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miR-4443/MMP2 suppresses the migration and invasion of trophoblasts through the HB-EGF/EGFR pathway in preeclampsia

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ABSTRACT

Preeclampsia (PE) is a pregnancy-associated disease that may cause maternal and fetal morbidity and mortality. The dysregulation of microRNAs (miRNAs) and their potential functions has been an important direction for elucidating the mechanism of preeclampsia in recent years. The present study investigated whether miR-4443 was significantly increased in the placentas of severe preeclamptic patients, and the upregulation of miR-4443 inhibited the migration and invasion of HTR-8/SVneo cells according to transwell assays. Matrix metalloproteinase 2 (MMP2), which is involved in the degradation of extracellular matrix (ECM) components and harbors a miR-4443-binding site within its 3'-UTR as confirmed by a luciferase reporter assay, was identified to be directly inhibited by miR-4443. Moreover, siRNA targeting MMP2 imitated the effects of over-expressed miR-4443 on HTR-8/SVneo cell invasion and migration, whereas rescue experiments showed that MMP2 reversed this inhibitory function of miR-4443. Heparin-binding EGF-like growth factor (HB-EGF), as the downstream gene of MMP2, plays an important role in trophoblast invasion, and we confirmed that the expression of HB-EGF/EGFR pathway-related biomolecules was consistent with MMP2 influenced by upregulating and downregulating miR-4443 and that activated EGFR further transmitted intracellular downstream signaling via the MAPK pathway according to western blot assay. In conclusion, we demonstrated that miR-4443 suppresses the migration and invasion of trophoblasts, and its inhibitory effects are at least partially mediated by the suppression of MMP2. This inhibition might further affect the progression of preeclampsia through the HB-EGF/EGFR pathway, thus providing a new clue on the role of miR-4443 in the pathogenesis of preeclampsia.

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Introduction

Preeclampsia (PE) is a pregnancy-specific disease that might cause maternal and fetal morbidity and mortality, which is present in 2–8% of pregnancies worldwide [1,2]. It is widely accepted that the pathogenesis of PE is associated with two stages: 1) abnormal placentation in the first trimester, 2) followed by a maternal syndrome in the later second and third trimesters characterized by an excess of anti-angiogenic factors [3,4]. The invasion of extravillous trophoblasts plays an important role in reshaping the uterine spiral artery and developing into a large vessel with high volume and low resistance to meet the increased blood supply demand of placental formation in early pregnancy [5]. In preeclampsia, cytotrophoblast invasion of the interstitial uterine

compartment is frequently shallow and incomplete. There are fewer endovascular cytotrophoblasts than in normal pregnancy, but some vessels retain portions of their endothelial lining with relatively intact muscular coats, which in turn frequently causes poor blood supply to the intervillous spaces [6]. In the studies reported thus far, the poor invasion of extravillous trophoblasts is an important reason for the pathogenesis and progression of preeclampsia, while the underlying mechanisms involved remain incompletely understood.

MicroRNAs (miRNAs) are short RNA molecules with 19 to 25 nucleotides that can regulate posttranscriptional silencing of target genes by targeting the three prime untranslated regions (3'-UTR) of mRNA, thereby regulating gene expression [7]. Some studies

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have shown that the dysregulation of miRNAs might be related to some obstetric complications during pregnancy, indicating a possible association between miRNAs and the placental pathology of preeclampsia [8–11]. In our previous studies, a significant increase in some miRNAs in human umbilical vein endothelial cells (HUVECs), including miR-4443, was found in patients with severe preeclampsia (sPE) [12]. Subsequently, the increase in placental tissue miR-4443 was also confirmed in patients with sPE, which suggested the importance of miR-4443 in the pathogenesis of preeclampsia. Some studies have shown that the dysregulation of miR-4443 affects the diagnosis and prognosis of patients with tumors of glial origin [13], colorectal cancer [14], esophageal squamous cell carcinoma [15] and Graves' disease [16]. However, the potential function of miR-4443 in placental development and its predictive value in the development of PE have not been investigated.

The present study found a change in miR-4443 expression in sPE placentas and the roles of miR-4443 in trophoblast invasion and migration for the first time. Combined with target prediction and RNA-Seq, we confirmed that matrix metalloproteinase 2 (MMP2) is a candidate miR-4443 target gene for further evaluation. Furthermore, we confirmed that MMP2 was a target gene directly inhibited by miR-4443 by a luciferase reporter assay. Finally, our studies showed that the expression of HB-EGF/EGFR pathway-related biomolecules was consistent with MMP2 influenced by upregulating and downregulating miR-4443 expression. Activated EGFR further transmitted intracellular downstream signaling via the mitogen activated protein kinases (MAPK) pathway, thus controlling the functions required for trophoblast cell migration and invasion. In conclusion, we first found that by targeting MMP2, miR-4443 might further affect the HB-EGF/EGFR pathway and the occurrence of preeclampsia.

Materials and methods

Sample collection

According to our previous sequencing results, the relative miR-4443 expression in HUVECs with severe preeclampsia patients and normal pregnant woman were 6.85 ± 0.48 and 6.05 ± 0.44 (means \pm standard deviation), and we set the target power and

alpha as 0.9 and 0.05 for collecting validation samples. The results showed that the group sample sizes of 8 and 8 can reach 0.9 power when using two-sided two-sample equal-variance t-test by PASS 2021. Considering the quality of the samples, matched placenta samples were collected with informed consent from women experiencing severe preeclamptic pregnancies (sPE group; $n = 10$) and normal pregnancies (normal group; $n = 10$). The diagnosis of sPE was based on the guidelines of the American College of Obstetricians and Gynecologists (ACOG): SBP \geq 160 mmHg or DBP \geq 100 mmHg on 2 occasions at least 4 h apart after 20 weeks of gestation with previously normal BP with proteinuria or other end-organ effects, including thrombocytopenia $< 100 \times 10^9/L$, renal insufficiency with serum Cr > 1.1 mg/dL or doubling from baseline, impaired liver function with transaminases greater than twice normal, pulmonary edema, and new onset of headache that is unresponsive to medications or visual symptoms [1,17]. Patients with cardiovascular diseases, diabetes, kidney diseases, congenital malformations, multiple pregnancy, pregnancies conceived by fertility treatment and chromosomal anomalies were all excluded. All pregnancies were treated by elective cesarean delivery. Within 0.5 h of cesarean birth, 4 tissue blocks (~ 0.5 cm³ each) were sampled randomly around the umbilical cord insertion site near the maternal surface of each placenta to achieve adequate and uniform sampling. Placental tissues were stored at -80°C until use. The research protocols were approved by the International Peace Maternity and Child Health Hospital Ethics Committee, School of Medicine, Shanghai Jiaotong University, Shanghai, China. The clinical data of the patients are presented in Table 1.

Cell culture and treatment

The human trophoblast cell lines JAR and JEG-3 were obtained from the Cell Bank of Chinese Academy of Sciences (China), and the human trophoblast cell line HTR-8/SVneo was a gift. All three cell lines were maintained in RPMI-1640 medium (Gibco, United States) containing 10% fetal bovine serum (FBS, Transgen, China), 100 $\mu\text{g}/\text{mL}$ streptomycin (Sangon Biotech, China) and 100 U/mL penicillin (Sangon Biotech, China) in a humidified incubator with 5%

Table 1. Clinical characteristics of samples for validation.

Characteristics	CON (n = 10)	sPE (n = 10)	P-value
Age, years	29.70 ± 3.40	30.80 ± 2.53	0.4226
BMI, kg/m ²	22.60 ± 2.11	21.44 ± 3.04	0.4843
Gestation, weeks	38.80 ± 0.45	37.23 ± 1.55	0.0064
Systolic blood pressure, mmHg	110.90 ± 6.92	153.10 ± 10.52	<0.0001
Diastolic blood pressure, mmHg	72.50 ± 7.31	95.30 ± 11.26	<0.0001
Proteinuria, g/24 h	-	0.90 ± 0.75	0.0013
Birth weight, g	3238.00 ± 331.05	2690.50 ± 658.36	0.0304

Values are expressed as the mean ± SD.

CON: control; sPE: severe preeclampsia.

CO₂ at 37°C. The culture medium was routinely changed, and the cells were passaged every 2 days.

Oligonucleotide, plasmid construction and transfection

Hsa-miR-4443 mimic and inhibitor and their corresponding negative controls were obtained from RiboBio (RiboBio, China). The siRNAs for MMP2 (siRNA-MMP2) and the corresponding negative controls (siRNA-NC) were purchased from GenePharma company (GenePharma, China). The MMP2-overexpressing plasmid (pEX-2-MMP2) and the corresponding empty vectors (pEX-2-EV) were also constructed by GenePharma. Cell transient transfections were performed by using Lipofectamine 3000 reagent (Invitrogen, United States) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from tissue samples or cells by TRIzol reagent (Invitrogen, United States), and a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, United States) was used to measure the concentration and purity of total RNA. Subsequent qRT-PCR was performed using a QuantStudio 7 Flex instrument (Thermo Fisher Scientific, United States). Reverse transcription of miR-4443 and MMP2 were performed using a miRNA First Strand cDNA Synthesis Kit (Accurate Biology, China) and TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen, China), respectively. The expression of miR-4443 and MMP2 were examined with SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China) and were normalized to small nuclear RNA U6 and 18S RNA

Table 2. Primers used in qRT-PCR analysis.

Genes	Primers	Sequence (5'-3')
hsa-miR-4443	Forward	GCTTGAGGGCTGGGTTTTA
	Reverse	#
U6	Forward	GGAACGATACAGAGAAGATTAGC
	Reverse	TGGAACGCTTCACGAATTTGCG
MMP2	Forward	TACAGGATCATTGGCTACACACC
	Reverse	GGTCACATCGCTCCAGACT
18S	Forward	GTAACCCGTTGAACCCCAT
	Reverse	CCATCCAATCGGTAGTAGCG

#The 3' primer of hsa-miR-4443 was provided by the miRNA First-Strand cDNA Synthesis Kit (Accurate Biology, China).

expression as respective internal reference. Primers for mRNA expression are shown in Table 2. The relative expression levels of miR-4443 and MMP2 were calculated by using the comparative Ct method.

Migration/invasion assay

For the migration assay, 5×10^4 cells were seeded with 200 μ L of RPMI-1640 medium without fetal bovine serum in the transwell upper chamber (8- μ m pore size membrane; Corning, United States), whereas the lower chamber was filled with 600 μ L of RPMI-1640 medium supplemented with 10% FBS. After 24 h of incubation with 5% CO₂ at 37°C, the nonmigrating cells in the upper chamber were removed with a cotton bud, and the cells migrating to the bottom side of the insert membrane were gently cleaned with phosphate buffered saline (PBS, Sangon Biotech, China). The inserts were then fixed in cold 4% paraformaldehyde (Sangon Biotech, China) for 30 min at room temperature and stained with 1% crystal violet (Sigma-Aldrich, United States) for 20 min. The number of migrated cells was quantified using three random fields per sample (at 100 \times original magnification).

The invasion assays were performed similarly to the migration assays, except the upper chamber

was precoated with Matrigel (BD Bioscience, United States) at 37°C for 3 h to allow gelling, and the density of seeded cells was 1.5×10^5 cells per 200 μ L of RPMI-1640 medium without fetal bovine serum. In addition, the incubation time was increased to 48 h with 5% CO₂ at 37°C. The number of invaded cells was also quantified using three random fields per sample (100 \times original magnification), and representative fields of invaded cells are shown.

Cell counting Kit-8 (CCK-8) assay

Cells were seeded in 96-well plates at a density of 3×10^3 cells per well (five replicate wells for each treatment). Then, CCK8 solution (Yeasen, China) was added according to the manufacturer's protocol, and the cells were cultured at 37°C for another 2 h in the incubator. The cell proliferation curves were plotted using the 450-nm absorbance at each time point (0, 24, 48, and 72 h). The absorbance of replicate wells per treatment was recorded.

Target prediction and RNA-Seq

The collected cells were processed for RNA isolation using TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, United States), and samples with an RNA integrity number (RIN) ≥ 7 were subjected to subsequent analysis. Libraries were constructed using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) according to the manufacturer's instructions, and then, an Illumina sequencer was used for sequencing. A *P* value < 0.05 and fold change > 2 or fold change < 0.5 were set as the cutoff criteria for significantly differential expression. Hierarchical cluster analysis of differentially expressed genes (DEGs) was performed to explore gene expression patterns. miR-4443 target genes were predicted by TargetScan 7.2 (<https://www.targetscan.org/>). RNA sequencing was subsequently carried out to confirm the change in the target gene of the cells transfected with the miR-4443 mimic or the corresponding negative control in collaboration with Oebiotech Company (Oebiotech, China).

Western blot assay

Radioimmunoprecipitation assay lysis buffer (RIPA, Invitrogen, United States) containing 1% protease inhibitor (Yeasen, China) and 1% phosphatase inhibitor (Yeasen, China) was used to lyse the protein of the placentas and the cells. The concentration of lysates was then quantified using a bicinchoninic acid (BCA) protein assay kit (Invitrogen, United States). Proteins were separated on a 10% SDS-PAGE gel by electrophoresis, and separated proteins were subsequently electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, United States). After 1 h of 5% bovine serum albumin (BSA, Sigma-Aldrich, United States) blocking at room temperature, the PVDF membranes were incubated with primary antibodies against MMP2 (anti-human MMP2, 1:1000, 10,373-2-AP, Proteintech), HB-EGF (anti-human HB-EGF, 1:10,000, ab185555, Abcam), EGFR (anti-human EGFR, 1:1000, bs-34,018 R, Bioss), p-EGFR (anti-human p-EGFR, 1:1000, ab40815, Abcam), MEK (anti-human MEK, 1:20,000, ab178876, Abcam), p-MEK (anti-human p-MEK, 1:1000, 9154S, CST), ERK (anti-human ERK, 1:1000, 4695 T, CST) or p-ERK (anti-human ERK, 1:2000, 4370 T, CST) at 4°C overnight. Glyceraldehyde 3-phosphate dehydrogenase (anti-human GAPDH, 1:3000, ab181602, Abcam) was used as a control. Then, the PVDF membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG-HRP secondary antibody (1:10,000, ab6721, Abcam) for 1 h at room temperature. The protein bands were detected by ECL Detection Reagent (Bio-Rad, United States) with an Amersham Imager 600 gel imaging system (Cytiva, United States), and the signal intensities were quantitated by ImageJ.

Luciferase reporter assay

The wild-type (WT) MMP2 3'-UTR as well as its corresponding mutant-type (MUT), wherein the putative binding site was mutated, were synthesized and inserted into the pmirGLO vector (GenePharma, China). The synthesized vectors were verified by DNA sequencing. HTR-8/SVneo cells (50–70% confluency in 12-well plates) were cotransfected with WT or MUT plasmid and hsa-miR-4443 mimics/inhibitors or the corresponding

negative control using Lipofectamine 3000. Forty-eight hours after transfection, firefly and Renilla luciferase activities were determined using the Dual Luciferase Assay (Promega, United States). The activity of Renilla luciferase was detected as the control for normalization.

Immunohistochemistry

Paraffin-embedded placental tissue specimens were dewaxed under xylene (Sinopharm, China), rehydrated with absolute alcohol (Sangon, China) and 95% and 75% alcohol and finally washed in PBS 3 times. The samples were then incubated with a primary antibody against HB-EGF (anti-human HB-EGF, 1:1000, ab185555, Abcam) at 4°C overnight and visualized using a mouse- and rabbit-specific HRP/DAB detection IHC Kit (ab64264, Abcam). After incubation with DAB for 20s, the sections were counterstained with hematoxylin (Servicebio, China) for 20s.

Statistical analysis

The data are expressed as the means \pm standard deviation (SD) based on at least three independent experiments unless specifically otherwise indicated. Statistically significant differences were estimated by Student's *t* test, and all statistical analyses were established by GraphPad Prism 8.3. $P < 0.05$ was considered to represent a significant difference.

Results

Clinical characteristics of the samples

As presented in Table 1, 20 women were recruited, consisting of 10 patients with severe preeclampsia and 10 healthy pregnant women. No significant difference was found in maternal age or BMI between healthy pregnancies and women with sPE. Compared with the control group, the systolic and diastolic blood pressures were both significantly higher in the sPE group. In addition, the pregnant women with sPE had significant proteinuria, while it was not detectable in normal pregnant women. Furthermore, maternal gestational age and fetal weight with sPE were also lower

than those in healthy pregnant women, which might be related to the preeclampsia.

miR-4443 was upregulated in placental tissues of severe preeclampsia

Our previous study demonstrated changes in 11 miRNAs in the HUVECs of sPE patients compared to those with normal pregnancies (Figure 1a). Notably, among these differentially expressed miRNAs, miR-4443 was the most upregulated. The relative expression levels of miR-4443 in the placental tissues of pregnant women were subsequently measured, and we confirmed that the expression level of miR-4443 in the sPE group was higher than that in the healthy control group (Figure 1b). The expressions of miR-4443 in three trophoblast cell lines, HTR-8/SVneo, JAR and JEG-3, were further tested, and the results revealed little difference (Figure 1c). We then observed the migration and invasion capacities of the above three trophoblast cell lines (Figure 1d, e). The results showed that JEG-3 had the highest migration and invasion capacities, and the cells migrating to the bottom side of the insert membrane were gathered too tightly, which affected the count. JAR had the weakest migration and invasion capacities and had a tendency to aggregate. Compared to JAR and JEG-3 cells, HTR-8/SVneo cells had moderate capacities for migration and invasion, which might be more suitable for subsequent cell experiments.

miR-4443 inhibited HTR-8/SVneo cell migration and invasion abilities in vitro

To further elucidate the potential functions of miR-4443 in trophoblast cells, we measured the migration and invasion abilities of the HTR-8/SVneo cell line via transfection of miR-4443 mimics or the corresponding negative control before using transwell assays. The expression of miR-4443 was confirmed by qRT-PCR after transfection with mimics or the inhibitor of miR-4443 and the corresponding negative control (Figure 2a, b). The significantly reduced number of cells passing through the upper chamber after transfection with miR-4443 mimics revealed that miR-4443 suppressed the migration and invasion of trophoblast cells (Figure 2c, e). Subsequently, inhibiting miR-4443 expression with the miR-4443

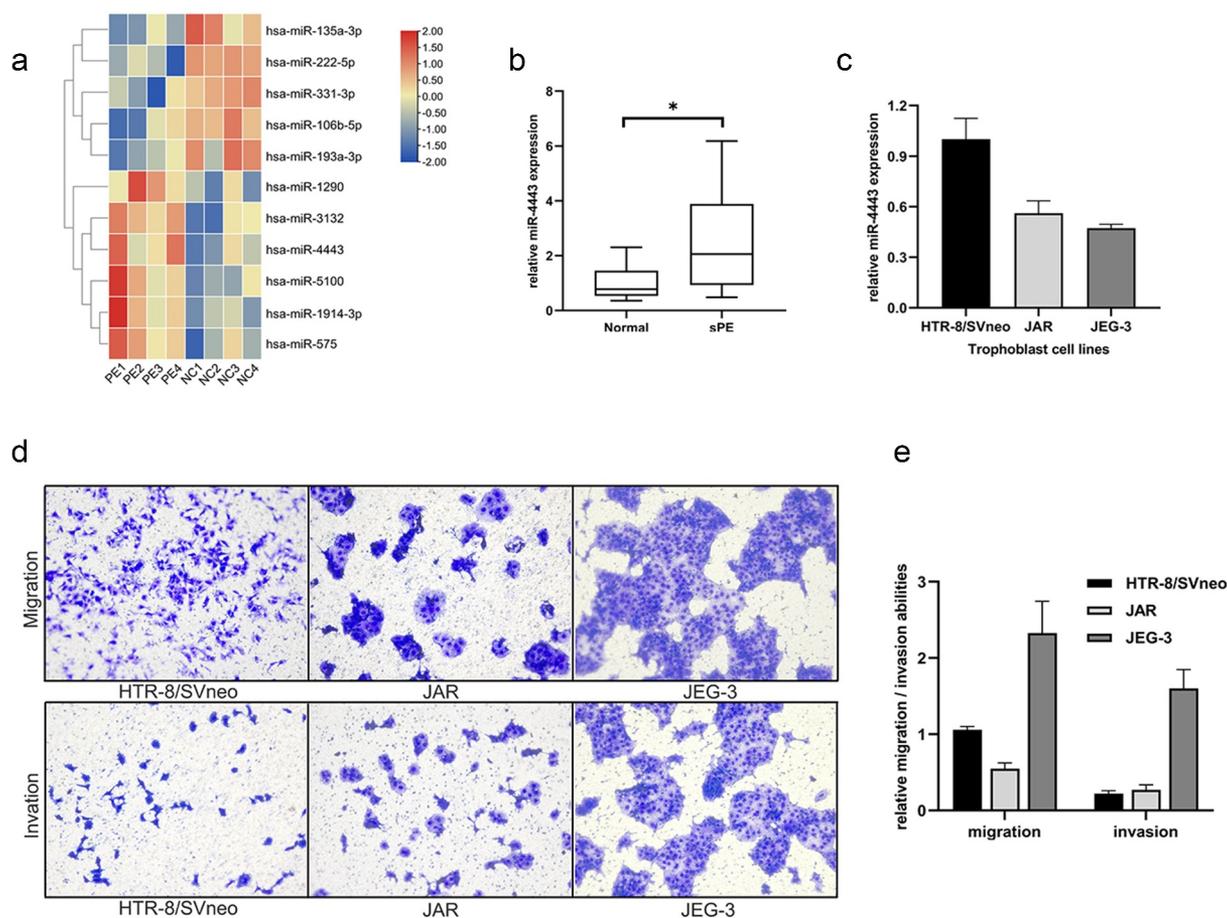


Figure 1. The expression of miR-4443 in human placentas and trophoblast cells. (a) The heatmap of differential miRNA expression in HUVECs between severe preeclampsia patients ($n = 4$) and normal pregnancies ($n = 4$). (b) The miR-4443 expression in severe preeclampsia placentas ($n = 10$) and normal placentas ($n = 10$). (c) The miR-4443 expression in three trophoblast cell lines. (d) Migration and invasion abilities of three trophoblasts by transwell assays at 100 \times original magnification. (e) The results were shown as the mean \pm SD based on three independent experiments. HUVECs: human umbilical vein endothelial cells; * $P < 0.05$.

inhibitor significantly promoted HTR-8/SVneo cell invasion and migration compared to its negative control, as anticipated (Figure 2d, f). The CCK8 assay was also used to evaluate the proliferation ability of trophoblast cells. Compared to their respective negative controls, absorbance value detection revealed that the miR-4443 inhibitor promoted the proliferation of trophoblast cells (Figure 2h), while the miR-4443 mimics had no obvious effect on cell proliferation at 72 h after transfection (Figure 2g).

miR-4443 inhibited the expression of MMP2

To explore the potential mechanism involved in miR-4443 suppressing the invasion and

migration of trophoblasts, TargetScan 7.2 was used to predict miR-4443 target genes. We subsequently performed microarray analysis to identify the genes that were differentially expressed in HTR-8/SVneo cells transfected with the miR-4443 mimics. The results showed that approximately 38 genes were differentially expressed (Figure 3a). Both the results of microarray analysis and target gene prediction showed that MMP2 was a candidate miR-4443 target for further evaluation. To confirm whether the expression of MMP2 is correlated with miR-4443 expression in placental tissue of sPE, we tested the expression of MMP2 in existing matched placental samples by qRT-PCR. MMP2 expression was inversely correlated with

miR-4443 expression in sPE placentas (Figure 3b, c). This conclusion was reinforced by the western blot results (Figure 3d, e). Furthermore, the studies yielded the consistent conclusion that the same

inverse correlation between the expression of miR-4443 and MMP2 also existed when HTR-8/SVneo cells were treated with mimics or inhibitors (figure 3f-i).

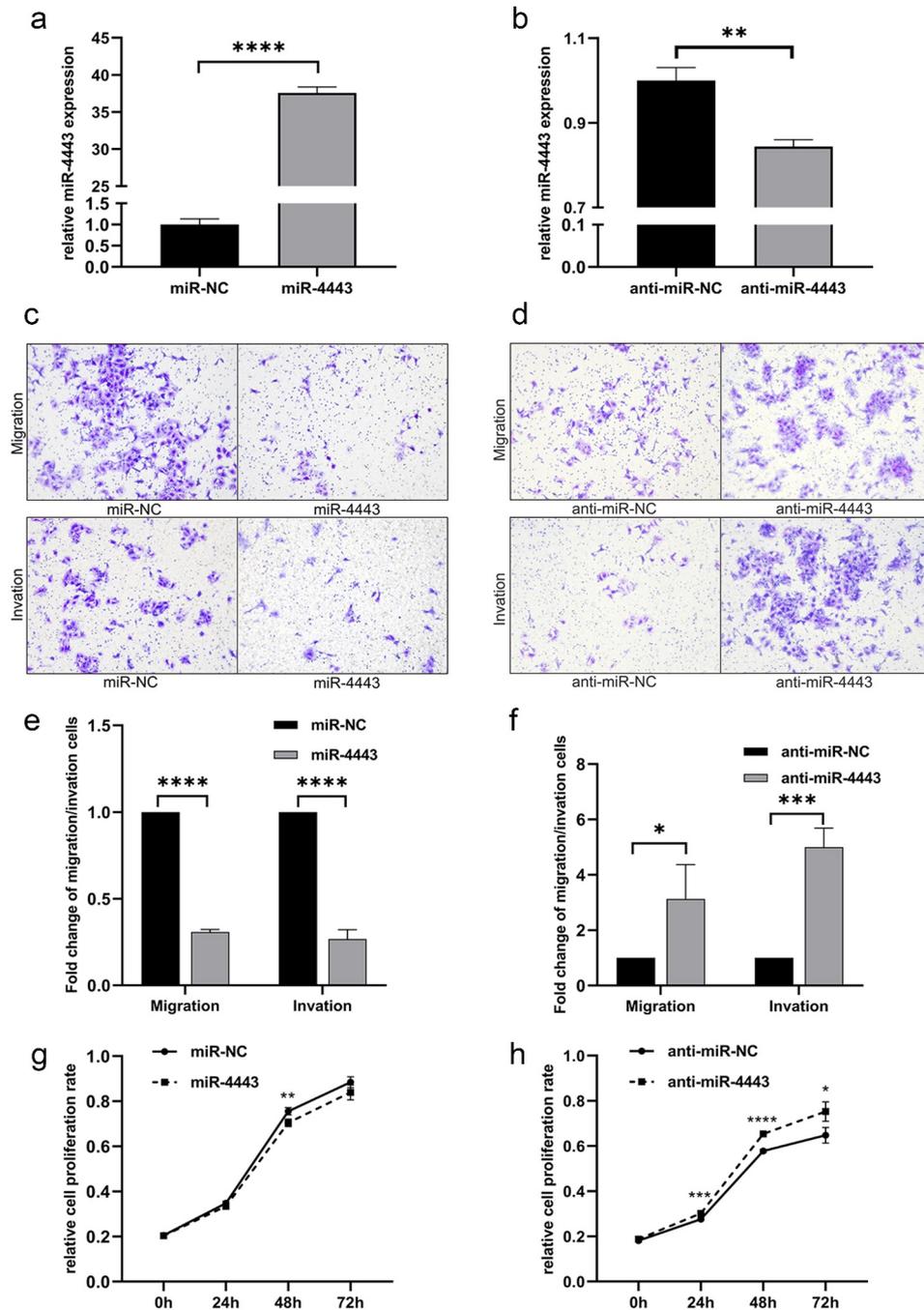


Figure 2. miR-4443 inhibits HTR-8/SVneo cell migration and invasion. (a), (b) The miR-4443 expression measured by qRT-PCR after transfecting with miR-4443 mimics and inhibitor, respectively. (c), (d) Migration and invasion abilities of HTR-8/SVneo cell transfected with miR-4443 mimics and inhibitor at 100 \times original magnification, respectively. (e), (f) The results were statistically based on the mean \pm SD of three independent experiments. (g), (h) Proliferation ability of HTR-8/SVneo cell by CCK8 assays after transfected with miR-4443 mimics and inhibitor, respectively. The absorbance at 450 nm were measured at 0, 24, 48, 72 h. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

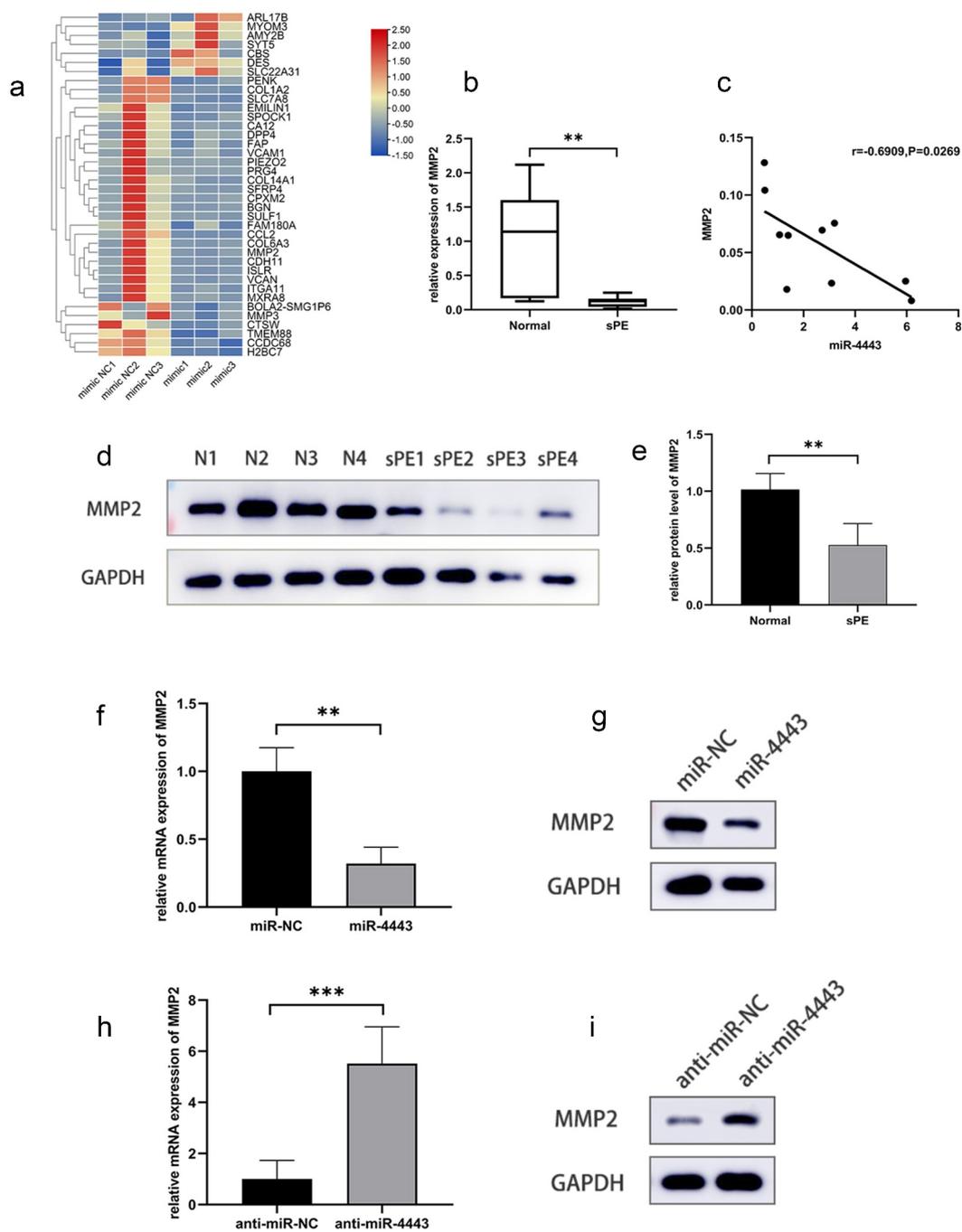


Figure 3. miR-4443 inhibits the expression of MMP2. (a) The heatmap of differential mRNA expression in HTR-8/SVneo cell between transfected miR-4443 mimics ($n = 3$) and its negative control ($n = 3$). (b) The MMP2 expression in severe preeclampsia placentas ($n = 10$) and normal placentas ($n = 10$) measured by qRT-PCR. (c) Relationship between MMP2 mRNA and miR-4443 levels in placentas from women with preeclampsia. $r = 0.6909$, $p < 0.05$. (d) MMP2 protein level of four paired placentas measured by western blotting. MMP2 protein level was statistically analyzed by quantitating the relative intensity of the bands. (e),(f) The MMP2 mRNA and protein levels after miR-4443 mimics or inhibitor transfection compared to their respective negative control. $**P < 0.01$; $***P < 0.001$.

miR-4443 directly inhibited the expression of MMP2 by binding to the 3'-UTR of MMP2 mRNA

To further verify whether the suppression of MMP2 by miR-4443 occurs via the predicted binding site,

the wild-type 3'-UTR of MMP2 and the mutant 3'-UTR of MMP2 were cloned into the pmiR-GLO reporter vector (Figure 4a). The two plasmids were subsequently cotransfected with miR-4443 mimics

or its negative control into HTR-8/SVneo cells, and luciferase reporter assays indicated that the luciferase activity of the plasmid containing the wild-type MMP2 3'-UTR was significantly decreased in the presence of miR-4443 mimics compared to the mutant one (Figure 4b). Consistently, the luciferase activity was increased after cotransfection with the wild-type MMP2 3'-UTR and miR-4443 inhibitor (Figure 4c). These results suggested that miR-4443-mediated MMP2 expression suppression might occur by binding to the 3'-UTR of MMP2 mRNA.

Decreased MMP2 inhibited HTR-8/SVneo cell migration and invasion, similar to the response with the overexpression of miR-4443

To ascertain whether the effects of miR-4443 on trophoblast migration and invasion were mediated by its target gene MMP2, HTR-8/SVneo cells were transfected with MMP2 siRNA or corresponding control siRNA for further transwell assays. MMP2 expression was detected by qRT-PCR and western blotting

synchronously after transfection (Figure 5a, b). The results showed that suppressing MMP2 expression significantly inhibited HTR-8/SVneo cell invasion and migration ability compared to its negative control (Figure 5c, d). Conversely, we verified these phenotypic changes mediated by MMP2 by transfecting the MMP2 overexpression plasmid, and the conclusion was consistent with the inhibition of miR-4443 expression (Figure 5e-h). These results suggested that the effect of the change in MMP2 expression on the migration and invasion of trophoblast cells was consistent with the effect of miR-4443 in the pre-eclampsia pathological state.

miR-4443 overexpression suppressed trophoblast migration and invasion by inhibiting MMP2 expression

To verify whether miR-4443 directly modulates sPE progression via MMP2, further rescue experiments were performed. MMP2 levels were detected by qRT-PCR and western blot in parallel after

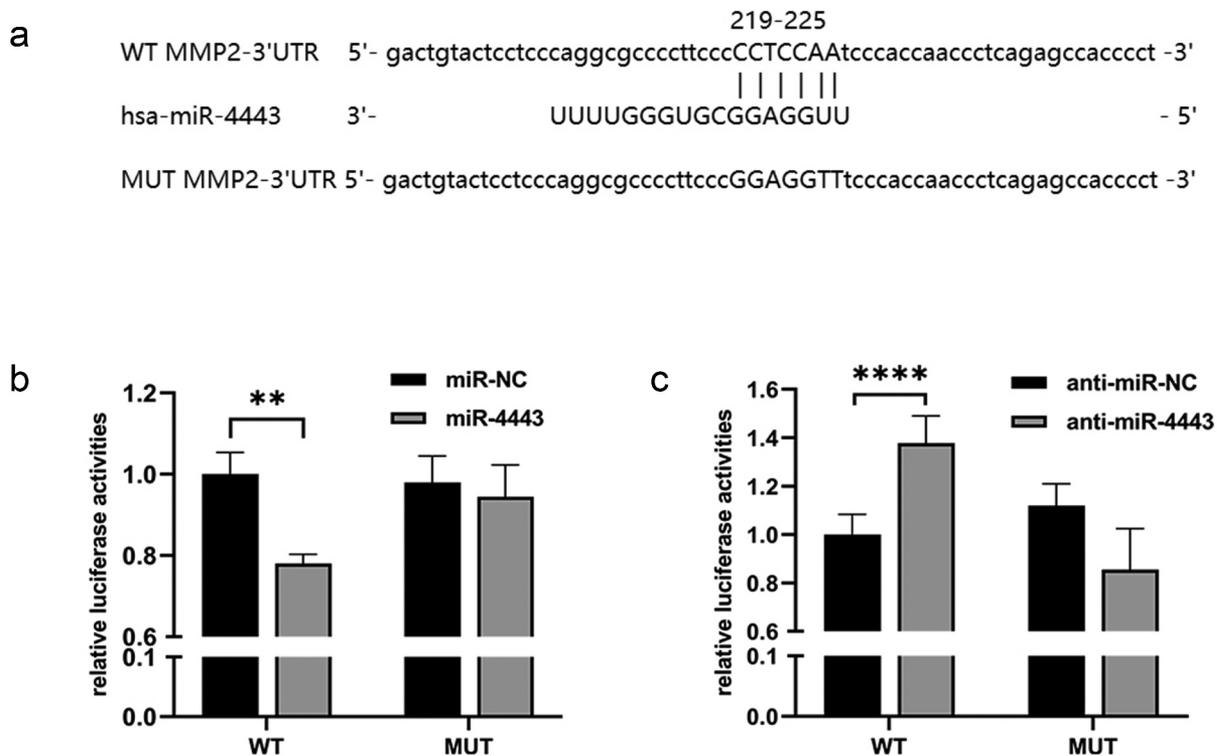


Figure 4. miR-4443 directly inhibits the expression of MMP2 by binding to the 3'-UTR of MMP2 mRNA. (a) The predicted binding site of miR-4443 to 3'-UTR of MMP2 mRNA using TargetScan and the WT/MUT 3'-UTR of MMP2 mRNA. (b), (c) The relative luciferase activity of reporter vectors containing either the WT or MUT 3'-UTR in the presence of miR-4443 mimics or inhibitor. WT: wild-type; MUT: mutant-type; ** $P < 0.01$; **** $P < 0.0001$.

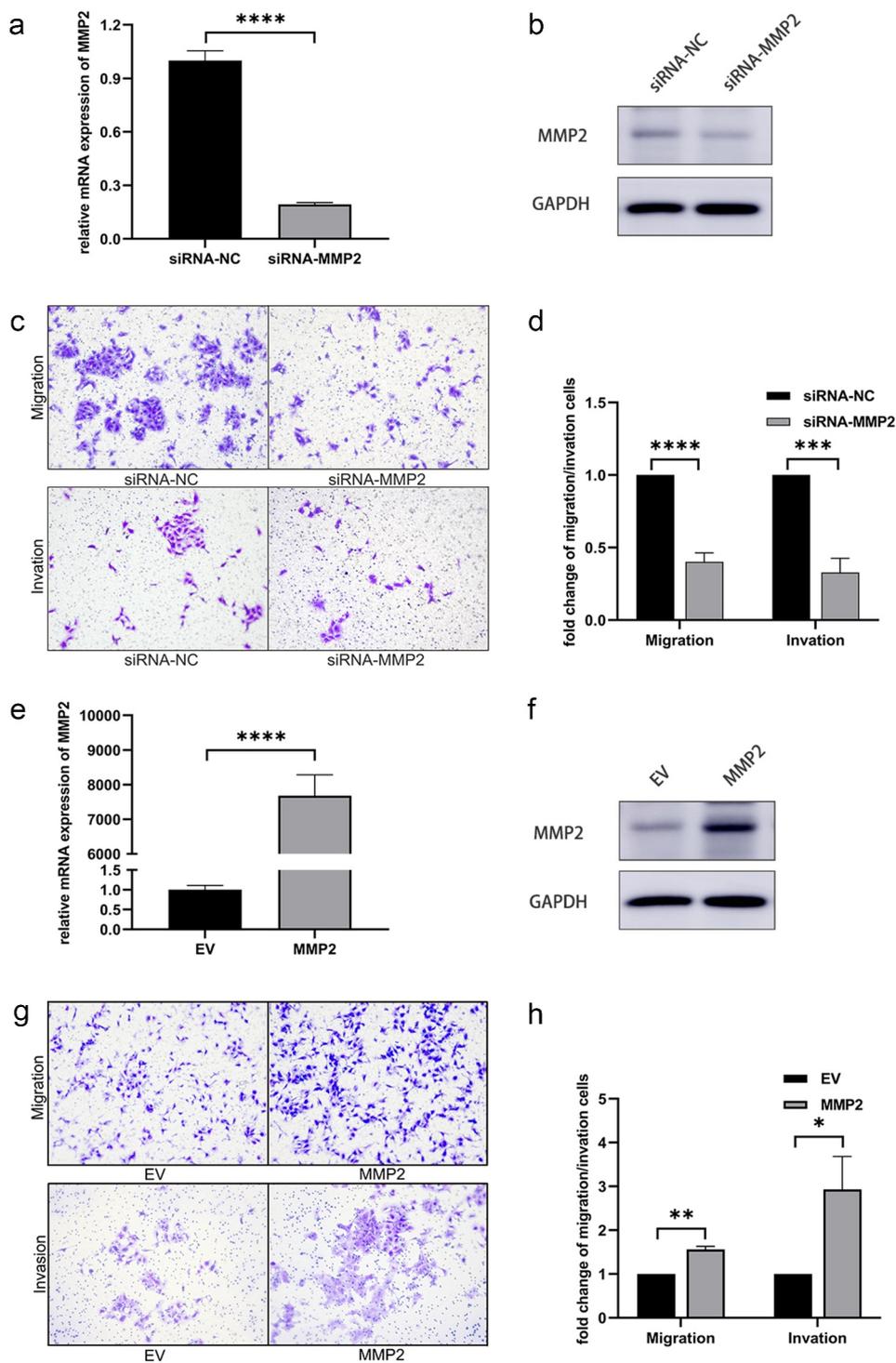


Figure 5. Decreased MMP2 inhibits HTR-8/SVneo cell migration and invasion, similar to the response with the overexpression of miR-4443. (a), (b) The MMP2 mRNA and protein levels after transfecting with MMP2 siRNA. (c) Changes of migration and invasion of HTR-8/SVneo cell transfected with MMP2 siRNA. (d) The results were statistically based on the mean \pm SD of three independent experiments. (e), (f) The MMP2 mRNA and protein levels after transfecting with overexpression MMP2 plasmid. (g) Changes of migration and invasion of HTR-8/SVneo cell transfected with overexpression MMP2 plasmid. (h) The results were statistically based on the mean \pm SD of three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

cotransfection (Figure 6a, b). It was inspiring that the effects of miR-4443 upregulation on trophoblast

invasion and migration were significantly reversed by cotransfection with the MMP2 overexpression

plasmid (Figure 6c-e). Similarly, cotransfection of miR-4443 inhibitor and MMP2 siRNA led to the same conclusion: miR-4443 suppresses trophoblast invasion and migration by directly inhibiting MMP2 expression (figure 6f-j).

miR-4443 repressed HB-EGF/EGFR signaling by inhibiting the expression of MMP2

We then studied whether the pathogenetic progress mediated by miR-4443 had an inhibitory effect on the expression of HB-EGF and its possible mechanism, including the role of HB-EGF/EGFR signaling and its downstream mediator MAPK signaling in preeclampsia. HTR-8/SVneo cells were transfected with miR-4443 mimics or inhibitors, and the protein expressions of pathway-related genes MMP2, HB-EGF, EGFR, p-EGFR, MEK, p-MEK, ERK and p-ERK were detected. The results showed that miR-4443 expression was also reversely correlated with the expressions of HB-EGF, p-EGFR, p-MEK and p-ERK, in addition to MMP2 (Figure 7a, b). We subsequently confirmed this conclusion by cotransfecting miR-4443 mimics and the MMP2 overexpression plasmid or miR-4443 inhibitor and MMP2 siRNA (Figure 7c, d). Finally, we detected the expression of HB-EGF in the placenta by IHC and found that HB-EGF expression was significantly decreased in preeclampsia placentas compared with in normal placentas (Figure 7e). These results suggested that miR-4443 might repress HB-EGF/EGFR signaling by inhibiting the expression of MMP2 (Figure 8).

Discussion

Preeclampsia, a common pregnancy-associated disease, is one of the major causes of maternal and perinatal mortality. Although there are numerous studies committed to the etiology of preeclampsia, the pathogenesis has been inadequately identified. Among the many etiologies that explain the pathogenesis of preeclampsia, the dysregulation of miRNAs has become increasingly attractive in recent years [18]. Many previous studies found that abnormally expressed miRNAs have significant effects on the function of trophoblast cells. For example, miR-30a-3p [8] and miR-4421 [11] negatively regulated the function of trophoblasts and then affected the

development of preeclampsia. Wu L et al. found that miR-181a-5p targeted IGF2BP2, thereby inhibiting the migration and invasion of trophoblast cells leading to preeclampsia [10]. Xu P et al. found that miR-18a inhibited the expression of Smad2 (FL) and TGF- β signal transduction in patients with preeclampsia [9]. In addition, many miRNAs are involved in regulating the expression of angiogenesis-related factors. The overexpression of miR-346 or miR-582-3p significantly reduced the level of VEGF in trophoblast cells [19], and the downregulation of miR-126 significantly reduced VEGF expression [20]. Wang Y et al. found that the expression of miR-141-5p in preeclampsia patients was significantly downregulated, which affected MAPK1/ERK2 signaling by targeting ATF2 and then influenced the pathogenesis of preeclampsia [21].

In our previous work, a significant increase in miR-4443 was found in the HUVECs of patients with severe preeclampsia. We subsequently confirmed that the expression of miR-4443 was also higher in severe preeclampsia placental tissues than in normal pregnancy tissues, suggesting that miR-4443 might have a pathogenic role in preeclampsia. Recent studies have shown that the dysregulation of miR-4443 affects the diagnosis and prognosis of patients with tumors of glial origin [13], colorectal cancer [14], esophageal squamous cell carcinoma [15] and Graves' disease [16], which indicates that miR-4443 might act as a pathogenic factor, while its role in the pathogenesis of preeclampsia has not yet been studied. To further elucidate the potential functions of miR-4443 in preeclampsia, HTR-8/SVneo cells were selected for functional studies, and the results showed that miR-4443 suppressed trophoblast migration and invasion in vitro.

MMP2 was one of the targets of miR-4443 predicted by TargetScan 7.2, and there was a predicted binding site of miR-4443 in the 3'-UTR of MMP2 mRNA. Microarray analysis of HTR-8/SVneo cells transfected with miR-4443 mimics demonstrated that MMP2 expression was also decreased. Combined with target prediction and RNA-Seq, we confirmed that MMP2 was a candidate miR-4443 target for further evaluation. Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent proteases that have a wide tissue distribution and are responsible for

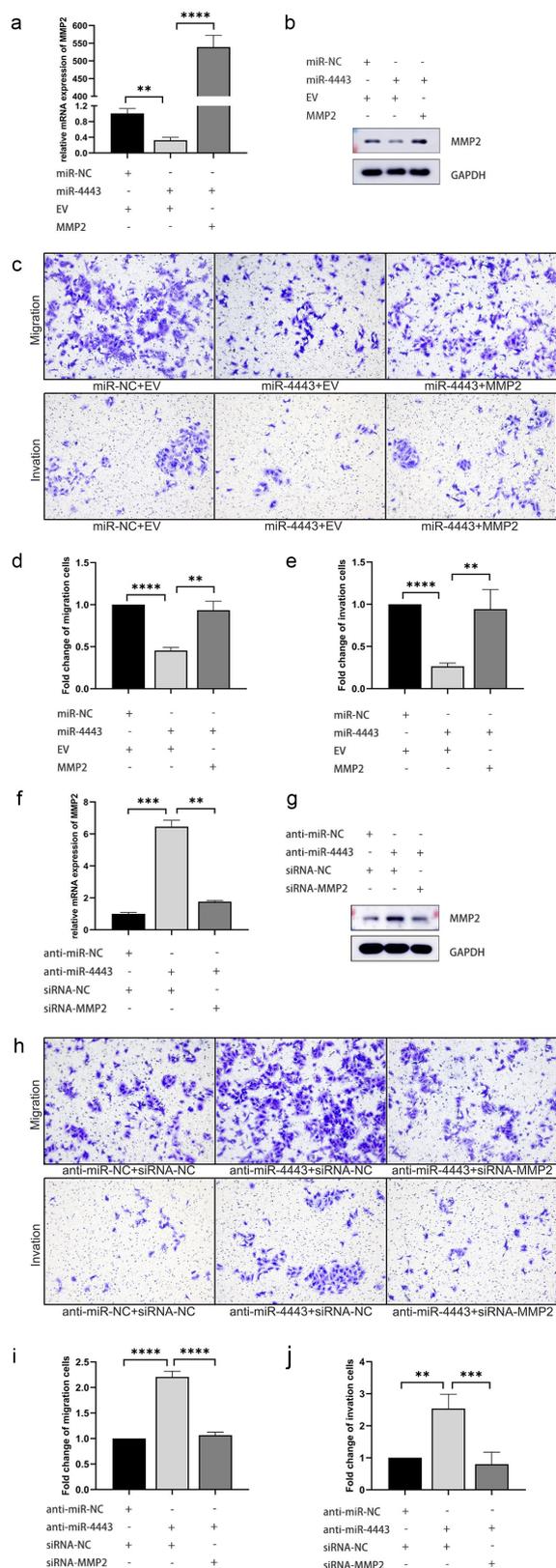


Figure 6. miR-4443 overexpression suppresses trophoblast migration and invasion by inhibiting MMP2 expression. (a), (b) The MMP2 mRNA and protein levels after MMP2 reintroduction. (c) The effects of miR-4443 overexpression on HTR-8/SVneo cell could be reversed via reintroduction of MMP2. Representative fields of invaded/migrated cells are shown (at 100× original magnification). (d), (e) The abilities of migration and invasion of HTR-8/SVneo cell were statistically based on the mean ± SD of three independent experiments. (f), (g) The MMP2 mRNA and protein levels after MMP2 inhibition. (h) The effects of miR-4443 knockdown on HTR-8/SVneo cell could be reversed via inhibition of MMP2. Representative fields of invaded/migrated cells are shown (at 100× original magnification). (i), (j) The abilities of migration and invasion of HTR-8/SVneo cell were statistically based on the mean ± SD of three independent experiments. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

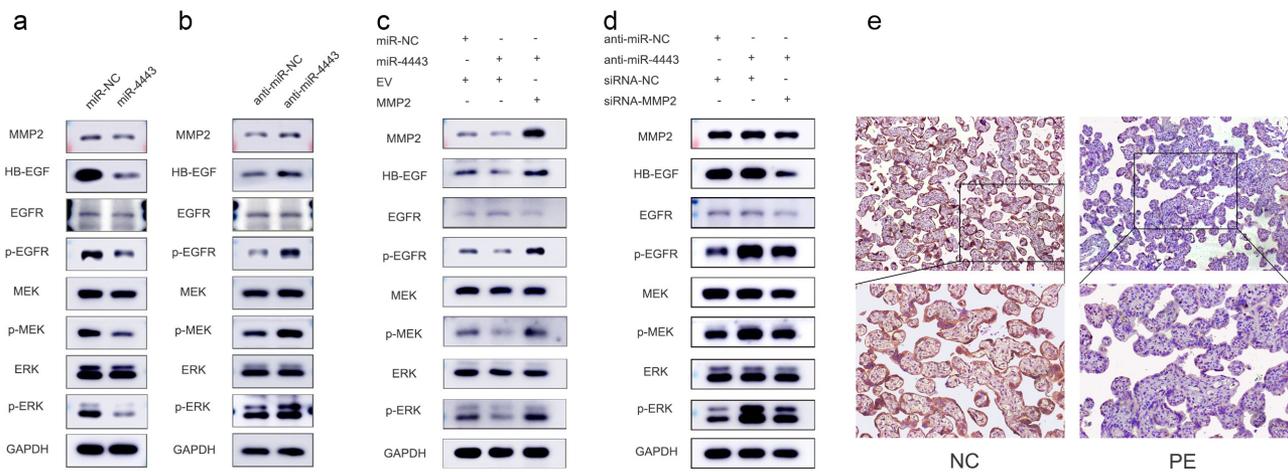


Figure 7. miR-4443 represses the HB-EGF/EGFR pathway by inhibiting the expression of MMP2. (a), (b) The protein levels of HB-EGF/EGFR pathway-related biomolecules after transfecting with miR-4443 mimics and inhibitor, respectively. (c), (d) The protein levels of HB-EGF/EGFR pathway-related biomolecules after co-transfecting of miR-4443 mimics and the plasmid overexpressing MMP2 or miR-4443 inhibitor and MMP2 siRNA. (e) HB-EGF protein level of preeclampsia and normal placentas measured by IHC at 100× original magnification and 200× original magnification.

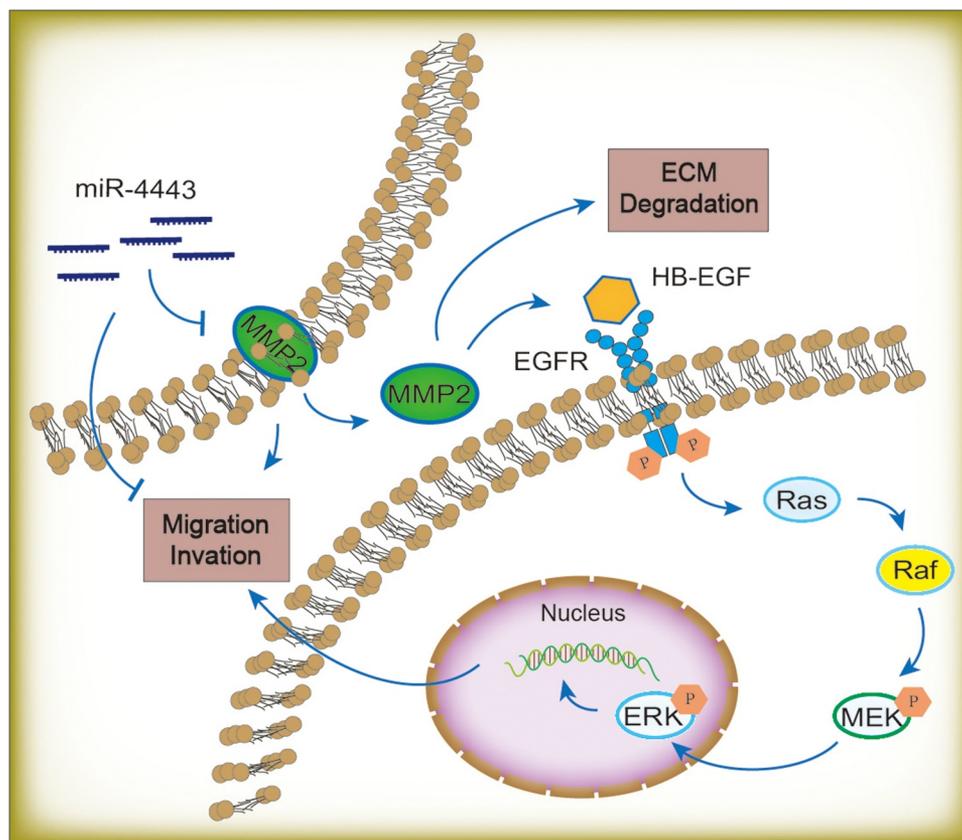


Figure 8. miR-4443/MMP2 suppresses migration and invasion of trophoblast cells through HB-EGF/EGFR pathway. miR-4443 suppresses the migration and invasion of trophoblasts, and its inhibitory effects are at least partially mediated by the suppression of MMP2, this inhibition further affects the progression of the preeclampsia through the HB-EGF/EGFR pathway, thus providing a new clue on the role of miR-4443 in the pathogenesis of severe preeclampsia.

the degradation and turnover of extracellular matrix (ECM) components in several physiological

processes [22]. As a key member of the MMP family, MMP2 is abnormally expressed in

pathological conditions, including cancer, chronic inflammation, and neurological and reproductive disorders [23]. Normal pregnancy is accompanied by several changes in the utero placenta and hemodynamics to meet the growth and metabolic demands of the developing fetus. Placental remodeling and cytotrophoblast invasion of spiral arteries maintain adequate blood supply for the developing fetus. MMP2, as an important proteolytic enzyme that degrades ECM, also plays a significant role in the process of trophoblast invasion into the decidual stroma during pregnancy [22,24]. There have been many reports about abnormal MMP2 expression in preeclampsia, but interestingly, based on previous studies, there is no clear consensus on the upregulation or downregulation of MMP2 expression in pregnant women with preeclampsia [24–27]. In our study, the expression of MMP2 was decreased in preeclampsia placentas. Subsequent luciferase reporter assays verified that miR-4443 directly inhibited MMP2 expression by binding to the 3'-UTR of MMP2 mRNA. In addition, we demonstrated that miR-4443 suppressed the invasion and migration of trophoblasts at least partly by directly targeting MMP2 via siRNA imitation assays and rescue experiments.

HB-EGF, which is downstream of MMP2 in the GnRH signaling pathway [28], is a member of the epidermal growth factor (EGF) family, which is expressed at lower levels in preeclampsia placentas and plays an important role in trophoblast survival, differentiation, and invasion, leading to poor placental perfusion [29,30]. Some studies have shown that the processed HB-EGF liberated by MMP2 action causes phosphorylation of EGFR and initiates downstream Ras-dependent signaling, leading to activation of the mitogen-activated protein kinase (MAPK) pathway [31,32]. Recent studies have found that the HB-EGF/EGFR pathway is involved in the development of many diseases [33–36], however, whether miR-4443 can also trigger this signal by targeting MMP2 in preeclampsia has not yet been studied. Therefore, we detected the protein expression of HB-EGF/EGFR pathway-related genes and found that miR-4443 expression was also reversely correlated with the expression of HB-EGF, p-EGFR, p-MEK and p-ERK.

To date, our findings demonstrate for the first time that the dysregulation of miR-4443 in placentas affects the migration and invasion of trophoblasts. In addition, we first showed that by targeting MMP2, the upregulation of miR-4443 might further affect HB-EGF/EGFR signaling and have an effect on the occurrence of preeclampsia. However, the main shortcoming of this study was the absence of animal experiments to further confirm the potential roles of miR-4443 *in vivo* in the pathogenesis of PE. In addition, in recent years, although HTR-8/SVneo has been increasingly used as an *in vitro* research cell line of trophoblast diseases [37–40], studies have shown that HTR-8/SVneo hardly expresses several key markers of some trophoblast cells, including HLA-G, CK7 and E-cadherin [41], which is a deficiency of this study. Another limitation is that there was no evaluation of whether miR-4443 could serve as a biomarker for the prediction and diagnosis of sPE as early as the first trimester. Thus, we will conduct more experiments in the future to investigate the possibility of miRNA detection for the early diagnosis of PE.

Author contributions

CC contributed to the experimental design, experimental operation, experimental validation, data curation, and writing of the original draft. JG contributed to the experimental operation, experimental validation and data curation. DC, JL, BH, YC and HZ contributed to the experimental guidance and experimental operation. XY and WC contributed to the experimental supervision, project administration and funding acquisition. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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