EPHB4, a down stream target of IFN-γ/STAT1 signal pathway, regulates endothelial activation possibly contributing to the development of preeclampsia

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Problem: Preeclampsia is characterized by endothelial activation and excessive inflammation, of which interferon (IFN)-γ is a potent inducer. Eph receptor B4 (EPHB4) also involved in endothelial activation in inflammation. Their role and relationship in preeclampsia remain unclear.

Method of study: Intercellular adhesion molecular (ICAM)-1 was employed as the hallmark of endothelial activation. The serum levels of IFN-γ and the expression of EPHB4 and ICAM-1 were assessed by ELISA, qRT-PCR and WB, respectively. Primary human umbilical vein endothelial cells (HUVECs) were treated with IFN-γ of different concentration or for different times to determine the effect of IFN-γ on EPHB4 and ICAM-1 expression. Overexpression and shRNA constructs, chromatin immunoprecipitation (ChIP) and luciferase assays were conducted to clarify the regulation mechanism of IFN-γ/STAT1 on EPHB4 resulting in HUVECs activation. Endothelial–trophoblast co-culture model was used to illustrate the role of EPHB4 in the process of activated endothelial cells resisting trophoblast invasion.

Results: IFN-γ, EPHB4 and ICAM-1 expression were elevated in preeclampsia. IFN-γ induced HUVECs activation through EPHB4 expression. ChIP and luciferase assays revealed that IFN-γ promoted EPHB4 transcription by STAT-1 binding to EPHB4 promoter. EPHB4 probably involved in resisting trophoblasts displacement by IFN-γ-activated HUVECs.

Conclusion: This study uncovered the character of EPHB4-regulating endothelial activation in the pathogenesis of preeclampsia.

KEYWORDS
endothelial activation, endothelial–trophoblasts coculture, EPHB4, IFN-γ/STAT1, preeclampsia

1 | INTRODUCTION

Preeclampsia is pregnancy-specific systemic disease that is unique to humans and is clinically characterized by the development of new onset hypertension and proteinuria after the 20th week of gestation. Preeclampsia affects approximately 5%–8% pregnancies worldwide and remains a leading cause of maternal and perinatal morbidity and mortality. However, its etiology and pathogenesis are not well understood, and currently, there are no effective interventions and treatments.

As is known, an appropriate ratio of Th1/Th2 at the maternal–fetal interface and in peripheral blood is necessary for the maintenance of pregnancy. Multiple implantation failures resulting in adverse pregnancy outcomes are associated with a predominance of Th1 cells and an
absence of Th2 cells. When maternal vascular cells are exposed to immune imbalance, they are readily to dysfunction, which further aggravate immune disorders. Mounting evidence suggested that preeclampsia is preceded by generalized maternal endothelial cell activation and exaggerated inflammation. Many extracellular signals have been implicated in triggering and/or sustaining the process. Among them, interferon-γ (IFN-γ), as a Th1-derived cytokine, is the most potent inducer of endothelial cell activation. Therefore, we focused on the mechanism of IFN-γ inducing endothelial cell activation in the pathogenesis of preeclampsia.

In addition, normal spiral artery remodelling is essential for placentation during early pregnancy. Otherwise, adverse pregnancy outcome such as preeclampsia may occur. However, most researches focused on the underlying assumption that trophoblasts predominantly control the extent of vascular remodelling by producing adhesion molecular, nitric oxide, vascular endothelial growth factor (VEGF) and so on. However, maternal vascular cells, particularly endothelial cells, are not passive targets during trophoblasts invasion. They were capable to sense stimuli from inflammatory cytokines including IFN-γ or deported trophoblast debris and then became activated state. Demonstratively, activated endothelial cells showed a rejection for trophoblasts invading. Therefore, that endothelial cells became activated as not only a sequence but also an incentive to superficial placenta implantation. The role of endothelial cells in the pathogenesis of preeclampsia is worth further exploring.

Erythropoietin-producing hepatocellular receptor B4 (EPHB4) belongs to the largest family of receptor tyrosine kinases. In mammals, there are nine glycosylphosphatidylinositol-linked EPHA receptors (EPHA1-8, EPHA10) and five EPHB transmembrane receptors (EPHB1-4, EPHB6). EPHB4 and its sole ligand Eph-family receptor-interacting protein B2 (EFNB2) represent venous and arterial markers, respectively. Yet in mammals, EPHB4 is also expressed in some artery, such as human umbilical arterial ECs, arterial tumor and so on. Recent evidence indicated that EPHB4 plays an important role in inflammation, including airway capillaries inflammation, pyogenic granuloma of human gingival, rheumatoid arthritis and osteoarthritis, which all involve in endothelial cells dysfunction. As preeclampsia is similarly characterized by endothelial cell activation and exaggerated inflammation, whether EPHB4 plays a role in maternal endothelial cell activation in preeclampsia remains to be explored.

In this study, we adopted intercellular adhesion molecule-1 (ICAM-1) as endothelial activation marker. First, we investigated the involvement of IFN-γ, EPHB4 and ICAM-1 expression in the pathogenesis of preeclampsia by detecting associated factors expression. Second, we examined the role of IFN-γ signaling in the regulation of EPHB4 in endothelial activation and further uncovered the causality of IFN-γ signaling and EPHB4 via endothelial–trophoblast co-culture model. Finally, we explored the molecular mechanism of IFN-γ signaling modulating EPHB4.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was approved in advance by the Ethics Committee of Union Hospital, Huazhong University of Science and Technology (HUST), Wuhan, China. And written informed consent was obtained from all patients.

2.2 | Study population and biological sample collection

The study was conducted at the Department of Obstetrics and Gynecology, Union Hospital, HUST, Wuhan, China, from October 2015 to February 2016. The study groups consisted of 10 nulliparous women with preeclampsia and 10 nulliparous women with uncomplicated pregnancy. All subjects were in their preterm (<37 weeks of gestation) and underwent cesarean sections. Subjects had routinely undergone detailed history collection, systemic and obstetric examinations, ultrasonographic evaluations, hematologic testing and urine analyses. Preeclampsia was diagnosed as a systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg after 20 weeks, as well as proteinuria of >300 mg/24 h in previously normotensive women. The exclusion criteria included tobacco use, cardiovascular disease history, diabetes mellitus, other severe preexisting metabolic disorders, membrane rupture, fetal anomaly and multiple pregnancies. Blood samples were obtained from 10 nulliparous women with preeclampsia and 10 nulliparous women with uncomplicated pregnancy (mentioned above) in their 16–18 weeks of gestational age. Fasting blood samples were collected from an antecubital vein into plain tubes and then centrifuged at room temperature with a relative centrifugal force of 3000 g for 10 minutes. The aliquots of serum were stored at −80°C for the analyses of IFN-γ level. After delivery of the placenta, placental specimens were taken from the center area around the umbilical cord attachment site and then washed with sterile phosphate-buffered saline (PBS) before storage in liquid nitrogen for RNA and protein extraction.

2.3 | Isolation and culture of human umbilical vein endothelial cells (HUVECs)

Human umbilical cords were collected from term (gestational age of 38–41 weeks), singleton pregnancies with no complications derived by cesarean section. HUVECs were extracted as previously described. Both umbilical veins were isolated, cannulated, purged with cold phosphate-buffered saline (PBS) and incubated for 20–30 min following injection with 0.05% type I collagenase solution at 37°C in circulation water bath. The vessels were then purged with 20 mL PBS. This PBS containing the endothelial cells was collected and centrifuged at 288 g for 5 min. The supernatant was discarded, and the cells were resuspended in DMEM/F-12 (Hyclone, USA) supplement with 15% fetal bovine serum (FBS; Gibco-BRL-Life Technologies, USA), 1% endothelial cell growth supplement (ECGs; ScienCell, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and then plated in gelatin-coated flasks and incubated at 37°C with 5% CO2 in a humidified atmosphere. HUVECs were identified based on their cobblestone morphology and the presence of CD31, which was detected by immunohistochemistry using a specific antibody. The primary cells reached 80-90% confluence after 3 days, and the cells were then subcultured
at a ratio of 1:3 every 4–7 days. Passages two through six were used for the assays.

2.4 | Reagents and antibodies

Recombinant human IFN-γ was purchased from PeproTech (USA). EPHB4, STAT-1 and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (USA). ICAM-1 and β-actin mouse monoclonal antibody was purchased from Origene and Santa Cruz, respectively.

2.5 | Plasmid transfection

Over-expression and inhibition of EPHB4 were achieved by transfection of pG/CMV/EPHB4/IREs/EGFP (pEGFP-EPHB4) and MCS-shEPHB4-EGFP-IREs (EPHB4 shRNA) plasmids separately, which were obtained from GeneChem (Shanghai, China). For STAT1 response, a STAT1 knockdown short hairpin RNA construct MCS-shSTAT1-EGFP-IREs (sh-STAT1) was purchased from GenePharma (Shanghai, China). And the empty vectors were used as a negative control. The transient transfection of HUVECs was performed using Neofect™ reagent (Neofect Bio-technologies, Beijing, China). For STAT1 response, EPHB4 promoter plasmids, pRL-SV40 plasmids as internal control and sh-STAT1 plasmids were co-transfected into HUVECs. After the recommended time, IFN-γ was then added into the media for approximately 24 h. Then, cells were collected with 70 μL of passive lysis buffer. Luciferase reporter assay was implemented with a dual-luciferase reporter assay system (Promega) and measured using a Veritas Microplate Luminometer (Turner BioSystems, USA). The transfections were conducted in triplicate, and the activity of promoter was recorded as Firefly/Renilla ratio using mean ± SD of three independent experiments.

2.6 | RNA extraction, cDNA preparation and RT PCR

RNA was extracted; reverse transcription PCR and data analysis were conducted as previously described. The sequences of primers used herein were provided in Supporting information. PCR amplification was performed in triplicate using the StepOne™ Real-Time PCR system under the following conditions: 95°C for 30 seconds, followed by 40 cycles at 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 60 seconds. Relative mRNA expressions levels were normalized to the constitutive transcriptional level of β-actin in using the comparative Ct \((2^{-\Delta\Delta C_t}})\) method.

2.7 | Western blot analysis

Total proteins from cultured cells were extracted using RIPA buffer, and protein concentrations were measured using the BCA method. Thirty micrograms of total proteins per sample was subjected to SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST [20 mmol/L Tris (pH 7.4), 150 mmol/L sodium chloride, 0.1% Tween-20] for 60 min at room temperature. The membranes were incubated with specific primary antibodies at 4°C overnight. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and developed using an enhanced chemiluminescence detection system.

2.8 | Dual-luciferase reporter assays

HUVECs were cultured in a 24-well plate in advance and co-transfected with EPHB4 promoter plasmids (the firefly luciferase gene ligated to the EPHB4 promoter was performed with pGL3 plasmids) and endogenous control pRL-SV40 (Promega) containing the Renilla luciferase gene using jetPRiMe™ (Polyplus Transfection, Illkirch, France) according to the manufacturer's protocol. For STAT1 response, EPHB4 promoter plasmids, pRL-SV40 plasmids as internal control and sh-STAT1 plasmids were co-transfected into HUVECs. The red “island” areas were calculated as the number of pixels, which was used to value HTR-8/SVneo cells ability to displace HUVECs.

2.9 | Chromatin immunoprecipitation (ChIP)

The HUVECs were cultured to 80% confluence in 150-mm² dishes in DMEM/F-12 medium supplemented with 15% FBS. Cells were then incubated with PBS, 10 ng/mL IFN-γ, 10 ng/mL IFN-γ + sh-STAT1 for 48 h. ChIP assays were performed using the ChIP Express kit (Millipore) following the manufacturer’s protocol. DNA was extracted and analyzed. A rabbit anti-STAT1 antibody (Cell Signaling Technology, USA) and rabbit IgG (supported by ChIP Express kit) were used in these experiments. Real-time quantitative PCR (qPCR) was performed with PCR primer sets targeting the STAT1 binding sites within the EPHB4 promoter.

2.10 | Trophoblast-endothelial co-culture

Primary HUVECs were grown onto plastic microscope slide coverslips in six-well plates until confluent and then underwent different treatments for 24 h. After HUVECs were labeled with 1 μM CMFDA (cell tracker green, Molecular Probes, Inc., UK) for 2 h, HTR-8/SVneo cells were labeled with 1 μM CMTPX (cell tracker red, Molecular Probes, Inc., UK) and then added to the HUVEC monolayer \((2 \times 10^5/ \text{well})\) for another 24 h of culturing. All co-cultures were then washed and fixed with 4% paraformaldehyde. Coverslips were mounted onto glass microscope slides and examined with an Olympus IX71 confocal microscope. The area of the HTR-8/SVneo cell “island” (red) within the HUVEC monolayer (green) was then quantified by ImageJ software. All images were converted to 8-bit gray scale with a defined preset threshold and made into binary images of black and white only. The red “island” areas were calculated as the number of pixels, which was used to value HTR-8/SVneo cells ability to displace HUVECs.
2.11 | Statistical analysis

The data were presented as the mean ± SD from at least three independent experiments. The one-way ANOVA and Student’s t tests were applied to analyze between groups in normal distribution. For variables in non-normal distribution, the Mann–Whitney U test was used for statistical significance of differences. P<.05 was valued as statistically significant (*P<.05, **P<.01, ***P<.001).

3 | RESULTS

3.1 | IFN-γ, EPHB4 and ICAM-1 expression are elevated in preeclampsia

Clinical characteristics of the patient are summarized in Table 1. Figure 1a showed that serum level of IFN-γ was significantly increased in placenta from preeclamptic pregnancy compared with normal group (P=.012, by Mann–Whitney U test). The results in Fig. 1b indicated that, compared with the normal pregnancy, the preeclamptic placenta showed significantly higher EPHB4 and ICAM-1 mRNA expression levels. To analyze EPHB4 and ICAM-1 more quantitatively and objectively, we performed Western blot analysis of placenta sample lysates. Figure 1c shows an example for protein expression of EPHB4 and ICAM-1 in normal placenta and in preeclamptic tissues. The levels were compared between normal pregnancy (labeled normal in the figure) and preeclampsia (labeled preeclampsia in the figure). A significant difference was observed between the two groups: EPHB4 increased about 6.2-fold (P=.0028) and ICAM-1 increased 12.0-fold (P<.0001) in preeclamptic placenta.

3.2 | IFN-γ induces primary HUVECs activation through EPHB4 expression

To investigate the effect of IFN-γ on HUVECs activation, HUVECs were treated with different concentrations of IFN-γ (from 10 to 100 ng/mL) for 24 h. As shown in Fig. 2a,b, IFN-γ enhanced EPHB4 and ICAM-1 expression in HUVECs, as determined by Western blots. The effect was dose dependent. To further characterize the effects of IFN-γ on ICAM-1 expression in HUVECs, time course experiment was carried out in HUVECs stimulated with 10 ng/mL IFN-γ (Fig. 2d,e). The maximal effect was observed at 72 h. Furthermore, EPHB4 increased coincidently with ICAM-1 under different concentrations of IFN-γ (Fig 2c) and different time courses (Fig 2f), which prompted us to investigate the effect of EPHB4 on HUVECs activation. First, EPHB4 shRNA and pEGFP-EPHB4 overexpression plasmids were transiently transfected into HUVECs to silence and upregulate EPHB4 expression in HUVECs, respectively. EPHB4 mRNA expression in sh-EPHB4 group was substantially decreased than that in control HUVECs (Fig 3a, P<.01), and distinctly elevated in pEGFP-EPHB4 group (Fig 3b, P<.01). There was no significant difference between empty control and negative control (Fig. 3a,b). Similarly, EPHB4 protein expression dropped in sh-EPHB4

TABLE 1 Baseline characteristics of study population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Preeclampsia (n=10)</th>
<th>Normal (n=10)</th>
<th>P* value preeclampsia vs normal</th>
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<tr>
<td>Maternal age (years)</td>
<td>29.9 ± 1.1</td>
<td>28.8 ± 1.1</td>
<td>P&gt;.05</td>
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<tr>
<td>Maternal weight (kg)</td>
<td>67.9 ± 1.2</td>
<td>67.2 ± 2.4</td>
<td>P&gt;.05</td>
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<tr>
<td>Gestational age (weeks)</td>
<td>34.2 ± 0.5</td>
<td>35.1 ± 0.5</td>
<td>P&gt;.05</td>
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<td>Systolic blood pressure (mm Hg)</td>
<td>166 ± 5</td>
<td>113 ± 3</td>
<td>P&lt;.05</td>
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<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>110 ± 3</td>
<td>64 ± 3</td>
<td>P&lt;.05</td>
</tr>
<tr>
<td>Proteinuria (g/d)</td>
<td>100% (10/10)</td>
<td>0 (0/10)</td>
<td>P&lt;.05</td>
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<tr>
<td>Body weight of infant (g)</td>
<td>1826 ± 66</td>
<td>2234 ± 99</td>
<td>P&lt;.05</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM or percentage (number/total). *P-value<.05 was considered as significant difference.
HUVECs (Fig. 3c, P < .01) and increased in the pEGFP-EPHB4 group (Fig. 3D, P < .001). Next, Western blotting was used to detect the expression of ICAM-1 in HUVECs after transfection with EPHB4 plasmids. Quantitative analysis showed that ICAM-1 protein expression was reduced in sh-EPHB4 HUVECs and increased significantly in pEGFP-EPHB4 than in respective control HUVECs (Fig. 3e, P < .05, P < .001). Besides, ICAM-1 reached its maximum expression trend in HUVECs transfected with the EPHB4 overexpression plasmid in the presence of IFN-γ without statistical significance (Fig. 4d, f, P < .001), and this effect was notably blocked by cotransfection with EPHB4 shRNA (Fig. 4a, c, P < .001). Taken together, these results suggested that EPHB4 might involve in the process of IFN-γ inducing HUVECs activation.

3.3 IFN-γ/STAT1 signaling promotes EPHB4 expression, contributing to HUVECs activation

As is known, signal transducers and activators of transcription (STAT) 1 signal transduction are initiated upon IFN-γ binding to its receptor, which activates Janus tyrosine kinases (JAKs). STAT1 is phosphorylated forming dimerization, which translocate to the nucleus and bind to specific sequences within the promoters of target genes to modulate transcription. Gene expressions induced by STAT1 signaling may be associated with physiological changes, such as cell growth inhibition, differentiation and apoptosis. It has been reported that IFN-γ induces the JAK/STAT1 signaling pathway in endothelial cells. Therefore, in our study, silencing STAT1 expression by shRNA plasmid in HUVECs was employed to explore whether IFN-γ/STAT1 signal played a role in modulating EPHB4 and ICAM-1 expression. As was seen in Fig. 4, no matter in sh-EPHB4 group or in pEGFP-EPHB4 group, when IFN-γ signaling was blocked, both EPHB4 expression and ICAM-1 expression in HUVECs were significantly declined compared to that under IFN-γ condition (P < .001, P < .05, respectively).

3.4 STAT1 binds directly to the EPHB4 promoter

To determine whether STAT1 could increase the transcription of EPHB4, the EPHB4 promoter luciferase reporter and its truncation vectors were transfected into HUVECs (Fig. 5a). Dual-luciferase assay indicated that the fragment of ~355/~131 bp relative to transcription initial site was essential for the EPHB4 promoter activities (Fig. 5b). Knockdown of STAT1 resulted in significantly decreased promoter activities of EPHB4 in HUVECs (Fig. 5c, P < .001). Luciferase activity was measured following incubation with IFN-γ (10 ng/mL) for 24 h. The fragment of ~355/~131 bp contains two putative response element box “TTCCC.” To further determine the key transcription factor binding sites involved in our system, site-directed mutagenesis was conducted to create single STAT1 binding site mutants within the EPHB4 promoter (mut1 and mut2 in Fig. 5d), as well as a double mutant in which both binding sites were mutated (mut3 in Fig. 5d). Then, the wild-type EPHB4 promoter (serve as control) and the three mutants were transfected into HUVECs individually, and the Renilla luciferase gene was co-transfected as an internal control. Firefly and Renilla luciferase activities were measured after incubation with IFN-γ (10 ng/mL) for 24 h. Compared with control group, firefly/Renilla luciferase activities in mut1 and mut3 dropped distinctly, while mut2 group showed...
no significant difference (Fig. 5e, P < .01, ns: not significant). Results indicated the effective binding sites may locate in −166 to −162 bp.

Next, ChIP and qPCR were applied to measure the enrichment of STAT1 on the EPHB4 promoter with primer sets spanning its binding sites. Knockdown of STAT1 with shRNA construct decreased the binding of STAT1 on the −232/−91 region of the EPHB4 promoter in HUVECs (Fig. 5f, P < .001, P < .05). These results showed that STAT1 directly interacted with the binding site within the EPHB4 promoter to increase its transcription.

3.5 | IFN-γ-activated HUVECs resist trophoblasts displacement via promoting EPHB4 expression in endothelial cells

Our results above confirmed the hypothesis that IFN-γ regulated HUVECs activation through EPHB4 expression. As mentioned in introduction part, endothelial activation is not only a sequence but also an incentive to superficial placentation implantation, which is widely recognized as one of the pathogenesis of preeclampsia. What’s more, endothelial–trophoblast interaction is crucial for vascular remodeling during placentation. Thus, we used endothelial–trophoblast co-culture model to further study the role of EPHB4 expression in cell biological function of IFN-γ-activated HUVECs. After HUVEC monolayer was treated with different conditions for 24 h in advance, HTR-8/SVneo cells were added to the co-culture system for another 24 h. Results revealed that the area of trophoblasts replacement within the HUVECs monolayer in IFN-γ treatment group decreased significantly compared to PBS group (Fig. 6a, P < .001). Then, EPHB4 was knocked down by a specific shRNA in HUVECs. We then transfected HUVECs with empty vector (NC1) or EPHB4 silencing plasmids (shEPHB4). The results showed that EPHB4 downregulation in HUVECs...
led to a significant increase of trophoblasts invasion and displacement (Fig. 5b, \( P < .01 \)). Furthermore, EPHB4 overexpression in HUVECs obviously resisted trophoblasts invasion and occupation (Fig. 5c, \( P < .001 \)). Thus, the IFN-γ-activated HUVECs showed an inhibited effect on trophoblasts invasion and replacement, which may be associated with EPHB4 enhancement in HUVECs.

4 | DISCUSSION

It is widely accepted that preeclampsia features systemic maternal endothelial dysfunction. When exposed to inflammatory stimuli, endothelial cells turned activated and then highly expressed ICAM-1.29,30 Cross talk between ICAM-1 and its ligand LFA-1 (leukocyte adhesion molecule-1) recruited increased numbers of abortogenic Th-1 cytokine-expressing cells at the implantation site, resulting in the predominance of Th-1 cytokine profile, IFN-γ included.21 This forms focal damage of the placental barrier and a vicious cycle of excessive inflammation, which may account for high levels of IFN-γ and ICAM-1 in preeclamptic pregnancies.

In fact, mounting evidence identified the role of IFN-γ/STAT1 signaling in modulating ICAM-1 expression.32–34 Here, we identified a unique molecular mechanism for regulation of endothelial activation in preeclampsia. Evidence in support of this pathway includes the following: (i) elevated serum level of IFN-γ and increased expression of EPHB4 and ICAM-1 in placental tissues from preeclampsia patients, (ii) after treating HUVECs with IFN-γ, the EPHB4 expression changes coincided with the ICAM-1 expression changes, (iii) ICAM-1 enhanced its expression following transfection with EPHB4 overexpression plasmid under IFN-γ treatment while EPHB4 shRNA blocked this effect, (iv) IFN-γ/STAT1 signal was blocked, EPHB4 was downregulated, contributing to reduced ICAM-1 expression in HUVECs. (v) EPHB4 promoter deletional and mutational analyses uncovered a regulatory region (−166 to −162 bp) that contains STAT1 response elements in HUVECs, and ChIP analysis revealed that STAT1 binds directly to the EPHB4 promoter. Together, these observations provide a new mechanistic model linking IFN-γ/STAT1, EPHB4 and ICAM-1 to the pathogenesis of preeclampsia. Although EPHB4 was reported to play an important role in various inflammatory diseases related to endothelial cells dysfunction,23 this is the first time we uncovered its role in endothelial activation in preeclampsia.

More importantly, as conflicting results demonstrated IFN-γ mediated the resistant effect of activated endothelial cells on trophoblasts invasion,17,36 the mechanism remains to be further studied. Interestingly, we further discovered that IFN-γ-activated HUVECs resisted trophoblasts invasion via regulating EPHB4 expression in...
endothelial cells. In fact, HUVECs with EPHB4 upregulation showed a rejection for trophoblasts invasion and replacement, which may be favored by the following results. Researches ex vivo show that EPHB4 over-expressing in endothelial cells controls cellular repulsion and segregation from adjacent cells in angiogenesis and vessel assembly.  

![FIGURE 5](image)

**FIGURE 5** IFN-γ increased the transcription of EPHB4 through STAT1 binding directly to the EPHB4 promoter. (a) Schematic of the EPHB4 promoter showing the five putative STAT1-binding sites and schematic diagram of the EPHB4 promoter constructs utilized for dual-luciferase assays. (b) Dual-luciferase assay indicated that −335/−131 fragment relative to transcription initial site was essential for the promoter activities of EPHB4 (**P<.001 by one-way ANOVA). (c) When incubating with IFN-γ for 24 h, HUVECs showed decreased luciferase activities of pGL3-EPHB4 (~999/+100) after transfection of sh-STAT1 compared to those transfected with empty vector (**P<.001 by unpaired Student’s t test). (d) A schematic representation of −335/−131 fragment in the EPHB4 promoter containing two possible binding sites. Mutant EPHB4 promoters were designed as following: mut1 for mutant at −165 & −163 bp site of EPHB4 promoter, mut2 for −131 & −129 bp and mut3 for both. (e) Levels of luciferase expression in HUVECs transfected with mutant EPHB4 promoter. HUVECs were cotransfected with plasmids expressing the 435 bp wild-type EPHB4 promoter ligated to a firefly luciferase reporter gene and a plasmid encoding the Renilla luciferase reporter gene. The cells were then treated with IFN-γ for 24 h before luciferase activity was measured. Next, comparisons were made between HUVECs transfected with plasmids expressing the 435 bp wild-type EPHB4 promoter and the EPHB4 mutant promoter. Decreased luciferase expression was seen in mut1 and both mutant groups. (f) The human EPHB4 gene promoter was analyzed, and primers for chromatin immunoprecipitation (ChIP) were designed according to the sequences of potential binding sites. ChIP assays were conducted in HUVECs treated with PBS, IFN-γ, IFN-γ plus sh-STAT1 using the indicated antibodies, respectively. qPCR was performed with primer set1, set2 and set3 (**P<.05 and ***P<.001 by two-way ANOVA). Data from three independent experiments were reported as mean ± SD.
spirals arteries shifts from EFNB2+/EPHB4− at gestation day (gd) 6.5 to EFNB2+/EPHB4+ at gd10.5 and onwards. These evidence prompted that EPHB4 may be a vital regulator for spiral arteries to circumvent uncontrolled invasion of trophoblasts. The possible mechanism may attribute to bidirectional identification between trophoblasts expressing EFNB2 and endothelial cells labeling EPHB4. Thus, we are...
convinced that EPHB4, as a target of IFN-γ/STAT1 signaling, was not only an inducer of endothelial activation, but also involve in poor trophoblasts invasion during vascular remodeling.

Therefore, our results indicate again that endothelial cell dys-function could not only be sequent to the maternal symptoms of preeclampsia seen in later gestation, but also may be one of the underlying pathogenic triggers contributing to the superficial invasion of the spiral artery by trophoblasts in early pregnancy.

In summary, this study found that over-expressed EPHB4 was associated with increased ICAM-1 expression in preeclampsia patients, and EPHB4 expression was enhanced by IFN-γ in a dose-dependent manner confirmed in a cell culture system in vitro. Furthermore, IFN-γ increased the transcription of EPHB4 through STAT1 binding directly to the EPHB4 promoter. Eventually, we identified EPHB4 involved in the process of IFN-γ-activated HUVECs resisting trophoblasts displacement. Thus, this work uncovered the character of EPHB4-regulating endothelial activation in the pathogenesis of preeclampsia, which may provide several clinical applications and open up new therapeutic strategies for the treatment of preeclampsia.

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REFERENCES


**SUPPORTING INFORMATION**

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