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Original Article

Retinol-binding protein 4 regulates the biological functions and molecular mechanisms of JEG-3 cells

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Abstract: Objective: Preeclampsia (PE) is a disorder of pregnancy that is associated with maternal and fetal mortality and morbidity. Abnormal placental Retinol-binding protein 4 (RBP4) expression has been found in cases of PE. This study aimed to further investigate the possible role of RBP4 in the pathophysiology of PE. Study Design: RBP4 serum concentration was detected using enzyme-linked immunosorbent assays in eighteen patients with severe PE and twenty-five individuals with healthy pregnancies. JEG-3 cells were transiently transfected with a plasmid construct expressing RBP4 (pCMV-RBP4), an empty plasmid (p-CMV) or siRNA, and the MMP2 and MMP9 protein levels were analysed 48 h after transfection. MTT assays and transwell assays were used to explore the functional role of RBP4 in the proliferation and invasion of JEG-3 cells. Results: RBP4 serum concentration in patients with severe PE was significantly lower than that in individuals with healthy pregnancies. RBP4 over-expression enhanced the invasion and proliferation of JEG-3 cells and increased MMP2 and MMP9 in JEG-3 cells. Conclusions: RBP4 plays an important role in the regulation of trophoblast invasion and migration and represents a possible underlying pathological and molecular mechanism of PE.

Keywords: Retinol-binding protein 4, trophoblast invasion, preeclampsia, matrix metalloproteinase

Introduction

Preeclampsia (PE) is a specific complication of pregnancy that occurs after 20 weeks of gestation and is characterised by the onset of hypertension and proteinuria during pregnancy, and it continues to be a leading cause of maternal and perinatal morbidity and mortality worldwide [1-3]. Although the specific etiology and pathogenesis of PE are unclear, it is widely accepted that PE is associated with inadequate invasion of trophoblasts and spiral artery remodeling [4, 5]. Trophoblast dysfunction reduces the invasion and migration capacities of EVTs and placental hypoperfusion, hypoxia, or ischaemia may occur and thus induce the occurrence and deterioration of PE [6-8]. However, the molecular mechanisms for the regulation of trophoblast behavior are still unclear.

Therefore, researchers have speculated that the pathophysiologic changes of PE may result from the abnormal expression of certain proteins [1, 6, 9]. Several biochemical markers, including RBP4, are involved in placental development, and their altered expression and dysfunction may play a role in the development of PE. Retinol-binding protein 4 (RBP4) is a single polypeptide protein that was first discovered in 2005 and was originally described as the sole transport protein carrying retinoids such as vitamin A in the blood [10]. RBP4, a 21-kDa protein secreted from adipocytes and liver, was subsequently identified as an adipokine that impaired insulin signaling in experimental animals [6, 11, 12].

RBP4 has been shown by several researchers to be dysregulated during preeclampsia and may serve as a potential marker for preeclmapsia. Studies have also shown that dysregulated placental RBP4 levels are associated with intrauterine growth restriction and severe early-onset PE, indicating that changes in RBP4 lev-

els may correlate with the severity of PE [13]. However, a mechanistic study has not been done to understand the role of RBP4 in placental development especially during first trimester. Whether RBP4 contributes to the pathogenesis of PE has not been confirmed [14-16]. Our study aimed to investigate the possible role of RBP4 in the pathophysiology of PE and its function in trophoblast biology.

Materials and methods

Subjects

The research protocol was based on the guidelines of the World Medical Association's Declaration of Helsinki and was approved by the Medical Ethics Committee of Capital Medical University Beijing Chao-Yang Hospital. Written informed consent was obtained from each patient prior to participation. Eighteen patients with severe PE and twenty-five healthy pregnant women, all from the inpatient population of Capital Medical University of Chao-yang Hospital, were recruited for enzyme-linked immunosorbent assay (ELISA) analysis. Women were diagnosed with severe PE based on the World Health Organization recommendations: blood pressure ≥ 160/110 mmHg on two occasions at least 4 h apart after 20 gestational weeks in a pregnant woman with previously normal blood pressure and at least one of the following: proteinuria > 5 g/24 h or symptoms such as abdominal pain, headache, and visual disturbance. Body mass index was calculated as weight before pregnancy divided by squared height. The exclusion criteria were as follows: intrauterine fetal demise, rupture of membranes, clinical chorioamnionitis, multiple gestation, acute or chronic infection and history of diabetes, chronic hypertension, maternal immune diseases, maternal inflammatory bowel diseases or previous exposure to $MgSO_4$ treatment.

ELISA

Blood samples drawn via venipuncture were collected in a serum-separator tube from PE patients after diagnosis and admission. The serum was removed after centrifugation at 3000 r/min for 15 min at 4°C within 2 h of collection and stored at -80°C until analysis. Serum RBP4 concentration was measured with an ELISA according to the manufacturer's instructions (R&D Systems).

Cell culture

JEG-3 cells, purchased from the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences & School of Basic Medicine Peking Union Medical College (Beijing, China), were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) at 37°C in a 5% CO_2 incubator. When the cells reached 80-90% confluence, they were trypsinised and subcultured into new culture flasks.

Construction of RBP4 Over-expression recombinant plasmid and RBP4 siRNA interference sequences

The nucleotide sequence of the RBP4 coding region was used to design amplification primers with Primer-BLAST software (U.S. National Library of Medicine, Rockville Pike, Bethesda MD, USA). The primer sequences were as follows: forward, 5'-TCGAAGAACAAGTTTG TACAAAAAAGATGAAGTGGGTGTGGGCGCT-3' and reverse, 5'-ATCAACCACTTTGTACAAGAAAGCTACAAAAGGTTTC TTTCTGATCT-3'. The template used to amplify the coding region of the RBP4 gene was human cDNA. The resulting PCR product was ligated into the NspvI/NotI enzyme restriction sites of the p-CMV plasmid. The positive recombinant plasmids were double-digested, and the sequence was termed pCMV-RBP4. The empty plasmid (p-CMV) was used as a negative control. siRNAs were constructed by Suzhou GenePharma. Co., Ltd. (Suzhou, China). The siRNA sequences were as follows: RBP4-homo-577, sense: 5'-GAAGCGCAGAAGAUUGUAATT-3', antisense: 5'-UUACAAUCUUCUGCGCUUCTT-3'; RBP4-homo-247, sense: 5'-GCCUCUUUCUGCAGGACAATT-3', antisense: 5'-UUGUCCUGCAGAAAGAGGCTT-3'; RBP4-homo-432: sense: 5'-GGAUCGUCGACACAGACUATT-3', antisense: 5'-UAGUCUGUGUCGACGAUCCTT-3'; negative control: sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'.

JEG-3 cells were transfected with Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The best interference sequence was selected by western blot analysis.

Table 1. Clinical Characteristics of Study Patients Data are shown as the mean ± SD, and differences between groups were analysed with t-tests

Characteristics	Normal pregnancy (n = 25)	Preeclampsia (n = 18)	*P*-value
Maternal age (years)	31.18 ± 4.05	28.85 ± 4.72	0.09
BMI (kg/m^2)[a]	22.16 ± 3.15	21.38 ± 3.2	0.43
Systolic blood pressure (mmHg)	115.58 ± 6.22	154.45 ± 14.24*	< 0.001
Diastolic blood pressure (mmHg)	76.84 ± 5.62	101.40 ± 8.24*	< 0.001
24 h urine protein (g)	0.1 ± 0.05	4.48 ± 0.62*	< 0.001
Gestational day at delivery (day)	264.24 ± 12.78	258.63 ± 5.73	0.088
Infant birth weight (g)	3285.32 ± 482.62	2604.14 ± 592.16*	0.0002

*Compared with normal pregnancy, P < 0.05. [a]BMI, body mass index, indicating the weight in kilograms divided by the square of the height in metres.

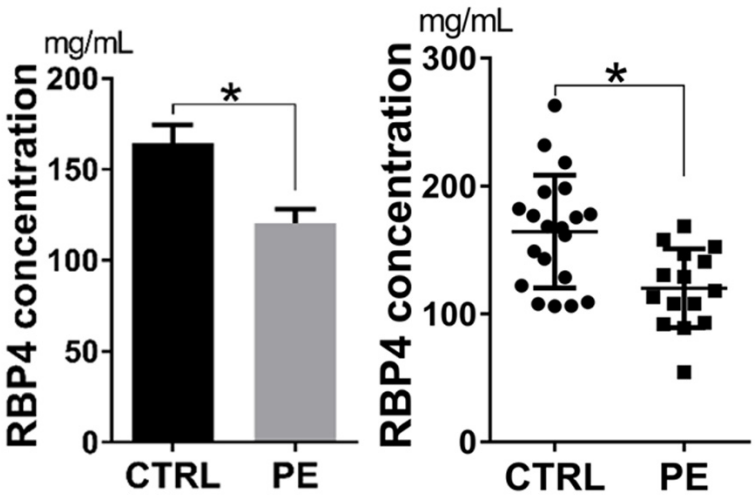

Figure 1. Serum RBP4 concentration was significantly decreased in the preeclampsia group compared with the control group (P < 0.05).

Western blotting

Cell lysates were harvested, and protein concentration was determined via the bicinchoninic acid protein quantification method [17]. Then, 30 μg of total protein was separated by SDS-PAGE and electrotransferred onto NC membranes (Millipore, Bedford, MA, USA) using a semi-dry western blot transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primary rabbit anti-RBP4 polyclonal antibody (1:2,000 dilution), rabbit anti-matrix metalloproteinase 2 (MMP2) monoclonal antibody (1:1,000 dilution) and rabbit anti-MMP9 monoclonal antibody (1:2,000 dilution) (all from Abcam, Cambridge, MA, USA) and anti-GAPDH (loading control, 1:3,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) were incubated at 4°C overnight. After three washes with PBST, secondary HRP-conjugated goat anti-rabbit antibody (1:10,000 dilution; Cell Signaling Technology, Inc.) incubation was performed for 2 h at room temperature. Finally, detection was performed by chemiluminescent ECL reagent (Millipore), and signals were exposed to X-ray film. Analysis of total signal intensity of western blot bands was performed with Image Studio Lite version 4.0 (Li-Cor Biosciences, Lincoln, NE, USA).

Transwell assay

Cell motility was evaluated with a 24-well Boyden chamber with an 8-μm pore size polycarbonate membrane (Corning Costar Inc., Corning, NY, USA) and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to simulate the matrix barrier. Twenty-four hours after transfection, 1 × 10^5 cells were resuspended in 100 μl serum-free medium comprising 1% bovine serum albumin, seeded on top of the transwell membrane in the upper chamber and incubated with 650 μl culture medium containing 10% FBS in the bottom chamber. After a 24-h incubation, the membranes were fixed with 95% ethyl alcohol and stained with 0.1% crystal violet at room temperature (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Three visual fields were randomly selected from each membrane, the migrated cells were counted underneath an inverted microscope (IX-71; Olympus, Tokyo, Japan), and each assay was repeated at least 3 times.

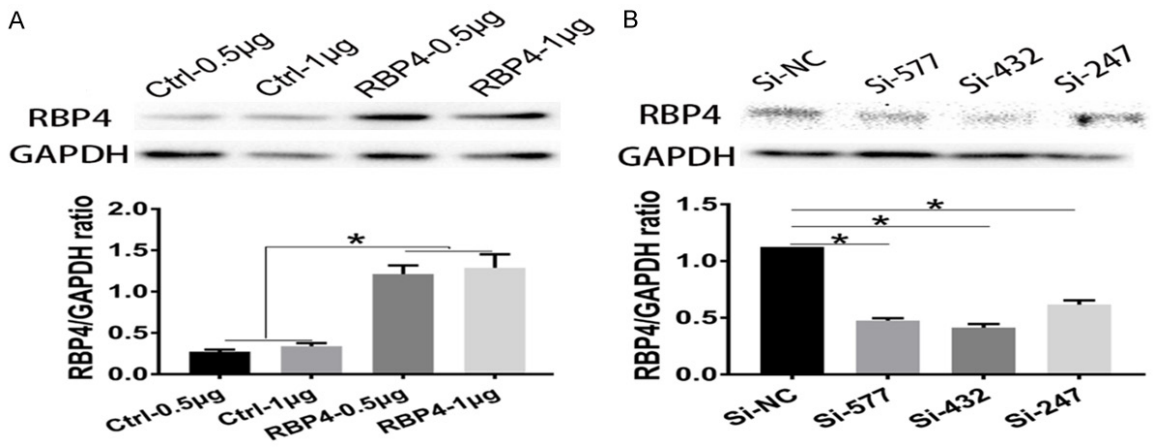

Figure 2. RBP4 expression determined by western blot analysis. A. RBP4 expression in JEG-3 cells transfected with pCMV-RBP4 or pCMV. Ctrl-0.5 μg, the pCMV quantity is 0.5 μg; Ctrl-1 μg, the pCMV quantity is 1 μg; RBP4-0.5 μg, the pCMV-RBP4 quantity is 0.5 μg; RBP4-1 μg, the pCMV-RBP4 quantity is 1 μg. B. RBP4 expression in JEG-3 cells transfected with RBP4 siRNA (si-577, si-432, si-247) or negative control (si-NC). Bar chart shows the RBP4 level based on the statistical analysis of the western blotting results. Data are expressed as the mean ± SD of three independent biological repeats. *, compared with corresponding data in the control, $P < 0.05$.

MTT assay

JEG-3 cells transfected after 24 h were seeded into 96-well plates at a density of 5×10^3 cells/well and a final volume of 150 μl/well in triplicates per experiment. After 0, 24, 48, and 72 h, MTT reagent (Sigma-Aldrich, Shanghai, China) (20 μl) was added, and cells were incubated for 4 h at 37°C. The medium was then discarded, and cells were oscillated in 150 μl/well dimethyl sulphoxide for 15 min. The absorbance was measured at 490 nm using a Fluoroskan Ascent FL Microplate Fluorometer (Thermo Scientific, Sunnyvale, CA, USA), and each assay was repeated at least 3 times.

Statistical analysis

Data are presented as the mean ± SD. One-way analysis of variance (ANOVA) was conducted to compare multiple groups, followed by the Bonferroni post hoc test. GraphPad Prism software, version 4.0 (GraphPad Software, Inc., San Diego, CA, USA), was used for data analysis and plotting. $P < 0.05$ was considered a significant difference.

Results

Subject characteristics

The clinical characteristics of the patients are summarised in **Table 1**.

Serum RBP4 expression

RBP4 concentration in patients with severe PE was significantly decreased compared with that in individuals with healthy pregnancies ($P = 0.0022$; **Figure 1**). Mean (SD) serum RBP4 concentrations were higher among PE cases 164.5 (44.07) mg/ml compared with controls 120.25 (30.79) mg/ml ($P < 0.05$).

RBP4 protein expression by western blotting

RBP4 was substantially overexpressed in JEG-3 cells transfected with pCMV-RBP4 compared with that in JEG-3 cells transfected with pCMV (**Figure 2A**), which confirmed that pCMV-RBP4 plasmids were constructed successfully. Compared with the negative control group (si-NC), the RBP4 siRNA fragments could significantly inhibit RBP4 expression. The best interference sequence was RBP4-homo-432 (**Figure 2B**), so RBP4-homo-432 was used as an interference fragment in follow-up experiments.

RBP4 regulates the proliferation, migration and invasion of JEG-3 cells in vitro

The migration and invasion of JEG-3 cells transfected with pCMV-RBP4 were significantly increased compared to those of JEG-3 cells transfected with pCMV ($P < 0.05$) (**Figure 3A**, **3B**). Silencing of RBP4 inhibited JEG-3 cell migration and invasion in vitro compared to those of the

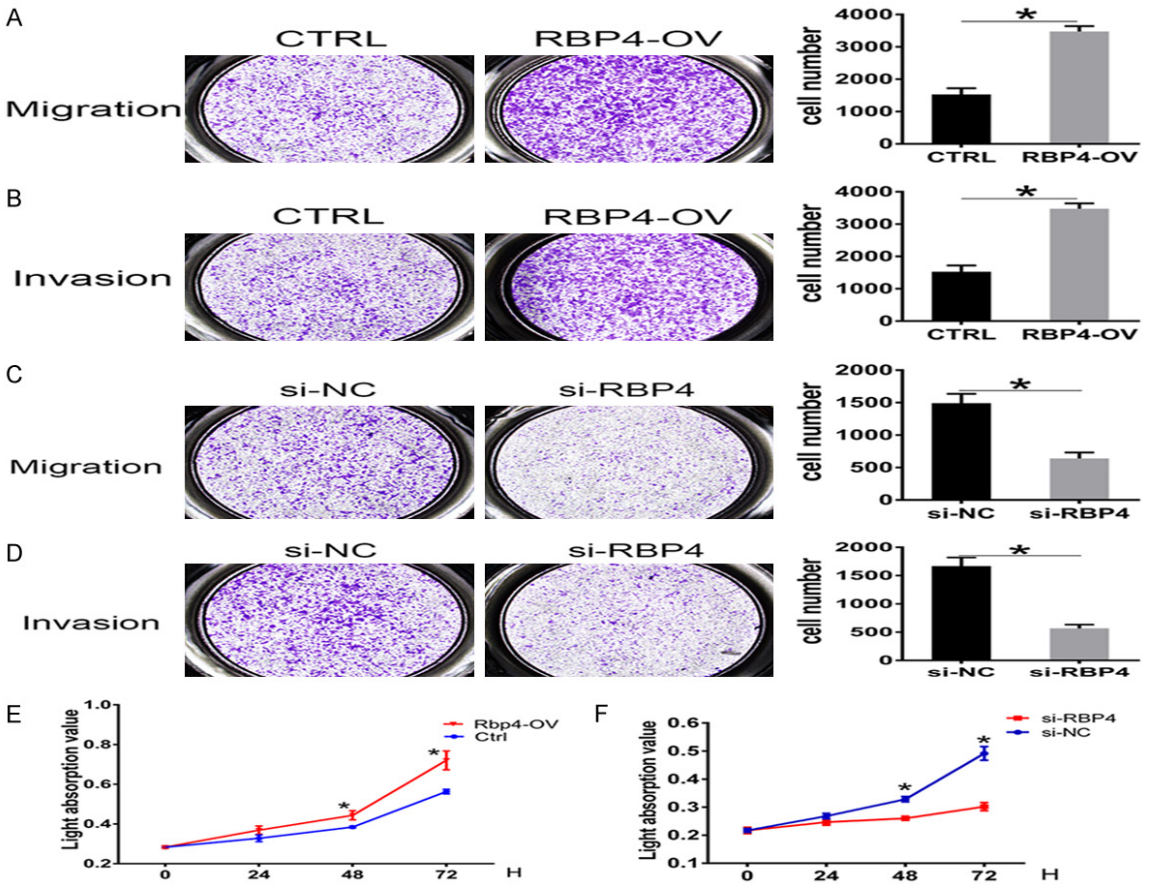

Figure 3. RBP4 regulates the migration and invasion of JEG-3 cells. A. The migration of JEG-3 cells was compared between the CTRL and RBP4-OV groups by transwell migration assays. CTRL, JEG-3 cells transfected with the empty plasmid. RBP4-OV, JEG-3 cells transfected with pCMV-RBP4. B. Matrigel-based transwell invasion assays of CTRL and RBP4-OV cells. C. Transwell migration assays of si-NC and si-RBP4 cells. D. Matrigel-based transwell invasion assays of si-NC and si-RBP4 cells. Representative images are shown on the left bar chart. E. MTT assays detected the fold change in JEG-3 cell growth rate for RBP4-OV cells at 0, 24, 48 and 72 h compared with the CTRL. F. MTT assays detected the fold change in JEG-3 cell growth rate for si-NC cells at 0, 24, 48 and 72 h compared with the si-RBP4 cells. Data are expressed as the mean ± SD of three independent biological repeats. *, compared with the corresponding data in the control, $P < 0.05$.

negative control ($P < 0.05$) (**Figure 3C**, **3D**). Furthermore, the proliferation of JEG-3 cells transfected with pCMV-RBP4 was strongly increased compared to that of pCMV-treated JEG-3 cells at 48 and 72 h after transfection ($P < 0.05$) (**Figure 3E**); similarly, RBP4 silencing dramatically inhibited the proliferation of JEG-3 cells at 48 and 72 h after transfection ($P < 0.05$) (**Figure 3F**).

RBP4 regulates MMP2 and MMP9 in JEG-3 cells

To explore the molecular mechanisms of RBP4 in mediating trophoblast invasion, we analysed the MMP2 and MMP9 protein levels 48 h after transfection. The results showed that RBP4 overexpression substantially increased the MMP2 and MMP9 levels, and silencing of RBP4 significantly downregulated the MMP2 and MMP9 levels (**Figure 4**).

Discussion

A relationship between RBP4 and pre-eclampsia (PE) was previously observed in several studies, but the results were controversial. Stepan et al. [14, 17] found no link between serum RBP4 levels and the risk of PE. Yliniemi et al. [6] found that RBP4 levels in first trimester serum were significantly higher in females who subsequently developed EO-PE than controls in

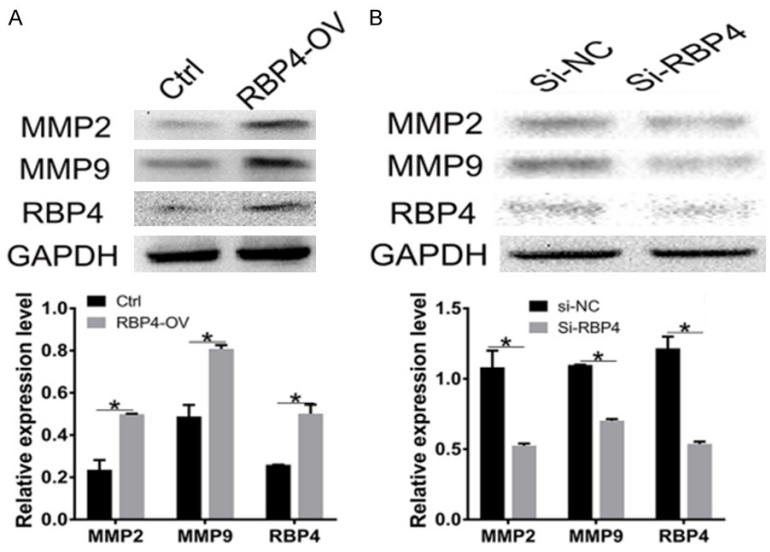

Figure 4. RBP4 influences MMP2 and MMP9 in JEG-3 cells. A. The expression of MMP-2 and MMP-9 was compared between RBP4-OV cells and CTRL cells. B. The expression of MMP-2 and MMP-9 was compared between the si-NC cells and siRNA cells. Bar chart shows the MMP-2 and MMP-9 expression levels based on the statistical analysis of the western blotting results. Data are expressed as the mean ± SD of three independent biological repeats. *, compared with corresponding data in the control, $P < 0.05$.

a retrospective case-control study. Park et al. [13, 16] detected that the serum level of RBP4 was reduced in the PE group compared to that in the normal group. Our findings are similar to some previous reports of associations of RBP4 with PE in pregnant populations. We analysed the serum RBP4 concentrations of eighteen patients with severe PE and twenty-five healthy pregnant women with ELISA, and observed that RBP4 concentration in patients with severe PE was significantly decreased compared with that in individuals with healthy pregnancies. Overall, there is modest evidence to support that PE is associated with decreased maternal RBP4 concentration compared with normal pregnancy. Our studies showed that reduced RBP4 may be responsible for the occurrence of PE. However, it remains unclear how RBP4 is involved in the pathogenesis of PE.

JEG-3, phenotype similar to extravillous trophoblasts (EVTs), is a line of choriocarcinoma cells that originated from Choriocarcinoma explants. They express human choriogonadotropin; human leukocyte antigen-G, integrin α (ITGA) 1, 5 and 6; and MMP2 and MMP9; and have been widely used as an in vitro model to study the functions of trophoblast migration and invasion [18, 19].

Our previous studies found that the content of RBP4 in placenta with severe PE was distinctly lower than that in placenta with normal pregnant women [20]; therefore, we speculate that RBP4 might play an important role by affecting the function of trophoblast cell migration and invasion. Review of RBP4 related literature showed a lack of a role of RBP4 in the biological behavior of trophoblastic cells and the pathogenesis of preeclampsia. In this study, we constructed RBP4 overexpression plasmid and RBP4 siRAN to transfect JEG-3 cells, and further, we observed the effect of overexpression and knockdown of RBP4 on the biologic function of trophoblast cells in JEG-3 cells. Through invasion, migration and MTT assays, we showed that RBP4 over-expression enhanced the proliferation and invasion of JEG-3 cells. In addition, silencing of RBP4 dramatically suppressed the proliferation and invasion of JEG-3 cells. Therefore, we hypothesized that RBP4 reduction leads to inhibition of the migration and invasion of trophoblasts in PE patients, resulting in shallow trophoblast invasion. These changes further cause placental hypoxia and ischemia, which eventually leads to the occurrence of PE. Our studies confirmed the role of RBP4 in the pathogenesis of PE, but further analyses are needed to elucidate the molecular mechanisms.

MMPs are a family of zinc-containing endopeptidases capable of degrading a wide range of extracellular matrix components [21, 22]. Of these, MMP2 and MMP9, respectively called gelatinase A and B, are the most common MMPs and are believed to play important roles in trophoblast invasion. Studies have also shown that MMPs are involved in the remodelling of uterine spiral arteries, and reduced MMP activity may affect the invasion of trophoblast cells, resulting in abnormal placental development and onset of PE [23-26].

Our results showed that RBP4 expression significantly regulated the protein levels of MMP2

and MMP9, indicating that RBP4 likely promotes the migration and invasion of trophoblasts by increasing the secretion of MMP2 and MMP9. The decreased levels of RBP4 may reduce the secretion of MMP2 and MMP9 in PE patients and subsequently inhibit the migration and invasion of trophoblasts. Shallow trophoblast invasion contributes to placental hypoxia and ischemia, eventually leading to the occurrence of PE.

In conclusion, we demonstrated that RBP4 is significantly reduced in the sera of patients with PE, and further data indicated that RBP4 over-expression effectively promotes the proliferation and invasion of JEG-3 cells, whereas RBP4 knockdown significantly inhibits JEG-3 cell proliferation and invasion, which is associated with changes in the protein levels of MMPs. Our findings support an important role for RBP4 in regulating trophoblast invasion and proliferation, representing a possible underlying pathological and molecular mechanism of PE.

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Disclosure of conflict of interest

None.

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