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ORIGINAL ARTICLE

CircRNA_0088196 Regulates Trophoblast Proliferation and Apoptosis in Preeclampsia Through the miR-379-5p/HSPA5 Axis

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Abstract

Existing research has confirmed the dysregulation of circular RNA (circRNA) in a wide variety of human diseases. Thus, in this study, we explored the potential mechanism of circRNA_0088196 in preeclampsia (PE). We performed quantitative real-time PCR to examine circRNA_0088196 expression and verified the function of circRNA_0088196 in vitro using CCK-8, TUNEL, flow cytometry, and Western blotting analyses. Additionally, we studied the mechanism using dual-luciferase reporter gene experiments. The results of our research revealed the up-regulation of circRNA_0088196 in PE patients' placentas and Heat Shock 70 kDa Protein 5 (HSPA5)-stimulated trophoblast (HTR-8/SVneo) cells. An investigation of the mechanism also showed that there was a binding between miR-379-5p and circRNA_0088196. Additionally, circRNA_0088196 inhibited HTR-8/SVneo cell proliferation and promoted cell apoptosis via the miR-337-3p/HSPA5 axis, thereby facilitating PE. In vivo experiments indicated that circRNA_0088196 regulated HTR-8/SVneo cell production through miR-379-5p. Overall, the findings of this study illustrate that circRNA_0088196 interference promotes cell apoptosis and inhibits HTR-8/SVneo proliferation via the miR-379-5p/HSPA5 axis, thereby accelerating the development of PE.

Keywords HSPA5 · HTR-8/SVneo · circRNA_0088196 · miR-379-5p · Preeclampsia

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Impact Statement

Increasing evidence expounds that the dysregulation of circRNAs is associated with the progress of various human diseases. Nevertheless, it is currently unclear whether circRNA_0088196 acts as a ceRNA in the progress of PE. This study expounded that the interference with circRNA_0088196 restrained HTR-8/SVneo proliferation and boosted cell apoptosis through the miR-379-5p/HSPA5 axis, eventually accelerating the progress of PE.

Introduction

Preeclampsia (PE) refers to a complex systemic disease that involves clinical features such as proteinuria and new-onset hypertension. It affects approximately 3–5% of women and can lead to maternal mortality and fetal morbidity (Chappell et al. 2021). However, this condition has a complex background and unknown etiology. The exact cause of PE is still unknown, but it has significant implications for both short-term and long-term maternal and fetal health. Early prediction of PE is crucial in order to implement preventive measures and reduce the risk. However, diagnosing PE can be challenging due to its diverse initial symptoms (Vidaeff et al. 2021). Currently, termination of pregnancy is the most effective treatment for PE. However, the decision to terminate should consider the potential benefits and risks to both mother and infant, as well as predicting disease severity and adverse outcomes. Timely administration of appropriate treatment, including determining when to terminate the pregnancy, is essential. After the onset of PE, various pathological factors directly or indirectly affect trophoblasts—cells that play a critical role in placental development. These factors include suppressing inflammatory responses and promoting trophoblast apoptosis, progress (Zhang et al. 2021; Cheng et al. 2019) as emerging evidence indicates. Accordingly, studying trophoblast proliferation mechanisms as well as understanding how apoptosis influences PE progression is an important area for further research with significant implications for managing this condition effectively.

Circular RNA (circRNA) belongs to a special endogenous non-coding molecule class and is the primary focus of current RNA research. Unlike linear RNA, circRNA molecules have no 5′ end cap or 3′ end polyadenylation tail, and they exhibit tissue-specific expression and a stable structure (Szabo and Salzman 2016; Memczak et al. 2013). According to a growing number of reports, circRNA dysregulation is closely associated with the progression of a wide range of human diseases. Ye et al. illustrated that PE placental tissue raises circ-AK2 levels, and high circ-AK2 expression can boost apoptosis and restrain trophoblast proliferation (Ye et al. 2021). Besides, Hu et al. discovered that circ_0015382 overexpression impedes PE progression by inhibiting human trophoblast cell growth, migration, and invasion (Hu et al. 2021). In this study, we focused on circRNA_0088196, specifically known as circ-TNC, which is upregulated in PE

cases (Liu et al. 2019), suggesting a possible connection between PE occurrence and circRNA_0088196. Additionally, growing evidence suggests that the binding sites of miRNA contain a considerable amount of circRNA, affecting downstream target mRNA expression through competing endogenous RNA (ceRNA) interaction with miRNA (Hansen et al. 2013; Zheng et al. 2016). Notably, circRNA_0088196 is derived from the exon 15 and 16 regions. Nevertheless, it remains unclear whether circRNA_0088196 is a ceRNA in PE development.

MicroRNA (miRNA) refers to a type of single-strand noncoding RNA with a length of 20–22 nucleotides. It exists extensively in eukaryotic organisms and can be combined with 3′UTR (untranslated region) of the target mRNA through complete or incomplete complementary pairing, resulting in the degradation of target genes or inhibition of protein translation. On that basis, a wide variety of biological processes are regulated, such as cell apoptosis, tissue differentiation, and organ development (Sheikh et al. 2016). Besides, existing research has suggested a correlation between PE development and abnormal miRNA expression (Brodowski et al. xxxx; Rokni et al. 2019). Further studies have also suggested that miR-30b-5p undergoes abnormal up-regulation after PE occurs, and the PE symptoms in rat models are reduced by suppressing miR-30b-5p expression and supplementing ferroptosis inhibitors (Zhang et al. 2020). Besides, Chen et al. reported that miR-203a-3p plays an anti-inflammatory role in pregnant women with PE through the down-regulation of IL24 (Ma et al. 2020). Notably, our previous research suggested the down-regulation of miR-379-5p in PE placental tissue (Inno et al. 2021). Nevertheless, the mechanism of miR-379-5p in PE requires further investigation.

HSPA5 is also known as glucose-regulated protein 78 (GRP78) or the immunoglobulin heavy chain binding protein. It belongs to the heat shock protein 70 (Hsp70) family and principally exists in the endoplasmic reticulum (ER). Besides, HSPA5 participates in the genesis and development of numerous ailments (Lee 2014) and is also a therapeutic target for various diseases (Booth et al. 2015; Rezanezhad et al. 2013). The ER signal sequence at the N end and the KDEL search sequence at the C end of HSPA5 are structural features that are unique from other Hsp70 proteins. This distinctive structure enables HSPA5 to transfer to the ER and maintain the form of an ER protein (Wang et al. 2017). Moreover, HSPA5 regulates endoplasmic reticulum homeostasis, mediates endoplasmic reticulum-related cell apoptosis, controls the inflammatory signal pathway, and performs other functions related to the pathogenesis of PE. The results of bioinformatics analysis have indicated that the target molecule hsa-mir-379-5p binds to hsa-circ_0088196, while hsa-mir-379-5p has the potential to exert the negative targeted regulation of HSPA5.

The expressions of circRNAs were screened in healthy controls and patients with PE. We established that hsa-circ_0088196 was the most significantly up-regulated circRNA, and RT-qPCR further verified that it was up-regulated by a factor of approximately four in the placentas of patients with PE. This suggested that hsa-circ_0088196 may participate in the regulation of the PE pathophysiological process. Subsequently, after we transfected trophoblast cells with adenovirus-encapsulated circRNA_0088196, cell apoptosis increased while the proliferation capacity fell drastically. In contrast, using shRNA to knock down circRNA_0088196 significantly promoted the proliferation of trophoblast cells

and inhibited the number of apoptotic cells. Thus, circRNA_0088196 may be a new target for regulating the proliferation and apoptosis of trophoblast cells. However, the molecular signal pathway for regulating the function of trophoblast cells still requires further clarification.

Materials and Methods

Patients and Tissue Samples

The Tongde Hospital of Zhejiang Province provided a total of 20 participants between January 2020 and January 2021, including ten patients with preeclampsia (Group I) and ten healthy pregnancy controls (term delivery) (Group II). The demographic characteristics of participants are shown in Table 1. Preeclampsia refers to systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on at least two occasions after the 20-week gestation period, as well as significant proteinuria (> 2 g/24 h and/or 2+ on dipstick testing) or evidence of multiorgan problems, such as central nervous system perturbations, liver dysfunction, thrombocytopenia, oliguria, seizures, or pulmonary edema (Brown et al. 2018).

All participants in the study opted for cesarean section delivery. The exclusion criteria included HELLP syndrome, fetal malformation, thrombophilic conditions, autoimmune diseases, heart diseases, chronic hypertension, chronic nephritis, diabetes, and multiple pregnancies. When the placenta was extracted from the uterus, the excision of placental tissue pieces (~ 1.0 cm^3) was performed in random regions, such that areas of infarction, calcification, or vessels were avoided. Sterilized saline water was utilized to wash the samples three times, then the samples were kept at − 80 ℃ for subsequent application. Informed consent was obtained from all placenta donors and approval was given by the Ethics Committee of the Tongde Hospital of Zhejiang Province.

Table 1 The clinical characteristics of the pregnant women ($N = 20$)

Group	PE ($N = 10$)	Healthy pregnancy controls ($N = 10$)
Maternal age	28.3 ± 3.4	27.5 ± 4.2
Gestational age (weeks)	37.8 ± 2.6	38.6 ± 3.5
Mode of delivery	Cesarean section	Cesarean section
Proteinuria (g/l)	> 0.34	< 0.34
Systolic blood pressure (mmHg)	165 ± 6.8	127 ± 4.7
Diastolic blood pressure (mmHg)	128 ± 7.3	93 ± 6.4
Infant birth weight (g)	2693 ± 408	3354 ± 396
Placental weight (g)	374 ± 96.2	579 ± 92.3

RNA Extraction and Reverse Transcription-Quantitative Real-Time PCR (RT-qPCR)

The isolation of RNA from placental tissue specimens or HTR-8/SVNEO cells was achieved using TRIzol reagent (#10606ES60; Yeasen, Shanghai, China). The thermal conditions used for RT-qPCR are as follows. Amplification: 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Extension at 72 °C for 5 min and termination. Subsequently, the synthesis of complementary DNA (cDNA) was conducted with First-Strand cDNA Synthesis Master Mix (#11123ES60; Yeasen, Shanghai, China). Besides, qPCR was carried out using SYBR Green Master Mix (#11202ES08; Yeasen, Shanghai, China). The fold change was examined by applying the $2^{-\Delta\Delta C_t}$ method with U6 acting as an internal control of circRNA_0088196 and miR-379-5p or GAPDH as an internal reference of HSPA5. We designed the primers in the primer 3.0 (https://primer3.ut.ee/), t he specific primer sequences were shown in Table 2.

About quantification of miR-379-5p expression, $2^{-\Delta\Delta C_t}$ method was applied to represent the relative expression. Firstly, ΔC_t was calculated by subtracting the C_t value of U6 from the C_t value of miR-379-5p. Then, subtract the average ΔC_t of healthy pregnant subjects from the ΔC_t value of every sample to obtain $\Delta\Delta C_t$ value. Finally, calculate the $2^{-\Delta\Delta C_t}$ value to represent the relative expression of miR-379-5p.

Western Blotting

The extraction of total protein was conducted using radioimmunoprecipitation assay (RIPA) buffer (#P0013B; Beyotime, Beijing, China). Also, 30 μm protein was utilized for SDS-PAGE, followed by transference to a polyvinylidene fluoride (PVDF) membrane. The non-specific protein signal was blocked with 5% skim milk for 1 h. The primary antibodies (HSPA5; #11,587-1-AP/66574-1-Ig; 1/3000; Proteintech, Wuhan, China; Cyclins; #4656T; 1/2000; CST, China; and GAPDH; #ab186930; 1/5000; Abcam, USA) were incubated at 4 ℃ overnight. Next, the protein was held for 1 h at 37 ℃ with HRP-conjugated goat anti-rabbit secondary antibodies

Table 2 Sequences of primers used in this study. *HSPA5* heat shock protein 5

Gene	Primer sequences
circRNA_0088196	F: 5′-CTGAGCCACTGGAAATAA-3′
	R: 5′-GTAATGGGCACATAGGTAAT-3′
miR-379-5p	F: 5′-GCGCGTGGTAGACTATGGAA-3′
	R: 5′-ACTGAGAGTTAGTGGACCAT-3′
HSPA5	F: 5′-TTGGAGGTGGGCAAACAAAG-3′
	R: 5′-CCAGCAATAGTTCCAGCGTC-3′
GAPDH	F: 5′-TCAAGAAGGTGGTGAAGCAGG-3′
	R: 5′-TCAAAGGTGGAGGAGTGGGT-3′
U6	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′
	R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′

(#SA00001-2; 1/5000; Proteintech, Wuhan, China). ECL reagent was used to capture the protein band image and the gray value was examined using ImageJ software.

ELISA

The placenta samples of both Group I ($N = 10$) and Group II ($N = 10$) were quantitatively analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA) for pro-inflammatory HSPA5. A quantikine analysis of HSPA5 was performed according to the protocols of the ELISA kit manufacturer (Xiamen Lunchangshuo Biotechnology Co., Ltd; catalog no. ED-10036). The minimum detectable dose for HSPA5 was typically < 10 pg/ml.

Dual-Luciferase Report

The amplification of specific circRNA_0088196 and HSPA5 3′-untranslated region (3′-UTR) sequences harboring miR-379-5p binding sites was realized. They were then cloned as the pmirGLO vectors for the production of the corresponding wild-type luciferase reporter system (circRNA_0088196-WT or HSPA5-WT). Mutation of the putative binding site sequences was achieved, and these sequences were introduced into pGL3-basic to build a mutated luciferase reporter system (circRNA_0088196-MUT and HSPA5-MUT). Thereafter, 50 ng pGL3 vectors, 10 ng pRL-TK Renilla plasmids, and 50 nM miR-379-5p mimics were co-transfected into HTR8 cells. To examine relative luciferase activity, the Dual-Luciferase Reporter Assay System (Promega, USA) was applied.

Cell Culture

Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 100 µg/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (FBS) was used to maintain the HTR-8/SVNEO human trophoblast cells. A 5% CO_2 incubator was employed to grow cells at 37 ℃.

Transfection of Oligonucleotides and Overexpression Plasmids

The specific oligonucleotides and plasmids of circRNA_0088196 and HSPA5 were sourced from GenePharma (Shanghai, China). The specific small interfering RNAs (siRNAs) at a final concentration of 50 nM targeting circRNA_0088196 and HSPA5 were shown in Table 3. Full-length circRNA_0088196 and HTRA1 were separately cloned into pLX2 and pVL3 overexpression vectors. Moreover, GenePharma (Shanghai, China) provided mimics and inhibitors to match the negative controls (NCs) of miR-379-5p. Using Lipofectamine® 2000 reagent (Thermo Fisher, USA), the above plasmids and oligonucleotides were transfected into the HTR-8/SVNEO cell line. After 48 h, the transfection effects were estimated using RT-qPCR.

Table 3 Sequences of siRNAs used in this study

Definition	Sequences
Hsa circ-0088196 siRNA1	F: 5′-CACAGGCGAGAAAGCCAAATT-3′
	R: 5′-UUUGGCUUUCUCGCCUGUGTT-3′
Hsa circ-0088196 siRNA2	F: 5′-GGCGAGAAAGCCAAAGAACTT-3′
	R: 5′-GUUCUUUGGCUUUCUCGCCTT-3′
Human HSPA5 siRNA1	F: 5′-GAAUCAGAUUGGAGAUAAATT-3′
	R: 5′-UUUAUCUCCAAUCUGAUUCTT-3′
Human HSPA5 siRNA2	F: 5′-GGGCAAAGAUGUCAGGAAATT-3′
	R: 5′-UUUCCUGACAUCUUUGCCCTT-3′
Human HSPA5 siRNA3	F: 5′-GAGUGACAGCUGAAGACAATT-3′
	R: 5′-UUGUCUUCAGCUGUCACUCTT-3′
NC siNRA	F: 5′-UUCUCCGAACGUGUCACGUTT-3′
	R: 5′-ACGUGACACGUUCGGAGAATT-3′

Cell Proliferation Assay

Based on the manufacturer's guidelines, Cell Counting Kit-8 (CCK-8) kits were utilized to assess cell proliferation. To achieve this, the HTR-8/SVNEO cell line was seeded onto 96-well plates and incubated for 48 h. Next, 10 μm CCK-8 solution was added to the respective wells and the cells were incubated for another 3 h. A microplate reader was employed to examine optical density values at 450 nm.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Assay

Cell apoptosis was evaluated with a TUNEL kit (#ATK00001; AtaGenix, Wuhan, China). After PBS was used to wash the HTR-8/SVneo cells, the cells were fixed with 4% paraformaldehyde and ethanol was used to permeabilize the cells. Subsequently, 50 μl TUNEL assay solution was administrated to the HTR-8/SVneo cells for 60 min at 37 ℃ without light, and then anti-fluorescence quenching liquid was used to seal the cells. A fluorescence microscope was used to acquire the TUNEL results, with 515–565 nm emission light and 450–500 nm excitation light. The apoptotic cells were determined in five random regions, and the experiments were performed in triplicate.

Cell Cycle Assay

The HTR-8/SVNEO cells were cleaned with PBS, and then 0.25% trypsin was added to digest the cells. When the cells adhering to the wall were rounded or detached from the wall, the digestion process was terminated by adding complete culture medium. Then, centrifugation of cells was performed for 5 min at 1500 × *g*

and PBS was used to wash the cells at 4 ℃. The precipitates were resuspended with pre-cooled anhydrous ethanol and fixed overnight. Next, the cells were placed in a centrifuge at $350 \times g$ for 5 min, before the supernatant was removed. The cells were exposed to PI solution (#421301; Biolegend, Beijing, China) and RNase A solution (#B500474; Sangon Biotech, Shanghai, China) at ambient temperature for 30 min without light. The cell cycle was estimated with FACScan flow cytometry.

Transwell Assay

For the preparation of the cell suspension (3×10^5 cells/mL), HTR-8/SVNEO cells were trypsinized and administrated with serum-free RPMI 1640 medium. Next, 200 µl cell suspension was pipetted into the upper chamber of a 24-well transwell with a precoated Matrigel matrix (#356234; BD Biocoat, Beijing, China). Following that, 600 µl RPMI 1640 medium and 10% FBS were introduced into the lower chamber of each respective well. A 5% CO_2 cell incubator was used to incubate the transwell chamber at 37 ℃ for 24 h. Afterward, the chamber was removed and a cotton swab was used to wipe the non-migrated cells from the upper layer. Next, the chamber was washed three times with sterile PBS, and 4% paraformaldehyde was used to fix the cells for 20 min (#P0099; Beyotime, Shanghai, China). Subsequently, we used 0.1% crystal violet for 15-min staining of the cells (#C0121; Beyotime, Shanghai, China). Finally, we calculated the invaded cell number at five randomly selected locations.

Statistical Analysis

SPSS 23.0 was used to perform the statistical analysis. The normality of the data for the quantitative variables was assessed using the Shapiro–Wilk test. If the data followed a normal distribution, the mean and standard deviation were used to represent the data. For the comparison between two groups, an independent samples *t*-test was employed. For the comparison among multiple groups, a one-way analysis of variance (ANOVA) was conducted. Post hoc comparisons were performed using the least-significant difference (LSD) test. The results were considered statistically significant when $p < 0.05$.

Results

CircRNA_0088196 and HSPA5 are Overexpressed and miR379-5p is Reduced in PE Placental Tissue

The expression of circRNA_0088196 (chr9:117800539-117819704), miR379-5p, and HSPA5 was measured by RT-qPCR in PE placental tissue from ten patients with PE and ten healthy pregnant subjects. Compared to the healthy controls, circRNA_0088196 expression was considerably up-regulated in PE patients (Fig. 1A). Conversely, there was significant down-regulation of miR379-5p expression in PE patients (Fig. 1B). Furthermore, HSPA5 expression was substantially up-regulated

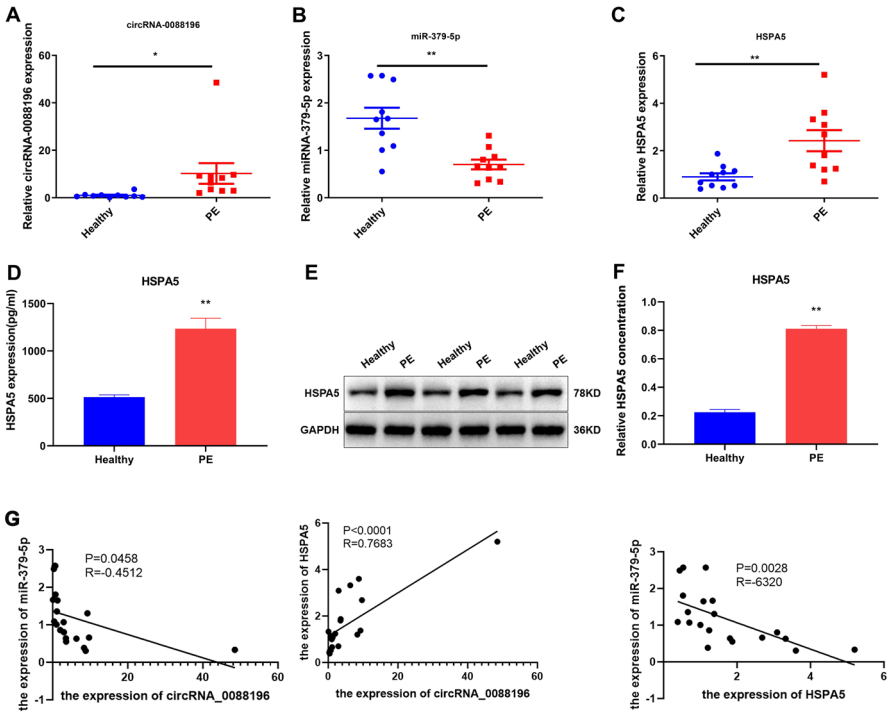


Fig. 1 Circ-0088196 and HSPA5 are overexpressed, and miR-397-5p is down-regulated in PE placental tissues. **A–C** RT-qPCR analysis of the expression of **A** circ-0088196, **B** miR-397-5p, and **C** HSPA5 in placental tissue specimens from PE patients and healthy pregnancy subjects. **D** Levels detection of HSPA5 expression levels in PE patients and healthy pregnancy subjects using ELISA. **E, F** Western blotting of HSPA5 expression in placental tissue specimens from PE patients and healthy pregnancy subjects. **G** The correlation of the expression of circRNA_0088196, miR-379-5p, and HSPA5. The statistical analysis was conducted using Independent-Sample *t*-test. Mean ± MES, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$

in PE placental tissue (Fig. 1C). HSPA5 expression was further verified by western blotting and ELISA, The western blotting and ELISA results were consistent with the trend of RT-qPCR (Fig. 1D–F). Generally, circRNA_0088196 and HSPA5 were abnormally expressed in patients with PE. The correlation of the expression of circRNA_0088196, miR-379-5p, and HSPA5 was presented in Fig. 1G.

CircRNA_0088196 Enables the Sponging of miR379-5p to Up-regulate HSPA5

Dual-luciferase reporter experiments were conducted to verify the above interactions between circRNA_0088196 and miR379-5p and between miR379-5p and HSPA5. The relative luciferase activity fell markedly following the co-transfection of circRNA_0088196-WT and miR379-5p mimics but not the co-transfection of circRNA_0088196-MUT or miR379-5p (Fig. 2A, B). This suggested that circRNA_0088196 exerted a sponge effect on miR379-5p. Moreover, there was a

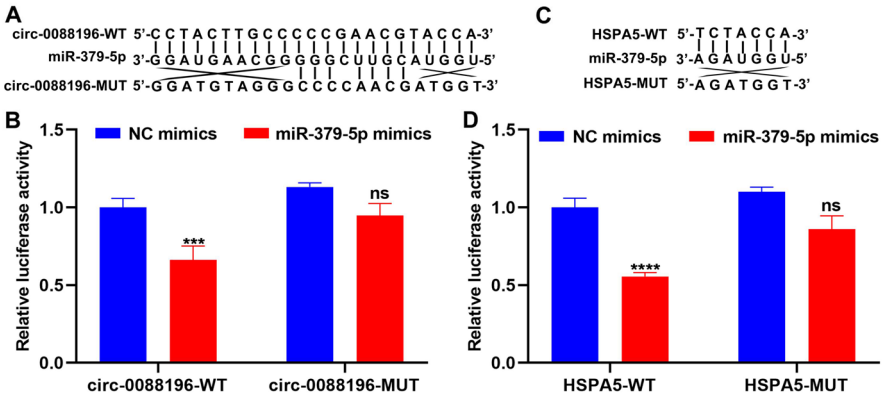


Fig. 2 Circ-0088196 sponges miR-397-5p to up-regulate HSPA5. **A** Schematic diagram of the binding sites of circ-0088196 with miR-397-5p. **B** Relative luciferase activity in HTR-8/SVNEO cells when co-transfection of circ-0088196-WT or circ-0088196-MUT with miR-397-5p mimics. **C** Schematic diagram of the binding sites between miR-397-5p and HSPA5. **D** Relative luciferase activity in HTR-8/SVneo cells when co-transfection of HSPA5-WT or HSPA5-MUT and miR-397-5p mimics. The statistical analysis was conducted using one-way ANOVA and LDS test. Mean ± MES, $n = 3$; ***$p < 0.001$; ****$p < 0.0001$

notable decrease in relative luciferase activity after the co-transfection of HSPA5-WT and miR379-5p mimics (Fig. 2C, D). However, relative luciferase activity did not change significantly after the co-transfection of HSPA5-MUT and miR379-5p mimics, confirming that miR379-5p was directly bound to HSPA5. In summary, circRNA_0088196 enabled the up-regulation of HSPA5 expression by sponging miR379-5p.

MiR379-5p Interacts with CircRNA_0088196 and HSPA5 in Trophoblast Cells

In order to explore up-and-down relationships of circRNA_0088196, miR-379-5p, and HSPA5, the specific overexpression plasmids and oligonucleotides of circ-0088196 were separately transfected into HTR-8/SVNEO cells to overexpress or silence circ-0088196. RT-qPCR suggested that circ-0088196 expression was notably elevated by overexpression plasmids (Fig. 3A) and was notably silenced by two specific siRNAs targeting circ-0088196 (Fig. 3B). Moreover, we transfected specific overexpression plasmids and three oligonucleotides of HSPA5 into HTR-8/SVNEO cells. As shown in Fig. 3C, D, compared to the empty, HSPA5 expression was substantially up-regulated in OE-HSPA5. Western blotting indicated that three oligonucleotides of HSPA5 reduced the levels of HSPA5 (Fig. 3E, F).

To further demonstrate that miR-379-5p is involved in crossregulation between circ-0088196 and HSPA5, we evaluated the expression of HSPA5. We transfected specific overexpression plasmids and oligonucleotides of circ-0088196 into HTR-8/SVNEO cells to overexpress and knock down circ-0088196 (Fig. 4A). Compared to NC mimics group, OE-circ_0088196 reduced the level of miR-379-5p expression,

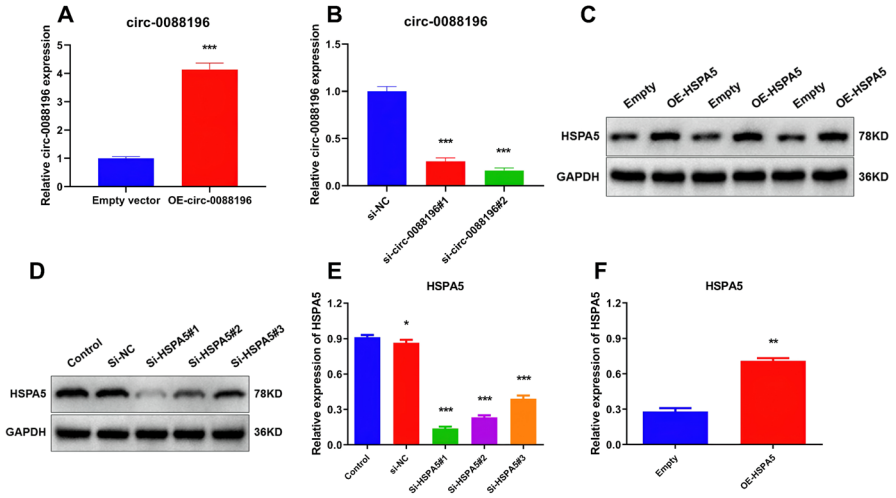


Fig. 3 Overexpress or silence circ-0088196 and HSPA5 in the trophoblast cell line. **A**, **B** RT-qPCR analysis of the expression of circ-0088196 in HTR-8/SVNEO cells transfected with specific overexpression plasmids or oligonucleotides of circ-0088196. **C**–**F** Western blotting analysis of HSPA5 expression in HTR-8/SVneo cells transfected with specific overexpression plasmids or oligonucleotides of HSPA5. The statistical analysis was conducted using one-way ANOVA and LDS test. Mean ± MES, $n = 3$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$

while si-cir-0088196 increased the level of miR-379-5p expression (Fig. 4B). As depicted in Fig. 4C–E, miR-379-5p mimics inhibited HSPA5 expression, and its expression was elevated when transfection with miR-379-5p inhibitor. Whereas, OE-circ_0088196 increased the level of HSPA5 expression. In contrast, Knockdown of circ_0088196 suppressed the mRNA and protein expression of HSPA5 in HTR-8/SVNEO cells. In general, circ-0088196 enabled to elevate HSPA5 expression through sponging miR-379-5p in trophoblast cells.

CircRNA_0088196 Weaken Proliferation and Facilitates Trophoblast Cell Apoptosis Through miR-397-5p/HSPA5 in Trophoblast Cells

To assess whether CircRNA_0088196 could affect trophoblast cells growth, the CCK8 assay was performed after treatment. In Fig. 5A, overexpressed circ-0088196 or HSPA5 notably inhibited proliferation of HTR-8/SVNEO cells, with opposite effects when circ-0088196 or HSPA5 was knocked out. Moreover, miR-379-5p mimics prominently enhanced proliferation of HTR-8/SVNEO cells, with opposite effects when miR-379-5p inhibitors were transfected. TUNEL staining analysis was conducted to observe the functions of circ-0088196, miR-379-5p, and HSPA5 on trophoblast cell apoptosis. As illustrated in Fig. 5B, C, both overexpressed circ-0088196 and HSPA5 significantly strengthened HTR-8/SVneo cell apoptosis, while knockdown of both significantly inhibited HTR-8/SVneo cell apoptosis. Furthermore, HTR-8/SVNEO cell apoptosis was suppressed by miR-379-5p mimics, while

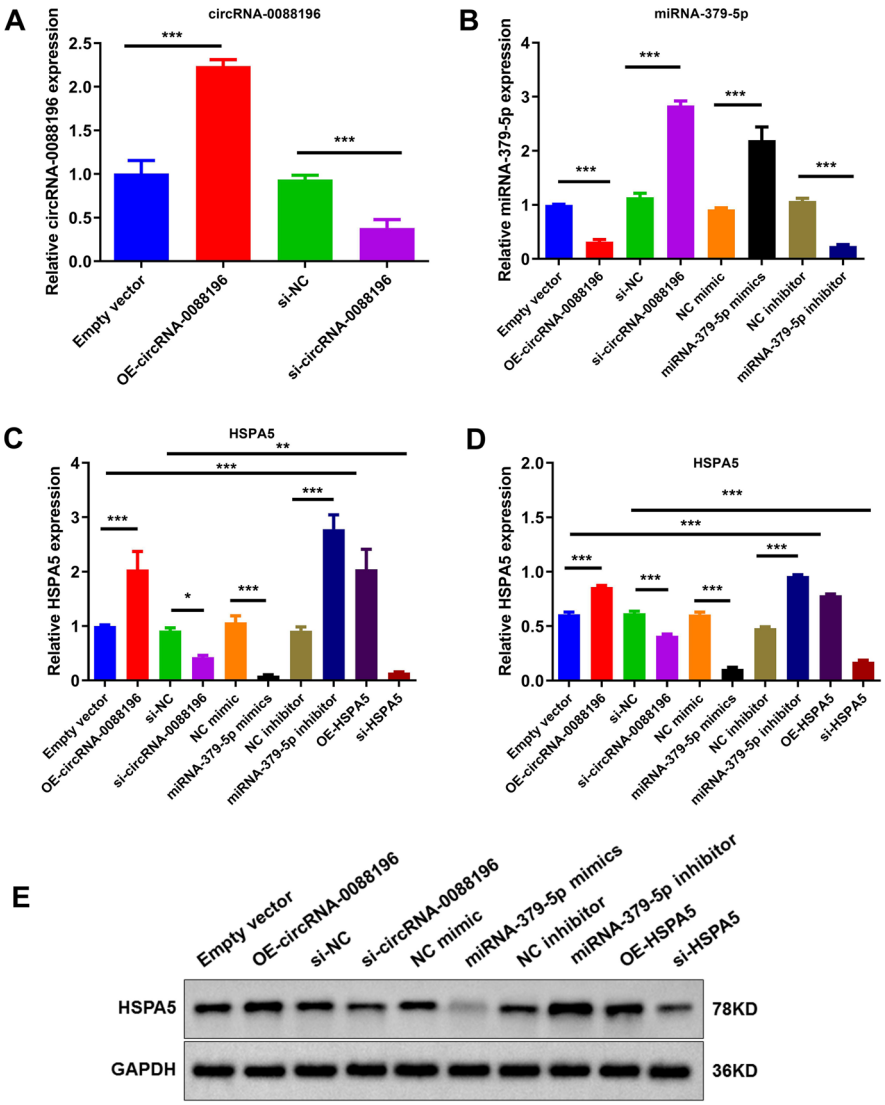


Fig. 4 Circ-0088196 enables to sponge miR-397-5p to up-regulate HSPA5 in trophoblast cells. **A** RT-qPCR analysis of the expression of circ-0088196 in HTR-8/SVneo cells transfected with specific overexpression plasmids or oligonucleotides of circ-0088196. **B** RT-qPCR analysis of the expression of miR-379-5p in HTR-8/SVneo cells. **C** RT-qPCR analysis of the expression of HSPA5 in HTR-8/SVneo cells. **D**, **E** Western blotting analysis of the expression of HSPA5 in HTR-8/SVneo cells with specific overexpression plasmids and oligonucleotides. The statistical analysis was conducted using one-way ANOVA and LDS test. Mean ± MES, $n = 3$;*$p < 0.05$; **$p < 0.01$; and ****$p < 0.0001$

transfection with miR-379-5p inhibitors yielded opposite results. In summary, circ-0088196 enabled restraint of proliferation and facilitated trophoblast cell apoptosis through miR-379-5p/HSPA5 signaling.

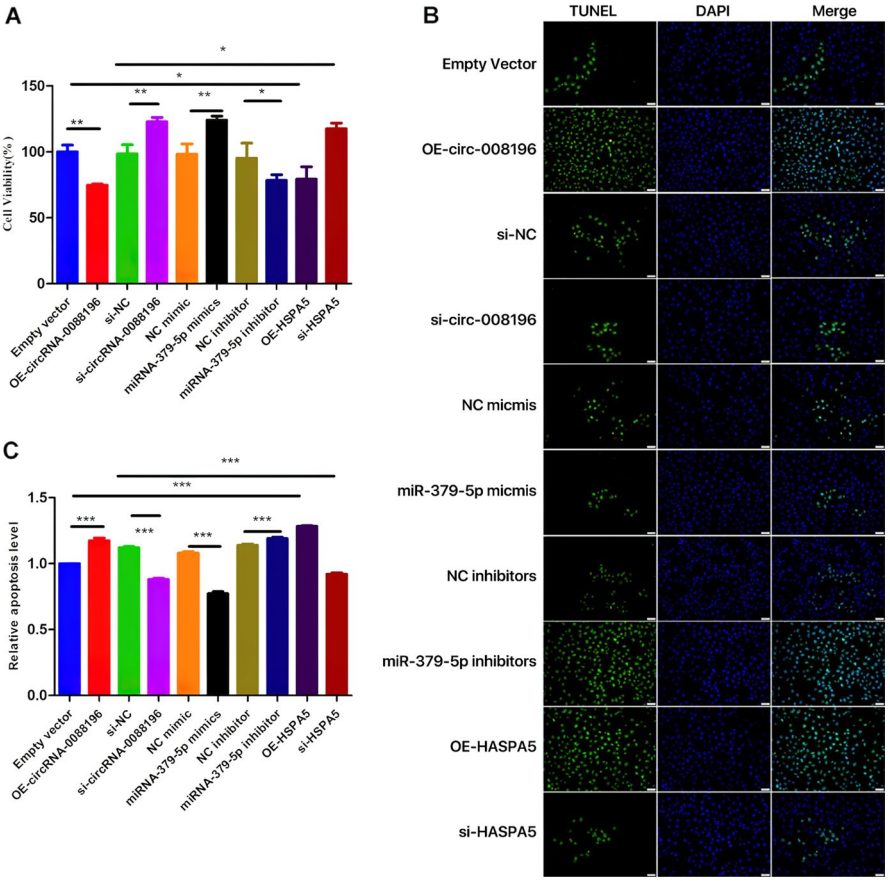


Fig. 5 Circ-0088196 weaken proliferation and facilitates trophoblast cell apoptosis through miR-397-5p/HSPA5 signaling. **A** CCK-8 analysis of the proliferation of HTR-8/SVneo cells transfected with specific overexpression plasmids or oligonucleotides of circ-0088196 or HSPA5, or miR-397-5p mimics or inhibitors. **B**, **C** TUNEL staining analysis of HTR-8/SVneo cells transfected with specific overexpression plasmids or oligonucleotides of circ-0088196 or HSPA5, or miR-397-5p mimics or inhibitors. *Scale bar* 50 μm; and magnification × 200. The statistical analysis was conducted using one-way ANOVA and LDS test. Mean ± MES, $n = 3$; $^{*}p < 0.05$; and $^{**}p < 0.01$

CircRNA_0088196 Induces G0/G1 Phase Arrest in Trophoblast Cells via miR379-5p/HSPA5 Signaling

The effects of circRNA_0088196, miR379-5p, and HSPA5 on the cell cycle of trophoblast cells were further evaluated. The results of this study demonstrated that both overexpressed circRNA_0088196 and HSPA5 significantly prolonged the G0/G1 phase of HTR-8/SVNEO cells, with the opposite effect when circRNA_0088196 and HSPA5 were knocked out (Fig. 6A, B). Nevertheless, miR379-5p mimics significantly shortened the G0/G1 phase of HTR-8/SVneo cells, while its inhibitors prolonged the G0/G1 phase. According to Fig. 6C, the S phase of HTR-8/

Fig. 6 Circ-0088196 induces G0/G1 phase arrest in trophoblast cells via miR-397-5p/HSPA5 signaling. ▶ **A** Cell cycle analysis of HTR-8/SVneo cells transfected with specific overexpression plasmids or oligonucleotides of circ-0088196 or HSPA5, or miR-397-5p mimics or inhibitors. **B–D** Evaluation of **B** G0/G1, **C** S and **D** G2/M phases of HTR-8/SVneo cells with specific overexpression plasmids or oligonucleotides of circ-0088196 or HSPA5, or miR-397-5p mimics or inhibitors. **E**, **F** Western blotting analysis of CyclinD1 protein levels in HTR8 cells. The statistical analysis was conducted using one-way ANOVA and LDS test. Mean ± SD, $n = 3$; Ns: no significance; ***$p < 0.001$; and ****$p < 0.0001$

SVneo cells was markedly shortened by the overexpression of circRNA_0088196 or HSPA5 and was prolonged by the knockdown of circRNA_0088196 or HSPA5. The effect of miR379-5p on the cell cycle of HTR-8/SVNEO cells was the opposite of circRNA_0088196 and HSPA5. However, circRNA_0088196, miR379-5p, and HSPA5 did not affect the G2/M phase of HTR-8/SVneo cells (Fig. 6D). Besides that, western blotting analysis of CyclinD1 protein levels in HTR8 cells was consistent with flow cytometry (Fig. 6E, F). Therefore, circRNA_0088196 triggered G0/G1 phase arrest in trophoblast cells by modulating miR379-5p/HSPA5 signaling.

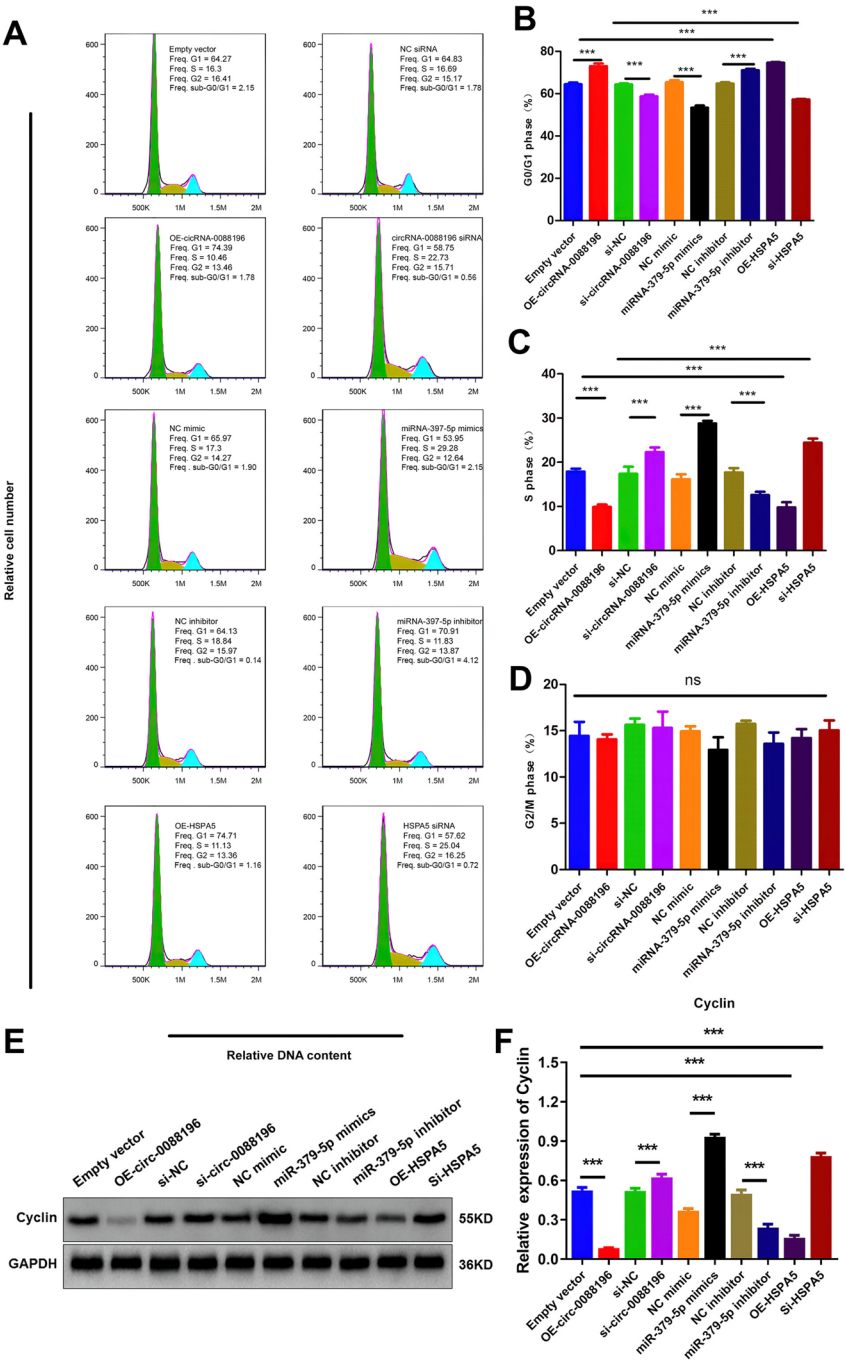
CircRNA_0088196 Restrains Trophoblast Cell Invasion Through miR379-5p/HSPA5 Signaling

Further analysis demonstrated that overexpression of circRNA_0088196 or HSPA5 inhibited HTR-8/SVneo cell invasion (Fig. 7A, B). In contrast, circRNA_0088196 or HSPA5 knockdown enhanced HTR-8/SVneo cell invasion. Moreover, the invasive capacity of HTR-8/SVneo cells was noticeably strengthened by miR379-5p mimics, while the opposite occurred with miR379-5p inhibitor transfection. Hence, circRNA_0088196 restrained trophoblast cell invasion by regulating miR379-5p/HSPA5 signaling.

Discussion

There are few effective strategies for treating PE (Phipps et al. 2019). Therefore, to investigate effective therapy options, there is an urgent need to investigate the underlying molecular mechanisms that enhance PE progress and it is necessary to identify new biological targets. In this study, we discovered that circRNA_0088196 was a key up-regulator of circRNAs, which are linked to the progression of PE. Additionally, further research revealed that circRNA_0088196 exerted a vital role in trophoblast cell proliferation, cell cycle progression, invasion, and apoptosis by modulating miR379-5p/HSPA5 signaling. Therefore, the findings of this study suggested that circRNA_0088196, miR379-5p, and HSPA5 were potential biomarkers and targets for the clinical diagnosis and treatment of PE.

In contrast to the controls, circRNA_0088196 expression was distinctly up-regulated and miR379-5p was significantly down-regulated in PE placental tissue. However, the expression and function of circRNA_0088196 and miR379-5p have already been widely reported. The HSPA5 gene (i.e., GRP78) encodes the Hsp70 chaperone binding immunoglobulin protein within the endoplasmic reticulum


A
Relative cell number
Empty vector
Freq. G1 = 64.27
Freq. S = 16.3
Freq. G2 = 16.41
Freq. sub-G0/G1 = 2.15
NC siRNA
Freq. G1 = 64.83
Freq. S = 16.69
Freq. G2 = 15.17
Freq. sub-G0/G1 = 1.78
OE-circRNA-0088196
Freq. G1 = 74.39
Freq. S = 10.46
Freq. G2 = 13.46
Freq. sub-G0/G1 = 1.78
circRNA-0088196 siRNA
Freq. G1 = 58.75
Freq. S = 22.73
Freq. G2 = 15.71
Freq. sub-G0/G1 = 0.56
NC mimic
Freq. G1 = 65.97
Freq. S = 17.3
Freq. G2 = 14.27
Freq . sub-G0/G1 = 1.90
miRNA-397-5p mimics
Freq. G1 = 53.95
Freq. S = 29.28
Freq. G2 = 12.64
Freq. sub-G0/G1 = 2.15
NC inhibitor
Freq. G1 = 64.13
Freq. S = 18.84
Freq. G2 = 15.97
Freq . sub-G0/G1 = 0.14
miRNA-397-5p inhibitor
Freq. G1 = 70.91
Freq. S = 11.83
Freq. G2 = 13.87
Freq. sub-G0/G1 = 4.12
OE-HSPA5
Freq. G1 = 74.71
Freq. S = 11.13
Freq. G2 = 13.36
Freq . sub-G0/G1 = 1.16
HSPA5 siRNA
Freq. G1 = 57.62
Freq. S = 25.04
Freq. G2 = 16.25
Freq. sub-G0/G1 = 0.72
500K 1M 1.5M 2M
B
G0/G1 phase (%)
C
S phase (%)
D
G2/M phase (%)
ns
Empty vector
OE-circRNA-0088196
si-NC
si-circRNA-0088196
NC mimic
miRNA-379-5p mimics
NC inhibitor
miRNA-379-5p inhibitor
OE-HSPA5
si-HSPA5
E
Relative DNA content
Cyclin
55KD
GAPDH
36KD
F
Relative expression of Cyclin

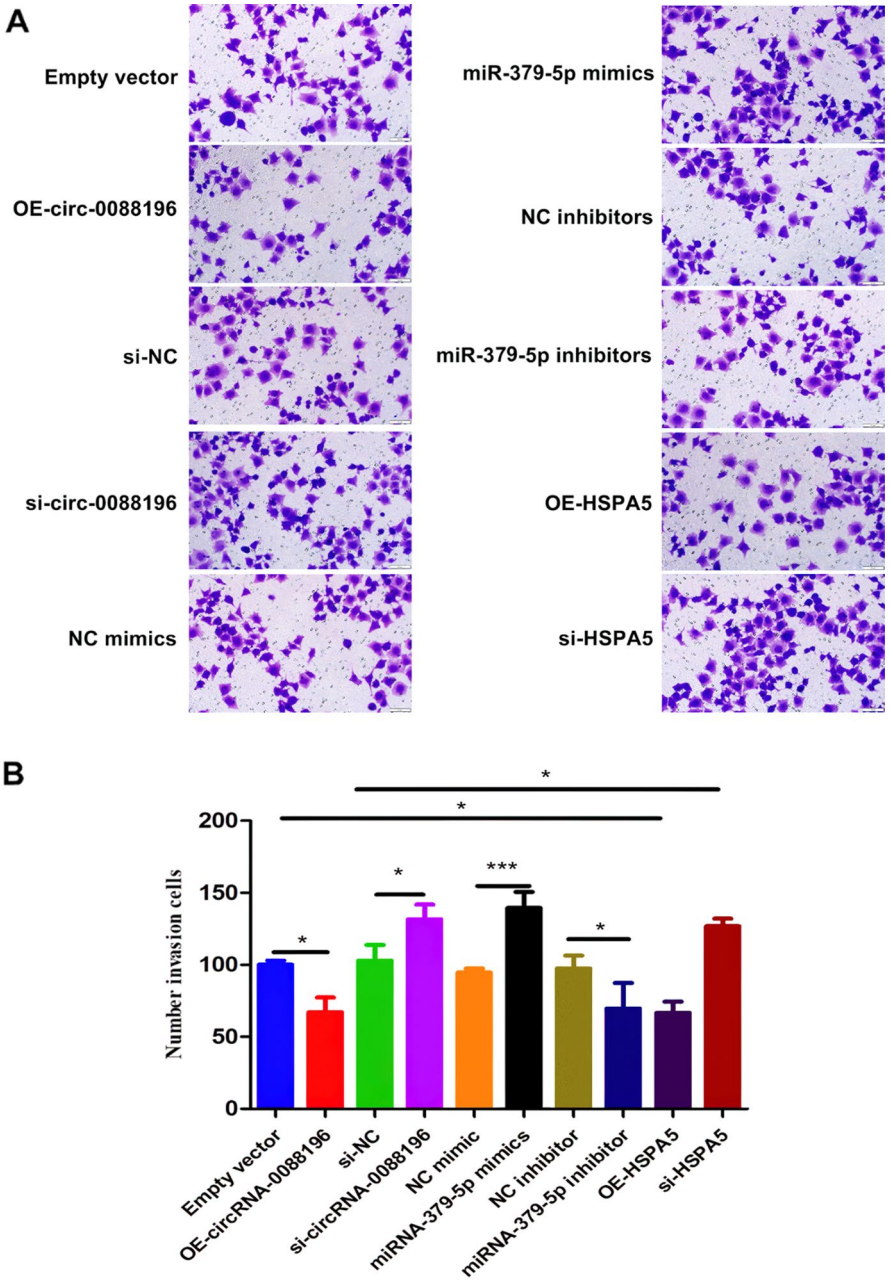


Fig. 7 Circ-0088196 restrains trophoblast cell invasion through miR-397-5p/HSPA5 signaling. **A**, **B** Transwell analysis of the invasion of the HTR-8/SVneo cell line that was transfected with specific overexpression plasmids or oligonucleotides of circ-0088196 or HSPA5, or miR-397-5p mimics or inhibitors. *Scale bar* 50 μm; and magnification × 200. The statistical analysis was conducted using one-way ANOVA and LDS test. Mean ± SD, $n = 3$;*$p < 0.05$; and ***$p < 0.001$

(Wang et al. 2017). Moreover, it exhibits substantial up-regulation in PE placental tissue compared to the controls, which is consistent with previous research (Du et al. 2017). Circulating HSPA5 may be an early predictive biomarker of PE in pregnant women (Laverriere et al. 2009). Results from the dual-luciferase reporter assay in this study suggested that circRNA_0088196 enabled the sponging of miR379-5p to up-regulate HSPA5 expression in trophoblast cells. By combining our findings with previous evidence, circRNA_0088196 may act as a sponge for miR379-5p and up-regulate endoplasmic reticulum stress by elevating HSPA5 during PE. Besides that, HSPA5 overexpression reversed the inhibitory action of miR379-5p on the biological behaviors of trophoblast cells. Du et al. argued that placental protein expression of the endoplasmic reticulum (ER) stress-related markers, including HSPA5, was higher in PE, suggesting that the exaggerated ER stress response was associated with increased apoptosis in placenta of PE patients (Du et al. 2017). Abdollahi et al. found miR-379 plays an important role in high-fat diet (HFD)-induced obesity through increased adipose inflammation, mitochondrial dysfunction, and ER stress as well as impaired adipogenesis and angiogenesis (Abdollahi et al. 2022). In PE, Xu's team found miR-379 was significantly downregulated in severe PE placentas, compared with normal pregnant controls (Xu et al. 2014). Thus, we assumed that HSPA5 could regulate miR-379 through ER stress, and it was proved that miR-379-5p directly bound to circRNA_0088196 in trophoblast cells. By quantitatively assessing apoptosis in the placenta through electron microscopy, placental apoptosis rose noticeably as pregnancy progressed (Smith et al. 2000). Cytotrophoblast cell apoptosis in PE patients was considerably higher than that in full-term pregnancies with normal blood pressure (Longtine et al. 2012). Thus, an enhanced apoptotic level of trophoblast cells is a prominent pathological feature of PE. Our findings revealed that circRNA_0088196 facilitated apoptosis by modulating miR379-5p/HSPA5 signaling. Furthermore, circRNA_0088196 inhibited trophoblast cell proliferation and facilitated G0/G1 phase arrest through miR379-5p/HSPA5 signaling, implying that targeting circRNA_0088196 may exert a protective role on trophoblast cells.

The invasion of trophoblasts into the endometrium and vasculature is a crucial step in human placenta formation (Abbas et al. 2020). Patients with PE exhibit superficial trophoblast invasion and unconverted narrow spiral arteries (Zha et al. 2020). Trophoblasts develop from the trophectoderm, forming the blastocyst wall and creating both villous and extra-villous trophoblasts (Jia et al. 2017). From a physiological perspective, extra-villous trophoblasts attach the placenta to the uterine wall, thereby reshaping the spiral artery. Here, the interstitial trophoblasts destroy the middle layer of the artery by surrounding the spiral artery (Peng et al. 2019). Subsequently, the trophoblasts in the blood vessels move down the lumen of the artery from the cytotrophoblast shells, forming a loose embolus in the first trimester and temporarily congregating in the artery (Zhang et al. 2020). In the first half of pregnancy, the invasive abilities of trophoblasts are strictly modulated (Sun et al. 2021). In the tenth week, the basal decidua includes numerous extra-villous trophoblasts, extending into the myometrium during the 15th week (Su et al. 2021). In normal pregnancies, extra-villous trophoblasts further fuse to multinucleated giant cells, stopping in the inner third of the myometrium (Chen et al. 2020). However, the invasive capacity

of trophoblasts falls considerably in pathological pregnancies, such that insufficient conversion of the spiral artery and blood flow disorders occur in the intervillous space (Ridder et al. 2019). CircRNA_0088196 and HSPA5 both inhibit the invasion of HTR-8/SVneo cells, while miR379-5p enhances the invasive capacity, implying that targeting circRNA_0088196 represents a potential strategy for improving the invasive capacity of trophoblasts in PE. Although we conducted a comprehensive series of studies to dissect the role of circRNA_0088196, miR379-5p, and HSPA5 in the pathogenesis of PE, further research is still necessary. In our future research, we will investigate the functions of circRNA_0088196, miR379-5p, and HSPA5 in distinct cell locations within the trophoblasts. Additionally, we will establish in vivo models to verify the function of circRNA_0088196, miR379-5p, and HSPA5 in PE and conduct a larger epidemiological survey to evaluate clinical diagnoses and the therapeutic significance of circRNA_0088196, miR379-5p, and HSPA5.

Conclusion

In summary, our study provides the first evidence that circRNA_0088196 is highly expressed in human PE placental tissue, and it restricts proliferation and invasion while triggering the apoptosis of trophoblast cells through miR379-5p/HSPA5 signaling. Our findings imply that circRNA_0088196 can be a promising therapeutic target for therapeutic interventions in PE.

Author Contributions ZX and HZ played a guiding role in carrying out the studies, collecting data and drafting the manuscript. SP helped to draft the manuscript. QW and WW was responsible for revision of the paper and the finalization of the paper.

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Declarations

Conflict of interest The author(s) declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Ethical Approval All patients signed an informed consent with approval from the Institutional Ethic Review Committee of Tongde Hospital of Zhejiang Province.

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