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LncRNA PRNCR1 promoted the progression of eclampsia by regulating the MAPK signal pathway

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Abstract. – OBJECTIVE: We aimed to explore the expression level and biological function of lncRNA PRNCR1 in preeclampsia (PE).

PATIENTS AND METHODS: 57 PE patients and 57 normal pregnant women were enrolled in this study. The expression level of PRNCR1 in the maternal placenta of PE patients and normal pregnancy was detected by qRT-PCR. The CCK-8 assay was carried out to determine the cell viability after interference and overexpression of PRNCR1 on trophoblasts. We utilized Western blot to examine the protein level of PRNCR1.

RESULTS: Higher systolic blood pressure, diastolic blood pressure and urinary protein levels in PE patients were observed in comparison with those in normal pregnant women, while the neonatal weight in PE group was markedly lower than that in normal pregnant women. LncRNA PRNCR1 was overexpressed in PE patients, which was positively correlated with systolic blood pressure, diastolic blood pressure and urine protein, whereas negatively correlated with fetal birth weight of PE patients. In addition, the expression of PRNCR1 in BeWo trophoblast cells was significantly decreased after the interference with PRNCR1, while the cell viability increased. However, overexpression of PRNCR1 in HTR-8 cells significantly reduced the viability of cells. Expression levels of p-p38 and p-JNK in PE patients were higher than those in normal pregnancy women, and the expression level of p-ERK was decreased, which suggested that PRNCR1 promoted the progression of PE by modulating the MAPK signaling pathway.

CONCLUSIONS: PRNCR1 is highly expressed in PE and promotes the progression of PE by modulating the MAPK signaling pathway.

Key Words:
Preeclampsia, LncRNA, PRNCR1, MAPK signal pathway.

Introduction

Preeclampsia (PE) infers that pregnant women with normal pre-pregnancy blood pressure exhibit high blood pressure, proteinuria and other performance after 20 weeks of pregnancy, which is an idiopathic placenta-derived disease during pregnancy. The average prevalence of PE in pregnant women is about 5-8%[1]. PE can cause serious maternal-fetal complications and is the leading cause of maternal and perinatal deaths[2]. Studies have indicated that PE patients experience a higher prevalence of hypertension, myocardial ischemia and stroke in the following decade. More seriously, hypertension risk in their offspring is also exponentially increased. PE is a polygenic disease and its pathogenesis not fully elucidated. There are many studies on placental angiogenesis in PE at the genetic level, including cytokines released from the placenta, biologically active cell debris released by cytotrophoblasts, as well as a series of anti-angiogenic genes, such as soluble endothelial factor (sEng) and soluble vascular endothelial growth factor receptor (Flt-1)[4].

Long non-coding RNAs (lncRNAs) are RNA transcripts of more than 200 nucleotides with no protein-coding function and gradually becoming key regulators of different cellular activities[5]. It has been found that lncRNAs play significant roles in inactivation of X chromosome, chromatin modification, transcription, translation, dose compensation effect, gene imprinting, regulation of protein activity and alteration of RNA. Accumulating researches[7] showed that lncRNAs regulate the growth and development of the body and its disorders are associated with many human diseases. It has been reported that the expression of lncRNA MEG3 is down-regulated in placenta

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of PE patients. This down-regulation could inhibit the migration of trophoblasts, promote its apoptosis and increase the expression of NF-κB, Caspase3 and Bax, which might be related to the PE-induced failure of uterine spiral artery remodeling[8]. Zou et al[9] reported that lncRNA SPRY4-IT1 is overexpressed in placenta tissue and regulate the proliferation, migration, apoptosis and tube formation of the trophoblast cell line HTR-8/SVneo. This study, for the first time at the cellular level, demonstrated that lncRNAs play vital roles in PE.

Prostate cancer non-coding RNA 1 (PRNCR1) is a lncRNA found in recent years. Researchers from UC Davis, San Diego (CA, USA) and other research institutes have identified it as a pivotal factor in invasive prostate cancer[10]. PRNCR1 locates on chromosome 8q24 with a size of 13 kB, and has been shown to be greatly involved in prostate cancer and colon cancer[11]. However, the role of PRNCR1 in PE has not been studied yet.

At present, researches on the signaling pathways of PE mainly focus on adhesion proteins, PI3K, TGF-Smad pathway, and on immune factors such as HLA-DR4, sVCAM-1, Th1/Th2, etc. PI3K is related to the differentiation and invasion of lacental trophoblast[12,13]. Mitogen-activated protein kinase (MAPK) is a kind of intracellular serine/threonine protein kinase. These kinases are activated by different molecular signals and participate in the signal transduction of upstream and downstream, thus causing cell proliferation, differentiation, transformation, apoptosis and other biological reactions[14]. Yong et al[15] have found that a variety of stimuli cause the activation of p38 MAPK signaling pathway in PE patients and upregulates the expression level of matrix metalloproteinases and their inhibitors (MMPs/TIMPs), urokinase-type plasminogen activator and its inhibition (UPA/PAI) expression, thus affecting the ability of trophoblasts invasion. Our study investigated the important effect of PRNCR1 on PE by detecting key genes in MAPK pathway for the first time, thus providing new direction for preventing and treating PE.

Patients and Methods

Specimen Collection

Totally, 57 pairs of placental samples from PE patients and normal pregnant women during the same period were collected rapidly within 5 min after the placenta was delivered. A few pieces of about 1 cm^2 placental tissue were cut in different regions of the placenta (to avoid calcified areas) and washed with diethyl pyrocarbonate PBS twice. The tissues were frozen with liquid nitrogen immediately after labeling, and then stored in a -70°C freezer. This work was approved by the Ethics Committee of Nanhai People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Trophoblast cells, including Wish, HUVEC-C, JEG3, BeWo, and HTR-8 cell lines, were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco, Rockville, MD, USA) containing 5% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). Cells were cultured in a 5% CO_2 incubator at 37°C. Cells were passaged at 80% confluence after being trypsinized and then seeded into 6-well plates for 24 h followed by transfection with si-NC, si-PRNCR1 (1 #, 2 #), pcDNA-NC and pcDNA -PRNCR1.

QRT-PCR

Trophoblast cells were collected 48 h post-transfection, and the total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). We used a reverse transcription kit (TaKaRa, Dalian, China) to transcribe the RNA into cDNA following manufacturer's instruction.

Cell Counting Kit-8 (CCK-8) Assay

BeWo cells and HTR-8 cells were cultured in 96-well plates. 24 h later, cells were transfected with si-PRNCR1 and pcDNA-PRNCR1, respectively, followed by the manufacturer's protocol. We added 10 μL of CCK-8 solution into each well and then cells were incubated at 37°C for 2 h. The absorbance at 450 nm was recorded by spectrophotometry. The experiments were repeated 3 times independently.

Western Blot

The trophoblast cells were washed with phosphate-buffered saline (PBS), and then an appropriate amount of cell lysate was added to lyse the cells to obtain the total cell protein solution. Each protein sample was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane. 1 h after blocking, we used the primary antibody for incubation at 4°C overnight. The corresponding secondary

antibody (goat anti-rabbit) was used for incubation with above membrane for 2 h at room temperature. All protein bands were exposed after enhanced chemiluminescence (ECL) reaction. The gray level of the blot was analyzed by Gel-ProAnalyzer software (United Bio, Marlton, NJ, USA) and the gray value of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as an internal control. Primary antibodies were: p38, p-p38, JNK, p-JNK, ERK, p-ERK, GAPDH antibody.

Statistical Analysis

We used statistical product and service solutions (SPSS) 22.0 statistical software (Chicago, IL, USA) for data analysis. The GraphPad Prism5.0 (Version X; La Jolla, CA, USA) was introduced for image editing. Independent-sample *t*-test was performed for the analysis of two groups. $p < 0.05$ was considered statistically significant ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

Results

Relationship Between Clinical Data and PE

By analyzing the clinical data of the study subjects, we found no significant difference in reproductive weight and reproductive age between PE patients and normal pregnant women (Figure 1A, B). Besides, we observed higher systolic blood pressure, diastolic blood pressure and urinary protein levels (Figure 1C, D, F), as well as lower neonatal weight (Figure 1E) in PE patients than those of normal pregnancies.

PRNCR1 Expression was Upregulated in the Placenta from PE Patients

The results of qRT-PCR revealed higher expression of PRNCR1 in placenta of PE patients compared with normal pregnant women ($p < 0.001$) (Figure 2A). According to the expression level of PRNCR1, we assigned PE patients into high and low expression group, and the systolic pressure, diastolic pressure and urinary protein level were much higher in high PRNCR1 expression group than that in low expression group (Figure 2A, B, C). However, the neonatal weight in high expression group showed a significant decrease compared to the low expression group (Figure 2D), which indicated that PRNCR1 might participate in the progression of preeclampsia.

PRNCR1 Promoted the Progression of PE

To investigate the effect of PRNCR1 expression on PE, we extracted the total RNA from trophoblast cell lines (Wish, HUVEC-C, JEG3, BeWo, and HTR-8) and detected the relative expression of PRNCR1. We found that PRNCR1 was lowly expressed in HTR-8 cells, but highly expressed in BeWo cells, which were then selected for our overexpression and knockdown experiments (Figure 3A). The results illustrated that si-PRNCR1 had the most significant effect on knockdown of PRNCR1 (Figure 3B), whereas the PRNCR1 expression was significantly elevated in HT-18 cells after transfection of pcDNA-PRNCR1 (Figure 3B). Moreover, the viability of BeWo cells was significantly increased after interfering with PRNCR1 (Figure 3C), while overexpression of PRNCR1 markedly reduced the HTR-8 cells' viability (Figure 3D). These findings suggested that PRNCR1 might promote the progression of PE.

PRNCR1 Promoted the Progression of Eclampsia by Regulating the MAPK Signaling Pathway

Western blot results elucidated higher levels of p-p38 and p-JNK, as well as lower levels of p-ERK in PE patients were remarkably higher than that of normal pregnancy group ($p < 0.05$). Meanwhile, overexpression of PRNCR1 in HTR-8 cell line significantly enhanced the expressions of p-p38 and p-JNK ($p < 0.05$) and downregulated the expression of p-ERK ($p < 0.05$) (Figure 4). Besides, knockdown of PRNCR1 in BeWo cells attenuated the expressions of p-p38 and p-JNK but enhanced the expression of p-ERK (Figure 5). Above results demonstrated that lncRNA PRNCR1 promotes the progression of PE by regulating the MAPK signal transduction pathway.

Discussion

LncRNAs can regulate many key life activities such as cell development and metabolism at epigenetic, transcriptional and post-transcriptional levels. LncRNAs are closely related to the occurrence of human diseases and increasingly become a hot topic at home and abroad[16]. A number of studies have shown that abnormal expression of lncRNAs in placenta is associated with the pathogenesis of PE. Zou et al[9] reported that lncRNA SPRY4-IT1 is overexpressed in the

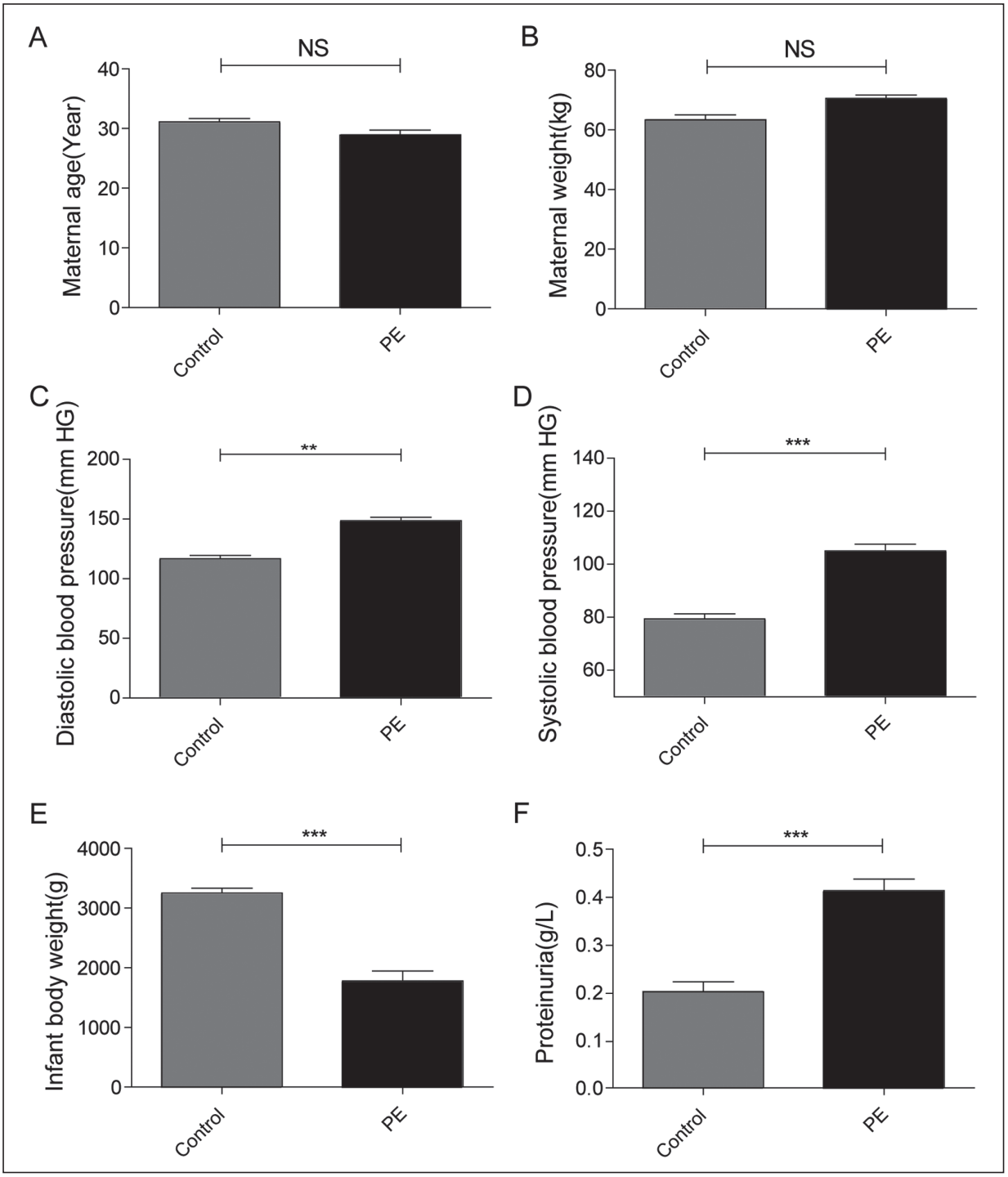

Figure 1. Relationship between clinical data and PE. **A-B,** There is no significant difference in the age and weight of PE maternal and normal pregnancy. **C,** The systolic blood pressure in PE patients was significantly higher than that in normal pregnant women. **D,** The level of diastolic blood pressure in PE patients was significantly higher than that of normal pregnant women. **E,** The newborn weight in PE patients was significantly lower than that in normal pregnant women. The level of urinary protein in PE patients was significantly higher than that of normal pregnant women.

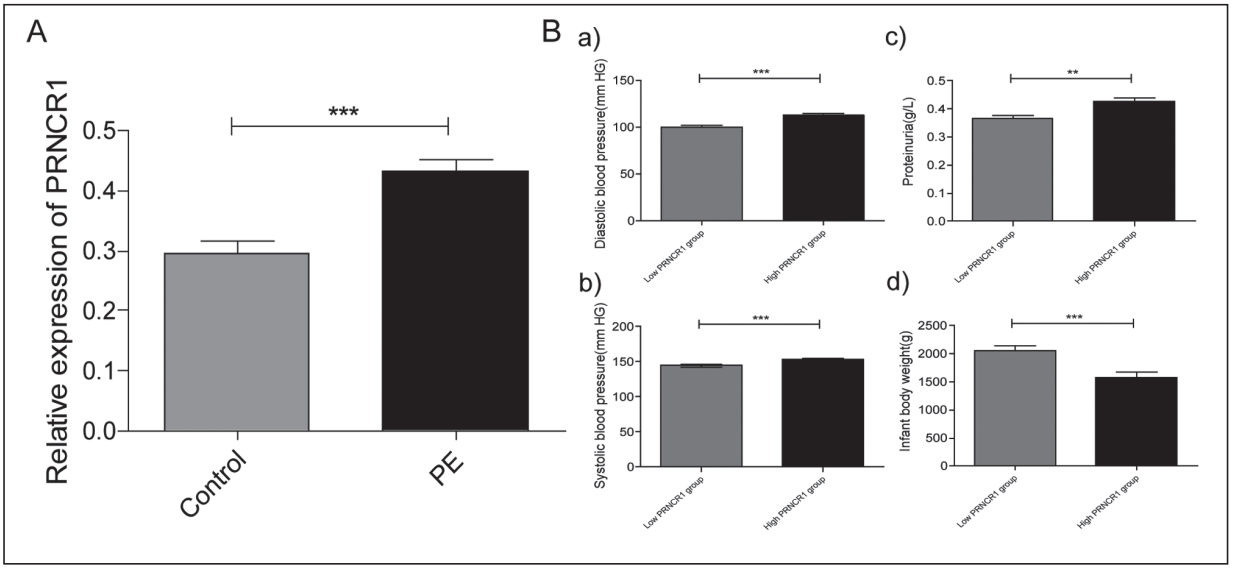

Figure 2. PRNCR1 expression was upregulated in the placenta from PE patients. **A,** PRNCR1 expression in placenta of PE patients was significantly higher than that in the control group. **B** a) and b), The systolic blood pressure and diastolic blood pressure in the PRNCR1 high expression group was significantly higher than that in the low expression group. c) The urine protein in PRNCR1-overexpressing group was significantly higher than that in low-expression group. d) Newborns in PRNCR1-overexpressing group had significantly lower body weight than those in the low-expression group.

placenta of PE patients and regulates the proliferation, migration, apoptosis and tube formation of the feeder cell line HTR-8/SVneo. Zhang et al[8] found that lncRNA MEG3 is downregulated in PE placenta and inhibits the migration of trophoblasts. According to Chen et al[17], lncRNA MALAT-1 is significantly decreased in PE placenta and overexpression of MALAT-1 promotes the apoptosis of JEG-3 cells and inhibits its migration and invasion. The abnormal expression of lncRNAs participates in the biological function of trophoblast proliferation, migration, invasion and angiogenesis, and then participates in the pathogenesis of PE.

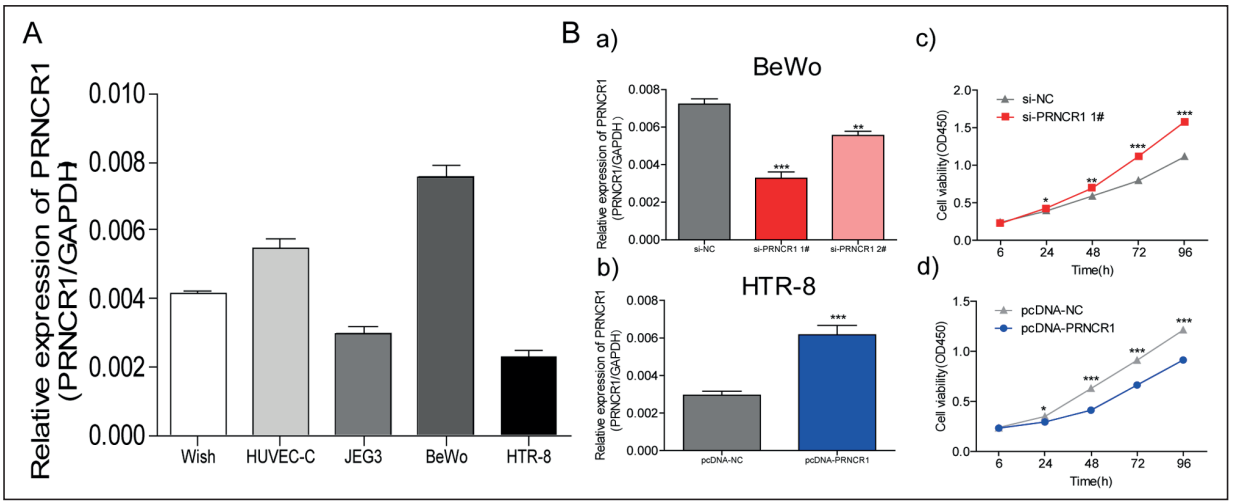

Figure 3. PRNCR1 promoted the progression of preeclampsia. **A,** PRNCR1 was lowly expressed in HTR-8 cells and highly expressed in BeWo cells. **B** a), After transfected with si-PRNCR1 1 #, the expression of PRNCR1 in BeWo cells was significantly decreased, of which si-PRNCR1 1 # showed the best knockdown efficiency. b), The expression of PRNCR1 in HTR-8 cells was significantly increased after transfection of pcDNA-PRNCR1. c), After interference with PRNCR1, the viability of BeWo cells was significantly increased. d), Overexpression of PRNCR1 significantly reduced the viability of HTR-8 cells.

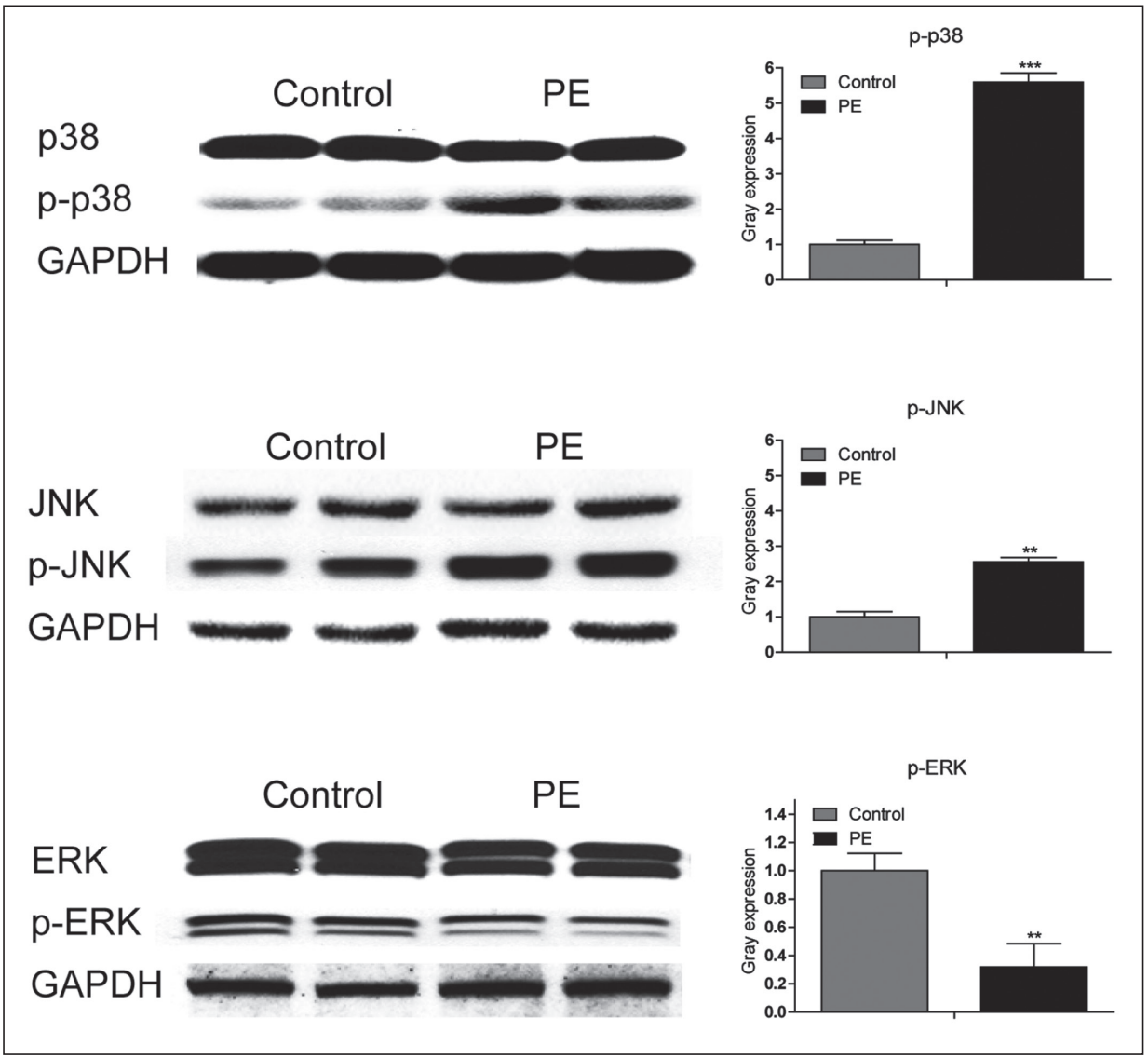

Figure 4. The expression of MAPK related protein in PE. The expression of p-p38 and p-JNK in patients with PE was higher than that in normal group, while p-ERK was significantly decreased.

The latest work founds that gene polymorphism of PRNCR1 is associated with the risk of gastric cancer[18]. PRNCR1 was first discovered to have a relationship with prostate cancer. Yang et al[11] reported that PRNCR1 and PCGEM1 are overexpressed in many invasive prostate cancer tissues, which are capable of stimulating androgen receptor-related transcriptional program and tumor growth. Although researches on the regulatory mechanism of trophoblasts by lncRNAs are still in progress, the effects of lncRNAs on PE remain largely unknown. Therefore, it is of great significance to study the role of lncRNAs in PE.

In this study, PRNCR1 in PE placental tissue showed a significant high expression, indicating that PRNCR1 might be involved in the progress of PE. Our report enriched the evidence of the differential expression of lncRNA PRNCR1 in PE tissue and explored the effects of PRNCR1 and PRNCR1 on the trophoblast cells. Interference of PRNCR1 significantly increased the viability of BeWo cells, while overexpression of PRNCR1 reduced the viability of HTR- 8 cells. MAPK is early proved to be associated with complications related to PE. Lappas et al[19] observed enhanced expression of phosphorylated MAPK (p-ERK, p- JNK, p-p38) in fetal membrane near the cervix of premature

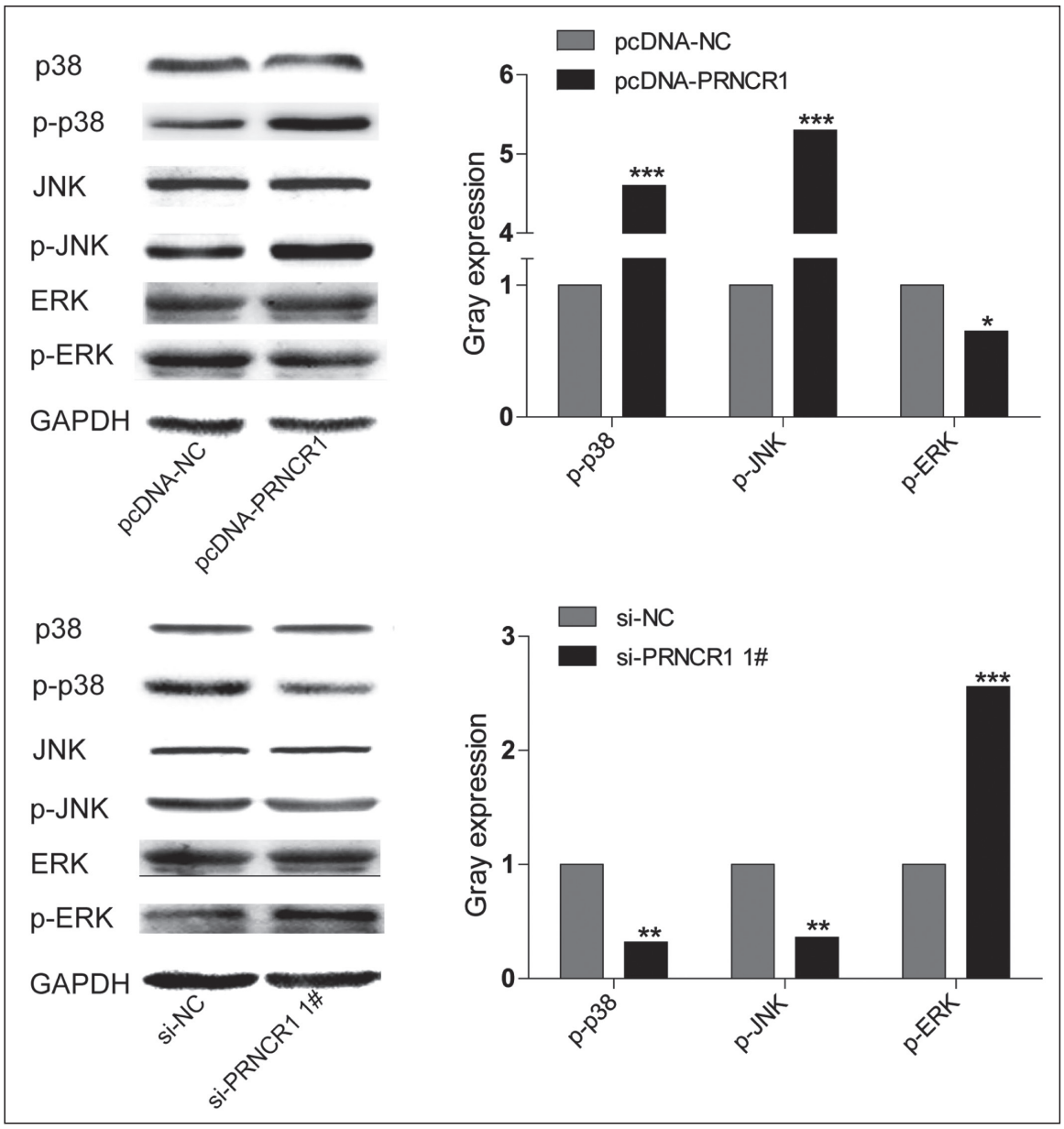

Figure 5. PRNCR1 promoted the progression of PE by regulating the MAPK signaling pathway. Overexpression of PRNCR1 increased the expression of p-p38 and p-JNK, but decreased p-ERK expression in HTR-8 cells. On the contrary, knockdown of PRNCR1 decreased the expression of p-p38 and p-JNK, but increased p-ERK expression in BeWo cells.

pregnant women, as well as the MAPK-activated downstream target proteins including AP-1 c-Fos and c-Jun. However, inhibition of MAPK signal pathway promotes IL-1β-dependent decrease of MMP-9 activity in amniotic cells. Otherwise, the deletion of c-Jun and JunB also causes early embryonic death[20,21]. MMPs regulated by MAPK are also involved in the pathological process of abortion. The changes of 735 C/T and 1562 C/T polymorphisms in MMP-2 enhance the probability of spontaneous abortion in pregnant women[22]. Therefore, this research explored the role of PRNCR1 in PE by regulating the MAPK signal transduction pathway, which may provide a new basis for the treatment of PE and its complications.

Conclusions

We showed that PRNCR1 is highly expressed in preeclampsia and promotes the progression of PE by modulating the MAPK signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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