Down-regulation of microRNA let-7d inhibits the proliferation and invasion of trophoblast cells in preeclampsia

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Abstract
Preeclampsia (PE) is a serious pregnancy complication that continues to adverse effects on mother and the fetus. The study investigated the effect of microRNA let-7d (miR-let-7d) on the proliferation and invasion of trophoblast cells (TC) in PE. In situ hybridization techniques were employed to evaluate the miR-let-7d expressions in the placental tissues from 63 PE patients as well as 65 normal placental tissues. Transfection of pre-let-7d, anti-let-7d, and their corresponding control sequences was performed in TCs obtained from PE. The blank group was composed of TCs no transfection and normal group consisted of normal TCs. Expressions of proliferation and invasion markers in TCs were detected by qRT-PCR. Proliferation, apoptosis, and the invasion ability of the TCs were determined using a CCK-8 assay, flow cytometry, and Transwell assay. In comparison to the normal placental tissues, PE placental tissues exhibited increased levels of let-7d expression. Following transfection of anti-let-7d, when compared with the blank group, the anti-let-7d group displayed increased levels of proliferation ability, expression of proliferation labeling proteins PCNA and Ki67, number of TCs as well as expressions of the invasion related proteins MMP-2, MMP-9, and TIMP-1. In contrast declines in cell apoptosis rate were observed. Compared with the blank group, the changes of the indexes were reversed in the pre-let-7d group. The study provided evidence suggesting that low expression levels of miR-let-7d plays a central role in suppressing apoptosis in addition to promoting the proliferation and invasion of PE TCs.

KEYWORDS
apoptosis, invasion, microRNA let-7d, preeclampsia, primary isolation, proliferation, psoriasis trophoblast cells, trophoblast cells

1 INTRODUCTION

Preeclampsia (PE), a serious clinical disorder syndrome, manifested by the onset of hypertension and proteinuria in the second half of pregnancy. PE is stereotypically accompanied with placental and maternal vascular malfunction in addition to the transformative failure of the spiral arteries in the placental bed. PE is currently a primary cause of both fetus and mother death. PE occurs in approximately 3-5% of all pregnancies, and has a particularly high incidence in the developing countries. PE is conventionally, clinically classified as severe in cases of fetal growth restriction (FGR) complications. At present, the pathogenesis of PE still eludes the medical world, however, it has been suggested that
shallow trophoblast invasion may have played an important role.\textsuperscript{5} Additionally impaired vascular remodeling of spiral arteries have been recognized in early onset preeclampsia.\textsuperscript{7} Trophoblast cells (TCs) are a heterogeneous type of fetal cells, are capable of developing into the feto-maternal interface as well as exerting a wide range of functions.\textsuperscript{8} Differentiation of trophoblast stem cells into particular subtypes of TCs plays an essential role in the formation and development of the placenta.\textsuperscript{9} Previous findings have indicated that declined levels of proliferation, abnormal differentiation of TCs in addition to insufficient invasion and migration of TCs in the uterus have all been closely associated with PE.\textsuperscript{10} Furthermore, malfunctioned TCs have been reported to serve as a possible developmental trigger of deterioration in PE.\textsuperscript{9}

MiRNAs are conserved genetic sequences of single-stranded RNA.\textsuperscript{8} Recently, miRNAs have been highlighted as being key regulators capable of influencing various cell processes including differentiation, proliferation, apoptosis, development, as well as angiogenesis and endothelial cell functions, all of which are associated with PE occurrence and manifestation. Furthermore, altered expression of miRNAs has been reportedly linked to multiple pathological diseases such as breast and cervical cancers.\textsuperscript{3,11} Mutations, dysfunction of miRNA biogenesis and their targets leads to the blockage of physiological and biochemical pathways.\textsuperscript{12} The concentration of the chromosome 19 miR clusters related to pregnancy, have been noted as exhibiting notable increased levels during pregnancy, while altered levels in preeclampsia or preterm labor placentas, have provided evidence that miRs may be potential serum markers for the normal function of the placenta.\textsuperscript{8} As a member of the lethal-7 (let-7) miRNA family, known to be responsible for normal development and disease, miR-let-7d has been highlighted as being capable of depressing the proliferation of neural stem cells and enhancing differentiation of neuronal outward migration, thus regulating the processes of cell fate management and functions.\textsuperscript{13} In the diagnosis and treatment of PE, the application certain biochemical markers including placental proteins and free fetal hemoglobin (HbF) have been shown to be of particular useful clinical significance.\textsuperscript{14} Thus, the central objective of this study was carried out with the purpose of exploring the possible effects of let-7d on the proliferation and differentiation of TCs in patients with PE, with the aim of ascertaining an improved understanding of the pathogenesis and treatment of PE.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Sixty-three respective sterile placental tissues (55 cases of early-onset PE, 8 cases of late-onset PE) were obtained from patients diagnosed with severe PE. The aforementioned patients included in this study, had previously undergone caesarean section procedures at The Fourth Hospital of Harbin Medical University from March 2012 to March 2016. These patients were included in our study as the PE group. The normal group of this study was consisted of patients whose pregnancies had been terminated between 34 and 40 gestational weeks. A total of 65 placental tissues from pregnant women free of any pregnancy complications were included in the study as the normal group. Patient PE status was confirmed as severe if they exhibited symptoms that were in accordance with the international society for the study of hypertension in pregnancy (ISSHP) standard including: after 20 gestational weeks, the systolic blood pressure (SBP) of the pregnant women with normal blood pressure was no less than 140 mmHg or their diastolic blood pressure (DBP) no less than 90 mmHg; the proteinuria was no less than 0.3 g/24 h; and the ratio between proteinuria and creatinine was 30 mg/mmol or remained increase.\textsuperscript{15} Patients with pregnancy complicated by essential hypertension, diabetes and chronic nephrosis were excluded from this study. All the subjects participating in this study did not enter the stages of labor and were caesarean section recipients. Furthermore the subjects had normal platelet counts as well as normal hepatic and renal function. Six weeks after delivery, the PE patients had recovered from hypertension and proteinuria. The age, body mass index and gestational weeks of the study subjects in the two groups were mutually and methodologically matched. All subjects in our study were selected on the premise that they were in strictly compliance with all medical ethics and authorized by the ethics committee of The Fourth Hospital of Harbin Medical University. The present study was carried out with the consent from all patients and their respective families. Clinical features are presented in Table 1.

2.2 | Separation, purification, and in vitro culture of TCs with PE

The purified TCs were extracted by trypsin digestion combined with Percoll density gradient centrifugation. Fresh placental tissues (normal human placenta, early-onset [26 ~ 31 weeks], pre-eclampsia human placenta) after caesarean sections (40 ~ 50 g) were collected from the two groups, washed with phosphate buffer saline (PBS) and cut into separate pieces. A mixture containing 0.25% pancreatic enzymes and 300 U/mL deoxyribonuclease I (DNase I) was used to digest the TCs for a 30-min period. Percoll density gradient separation was used for purification of TCs psoriasis. The Percoll separation included four density individual gradients (70%, 50%, 30%, and 10%) with each gradient of a 3 mL volume. The density of psoriasis TCs was 1.050 g/mL (between 1.039 ~ 1.065 g/mL; 50% ~ 30%). Following centrifugation, the gray cloud cell layer at the junction of two
concentrations of Percoll separating solution levels had been absorbed. After being washed twice by PBS, the suspension was again re-suspended in a full DMEM/F12 medium containing 20% FBS. Trypan blue was applied for the purpose of calculating the cell survival rate. In the scenario where the rate was greater than 90%, the cell suspension (ratio of 1:10) was subsequently added to a six-well plate with a coverslip. Dulbecco's modified eagle medium (DMEM/F12) was added, and the cells were cultured for 48 h at 37°C in 5% CO2.

After separation and purification, psoriasis TCs was cultured in the plates using DMEM (containing 20% fetal calf serum). After 24 h, TCs were all attached to the coverslip. After a 48-h culture period, the TCs were fixed with 4% paraformaldehyde and their membranes were lysed with Triton X-100. Mouse anti-human cytokeratin-7 and mouse anti-human vimentin were added to the cells, respectively. Next, the cells were stored in a wet box at 37°C for a duration of 60 min. Biotin-labeled secondary antibodies were incubated with the cells in the wet box at 37°C for another 60 min and the staining was observed under the microscope. The reaction was stopped once the cells were washed. Hematoxylin was used for counterstaining the nucleus. Cells were dehydrated in ascending series of ethanol. Xylene was used to allow the slips to become transparent prior to being mounted for further observation.

### 2.3 Transfection and grouping of TC cells

TCs with PE in the logarithmic growth phase were transfected using lipofectamine. Cells were divided into the normal (normal TCs), blank (TCs without transfection), pre-let-7d (TCs transfected with pre-let-7d), pre-negative control (TCs transfected with pre-negative control), anti-let-7d (TCs transfected with anti-let-7d), and anti-negative control (TCs transfected with anti-negative control) groups. Pre-let-7d and anti-let-7d as well as their respective nonsense controls were purchased from Shanghai GenePharma Co., Ltd. Next, the TCs were inoculated to a six-well plate. When the density reached 70~80%, the pre-let-7d, anti-let-7d and their respective nonsense controls were transfected with a final concentration of 100 nM under aseptic conditions in accordance with Lipofectamine 2000 instructions. After the TCs had been transfected for a 6 h period, the respective culture mediums were replaced with normal culture medium. Following continuous culture of 48 h, the cells were collected and the transfection efficiency was detected by quantitative real-time polymerase chain reaction (qRT-PCR).

### 2.4 In-situ hybridization assay

The frozen tissue specimens were cut into 16µm-thick sections, which were then immediately placed into precooled 4% paraformaldehyde, fixed for 15 min at 4°C. The sections were then placed into 0.5% H2O2 for 30 min at room temperature and dehydrated with 100% ethanol. Next, pre-hybridization solution was added and cells were incubated at 37°C for 4 h. Hybridization solution was diluted at 1:100 and 20 µL of the solution was added to each specimen, and incubated at 37°C overnight. The negative control was added to a dilution solution without probes. The cells were washed, cultured, and colorized with diaminobenzidine (DAB), observed and photographed.

MiR-let-7d was positively expressed when there were brown-yellow granules present in the cytoplasm. A semi-quantitative formula was adopted to evaluate the staining results: negative, no staining, (0 point); weakly positive, yellowish staining, (1 point); moderately positive, yellow staining, (2 points); strong positive, brown-yellow staining, (3 points). The average staining intensity was calculated using
the formula below: intensity score (IS) = Σ((0 × F 0) + (1 × F 1) + (2 × F 2) + (3 × F 3)), F x = 0.1 × n (“n” signifies the number of stained cells with each intensity in each specimen).

2.5 | QRT-PCR

Trizol (Invitrogen Inc., Carlsbad, CA) was used to extract total RNA of TCs from each group and the RNA was preserved at −80°C. PrimeScript® RT reagent Kit (Perfect Real Time) (TAKARA Biotechnology [Dalian] Co., Ltd., Dalian, China) was used to reverse transcribe the total RNA into cDNA, which was then preserved at −20°C. ABI7500 qRT-PCR machine (ABI Company, Oyster Bay, NY) was utilized to conduct the qRT-PCR experiment. The reaction conditions were as follows: 5-min pre-denaturation at 95°C; 30-s denaturation at 90°C; 40-s annealing at 60°C; 40-s extension at 72°C; 40 cycles of the above procedures were performed. qRT-PCR primers were designed with primer 3 (version 4) on-line primer design software and the primers used in the reaction are shown in Table 2. U6 was taken as the internal reference in the detecting of miR-let-7d, while β-actin as the internal reference in the detection of proliferation markers (PCNA, Ki67) and invasion markers (MMP-2/-9 and TIMP-1). Each specimen was measured at three times. Opticon Monitor 3 software (Bio-Rad, Inc., Hercules, CA) was adopted to analyze the PCR results. The cycle threshold (Ct) of all reaction tubes were manually set at the value corresponding to the bottom of the logarithmic amplification curve. The value of 2−ΔΔCt represented the ratio between the target gene expression in the experiment group and that in the control group. The formula was as follows: ΔΔCt = ΔCt_experiment group − ΔCt_control group (ΔCt = Ct_target gene − Ct_β-actin). The experiment was repeated three times.

2.6 | Western blotting

Total protein in each group was extracted and the protein concentration was subsequently determined according to the BCA Kit (Beyotime Biotechnology Co., Shanghai, China). After boiled with a sample buffer at 100°C for 10 min, the extracted protein was added to the samples (30 µg each hole) and separated by 10% polyacrylamide gel electrophoresis, followed by 80 V electrophoresis for 30 min and finally at 120 V till the end of electrophoresis process. Then the protein was transferred to PVDF film via the wet-transfer method at 100 V for 30 min and closed in 5% BSA at room temperature for 1 h. Afterwards, Cleaved caspase 3 (CST, #9664, diluted at 1:1000), Bax (CST, #2774, diluted at 1:1000), Bcl2 (CST, #2870, diluted at 1:1000 and GAPDH (CST, #2118, diluted at 1:1000) first antibody were added for incubation at 4°C overnight. The next day, the film was washed by TBST (3 times × 5 min), the corresponding secondary antibody was incubated at room temperature for 1 h, then the film was washed by TBST (3 times × 5 min), followed by chemiluminescent reagent development and photographing by Bio-Rad Gel Dol EZ imager (GEL, DOC, EZ, IMAGER, Bio-rad, CA).

2.7 | CCK-8 assay

The TCs in all the groups were inoculated in 96-well plates at a density of 2 × 10³/mL with each well containing 100 µL of culture medium. Cell activity was tested at zero-time, 24, 48, 72, and 96 h. A volume of 10 µL CCK-8 (1:10; Beyotime Biotechnology Co., Shanghai, China) was added to each cell. The cells were then incubated at 37°C for 1 ~ 2 h. Absorbance was measured at 450 nm/630 nm. A total of three parallel wells were selected for average value determination. Each experiment was repeated three times.

2.8 | Flow cytometry

An AnnexinV/PI double-staining method was adopted to detect cell apoptosis. TCs were collected 48 h after transfection with their density adjusted to 1 × 10⁶/mL. A 0.5 mL cell suspension and 1.25 µL AnnexinV-FITCs (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China) were added to a centrifuge tube to react in the dark for 15 min at room temperature. Next, the tube was centrifuged at 1000 rpm for 5 min. Cells were subsequently suspended using 0.5 mL precooled binding buffer and 10 µL of Propidium iodide (PI) were added. Flow

<table>
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<tr>
<th>TABLE 2 The primer sequences for quantitative real-time polymerase chain reaction</th>
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<tr>
<td><strong>Gene</strong></td>
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<tr>
<td>U6</td>
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<td>let-7d</td>
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<td>PCNA</td>
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PCNA, proliferating cell nuclear antigen; MMP-2, matrix metalloprotease-2; MMP-9, matrix metalloprotease-9; TIMP-1, tissue inhibitor of metalloproteinase-1; F, forward; R, reverse.
cytometry (Becton, Dickinson and Company, NJ) was immediately used for analysis. According to the results, healthy and living TCs were in the 4th quadrant (Q4) and were labeled with FITC's-/PI-. Cells in early apoptosis were in the 3rd quadrant (Q3) and labeled with FITC's+/PI−. Cells in late apoptosis were in the 2nd quadrant (Q2) and labeled with FITC's+/PI+. Dead cells were in the 1st quadrant (Q1) and were labeled with FITC's−/PI+. Apoptosis rate = Percentage of cells in early apoptosis (Q3) + Percentage of cells in late apoptosis (Q2).19

2.9 | Transwell assay

A total of 40 µL of Matrigel dissolved at 4°C were added to the precooled Transwell chambers and stored in an incubator for solidification. Seventy-two hours after transfection, TCs were digested by pancreatin enzymes and cell suspension was collected. A total of 1 × 10^5 cells were inoculated in the upper chamber of Transwell (Corning Glass Works, Corning, NY). The upper chamber contained a serum-free medium, while there was 10% fetal bovine serum medium in the lower chamber. The cells were kept in the incubator for 24 h. Unpenetrated cells in the upper chamber were wiped away using a cotton swab and the penetrated cells were fixed with paraformaldehyde for 20 min, then stained with 1% crystal violet solution for 10 min and washed three times with PBS. Cells were observed under a high power lens and then photographed for tallying purposes. The number of penetrated cells in 10 view fields was counted for each membrane. Statistical analysis was conducted on the average numbers. A total of three parallel tests were performed for each group.

2.10 | Statistical analysis

Statistical analysis was performed using SPSS 21.0 software (SPSS Inc., Chicago, IL). Enumeration data were expressed as rate or percentage and verified using a chi-square test. Measurement data were expressed as mean ± standard deviations. Comparisons between any two groups were evaluated via t test and comparisons among multiple groups were completed using one-way analysis of variance (ANOVA). P < 0.05 was regarded as being statistically significant.

3 | RESULTS

3.1 | LET-7D expression in PE and normal placental tissues

Expression of miR-let-7d in PE placental tissues and normal placental tissues were analyzed by in situ hybridization. The positive signals of miR-let-7d were observed in the cytoplasm and were yellowish-brown in color with their nucleus not being stained. Strong signals of miR-let-7d in yellowish-brown were detected in the PE placental tissues (Figure 1A). MiR-let-7d exhibited lesser expression in normal placental tissues, generating few weakly positive yellowish signals (Figure 1B). There was little signal in the negative control group (Figure 1C). Compared with normal placental tissues, miR-let-7d expression increased in the PE placental tissues (1.02 ± 0.20 vs 1.67 ± 0.13, t = 4.720, P = 0.009). qRT-PCR demonstrated that the expression of miR-let-7d had increased in the PE placental tissues compared with normal placental tissues (P < 0.001) (Figure 1D).

3.2 | Observation and immunohistochemical staining of TCs with PE

Observations made using an inverted microscope exhibited that the separated middle layer cells were mainly mononuclear cells, with few cytoplasm and no microvilli within the general vicinity. The TCs displayed characteristics of proliferating cell structure, with little poly nuclear trophoblasts (Figure 2A). Furthermore, it was observed that the isolated cells were mainly TCs with high proliferation and invasive activity. The cytokeratin in TCs was positive, the
positive cytokeratin cells accounted for over 90% of all the TCs (Figure 2B). However, in vascular endothelial cells and mesenchymal cells, the cytokeratin was negative (Figure 2C). The yield of purified TCs was greater than 90%.

### 3.3 | Expressions of MIR-LET-7D in each group

As presented in Figure 3, in comparison to the normal group, significant increases in the expression of miR-let-7d were detected in the blank group ($P < 0.05$). Compared with the blank group, expression of miR-let-7d increased in the pre-let-7d group, however, reduced levels were seen in the anti-let-7d group. Thus suggested that pre-let-7d could promote the expression of miR-let-7d, while anti-let-7d could inhibit the expression of miR-let-7d. No significant differences were detected in the pre-negative control and anti-negative control groups when compared with the blank group ($P > 0.05$).

### 3.4 | Effect of LET-7D on the biological behaviors of TCs with PE

The CCK-8 assay indicated that the absorbance values of the TCs throughout all groups in the study, at 24 h after transfection were not significantly different (Figure 4) (all $P > 0.05$). At the 48th, 72nd, and 96th h after transfection, compared with the normal group, proliferation in the blank group evidently inhibited (all $P < 0.05$). Compared with the blank group, suggested that transfection of pre-let-7d (high expression) noticeably inhibited the proliferation of TCs with PE (all $P < 0.05$). However, transfecting anti-let-7d (low expression) had a prominent effect in relation to the promotion of the proliferation of TCs with PE (all $P < 0.05$). There were no significant differences detected in the pre-negative control and anti-negative control groups, when compared with the blank group ($P > 0.05$). Results obtained from the qRT-PCR analysis indicted that in comparison to the normal group, the expression of proliferating labeling proteins PCNA and Ki67 were significantly down regulated in the blank group (all $P < 0.05$). In comparison to the blank group, expressions of PCNA and Ki67 decreased evidently in the pre-let-7d group (all $P < 0.05$), however, a sharp increase in the anti-let-7d group was observed (all $P < 0.05$). There were no significant differences detected in the pre-negative control and anti-negative control groups when compared with the blank group ($P > 0.05$).

The flow cytometry results revealed that when compared with the normal group, the rate of apoptosis in the blank group had significantly increased (all $P < 0.05$). In comparison to the blank group, the apoptosis rate increased evidently in the
pre-let-7d group (all $P < 0.05$) but a distinct decrease was observed in the anti-let-7d group (all $P < 0.05$). There was no significant difference detected in the pre-negative control and anti-negative control groups, when compared with the blank group ($P > 0.05$) (Figure 5A). Western blotting technique indicated that compared with the normal group, protein expressions of Bax and caspase-3 significantly increased, while protein expressions of Bcl2 had rather evidently declined (all $P < 0.05$). In comparison to the blank group, protein expressions of Bax and caspase-3 significantly increased in the pre-let-7d group ($P < 0.05$) but displayed a sharp decreased in the anti-let-7d group (all $P < 0.05$). The protein expressions of Bcl2 decreased notably in the pre-let-7d group, but displayed significantly increased levels in the anti-let-7d group ($P < 0.05$). There was no significant difference in the protein expressions of Bcl2 and anti-negative control groups, in comparison to the blank group ($P > 0.05$) (Figure 5B).

The Transwell assay results suggested that compared with the normal group, the number of migrated TCs decreased obviously in the blank group ($P < 0.05$). In comparison to the blank group, the number of migrated TCs noticeably decreased in the pre-let-7d group, however sharp increases in the anti-let-7d group were detected ($P < 0.05$). There was no significant difference found in the number of migrated TCs among the pre-negative control, anti-negative control and blank groups (all $P > 0.05$) (Figure 6A ∼ B). qRT-PCR demonstrated that when compared with the normal group, expressions of MMP-2, MMP-9, and TIMP-1 in the blank group were significantly decreased (all $P < 0.05$). In comparison to the blank group, expressions of MMP-2, MMP-9 and TIMP-1 displayed noticeably increased levels in the anti-let-7d group (all $P < 0.05$). Difference of expressions regarding MMP-2, MMP-9, and TIMP-1 among the blank, pre-negative control and anti-negative control groups was confirmed to be of no statistical significance ($P > 0.05$) (Figure 6C).

4 | DISCUSSION

Characterized by widespread vascular dysfunction, PE is a systemic syndrome originating in the placenta, which usually begins with insufficient trophoblast invasion causing severe endothelial malfunction during pregnancy. PE contributes to approximately 18% of all maternal deaths and as much as 40% of fetal mortality globally. Recent findings have indicated that certain miRNAs have play a regulatory role in placenta which, in the scenario of abnormal development, is manifested by limited invasion of trophoblast cells into the uterus, leading to the occurrence of PE. In addition, miR-let-7d has been demonstrated in studies as playing a role in the proliferation and differentiation of neural stem cells. Hence, our study was carried out with the purpose of investigating relevant regulatory functions of miR-let-7d, and our obtained results suggested that lowly expressed miR-let-7d is associated with the inhibition of apoptosis as well as in the promotion of proliferation, differentiation, and invasion ability of trophoblast cells in PE.

Initially, our study revealed that when compared with normal placental tissues, the miR-let-7d expression had increased in the placental tissues of patients with PE. Despite the pathophysiology of PE being generally unclear, many factors including the dysfunction of differentiated endothelial cells (trophoblast) have been reported to be associated with the occurrence of this disease. Previous findings illustrated that miRNAs in epithelial-mesenchymal transition (EMT) can regulate post-transcriptional genes by preventing their target genes from translating in addition to rapidly
degrading their target transcripts. This further confirmed our findings, indicating that the comparison of healthy normal placentas, with the placentas of PE patients were likely to exhibit far greater endothelial gene dysfunction, which were further inhibited by the increased expression of miR-let-7d.

In comparison to the normal group, expression of miR-let-7d had significantly increased in the blank group. Compared with the blank group, expression of miR-let-7d increased in the pre-let-7d group, but declined in the anti-let-7d group, indicating that pre-let-7d could promote the expression of miR-let-7d, while anti-let-7d could inhibit expression of miR-let-7d. Various reported research and scientific literature evidences have indicated that miRNAs are able to inhibit the expression of their target genes, miR-let-7 has been especially highlighted as a participator in the regulation and developing of cell cycles and as an inhibitor in modulating plentiful mitogenic pathways related to cell proliferation. The aforementioned findings further verified the involvement of apoptosis in PE. Furthermore, TCs of pregnant women complicated by PE have also been found to possess more severe apoptosis when compared with those of normal pregnant women. Therefore, when miR-let-7d was highly expressed, it led to a proliferation decrease and an apoptosis increase in TCs. When let-7d was lowly expressed, it promoted proliferation while inhibited apoptosis of TCs. It has been well documented that human placenta TCs can provide the developing fetus with nourishment through proliferation, migration and invasion into the pregnant uterus, hence the number of invaded TCs increased in the placenta of patients with PE. These findings were in accord with the results of this study in when evaluating the blank group the apoptosis rate had evidently increased while in the pre-let-7d group, sharp decreases in the anti-let-7d group were displayed. In comparison to the blank group, protein expressions of Bax and caspase-3 had noticeably increased in the pre-let-7d group but decreased sharply in the anti-let-7d group. The protein expressions of Bcl2 decreased notably in the pre-let-7d group, however, displayed significant increased

FIGURE 5  Apoptosis and expression of apoptosis related proteins in each group. Note: TCs, trophoblast cells; PE, preeclampsia; A, apoptosis of TCs in PE placenta tissues in each group after 48 h transfection; B, protein expressions of Bax, cleaved caspase3 and Bcl2 in each group; **P < 0.05 compared with the normal group; ##P < 0.05 compared with the blank group
levels in the anti-let-7d group. As a cytosolic protein, Bcl2 targets the nucleus and inhibits apoptosis with a lipid-anchoring domain, while Bax, one member of the bcl-family, homodimerizes, and takes shape heterodimers with bcl2 protein and thus lower anti-apoptotic effects of bcl2 and leading to apoptotic death. A transcriptional factor, Ap-2α may enhance Bax expression and decrease Bcl2 expression by overexpression while reduce Bax expression and increase Bcl2 expression through down-regulation, indicating that regulation of Bcl2 and Bax mediated by AP-2α affects apoptosis and thus in turn results in pathogenesis of preeclampsia. As one of the main executioners of apoptosis, caspase-3 expressed in many tissues containing human placental trophoblast displays changes in the promoter region of the caspase genes might modulate apoptotic signaling, thereby increasing the risk of preeclampsia. QRT-PCR demonstrated that when compared with the normal group, the expressions of MMP-2, MMP-9, and TIMP-1 in the blank group had significantly decreased. In comparison to the blank group, expressions of MMP-2, MMP-9, and TIMP-1 had evidently declined in the pre-let-7d group but significantly increased in the anti-let-7d group. As proteinases involved in ECM degradation as well as the loss of activity control of MMPs, are collectively referred to as matrixins, have been reported as being causative factors in diseases such as arthritis, aneurysms, cancer, atherosclerosis, tissue ulcers and nephritis, while TIMPs, specific inhibitors of matrixins, regulate local activity of MMPs in tissues. Preeclampsia is associated with an accumulation of collagen and proteoglycan of umbilical cord tissues, which is due to an increased
biosynthesis and reduced levels of degradation in regards to these components, while MMPs proteins have been highlighted as being involved in the degradation of collagen and the protein core of proteoglycan. In addition, MMP-2, MMP-9, and TIMP-2 have been reported to be continuously expressed in the placenta during pregnancy, which is in correlation with gestational trophoblastic disease, spread of labor in both short and preterm labor associated with pathological processes.

In conclusion, the present study explored the regulation and potential role of miR-let-7d in the proliferation and differentiation of TCs with PE. Taken together, our findings have suggested that, poorly expressed miR-let-7d, can promote the proliferation, differentiation, and invasion abilities of TCs with PE while inhibit their apoptosis. In addition, miR-let-7d has shown promise in its potential to serve as a target gene in the investigation of PE treatment approaches. However, this study focused particularly on the events that occur during the early stages of pregnancy, using TCs from late pregnancy, which has consequently brought about certain limitations to this study. Further research and investigation into how miR-let-7d is directly involved in PE processes through in vivo experimentation and clinical studies are required, which may further verify the findings of this study.

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CONFLICTS OF INTEREST

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