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Hypoxia-inducible microRNA-218 inhibits trophoblast invasion by targeting LASP1: Implications for preeclampsia development

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ABSTRACT

Preeclampsia (PE) is a major contributor to maternal morbidity and mortality. However, the molecular mechanisms underlying PE progression are not well characterized. Here, we investigated the role of miR-218 in PE development. The expression of miR-218 and its host genes SLIT2 and SLIT3 was up-regulated in preeclamptic placentae compared to normal placentae. miR-218 expression was induced by hypoxia and decreased after knockdown of HIF-1α in an extravillous trophoblast cell line (HTR-8/SVneo). Chromatin immunoprecipitation assays showed direct binding of HIF-1α to the promoters of SLIT2 and SLIT3. Bioinformatics analysis identified LASP1 as a direct target of miR-218. Overexpression of miR-218 repressed the expression of LASP1 at both the mRNA and protein level. Meanwhile, miR-218 repressed the activity of a luciferase reporter containing the 3′-untranslated region of the LASP1 gene. Furthermore, expression of LASP1 rescued the inhibitory effect of miR-218 on HTR-8/SVneo cell invasion. Together, these results indicated that miR-218 contributes to PE by targeting LASP1 to inhibit trophoblast invasion.

1. Introduction

Preeclampsia (PE), which is characterized by elevated blood pressure and proteinuria, develops after 20 weeks of gestation, affects 5–7% of pregnant women worldwide, and is a major contributor to maternal morbidity and mortality. Severe PE is associated with maternal morbidity and adverse neonatal and fetal outcomes, including perinatal death, preterm birth, infants that are small for gestational age, and intrauterine growth restriction (Steegers et al., 2010). Delivery of the placenta remains the only known treatment for PE, suggesting that the placenta plays a central role in the development of PE (Huppertz, 2008). Despite extensive research investigating the pathogenesis of PE, the underlying mechanisms of the disease remain unknown.

During early pregnancy, trophoblast cells invade the placental bed, leading to remodeling of the spiral arteries into maximally dilated low-resistance vascular channels. Placenta artery remodeling guarantees a high flow volume to the uteroplacental bed (Meekins et al., 1994). However, in patients with PE, trophoblast functions are impaired, and the spiral arteries maintain their endothelial lining and musculature, inducing narrow and reactive vessels. Defective artery remodeling thereafter induces placental disorders, including hypoxia (Soleymanlou et al., 2005) and an imbalance of angiogenesis (Maynard et al., 2003). These pathological changes result in endothelial dysfunction, leading to the clinical syndrome of PE (Chaiworapongsa et al., 2014; Huppertz, 2008; VanWijk et al., 2000). Gene expression deregulation contributes to the trophoblast dysfunction seen in PE (Sitras et al., 2009). However, the regulatory mechanisms controlling these gene expression changes remain unknown.

MicroRNAs (miRNAs) are 20–25 nucleotide RNAs that regulate gene expression at the post-transcriptional level by binding to target mRNAs for translational repression or mRNA cleavage (Bartel, 2004). miRNAs have been reported to participate in PE development by regulating a series of processes, including trophoblast cell invasion (Ospina-Prieto et al., 2016; Sun et al., 2015), proliferation (Lu et al., 2016), apoptosis (Ding et al., 2016), intercellular communication (Ospina-Prieto et al., 2016) and angiogenesis (Escudero et al., 2016). However, considering the complexity of the process, these identified PE-related miRNAs do not explain the mechanisms of PE development.

MicroRNA-218 (miR-218) is a vertebrate-specific intronic miRNA. The mature form of miR-218 is generated from two separate loci that

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are located on chromosomes 4p15.31 and 5q35.1 within the introns of SLIT2 and SLIT3 (Tatarano et al., 2011). Evidence suggests that miR-218 is frequently down-regulated and acts as a tumor suppressor in various cancers, such as bladder cancer, colorectal cancer, and breast cancer (Lu et al., 2015; Tatarano et al., 2011). We have reported that miR-218 targets adiponectin receptor 2 to regulate adiponectin signaling, indicating that miR-218 is a multifunctional miRNA (Du et al., 2015). However, whether miR-218 is involved in PE progression remains unclear. In this study, we evaluated miR-218 expression in preeclamptic placentae and studied the functions of miR-218 in trophoblast invasion. Moreover, we identified LIM and SH3 protein 1 (LASP1) as a new functional target of miR-218.

2. Materials and methods

2.1. Tissue samples

Placenta tissues were obtained from 12 women with normal pregnancies and 12 women with PE at delivery at the Shaoxing Women and Children's Hospital (Shaoxing, Zhejiang, China). The 12 PE patients included 1 with early-onset and 11 with late-onset PE. All experimental protocols were approved by the Ethics Committee of the Shaoxing Women and Children's Hospital. All studies were performed in accordance with the ethical guidelines of the Ethics Committee of the Shaoxing Women and Children's Hospital. Informed consent was obtained from all patients.

2.2. Isolation of human trophoblast cells

Human trophoblast cells were isolated as described previously (Kliman et al., 1986; Zhou et al., 2008). The placenta was digested with 0.25% trypsin (Life Technologies, Grand Island, NY, USA) and 0.02% DNase type I (Sigma, St Louis, MO, USA) at 37 °C for 5 min. The digested suspension was discarded, and the residual tissue was then digested for 30 min. The cell suspensions were pooled and centrifuged at 1000*g* for 20 min over a 20–65% Percoll gradient (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The cells sedimenting at densities between 1.048–1.062 g/ml were collected and subjected to RNA extraction and qPCR analysis.

2.3. Cell culture and treatment

The extravillous trophoblast cell line HTR-8/SVneo and the trophoblast-derived choriocarcinoma cell line JEG-3 were from ATCC and maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies). The cells were incubated at 37 °C with 5% CO_2. For the hypoxia treatment, cells were exposed to 0.5% O_2 in a hypoxic incubator (BioSpherix, Redfield, NY, USA) for the indicated time.

2.4. Transfection

For miR-218 expression, HTR-8/SVneo cells were transiently transfected with 10 pmol of chemically synthesized miR-218 (sense sequence, 5′-UUGUGCUUGAUCUAACCAUGU-3′) or negative control RNA (sense sequence, 5′-UUCUCCGAACGUGUCACGU-3′) (Genepharma, Shanghai, China) using Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions. The cells were treated for additional experiments 48 h after transfection.

For LASP1 knockdown, the HTR-8/SVneo cells were transiently transfected with 10 pmol of the chemically synthesized siRNA targeting LASP1 (sense sequence, 5′-CUUAUCCAGACAGUUCACC-3′) or negative control RNA (Genepharma) using Lipofectamine 2000. The cells were treated for further experiments 48 h after transfection.

2.5. Immunoblotting analysis

Proteins were quantified using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA) and applied to immunoblotting analysis as described previously (Gao et al., 2014; Lin et al., 2016). Briefly, 50 μg of total protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 3% bovine serum albumin in TBS-T buffer, probed with antibodies targeting SLIT2 (Abcam, Cambridge, MA, USA, rabbit polyclonal, ab155605), SLIT3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, goat polyclonal, Cat#: sc-16624), HIF-1α (Abcam, rabbit monoclonal, Cat#: ab51608), LASP1 (Abcam, rabbit monoclonal, Cat#: ab191022), or β-actin (ACTB) (Santa Cruz Biotechnology, mouse monoclonal, Cat#: sc-47778). The membrane was then incubated with horseradish-conjugated secondary antibodies, probed with the SuperSignal West Pico chemiluminescence substrate (Thermo Fisher Scientific, Waltham, MA, USA), and then exposed to an X-ray film.

2.6. Immunohistochemistry (IHC)

Embedded tissues were deparaffinized with xylene, rehydrated in ethanol, and boiled in 10 mM citrate buffer (pH 6.0) for 30 min for antigen retrieval. Tissues were treated with 3% H_2O_2 to block endogenous peroxidase. After blocking in goat serum for 30 min at room temperature, tissues were incubated with HIF-1α antibody at 4 °C overnight. The slides were then visualized with an Envision System (DAKO Corporation, Carpinteria, CA, USA) and counterstained with hematoxylin.

2.7. Immunostaining evaluation

HIF-1α immunostaining was graded according to both intensity and the percentage of nuclear-positive cells. HIF-1α staining intensity was scored as 0, 1, 2, or 3. The percentage of HIF-1α-positive cells was scored into four categories: 1 (0–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The level of HIF-1α staining was evaluated with an immunoreactive score, which was calculated by multiplying the staining intensity scores and the percentage of positive cells (Remmele and Stegner, 1987).

2.8. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using a ChIP assay kit (Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, HTR-8/SVneo cells were cross-linked with 1% formaldehyde for 10 min at 37 °C. After sonication to yield DNA fragments of 1000 base pairs, the lysates were cleared by centrifugation, diluted with ChIP dilution buffer, and precleared with salmon sperm DNA/protein A-agarose at 4 °C for 1 h. For each immunoprecipitation assay, the lysates were incubated with 5 μg of anti-HIF-1α (Abcam) antibody or control IgG (Santa Cruz Biotechnology) at 4 °C overnight. The immune complexes were then collected with the protein A-agarose slurry, eluted, and de-crosslinked at 65 °C. After RNase digestion and proteinase digestion, immunoprecipitated DNA was extracted. The purified DNA was amplified using real time quantitative PCR.

The sequences of the primers used for SLIT2 and SLIT3 promoter amplification are listed in Supplemental Table 1.

2.9. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from lung tissues using TRIzol reagent (Life Technologies) following the manufacturer's instructions. For the detection of miR-218, qPCR assays were performed using the TaqMan miRNA Assay (Life Technologies) following the manufacturer's instruc-

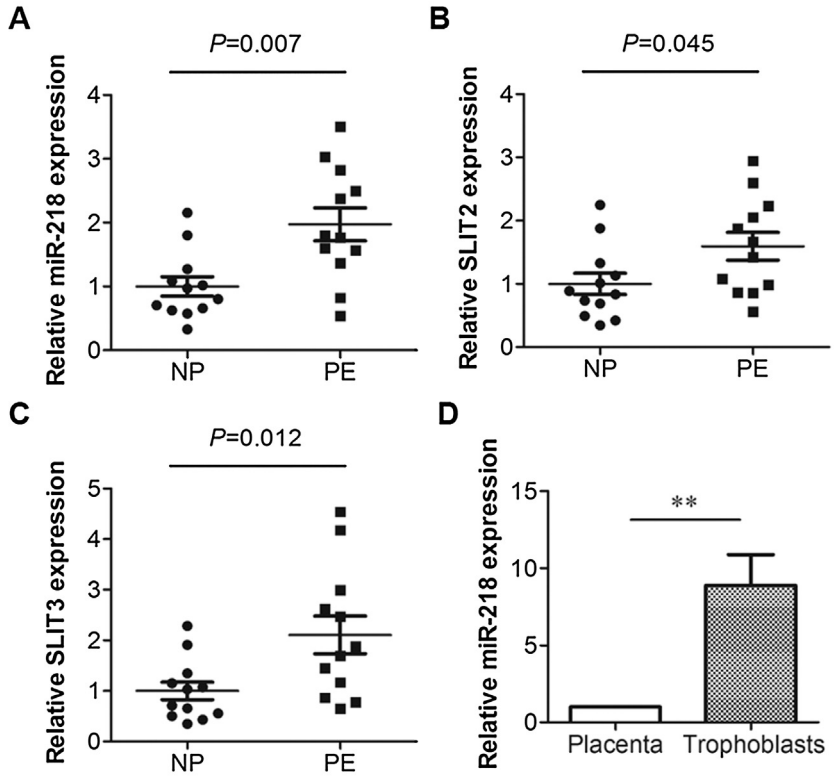

Fig. 1. The expressions of miR-218 and its host genes SLIT2 and SLIT3 are increased in preeclamptic placentae.(A) Scatter plot of miR-218 expression in placentae from normal pregnancies (NP, n = 12) or preeclamptic pregnancies (PE, n = 12). miR-218 expression was calculated as fold change with U6 snRNA as an endogenous control and normalized to the mean NP values. (B, C) Scatter plot of SLIT2 (B) and SLIT3 (C) expression in placentae from normal pregnancies (NP, n = 12) or preeclamptic pregnancies (PE, n = 12). SLIT2 or SLIT3 expression was calculated as fold change with ACTB as the endogenous control and normalized to the mean NP values. Statistical analysis was performed using Mann-Whitney *U* test. (D) miR-218 expression in the isolated trophoblast cells and the whole placenta from 3 PE patients were detected using qPCR (**$P < 0.01$, Student's *t*-test)

tions. The expression levels of miR-218 were normalized to the expression level of U6 small nuclear RNA (snRNA).

For detection of the *SLIT2*, *SLIT3*, and *LASP1* mRNA levels, 1 μg of total RNA was reverse transcribed using random hexamers and the High Capacity cDNA Reverse Transcription Kit (Life Technologies), followed by conventional quantitative PCR (qPCR) with SYBR Premix Ex Taq (TaKaRa, Dalian, China). The *SLIT2*, *SLIT3*, or *LASP1* mRNA level was normalized to the *ACTB* mRNA level. Data were analyzed using the $2^{-D\Delta Ct}$ method (Livak and Schmittgen, 2001). The sequences of all the primers used for PCR amplification are listed in Supplemental Table 1.

2.10. Cell invasion assay

Cell invasion was measured using Transwell chambers (Millipore Corporation, Billerica, MA, USA). Transwell inserts were coated with Matrigel (Life Technologies). The HTR-8/SVneo cells transfected with control RNA or miR-218 were seeded into the upper chambers in RPMI 1640 with FBS. The same medium was also placed in the lower wells. After 18 h, the cells migrating to the lower surface of the Transwell membrane were fixed and stained with 0.2% crystal violet solution. Images were captured with a wide-field microscope (Eclipse TS100; Nikon, Melville, NY, USA). Crystal violet was resolved in 10% acetic acid, and the absorption at 590 nm was measured. The absorption of the control group was normalized to 1.0.

2.11. CCK8-based viability assay

The cells were transfected with control RNA or miR-218. At 24 h post-transfection, the cells were seeded in 6-well plates at a density of 2×10^5 cells per well. Cell viability was assessed with a Cell Counting Kit-8 (CCK8) assay (Dojindo Laboratory, Japan). Briefly, 10 μl of CCK8 reagent was added to each well, and the cells were incubated for 2 h at 37 °C. The optical density (OD) at 450 nm was measured.

2.12. Plasmid construction

The 3′-untranslated region (3′-UTR) of the *LASP1* gene containing the predicted miR-218 target sites was amplified using PCR. The 3′-UTR fragments were cloned downstream of the firefly luciferase coding region into the XbaI site of the pGL3-control plasmid (Promega, Madison, WI, USA). To construct a seed sequence-mutated LASP1 3′-UTR, the 5′ fragment and 3′ fragment of the LASP1 3′-UTR were amplified using the primer sets LASP1UTR-F/LASP1-mut-R and LASP1-mut-F/LASP1UTR-R. These fragments were digested with BamH I, ligated together and inserted into the pGL3-control plasmid. The *LASP1* coding region was amplified from the cDNA library and inserted into a pcDNA3.1 vector (Life Technologies). The primers used for cloning are listed in Supplementary Table 1.

2.13. Luciferase assays

HTR-8/SVneo cells in 24-well plates were transfected with 100 ng of firefly luciferase reporter plasmid, 10 ng of pRL-TK plasmid (Promega) and 10 pmol of miR-218 mimic or control RNA. The cells were harvested 48 h post-transfection, and firefly luciferase activity was measured and normalized to Renilla luciferase signals.

2.14. Statistical analysis

The mRNA levels of *miR-218*, *SLIT2* and *SLIT3*, and HIF-1α IHC scores in the tissue samples were analyzed using the Mann-Whitney *U* test. Other data were expressed as the mean ± standard deviation and were evaluated with a double-sided Student's *t*-test. Statistical significance among more than 3 groups was determined using one-way

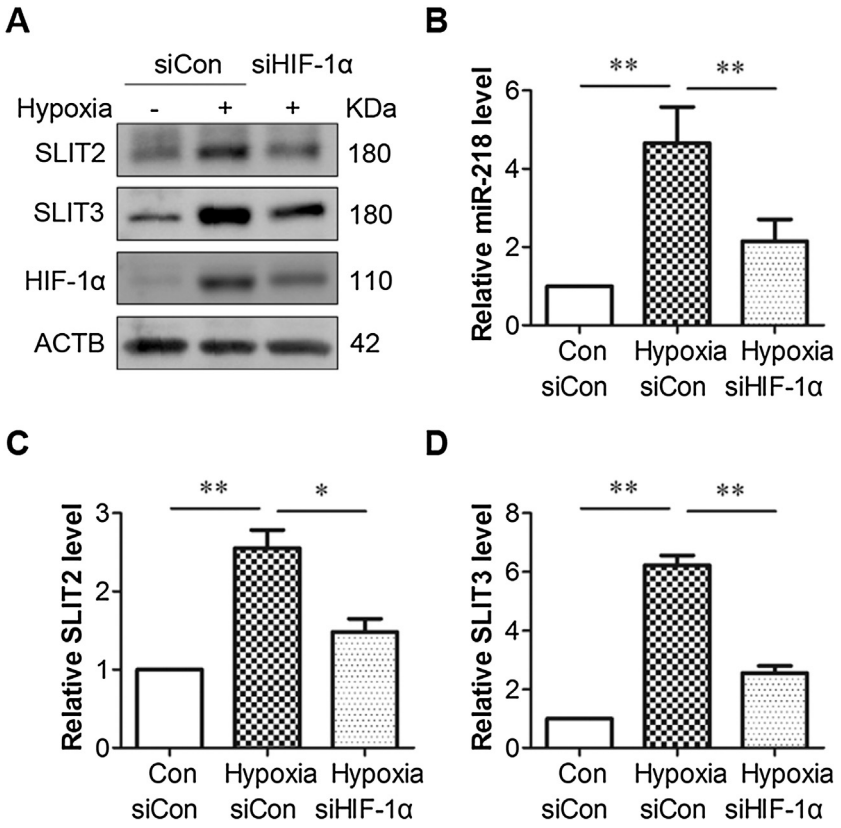

Fig. 2. Hypoxia induces miR-218 expression in HTR-8/SVneo cells through HIF-1α.HTR-8/SVneo cells were transfected with negative control RNA or siRNA targeting HIF-1α (siHIF-1α) and then exposed to normoxic condition or 0.5% oxygen (hypoxic condition) for 24 h. (A) Cell lysates were blotted with anti-HIF-1α, anti-SLIT2, anti-SLIT3, or anti-ACTB. (B, C, D) The expression level of miR-218 (B), SLIT2 (C), or SLIT3 (D) was detected using qPCR. miR-218 expression was calculated using U6 snRNA as the endogenous control and normalized to the control group. SLIT2 or SLIT3 expression was calculated using ACTB as the endogenous control and normalized to the control group. Statistical analysis was performed using one-way ANOVA. **, $P < 0.01$; *, $P < 0.05$.

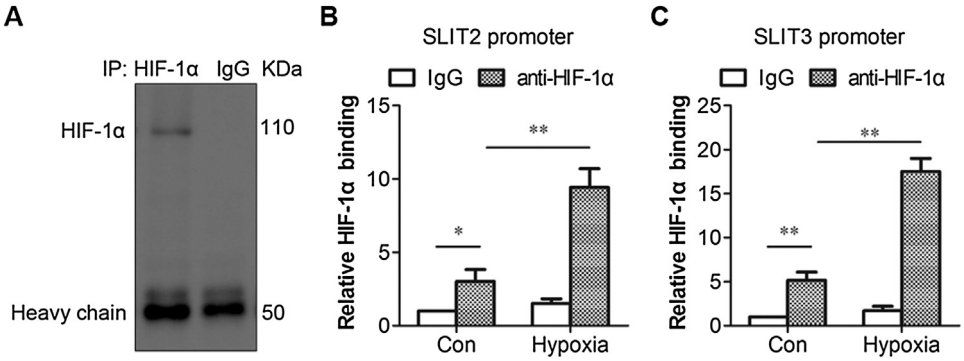

Fig. 3. HIF-1α binds to the promoters of SLIT2 and SLIT3.(A) Sonicated chromatin samples from HTR-8/SVneo cells exposed to hypoxia for 24 h were immunoprecipitated overnight with anti-HIF-1α antibody or normal IgG. The immunoprecipitates were blotted with anti-HIF-1α antibody. (B, C) HTR-8/SVneo cells were exposed to normoxic or hypoxic conditions for 24 h and then subjected to ChIP assays with anti-HIF-1α antibody or normal IgG. Enrichment of the SLIT2 promoter (B) and SLIT3 promoter (C) was detected with qPCR. Data were calculated using the ACTB promoter as the endogenous control and normalized to the IgG group exposed to normoxic conditions. Statistical analysis was performed using one-way ANOVA. **, $P < 0.01$; *, $P < 0.05$.

analysis of variance (ANOVA). Correlations between miR-218 expression and HIF-1α expression were analyzed with a Pearson analysis. $P < 0.05$ was used to indicate a statistically significant difference.

3. Results

3.1. miR-218 is up-regulated in preeclamptic placentae

miR-218 is generated from the introns of SLIT2 and SLIT3 (Tatarano et al., 2011), and SLIT2 and SLIT3 have been reported to be up-regulated in placentae from PE pregnancies (Liao et al., 2012). We hypothesized that miR-218 is also abnormally expressed in preeclamptic placentae. Therefore, we assessed the expression levels of miR-218 in placentae from 12 patients with PE and 12 women with normal pregnancies. The data showed that the miR-218 level was significantly higher in PE placentae than in normal pregnant controls (Fig. 1A). Consistent with the previous reports, SLIT2 and SLIT3 mRNA levels were elevated in PE placentae (Fig. 1B, C). These data suggested that miR-218 expression is associated with PE development. Furthermore, we isolated trophoblast cells from 3 PE placentae and detected miR-218 expression in these cells. The data showed that miR-218 expression was much higher in isolated trophoblast cells than in whole placenta (Fig. 1D), suggesting that miR-218 is primarily expressed in trophoblast cells and might play important roles in regulating trophoblast cell

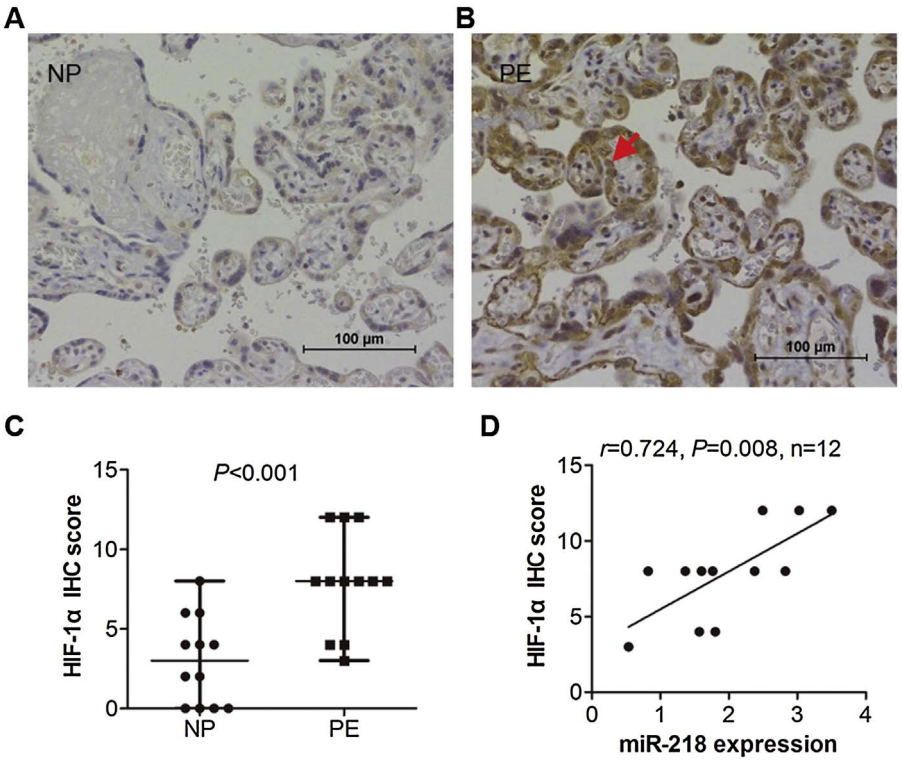

Fig. 4. miR-218 expression is positively correlated with HIF-1α expression in preeclamptic placentae.Normal placenta (A) and preeclamptic placenta (B) tissues were stained with HIF-1α antibody and visualized using Dako's Envision kit. The arrow indicates positive nuclear staining of HIF-1α. (C) HIF-1α staining in normal placentae and preeclamptic placentae were scored. Differences between the two group IHC score were analyzed with Mann-Whitney *U* test. (D) Correlation analyses of HIF-1α and miR-218 expression were performed using a Pearson analysis.

behavior.

3.2. Hypoxia induces miR-218 expression through HIF-1α

Insufficient uteroplacental oxygenation is believed to be responsible for the molecular events leading to the development of PE (Soleymanlou et al., 2005). To investigate whether hypoxia regulates miR-218, trophoblast cells (HTR-8/SVneo) were exposed to 0.5% O_2. HIF-1α, the marker for hypoxia, was induced by hypoxia (Fig. 2A). Compared with the normoxic control group, both the protein levels and the mRNA levels of SLIT2 and SLIT3 were significantly elevated during hypoxia (Fig. 2A, C, D). Remarkably, hypoxia significantly increased the expression level of miR-218 (Fig. 2B). To explore the association between increased expression of miR-218 and HIF-1α signaling, HIF-1α protein was silenced using siRNA (Fig. 2A). Knockdown of HIF-1α significantly decreased the expression of miR-218 and the host genes SLIT2 and SLIT3 under hypoxia (Fig. 2A–D). These data indicated that hypoxia up-regulates the expression of miR-218 and the host genes SLIT2 and SLIT3 through HIF-1α.

3.3. HIF-1α binds to the promoters of the miR-218 host genes SLIT2 and SLIT3

HIF-1α acts as a transcription factor (Jaakkola et al., 2001). To determine whether HIF-1α regulates miR-218 expression by binding to the promoters of its host genes SLIT2 and SLIT3, ChIP assay was performed. The eluted HIF-1α/DNA complexes were applied to immunoblotting analysis. HIF-1α was detected in the group immunoprecipitated by anti-HIF-1α antibodies but not in the IgG group (Fig. 3A). The purified DNA sequences from the ChIP experiments were then analyzed with qPCR. An enrichment of the sequence located on the SLIT2 and SLIT3 promoter regions was detected in the anti-HIF-1α group compared with the IgG group (3.02-fold and 5.15-fold, respectively, Fig. 3B, C), indicating a direct interaction between the transcription factor HIF-1α and SLIT2 and SLIT3 promoters. The binding of HIF-1α to the promoters of SLIT2 and SLIT3 significantly increased under hypoxic conditions (9.43-fold and 17.53-fold, respectively, Fig. 3B, C).

3.4. miR-218 expression is positively correlated with HIF-1α expression in preeclamptic placentae

Hypoxia contributes to PE development (Soleymanlou et al., 2005). To detect whether hypoxia exists in the 12 PE placentae, we performed immunohistochemistry using HIF-1α as a marker. The data showed that HIF-1α expression was low in the control placentae, while its expression was significantly increased in the PE placentae (Fig. 4A–C). The up-regulated HIF-1α was predominantly localized in the nucleus of trophoblast cells (Fig. 4B). Moreover, HIF-1α expression was positively correlated with miR-218 expression in PE patients ($r = 0.724$, $P = 0.008$, Fig. 4D). These observations in patient samples are consistent with the above finding that HIF-1α regulates miR-218 expression in trophoblast cells.

3.5. miR-218 suppresses trophoblast cell invasion

Because decreased trophoblast cell invasion is involved in PE development, we asked whether miR-218 regulates this process. Therefore, we detected the effects of miR-218 on the invasion of JEG-3 and HTR-8/SVneo cells using Matrigel assays. The data showed that JEG-3 or HTR-8/SVneo cells transfected with miR-218 displayed markedly decreased invasion ability compared with negative control cells (Fig. 5A–D). The effects of miR-218 on trophoblast cell invasion were likely not due to cell proliferation because overexpression of miR-218 slightly inhibited the growth of HTR-8/SVneo cells on day 3 after transfection, while the Matrigel assays were detected on day 1 after transfection (E).

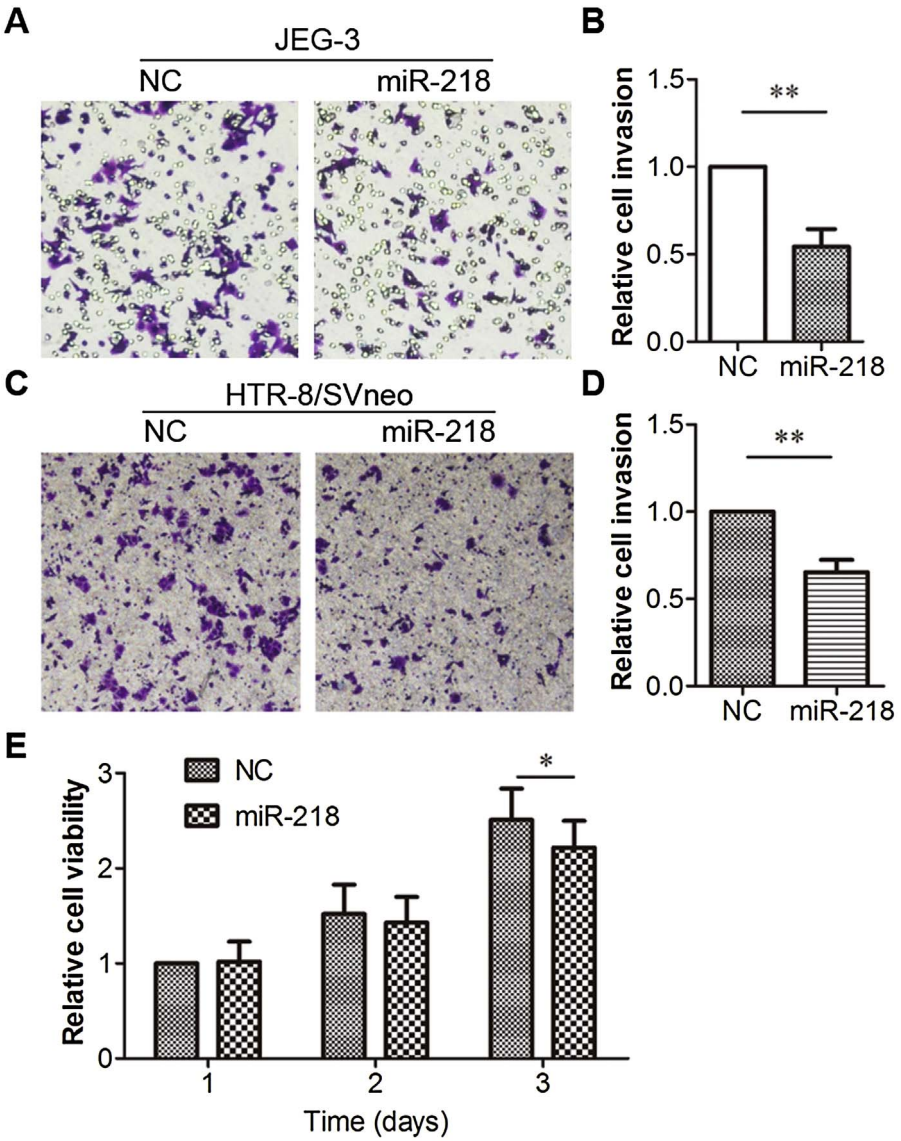

Fig. 5. miR-218 suppresses the invasion of trophoblast-derived cells.JEG-3 cells (A, B) or HTR-8/SVneo cells (C, D) were transfected with control RNA or miR-218. (A, C) Cells were subjected to Matrigel migration assays for 12 h. The migrated cells were stained with crystal violet. (B, D) The invaded cells were quantified. (E) HTR-8/SVneo cell proliferation rate was assessed using a CCK8 assay. Statistical analysis was performed using Student's *t*-test. **, $P < 0.01$; *, $P < 0.05$.

3.6. LASP1 is a direct target of miR-218 in trophoblast cells

To identify direct target genes of miR-218, we applied the bioinformatics algorithms TargetScan (Lewis et al., 2005) and miRanda (Wang and El Naqa, 2008). LASP1 drew our attention because it was reported to regulate HTR-8/SVneo cell invasion (Segerer et al., 2016). We therefore validated the targeting of LASP1 by miR-218. Overexpression of miR-218 reduced mRNA level and protein level of LASP1 in both JEG-3 and HTR-8/SVneo cells (Fig. 6A–D). To address whether LASP1 is a direct target of miR-218, the 3′-UTR of the LASP1 gene containing the miR-218 binding site was cloned into a luciferase reporter vector and subjected to a dual-luciferase assay. The data showed that miR-218 reduced the luciferase activity of the wild-type LASP1 3′-UTR reporter by 58% (Fig. 6F). TargetScan predicted that the 3197–3203 nt region of the LASP1 3′-UTR (NM_006148) is the seed sequence of miR-218 (Lewis et al., 2005) (Fig. 6E). Mutation of the seed sequence on the LASP1 3′-UTR abolished the inhibitory effect of miR-218 on luciferase activity (Fig. 6F). Overall, these data suggested that miR-218 regulates LASP1 expression by directly targeting its 3′-UTR.

3.7. Overexpression of LASP1 rescues the inhibitory effect of miR-218 on HTR-8/SVneo cell invasion

LASP1 was reported to regulate JEG-3 cell invasion (Segerer et al., 2016). Consistent with this report, our data showed that knockdown of LASP1 inhibited HTR-8/SVneo cell invasion (Fig. S1). To test whether LASP1 mediated the functions of miR-218 during HTR-8/SVneo cell invasion, we performed rescue experiments. The LASP1 gene lacking the 3′-UTR was co-expressed with miR-218 in HTR-8/SVneo cells (Fig. 7A), and cell invasion was then examined. The results showed that in the miR-218-expressing group, LASP1 rescued the impaired cell invasion almost to the basal level (Fig. 7B, C), suggesting that LASP1 indeed mediates the inhibitory effect of miR-218 on HTR-8/SVneo cell invasion.

4. Discussion

In the present study, we considered the regulation and the function of miR-218 in the development of PE. Our data showed that miR-218 expression was elevated in preeclamptic placentae and under hypoxic conditions. Expression of miR-218 inhibited the invasion of trophoblast

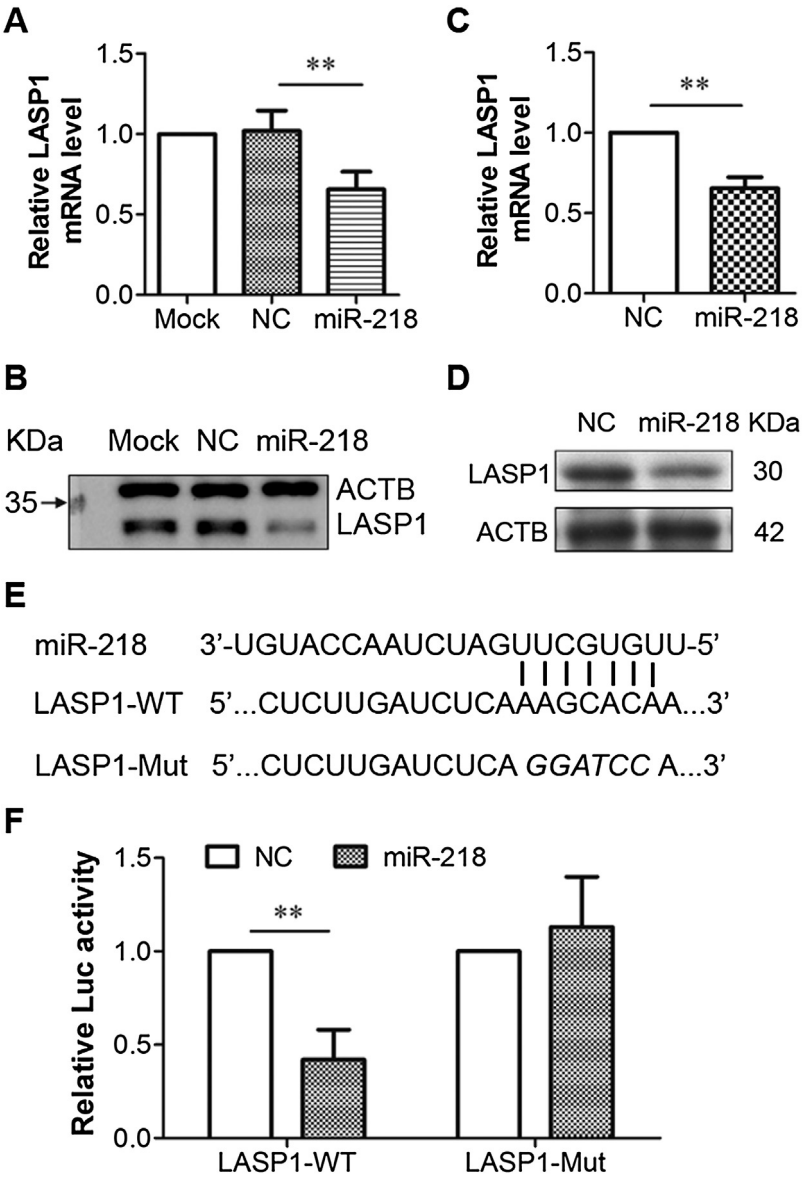

Fig. 6. miR-218 represses the expression of LASP1.JEG-3 cells (A, B) or HTR-8/SVneo cells (C, D) were transfected with control RNA or miR-218. (A, C) The mRNA level of LASP1 was detected using RT-qPCR, and (B, D) the cell lysates were blotted with anti-LASP1 or anti-ACTB antibodies. (E) Schematic illustration of miR-218, LASP1 3′-UTR and seed sequence-mutated 3′-UTR. (F) HTR-8/SVneo cells were transfected with wild-type or seed sequence-mutated LASP1-Luc plasmid together with miR-218. Luciferase activities were detected 24 h after transfection. Statistical analysis was performed using Student's *t*-test. **, $P < 0.01$.

cells *in vitro*. Since limited invasion of trophoblast cells is essential for PE development, our data suggested a promotive role of miR-218 in PE development (Fig. 7D).

Clinical studies have shown that the expression of miRNAs is deregulated in preeclamptic placentae. For example, miR-141 was reported to be up-regulated in PE placentae (Ospina-Prieto et al., 2016). Furthermore, several miRNAs were found to be aberrantly expressed in plasma from patients with PE and therefore may serve as potential serum biomarkers for PE. For example, miR-885-5p was reported to increase in plasma from patients with PE compared with the levels in plasma from healthy pregnant women and was released into circulation mainly inside exosomes (Sandrim et al., 2016). Our study showed that the miR-218 level was significantly higher in preeclamptic placentae than in normal placentae (Fig. 1A). However, Xu et al. identified down-regulation of miR-218 in severe PE placentae when compared with normal placenta controls (Xu et al., 2014). This inconsistency might be due to the features of PE in the patients that participated in the two studies, including the types and stages. Systematic analysis of miR-218 expression in a large-sized sample and in different cohorts will help elucidate the regulation of miR-218 during PE development.

Hypoxia is an important pathological factor during PE development. HIF-1 is a heterodimeric transcription factor composed of an oxygen-dependent α-subunit and a constitutively expressed β-subunit. Under normal conditions, HIF-1α is constantly expressed and rapidly degraded through a mechanism that involves ubiquitylation by the von Hippel-Lindau (pVHL) E3 ligase complex. The interaction between pVHL and the HIF-1α subunit is regulated by prolyl hydroxylase (Jaakkola et al., 2001). During hypoxia, prolyl hydroxylase activity decreases, and HIF-1α is stabilized. Then, HIF-1α dimerizes with HIF-1β, is translocated into the nucleus, and activates the transcription of target genes (Bruick and McKnight, 2001). Our data showed that hypoxia significantly increased the expression level of miR-218. Knockdown of HIF-1α significantly decreased the expression of miR-218, indicating that HIF-1α mediates miR-218 expression under hypoxic conditions (Fig. 2B). Since miR-218 lies within the introns of SLIT2 and SLIT3

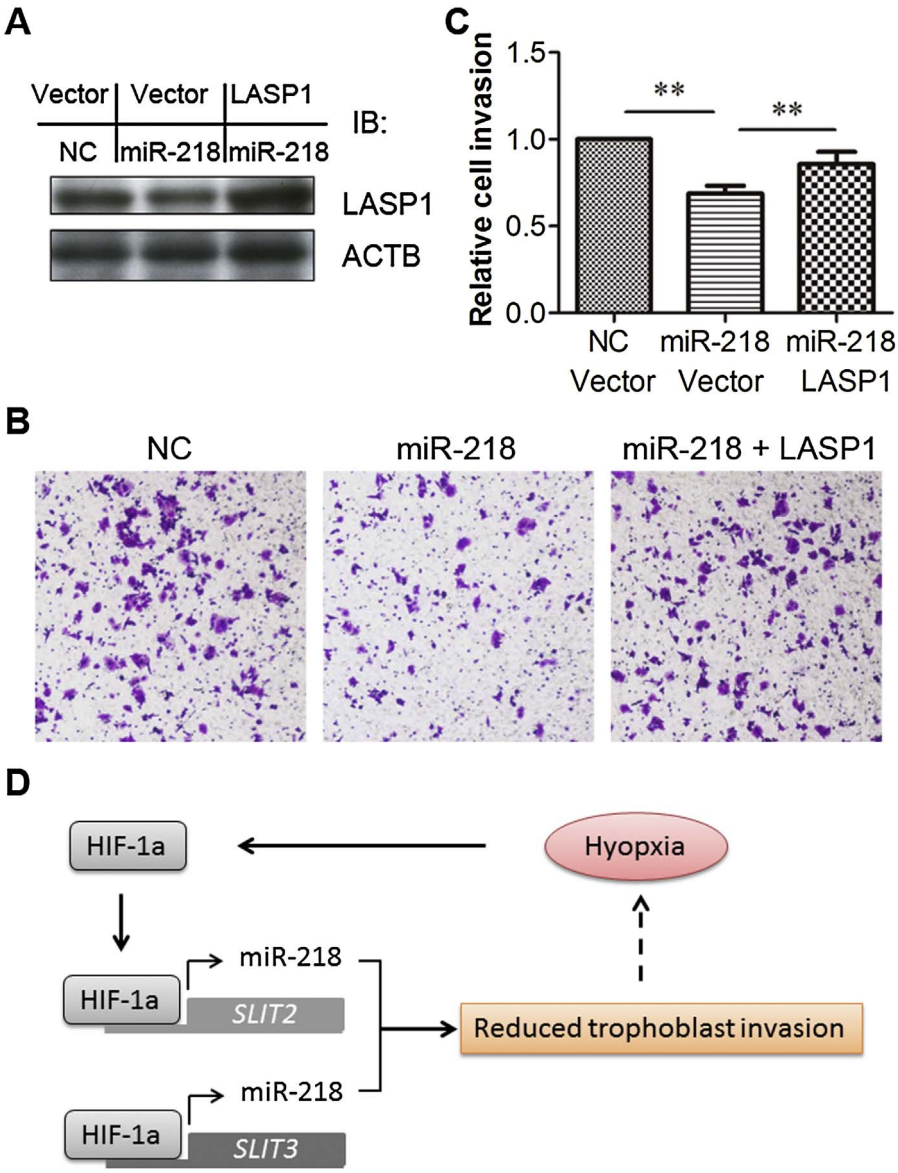

Fig. 7. Overexpression of LASP1 rescues the inhibitory effect of miR-218 on HTR-8/SVneo cell invasion.HTR-8/SVneo cells were transfected with control RNA or miR-218 together with plasmid expressing LASP1 or empty vector. (A) The cell lysates were blotted with anti-LASP1 or anti-ACTB antibodies. (B) Cells were subjected to Matrigel migration assays for 12 h. The migrated cells were stained with crystal violet. (C) The invaded cells were quantified (**$P < 0.01$, one-way ANOVA). (D) Schematic model for the regulation of miR-218 and its role in preeclampsia development. Defective placenta artery remodeling induces hypoxia, which activates the transcription of miR-218 through the transcription factor HIF-1α. miR-218 inhibits trophoblast invasion and thereby further reduces artery remodeling. This positive feedback loop contributes to the development of preeclampsia.

(Tatarano et al., 2011), it is not surprising that SLIT2 and SLIT3 are co-expressed with miR-218. ChIP assays showed that HIF-1α binds to the promoters of SLIT2 and SLIT3, supporting the notion that HIF-1α regulates the expression of SLIT2, SLIT3 and miR-218 at the transcriptional level. Moreover, we found that HIF-1α expression is positively correlated with miR-218 expression in preeclamptic placentae, further supporting the regulation of miR-218 expression by HIF-1α in preeclamptic placentae. SLIT2-Robo signaling has been reported to play important roles in vascular remodeling and placenta angiogenesis (Kliman et al., 1986; Zhou et al., 2008). Therefore, aberrantly expressed SLITs and miR-218 might work coordinately to contribute to PE development.

Trophoblast cells play important roles in placentation and in remodeling spiral arteries. miRNAs have been reported to regulate trophoblast behaviors, including cell invasion, proliferation, and apoptosis. For example, miR-34a, miR-210, and miR-141 inhibit human trophoblast cell invasion (Luo et al., 2016; Ospina-Prieto et al., 2016; Sun et al., 2015). In addition, microRNA-128a-induced trophoblast cell apoptosis has been reported to contribute to PE (Ding et al., 2016). Our data revealed that miR-218 targets LASP1 to suppresses trophoblast invasion, indicating that miR-218 contributes to PE. Moreover, miRNAs were reported to regulate angiogenesis in the placenta, another key factor in placental vascular development in pregnancy (Yan et al., 2013). These miRNAs and their target genes form a complex network to maintain placenta homeostasis. Deregulation of these miRNAs will lead to vascular dysfunction in the placenta and PE development.

A single miRNA can regulate hundreds of mRNAs. Consistent with this characteristic, a series of targets of miR-218 have been reported (Du et al., 2015; Lu et al., 2015; Nishikawa et al., 2014; Tatarano et al., 2011). Here, using RT-qPCR, immunoblotting, and luciferase reporter assays, we demonstrated that miR-218 inhibits LASP1 expression in trophoblast cells. More importantly, overexpression of LASP1 rescues the inhibitory effect of miR-218 on cell invasion, indicating that LASP1 is a functional target of miR-218 in trophoblast cells. Consistent with this finding, miR-218 was reported to inhibit prostate cancer cell migration and invasion via targeting of LASP1 (Nishikawa et al.,

2014). LASP1 is an actin-binding protein that can interact with various binding partners within the cytoskeleton and can transmit signals from the cytoplasm into the nucleus. LASP1 is ubiquitously expressed in normal tissues, including the placenta (Segerer et al., 2016). Both the previous report and our study have demonstrated the function of LASP1 in trophoblast invasion, suggesting an important role of LASP1 in placenta artery remodeling. Therefore, down-regulation of LASP1 expression by miR-218 might be a novel mechanism for PE progression.

In conclusion, our data revealed up-regulation of miR-218 in preeclamptic placentae and under hypoxic condition. Furthermore, *in vitro* studies showed that inhibition of LASP1 expression by miR-218 is critical for reduced invasion of trophoblast cells. This study provides novel insights into the mechanism of PE progression and suggests that targeting miR-218 might be a new therapeutic strategy against PE.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2017.04.005.

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