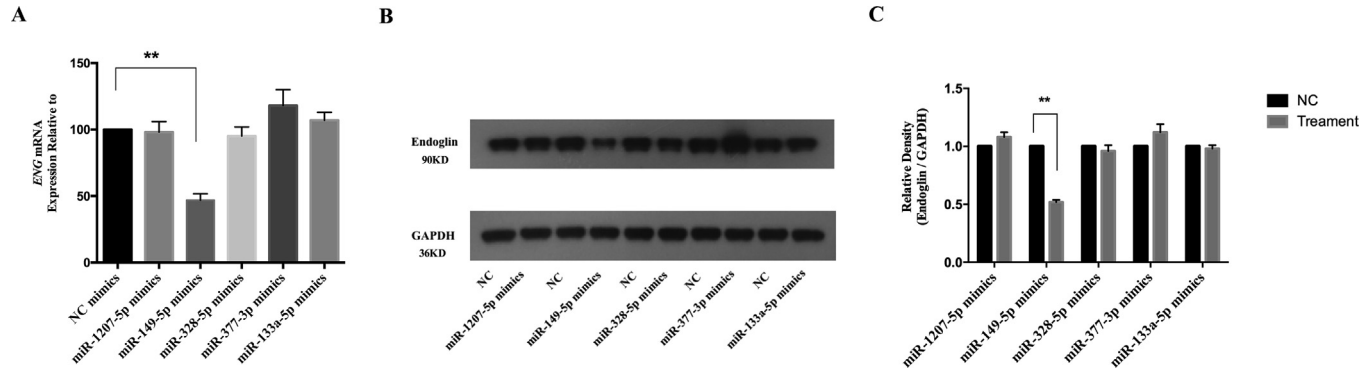

Fig. 2. *ENG* is directed inhibited by miR-149-5p. A. Real-time PCR to detect changes in *ENG* mRNA levels in HTR–8/SVneo cells transfected with scrambled control (NC), miR-1207-5p mimics, miR-149-5p mimics, miR-328-5p mimics, miR-377-3p mimics, or miR-133a-5p mimics. The data were calculated by the $2^{-\Delta\Delta CT}$ method and are presented as the means ± SEM according to three independent experiments. **Compared with NC, $P < 0.01$; B. A typical result of Western blotting, showing the change of endoglin levels in HTR-8/SVneo cells transfected with NC, miR-1207-5p mimics, miR-149-5p mimics, miR-328-5p mimics, miR-377-3p mimics, or miR-133a-5p mimics. C. Bar chart representing the statistical analysis by *t*-test according to three independent experiments. The density of endoglin was adjusted by that of GAPDH in the same blot, and the values are presented as the means ± SEM. **Compared with NC, $P < 0.01$.

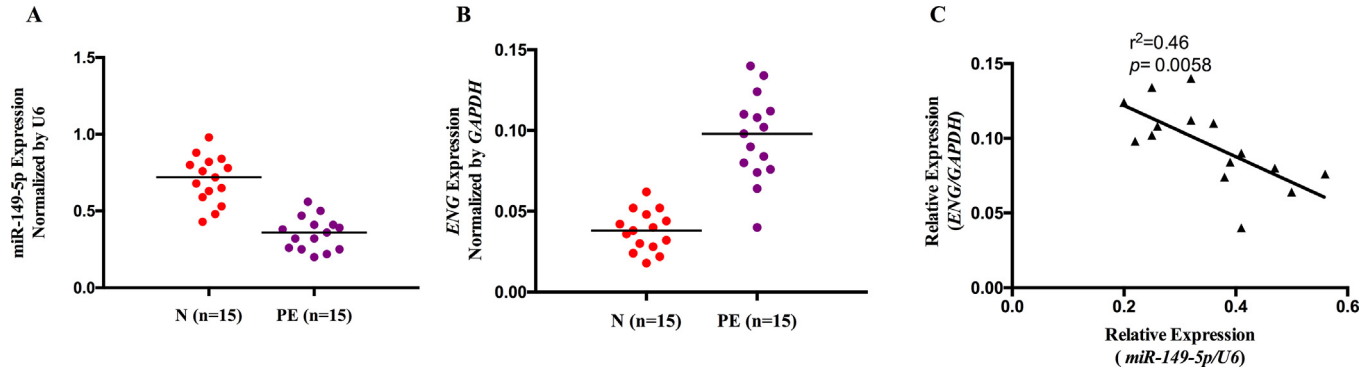

Fig. 3. Differential expression patterns of miR-149-5p and *ENG* in placentas derived from preeclamptic patients and normotensive pregnant women. Placentas of preeclamptic and normotensive pregnant women were collected after delivery (n = 15 each). MiR-149-5p (A) and endoglin (*ENG*) (B) expression levels in the placental chorionic villi were determined by quantitative real-time PCR using *U6* and *GAPDH* as the internal reference, respectively. The data were calculated by the $2^{-\Delta CT}$ method and are presented as medians with ranges. The comparisons were performed by using the Mann-Whitney *U* test. **, compared with normotensive pregnant women, $P < 0.01$. Spearman's correlation analysis was used to show the correlation between the miR-149-5p and *ENG* mRNA levels in the placentas of preeclamptic pregnant women (C). PE: preeclampsia; N: normotensive.

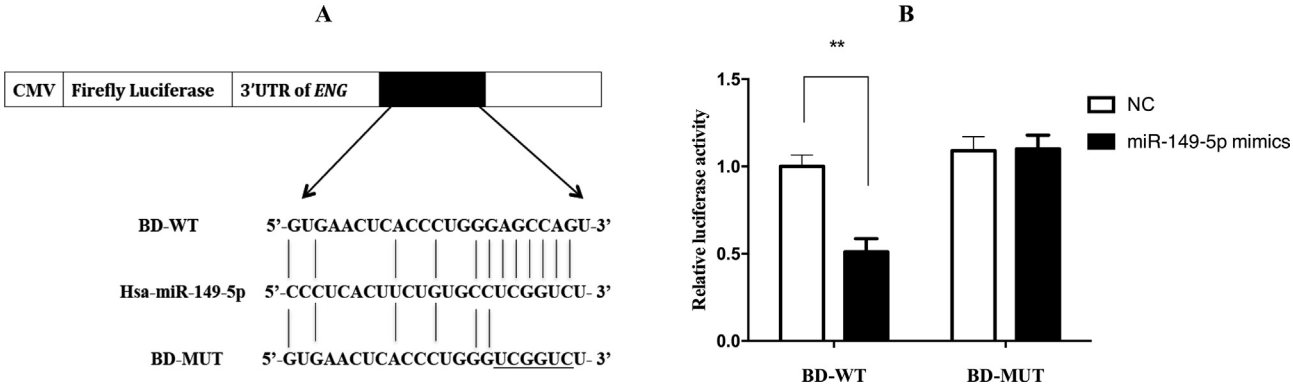

Fig. 4. Validation of *ENG* as a target gene of miR-149-5p in HTR-8/SVneo cells. A. The miR-149-5p target site within the 3′ UTR of *ENG* is shown as a black box. The constructs containing the region complementary to the seed sequence for miR-149-5p in the 3′ UTR segment of the human *ENG* gene are shown as BD-WT, and the mutant constructs are shown as BD-MUT with "__" indicating the mutation sites.B. HTR-8/SVneo cells were transfected with BD-WT or BD-MUT luciferase constructs together with miR-149-5p or NC. The value of the corresponding control group (NC) was set as 1.0, and the results are presented as the means ± SEM according to three independent experiments. **Compared with NC, $P < 0.01$.

4. Discussion

PE is a heterogeneous disorder of pregnancy whose etiology may stem from a variety of underlying mechanisms. However, a dysfunctional placenta has been acknowledged as a major common pathophysiological pathway for triggering the condition [25].

Endoglin and its soluble form, sENG, are angiogenesis-modulatory factors that are thought to have a role in the development of PE, and associations have been found at the gene, mRNA and protein levels. In the present study, we initially redemonstrated the significant increase of endoglin expression in the placentas of women with PE compared with normotensive pregnant women. In addition, we found a more enhanced expression of endoglin in E-PE patients than that of L-PE. It is now common to classify PE into E-PE and L-PE. It is believed that the two forms may have different modes of pathogenesis. Shallow invasion of trophoblast cells into the decidual stroma and spiral arteries elicits incomplete spiral artery remodeling during early gestation and is thought to be mainly associated with E-PE. Our finding is consistent with ideas from previous studies showing that inhibition of *ENG* translation in first-trimester human villous explants [26] or a human extravillous trophoblast cell line improves the invasive capacity of extravillous trophoblast [14], which is essential for uterine spiral artery remodeling in pregnancy [4]. We next aimed at exploring the regulation of this process.

MiRNAs, a class of 20- to 22-nt, endogenous and noncoding RNAs, are involved in a diverse range of cell events. A great number of differentially expressed miRNAs can be found in human placenta [18,23]. Some of them have been demonstrated to participate in the regulation of trophoblast cell invasion [28], placental immune activation [29], and platelet aggregation [30], among which there is miR-149-5p [22]. In this study, we confirmed the decreased expression of miR-149-5p in preeclamptic placentas from Chinese Han women. Previous functional studies on miR-149-5p have mainly focused on cancer, whereas little is known about its role in human placenta. We are interested in this small molecule for two reasons: first, miR-149-5p is one of the most abundant placental-specific miRNAs, and its concentration in the placenta is 10-fold higher than in maternal blood cells [31]; second, both Targetscan7.2 and Miranda have forecasted that *ENG* is one of the possible targets of miR-149-5p. In addition, our study has revealed that the expression of miR-149-5p was inversely associated with *ENG* mRNA levels in PE placentas.

Based on the evidence above, we evaluated whether *ENG* is functionally involved in trophoblast cell invasion regulated by miR-149-5p. Evidence has suggested that *ENG* is a target of miR-149-5p in human trophoblasts. First, *ENG* expression was suppressed by miR-149-5p in HTR-8/SVneo cells. Second, a luciferase reporter construct containing the putative miR-149-5p binding sequence in *ENG* mRNA was specifically responsive to miR-149-5p overexpression, as shown by inhibition of the luciferase activity, and the inhibitory effect of miR-149-5p was abolished following mutation of the binding sites paired by the seed

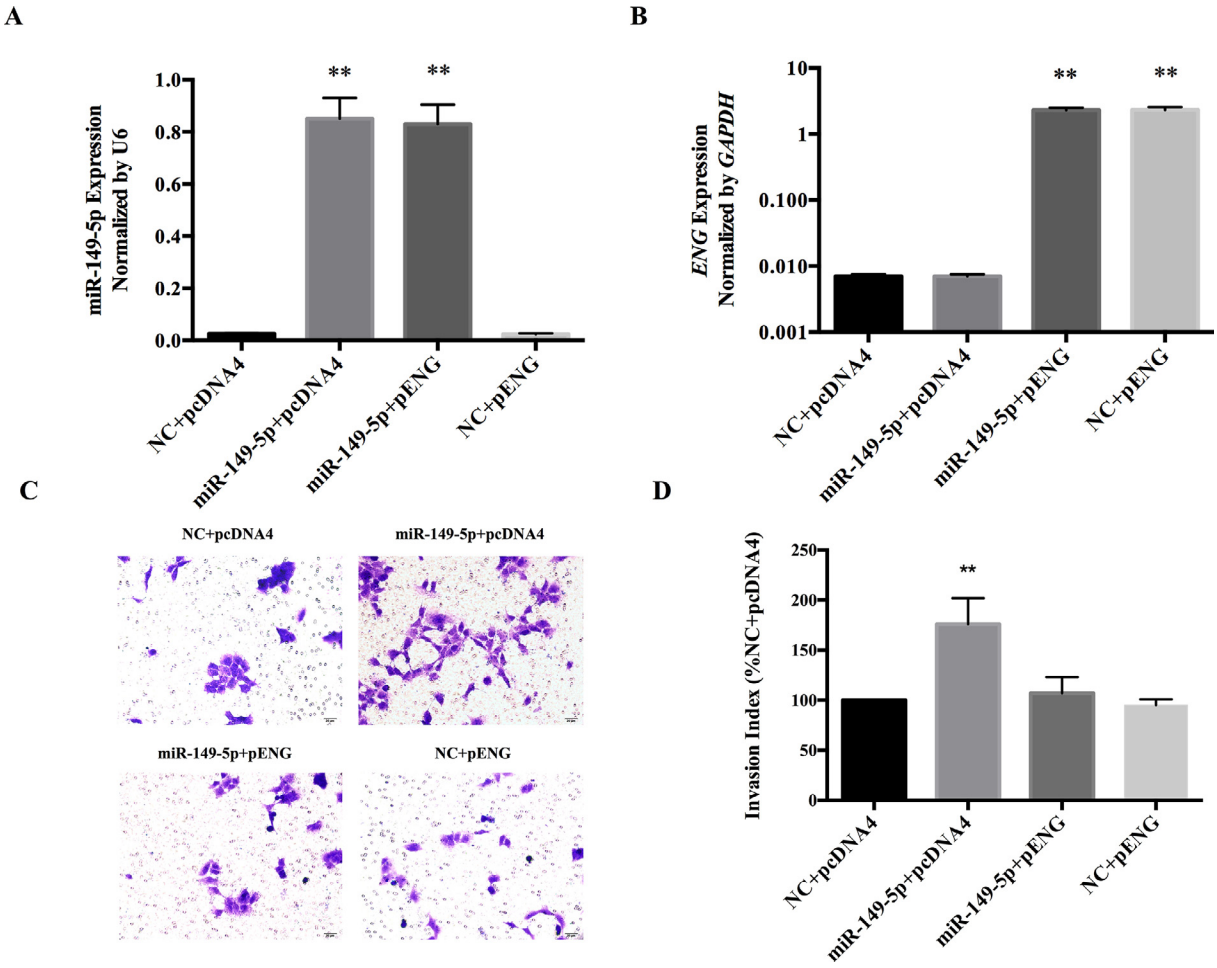

Fig. 5. *ENG* rescued the effect of miR-149-5p on cell invasion in HTR-8/SVneo cells. The expression levels of miR-149-5p (A) and *ENG* (B) in HTR-8/SVneo cells transfected with scrambled negative control (NC) and pcDNA4 vector (pcDNA4), miR-149-5p mimics and pcDNA4, miR-149-5p mimics and *ENG* expressing plasmids (pENG), NC and pENG were measured by real-time PCR. The levels of miR-149-5p and *ENG* were adjusted by those of *U6* and *GAPDH,* respectively. The data were calculated by the $2^{-\Delta CT}$ method and are presented as the means ± SEM according to three independent experiments. **, compared with the NC + pcDNA4 group, $P < 0.01$. C, a typical result of transwell insert assays to monitor cell invasiveness in HTR-8/SVneo cells transfected with NC and pcDNA4, miR-149-5p mimics and pcDNA4, miR-149-5p mimics and pENG, NC and pENG. D, the value of the invasion index in the corresponding NC + pcDNA4 group was set as 100, and the results are presented as the means ± SEM according to three independent experiments. **, compared with NC + pcDNA4 group, $P < 0.01$. Scale bars: 20 μm.

sequence of miR-149-5p. Third, a functional study of HTR-8/SVneo cells demonstrated an invasion-promoted effect of miR-149-5p, where the ectopic overexpression of *ENG* could substantially abrogate the effect of miR-149-5p on cell invasion. These findings indicated that *ENG* is one of the critical targets of miR-149-5p.

Endoglin is a coreceptor for TGF-β 1 and TGF-β 3. When TGF-β 1 and TGF-β 3 bind to TGF-β type I receptor, TGF-β type II receptor, betaglycan and endoglin, PAI-I and MMP-9 inhibitors are expressed to inhibit trophoblast invasion in the syncytium as well as in the extravillous trophoblasts that have invaded maternal tissue and anchored the placenta. MiR-149-5p promotes invasion of HTR-8/SVneo cells, most likely through interference of TGF-β signaling mediated by suppressing *ENG* expression. In PE placenta, reduced miR-149-5p expression may result in increased *ENG* expression, which can boost TGF-β signaling to inhibit trophoblast invasion.

Functional studies of miR-149-5p have been performed in cancer cells. For instance, miR-149-5p was found to suppress melanoma cell proliferation and colony formation and to promote cell apoptosis by targeting *LRIG2* [32]. In nasopharyngeal carcinoma, miR-149-5p inhibited cell invasion by targeting *IL-6* [33]. Human placental trophoblast cells share similar properties as cancer cells concerning active cell proliferation and invasion, although their behaviors are temporally and spatially restricted during pregnancy. However, miR-149-5p exhibits a strong invasion-promoting effect on HTR-8/SVneo cells in human trophoblast cell lines. This is absolutely opposite to the results observed in melanoma cells, nasopharyngeal carcinoma cells and renal carcinoma cells [34]. It is most likely that miR-149-5p works in a unique way in human trophoblast cells because one miRNA may regulate diverse targets in different cellular contexts or cell lines.

In this study, we revealed that miR-149-5p can inhibit *ENG* expression and was negatively correlated with *ENG* expression in placentas from PE patients. However, the r^2 was only 0.46, which suggested that there may be other mechanisms involved in the upregulation of endoglin in PE. Previous studies discovered that TGFβ-3, the LXR pathway and genetic variations in the *ENG* gene might be involved in the upregulation of endoglin in PE. Caniggia et al. [35] demonstrated that placental endoglin expression is upregulated by hypoxia via TGFβ-3 in intrauterine growth-restricted placenta. Reduced placental perfusion leading to placental hypoxia and TGFβ-3 overexpression is also true in PE, which might also contribute to the increased expression of endoglin in PE placenta. It has been reported that activation of liver X receptor (LXR) by oxysterols in JAR cells can upregulate both endoglin [36] and sEng [37]. In PE, oxysterol production is enhanced by hypoxia, and one can hypothesize that an increased internalization of oxysterol in trophoblasts might activate LXR to increase endoglin and sEng levels in PE. However, another group found

that oxysterols upregulated sEng production from human placental explants, but the increase was modest, suggesting this may not be the main mechanism for the very significant elevations in sEng seen in PE [38]. Mandy J Bell et al. [27]. found that genetic variation in *ENG* (rs10121110) was significantly associated with the development of PE in American Caucasian women. Given rs10121110′s location, the author inferred that this tagged single-nucleotide polymorphism (tSNP) might potentially affect either the capacity for transcription factor access/binding to the promoter of *ENG* or the degree of TGFβ 1 transmission, which might explain the differences in *ENG* expression between women with/without PE. In a later study, they failed to replicate their previous SNP-specific findings in a Norwegian cohort and a Latina cohort [39]. Our study complemented the findings that overexpression of endoglin in PE is not only at the transcription level but at the post-transcriptional levels.

The mechanism of miR-149-5p downregulation in preeclamptic placenta remains unknown. It was reported that miR-149-5p was inhibited by IL-1β plus IFN-γ in human pancreatic β-cells [40]. IL-1β and IFN-γ, which were also reported to be upregulated in PE [41], might be involved in the reduced expression of miR-149-5p in preeclamptic placenta. The rs2292832 C/T polymorphism is a pre-miRNA SNP that is located in the precursor of *miR-149*. It was reported that papillary thyroid cancer patients carrying the CC genotype had lower miR-149-5p expression than those with the TC genotype [42]. Since rs2292832 is located outside the seed region in mature *miR-149*, the authors inferred that it may influence the maturation process of miRNA and alter its expression level. Whether this genetic polymorphism can account for the reduced miR-149-5p expression in preeclamptic placentas needs further investigation.

Several limitations need to be clarified. First, the miR-149-5p levels in this study were restricted in the 3rd trimester placentas and were not extended to the whole gestation period. If the miR-149-5p levels decreased before the onset of PE, it could be strong evidence confirming that miR-149-5p is involved in PE. More studies will be required to examine the change pattern of miR-149-5p levels in different gestation periods, especially in early pregnancy while the placenta is in formation and undergoing invasion. Second, the functional assay of miR-149-5p was performed only on the HTR-8/SVneo cells that do not completely mimic the primary cells, so that further studies of primary cells are required. Third, from a diagnostic standpoint, prospective studies are expected to measure levels of miR-149-5p in serum or plasma in order to determine if the miRNA can be a predictor of PE. Fourth, given that one miRNA may target various genes, in addition to *ENG*, miR-149-5p can have other targets that also participate in PE development; further studies will consider this scenario to better understand its mechanism.

In summary, our findings provide new insight into the pathogenesis of PE by highlighting the important role of miR-149-5p. In exploring the relationship between miR-149-5p and PE, our study indicates that an aberrant decrease of miR-149-5p expression may contribute to PE through enhancing the pathological role of *ENG* in trophoblast cells.

5. Contribution to authorship

Zhao Xiaobo: designed the study and performed the experiments.
He Qizhi: performed IHC.
Wu Zhiping: Collected samples.
Duan Tao: performed manuscript editing.

Conflict of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.01.002.

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