Re-expression of circ_0043610 contributes to trophoblast dysfunction through the miR-558/RYPB pathway in preeclampsia

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Abstract. An increasing number of data have shown the pathogenesis of preeclampsia (PE) involves circular RNA (circRNA). The study aims to investigate the function and the potential mechanism of circ_0043610 in PE. The study was performed on two human placental trophoblastic cell lines (JEG-3 and HTR-8/SVneo). The expression of circ_0043610, microRNA-558 (miR-558), and RING1 and YY1 binding protein (RYBP) was detected by quantitative real-time polymerase chain reaction. The protein levels of N-cadherin, E-cadherin, and RYBP were assessed by Western blotting. Cell viability, proliferation, apoptosis, invasion, and migration were evaluated by cell counting kit-8, 5-Ethynyl-2'-deoxyuridine, flow cytometry analysis, transwell invasion assay, and wound-healing assay, respectively. Dual-luciferase reporter assay, RNA immunoprecipitation assay, and RNA pull-down assay were performed to identify the associations among circ_0043610, miR-558, and RYBP. Compared with normal placental controls, the increased expression of circ_0043610 and RYBP and the decreased miR-558 expression were detected in PE placental tissues. The overexpression of circ_0043610 led to decreased trophoblast cell proliferation, invasion, and migration but increased cell apoptosis. Mechanistically, circ_0043610 acted as a miR-558 sponge, and miR-558 bound to RYBP. Besides, miR-558 introduction remitted circ_0043610-mediated effects in JEG-3 and HTR-8/SVneo cells. Moreover, RYBP participated in the regulation of miR-558 on trophoblast cell behaviors. Further, the ectopic expression of circ_0043610 led to RYBP upregulation through miR-558. Circ_0043610 induced RYBP production to promote trophoblast dysfunction by binding to miR-558 in PE.

Key words: Preeclampsia (PE), circ_0043610, miR-558, RING1 and YY1 binding protein (RYBP)

Preeclampsia (PE) is a gestational disorder that has an increasing incidence in the world, affecting 3%–5% of pregnant women [1]. PE manifests as high blood pressure as well as proteinuria following 20 weeks of pregnancy [2]. The major risk factors for PE include chronic hypertension, obesity, advanced maternal age, nulliparity, and a history of preeclampsia [3]. Despite numerous basic and clinical studies on the pathophysiology, the etiology of preeclampsia remains obscure. As reported, dysfunction of the placenta is responsible for PE; however, the detailed pathophysiological mechanisms remain to be explored. It has been documented that extravillous trophoblast cell invasive and migratory activity is a leading cause of placentation failure [4]. As a result, clarifying the underlying mechanism of trophoblast cell migration and invasion is necessary.

Circular RNA (circRNA) can accumulate in specific cell types owing to its high stability [5]. CircRNA has a closed continuous loop structure without 5'-3' polarity [6]. CircRNA-microRNA (miRNA) duplex can form imperfect matches, which enables circRNA to function as a miRNA sponge, further preventing miRNA-induced mRNA degradation [7]. Emerging evidence shows that circRNA is significantly associated with the pathogenesis of human diseases [8], indicating that circRNA is a potential biomarker and therapeutic target. Investigators are now giving attention to the functions of circRNA in PE because it has been shown as an important biomarker of preeclampsia [9, 10]. Previous data have explained that circ-AK2 represses trophoblast cell growth and motility by the miR-454-3p/thrombospondin 2 (THBS2) pathway [11]. Circ_0007121 combined with miR-421 to promote trophoblastic cell migration and invasion [12]. Another circRNA, circ_0043610, was most significantly upregulated in PE in preliminary experiment among
the top ten upregulated circRNAs, as determined by GSE165324 dataset and qRT-PCR analysis of placental tissues from PE women; however, there is no report on the association of circ_0043610 and PE pathogenesis.

MiRNA is a small RNA and can combine with mRNA whose 3’-untranslated region (3’UTR) contains the complementary sites of miRNA 5’ terminal “seed sequence” to participate in various biological functions [13]. MiRNA abnormalities are found in multiple diseases like cancers, retinal disorders, and cardiovascular diseases [14]. Considerable reports demonstrate that miRNA is involved in pregnancy-related complications like PE as well as fetal growth restriction [15]. In particular, miR-519b reduced leptin (LEP) to promote HTR-8/SVneo cell migratory ability [16]. Zhang et al. reported that miR-30-5p was responsible for the ferroptosis of trophoblasts in PE [17]. MiR-558, a small RNA, is also associated with abnormal trophoblast cell proliferation and motility [18]. Through analysis of circinteractome online database, it was found that circ_0043610 potentially combined with miR-558, but whether circ_0043610 targeted miR-558 to regulate the biological behaviors of trophoblast cells needed further investigation.

Thus, the study was designed to analyze circ_0043610 function in PE. Competing endogenous RNA network that links the functions of noncoding RNAs and protein-coding mRNAs is an important mechanism through which circRNA and miRNA regulate mRNA expression [19]. So, we further assembled the circ_0043610/miR-558/mRNA pathway to disclose the detailed mechanism of circ_0043610 in PE.

Materials and Methods

PE patients and placental tissues

A total of 58 placental tissues near the maternal side (2 cm from the periphery, avoiding placental infarcts) were harvested from singleton pregnant women with PE (N = 29) and without PE (N = 29) in Zhongshan Hospital Xiamen University. Clinical information on patients with PE and healthy controls is shown in Table S1. All tissues were washed using sterile phosphate-buffered saline prior to being preserved at –80°C. The sampling was approved by the Ethics Committee of Zhongshan Hospital Xiamen University (IRB No.2021ZS331). All pregnant women were enrolled in the study with the written informed consent. The diagnosis of PE was performed according to the new criteria in the International Society for the Study of Hypertension (ISSHP) [20] and the new criteria of the American College of Obstetricians and Gynecologists (ACOG) [21].

Cell lines

Human placental trophoblastic cell lines (JEG-3 and HTR-8/SVneo) were purchased from Procell (Wuhan, China) and KALANG (Shanghai, China), respectively. Cells were cultured in DMEM (Biosun, Shanghai, China) plus 10% fetal bovine serum at 37°C in an atmosphere of 5% CO2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Placental tissues and cells were treated with TsingZol (Tsingke, Shanghai, China). cDNA synthesis was conducted based on the instruction of ABScript II cDNA synthesis reagents (ABclonal, Wuhan, China) and miRNA synthesis reagents (Tiangen, Beijing, China) and miRNA synthesis reagents (Tiangen, Beijing, China). Then, qRT-PCR was carried out using Talent qPCR PreMix (Tiangen) with an IQ5 thermocycler. The data were processed by the 2–ΔΔCt method with normalization to U6 or β-actin. Random primers and Oligo(dT)18 primers provided by Tsingke Biotech were used to identify the structure of circRNA. Primer sequences are listed in Table 1.

RNase R treatment assay

According to the published method [22], 2 μg RNA was incubated with 5 U of RNase R (CWBio, Beijing, China), regarding blank RNA as a control reference. Then, qRT-PCR was conducted to examine circ_0043610 expression. GAPDH served as a control reference.

Nuclear and Cytoplasmic separation

The assay regarding PARIS™ Kit (Thermo Fisher, Waltham, MA, USA) was performed for the separation of nucleoplasm fractions as per the guidebook. The nuclear and cytoplasmic fractions from trophoblastic cells were separated and lysed using Trizol reagent (Tsingke). At last, circ_0043610 expression in each fraction was analyzed by qRT-PCR with U6 and GAPDH serving as control references.

Cell transfection

For circ_0043610 overexpression, the full-length circ_0043610 sequence (648 bp) was introduced into the pCD5-ciR vector using T4 DNA Ligase (TaKaRa, Dalian, China). MiR-558 mimics (5’-UGAGCGUGUG UACCCAAA-U3’), miR-558 inhibitors (anti-miR-558, 5’-UAVUUUGGUAACGACGCUA-3’), the small interfering RNA of YY1 binding protein (si-RYBP, 5’-TGGG ATGTGACGTCTGCACCTCTA-3’), and the matched controls (miR-NC, anti-miR-NC, and si-NC) were synthesized by Ribobio Co., Ltd. (Guangzhou, China). Transfection of the above molecules into trophoblastic cells with 80% confluence was conducted by Lipofiter (Hanbio, Shanghai, China). 1.6 microgram plasmid, 100
nM of miRNA mimics, and 200 nM of miRNA inhibitors were utilized for transfecting cells in 12-well plates.

**Cell counting kit-8 (CCK-8) assay**
JEG-3 and HTR-8/SVneo cells were seeded in 96-well plates and transfected with pCD5-ciR, circ_0043610, miR-558, miR-NC, anti-miR-558, anti-miR-NC, si-NC, or si-RYBP alone or jointly. After 48 h of culture at 37°C, the cells were incubated with CCK-8 solution (Beyotime, Shanghai, China). Four hours later, cell viability was analyzed under a microprocessor (REAGEN, Shenzhen, China).

**5-Ethynyl-2'-deoxyuridine (EdU) assay**
With regard to analyzing cell proliferation, EdU staining kit (Ribobio) was performed as per the guidebook. In brief, 4000 cells per assay were seeded in 96-well plates and incubated with EdU-labeled DMEM (Biosun) for 2 h. Then, each well was added with glycine and Apollo® staining solution, followed by incubating with DAPI dihydrochloride. Finally, the cells were assessed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Flow cytometry analysis**
In brief, cells were harvested and suspended in Binding Buffer (Solarbio, Beijing, China). After centrifugation at 300 g for 10 min, cell supernatant was discarded, and Annexin V-FITC (Solarbio) and propidium iodide (PI; Solarbio) were utilized to incubate the cells in the dark. At last, apoptotic cells were examined using a flow cytometer.

**Transwell invasion assay**
In terms of cell invasion analysis, 12-well transwell chambers coated with Matrigel (Solarbio) were employed as instructed [23]. Briefly, trophoblastic cells with various transfections were placed into the top chambers added with serum-free DMEM (Biosun). In the lower chambers, we added the complete DMEM with 15% serum. After removing the cells on the top chambers, the cells on the lower membrane surface were fixed with methanol and stained using crystal violet. Finally, microscope (Olympus) was used to analyze cell invasive ability.

**Scratch test**
After transfection, trophoblastic cells were allowed to grow in 6-well plates. Plastic pipette tips were then used to create single-cell layers, and the cells were cultured in serum-free DMEM, allowing the cells to migrate into the denuded area. After the scratches were completed, floated cells and debris were removed. At 0 h and 24 h of the scratch, microscope (Olympus) was applied to capture images.

**Western blotting analysis**
Total proteins from placental tissues and cells were extracted using NP-40 lysis buffer (Beyotime). 20 μg protein, as quantified with BCA protein assay kit (Beyotime), was loaded onto bis-tris acrylamide gels and then transferred onto nitrocellulose membranes. The proteins were detected using the following primary antibodies: anti-N-cadherin (Cat# MA5-15633; 1:1000; Thermo Fisher), anti-E-cadherin (Cat# PA5-32178; 1:5000; Thermo Fisher), anti-RYBP (Cat# PA1-26492; 1:1000; Thermo Fisher), and anti-β-actin (Cat# PA5-16914; 1:500; Thermo Fisher) and secondary antibodies (Thermo Fisher). At last, protein visualization was performed using eyoECL Plus (Beyotime).

### Table 1 Primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers for qRT-PCR (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa_circ_0043610</td>
<td>Forward: CCCCCTGGGCAATCAATACA; Reverse: CTCCAGGGTTCTGCATGGTG</td>
</tr>
<tr>
<td>RYBP</td>
<td>Forward: TCCCTGTTCTGAGTGATGCC; Reverse: CACACGCGAGTGTAAGAG</td>
</tr>
<tr>
<td>miR-558</td>
<td>Forward: GTATGAGTGAGTCGGTATAC; Reverse: CTCATGCGTTGCTGGAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: CAATCCATGCGACGTCA; Reverse: GACCTCCACGACGTACTGC</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: CGCTTCACGAATTTGCGTGTAC; Reverse: GCTTGGCAGCACAATAACTAAAAT</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: CTTTGGCGGGGCACGAT; Reverse: CCACATAGGAATCTTGTGACC</td>
</tr>
</tbody>
</table>
**Dual-luciferase reporter assay**

Circinteractome (https://circinteractome.nia.nih.gov/index.html) and Targetscan (http://www.targetscan.org/vert_71/) online database were employed for the prediction of the binding sites between miR-558 and circ_0043610 or RYBP 3'UTR. Circ_0043610 and RYBP 3'UTR sequences were used to build the wild-type (WT) plasmids of circ_0043610 and RYBP 3'UTR, including WT-circ_0043610 and WT-RYBP 3'UTR. The binding sites of circ_0043610 and RYBP 3'UTR for miR-558 were mutated as per the guidebook of Sit Mutagenesis Kit (Yeasen, Shanghai, China), and mutant (MUT) sequences were employed for the construction of MUT-circ_0043610 and MUT-RYBP 3'UTR. Plasmids were transferred into the JEG-3 cells and HTR-8/SVneo cells with miR-558 or miR-NC using LipoFiter (Hanbio) for 48 h. Relative light unit (RLU) was detected using a Dual-Lucy Assay Kit (Solarbio).

**RNA immunoprecipitation (RIP) assay**

The assay regarding anti-Ago2 (Abcam, Cambridge, MA, USA) and anti-IgG (Abcam) was conducted as instructed [22]. Cells were lysed with lysis buffer (Beyotime), and the cell supernatant was collected. After incubation of cell lysates with the antibodies, Magna RIP Kit (Millipore, Bradford, MA, USA) was utilized to enrich RNA. At last, qRT-PCR was applied to analyze circ_0043610, miR-558, and RYBP expression.

**RNA pull-down assay**

MiR-558 labeled with biotin (bio-miR-558) and negative control (bio-miR-NC) were transfected into JEG-3 cells and HTR-8/SVneo cells. After 48 h of culture, the cells were exposed to lysis buffer (Beyotime), followed by the incubation with Streptavidin MagneSphere, lasting 4 h. At last, qRT-PCR was applied to analyze circ_0043610 and RYBP expression.

**Statistical analysis**

The data from three independent duplicate tests were analyzed by GraphPad Prism. Results were shown as means ± standard deviations (SD). Student’s t-tests or Mann-Whitney-Wilcoxon was used for the comparisons between the two groups. One-way analysis of variance was used to analyze the data among three or more groups. P < 0.05 indicated statistical significance.

**Results**

**Circ_0043610 expression was upregulated in placental tissues from PE women**

We first analyzed the expression of different circRNAs in PE women through the GEO dataset (GEO accession: GSE165324). The differentiated circRNAs were accounted for fold-change (FC ≥2.0) and values (≤0.05) in the upregulated groups. Circ_0043610 (ID: hsa_circ_0043610) expression was most significantly upregulated among the top ten upregulated circRNAs in PE compared with the normal placenta (Fig. 1A), which was further confirmed by qRT-PCR analysis of placental tissues from PE women (Fig. S1). We also collected 29 PE placental tissues and 29 normal placental tissues, and then assessed circ_0043610 expression by qRT-PCR. The data showed that circ_0043610 expression was increased in PE placental tissues compared with normal placental tissues (Fig. 1B). Subsequently, we confirmed the circular characteristic of circ_0043610 using a series of experiments. For instance, GAPDH expression was dramatically reduced, but circ_0043610 was resistant to RNase R digestion, compared with the control groups (Fig. S2A and B). Meanwhile, we found that circ_0043610 could be significantly amplified using random primers compared with using Oligo(dT)$_{18}$ primers; however, there was no significant difference in GAPDH expression after amplifying using random primers or Oligo(dT)$_{18}$ primers (Fig. S2C and D). Further, the
results showed that circ_0043610 was predominately distributed in the cytoplasm of JEG-3 and HTR-8/SVneo cells (Fig. S2E and F). The above data demonstrated that circ_0043610 was highly expressed in PE placental tissues and had a circular structure.

**Re-expression of circ_0043610 inhibited JEG-3 and HTR-8/SVneo cell proliferation, migration, and invasion but induced cell apoptosis**

We then overexpressed circ_0043610 to determine the consequent effects on the biological behaviors of JEG-3 and HTR-8/SVneo cells. The results first showed that circ_0043610 expression was significantly increased in JEG-3 and HTR-8/SVneo cells after transfection with circ_0043610 overexpression plasmid compared with the control group (Fig. 2A), indicating the success of circ_0043610 overexpression. Subsequently, we observed that the increased expression of circ_0043610 inhibited JEG-3 and HTR-8/SVneo cell viability and proliferation but induced cell apoptosis (Fig. 2B–D). Moreover, the ectopic expression of circ_0043610 repressed cell invasion and migration, accompanied by a decrease of N-cadherin expression and an increase of E-cadherin expression (Fig. 2E–H). Collectively, these data indicated that circ_0043610 overexpression repressed trophoblast cell proliferation, migration, and invasion and induced cell apoptosis.

**Circ_0043610 bound to miR-558 to regulate JEG-3 and HTR-8/SVneo cell processes**

Circinteractome online database was performed to identify circ_0043610-associated miRNAs. The results showed that miR-558, a candidate, contained the complementary sites of circ_0043610 (Fig. 3A), indicating that circ_0043610 might target miR-558. Subsequently, we conducted a series of assays to confirm the prediction.
results. At first, the results of qRT-PCR demonstrated the high efficiency of miR-558 mimics in increasing miR-558 expression (Fig. 3B). Subsequently, we observed that the luciferase activity of wild-type reporter plasmid of circ_0043610 was significantly inhibited after miR-558 introduction in JEG-3 and HTR-8/SVneo cells, whereas the result was not found after co-transfection with the mutant reporter plasmid and miR-558 mimics (Fig. 3C and D). Also, both miR-558 and circ_0043610 were dramatically enriched in the anti-Ago2 group compared with them in the anti-IgG group, as revealed by the RIP assay (Fig. 3E and F). Compared with bio-miR-NC, the binding of circ_0043610 to bio-miR-558 was dramatically increased (Fig. 3G and H). As shown in Fig. 3I, miR-558 expression was significantly reduced in PE placental tissues compared with normal placental tissues. Furthermore, the study revealed that circ_0043610 was negatively correlated with miR-558 expression in PE placental tissues (Fig. 3J).

We then overexpressed circ_0043610 and miR-558 to analyze the consequent effects on the biological behaviors of JEG-3 and HTR-8/SVneo cells. The data first showed that circ_0043610 introduction reduced miR-558 expression, inhibited cell viability and cell proliferation and induced cell apoptosis, whereas these effects were attenuated when miR-558 expression was increased (Fig. S3A–E). Subsequently, re-expression of miR-558 also restored the inhibitory effects of circ_0043610 introduction on cell invasion, cell migration, and N-cadherin expression and the promoting effect on E-cadherin expression (Fig. S3F–I). Thus, these data manifested that the circ_0043610/miR-558 pathway regulated trophoblast cell proliferation, apoptosis, invasion, and migration.

**Circ_0043610 regulated JEG-3 and HTR-8/SVneo cell processes through RYBP and miR-558**

We further predicted miR-558-associated mRNAs using Targetscan online database. As presented in Fig. 4A, RYBP contained the binding sites of miR-558, indicating that miR-558 might target RYBP. To confirm the prediction, we performed dual-luciferase reporter assay, RIP assay and RNA pull-down assay. As shown in Fig. 4B and C, miR-558 mimics significantly reduced the luciferase activity of wild-type reporter plasmid of RYBP 3'UTR but not that of mutant reporter plasmid in JEG-3 and HTR-8/SVneo cells. (A) The schematic illustration showed the potential binding sites of circ_0043610 for miR-558. (B) qRT-PCR was used to analyze the efficiency of miR-558 overexpression in JEG-3 and HTR-8/SVneo cells. (C–H) The regulatory relationship between circ_0043610 and miR-558 was identified by dual-luciferase reporter assay, RIP assay, and RNA pull-down assay in JEG-3 and HTR-8/SVneo cells. (I) MiR-558 expression was analyzed by qRT-PCR in 29 PE placental tissues and 29 normal placental tissues. (J) Spearman correlation analysis was conducted to demonstrate that circ_0043610 expression was negatively correlated with miR-558 expression in PE placental tissues. All experiments were performed in triplicate and repeated three times. ***p < 0.001 and ****p < 0.0001
JEG-3 and HTR-8/SVneo cells. The results of Fig. 4D and E showed that both miR-558 and RYBP were greatly enriched in the anti-Ago2 group compared to the anti-IgG group. Moreover, compared with bio-miR-NC, bio-miR-558 could dramatically bind to RYBP in JEG-3 and HTR-8/SVneo cells (Fig. 4F and G). In addition, we observed that RYBP expression was higher in PE placental tissues than in normal placental tissues (Fig. 4H and I). As revealed by Spearman correlation analysis, miR-558 expression had a negative correlation with RYBP expression in PE placental tissues (Fig. 4J). Furthermore, the results revealed that the increased expression of circ_0043610 promoted RYBP protein expression, whereas the effect was remitted by increasing miR-558 expression (Fig. 4K).

The study continued to analyze whether RYBP participated in miR-558-induced effects on the biological behaviors of JEG-3 and HTR-8/SVneo cells. The success of miR-558 knockdown was confirmed by qRT-PCR and shown in Fig. S4A. Subsequently, miR-558 depletion-induced upregulation of RYBP expression was attenuated by decreasing RYBP expression (Fig. S4B). The decreased cell viability and cell proliferation and the increased cell apoptosis by reducing miR-558 expression were remitted when RYBP expression was downregulated (Fig. S4C–E). Moreover, we observed that RYBP knockdown rescued the inhibitory effects of miR-558 inhibitors on cell invasion, cell migration, and N-cadherin expression and the promoting effect on E-cadherin expression (Fig. S4F–I). Further, we found that RYBP depletion relieved the promoting effects of circ_0043610 overexpression on RYBP expression and cell apoptosis and the inhibitory effects on cell viability, proliferation, invasion and migration (Fig. S5A–F). In conclusion, these data demonstrated that the regulation of circ_0043610 on the biological behaviors of trophoblast cells involved miR-558 and RYBP.

**Discussion**

PE can persist after delivery although delivery resolves most signs. Moreover, hypertensive disorder of
pregnancy is related to a substantial risk for vascular diseases in the long term [24]. More and more theories have been proposed for placental abnormalities including trophoblastic cell invasion, such as oxidative stress and genetic and environmental factors [3]. Some work has demonstrated that the dysregulation of circRNAs in the maternal-fetal interface regulates trophoblast cell growth, migration and apoptosis, finally increasing PE pathogenesis [25]. Given the unknown roles of most circRNAs in the onset of PE due to the limited investigation on the link between circRNAs and PE, the study was organized to analyze the role of circ_0043610 in PE using human placental trophoblastic cell lines (JEG-3 and HTR-8/SVneo). Herein, the circ_0043610/miR-558/RYBP pathway was proposed as the underlying mechanism of circ_0043610 regulating PE for the first time.

Our study showed that circ_0043610 expression was upregulated in PE placental tissues, as determined by qRT-PCR and GEO dataset (GEO accession: GSE165324) analysis. It has been documented that circRNAs can escape degradation from RNase R and that exonic circRNA is exported to the cytoplasm [26, 27]. Consistently, we found circ_0043610 was mainly expressed in the cytoplasm and could escape degradation from RNase R. We also confirmed that circ_0043610 lacked 5’-3’ polarity using random primers and Oligo(dT)18 primers. Extravillous trophoblast cell invasion is required for normal fetal growth as well as development as its ability to transform the uterine spiral arteries [4]. Inadequate invasion can cause the rupture of the anchoring villi, which will damage the villous architecture and placental function [28]. Thus, we analyzed the function of circ_0043610 in placental trophoblast cell invasion and migration. As a result, we found that the increased circ_0043610 expression repressed cell migration as well as invasion. Cadherins are a class of calcium-mediated membrane glycoproteins that participate in epithelial-to-mesenchymal transition, and E-cadherin and N-cadherin are two members of the family [29]. The increased cell migratory and invasive behaviors involve E-cadherin loss and N-cadherin elevation [30]. In the present work, the two proteins were employed to determine circ_0043610-induced effects on trophoblastic cell migration and invasion. Our data showed that circ_0043610 overexpression repressed N-cadherin expression but promoted E-cadherin expression, indicating the inhibitory effects of circ_0043610 on trophoblastic cell migration and invasion. In addition, we found that circ_0043610 re-expression inhibited cell proliferation and increased cell apoptosis. Thus, these results demonstrated circ_0043610 might contribute to the dysregulation of placenta.

CircRNA participates in miRNA inhibition by functioning as a miRNA sponge [26]. Previous work confirmed the abnormal expression of miRNAs in pregnant women with preeclampsia, and the deep investigation revealed that these miRNAs regulated trophoblast function, angiogenesis, and mesenchymal stem cell (MSC) function [31]. Our work identified that circ_0043610 targeted miR-558 in trophoblastic cells. As reported, miR-558 combined with TIMP metallopeptidase inhibitor 4 (TIMP4) and long noncoding RNA Linc00261 to increase HTR-8/SVneo cell invasion and migration [32]. Liu et al. revealed that circ_0001326 targeted miR-558 to inhibit HTR-8/SVneo proliferation, motility, and epithelial-mesenchymal transition [33]. MiR-558 expression was reduced in placental tissues of PE women and that miR-558 bound to circ_0007611 to inhibit cell apoptosis [34]. Consistently, our reports showed the down-regulation of miR-558 in placental tissues of PE patients. Moreover, miR-558 introduction increased trophoblastic cell proliferation, migration, and invasion but decreased cell apoptosis. Importantly, we demonstrated the circ_0007611/miR-558 axis regulated trophoblastic cell dysregulation.

Multiple investigations have confirmed that circRNA can compete with miRNA targets, leading to the decreased repression of miRNA on target gene expression [35]. We searched for the target gene of miR-558 and identified that miR-558 targeted RYBP. RYBP is a polycomb group protein that can inhibit gene transcription through epigenetic chromatin modification [36]. The protein can accelerate p53-induced cell apoptosis by modulating murine double minute 2 (MDM2) as well as the MDM2-p53 loop [37]. Moreover, RYBP is able to increase Hippi-triggered apoptosis through the regulation of caspase 8 and Hippi [38]. It has been shown that RYBP is overexpressed in PE placental tissues and inhibits HTR-8/SVneo cell migration and epithelial-to-mesenchymal transition [39]. Consistently, our data showed RYBP was highly expressed in PE placental tissues and inhibited trophoblastic cell migration and epithelial-to-mesenchymal transition. Additionally, we found that RYBP inhibited trophoblastic cell proliferation and invasion but induced cell apoptosis. Also, circ_0043610 and miR-558-induced effects in trophoblastic cells involved RYBP. Importantly, circ_0043610 induced RYBP expression through miR-558. Thus, we proposed a mechanism by which circ_0043610 targeted miR-558 to promote RYBP expression, thereby promoting the occurrence of PE.

However, some limitations should be considered. Firstly, the sample size of the study was relatively small. Secondly, an animal model should be established to validate the in vitro data. In addition, a previous study demonstrated that RYBP inhibited cell metastasis by inactivating EGFR-ERK and EGFR-AKT pathways [40].
Whether circ_0043610 participated in the pathogenesis of PE through the miR-558/RYBP/EGFR-ERK (EGFR-AKT) pathway needed further investigation.

Taken together, the occurrence of PE might involve circ_0043610 overexpression. In terms of mechanism, circ_0043610 seemed to serve as a miR-558 sponge to induce RYBP expression, thus inhibiting trophoblast cell proliferation, invasion, and migration and inducing cell apoptosis (Fig. 5). Our data indicate a functional role of circ_0043610 in placental dysfunction in PE. Repressing circ_0043610 expression may be a therapeutic strategy for PE.

Acknowledgements

None.

Disclosure of Interest

The authors declare that they have no conflicts of interest.

Funding

None.

Fig. 5  The schematic illustration showed the mechanism of circ_0043610 regulating trophoblast cell dysfunction in PE.

Fig. S1  The expression of ten circRNAs in the trophoblastic tissues of PE patients was analyzed by qRT-PCR. All experiments were performed in triplicate and repeated three times. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.
Fig. S2 Analysis of circ_0043610 structure and location. (A–D) The circular structure of circ_0043610 was analyzed using RNase R, random primers, and Oligo(dT)18 primers. (E and F) Cell fractioning and qRT-PCR analysis of circ_0043610 localization in JEG-3 and HTR-8/SVneo cells. All experiments were performed in triplicate and repeated three times. **** p < 0.0001.

Fig. S3 MiR-558 mediated the effects of circ_0043610 on JEG-3 and HTR-8/SVneo cell proliferation, apoptosis, invasion, and migration. JEG-3 and HTR-8/SVneo cells were transfected with pCD5-ciR, circ_0043610, circ_0043610+miR-NC, circ_0043610+miR-558, and miR-558 expression was analyzed by qRT-PCR (A), cell viability by CCK-8 (B), cell proliferation by EdU assay (C), cell apoptosis by flow cytometry analysis (D and E), cell invasion by transwell invasion assay (F), cell migration by wound-healing assay (G), and the protein expression of N-cadherin and E-cadherin by Western blotting (H and I). All experiments were performed in triplicate and repeated three times. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.
MiR-558 mediated the biological behaviors of JEG-3 and HTR-8/SVneo cells by targeting RYBP. JEG-3 and HTR-8/SVneo cells were transfected with anti-miR-NC, anti-miR-558, anti-miR-558+si-NC, or anti-miR-558+si-RYBP, and miR-558 expression was analyzed by qRT-PCR (A), RYBP protein expression by Western blotting (B), cell viability by CCK-8 (C), cell proliferation by EdU assay (D), cell apoptosis by flow cytometry analysis (E), cell invasion by transwell invasion assay (F), cell migration by wound-healing assay (G), and the protein expression of N-cadherin and E-cadherin by Western blotting (H and I). All experiments were performed in triplicate and repeated three times. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) and **** \( p < 0.0001 \).
Fig. S5 RYBP depletion mediated the effects of circ_0043610 on JEG-3 and HTR-8/SVneo cell proliferation, apoptosis, invasion, and migration. JEG-3 and HTR-8/SVneo cells were transfected with pCD5-ciR, circ_0043610, circ_0043610+si-NC, circ_0043610+si-RYBP, and RYBP expression was analyzed by Western blotting (A), cell viability by CCK-8 (B), cell proliferation by EdU assay (C), cell apoptosis by flow cytometry analysis (D), cell invasion by transwell invasion assay (E) and cell migration by wound-healing assay (F). * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.

References


