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Overexpression of lncRNA TCL6 promotes preeclampsia progression by regulating PTEN

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of long non-coding RNA (lncRNA) TCL6 in preeclampsia (PE) development and to investigate its underlying mechanism.

PATIENTS AND METHODS: The expression of TCL6 in 42 placental tissues of PE pregnancies and normal pregnancies was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Receiver Operating Characteristic (ROC) curve was applied to explore the relationship between TCL6 expression, urine protein level, blood pressure and neonatal weight of PE pregnancies. The proliferation and cell cycle of trophoblast cells after TCL6 knockdown were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Moreover, the specific role of TCL6 in cell cycle was detected by Western blot.

RESULTS: TCL6 was highly expressed in 42 placental tissues of PE pregnancies when compared with that of normal pregnancies. PE pregnancies with lower expression level of TCL6 exhibited significantly lower urinary protein level, as well as systolic and diastolic blood pressure than those with higher level. Besides, neonatal weight was significantly higher in PE pregnancies with lower expression level of TCL6. Meanwhile, downregulation of TCL6 resulted in remarkably increased proliferation and cell cycle of trophoblast cells. In addition, Western blot results indicated that TCL6 knockdown significantly upregulated CDK2 and downregulated PTEN in trophoblast cells.

CONCLUSIONS: TCL6 was highly expressed in placental tissues of PE patients. Overexpression of lncRNA TCL6 inhibited the proliferation of trophoblast cells and promoted PE development via targeting PTEN.

Key Words:
Preeclampsia, TCL6, Cell cycle, Cell proliferation.

Introducion

Preeclampsia (PE) is a serious pregnancy complication that may eventually lead to maternal multisystem dysfunction. The morbidity and mortality of pregnancies and newborns resulted from PE have been significantly increased. Globally, 50,000-60,000 pregnancies die from PE every year, accounting for 5%-8% of all pregnant diseases[1]. As a multi-system syndrome, both genetic and environmental factors are involved in the pathogenesis of PE. Abnormal immune responses in major organisms[2], oxidative stress[3], placental formation and dysfunction, inflammatory response and heredity factors[4,5] are closely related to PE development. However, the pathogenesis of PE is still not fully elucidated. Currently, termination of pregnancy is the only effective treatment for PE[6].

Placental defects, especially placental shallow implantation, are considered to be the major causes of PE in recent years[7,8]. Decreased invasion of extra-villous trophoblasts (EVT) leads to the inhibition of uterine spiral artery remodeling. Subsequently, a series of pathophysiological changes of PE will be resulted from increased resistance of placental blood flow and decreased perfusion[9]. Further studies have shown that differentially expressed long non-coding RNAs (lncRNAs) in the placenta can regulate the proliferation, apoptosis, migration and invasion of EVT, thereby inducing the development of PE[10,11].

LncRNAs are involved in the occurrence and development of multiple diseases. Previous studies have explored the role of lncRNAs in tumors, cardiovascular diseases, dyspnea and neurodegenerative diseases. However, the specific effects

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of lncRNAs on PE are rarely reported. LncRNA TCL6 was initially reported in T-cell leukemia, which is involved in the development of leukemia[12]. However, the exact role of TCL6 in the occurrence and progression of PE has not been fully elucidated.

Patients and Methods

Patients

From March 2012 to August 2017, totally 42 PE pregnancies without uterine contractions during cesarean delivery in Obstetrics, Jining No.1 People's Hospital were enrolled in this study. During the same period, 42 normal pregnancies were selected as controls. PE diagnosis was based on the following AGOG (American College of Obstetricians and Gynecologists) guidelines: 1. New-onset hypertension after 20 weeks of pregnancy, with blood pressure ≥ 140 mm Hg systolic or ≥ 90 mm Hg diastolic on two separate readings taken at least 4 h; 2. Urine protein > 0.3 g/24 h or random urine protein > 1+; 3. In the absence of proteinuria, the presence of new-onset hypertension and new onset of one or more of the following characteristics was suggestive of PE diagnosis: thrombocytopenia (platelet count <100,000/μL), kidney dysfunction (creatinine >1.1 mg/dl), pulmonary edema, impaired liver function, cerebral or visual disturbances. This study was approved by the Ethics Committee of the Hospital. The informed consent was obtained from each patient before the study.

Placenta Specimen Collection

Surgical instruments were routinely disinfected. Placenta tissues were surgically resected within 5 min after placental expulsion. Several placenta tissues (1 cm × 1 cm × 1 cm) on the placenta surface near the root of the umbilical cord were taken. After washing with phosphate-buffered saline (PBS) repeatedly until no blood remained, aseptic placenta tissues were stored at -80°C for further use.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) in accordance with the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was determined by a spectrophotometer (Hitachi, Tokio, Japan). The relative expression level of target genes was calculated by the $2^{-\Delta\Delta CT}$ method. Primers used in the study were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5′-CACCCACTCCTCCACCTTTG-3′, R: 5′-CCACCACCCTGTTGCTGTAG-3′; TCL6, F: 5′-TGTCTCATTCGCCTCTGGAT-3′, R: 5′-GTCTCCCTCCTTCTGCCTTT-3′.

Cell Culture

Human HUVEC-C, JEG-3, Wish and HTR-8 cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Meanwhile, cells were maintained in a 37°C, 5% CO_2 incubator.

Cell Transfection

Cells in good growth were selected and seeded into 6-well plates. When cell density was up to 50%-60%, cell transfection was performed according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Primers of constructed siRNAs were: si-TCL6 1# 5′-TTTCCTGGAAAACTCATGAATAATC-3′; si-TCL6 2# 5′-AAGTGATTCTTCTGCCTCAGCCTCC-3′; si-TCL6 3# 5′-TATTTTTAGTAGAGACAGGGTTTCAC-3′.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates, with 2000 cells per well. After culturing for 6 h, 24 h, 48 h, 72 h and 96 h, 10 μL CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added in each well, followed by incubation for 2 h in the dark. Optical density (OD) value at the wavelength of 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Cycle

Cells were first centrifuged at 800 rpm/min for 5 min, and the precipitant was re-suspended in pre-cooled 70% ethanol. After washing with PBS, the cells were stained with propidium iodide (PI) for 30 min. Subsequently, the cell cycle of stained cells was detected by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Western Blot

Transfected cells were lysed with cell lysis buffer, shaken on ice for 30 min and centrifuged at 4°C, 14,000 ×g for 15 min. The concentration of extracted protein was detected by the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Subsequently, extracted proteins were separated on 10% SDS- sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies of anti-CDK2 and anti-PTEN (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After rinsing with phosphate buffered saline-tween (PBST), the membranes incubated with corresponding secondary antibody. Finally, immunoreactive bands were exposed by the enhanced chemiluminescence method (ECL) (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software was used for all statistical analysis. GraphPad (La Jolla, CA, USA) was applied for figure editing. Receiver operating characteristic (ROC) curve was used to analyze the relationship between TCL6 expression and the prognosis of PE pregnancies. Experimental data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

TCL6 was Highly Expressed in PE Patients

Compared with normal pregnancies, the expression of TCL6 in 42 placental tissues of PE pregnancies was significantly higher ($p<0.001$, Figure 1A). The area under the ROC curve was 0.8625, suggesting that TCL6 could be served as a prognostic factor for PE (Figure 1B). The basic characteristics of the enrolled subjects were shown in Table I. No significant differences in age, body weight and Apgar score were found between PE pregnancies and normal pregnancies. Furthermore, all PE pregnancies were divided into two groups according to TCL6 expression. Results demonstrated that PE pregnancies with lower expression level of TCL6 presented significantly lower urinary protein level, systolic and diastolic blood pressure than those with higher level (Figure 1C-1E). Besides, neonatal weight was obviously higher in PE pregnancies with lower TCL6 expression (Figure 1F). The above results indicated that TCL6 overexpression might promote PE development.

TCL6 Inhibited the Proliferation of Trophoblast Cells

To explore the specific role of TCL6 in trophoblast cells, we detected TCL6 expression in HUVEC-C, JEG-3, Wish and HTR-8 cells by qRT-PCR. Results showed that HUVEC-C cells expressed the lowest level of TCL6, whereas JEG-3 and Wish cells expressed the highest level (Figure 2A). Subsequently, we constructed three siRNA sequences of TCL6, and found that si-TCL6 1# exhibited the highest efficiency in downregulating TCL6 (Figure 2B and 2C). Meanwhile, the overexpression plasmid of TCL6 remarkably upregulated TCL6 expression in HUVEC-C cells (Figure 2D). Subsequently, the proliferation of trophoblast cells was detected by CCK-8. Results showed that si-TCL6 transfection remarkably increased the proliferative capacity of JEG-3 and Wish cells (Figure 2E and 2F). However, TCL6 overexpression significantly reduced the proliferation of HUVEC-C cells (Figure 2G).

TCL6 Inhibited Cell Cycle of Trophoblast Cells

TCL6 knockdown prolonged the G0/G1 phase, whereas reduced the G2/M phase in JEG-3 and Wish cells (Figure 3A and 3B). Overexpression of TCL6 in HUVEC-C cells obtained the opposite results (Figure 3C). Furthermore, we detected the protein expression levels of cell cycle-related genes by Western blot. Interestingly, we found that CDK2 expression was negatively regulated by TCL6 (Figure 3D-3F). PTEN is a tumor-suppressor gene that arrests the cell cycle. In the present study, our findings demonstrated that TCL6 positively regulated PTEN in trophoblast cells (Figure 3D-3F).

Discussion

PE is a serious complication during pregnancy, and its pathological mechanism is related to multiple factors. PE can only be completely cured when the placenta is delivered[13]. Therefore, early

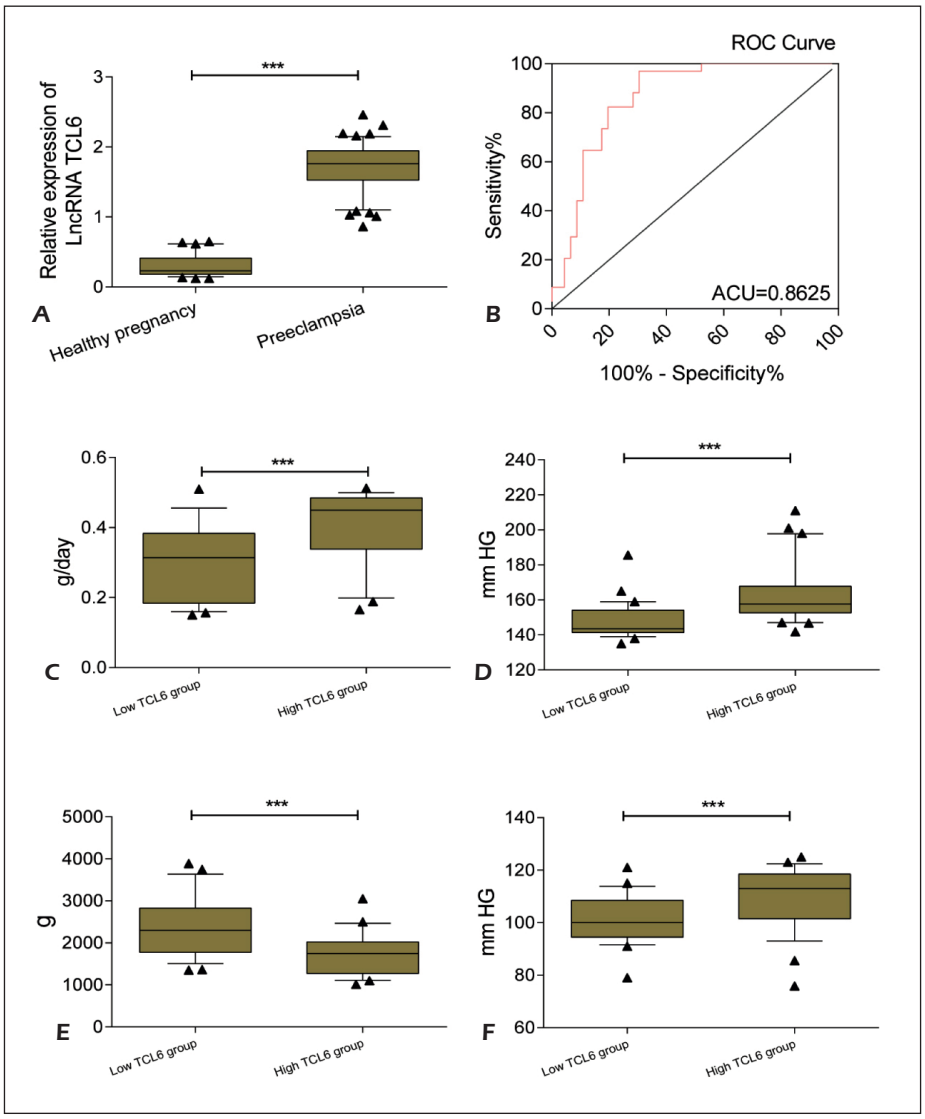

Figure 1. TCL6 was highly expressed in PE patients. **A**, TCL6 was highly expressed in 42 placental tissues of PE pregnancies compared with that of normal pregnancies. **B**, The area under the ROC curve of TCL6 expression in PE pregnancies. **C-E**, PE pregnancies with lower expression level of TCL6 exhibited significantly lower urinary protein (**C**), systolic (**D**) and diastolic blood pressure (**F**) than those with higher level. **F**, Neonatal weight was significantly higher in PE pregnancies with low TCL6 expression.

Table I. Basic characteristics of enrolled PE pregnancies and normal pregnancies.

Variable	Preeclampsia (n=42)	Healthy pregnancy (n=42)	*p*-value
Maternal age (year)	28.21±4.12	30.36±3.17	>0.05
Maternal weight (kg)	67.56±9.84	69.71±8.75	>0.05
Apgar (1 min)	7.87±2.02	8.73±1.02	>0.05
Apgar (5 min)	8.91±1.01	10	>0.05
Proteinuria (g/day)	>0.3	<0.3	<0.05
Systolic blood pressure (mm Hg)	165.44±16.33	110.31±9.07	<0.05
Diastolic blood pressure (mm Hg)	105.69±9.14	77.12±5.12	<0.05
Body weight of infant (g)	1531.80±641.27	3275.55±487.61	<0.05

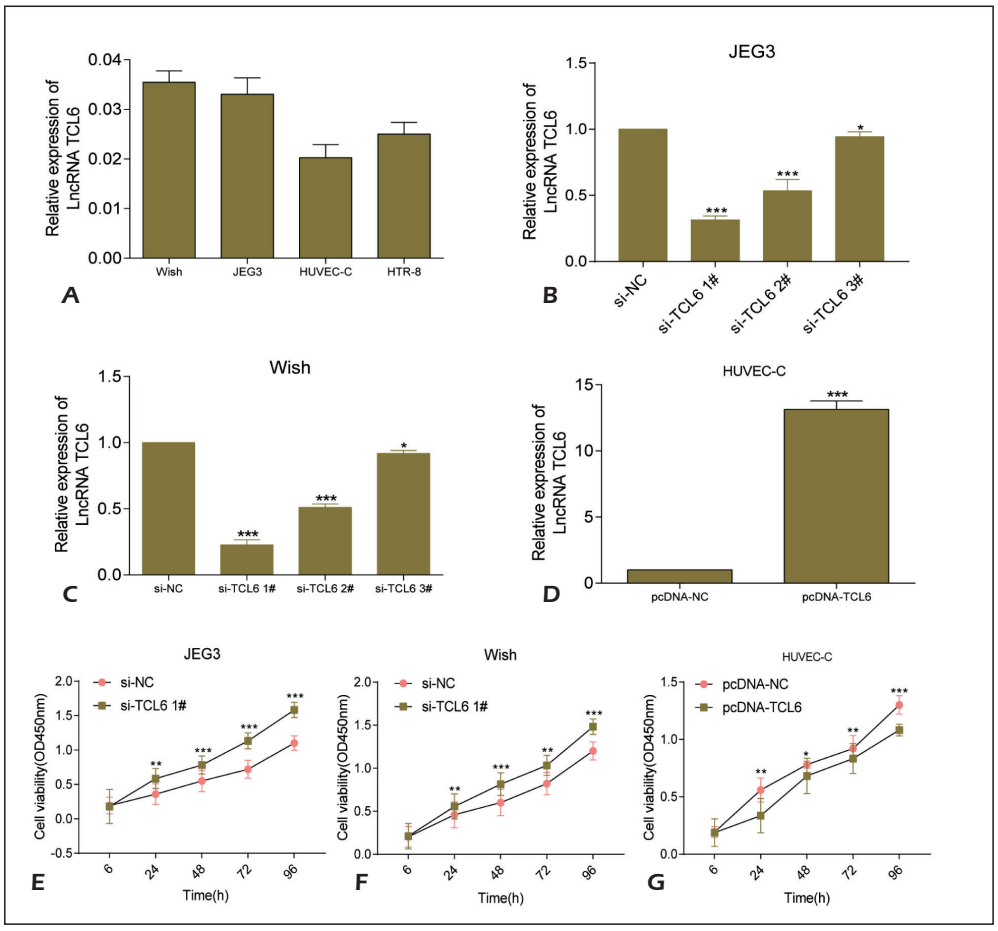

Figure 2. TCL6 inhibited the proliferation of trophoblast cells. **A**, TCL6 expression in HUVEC-C, JEG-3, Wish and HTR-8 cells detected by qRT-PCR. **B-C**, Si-TCL6 1# exhibited the highest transfection efficiency in downregulating TCL6. **D**, Overexpression plasmid of TCL6 remarkably upregulated TCL6 expression in HUVEC-C cells. **E-F**, Transfection of si-TCL6 remarkably increased the proliferative abilities of JEG-3 and Wish cells. (**G**) TCL6 overexpression significantly reduced the proliferation of HUVEC-C cells.

recognition and clinical intervention of high-risk factors for PE can significantly improve pregnancy outcomes. A large number of studies have shown that abnormal trophoblastic invasion, inadequate remodeling of uterine spiral arteries, as well as increased apoptosis of trophoblast cells are the major pathological features of PE[14].

Recent whole-genome sequencing results have shown that most of the stably transcribed RNAs are non-coding RNAs. Among them, lncRNAs are a kind of non-protein-coding RNAs with over 200 nucleotides in length[15]. Functionally, lncRNAs are involved in cell proliferation, differentiation and metabolism. Meanwhile, lncRNAs participate in many pathological processes of the body, such as tumors, diabetes and immune diseases[16,17]. Accumulating evidence has proved that lncRNAs are involved in the occurrence and development of PE. It has been reported that lncRNA SPRY4-IT1, MEG3, LOC391533, LOC284100, and MALAT-1 in placental tissue exert a crucial role in the pathogenesis of PE[18]. TCL6 is lowly expressed in suprarenal epithelioma, which is correlated with poor prognosis in affected patients[19]. In the present study, we found that TCL6 was highly expressed in placental tissues of PE pregnancies than that of controls. Meanwhile, PE pregnancies with lower expression level of TCL6 exhibited significantly lower urinary protein, as well as systolic and diastolic blood pressure. In addition, neonatal weight was obviously higher in PE pregnancies with lower level of TCL6.

Trophozoites, derived from the trophoblast ectoderm, are one of the first differentiated and developed cells in human embryos. During embryo implantation and maternal immunization,

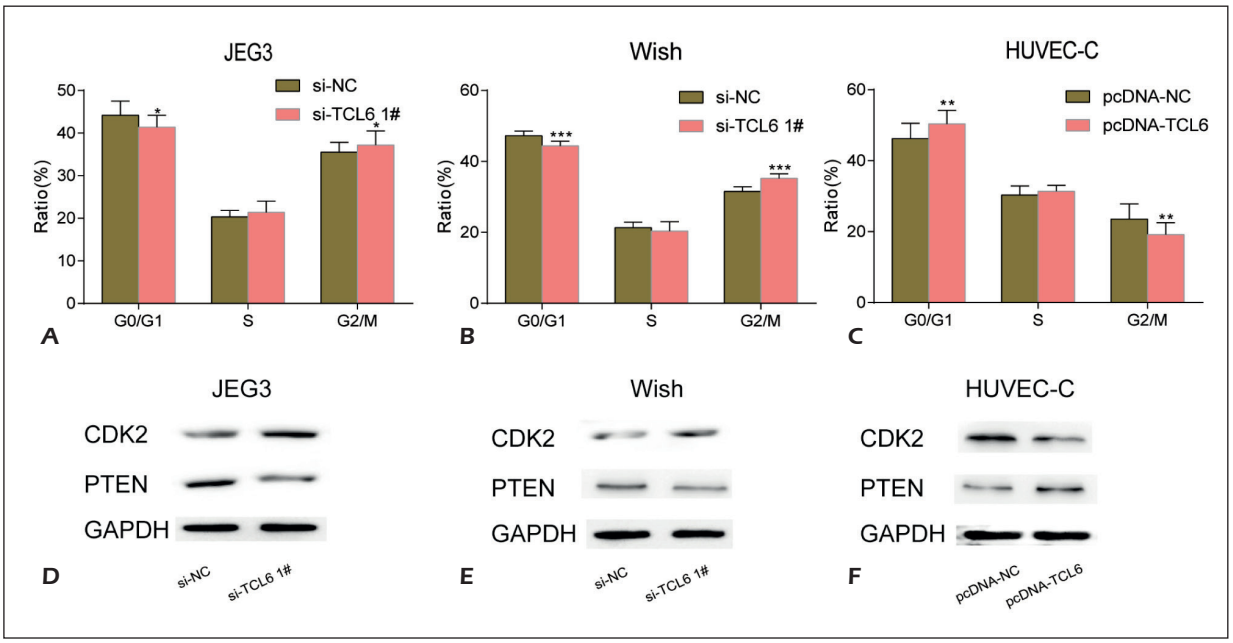

Figure 3. TCL6 inhibited cell cycle of trophoblast cells. **A-B**, TCL6 knockdown prolonged the G0/G1 phase, whereas reduced the G2/M phase in JEG-3 and Wish cells. **C**, Overexpression of TCL6 in HUVEC-C cells obtained the opposite results. **D-E**, CDK2 expression was negatively regulated by TCL6, while PTEN expression was positively regulated by TCL6 in trophoblast cells.

trophozoites are the only fetal cells in direct contact with the maternal immune system at the maternal-fetal interface. The proliferation, differentiation, apoptosis, migration and invasion of trophoblast cells are the basic elements of placental formation and embryonic development[20-23]. Moreover, the insufficient proliferation of trophoblasts is the central link of PE in the early stage. In our study, we explored the effect of TCL6 on the proliferation and cell cycle of trophoblasts. Meanwhile, we also evaluated whether TCL6 could regulate cell cycle-related genes in trophoblasts. Our results showed that TCL6 knockdown resulted in increased cell proliferation and cell cycle.

Conclusions

Our results indicated that TCL6 was highly expressed in placental tissues of PE patients. Overexpression of lncRNA TCL6 inhibited trophoblast cell proliferation and promoted PE development *via* targeting PTEN.

Conflict of Interests

The authors declare that they have no conflict of interest.

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