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LncRNA HOXD-AS1 promotes preeclampsia progression via MAPK pathway

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Abstract. – OBJECTIVE: To investigate the role of HOXD-AS1 in preeclampsia and its underlying mechanism.

PATIENTS AND METHODS: A total of 50 preeclampsia primiparas and 34 normal pregnancies admitted in our hospital from July 2015 to July 2017 were selected as the study group and control group, respectively. Age, body weight, blood pressure, 24-h urinary protein and neonatal weight were compared between the two groups. HOXD-AS1 expression in the placenta tissues was detected by quantitative Real-time PCR (qRT-PCR). Preeclampsia patients were further assigned into high and low expression group according to their HOXD-AS1 expressions. The relationship between HOXD-AS1 expression and blood pressure, 24-h urinary protein and neonatal weight in preeclampsia patients were analyzed. For in vitro experiments, transfection efficacy of pcDNA-HOXD-AS1 and si-HOXD-AS1 were detected by qRT-PCR. Proliferative and colony formation abilities in BeWo and Wish cells were detected by CCK-8 and colony formation assay, respectively. Moreover, protein expressions of p-p38, p-JNK, and p-ERK were detected by Western blot.

RESULTS: The systolic blood pressure, diastolic blood pressure and urinary protein in preeclampsia patients were higher than those of normal pregnancies. However, neonatal weight in preeclampsia patients was lower than that of normal pregnancies. HOXD-AS1 expressions were gradually increased in normal pregnancies, patients with late onset preeclampsia and patients with early onset preeclampsia sequentially. Additionally, higher levels of systolic pressure, diastolic pressure and 24-h urinary protein, as well as lower neonatal weight, were observed in preeclampsia patients with high expression of HOXD-AS1 than those with low expression. In vitro results demonstrated that proliferative and colony formation abilities in trophoblasts were elevated after HOXD-AS1 knockdown. Western blot data illustrated that protein expressions of p-p38 and p-JNK were decreased, while p-ERK expression was increased after overexpression of HOXD-AS1 in trophoblasts.

CONCLUSIONS: HOXD-AS1 participates in the development and progression of preeclampsia by regulating trophoblast proliferation via the MAPK pathway.

Key Words:
Preeclampsia, HOXD-AS1, Proliferation, MAPK pathway.

Introduction

Preeclampsia is a multifactorial disorder unique to the third trimester of pregnancy, which is characterized by hypertension (≥ 140/90 mm Hg), proteinuria (≥ 0.3 g/24 h) and systemic dysfunction[1]. The incidence of preeclampsia in primiparas is 2% to 7%, which is one of the major causes of death in mother and child[2]. Perinatal mortality in preeclampsia patients is almost five times as those normal pregnancies in developed countries. More seriously, mortality in preeclampsia patients accounts for 20-80% in developing countries. The study of preeclampsia pathogenesis has been well recognized[3]. Researches have shown that the imbalance of immune tolerance, genetic factors, and placental ischemia and hypoxia are involved in the development of preeclampsia[4]. The specific mechanism, however, has not been fully elucidated. Long non-coding RNA (lncRNA) is a kind of non-coding RNA with 200 nucleotides in length, which is responsible for regulating cellular activities[5]. Researches[6] have shown that lncRNA exerts a crucial role in biological processes, such as chromatin modification, transcription, translation, dose-compensation effect, X chromosome inactivation, gene imprinting, variable cleavage of RNA, and regulation of protein activities. Abnormally expressed lncRNAs are related to the development of various human diseases[7]. It is reported that lncRNA is involved in cell proliferation, migration and invasion[8]. For example, lncRNA p21[9], DQ786243[10] and MALAT1[11] may participate in the abnormal activities of trophoblasts. Zou et al[12] first demonstrated that lncRNA SPRY4-IT1 regu-

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lates the proliferation, migration and apoptosis of trophoblast cell line HTR-8/SVneo. HOXD-AS1 is an antisense lncRNA encoded by the homeobox D family (HOXD) on chromosome 2, which belongs to the HOX family. The HOX gene family, as a class of evolutionarily conserved genes, was originally found in the Drosophila homomorphism. HOX gene is greatly involved in embryonic development[13]. Some investigations[14-19] have confirmed that HOX mutations can cause dysplasia, organ dysfunction, and even tumors. However, little study has been conducted on the role of HOXD-AS1 in preeclampsia. Our study aims to investigate the effect of HOXD-AS1 on preeclampsia and its underlying mechanism.

Patients and Methods

Sample Collection

50 preeclampsia primiparas admitted in the Second People's Hospital of Liaocheng from July 2015 to July 2017 were selected as the study group, and 34 cases of normal pregnancies in the same period were randomly selected as the control group. Blood pressure and 24-h urinary protein levels of pregnancies, as well as their neonatal weight were recorded. After the placenta was delivered, two placental tissues (1.0 cm×1.0 cm×0.6 cm) at the center of the placenta attached to the root of the umbilical cord were harvested. Placental tissues were required to be non-calcified without hemorrhage and necrosis. After being washed in DEPC water containing 0.1% normal saline, samples were then preserved at -80°C for further experiments. This study was approved by the Ethics Committee of the Second People's Hospital of Liaocheng and all subjects were informed consent. Pregnancies with other diseases, abnormal pregnancy hormone, medical history of antibiotic medication and adosculation were excluded. General characteristics of enrolled subjects were listed in Table I.

Cell Culture and Transfection

Human preeclampsia cell line was cultured in 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Rockville, MD, USA), and maintained in a 5% CO_2 incubator at 37°C. Cells in logarithmic growth period were seeded in 6-well plates. When cell confluence was up to 60-80%, pcDNA-HOXD-AS1 and si-HOXD-AS1 (GenePharma, Shanghai, China) were transfected into BeWo and Wish cells, respectively according to the instructions of Lipofatamine 2000 (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Transfection efficacy was verified by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

RNA Extraction and qRT-PCR

The mRNAs of cells were extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed to cDNAs. The reaction conditions were as follows: denaturation at 95°C for 30 s, followed by annealing at 95°C for 5 s and extension at 60°C for 31 s, for a total of 40 cycles. Each sample was repeatedly performed for 3 times. The mRNA level was calculated based on the $2^{-\Delta\Delta Ct}$ method. Primers used in this study were as follows: HOXD-AS1, F: GGCTCTTCCCTAATGTGTGG; R: CAGGTCCAGCATGAAACAGA; GAPDH, F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were collected and seeded into a 96-well plate at a dose of 2×10^3/mL. After 24 h-inoculation, 10 μL of CCK-8 solution were added into each well at 6 h, 24 h, 48 h, 72 h, 96 h, respectively. The absorbance (OD) values at the wavelength of 450 nm were accessed with a microplate reader.

Colony Formation Assay

BeWo and Wish cells were harvested and inoculated into a 6-well plate at a dose of 500/mL for 2-week culture in complete medium. 2 mL of 4% paraformaldehyde were applied to fix the colonies for 30 min and 0.1% crystal violet solution was added for 20-min staining. After washed with phosphate-buffered saline (PBS) for 3 times, 3 fields in each well were randomly selected for observing and capturing colonies using an inverted microscope.

Western Blot

The total protein of the transfected cells was extracted. The concentration of each protein sample was determined by a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Briefly, 50 μg of total protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with

5% skimmed milk, followed by the incubation of specific primary antibodies (p-p38, p-JNK and p-ERK) overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}$±s). Comparison of measurement data was conducted using *t*-test. $p<0.05$ was considered statistically significant.

Results

Clinical Data of Preeclampsia Patients and Normal Pregnancies

Clinical data of enrolled subjects were recorded, including the age, blood pressure and 24-h urinary protein levels, etc. No significant differences in age and body weight were found between the two groups ($p>0.05$). However, higher levels of systolic pressure (148.31±13.55 mmHg), diastolic pressure (107.89±17.35 mmHg) and 24-h urinary protein (0.42±0.16 g/day) were observed in preeclampsia patients than those of normal pregnancies (113.04±18.32 mmHg, 76.31±19.13 mmHg and 0.15±0.18 g/day, respectively). All the above differences were statistically significant (all $p<0.05$). We also recorded the neonatal weight of each subject. The data suggested that lower neonatal weight was found in preeclampsia patients (1785.25±530.17 g) compared with those of normal pregnancies (3317.13±389.16 g, $p<0.05$, Table I).

HOXD-AS1 Was Upregulated in Preeclampsia Patients

The expression of lncRNA HOXD-AS1 in the placenta tissues was detected by qRT-PCR. Our results demonstrated that HOXD-AS1 expressions were remarkably higher in placental tissues of preeclampsia patients than those of normal pregnancies (Figure 1A). Besides, HOXD-AS1 expressions were gradually increased in normal pregnancies, patients with late onset preeclampsia and patients with early onset preeclampsia sequentially (Figure 1B). Preeclampsia patients were further assigned into high and low expression group based on their HOXD-AS1 expressions. The data suggested that higher levels of systolic pressure (Figure 1C), diastolic pressure (Figure 1D) and 24-h urinary protein (Figure 1E) were observed in preeclampsia patients with high expression of HOXD-AS1 than those with low expression. Additionally, the neonatal weight in preeclampsia patients with high expression of HOXD-AS1 was remarkably lower than those with low expression (Figure 1F).

HOXD-AS1 Suppressed Cell Proliferation of Trophoblasts

We detected HOXD-AS1 expression in 5 trophoblast cell lines by qRT-PCR. The results found that HOXD-AS1 expression was the lowest in BeWo cells and the highest in Wish cells (Figure 2A). Therefore, BeWo cells and Wish cells were selected for the following experiments. HOXD-AS1 was overexpressed in BeWo cells by transfection of pcDNA-HOXD-AS1 (Figure 2B), whereas HOXD-AS1 was down-regulated in Wish cells by transfection of si-HOXD-AS1 (Figure 2C). CCK-8 and colony formation assay showed that proliferative (Figure 2D) and colony formation abilities (Figure 2F) in Wish cells transfected with si-HOXD-AS1 were remarkably in-

Table I. Clinical characteristics of normal and pre-eclamptic pregnacies

Variable	Healthy pregnancy (n=34)	Preeclampsia (n=50)	*p*-value
Maternal age (year)	31.12±4.03	31.54±3.59	>0.05
Maternal weight (kg)	69.56±7.84	68.71±8.05	>0.05
Proteinuria (g/day)	0.15±0.18	0.42±0.16	<0.05
Systolic blood pressure (mm Hg)	113.04±18.32	148.31±13.55	<0.05
Diastolic blood pressure (mm Hg)	76.31±19.13	107.89±17.35	<0.05
Body weight of infant (g)	3317.13±389.16	1785.25±530.17	<0.05

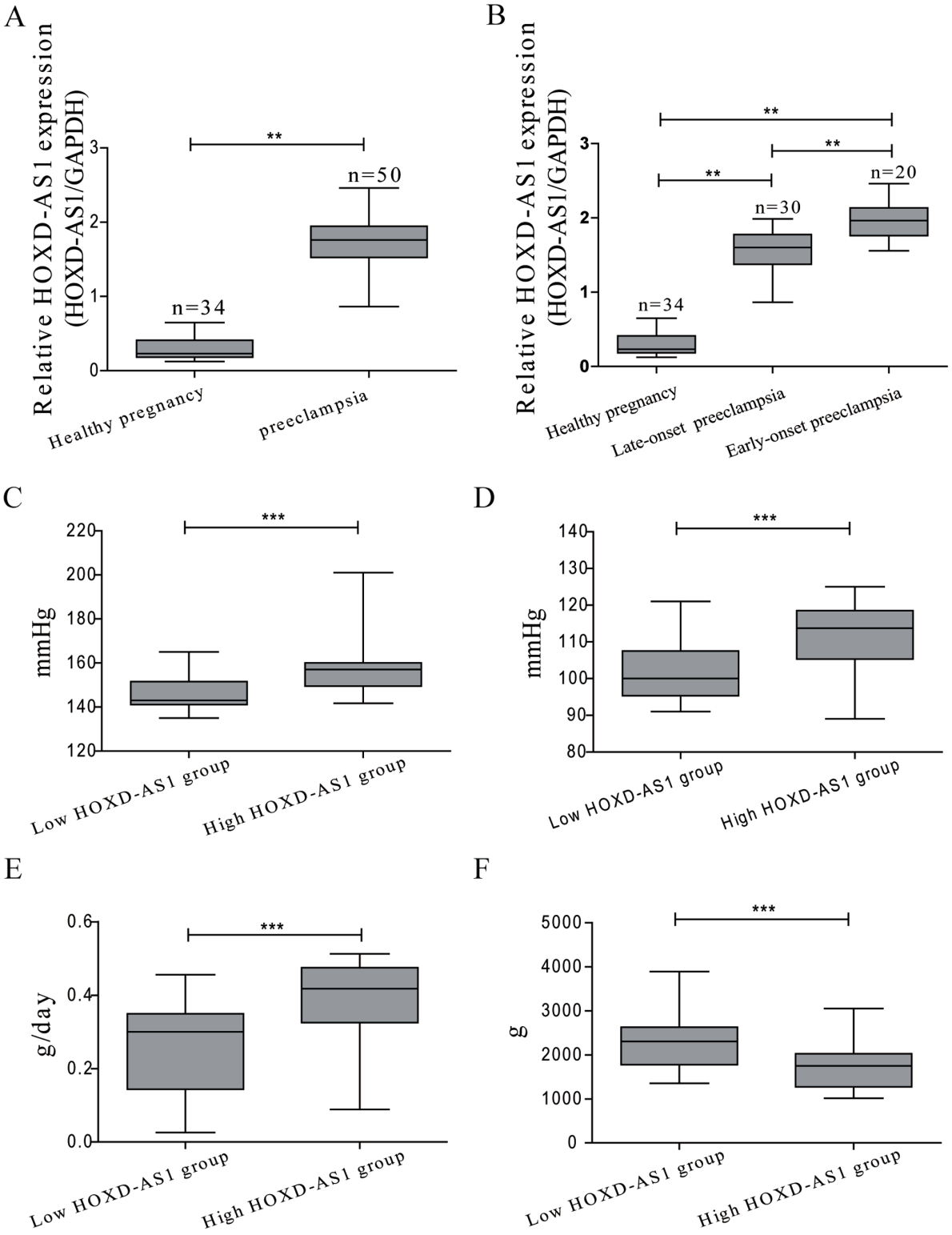

Figure 1. HOXD-AS1 was upregulated in preeclampsia patients. ***A,*** HOXD-AS1 expressions were remarkably higher in preeclampsia patients than those of normal pregnancies. ***B,*** HOXD-AS1 expressions were gradually increased in normal pregnancies, patients with late onset preeclampsia and patients with early onset preeclampsia sequentially. ***C-E,*** Levels of systolic pressure (Figure 1C), diastolic pressure (Figure 1D) and 24-h urinary protein (Figure 1E) were increased in preeclampsia patients with high expression of HOXD-AS1 than those with low expression of HOXD-AS1. ***F,*** Neonatal weight in preeclampsia patients with high expression of HOXD-AS1 was lower than those with low expression of HOXD-AS1.

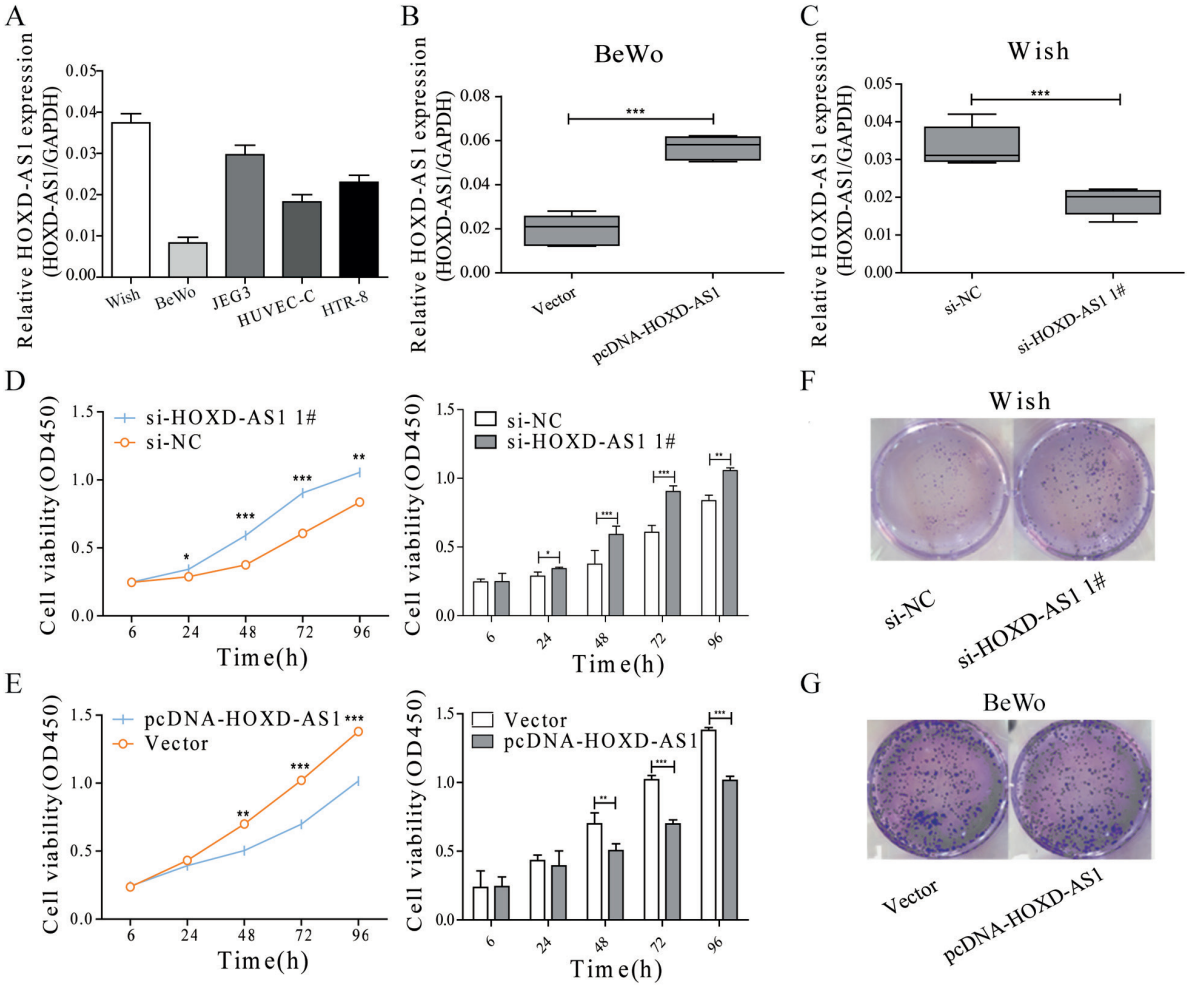

Figure 2. HOXD-AS1 inhibited cell proliferation of trophoblasts. ***A,*** HOXD-AS1 expression was the lowest in BeWo cells and the highest in Wish cells. ***B,*** HOXD-AS1 was overexpressed in BeWo cells by transfection of pcDNA-HOXD-AS1. ***C,*** HOXD-AS1 was down-regulated in Wish cells by transfection of si-HOXD-AS1. ***D,*** Proliferation of Wish cells was increased after transfection with si-HOXD-AS1. ***E,*** Colony formation ability of Wish cells was increased after transfection with si-HOXD-AS1. ***F,*** Proliferation of BeWo cells was decreased after transfection with pcDNA-HOXD-AS1. ***G,*** Colony formation ability of BeWo cells was decreased after transfection with pcDNA-HOXD-AS1.

creased compared with those negative controls. On the contrary, decreased abilities of proliferation (Figure 2E) and colony formation (Figure 2G) were found in BeWo cells transfected with pcDNA-HOXD-AS1.

HOXD-AS1 Promoted Preeclampsia Progression Via Regulating MAPK Pathway

Researches[20-22] have shown that trophoblasts exert a crucial role in blastocyst transfer, placental development and the establishment of the maternal and fetal circulation. Trophoblastic dysfunction and uterine spiral artery remodeling dysfunction are considered to be the leading pathogenesis of preeclampsia, spontaneous abortion and other pregnancy-related diseases. Investigations[23,24] have confirmed that mitogen-activated protein kinase (MAPK) pathway is involved in the regulation of trophoblast function, which mainly includes p38 MAPK, JNK and ERK. MAPK pathway is believed to participate in regulating cell proliferation, differentiation, apoptosis and migration. In this study, protein expressions of p-p38, p-JNK and p-ERK in the placenta tissues of preeclampsia patients and normal pregnancies were detected by Western blot. Lower expressions of p-p38 and p-JNK, as well as higher expression of p-ERK were observed in placental tissues of preeclampsia patients compared with those of

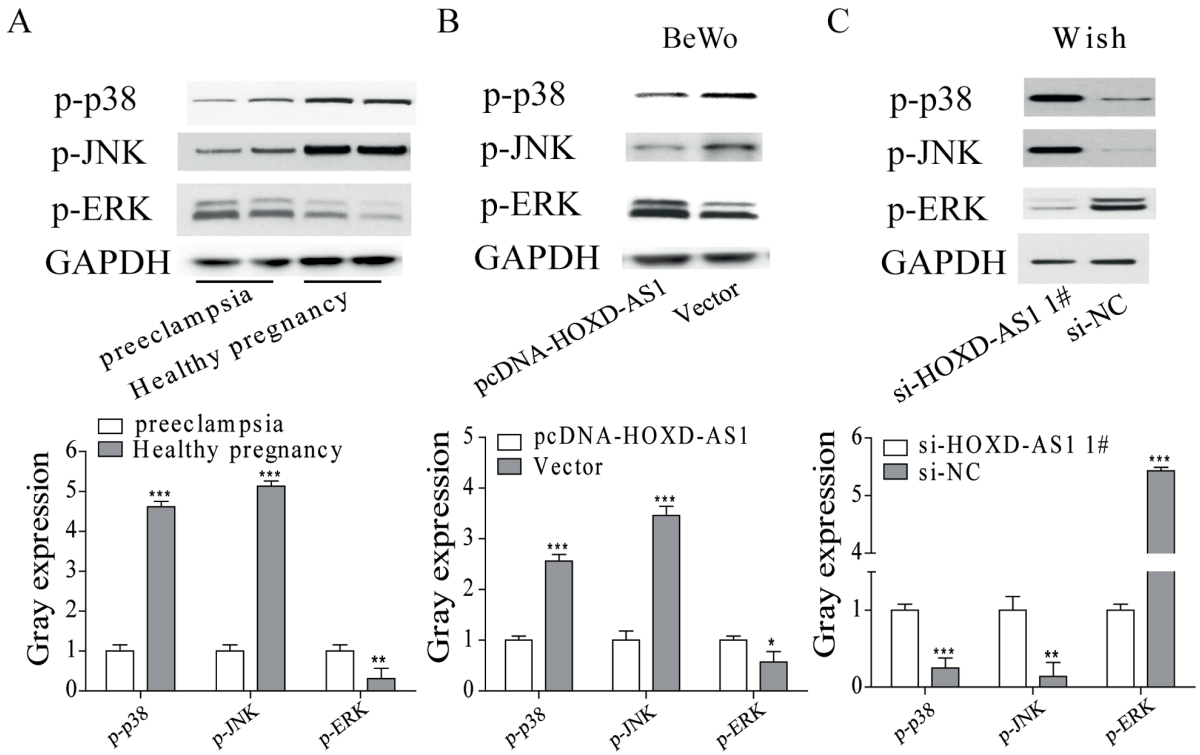

Figure 3. HOXD-AS1 promoted preeclampsia progression *via* regulating MAPK pathway. ***A,*** Lower expressions of p-p38 and p-JNK, and higher expression of p-ERK in preeclampsia patients compared with those of normal pregnancies. ***B,*** Protein expressions of p-p38 and p-JNK were decreased, whereas p-ERK expression was increased in BeWo cells after HOXD-AS1 overexpression. ***C,*** Knockdown of HOXD-AS1 in Wish cells increased expressions of p-p38 and p-JNK and decreased p-ERK expression.

normal pregnancies (Figure 3A). *In vitro* results suggested that protein expressions of p-p38 and p-JNK were decreased, whereas p-ERK expression was increased in BeWo cells after HOXD-AS1 overexpression (Figure 3B). Knockdown of HOXD-AS1 in Wish cells obtained the opposite results (Figure 3C). These results indicated that HOXD-AS1 may promote preeclampsia progression *via* regulating MAPK pathway.

Discussion

Ischemia and anoxia of placental trophoblasts induced by placental microvascular diseases could lead to placental tissue dysfunction, thereafter resulting in preeclampsia. During the course of disease, trophoblast erosion not only causes dysfunction of uterine spiral artery remodeling, but also leads to systemic inflammatory responses[25,26]. A great number of studies have found that cell apoptosis of trophoblasts is involved in preeclampsia. More importantly, the degree of cell apoptosis is closely related to the severity of preeclampsia. Various cytokines participate in the regulation of the immune inflammatory response, thereby regulating the proliferation, differentiation and apoptosis of trophoblasts. Excessive trophoblastic apoptosis directly leads to shallow implantation of placenta when cytokines are abnormally expressed, which in turn triggers clinical symptoms of preeclampsia[27-30]. In the present study, we found that HOXD-AS1 participates in the development of preeclampsia *via* inhibiting trophoblast proliferation. MAPK pathway is widely expressed in the human body, which is activated by multiple stimuli, including inflammatory factors, growth factors, neurotransmitters, neurotrophic factors and mitogens. MAPK pathway is involved in the pathophysiological processes of inflammation, cell proliferation, differentiation and apoptosis[31,32]. P38MAPK and JNK are key genes in MAPK pathway, which are generally considered to regulate vascular endothelial cells, cytokines and ROS system. Downregulated p38MAPK and JNK lead to decreased Caspase-3 activity and impaired cellular

DNA[33]. In addition, ERK is activated by different external stimuli, thereby protecting cell apoptosis *via* the promotion of cell proliferation[34-36]. In the present study, higher levels of systolic pressure, diastolic pressure and 24-h urinary protein were observed in preeclampsia patients than those of normal pregnancies. Moreover, neonatal weight of preeclampsia patients was lower than that of normal pregnancies. By detecting key factors in MAPK pathway, we found increased expressions of p-p38 and p-JNK, as well as decreased ERK expression in placental tissues of preeclampsia patients compared with those of normal pregnancies, which were regulated by HOXD-AS1. For *in vitro* experiments, transfection of pcDNA-HOXD-AS1 or si-HOXD-AS1 remarkably changed the proliferative and colony formation abilities *via* MAPK pathway, indicating that HOXD-AS1 is capable of regulating trophoblasts in preeclampsia patients.

Conclusions

We observed that HOXD-AS1 participates in the development and progression of preeclampsia by regulating trophoblast proliferation *via* MAPK pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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