Lowly expressed EGFR-AS1 promotes the progression of preeclampsia by inhibiting the EGFR-JAK/STAT signaling pathway

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Abstract. – OBJECTIVE: To detect the differentially expressed long non-coding RNA (lncRNA) EGFR-AS1 in the placenta tissues of preeclampsia (PE) patients and normal pregnancies. We also investigated the underlying mechanism of EGFR-AS1 in regulating PE development.

PATIENTS AND METHODS: 21 PE patients and 21 normal pregnancies admitted in our hospital were selected. The expression of EGFR Antisense RNA1 (EGFR-AS1) in the placenta tissues of PE patients and normal pregnancies was detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The regulatory effect of EGFR-AS1 on proliferation of HTR-8 cells was observed by Cell Counting Kit-8 (CCK-8) assay and colony formation assay. Cell cycle and apoptosis of HTR-8 cells after overexpression or knockdown of EGFR-AS1 were detected by flow cytometry. The expression changes of EGFR and proteins related to JAK/STAT signaling pathway after knockdown of EGFR-AS1 in HTR-8 cells were detected by Western blot. Rescue experiments were carried out after upregulating EGFR expression in HTR-8 cells.

RESULTS: QRT-PCR results showed that the mRNA expression of EGFR-AS1 in PE patients was lower than that in normal pregnancies. CCK-8 assay and colony formation assay showed that knockdown of EGFR-AS1 in HTR-8 cells inhibited cell proliferation. Flow cytometry results showed that knockdown of EGFR-AS1 blocked the cell cycle of HTR-8 cells. Overexpression of EGFR-AS1 obtained opposite results. Western blot results showed that the protein expressions of EGFR, p-JAK and p-STAT were decreased after knockdown of EGFR-AS1. Alterations in protein expressions of p-JAK and p-STAT, and proliferative ability of HTR-8 cells induced by EGFR-AS1 knockdown could be rescued after upregulation of EGFR.

CONCLUSIONS: The expression of lncRNA EGFR-AS1 is decreased in PE patients, which can promote the progression of preeclampsia by inhibiting EGFR-JAK/STAT signaling pathway.

Key Words:
Preeclampsia, lncRNA EGFR-AS1, Progression, JAK/STAT signaling pathway.

Introduction

Preeclampsia (PE) is a disorder of pregnancy characterized by hypertension, proteinuria, with or without multiple organ damage after 20 weeks of pregnancy. Fetal syndromes may be companied, including fetal growth restriction, fetal distress and oligohydramnios. Furthermore, the risks of placental abruption, coagulation disorders, stroke, respiratory distress syndrome, liver and kidney dysfunction are significantly increased in PE patients, especially in severe PE pregnancies. PE is the leading cause for pregnancies admitted to intensive care unit[1]. The prevalence of PE is 2-8% in developed countries, which is significantly higher in developing countries[1-3]. The most recent statistics have indicated that PE affects eight million pregnancies worldwide every year, which has become the main cause of the death of pregnant women and perinatal infants[4,5]. Therefore, it is of great significance to uncover the underlying regulation mechanism of PE pathology, thus providing novel targets in future therapy.

Many factors may be involved in the development and progression of PE, including abnormal immunomodulatory function, inflammatory theory, shallow placenta accreta, impaired vascular endothelium, and nutritional deficiency[6]. For now, the detailed causes and mechanisms of PE have not been clearly investigated. Currently, there has no effective treatment for PE except for the delivery of the placenta and fetus[7].

Long non-coding RNAs (lncRNAs) are a new class of transcripts first discovered by Okazaki et al[8] during the large-scale sequencing of mouse full-length complementary DNA (cDNA) libraries in 2002. LncRNAs are non-coding RNAs with over 200 nucleotides in length. LncRNAs are located in the cytoplasm or nucleus with little or no capacity to encode protein. Current researches[9,10] have shown that lncRNAs can widely participate in genome regulation, such as chromatin modifi-

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cation, transcription, translation and alteration of RNA. It is estimated that there are about 7000 to 23,000 lncRNAs in human genome exerts regulatory effects on biological evolution, embryonic development, material metabolism and tumorigenesis[11]. Functions of some certain lncRNAs in PE have also been explored. For example, lncRNA MEG-3[12] is down-regulated in PE placenta. It is involved in the regulation of migration and apoptosis of trophoblast cells, thereby intervening the process of helical artery remodeling and participating in PE. However, functions of most lncRNAs related to PE and their mechanisms are still not clear.

EGFR Antisense RNA1 (EGFR-AS1) is a lncRNA with 2.8 kb in length. In our study, we detected the expression of EFGR-AS1 in placenta tissues from 42 PE patients and normal pregnancies by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The results showed that EGFR-AS1 was lowly expressed in placenta tissues. As a vascular disease, the main characteristics of PE are systemic arteriole spasm, uterine spiral artery recanalization disorder and obvious angiogenesis disorder. Therefore, trophoblast cells were selected as the research object to explore the effect of EGFR-AS1 on cell proliferation and PE-related genes.

Patient and Methods

Tissue Samples

Tissues of 21 PE patients and 21 healthy donors were harvested from patients treated in our hospital from June 2015 to March 2017. This study was approved by the Ethics Committee of the Second People's Hospital of Liaocheng. Signed written informed consents were obtained from all participants before the study. 21 cases were diagnosed as PE according to united criteria. There was no significant difference in the average age between PE patients (25.8 ± 3.4 years) and healthy donors (25.2 ± 2.8 years).

Cell Culture and Transfection

Trophoblast cell line HTR-8 was cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. Trophoblast cells were seeded in a 6-well plate. When the cell confluence was up to 60%, plasmids were transfected with

Fugene-6 kit according to the instructions. Transfection of siRNAs was performed according to Lipofectamine 2000 kit instructions (Invitrogen, Carlsbad, CA, USA). Si-EGFR-AS1 sequence was 5'-AAGCCAGGUAUUU-CACAGCTT-3'.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) for reverse transcription. The reverse transcription reaction was performed according to the instructions of PrimeScript RT reagent kit (TaKaRa, Otsu, Shiga, Japan). Reverse transcribed cDNAs were diluted for further experiments. The appropriate amount of cDNAs, primers, pre-mixed solution and ultrapure water were mixed for preparing the PCR system. Polymerase chain reaction (PCR) amplification and analysis of fluorescence signals were carried out with ABI 7500 FAST Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) according to the instructions. Primers used in the study were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5′-AGAAG-GCTGGGGCTCATTTG-3′, reverse 5′-AGGG-GCCATCCACAGTCTTC-3′; EGFR-AS1 forward 5′-CCATCACGTAGGCTTCCTGG-3′, reverse 5′-GCATTCATGCGTCTTCACCTG-3; EGFR forward 5′-GCGTCTCTTGCCGGAATGT-3′, reverse 5′-CTTGGCTCACCCTCCAGAAG-3′.

Western Blot

Total protein was extracted using a cell lysate (RIPA) containing protease (Beyotime, Shanghai, China). The protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein samples were then separated by electrophoresis and incubated with primary antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) overnight at 4°C. After washed with Tris-buffered saline and Tween (TBS-T), the membranes were incubated with the corresponding secondary antibody for 2-3 h at room temperature. Enhanced chemiluminescence was performed and the integral optical density value of each band was determined by Gel imaging analysis system (Jackson, MI, USA).

Flow Cytometry

Cell supernatants were collected into labeled tubes and digested with ethylene diamine tetraacetic acid (EDTA)-free trypsin. The cell suspen-

sion was centrifuged and washed twice with phosphate-buffered saline (PBS). 200 µL of binding buffer containing calcium ions were added into the centrifuged precipitation. Corresponding antibodies were added and incubated in dark. Results were detected on a flow cytometer.

Statistical Analysis

Statistical product and service solutions (SPSS19.0, IBM, Armonk, NY, USA) statistical software was used for data analysis. GraphPad Prism 6.0 (La Jolla, CA, USA) was used for image editing. Comparison of measurement data was conducted using t-test. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Classification data were compared using x^2-test. $p<0.05$ was considered statistically significant.

Results

EGFR-AS1 was Lowly Expressed in Placenta and Serum of PE Patients

21 PE patients and 21 normal pregnancies admitted in our hospital from June 2015 to March 2017 were selected. The mRNA expression of EGFR-AS1 in the placenta tissues of PE patients and normal pregnancies was detected by RT-PCR. Results showed that the expression of EGFR-AS1 in placenta and serum of PE patien-

ts was lower than those of normal pregnancies ($p<0.01$, Figure 1A-B). The main characteristics of PE are systemic arteriole spasm, uterine spiral artery recanalization disorder and obvious angiogenesis disorder. We hypothesized that EGFR-AS1 may serve as a diagnostic molecular marker of PE and have biological effects on trophoblasts in the development of PE.

Knockdown of EGFR-AS1 Expression Inhibited the Proliferation and Blocked the Cell Cycle of HTR-8 Cells

To explore the effect of EGFR-AS1 on trophoblasts, HTR-8 cells were selected for the following experiments. EGFR-AS1 expression was knocked down in HTR-8 cells, which was confirmed by RT-PCR (Figure 2A). Colony formation ability of HTR-8 cells was significantly inhibited after EGFR-AS1 expression was decreased (Figure 2B). CCK-8 assay also showed that the proliferation ability of HTR-8 was significantly weakened after EGFR-AS1 knockdown (Figure 2C). Subsequently, flow cytometry results supported that HTR-8 cells were arrested in the G1 phase when EGFR-AS1 was down-regulated (Figure 2D). The above results demonstrated that EGFR-AS1 is involved in regulating proliferation activity and cell cycle of trophoblasts, implying its potential role in PE development.
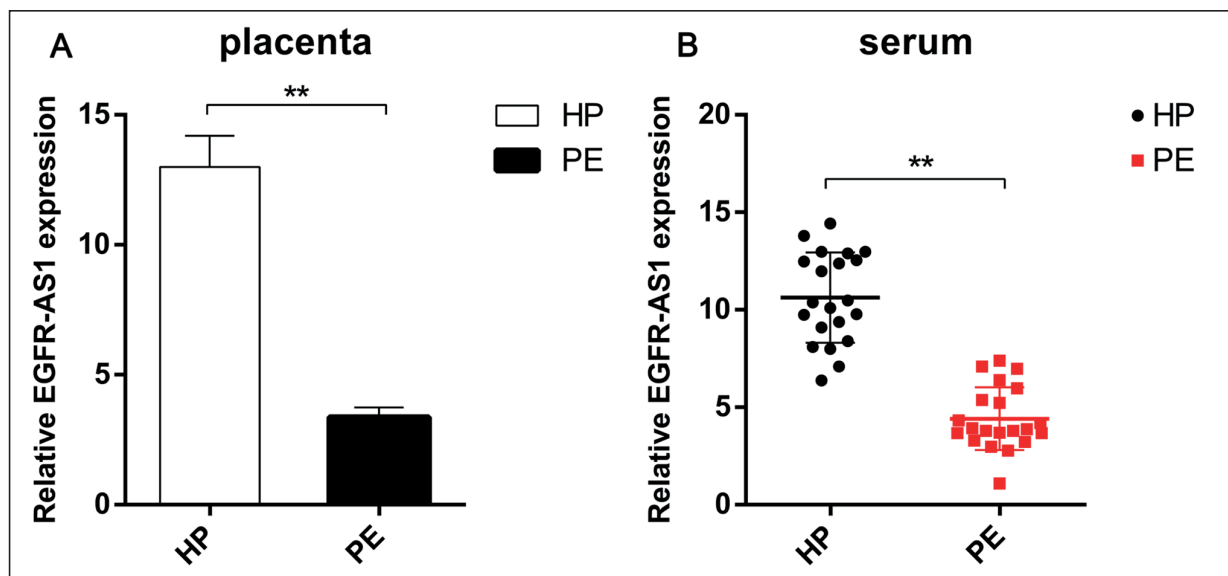
Figure 1. A, The EGFR-AS1 expression in placental tissues of PE patients was lower than that of normal pregnancies (n = 21). B, The EGFR-AS1 expression in serum samples of PE patients was lower than that of normal pregnancies (n = 21).
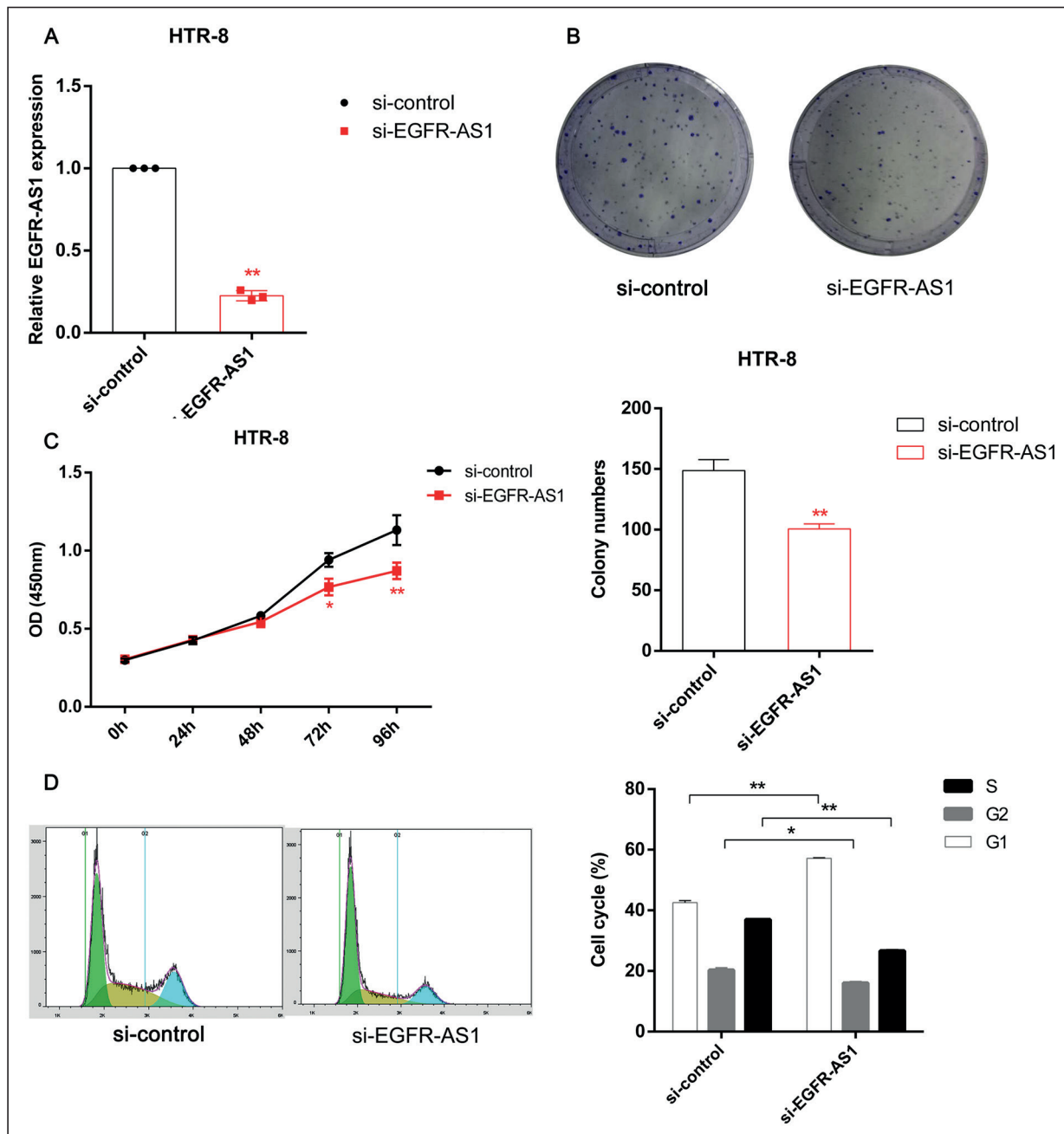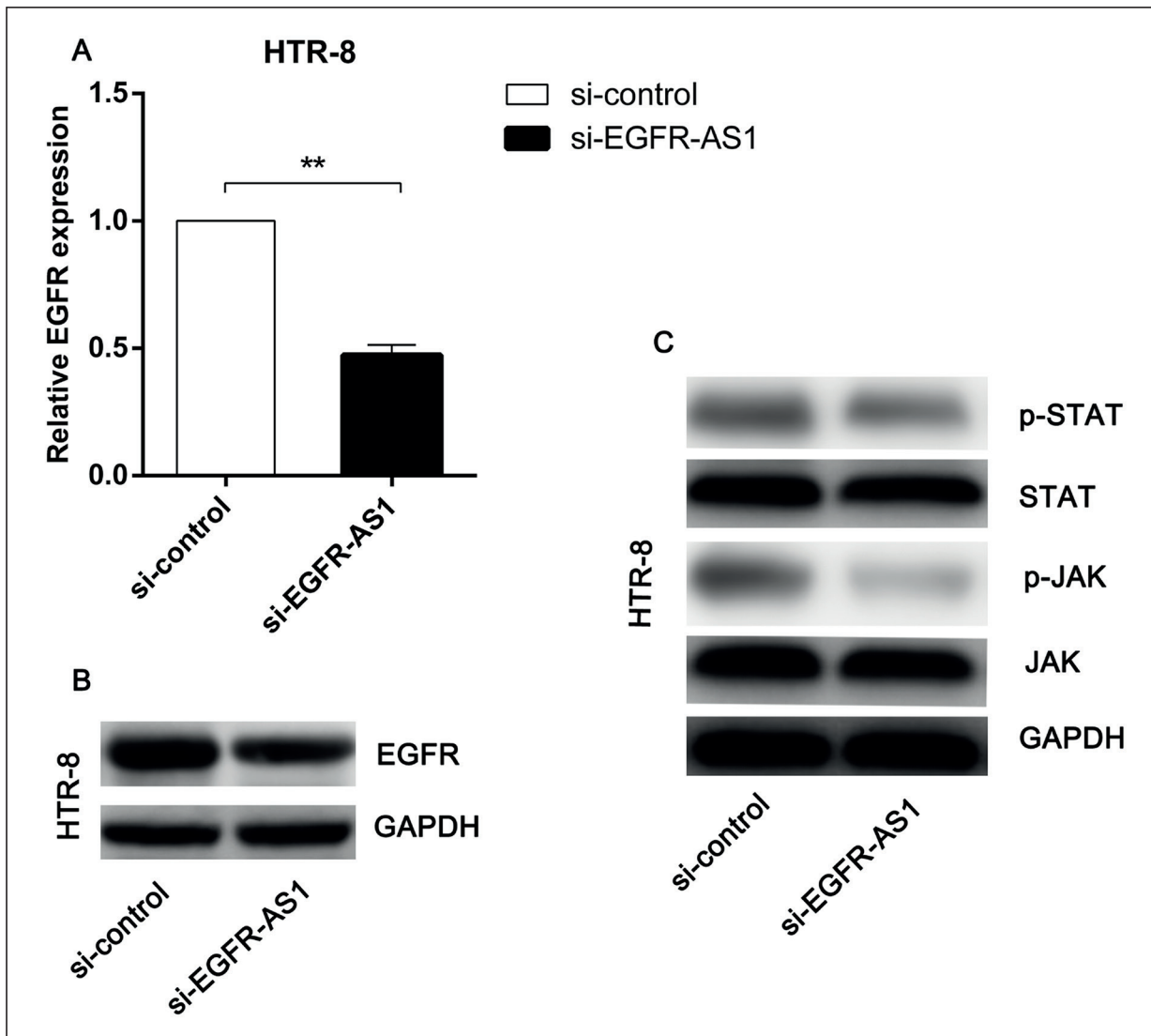
Figure 2. *A,* HTR-8 cells transfected with si-EGFR-AS1 significantly downregulated EGFR-AS1 expression in HTR-8 cells. *B,* The colony formation numbers of HTR-8 cells were significantly decreased after the EGFR-AS1 expression was decreased. *C,* CCK-8 assay showed that the proliferative ability of HTR-8 was significantly reduced after the EGFR-AS1 expression was decreased. *D,* HTR-8 cells were blocked in the G1 phase after the EGFR-AS1 expression was decreased.

Down-Regulation of EGFR-AS1 Led to the Decrease of EGFR Expression and Phosphorylation of Proteins Associated with JAK/STAT Signaling Pathway

Qi et al[13] indicated that down-regulation of EGFR-AS1 leads to decreased EGFR expression in liver cancer. We, therefore, examined the mRNA and protein expressions of EGFR in HTR-8 cells. The results showed that both the mRNA and protein expressions of EGFR-AS1 were decreased when EGFR-AS1 was knocked down (Figure 3A-B). Subsequently, the protein and phosphorylation levels of proteins associated with JAK/STAT signaling pathways were de-
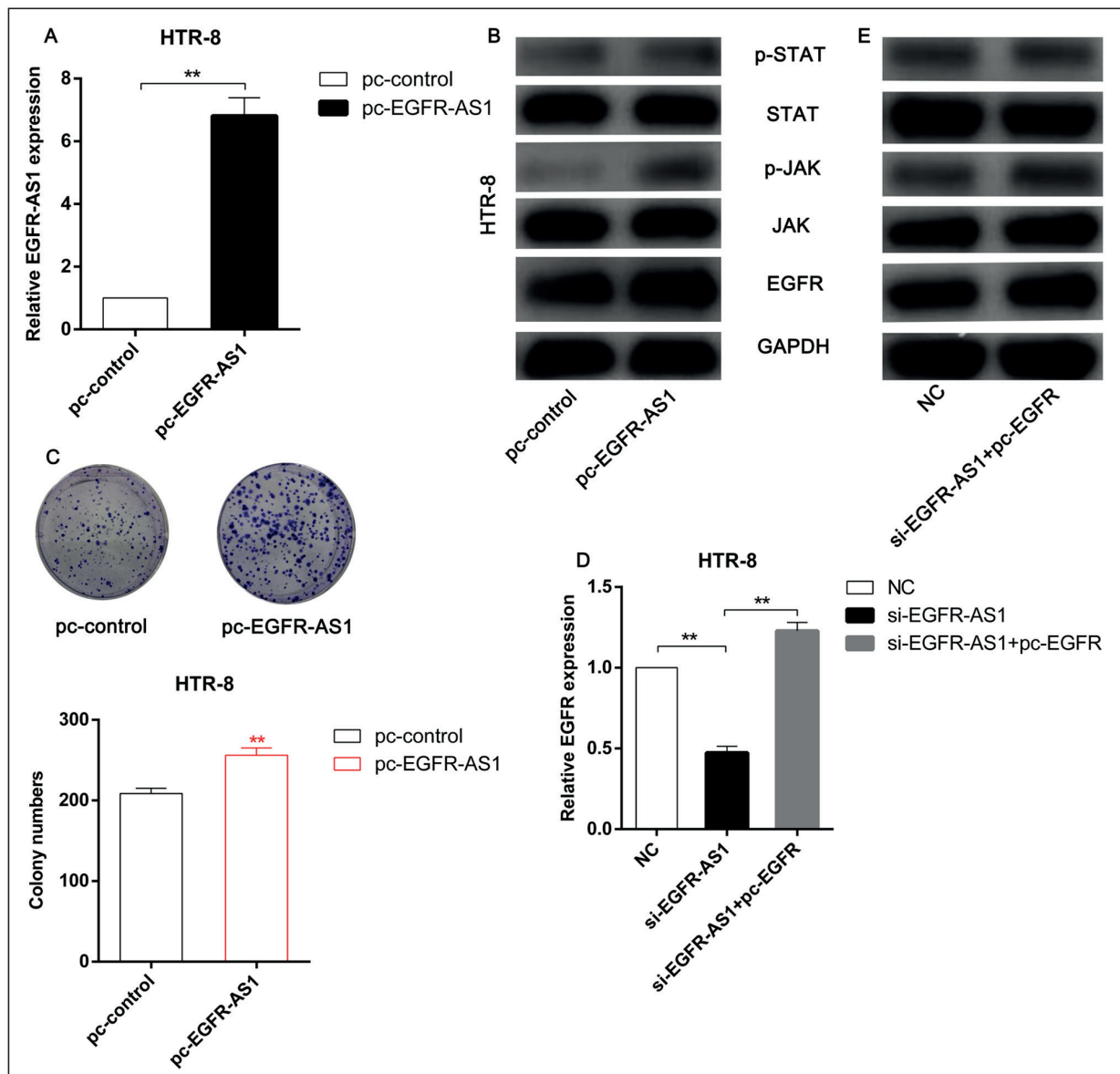
Figure 3. *A,* The mRNA level of EGFR in HTR-8 cells was significantly decreased after transfected with si-EGFR-AS1. *B,* The protein level of EGFR in HTR-8 cells was significantly decreased after transfection with si-EGFR-AS1. *C,* The expression levels of pJAK and pSTAT were significantly decreased after transfection with si-EGFR-AS1.

tected. The results showed that the phosphorylation levels of JAK and STAT were remarkably decreased accompanied by the decrease of EGFR expression (Figure 3C). It is indicated that JAK/STAT signaling pathway was inhibited after down-regulation of EGFR. JAK/STAT is a cytokine-stimulated signal transduction pathway involved in many crucial biological processes such as cell proliferation, differentiation, apoptosis and immunoregulation. Taken together, the down-regulation of EGFR led to the decrease of EGFR expression and phosphorylation levels of proteins associated with JAK/STAT signaling pathway.

Over-Expression of EGFR-AS1 in HTR-8 Cells Promoted Proliferation of HTR-8 Cells, and Up-Regulation of EGFR Up-Regulated Expressions of pJAK and pSTAT

Efficacy of over-expressing EGFR-AS1 in HTR-8 cells was first confirmed by RT-PCR (Figure 4A). Western blot showed that EGFR expression and phosphorylation levels of proteins related to JAK/STAT signaling pathways were increased after EGFR-AS1 over-expression (Figure 4B). In addition, colony formation ability of HTR-8 cells was enhanced after EGFR-AS1 over-expression (Figure 4C). Sub-

Figure 4. *A,* The expression level of EGFR-AS1 was significantly increased after transfection with pc-EGFR-AS1. *B,* The expression levels of EGFR, pJAK and pSTAT were significantly increased after transfection with pc-EGFR-AS1. *C,* The colony formation numbers of HTR-8 cells were significantly increased after transfection with pc-EGFR-AS1. *D,* The expression level of EGFR was rescued after pc-EGFR-AS1 was transfected in HTR-8 cells that were previously transfected with si-EGFR-AS1. *E,* The expression levels of pJAK and pSTAT were rescued after pc-EGFR-AS1 was transfected in HTR-8 cells that were previously transfected with si-EGFR-AS1.

sequently, EGFR expression was rescued in HTR-8 cells after transfection with pc-EGFR-AS1 (Figure 4D). Western blot showed that the decreased phosphorylation levels of JAK and STAT were rescued by pc-EGFR-AS1 transfection as well (Figure 4E). The above data indicated EGFR-AS1 promotes the activation of the JAK/STAT signaling pathway through EGFR in HTR-8 cells.

Discussion

Severe PE is a unique disease during pregnancy. Characteristics of PE include acute onset, rapid progression and poor prognosis of the mother and the baby[14]. Due to the high maternal and child mortality, PE has arouse immerse concerns among researchers. Although there have been many PE-related studies[15], the detai-

led pathogenesis of PE is still not clear. Currently, there are no effective screening methods of PE.

Evidence[16,17] has indicated that dysregulated lncRNAs are involved in a great number of human diseases. However, few researches have been carried out on the association between lncRNA and PE. Previous researches have explored the ability of EGFR-AS1 to promote tumor proliferation in hepatocellular carcinoma and its underlying mechanism in the cell cycle[13]. In addition, Tan et al[18] have confirmed the effect of EGFR-AS1 on regulating the development of squamous cell carcinoma. However, the effect of EGFR-AS1 on the occurrence of PE was rarely reported. In this study, we demonstrated the expression of EGFR-AS1, a lncRNA related to the pathogenesis of PE, is lowly expressed in PE patients than that of normal pregnancies. Additionally, since PE is a vascular disease, the main characteristics of which are systemic arteriole spasm, uterine spiral artery recanalization disorder and obvious angiogenesis disorder. We suggested that EGFR-AS1 may serve as a diagnostic molecular marker of PE and affect PE development via regulating biological functions of trophoblasts.

Previous studies have shown that many cytokines and growth factors transmit signals through the JAK/STAT signaling pathway, including EGFR[19-21]. Our work found that the proliferation of HTR-8 trophoblast cells was inhibited after EGFR-AS1 was down-regulated. EGFR expression and phosphorylation levels of proteins related to JAK/STAT signaling pathway were significantly decreased as well[22], whereas overexpression of EGFR-AS1 obtained the opposite results. These results indicated that lowly expressed EGFR-AS1 in placental tissues of PE patients can inhibit the expression of EGFR in HTR-8 cells. The phosphorylation levels of downstream proteins in JAK/STAT signaling pathway were down-regulated by EGFR knockdown, thereby promoting the progression of PE. However, *in vivo* experiments are still lacked to confirm the above results.

In conclusion, lowly expressed EGFR-AS1 in PE placenta has a close relationship with PE. *In vitro* experiments confirmed that EGFR-AS1 regulates the proliferation of trophoblast cells. Lowly expressed EGFR-AS1 can inhibit the expression of EGFR in HTR-8 cells and the phosphorylation levels of downstream proteins in JAK/STAT signaling pathway, thereby promoting the progression of PE.

Conclusions

We observed that expression of lncRNA EGFR-AS1 was decreased in PE patients. Lowly expressed EGFR-AS1 can promote the progression of PE by inhibiting EGFR-JAK/STAT signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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