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Long non-coding HOTTIP regulates preeclampsia by inhibiting RND3

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Abstract. – OBJECTIVE: Preeclampsia (PE) is an important disease affecting maternal and neonatal pregnancy. Long non-coding RNA plays an important role in preeclampsia. This study aims to explore the role of HOXA distal transcript antisense RNA (HOTTIP) in preeclampsia.

PATIENTS AND METHODS: The expression of HOTTIP in placentas of preeclampsia and normal pregnancy was detected by polymerase chain reaction (PCR). The difference of clinical data between high expression HOTTIP group and low expression HOTTIP group was compared to explore the relationship between HOTTIP and the progress of preeclampsia. Cell proliferation and cell cycle were detected after overexpression and interference with HOTTIP. Western blot was finally used for detecting protein expression.

RESULTS: By analyzing the clinical data, we found that systolic blood pressure, diastolic blood pressure, and urinary protein in preeclampsia patients were significantly higher than those in normal pregnant women. The birth weight of fetuses was significantly lower than that of normal pregnant women. Through PCR results, we found that the expression of HOTTIP in patients with preeclampsia was significantly lower, and the maternal systolic blood pressure, diastolic blood pressure and urinary protein in the low expression HOTTIP group were significantly higher than that in the high expression group. Fetal birth weight was significantly lower than the high expression group. After overexpression and interference with HOTTIP, it was found that overexpression of HOTTIP significantly increased the proliferation of trophoblast cells and markedly accelerated the cycle. Interfering with HOTTIP, trophoblast cell proliferation was significantly reduced and cell cycle was arrested. By Western blotting, we found that HOTTIP regulated the progression of preeclampsia via Rho family GTPase 3(RND3).

CONCLUSIONS: HOTTIP was lowly expressed in preeclampsia and was able to suppress the progression of preeclampsia.

Key Words
lncRNA, HOTTIP, Preeclampsia, RND3.

Introduction

Preeclampsia (PE) is a pregnancy-specific disease associated with polygenetic genetic background and environmental factors. Immune imbalance of maternal-fetal interface leads to insufficient placenta oxygen supply and placental dysplasia, resulting in local oxidative stress and various toxic factors and inflammatory mediators released. All these pathological changes cause systemic multiple organ vascular endothelial injury, vasospasm, and damages of multiple systems[1,2]. Effective measures for clinical preventive treatment are still lacked, the only cure is the fetus and placenta from the maternal childbirth[3], and preeclampsia is still one of the leading causes of maternal and perinatal death[4].

In recent years, non-coding RNAs have attracted more and more attention. There is no clear official consensus about the sequence, structure and biological characteristics of long noncoding RNAs (lncRNAs). To distinguish them from short non-coding RNA (<200 nt), RNAs whose transcripts are longer than 200 nucleotides and do not encode proteins are collectively referred to as lncRNAs[5]. Researches[6] have shown that certain lncRNAs can encode small polypeptides with important functional roles. The lncRNAs are classified into intergenic lncRNAs and intragenic lncRNAs according to their differences in position in the genome. The intragenic RNA can be subdivided into sense lncRNAs, anti-sense lncRNAs and bidirectional lncRNAs according to the position different between the lncRNAs and the protein-coding genes[7].

Numerous researches have proved that some lncRNAs have specific functions and played an important regulatory role in cell biology[8,9]. The diversity of mechanisms of lncRNAs is mainly due to numerous functional conformations, biochemical characteristics and specific subcellular localization[10]. Most of the reported functional ln-

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cRNAs were located in the nucleus. LncRNAs in the nucleus mainly regulate the gene expression at the epigenetic, transcriptional and post-transcriptional modifications, while cytoplasmic lncRNAs regulate the protein translation and mRNA stability maintenance[11-13]. Rashi et al[13] observed that cytoplasmic lncRNAs can regulate gene expression by regulating mRNA stability and protein modification after transcription and protein translation.

In preeclampsia, Zuo et al[14] found that the expression of sprouty RTK signaling antagonist 4 intronic transcript 1(SPRY4-IT1) in PE placenta was significantly increased, which affected epidermal mesenchymal transition of trophocytes through Wnt/β-catenin signaling pathway, thus inhibiting the invasion and migration of cells and finally leading to PE. Oudejans et al[15] showed that lncRNA storkhead box 2 intronic transcript 3 (STOX2-IT3) attenuated the differentiation and invasion of trophocytes through the regulation of storkhead box 2 (STOX2) gene expression, leading to PE. Chen et al[16] demonstated that the expression of lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) was significantly decreased in PE. After interrupting the lncRNA MAL-AT-1 in the trophoblast cell line JEG-3, it was found that the cell cycle arrested in the G0/G1 phase and the proapoptotic protein levels of caspase -3, caspase-9 and poly(ADP-ribose) polymerase 1(PARP-1) were significantly increased, cell invasion and migration were inhibited. This indicated that lncRNA MALAT-1 played an important role in the regulation of cell proliferation, cell cycle, apoptosis, migration and invasion of trophocytes, and the decrease of these expressions can lead to the occurrence of PE. Zhang et al[17] found that lncRNA maternally expressed 3 (MEG3) led to PE by affecting the migration, invasion of trophocytes and remodeling of uterine spiral arteries by affecting the expression of NF-κB, Caspase-3 and BCL2 associated X (Bax) in trophocytes. However, the function of a large number of lncRNAs in preeclampsia disease has not been reported. Therefore, the study on lncRNA in preeclampsia had important significance. HOTTIP has been reported playing important roles in diverse disease, but the role of HOTTIP is still unclear in PE.

Patients and Methods

Collection and Processing of Samples

Aseptic placenta after cesarean section was taken out, several placenta tissues on the placenta surface near the root of the umbilical cord sized (about 1 cm × 1 cm × 1 cm) were taken to avoid the organization, calcification, hemorrhage, etc. After the aseptic placenta was washed with phosphate-buffered saline (PBS) repeatedly until no blood remained, it was stored in -80°C refrigerator Thermo Fisher Scientific (Waltham, MA, USA) for further use. This study was approved by the Ethics Committee of People's Hospital of Linyi City. Signed written informed consents were obtained from all participants before the study.

Construction of Overexpression Vector and Interference Sequence of HOTTIP

Gene synthesis was performed by Invitrogen Company (Carlsbad, CA, USA). The splicing reaction of the single-stranded oligo was designed and synthesized according to the HOTTIP sequence analysis results. Next, the spliced PCR product was cloned into the vector. After that, the vector was transformed into host bacteria. At last, the target fragment was cloned into the target vector pcDNA-NC to form HOTTIP overexpression vector. At the same time, a series of small interfering oligo RNA was conducted to form si-HOTTIP, the sequence was: GCACAGAGAUAAUGGCAAAUU (Invitrogen, Carlsbad, CA, USA). After transfection, the expression of HOTTIP in cells was detected by PCR to verify the interference efficiency.

Cell Culture

Trophocytes HTR-8/SVNEO-8/SVneo, JEG-3 and BeWo cell lines were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) Gibco (Grand Island, NY, USA) medium containing 5% fetal bovine serum (FBS), Gibco (Grand Island, NY, USA) and placed in a 37°C, 5% CO_2 cell incubator. Cell culture medium was replaced according to cell growth conditions. The cells were passaged with trypsin enzyme-digesting technique at the concentration of 80%. The cells were inoculated into a 6-well plate ($2 \times 10^{\wedge 5}$/well), and after 24 h of incubation, the cell aggregation rate was over 70%. Cells were transfected with si-NC, si-HOTTIP, pcDNA-NC and pcDNA-HOTTIP, respectively.

Detection of the mRNA Expression of HOTTIP in Placenta Tissues and Cells by Real-Time Quantitative PCR (qRT-PCR)

Normal pregnancy, PE placental tissues and cell lines were treated with TRIzol Invitrogen (Carlsbad, CA, USA); total RNA was extracted by RNA extraction kit. After reverse transcription into cDNA, target genes were amplified using polymerase chain reaction to detect dif-

ferential mRNA expressions. The sequences of the primers were as follows: HOTTIP (Forward) 5'-CCTAAAGCCACGCTTCTTTG-3', HOTTIP (Reverse) 5'-TGCAGGCTGGAGATCCTACT-3'; GAPDH (Forward) AGGAGCGAGATCCCGCCAACA, GAPDH (Reverse) CGGCCGTCACGCCACATCTT.

Detection of Cell Proliferation After Transfection by CCK8 Assay

The four groups of cells were transfected for 24-48 h and then seeded into a 96-well plate, 5000 cells per well and five replicate wells were set. After cell were cultured for 6, 24, 48, 72 and 96 h, 10 μL cell count kit 8 (CCK-8) TaKaRa (Otsu, Shiga, Japan) were added in each well, the absorbance (A) value of each well was measured at 450 nm after incubation for 2 h. Each experiment was in triplicate.

Plate Clone Formation Assay

After 24-48 h transfection, cells at the concentration of $5*10^2$ were seeded into a 6-well plate per well. The cells were cultured in 10% fetal bovine serum (FBS) medium for 2 weeks. After that, cells were washed three times with phosphate-buffered saline (PBS) and then fixed by methanol and stained by 0.1% Crystal Violet (Sigma-Aldrich, St. Louis, MO, USA), respectively.

Cell Cycle Determination

Cells were seeded in a six-well plate and were divided into plasmid-transfected with pcDNA-NC, pcDNA-HOTTIP and si-NC and siHOTTIP groups. After cultured for 24 h, 1 × 10^6 cells were collected in each group, washed twice with PBS, and the supernatant was discarded. 70% ethanol (precooling at -20°C) was added and the cells were incubated for 30 min with propidium iodide (PI) 50 μg/mL in each sample after Rnase incubation for 15 min. The stained cells were detected by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Detection of the Protein Expression by Western Blot

RIPA (Beyotime, Shanghai, China) lysate was used to lyse cells to extract total protein after it was transfected for 24 to 48 h. The sample volume was determined. RND3 and cyclin dependent kinase 2(CDK2) primary antibodies (1:1000 from Cell Signaling Technology, CST, Danvers, MA, USA) were added by conventional electrophoresis and incubated at 4°C overnight. The cells were incubated with horseradish peroxidase (HRP)-based secondary antibody (Cell Signaling Technology, Danvers, MA, USA, goat anti-rabbit IgG 1:5000) for 2 h at room temperature and then developed by enhanced chemiluminescence (ECL) (Beyotime, Shanghai, China). The integral optical density (IOD) value of each band was measured with a gel imaging analysis system, and GAPDH was used as an internal reference. The relative expression of protein was calculated by the ratio of sample IOD to control IOD.

Statistical Analysis

Statistic package for social science (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) was used for picture editing. Survival analysis was performed using Kaplan-Meier survival curves. Measurement data were compared with *t*-test and presented as mean ± standard deviation ($\bar{x}\pm s$), categorical data were compared with x^2-test. $p<0.05$ indicated significant difference; *$p<0.05$, **$p<0.01$ and *** $p<0.001$.

Results

Relationship Between Clinical Data and Preeclampsia

Clinical data of 60 patients with preeclampsia and normal pregnancy in our center were collected and analyzed. Results showed that there was no significant difference in body weight at birth (Figure 1A) and childbearing age (Figure 1B) in preeclampsia. However, urinary protein level in patients with preeclampsia was significantly higher than those in normal pregnancy (Figure 1C). In addition, systolic blood pressure (Figure 1D) and diastolic blood pressure (Figure 1E) in preeclampsia group were significantly higher than the normal pregnancy group. In addition, the birth weight of preeclampsia patients was significantly less than the normal pregnancy group (Figure 1F).

Detection of HOTTIP Expression in PE Placenta

QRT-PCR results showed that the expression of HOTTIP in PE placenta was lower than that in normal placenta ($p<0.05$) (Figure 2A and 2B). In addition, patients with preeclampsia were divided into high and low expression groups. Systolic blood pressure (Figure 2C) and diastolic blood pressure (Figure 2D) in patients with HOTTIP

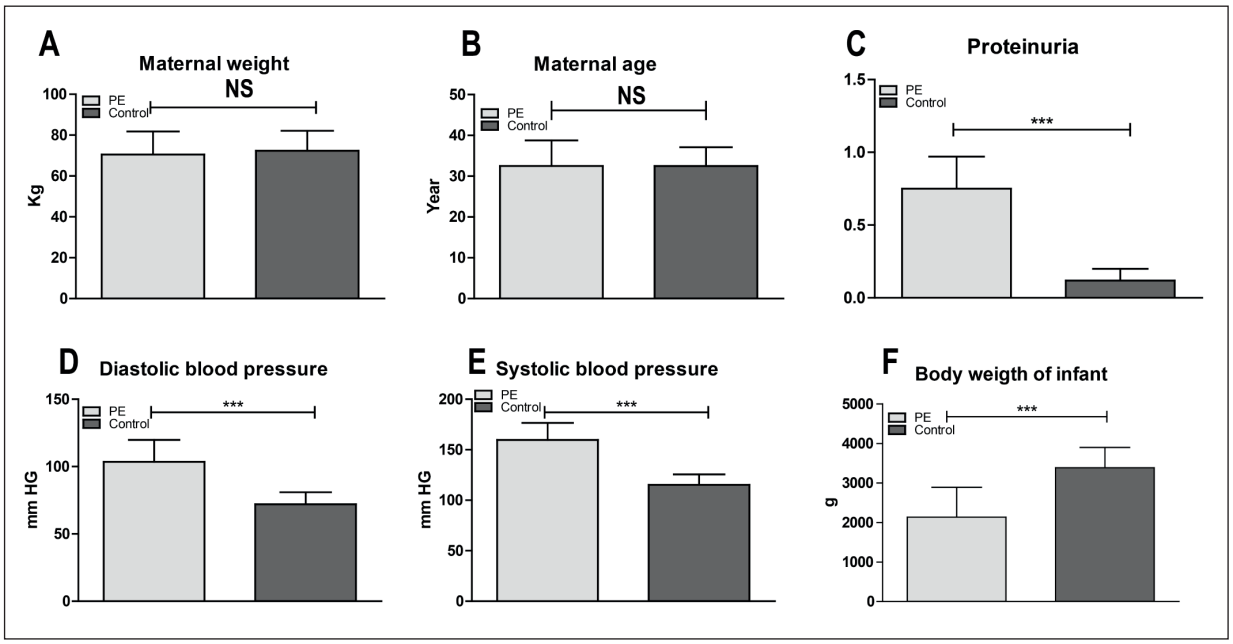

Figure 1. Similarities and differences between preeclampsia and normal pregnant women. **A**, There was no significant difference in body weight among women with preeclampsia and normal pregnancies. **B**, There was no significant difference in maternal age between preeclampsia and normal pregnancy. **C**, Urinary protein level in patients with preeclampsia was significantly higher than normal pregnant women. **D**, Systolic blood pressure in preeclampsia patients was significantly higher than normal pregnant women. **E**, Systolic blood pressure in preeclampsia patients was significantly higher than normal pregnant women. **F**, Neonatal weight in preeclampsia patients was significantly lower than normal pregnant women.

high expression group were significantly lower than those with low expression group analyzed by *t*-test. In addition, the level of proteinuria in the high expression group was significantly lower than in the low expression group (Figure 2E). The birth weight of neonates with high expression group was significantly higher than that of low expression group (Figure 2F). These data indicated that HOTTIP played an important role in the progression of preeclampsia and mainly suppressed the progression of preeclampsia.

HOTTIP Suppresses the Progression of Preeclampsia

The expression of HOTTIP in HTR-8/SVNEO-8/SVneo, BeWo and JEG-3 cell lines was detected firstly. It was found that HTTIV-8/SVNEO-8/SVneo had the lowest and JEG-3 had the highest expression of HOTTIP. The expression of pcDNA-HOTTIP in HTR-8/SVneo cell lines transfected with pcDNA-HOTTIP overexpression plasmid and empty vector for 24-48 h were significantly increased ($p<0.01$) (Figure 3C). After transfection of si-NC and si-HOTTIP into JEG-3 cell line, the interference ability of HOTTIP1 # was significantly higher than that of HOTTIP2 # group. After si-HOTTIP1 interference, the expression of HOTTIP was significantly decreased. The results showed that overexpression and knockout of HOTTIP were successful. Next, we found that after the interference of HOTTIP, cell proliferation of JEG-3 was significantly weakened, while after overexpression of HOTTIP, proliferation of HTR-8/SVneo was significantly enhanced via CCK-8 assay. By plate clone formation assay, similar results were also obtained.

HOTTIP Regulates Cell Cycle and Inhibits the Mechanism of Preeclampsia

By detecting the cell cycle by flow cytometry, we found that JEG-3 cell line was arrested in GO/G1 phase after interfering with HOTTIP (Figure 4A). After overexpression of HOTTIP, cell cycle transition of HTR-8/SVneo-8 significantly accelerated (Figure 4B). In order to explore the mechanism of HOTTIP in inhibiting preeclampsia, Western blot results found that the expression of CDK2 was significantly decreased and the expression of RND3 protein was significantly increased after interfering with HOTTIP (Figure 4C). Overexpression of HOTTIP markedly increased the expression of CDK2, and decreased the expression of RND3 in preeclampsia (Figure 4D). These studies showed that HOTTIP regulated the progression of preeclampsia by regulating RND3.

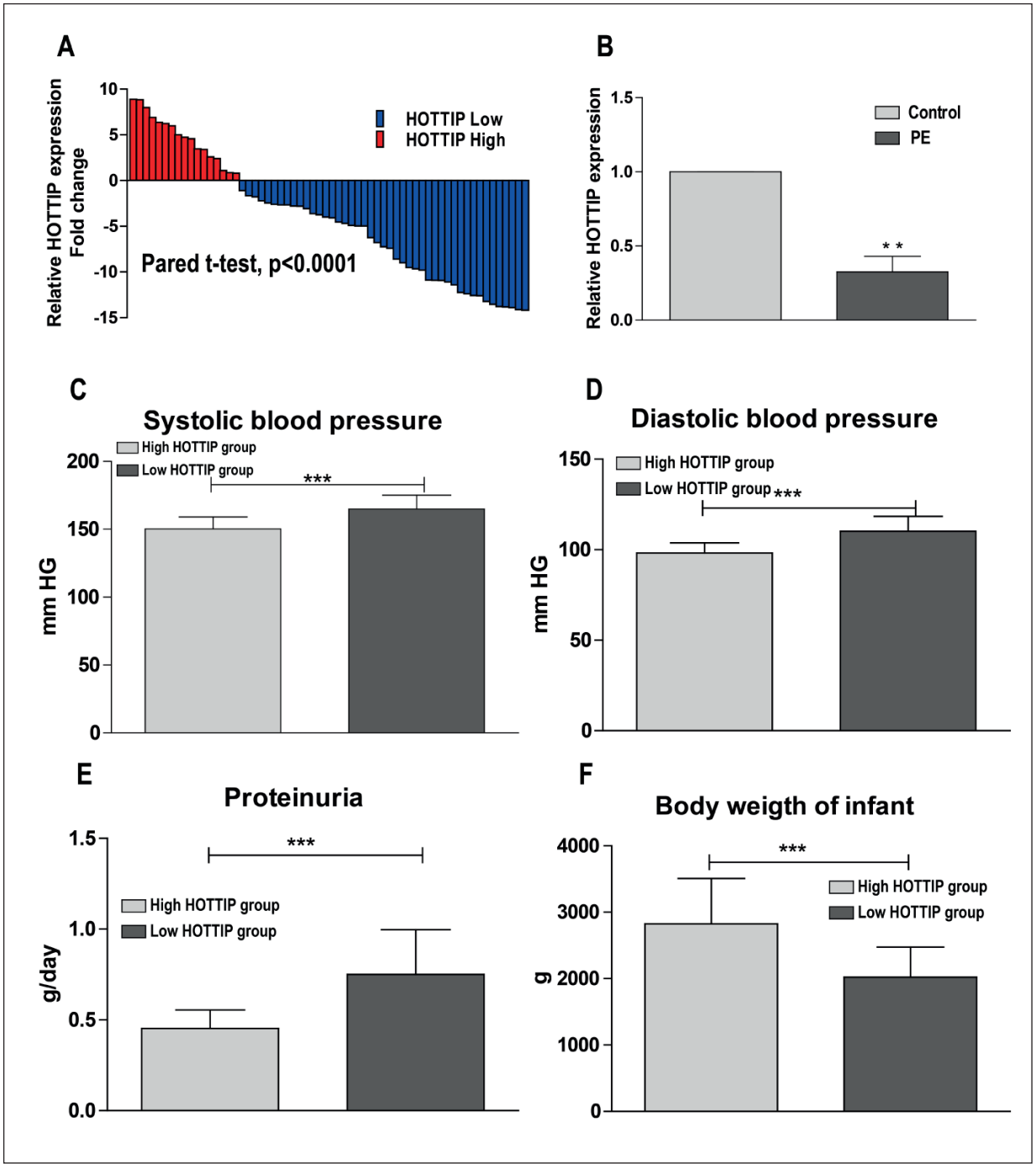

Figure 2. Relationship between moderate to low expression of HOTTIP in preeclampsia patients and the clinical data. **A**-**B**, The level of HOTTIP expression in placenta of preeclampsia patients was significantly lower than that in normal controls. **C**, Systolic blood pressure in patients from HOTTIP low expression group was significantly higher than that in high expression group. **D**, Diastolic pressure in patients from HOTTIP low expression group was significantly higher than the high expression group. **E**, Urinary protein level in patients from HOTTIP low expression group was significantly higher than the high expression group. **F**, Neonatal weight in patients from HOTTIP low expression group was significantly lower than the high expression group.

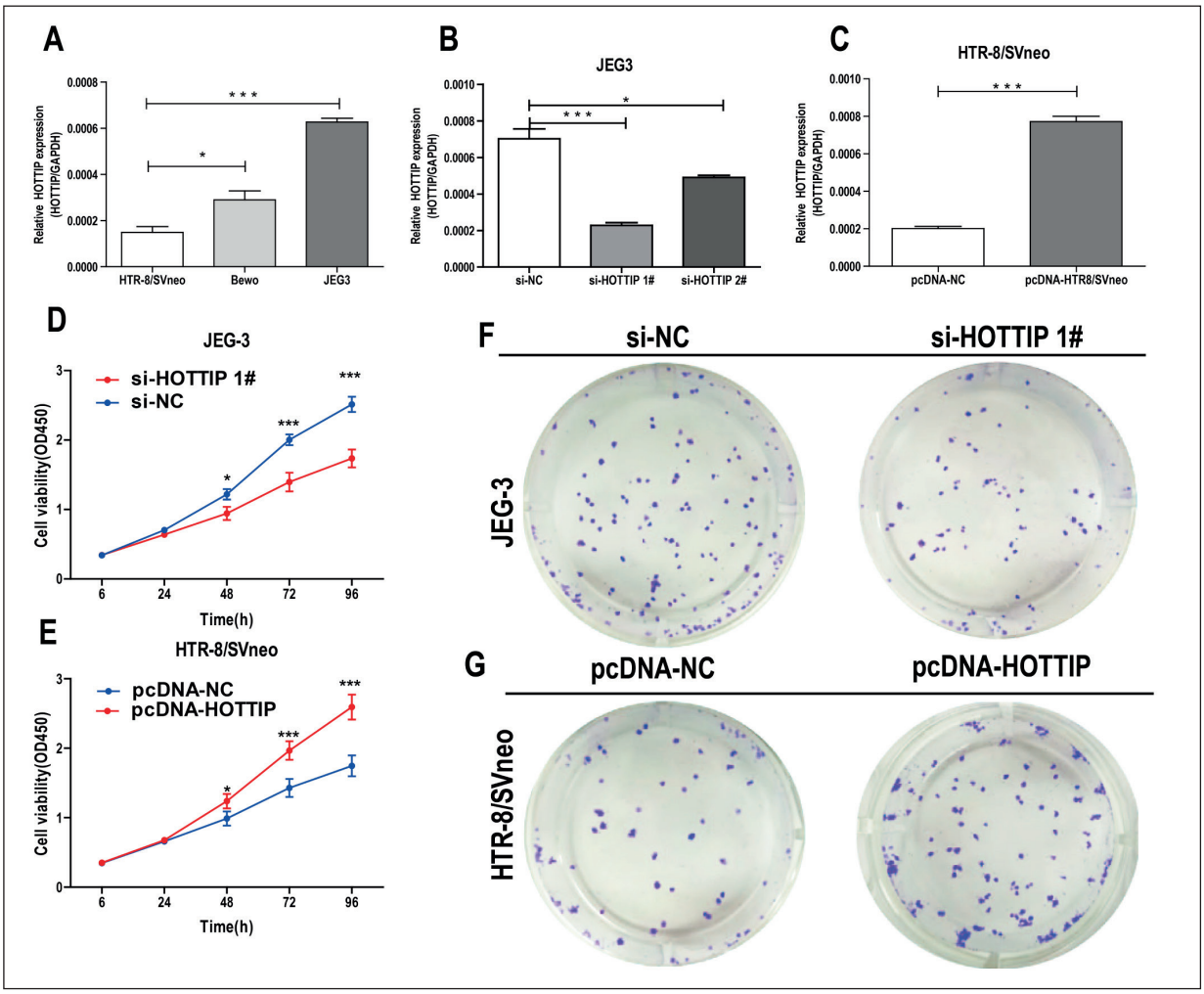

Figure 3. HOTTIP can promote proliferation of trophocytes. **A**, In trophocytes, HTR-8/SVneo had the highest expression of HOTTIP and JEG-3 had the lowest one. **B**, After transfection with si-HOTTIP, the expression of HOTTIP in JEG-3 cells was significantly decreased, especially in si-HOTTIP 1 #. **C**, After overexpressing HOTTIP, the expression of HOTTIP in HTR-8/SVneo cell line was significantly increased. **D**, After interfering with HOTTIP, JEG-3 cell proliferation was significantly reduced. **E**, After overexpressing HOTTIP, HTR-8/SVneo cell proliferation was significantly enhanced. **F**, After interfering with HOTTIP, JEG-3 cell clone ability was significantly reduced. G, After overexpressing HOTTIP, HTR-8/SVneo cell clone ability was significantly enhanced.

Discussion

Long non-coding RNA (lncRNA), as a kind of non-coding RNA, is universally expressed in mammalian cells. LncRNAs have a wide range of biological functions, such as genomic imprinting, chromatin modification, transcriptional activation, transcriptional interference, X chromatin inactivation, nuclear transport, splicing, translation, degradation process. Additionally, lncRNAs are widely involved in the regulation of individual and cell growth, proliferation, differentiation, apoptosis and other life activities. Investigations[18,19] have confirmed that lncRNA was closely correlated with biological evolution, embryonic development, metabolism and tumorigenesis.

Homeobox (HOX) gene is a kind of special transcription regulation factor, which plays a very important role in tumor development as a master gene of proliferation and differentiation[20]. HOX gene consists of four gene clusters: HOXA, HOXB, HOXC and HOXD, among which, HOXA plays an important role in female reproductive system development. HOTTIP was originally discovered by Wang et al[21] in human peripheral fibroblasts and located on 7p15.2. The HOTTIP transcription process begins roughly 300 bases upstream of the 5' end of HOXA13 and transfers

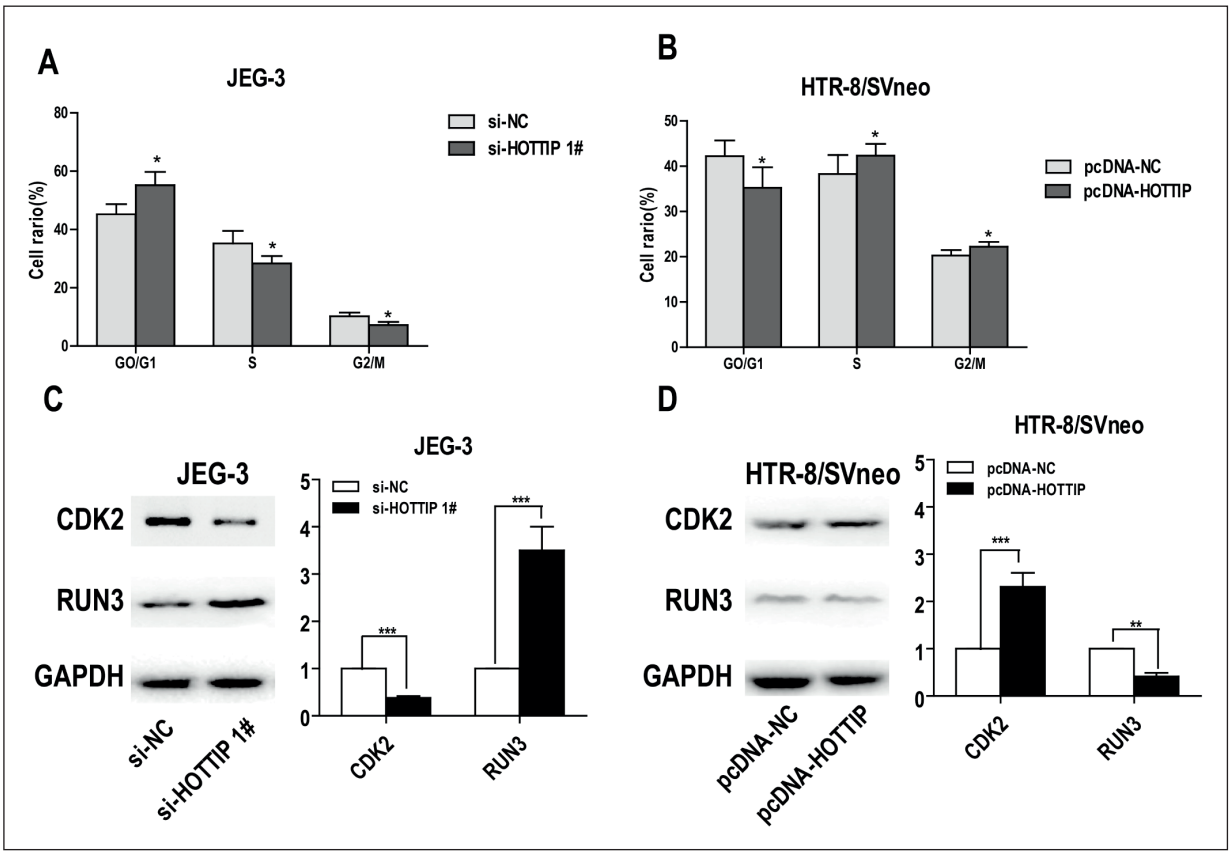

Figure 4. HOTTIP can promote cell cycle of trophocytes. **A**, JEG-3 cell line arrested in G0/G1 phase after interference with HOTTIP. **B**, After overexpressing HOTTIP, HTR-8/SVneo cell cycle was significantly accelerated. **C**, After interfering with HOTTIP, cdk2 expression in JEG-3 cell line was significantly reduced, rdn3 expression was significantly increased. **D**, After overexpression of HOTTIP, the expression of cdk2 in HTR-8/SVneo cell line was significantly increased, and the expression of rdn3 was significantly decreased.

RNA to the cytoplasm after shearing maturation at the 5' end to form a non-coding RNA of 3764 nucleotides in length.

HOTTIP is an important lncRNA whose function has been reported in many tumors. Heng et al[22] found that HOTTIP expression was significantly upregulated in gastric cancer tissues and cells, the expression was correlated with the tumor size, differentiation degree, clinical stage and survival rate. After silencing tumor cells HOTTIP, cell growth slowed down, cell cycle arrested, apoptosis increased, invasion and metastasis decreased. Lian et al[23] showed that HOTTIP in colon cancer inhibited the expression of cyclin dependent kinase inhibitor 1A (p21) protein, thereby promoting cell proliferation and cell cycle arrested in G0, suggesting that HOTTIP may be an important factor in the regulation of colon cancer progression. In addition, HOTTIP was correlated with the progress of esophageal cancer, lung cancer, pancreatic cancer, liver cancer and leukemia.

In this research, we found that HOTTIP expression in PE placenta tissue was lower than normal group, indicating that it may play a role in the pathogenesis of PE. To date, there have been many investigations on the role of lncRNA in development and oncology; however, few studies on other areas, especially pregnancy-related diseases. There is not much evidence on the association of lncRNAs with PE, focusing on differences in lncRNA expression and their effects on trophoblast function. Some previous studies reported the effect of lncRNA SPRY4-IT1 on trophoblast function; further experiments were carried out on its mechanism of affecting the pathogenesis of PE, which may have some guidance on early intervention of PE. This work further enriched the evidence of differentially expressed lncRNA in PE, and to a certain extent, enhanced the close relationship between lncRNA and PE. At the same time, we investigated the effect of HOTTIP on trophocytes by a variety of methods. The results showed that HOTTIP at the cellular level promoted the

growth of trophocytes and the cycle transformation to a great extent. An opposite effect was obtained after interfering with the expression. A research[24] reported that RND3 played an important role in the development of preeclampsia. Western blot showed that the expression of RND3 was significantly decreased after overexpression of HOTTIP, while the expression of RND3 was significantly increased after inhibition of HOTTIP. This result indicated that HOTTIP did play an important role in the regulation of trophoblast function in PE placenta as well as in the pathogenesis of PE.

However, the specific regulation and control mechanism of this factor on the development and progression of PE remains unclear. Therefore, expanding the sample size to explore the function of HOTTIP and its downstream regulatory mechanism will be helpful for the early diagnosis and treatment of PE.

As the powerful function of lncRNA and its mechanism remain unclear, the expression of lncRNA in large sample population by high-throughput sequencing and other techniques should be determined in order to make it a diagnostic marker for PE, as well as changes in clinical diagnostic significance. With the development of life sciences, more molecular biology, cell biology and animal holistic methods can be utilized to further study the expression of lncRNA during the development of PE and its mechanism, to provide a new target molecule for early detection and treatment of PE.

Conclusions

We demonstrated that HOTTIP is low expressed in preeclampsia, and is able to suppress the progression of preeclampsia.

Conflict of Interest:

The authors declared no conflict of interest.

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