HOXA9 transcriptionally regulates the EPHB4 receptor to modulate trophoblast migration and invasion

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ABSTRACT

Introduction: Functional placenta formation is crucially dependent on extravillous trophoblast migration and invasion. EPHB4 has been identified to play a negative but important role in regulating trophoblast biological function, whereas the upstream regulation mechanism remains unknown. As reported, there is a transcriptional stimulation of EPHB4 expression consequent to HOXA9 activation in endothelial cells (ECs). Therefore, this study is conducted to investigate the role of HOXA9 and its relationship with EPHB4 in trophoblast cells.

Method: Both mRNA and protein expression levels of HOXA9 and EPHB4 were measured in preeclamptic placenta (n = 15) and normal placenta (n = 15). Next, the expression and location of HOXA9 and EPHB4 in first-trimester villi were shown via immunohistochemistry. Trophoblast cell line HTR-8/SVneo was used to explore the effect of HOXA9 on EPHB4 expression and trophoblast bioactivity by gain- and loss-of-function studies. In addition, chromatin immunoprecipitation (ChIP) and luciferase assays were conducted to clarify the regulation mechanism of HOXA9 on EPHB4 expression in HTR-8/SVneo.

Result: HOXA9 and EPHB4 expression were increased in preeclamptic placenta compared with normal placenta. HOXA9 could promote EPHB4 expression and impaired HTR-8/SVneo cells migration and invasion. ChIP and luciferase assays revealed that HOXA9 could directly bind to EPHB4 promoter and promoted its transcription.

Conclusion: HOXA9 transcriptionally regulated EPHB4 expression to modulate trophoblasts migration and invasion, which may suggest a contribution of HOXA9-EPHB4 in the poor placentation in the pathogenesis of preeclampsia.

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1. Introduction

Preeclampsia is a specific hypertensive disorder that occurs during the second half of pregnancy and one of the leading causes of worldwide maternal-fetal morbidity and mortality [1,2]. It is well known that preeclampsia is often associated with superficial trophoblast invasion, insufficient spiral artery remodeling and subsequent placental hypoxia, as well as ischemia/reperfusion injury [3–5]. Thus, researches on the mechanism of trophoblast invasion are of great value for early diagnosis and intervention of pregnant complications.

Ephrins and their receptors (Ephs), identified in the late 1980s, are known as the largest family of receptor tyrosine kinases [6]. In mammals, the ephrins/Ephs system is composed of eight ephrin ligands (EFNA1–5, EFNB1–3) and fourteen Eph receptors (EPHA1–8, EPHA10, EPHB1–4, EPHB6) [7,8]. Recent researches have demonstrated ephrins/Ephs signal pathway is essential during placenta [9]. Among them, EFN2/EphB4 at maternal-fetal interface has been identified to play a critical role in directing trophoblast invasion [10]. In our previous study, we have confirmed EPHB4 downregulated HTR8/SVneo functions in the pathogenesis of preeclampsia [11]. However, the regulatory mechanism for EPHB4 expression in trophoblast cells still remains to be elucidated.

Homeobox morphoregulatory genes, first identified in Drosophila, encode for transcription factors–transcriptional DNA-binding motif and are mostly arranged in four paralogous clusters (A, B, C, and D) [12]. Homeobox genes regulate organogenesis [13], vessel remodeling as it occurs during angiogenesis and atherosclerosis [14], as well as the development of the cardiovascular system during embryogenesis [15]. The special pattern of homeobox genes in vascular development implicated their
essential roles in placentation. For example, previous studies have identified the role of HOXA11, HOXB6 and HOXC6 in the trophoblast differentiation process [16,17]. In this study, through mining of public databases, we focused on HOXA9 gene. The HOXA9 gene exhibits three exons resulting in the production of two kinds of proteins. One HOXA9 protein (HA-9A) is uniquely expressed in fetal development and a distinct HOXA9 isoform (HA-9B) is expressed in various tissues of the fetal and adult organism [18]. Both proteins share a common exon encoding the homeodomain. Mouse bone marrow cells with HOXA9 overexpression induced stem cell expansion, invariably progressing to acute myeloid leukemia within 3–10 months [19]. Knockdown of HOxa9 in bone marrow cells induces an elevated apoptosis of primitive thymocytes and contributes to a disorder of early T-cell development in mice [20]. Since HOXA9 plays an important role in controlling cancer cell biological behavior [21], we are convinced that it may work in modulating trophoblast functions, which share many striking similarities with the characteristics of malignant cells. Moreover, the regulation of HOXA9 on EPHB4 was demonstrated in endothelial cells [22]. Therefore, we sought to determine whether HOXA9 and EPHB4 have cooperating roles in regulating the trophoblast migration and invasion, which might involve in the pathogenesis of preeclampsia.

To data, the expression of HOXA9 has not been reported in first trimester placenta, and the relevance of HOXA9 to EPHB4 expression and invasiveness in trophoblast cells is unknown. In this study, we examined the HOXA9 and EPHB4 localization, expression and correlation in preterm preeclamptic placentas to that of normal controls, as well as in first-trimester human placenta. We also studied the role of HOXA9 in regulating EPHB4 expression and trophoblast invasion.

2. Materials and methods

2.1. Patient tissue samples

This study was approved in advance by the Ethics Committee of Union Hospital, Huazhong University of Science and Technology (HUST), Wuhan, China. And all patients provided written informed consents. The study subjects consisted of 15 nulliparous women with preeclampsia and 15 nulliparous women with uncomplicated pregnancy collected from October 2015 to February 2016. All of them underwent cesarean sections in preterm. During pregnancy, they had routinely undergone detailed history-collection, systemic and obstetric examinations, hematology testing, ultrasonographic evaluations, and uterine evaluation. Preeclampsia was diagnosed as a systolic blood pressure $\geq 140$ mmHg and/or diastolic blood pressure $\geq 90$ mmHg after 20 weeks, as well as proteinuria of $>300$ mg/24 h in previously normotensive women. The exclusion criteria included cardiovascular disease history, diabetes mellitus, other severe pre-existing metabolic disorders, fetal anomaly, multiple pregnancies and membrane rupture. After placenta delivery, placental specimens on the chorionic side were taken from the center area around the umbilical cord attachment site, washed with sterile phosphate-buffered saline (PBS) and then stored in liquid nitrogen for RNA, protein extraction and immunohistochemistry.

In addition, a total of 10 first-trimester (6–9 weeks) human placental villi were obtained from women undergoing elective termination of pregnancy. Samples were fixed in 4% formaldehyde and embedded in paraffin for sectioning. Pregnant women with the following complications were excluded: severe reproductive tract infection, hypertension, heart disease or other chronic diseases, pregnancy complication history (e.g., preeclampsia), other adverse pregnancy outcomes (e.g., recurrent miscarriage), and uterine surgery history.

2.2. Immunohistochemistry

Immunohistochemical staining was performed as previously described. After rehydration, paraffin-embedded placental tissues were heated in a citrate buffer (pH = 6.0) for antigen retrieval for 15 min, followed by blockade with 10% normal goat and rabbit serum for 1 h. Endogenous peroxidase activity was quenched by incubation in 3% H$_2$O$_2$ for 20 min. Sections were incubated for 1 h at room temperature with primary antibody (HOXA9, 1:250, D66686, Affinity; EPHB4, 1:250, #14960, Cell Signaling Technology; cytokeratin7, 1:200, MA1024, Boster). Slides were incubated for another 30 min with HRP-conjugated goat anti-rabbit and rabbit antimouse IgG (1:1000, Santa Cruz). Then, peroxidase reactivity was detected using a diaminobenzidine (DAB) substrate kit.

2.3. Cell culture

The HTR-8/SVneo immortalized EVT cell line was a kind gift from Dr. Charles Graham (Queen’s University, Canada). Cells were cultured in RPMI 1640 (Hyclone, USA) with 10% fetal bovine serum (FBS, Gibco-BRL-Life Technologies, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in 5% CO$_2$. Culture medium was changed every 48 h.

2.4. Plasmids constructs and transfection

Human HOXA9 expression vector (HOXA9) and the oligonucleotides encoding short hairpin RNA (shRNA) specific for HOXA9 (shHOXA9) were obtained from Genechem (Shanghai, China). Stable cell lines were constructed following the steps mentioned below. The HTR-8/SVneo cells were seeded into a 24-well plate at a concentration of $1.5 \times 10^5$ cells per well for 24 h before transfection. Transfection of HOXA9 expression vector and HOXA9 shRNA plasmid was performed by using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer’s instructions. The empty vector (mock) and scramble shRNA (sh-Scb) were applied as controls, respectively. After transfection for 6 h, fresh growth medium was changed and cells were passaged at a 1:10 dilution into fresh medium after the transfection for 24 h. Selective medium was used for screening colonies during the next two weeks. Then positive cells were seeded into a 96-well plate at one cell per well, cultured and expanded in selective medium. In vitro transfection efficiency was examined by detecting the expression of green fluorescent protein (GFP) by fluorescence microscope.

To restore the HOXA9-induced up-regulation of EPHB4, stable cell lines were transfected with the shRNA targeting the encoding region of EPHB4 (shEPHB4) by Genesilencer Transfection Reagent (Genlantis, San Diego, CA). The EPHB4 expression vector was transfected into cells stably transfected with shHOXA9. The empty vector (mock) and scramble shRNA (sh-Scb) were applied as controls, respectively.

2.5. Reverse transcription, semi-quantitative PCR and quantitative real-time PCR

RNA was extracted, reverse transcription, RT-PCR and data analysis were conducted as previously described [23]. The sequences of primers used herein were provided in Supplementary file. PCR amplification was performed in triplicate by the
StepOne™ Real-Time PCR System and conducted under the following conditions: 95 °C for 30 s, followed by 40 cycles (95 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s). Relative mRNA expressions were normalized to the constitutive transcriptional level of β-actin using the comparative Ct (2^−ΔΔCt) method.

2.6. Western blot analysis

Total proteins were extracted from cultured cells using RIPA buffer and protein concentrations were measured using the BCA method. Thirty micrograms of total proteins per sample was

<table>
<thead>
<tr>
<th>Clinical characteristics of normal and preeclamptic pregnancies</th>
<th>Preeclampsia (n = 15)</th>
<th>Normal (n = 15)</th>
<th>P value Preeclampsia vs Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>29.7 ± 0.9</td>
<td>29.1 ± 1.2</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Maternal weight (kg)</td>
<td>67.3 ± 1.0</td>
<td>66.6 ± 1.7</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Gestational age (week)</td>
<td>34.3 ± 0.4</td>
<td>34.6 ± 0.5</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>164 ± 3</td>
<td>112 ± 2</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>106 ± 2</td>
<td>65 ± 2</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>100% (10/10)</td>
<td>0 (0/10)</td>
<td>P &lt; 0.05</td>
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<tr>
<td>Body weight of infant (g)</td>
<td>1808 ± 46</td>
<td>2147 ± 75</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± SEM or percentage (Number/Total). *P-value < 0.05 was considered as significant difference.

Fig. 1. HOXA9 and EPHB4 localization and expression in different groups of placenta. Representative immunohistochemical images of HOXA9 and EPHB4 localization in normal (A and C) and preeclamptic placenta tissues (B and D), respectively. Photomicrographs were taken at magnification of ×100 (left panels) and ×200 (right panels). (E) RT-PCR analysis revealed that preeclamptic placenta produced significantly higher transcriptional level of HOXA9 and EPHB4 than that of normal control (P = 0.0004). (F) Representative western blot performed on protein extracts recovered from normal and preeclampsia placenta tissues. Results showed that the protein level of HOXA9 and EPHB4 were higher in preeclampsia placenta tissues than that of normal controls (P = 0.0004). mRNA and protein data were normalized to β-actin and are given as a multiple of the control value. (G) There was a positive correlation between HOXA9 and EPHB4 protein levels in preeclamptic placenta tissues (Pearson r = 0.8879, P < 0.001). Data presented as mean ± SD are from 15 independent experiments (***P < 0.001, Mann–Whitney U test).
Fig. 2. Representative microscopy images of placental villi. Photomicrographs of HOXA9 (A), EPHB4 (B) and CK7 (C) staining in placenta villi. Peptide-blocking reagent without antibody was applied as negative controls (D). Photographs were taken at magnification of ×100 (left panels) and ×200 (right panels).
subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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, then subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and eventually developed using an enhanced chemiluminescence detection system.

2.7. Dual-luciferase reporter assays

HTR-8/SVneo cells were cultured in a 24-well plates in advance and co-transfected with EPHB4 promoter plasmids (the firefly luciferase gene ligated to the EPHB4 promoter) was performed with pGL3 plasmids, 30 ng) and endogenous control pRL-SV40 (10 ng, Promega) containing the Renilla luciferase gene using jetPRiMe™ (Polyplus Transfection, Illkirch, France) following the manufacturer’s protocol. Twenty-four hrs post-transfection, firefly and Renilla luciferase activities were consecutively measured, according to the dual-luciferase assay manual (Promega). For EPHB4 promoter activities, the luciferase signal was normalized by firefly/Renilla ratio. The transfections were conducted in triplicate, and the activity of promoter was recorded as Firefly/Renilla ratio using mean ± SEM of three independent experiments.

2.8. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed according to the instructions of EZ-ChIP kit (Upstate Biotechnology, Temacula, CA). DNA was sonicated into fragments of an accordingly size (shown in supplemental file). Real-time quantitative PCR (qPCR) was performed with PCR primer sets (shown in supplemental file) targeting the HOXA9 binding sites within the EPHB4 promoter.

2.9. Cell migration and invasion assays

The migration assay was performed with 24-well transwell inserts that have 6.5-mm polycarbonate membranes with pores 8.0 μm in size (Corning, USA). A total of 2 × 10^5 transfected cells were resuspended in 200 μl serum-free RPMI1640 in the upper chamber and added to transwell insert with 500 μl complete culture medium in the lower chamber. After cells invaded for 24 h at 37 °C in a CO2 incubator, inserts were fixed in 4% paraformaldehyde and stained with 0.2% crystal violet. Migrating cells were counted at a magnification of 200× using an inverted phase-contrast microscope (Olympus, Japan). Average number of five random fields was calculated for each insert. Matrigel invasion assay was performed using membranes pre-coated with 50 μl Matrigel mix (BD Science,

Fig. 3. HOXA9 facilitated the expression of EPHB4 in HTR-8/SVneo cell lines. Western blot (A) and real-time quantitative RT-PCR (B) indicated that stable transfection of sh-HOXA9 into HTR-8/SVneo cells resulted in down-regulation of EPHB4, than those of scramble shRNA (sh-Scb) transfected cells (**P < 0.001 vs. sh-Scb by Newman-Keuls test). Western blot (C) and real-time quantitative RT-PCR (D) indicated that stable transfection of HOXA9 resulted in increased protein and transcript levels of EPHB4 in HTR-8/SVneo cells, when compared to those stably transfected with empty vector (mock) (*P < 0.05, **P < 0.01 vs. mock by Student’s-t-test). The empty vector (mock) and scramble shRNA (sh-Scb) were applied as controls.
2.10. Statistical analysis

Data are presented as the mean \( \pm \) SEM of at least three independent experiments. Results were analyzed by Student’s t-test, one-way ANOVA followed by Newman-Keuls multiple comparison test and two-way ANOVA using GraphPad Prism 5. P value < 0.05 was considered to be statistically significant.

3. Results

3.1. HOXA9 and EPHB4 expression are elevated in preeclamptic placental tissues

Clinical characteristics of the study population are summarized in Table 1. As can be seen, HOXA9 and EPHB4 protein was abundantly expressed in placenta from preeclampsia (Fig. 1B and D) compared with normal placenta (Fig. 1A and C). EPHB4 protein was almost in the cytomembrane, while HOXA9 protein entered into the nuclear of stroma cell of preeclamptic placenta. Results also indicated that compared with the normal pregnancy, placenta from preeclampsia showed significantly higher HOXA9 and EPHB4 mRNA expression levels (Fig. 1E, both \( P = 0.0004 \)). To analyze HOXA9 and EPHB4 more quantitatively and objectively, we performed Western blot analysis of placenta sample lysates. Results showed an example for protein expression of HOXA9 and EPHB4 in normal placenta (normal in figure) and in preeclamptic placenta tissues (preeclampsia in figure). A significant difference was observed between the two groups: HOXA9 increased about 1.6-fold (\( P < 0.001 \)) and EPHB4 increased 1.8-fold (\( P < 0.001 \)) in preeclamptic placenta (Fig. 1F). Besides, they exhibited a positive correlation between HOXA9 protein and EPHB4 protein levels in preeclamptic placenta tissues (Fig. 1G, \( P < 0.001, r = 0.8879 \)).

3.2. Location of HOXA9 and EPHB4 expression in human first-trimester villi

We examined HOXA9 and EPHB4 distribution in human placental of early gestation (6–9 week) by IHC. We can see, HOXA9

![Fig. 4] Knockdown of HOXA9 enhanced the migration and invasion of HTR-8/SVneo cells through targeting EPHB4. (A) Western blot indicated that stable transfection of sh-HOXA9 into HTR-8/SVneo cells decreased protein levels of HOXA9, when compared to those transfected with sh-Scb. Transfection of EPHB4 restored the down-regulation of EPHB4 induced by stable HOXA9 knockdown (\( **P < 0.01, ***P < 0.001 \) vs. sh-Scb by Newman-Keuls test). (B) Transwell assay showed the migration of HTR-8/SVneo cells with HOXA9 silencing was significantly increased when compared to those transfected with sh-Scb. Transfection of EPHB4 attenuated the migration of HOXA9 silencing cells (\( **P < 0.01, ***P < 0.001 \) vs. sh-Scb by Newman-Keuls test). (C) Matrigel invasion assay indicated the elevated invasion capabilities of HOXA9 knocking down HTR-8/SVneo cells than those transfected with sh-Scb. Moreover, transfection of EPHB4 impaired the invasion of HOXA9 silencing cells (\( **P < 0.01, ***P < 0.001 \) vs. sh-Scb by Newman-Keuls test). Photographs were taken at magnification of \( \times 200 \).
staining were exhibited in all nuclei of the villi, including in the cytotrophoblast cells (Fig. 2A), while only the trophoblast of first-trimester placental villi showed membrane EPHB4 immunoreactivity (Fig. 2B). Immunoreactivity to cytokeratin7, as positive control, was used to identify trophoblast cells in villi (Fig. 2C). Negative control was shown (Fig. 2D).

3.3. HOXA9 facilitated the expression of EPHB4 in HTR-8/SVneo cell lines

To explore the direct effects of HOXA9 on EPHB4 expression in HTR-8/SVneo cell lines, the sh-HOXA9 was stably transfected into HTR-8/SVneo cell lines, resulting in decreased protein and transcript levels of HOXA9 and EPHB4, when compared to those stably transfected with sh-Scb (Fig. 3A and B). On the other hand, stable transfection of HOXA9 into HTR-8/SVneo cell lines obviously up-regulated the expression of EPHB4, than those stably transfected with empty vector (mock: Fig. 3C and D). These results demonstrated that HOXA9 considerably facilitated EPHB4 expression at transcriptional and translation levels in HTR-8/SVneo cell lines.

3.4. Knockdown of HOXA9 enhanced the migration and invasion of HTR-8/SVneo cells through targeting EPHB4

We further investigated the effects of HOXA9 knockdown and EPHB4 restoration on biological behavior of HTR-8/SVneo cells. Ectopic expression of EPHB4 resulted in its up-regulation and restored the HOXA9 knockdown-induced down-regulation of EPHB4 in HTR-8/SVneo cells (Fig. 4A). In transwell assay, HOXA9 knockdown promoted the migration capabilities of HTR-8/SVneo cells, when compared to those stably transfected with sh-Scb (Fig. 4B). Matrigel invasion assay showed that HTR-8/SVneo cells stably transfected with sh-HOXA9 presented an enhanced invasion capacity than sh-Scb-transfected cells (Fig. 4C). In addition, restoration of EPHB4 expression prevented the HTR-8/SVneo cells from

**Fig. 5.** Over-expression of HOXA9 decreased the migration and invasion of HTR-8/SVneo cells in vitro. (A) Western blot indicated that stable transfection of HOXA9 vector into HTR-8/SVneo cells resulted in increased protein levels of HOXA9, when compared to those transfected with empty vector (mock). Transfection of sh-EPHB4 weakened the up-regulation of EPHB4 stimulated by stable HOXA9 over-expression (**P < 0.01, ***P < 0.001 vs. mock by Newman-Keuls test). (B) Transwell assay showed the migration of HTR-8/SVneo cells with HOXA9 over-expression was significantly reduced when compared to mock. Transfection of sh-EPHB4 vector rescued the migration of HOXA9 over-expressing cells (**P < 0.01, ***P < 0.001 vs. mock by Newman-Keuls test). (C) Matrigel invasion assay indicated the decreased invasion capabilities in HTR-8/SVneo cells of HOXA9 over-expression than those of mock. Moreover, transfection of sh-EPHB4 vector restored the invasion of HNF4a over-expressing cells (**P < 0.01 vs. mock by Newman-Keuls test). Photographs were taken at magnification of ×200.
Fig. 6. HOXA9 increased the transcription of EPHB4 through direct binding on its promoter. (A) Schematic of the EPHB4 promoter showing the three putative HOXA9-binding sites and schematic diagram of the EPHB4 promoter constructs utilized for dual luciferase assays. (B) Dual-luciferase assay indicated that −389 to −115 bp relative to transcription initial site (TIS) was essential for the promoter activities of EPHB4 (**P < 0.001 vs. pGL3-Basic by two-way ANOVA). (C) Stable transfection of sh-HOXA9 resulted in decreased luciferase activities of pGL3-EPHB4 (−999/+100) in HTR-8/SVneo cells, when compared with those stably transfected with sh-Scb (**P < 0.01, ***P < 0.001 vs. sh-Scb by Newman-Keuls test). (D) Stable over-expression of HOXA9 resulted in increased luciferase activities of pGL3-EPHB4 (−999/+100) in HTR-8/SVneo cells, when compared with those stably transfected with empty vector (mock; ***P < 0.001 vs. mock by Student’s t-test). (E) 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 HTR-8/SVneo cells transfected with sh-Scb, sh-HOXA9#1, sh-HOXA9#2, mock, HOXA9 plasmids, respectively. qPCR was performed with primer set 1, set 2 and set 3. The results indicated that stable knockdown of HOXA9 with shRNA constructs decreased the binding of HOXA9 on the −338/−96 region of EPHB4 promoter in HTR-8/SVneo cells (***P < 0.01 vs. sh-Scb by Newman-Keuls test). Meanwhile, stable transfection of HOXA9 into HTR-8/ SVneo cells resulted in enrichment of HOXA9 on the −338/−96 region of EPHB4 promoter (**P < 0.01 vs. mock by Student’s t-test).
their increase in the migration and invasion induced by stable knockdown of HOXA9 (Fig. 4B and C). These results indicated down-regulation of EPHB4 was possibly involved in HOXA9 knockdown-induced aggressiveness of HTR-8/SVneo cells.

3.5. Ectopic expression of HOXA9 decreased the migration and invasion of HTR-8/SVneo cells in vitro

To further explore the influence of HOXA9 on the aggressiveness of HTR-8/SVneo cells, we investigated the effects of HOXA9 over-expression and EPHB4 restoration on cultured HTR-8/SVneo cells. Transfection of sh-EPHB4 resulted in its down-expression and restored the up-regulation of EPHB4 induced by HOXA9 over-expression in HTR-8/SVneo cells (Fig. 5A). In transwell assay, HOXA9 over-expression decreased the migration capabilities of HTR-8/SVneo cells, when compared to those stably transfected with empty vector (mock) (Fig. 5B). Matrigel invasion assay showed that HTR-8/SVneo cells stably transfected with HOXA9 exhibited a reduced invasion capacity than mock cells (Fig. 5C). In addition, restoration of EPHB4 expression rescued the HTR-8/SVneo cells from their defects in the migration and invasion induced by stable over-expression of HOXA9 (Fig. 5B and C). These findings suggest that identification of EPHB4 as a HOXA9 target gene may explain, at least in part, why HOXA9 suppressed the migration and invasion of HTR-8/SVneo cells.

3.6. HOXA9 increased the transcription of EPHB4 through direct binding on its promoter

To determine whether HOXA9 could increase the transcription of EPHB4, the EPHB4 promoter luciferase reporter and its truncation vectors (shown in Fig. 6A) were transfected into HTR-8/SVneo cells. Dual-luciferase assay indicated that −389 to −115 bp relative to TIS was essential for the EPHB4 promoter activities (Fig. 6B). Knockdown or ectopic expression of HOXA9 attenuated and enhanced the promoter activities of EPHB4, respectively, in these HTR-8/SVneo cells (Fig. 6C and D). In addition, ChIP and qPCR were applied to measure the enrichment of HOXA9 on the EPHB4 promoter with primer sets spanning its binding sites. As is shown in Fig. 6E, stable knockdown of HOXA9 with shRNA constructs decreased the binding of HOXA9 on the −338/−96 region of the EPHB4 promoter in HTR-8/SVneo cells. Meanwhile, stable transfection of HOXA9 into HTR-8/SVneo cells resulted in enrichment of HOXA9 on the −338/−96 region of the EPHB4 promoter. These results indicated that HOXA9 directly interacted with the binding site within the EPHB4 promoter to increase its transcription.

4. Discussion

It has been demonstrated that up-regulation of EPHB4 impaired trophoblast migration and invasion during placentation, which may contribute to the pathogenesis of preeclampsia [11,24]. However, the upstream regulating mechanism of EPHB4 expression in trophoblast cells still remains largely unknown. Previous studies have verified that miR-17-family miRNAs (miR-17, miR-20a, and miR-20b) play key roles in trophoblast invasion during placentation development via directly targeting the 3’ UTR of EPHB4 and regulating EPHB4 expression [25]. In this study, we applied an integrative approach to analyze the public datasets of transcription factor binding, cis-regulatory elements, and transcription profiling of placenta specimens, and identified HOXA9 as the crucial modulator facilitating EPHB4 expression in trophoblasts for the first time. Notably, HOXA9 is highly expressed and positively correlated with EPHB4 levels in preeclamptic placenta tissues, which strongly suggested an important role for HOXA9 in placentation development and function.

Next, our immunostaining results demonstrated that HOXA9 and EPHB4 were co-expressed in trophoblast cells of first-trimester villi, while EPHB4 hardly existed in normal preterm placenta with lower HOXA9 expression. As we know, villi are a multifunctional structure and the basis of placenta formation. Indeed, it would be more convincing if villous samples of different gestational age could be collected to know the dynamic expression of HOXA9 and EPHB4. However, we had no access to obtain enough samples due to clinical practice. Based on our and other’s researches, we can largely infer that placental EPHB4 expression gradually decreased even appeared none as uncomplicated pregnancy went on. On the other hand, we can also explain this deduction from the perspective of the subcellular localization. EVT-dependent spiral artery remodeling is the key to placentation and EVTs role is of great value in this process [26]. In our previous work, EPHB4 expression in decidual EVTs showed a distinct decrease compared to villous trophoblast [11]. Consistently, we speculated that trophoblasts in villi differentiate into extravillous trophoblasts probably along with the down-regulation of EPHB4 during placentation. Herein, in this work, we adopted an immortalized EVTs cell line HTR8/SVneo to investigate the effect of EPHB4 expression on cell migration and invasion, since it was really difficult to extract and culture primary EVTs. Whereas HTR-8/SVneo cells respond to hypoxia differently with primary trophoblasts [27], we evaluated the changes of EVTs biological function by exogenously transfecting plasmids irrespective of hypoxia condition. Therefore, it seems to be feasible and meaningful to explore the upstream mechanism of EPHB4 regulation during placentation.

Then, gain- and loss-of-function studies were employed to identify whether HOXA9 affected the bioactivity of HTR-8/SVneo cells by modulating EPHB4 expression. The results revealed that knockdown of HOXA9 promoted the migration and invasion of HTR-8/SVneo cells in vitro. Conversely, ectopic expression of HOXA9 suppressed the migration and invasion of HTR-8/SVneo cells. Importantly, restoration of EPHB4 expression prevented HTR-8/SVneo cells from HOXA9-mediated changes in these biological features. In summary, molecular and functional analyses confirmed that HOXA9-EPHB4 axis plays a negative role in placentation.

Actually, mounting evidence indicated the expression of HOX genes in the normal and abnormal trophoblastic tissues and their role in the differentiation of trophoblast cells [16,17,28]. However, until now, few researches were reported on the expression and role of HOXA9 in the control of placental developmental processes. Emerging data showed HOXA9, as the functional targets of miR-210, was positively related with trophoblast migration and vascular remodeling [29]. In addition, HOXA9 was demonstrated to be necessary for endometrial receptivity to blastocyst implantation [30]. These results indicated the expression of HOX genes is a meaningful to explore the upstream mechanism of EPHB4 expression during placentation.
complexes, along with cofactor and collaborator proteins that provide target specificity and stabilization of the DNA, and epigenetic modifiers and transcriptional machinery [39,40]. Previous studies suggested that HOXA9 form triple complexes with members of the PBX or MEIS family, which enhances DNA binding affinity and provides selectivity [41,42]. However, the target genes of HOXA9 involved in trophoblast behavior still warrant investigation. In this study, direct evidence for an effect of HOXA9 on the EPHB4 transcription was provided by luciferase reporter and chromatin immunoprecipitation assays, which demonstrated that HOXA9 activated the EPHB4 promoter in HTR-8/SVneo cells. What’s more, since restoration of EPHB4 expression rescued the HTR-8/SVneo cells from HOXA9-mediated phenotypes in migration and invasion, our studies indicate that HOXA9 may exert its functions, at least in part, through activating the transcription of EPHB4 in HTR-8/SVneo cells.

In summary, our studies described, for the first time, the expression and localization of HOXA9 and EPHB4 in first-trimester and preterm human placenta. Furthermore, by gain- and loss-of-function studies, we showed that HOXA9 regulated EPHB4-mediated trophoblast invasion. Besides, HOXA9 was found to increase the transcription of EPHB4 through binding directly to the EPHB4 promoter.

Conflict of interest statement

All the authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2017.01.127.

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