

## Original Article

# MicroRNA-148a affects functions of placental trophoblast cells in preeclampsia by regulating HLA-G

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**Abstract:** Preeclampsia (PE) is a leading cause of perinatal maternal-fetal mortality and morbidity, while the pathogenesis has not been completely understood and no fundamental therapeutics are available. Low expression of human leukocyte antigen (HLA)-G in placentas is believed to be associated with PE; however, the mechanism underlying aberrant HLA-G expression has not been fully elucidated. microRNAs are negative regulators of gene expression by binding to 3'-UTR of mRNA for either degradation or translation repression. This study was performed to determine the functions of miR-148a in trophoblast cells and to discover its underlying role in the pathogenesis of PE. We found that miR-148a was decreased in placentas from PE and miR-148a positively regulated HLA-G at post-transcription level. To further investigate the functions of miR-148a, we tested the proliferation, apoptosis, and invasion abilities of HTR-8 cells after transferring miR-148a. In the miR-148a mimics group, the apoptosis of HTR-8 was decreased while the invasion was increased. Besides, we found that DNMT1 could down-regulate HLA-G. Considering that DNMT1 is the target gene of miR-148a, we confirm that miR-148a regulates the expression of HLA-G by DNMT1 and miR-148a may be a novel potential biomarker and therapeutic target for PE.

**Keywords:** MiR-148a, preeclampsia, HTR-8, HLA-G

## Introduction

Preeclampsia (PE) is a common pregnancy-induced disorder characterized by hypertension and proteinuria. PE affects 2%-8% of all pregnancies worldwide, and is a leading cause of perinatal maternal-fetal mortality and morbidity [1]. Numerous theories on the pathogenesis of PE have been elucidated, such as genetic, epigenetic factors, nutritional and immunological factors are all believed to contribute to the mechanism of PE [2, 3], but there is no single factor that sufficiently explains the exact etiology and pathogenesis of PE. Many studies have reported that the disorder of the trophoblast, especially the decline in its invasion function leading to poor placentation in early pregnancy is exactly associated with PE [4, 5]. The expressions of HLA-G in the peripheral blood and placenta in women with preeclampsia are significantly lower than those in normal pregnant women, and it is generally accepted that the abnormal expression of HLA-G is associat-

ed with the pathogenesis of preeclampsia [6]. Our early research indicated that DNMT1 is up-regulated in PE and is accompanied by high methylation level of HLA-G [7].

MicroRNAs (miRNAs) are conserved, small non-coding RNAs of 21-24 nucleotides that function as negative regulators of gene expression by binding to 3'-UTR of mRNA for either degradation or translation repression [8]. A lot of studies on cancers showed that miRNAs play an important role in diverse biologic and pathologic processes like cell proliferation, differentiation and apoptosis [9]. miRNAs are abundantly in human placentas and the expressions of miRNAs in placentas of PE patients are significantly different compared with that in women of normal pregnancy [10]. Zhu et al used microarray analysis and real-time PCR demonstrated thirty-four differentially expressed mRNAs in preeclamptic placentas, compared with normal placentas [11]. Recent studies reversed that miRNAs can be accurately measured in body

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**Table 1.** Characteristics of the study population

	Con (15)	PE (23)	p-value
Maternal age (Y)	28.1 ± 0.9	30.6 ± 1.0	> 0.05
Gestational age (wk)	38.6 ± 0.2	33.1 ± 0.2	< 0.05
Neonatal weight (g)	3382 ± 136.4	2039 ± 386.1	> 0.05
BMI (kg/m <sup>2</sup> )	24.8 ± 1.8	25.2 ± 2.3	> 0.05
BP systolic (mmHg)	118 ± 2.7	164 ± 4.8	< 0.05
BP diastolic (mmHg)	78 ± 2.2	106 ± 3.2	< 0.05

All results are presented as mean ± SD. P values are obtained using the Mann-Whitney U test on Graph Pad version 5.0.

**Table 2.** Primer sequences (5'-3') used in this study

HLA-G	FW: CTGGTTGTCCTTGCAGCCTGTAG
	RV: CCTTTCAATCTGAGCTCTTCTTTTCT
GAPDH	FW: TTCGACAGTCAGCCGCATCTT
	RV: CCCAATACGACCAATCCGTT

FW, forward; RV, reverse.

fluids, including serum for their highly stable forms, and serum miRNAs could play as a new group of diagnostic, prognostic, and predictive biomarkers [12]. In other studies, miRNAs specifically expressed in human placentas were detected in maternal plasma [13], and the circulating miRNAs are also differently expressed from PE patients to normal pregnancies [14].

It has been reported previously that miR-148a plays as a tumor-suppressor factor in gastric cancer, ovarian cancer and colorectal cancer by inhibit the proliferation, invasion and metastasis of tumor cells and arrest the cell cycle at the S-G2/M phase [9, 15, 16]. However, the function of miR-148a in PE is poorly understood. To date no studies have addressed the effects of miR-148a on trophoblast cell functions. Hence, it is necessary to investigate the potential role of miR-148a in the physiopathologic mechanism of PE. The aim of present study was to compare the expression of miR-148a in placentas from PE patients with that in normal pregnancy placentas and to explore the function of miR-148a in HTR-8/SVneo cells. Our findings increase our understanding of the role of miR-148a in PE.

## Materials and methods

### Ethics approval and clinical sample collection

Placentas from 15 normal and 23 PE pregnancies were collected for this case-control study.

This study was approved by the local ethics committees of Obstetrics and Gynecology Hospital of Fudan University (Shanghai, China), and informed consents were obtained from all the patients. The inclusion of PE patients followed the Criteria of the American College of Obstetricians and Gynecologists. The normal pregnancies consisted of healthy women not having preeclampsia or any other complications, such as maternal history of hypertension and/or renal or cardiac disease, maternal infection, multiple pregnancies, premature rupture of membranes or fetal anomalies. The clinical characteristics of the recruited pregnancies are shown in **Table 1**. Soon after each selective cesarean section, several tissue samples from the maternal side of the placentas (0.5 cm × 0.5 cm) were obtained; we washed the tissues in sterile phosphate-buffered saline to remove the blood cells and transferred samples to storage at -80°C for later use.

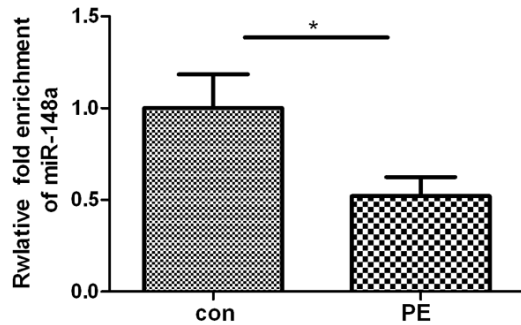
### Cell culture and transfection

HTR-8/SVneo (a gift from Professor Graham at the University of Toronto, Canada) was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum under standard culture conditions (37°C and 5% CO<sub>2</sub> incubator). Hiperfect transfection reagent (QIAGEN, Germany) was used to transfect the HTR-8 cells with miR-148a mimics, NC, miR-148a inhibit and inhibit NC (JIMA, Shanghai, China) according to the manufacturer's protocol. The efficiency of cell transfection was evaluated by performing real-time PCR analysis. Forty-eight hours or seventy-two hours after the transfection, the cells were collected for further experiments.

### Apoptosis assay

Forty-eight hours after the transfection, cells were washed with cold PBS twice, trypsinized using trypsin without EDTA, and then resuspended in 100 ml of 1 × binding buffer at a concentration of 1 × 10<sup>6</sup> cells/ml, and double-stained with PE and fluorescein isothiocyanate (FITC)-conjugated annexin V according to the manufacturer's instructions. Gently vortex the cells and incubate for 15 min at room temperature in the dark, added 400 µl of 1 × loading buffer to each tube. Samples were analyzed by

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**Figure 1.** The relative expression level of miR-148a in placental tissues from patient with preeclampsia (PE). As determined by qRT-PCR, miR-148a is lower in placental tissues from patients with PE compared to normal pregnancies (Control). Data are presented as mean  $\pm$  SD (\* $P < 0.05$ , Student's t test).

flow cytometry within 1 hour. The tests were repeated at least three times.

### Transwell assays

Twenty-four hours after transfection in trophoblast cells of each group, 200  $\mu$ l single-cell suspension at a concentration of  $25 \times 10^4$  cells/ml with a serum-free DMEM were added into each wells, 500  $\mu$ l of DMEM medium containing 20% FBS was added into the culture plates in the lower chamber. 48 or 72 hours after incubation in standard state, abandoned the upper chamber liquid, washed the chambers in clean water, fixed the cells in 4% paraformaldehyde for 10-20 minutes, added desired volume of crystal violet staining solution and incubated for 15 minutes, membrane-penetrating cell number was counted by 5 randomly selected fields under  $100 \times$  light microscope.

### Proliferation assay

The proliferation assay was performed using the Cell Counting kit-8 (DOJINDO), according to the manufacturer's instructions. Briefly, HTR-8 cells cultured in 96-wells plates with total 200  $\mu$ l in each group were transfected for 48 hours, and 10  $\mu$ l CCK8 reagent was added into each well. The cells were incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 1 hour, then A microplate reader (ABI company) was used to measure absorbance values of each well at 450 nm wavelength, the average value of 5 holes were calculated. Results are representative of more than three individual experiments.

### RNA isolation and real time reverse transcription PCR (qRT-PCR)

Total RNA from tissues and cultured cells were extracted using Trizol Reagent (Invitrogen), purified RNA with an optical density (OD) value between 1.8 and 2.0 was assumed to be of good quality. Five hundred nanograms of total RNA was then reverse transcribed. The reverse transcription of miR-148a was performed by QIAGEN RT Kit, quantitative real-time PCR (qRT-PCR) was conducted to detect the relative mRNA levels of genes using QIAGEN PCR Kit in a 7900HT Fast Real-time PCR System. U6 was used as an internal control. Revert Aid First Strand cDNA Synthesis Kit (THERMO, USA) was used to synthesize the first-strand complementary DNA of HLA-G, and SYBR Premix Ex Taq II (TAKARA) was used for qRT-PCR, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Relative expression levels were calculated using the comparative Ct method ( $2^{-\Delta\Delta\text{Ct}}$  method),  $\Delta\text{Ct} = \text{Ct}_{\text{gene}} - \text{Ct}_{\text{internal control}}$ ,  $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{experiment group}} - \Delta\text{Ct}_{\text{control group}}$ . The primers for qRT-PCR are shown in **Table 2**.

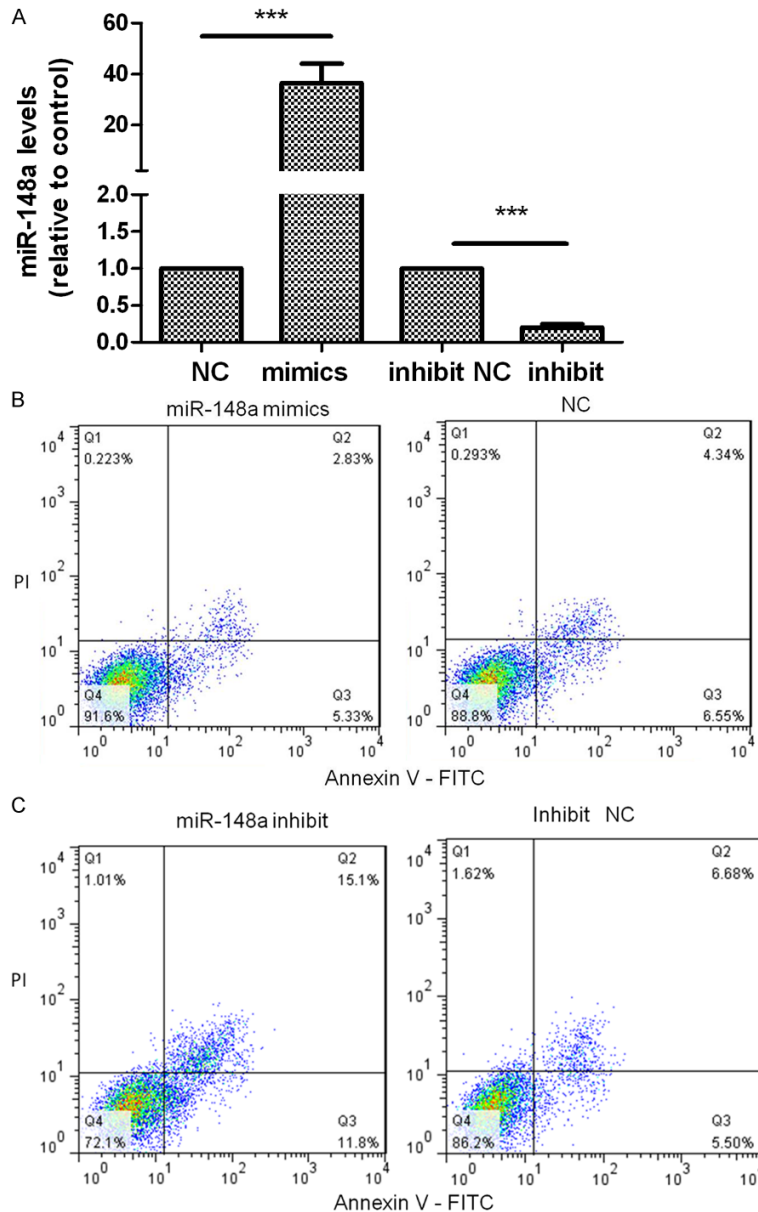
### Protein extraction and western blotting

After 48 hours of the transfection, cells were washed by cold PBS three times, and lysed with RIPA buffer on ice 30 minutes. The protein concentration was determined using the bicinchoninic acid (BCA) assay. Each sample containing equal amount of 30  $\mu$ g was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to PVDF membranes at 300 mA for 45 minutes. Then the PVDF membranes were blocked in 5% fat-free milk one hour at room temperature then incubated overnight at  $4^\circ\text{C}$  with the first antibodies (Abcam, 1:1000). The membranes were subsequently washed with TBST and incubated with secondary antibody for 1 h at room temperature. Tublin was used as a control to verify equal protein loading. By dividing gray values in each group compared to the gray value of control group, we then obtained the relative gray values of each gene, and thus the expressions of proteins were determined.

### Statistical analysis

The results were presented as mean  $\pm$  standard deviation (SD), values of  $P < 0.05$  were

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**Figure 2.** MiR-148a reduces the apoptosis of HTR-8/SVneo cells. The x-axis represents annexin V-FITC-positive cells, whereas the y-axis represents PI-positive cells. A: The fold change of miR-148a in HTR-8 cells transfected with miR-148a mimics, NC, miR-148a inhibit and inhibit NC. The miR-148a expression level increases significantly in the mimics group while decreases in the inhibit group. B, C: The apoptosis of HTR-8 cell transfected with miR-148a mimics, NC, miR-148a inhibit and inhibit NC for 48 hours. The apoptosis in miR-148a group is lower compared to the NC group and it is higher in the inhibit group compared to inhibit NC group.

considered statistically significant. Student's t-test and non-parametric Mann-Whitney U test were used for comparisons. Statistical analyses were performed using GraphPad Prism5.1 (GraphPad Software, Inc, USA). Image J was

used to analyze the electrophoretic band gray values of different groups.

### Results

*MiR-148a is down-regulated in placentas from PE placentas*

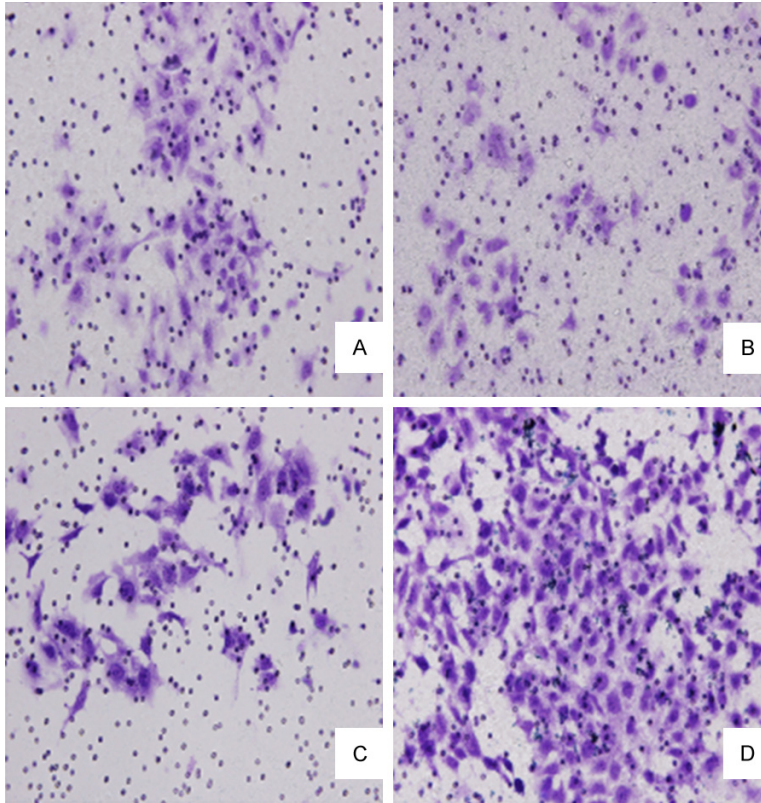
We examined the expression level of miR-148a in placental tissues from 23 PE patients by real-time qRT-PCR and compared the results with those from 15 normal placental tissues. The expression level of miR-148a was significantly lower (0.52 fold,  $P < 0.05$ ) in placentas from patients with preeclampsia compared to those in the control group (**Figure 1**).

*MiR-148a decreases the apoptosis of HTR-8*

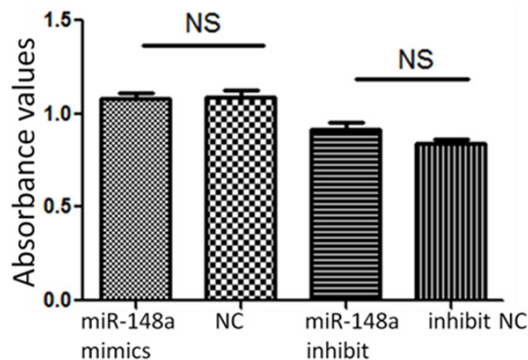
We transfected HTR-8 with the miR-148a mimics, NC, miR-148a inhibit and inhibit NC. Those cells transfected with the miR-148a mimics had significantly increased levels of miR-148a compared with the control group, while cells transfected with the miR-148a inhibit had significantly decreased levels of miR-148a compared with the control group ( $P < 0.05$ , **Figure 2A**). 48 hours after the translation, we labeled HTR-8 cells with annexin V-FITC and PE and analyzed them by flow cytometry, the percentage of annexin V-positive cell represented the percentage of the early apoptosis in the total cells. As depicted in **Figure 2B** the percentage of annexin

V-positive cells decreased from  $6.8 \pm 0.14\%$  to  $5.4 \pm 0.11\%$  after up-regulating miR-148a in HTR-8 cells, and increased from  $5.6 \pm 0.38\%$  to  $12.3 \pm 0.50\%$  after down-regulating miR-148a in HTR-8 cells ( $P < 0.05$ , **Figure 2C**).

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**Figure 3.** MiR-148a increases the invasion of HTR-8/SVneo cells. A, B: The membrane-penetrating cells after transfected with miR-148a mimics and NC for 48 hours. C, D: The membrane-penetrating cell after transfected with miR-148a inhibit and inhibit NC for 72 hours.



**Figure 4.** MiRNA-148a has no effect on the proliferation of HTR-8.

### *miRNA-148a increases the invasion of HTR-8*

The effect of miR-148a expression on trophoblast cell invasion is shown in **Figure 3**. The relative cell membrane permeable rate was higher in the miR-148a mimics group than that in the NC group after 48 hours of transfection, while the relative cell membrane permeable rate was significantly lower in the miR-148a

inhibit group compared to that in the inhibit NC group (all  $P < 0.05$ ) after 72 hours of transfection.

### *miRNA-148a has no effect on the proliferation of HTR-8*

The absorbance values are shown in **Figure 4**. The absorbance values have no differences between miR-148a mimics group ( $1.08 \pm 0.03$ ) and NC group ( $1.09 \pm 0.04$ ), either difference between miR-148a inhibit group ( $0.92 \pm 0.04$ ) and inhibit NC group ( $0.84 \pm 0.02$ ), indicating that miR-148a has no effect on the proliferation of HTR-8.

### *MiR-148a up-regulates HLA-G protein levels*

48 hours after the translation, we measured the mRNA and protein levels of HLA-G in HTR-8 cells. Whatever up regulate or down regulate miR-148a, the mRNA levels of HLA-G have no differences (not shown); while up-regulate

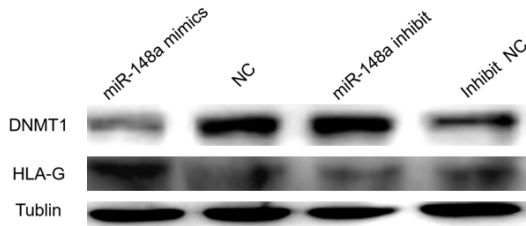
miR-148a can increase the protein level of HLA-G, and down-regulate miR-148a can reduce the protein level of HLA-G. These results strongly suggested that miR-148a up-regulate the expression of HLA-G on protein levels rather than RNA levels (**Figure 5**).

PicTar, TargetScan, miRanda have not been predicted any complementary binding regions between miR-148a and HLA-G. This suggests that miR-148a may not directly target HLA-G. While many previous studies have already confirmed that DNMT1 is the target gene of miR-148a [17] and in reverse miR-148a can be silenced by DNMT1 [18]. We find that miR-148a can decrease the protein expression of DNMT1 rather than mRNA levels (**Figure 5**). Therefore, our results show that low-expression of miR-148a in HTR-8 cells might down-regulate HLA-G expression through the modulation of DNMT1.

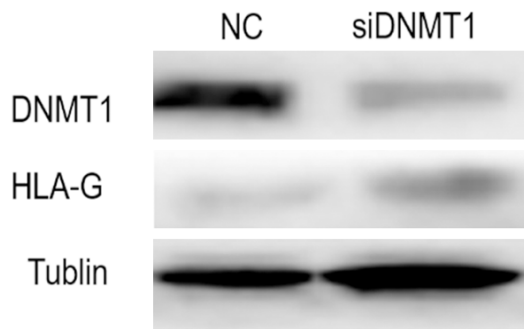
### *DNMT1 contributes to the low-expression of HLA-G*

To further study a DNMT-dependent mechanism of HLA-G alteration, we transfected HTR-8

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**Figure 5.** MiR-148a up-regulates HLA-G protein levels and down-regulates DNMT1 protein levels. HLA-G protein level increased while DNMT1 protein levels decreased after transfecting HTR-8 cells with miR-148a mimics compared to NC; HLA-G protein level decreased while DNMT1 protein levels increased after transfected with miR-148a inhibitor to inhibit NC.



**Figure 6.** DNMT1 down-regulates HLA-G protein levels. HLA-G protein level increased after transfecting HTR-8 cells with DNMT1 siRNA compared to NC.

cells with DNMT1 siRNA before Western blot analysis. An increase in HLA-G protein expression was observed in DNMT1 siRNA-treated cells (**Figure 6**). Altogether, our studies confirm that miR-148a can indirectly regulate HLA-G gene expression via modulation of DNMT1-dependent DNA methylation.

### Discussion

Recent evidence has indicated that epigenetic factors contribute to the mechanism of PE. Mean while, accumulating researches have illustrated the differential expression of microRNAs in PE patients compared to normal placentas [20]. For example, over-expressed miR-210 in placenta inhibited trophoblast invasion, in an extracellular signal-regulated kinase/mitogen-activated protein kinase-dependent manner [19]. In this study, we have shown that the relative expression of miR-148a was significantly lower in the preeclampsia groups. Down-expression of miR-148a in preeclampsia can

inhibit the invasion and induce the apoptosis of HTR-8 cells. Furthermore, miR-148a can positively regulate HLA-G expression by reduce the protein level of DNMT1.

In the present study, miR-148a was significantly lower in the preeclampsia groups, indicating that miR-148a was related to preeclampsia and might aggravate the development of preeclampsia. MiR-181a blocked activation of the TGF- $\beta$  signaling pathway in placentas from women with severe preeclampsia and inhibit proliferation and immunosuppressive properties of mesenchymal stem cells [20]. MiR-137 affects proliferation and migration of placenta trophoblast cells in preeclampsia by targeting ERRA [23]. MiR-148a expression was deregulated in gastric cancer, hepatocellular carcinoma, pancreatic cancer, and play as a tumor suppressor by inhibiting the proliferation, migration and invasion of cancer cells [21-23]. While miR-148a was over-expressed in osteosarcoma and promotes cancer cell growth by targeting PTEN [24]. The present study has determined that miR-148a can inhibit the apoptosis of HTR-8 but increase the invasion capacity of HTR-8 cells. In combination with previous reports of miR-148a in cancer biology, we speculate that miR-148a may play different roles in different types of cells, and the target genes and corresponding mechanisms are also likely to be different.

Another important result in our study revealed that miR-148a can up-regulate the protein expression of HLA-G by reducing the protein level of DNMT1. HLA-G is a non-classical HLA-Ib molecule characterized expressed in EVT, and played an important role in establishing a normal pregnancy for not only responsible for inducing maternal immune tolerance but also directly affect the functions of trophoblast cells, reduce the expression of HLA-G regulating prefoldin and MMP to regulate trophoblast invasion [4]. HLA-G is down expressed in EVT from pregnancy of PE with high hypermethylation of the HLA-G promoters [7]. Gao found the global DNA methylation level was significantly higher in the early-onset preeclamptic placentas compared with the normal controls [25]. DNMTs are the key enzymes that regulate DNA methylation and DNMT3a/3b act as 'de novo' methyltransferases while DNMT-1 acts as a 'maintenance' methyltransferase in mam-

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mals. DNMT1 are overexpressed consistent with high hypermethylated promoter region of HLA-G in placentas from PE pregnancies [7]. MiR-148a can deduce the expression of DNMT1 by binding to the 3'-UTR [17], meanwhile, many researches illuminated the existence of a negative feedback regulatory loop between miR-148a and DNMT1, DNMT1 is responsible for the hypermethylation of miR-148a gene promoters [17, 26, 27]. Thus we can infer that miR-148a can increase the expression of HLA-G by down-regulating DNMT.

It has been widely accepted that dysfunctions especially the decline in the invasion of trophoblast cells, leading to shallow implantation of placenta and uterine spiral artery recasting obstacles, trophoblast cells are ischemic and hypoxia is the fundamental pathological mechanism of PE [28], a lack of knowledge regarding the precise molecular mechanisms underlying PE progression limits the ability to treat advanced disease. Our results indicate that miR-148a can modulate the invasion and apoptosis of trophoblast cells through regulating the expression of HLA-G. Interestingly, a research on asthma showed that miR-152 family could directly up-regulating HLA-G expression with +3142G allele [29, 30], this was consistent with Zhu's research in JEG-3 cells [31], as the JEG-3 cell line has the +3142GG genotype, and therefore has a target to miR-148a. The discrepancy between the earlier works and our work probably arises from the differences in the cell lines researched.

Therefore, understanding the molecular mechanism involved in apoptosis, proliferation, and invasion of PE seems very important for the development of more effective diagnostic and therapeutic strategies.

There are several limitations to our study. First the study tissues were all in the late stages of pregnancy, while the onset of PE begins at 20<sup>th</sup>-week of the pregnancy; Second, we only test miR-148a expression in the placenta, but didn't test that in peripheral blood plasma, so whether miR-148a can be used as a biomarker is not sure yet. Third, since JEG-3 cell line and HTR-8 are the two most used cell line to research PE, and the regulate relationship is conversed, it is better to research the allele genotype of HLA-G in HTR-8.

In conclusion, we have characterized the expression of miR-148a in PE placentas, demonstrated the involvement of miR-148a mediated the expression of HLA-G in PE, and further illuminate miR-148a's impacts on the trophoblast cells' biological functions. This study provides new insights into the pathogenesis of PE, and miR-148a could be a novel prognostic marker or potential target for PE therapeutics.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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