Received: 30 March 2022 | Revised: 29 April 2022 | Accepted: 22 May 2022

DOI: 10.1002/jcla.24540

RESEARCH ARTICLE

WILEY

Hsa_circ_0008726 regulates the proliferation, migration, and invasion of trophoblast cells in preeclampsia through modulating the miR-1290-LHX6 signaling pathway

Yongyan Zhang[1] | Shuai Fang[2] | Jiayi Wang[1] | Siqian Chen[1] | Rongrong Xuan[1]

[1]Department of Obstetrics and Gynecology, The Affiliated Hospital of Medical School of Ningbo University, Ningbo, China

[2]Department of Thoracic Surgery, The Affiliated Hospital of Medical School of Ningbo University, Ningbo, China

Correspondence
Rongrong Xuan, Department of Obstetrics and Gynecology, The Affiliated Hospital of Medical School of Ningbo University, No. 247, Renmin Road, Jiangbei District, Ningbo 315020, Zhejiang Province, China.
Email: fyxuanrongrong@nbu.edu.cn

Funding information
This work was funded by the Ningbo Natural Science Foundation Project (No.2021J239) and Ningbo Public Welfare Technology Plan Project (No.2021S101)

Abstract

Background: Preeclampsia (PE) is a serious complication of pregnancy, with a global incidence of about 2%–8%. It is one of the important causes of morbidity and mortality among the pregnant women and perinatal infants. Circular RNA (circRNA) has been confirmed to play an important regulatory role in PE. This study aimed to evaluate the role of hsa_circ_0008726 in the occurrence and development of PE.

Methods: The expression of hsa_circ_0008726 in PE placental tissue and blood was detected by qRT-PCR. CCK-8, wound closure, and Transwell assay were used to measure cell proliferation, migration, and invasion. Bioinformatics prediction, Western blotting, and dual-luciferase reporter gene detection were used to explore the mechanism of hsa_circ_0008726 in HTR8/SVneo cells.

Results: The expression level of circ_0008726 in the placental tissue and blood samples of PE patients was significantly higher than that of normal controls. The overexpression of circ_0008726 can significantly inhibit the proliferation, migration, and invasion ability of HTR-8/SVneo cells. While the silence of circ_0008726 showed an opposite effect. Furthermore, hsa_circ_0008726 can modulate the expression of LHX6 by adsorbing miR-1290.

Conclusion: Hsa_circ_000872 can regulate LHX6 by adsorbing miR-1290 to inhibit PE progression, thus establishing hsa_circ_000872 as a potential target for predicting and treating PE.

KEYWORDS
hsa_circ_0008726, LHX6, miR-1290, preeclampsia

1 | INTRODUCTION

Preeclampsia (PE) is a serious complication associated with pregnancy and one of the important causes of morbidity and even death of the pregnant women as well as perinatal infants.[1] PE is the third leading cause of maternal death and, annually, more than 60,000 pregnant women die of PE globally, second only to bleeding and embolism.[2] PE can exhibit significant adverse effects on both the mother and the fetus. Moreover, if the fetus is not treated in time, complications such as intrauterine growth restriction (IUGR),

Yongyan Zhang and Shuai Fang are equally contributed to this work.

placental abruption, premature birth, neonatal respiratory distress syndrome, retinopathy of prematurity, and even death may occur. In addition, various complications such as hypertension, kidney damage, liver damage, central nervous system damage, stroke, cardiomyopathy, pulmonary edema, and respiratory distress syndrome may also occur. When PE occurs in combination with HELLP syndrome (hemolysis, elevated liver enzymes, and lower platelets), liver hemorrhage or rupture, acute kidney injury (AKI), diffuse intravascular coagulation (DIC), and pulmonary edema might occur, and hence the fatality rate can increase significantly.[3] Antiphospholipid antibody syndrome, hypertension, diabetes, chronic kidney disease, obesity, family history of PE, multiple pregnancy, advanced age of pregnant, and lying-in women are potential risk factors that can lead to PE.[4] The proliferation, invasion, and migration of trophoblast cells serve as important factors to ensure the normal development of the placenta and to maintain an optimal nutrient supply.[5] In PE, trophoblast cell cycle changes, apoptosis, and loss of invasion ability have been reported to promote the occurrence and development of PE.[6,7]At present, the pathogenesis underlying PE is not fully understood, so it is of great significance to explore the molecular mechanisms behind its occurrence and development, which can help to develop potentially novel treatment methods to prevent the progression of this disease.

Accumulating evidences suggest that some specific noncoding RNAs (ncRNAs) are involved in the occurrence and development of PE.[8–10] Among them, circular RNA (circRNA) has emerged as an important regulatory molecule that has been implicated in PE and may mediate the pathological process of a variety of complications linked to pregnancy.[11,12] For example, CircSFXN1 can mediate the development of PE by recruiting and combining with sFLT1 to negatively regulate the biological behavior of the trophoblast[13]; CircSFXN1 may also serve as a new biomarker of PE, by regulating the MSP/RON axis in the trophoblast cells in PE[14] and CircHIPK3 has been reported to participate in the PE process by deregulating the invasion, proliferation, angiogenesis, and apoptosis of trophoblast cells.[15]

LHX6 is a member of the LIM homeobox transcription factor family. It has been shown the potential to be a biomarker and therapeutic target for multiple diseases as a result of its ability to regulate cell growth, proliferation, and invasion.[16] A number of previous studies have indicated that LHX6 is expressed in the medial ganglion bulge of rodents and is essential for the migration of human pluripotent stem cell-derived GABA interneurons.[17] LHX6 can also participate in the regulation of methylation silence and can functionally promote the proliferation of pancreatic cancer cells[18] and can attenuate the growth of colorectal cancer cells by inhibiting the expression of miR-346.[19] These studies have indicated that LHX6 can exhibit an important regulatory role in inhibiting cell growth, proliferation, and invasion.

In this study, we screened out several differentially expressed circRNAs by mining and sorting out PE gene chip data. Thereafter, by using different assays, we have found that hsa_circ_0008726 is highly expressed in PE. At the same time, our findings indicate that hsa_circ_0008726 can effectively regulate LHX6 through modulating miR-1290 to potentially inhibit the occurrence and development of PE in vitro. Overall, findings of our research provide new insights into the pathogenesis of PE.

2 | MATERIALS AND METHODS

2.1 | Sample collection

In this study, we recruited 17 patients diagnosed with PE and 17 control pregnant women in the Affiliated Hospital of Medical School of Ningbo University. All the recruited pregnant women chose Caesarean delivery. Blood samples were collected from patients on an empty stomach before surgery, and samples were stored in a −80°C freezer. Inclusion criteria: PE was new-onset hypertension after 20 weeks of gestation, diastolic blood pressure (mmHg ≥90), or systolic blood pressure (mmHg ≥140), proteinuria ≥300 mg/day, excluding twin pregnancy, gestational diabetes mellitus, kidney disease, chronic hypertension, acute and chronic hepatitis, hyperthyroidism. The research protocol carried out was approved by the Ethics Committee of the Affiliated Hospital of Medical School of Ningbo University, and a written informed consent form was signed with each participant.

2.2 | qRT-PCR

Trizol method was used to extract total RNA from the placental tissue, blood, and cells. The concentration and purity of RNA were detected by a trace nucleic acid protein analyzer. Take 500 ng RNA was used and cDNA was synthesized using TaKaRa Reverse Transcription Kit. Using cDNA as a template. The reaction was carried out according to the instructions of the TaKaRa fluorescence quantitative PCR detection kit. The primers are listed in Table S1.

2.3 | Cell culture and transfection

HTR8/SVneo cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and placed in an incubator at 37°C and 5% CO_2. When the cell confluence reached 90%, only cells in good condition were used for subsequent experiments. HTR8/SVneo cells were first inoculated on a 6-well plate. When the cell confluence was about 70% observed under a microscope, serum-free DMEM medium was added to the cells, and based on instructions provided for the use of lip2000 transfection reagent to replace the recombinant plasmid or siRNA, lip2000, and Opti-MEM was mixed, and the cells to be transfected were added to the culture wells. After culturing for 48 h, the cell samples of each group were collected for subsequent experiments. The oligos are shown in Table S2.

2.4 | CCK-8 assay

HTR8/SVneo cells were diluted in the logarithmic growth phase and spread at a volume of 200 μl per well in a 96-well plate. Each group consisted of 3 multiple wells. When the cells grew to about 70% confluence, the transfected recombinant plasmids or siRNA were added to the cells. After culturing for 0, 24, 48, 72, and 48 h, 10 μl of the prepared CCK-8 solution was added to each well of the cells, and the cells were placed in a cell incubator away from light for 2 h. Thereafter, absorbance was measured at 450 nm in a microplate reader.

2.5 | Transwell assay

The matrix gel was diluted and spread on the upper chamber of transwell for 12 h. After successful transfection of HTR8/SVneo cells, a suspension containing 1×10^5 cells was spread on the upper chamber of transwell. The culture medium was added to the lower chamber, incubated for 24 h, fixed with 4% paraformaldehyde, washed with PBS, and then stained with the crystal violet dye. The cells were placed under a microscope and randomly selected 5 fields of view to observe and analyze the results.

2.6 | Wound-closure assay

HTR8/SVneo cells were inoculated in the logarithmic growth phase in a 6-well plate, and 3 parallel lines were drawn on the back of the 6-well plate as a marker for shooting. After 48 h, 200 μl pipette tip was used to draw 2 parallel lines in the 6-well plate. The straight line was perpendicular to the straight line on the back of the 6-well plate. After washing with PBS, 2 ml of serum-free DMEM medium was added, and the cells were observed under a microscope at 0 and 24 h to determine the migration distance of the cells to the middle of the scratch, and the pictures were captured.

2.7 | Dual-luciferase activity assay

HTR8/SVneo cells were diluted in the logarithmic growth phase and then spread in a 12-well plate. When the cell confluence was about 75%, lip2000 reagent was used to combine the circ_0008726-WT and circ_0008726-MUT plasmids with miR-1290 mimics and miRNA-NC, respectively. Transfect into HTR8/SVneo cells, and finally measure the intensity of luciferase activity.

2.8 | Western blotting assay

First RIPA was used to lyse the cell protein, and thereafter the protein concentration was detected by the BCA protein detection kit. 50 μg protein sample was used for SDS-PAGE, and the protein was transferred to PVDF membrane after electrophoresis. The PVDF membrane was placed in 5% skimmed milk powder diluted in TBST buffer, and blocked for 3 h. After the incubation, the PVDF membrane was placed in a specific primary antibody diluted with TBST, overnight at 4°C, washed in TBST buffer 5 times, for 5 min each time. Thereafter, the PVDF membrane was placed in the secondary antibody diluted with TBST, and the ECL reaction solution was added dropwise to the PVDF membrane for protein exposure at room temperature for 2 h. ImageJ software was employed to analyze the gray value of the protein.

2.9 | Statistical analysis

All the above-described experiments were repeated 3 times independently. GraphPad Prism 6.0 software was used for analysis, and the measurement data conforming to the normal distribution was expressed as ($\bar{x}\pm s$), among which pairwise comparisons were performed by LSD t test. $p<0.05$ or $p<0.01$ indicated that the difference was statistically significant.

3 | RESULTS

3.1 | circ_0008726 was highly expressed in PE tissues and blood

We extracted PE circRNA chip data (Accession No. GSE102897) from the GEO database and screened out 3 upregulated circRNAs in PE placenta tissue after analysis and comparison, namely circ_0044559, circ_0063108, and circ_0008726. Validated by qRT-PCR, the results showed that the expression of circ_0008726 in PE placenta tissue was significantly higher than that in normal tissue (Figure 1A), while there was no statistical difference between circ_0044559 and circ_0063108 (Figure S1A,B). Therefore, we further verified the expression of circ_0008726 in 17 pairs of blood samples. The results showed that the expression level of circ_0008726 in the blood samples of PE patients was significantly higher than that of the normal control group (Figure 1B). This finding suggested that circ_0008726 may play an important regulatory role in the occurrence and development of PE.

Bioinformatics analysis revealed that circ_0008726 was derived from exons 4, 5, and 6 of DNAJB6 gene, and the length was 281 nt (Figure 1C). We next used placental trophoblast cells (HTR-8/SVneo) to further explore the potential effects of overexpression or knockdown of circ_0008726 on cellular functions. The results of qRT-PCR showed that compared with the blank control (siRNA-NC), the expression level of circ_0008726 was significantly reduced after transfection of siRNA-1 and siRNA-2 interference sequences targeting circ_0008726 knockdown. Therefore, we selected siRNA-1 and siRNA-2 for knockdown experiments in our follow-up studies (Figure 1D). In addition, we constructed an overexpression vector based on the full-length

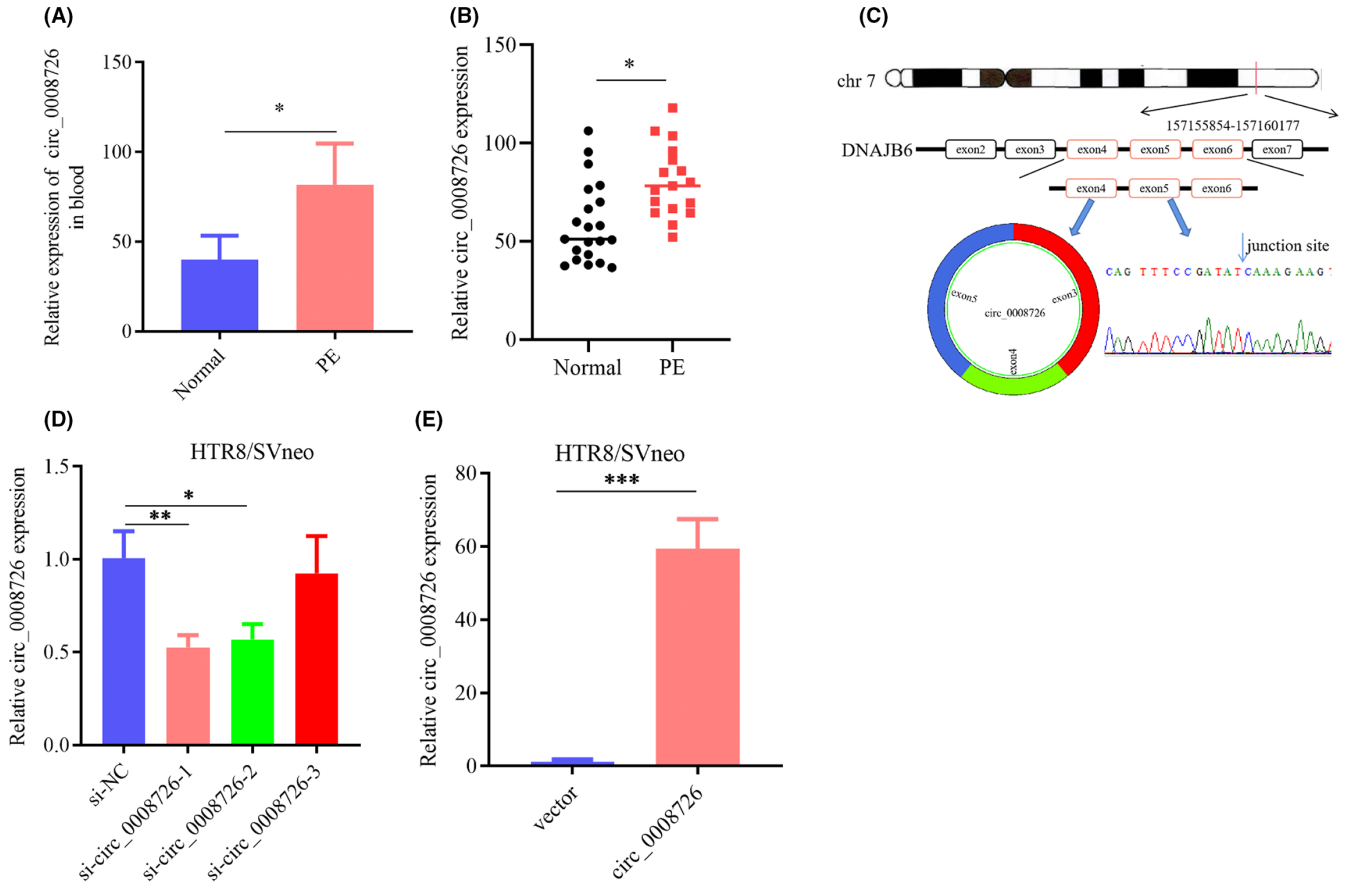

FIGURE 1 circ_0008726 was highly expressed in the tissues and blood of PE. (A) qRT-PCR was employed to examine the expression of circ_0008726 in the placenta of PE patients and normal pregnant women, $N = 3$. (B) qRT-PCR was used to detect the expression of circ_0008726 in the blood samples of PE patients and normal pregnant women, $N = 17$. (C) circ_0008726 is formed by back-splicing of exons 4, 5, and 6 of gene DNAJB6. (D) qRT-PCR was used to determine the knockdown efficiency of circ_0008726 in HTR-8/SVneo cells. (E) qRT-PCR was used to analyze the overexpression efficiency of circ_0008726 in HTR-8/SVneo cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

sequence of circ_0008726 and transfected it into HTR-8/SVneo cells. These results indicated that compared with the empty vector group (vector), the expression level of circ_0008726 was significantly increased after transfection of the circ_0008726 overexpression vector (Figure 1E).

3.2 | circ_0008726 can inhibit the proliferation, migration, and invasion of trophoblast cells

First, the results of CCK-8 experiments showed that compared with siR-NC cells, knocking down circ_0008726 could significantly promote the proliferation of HTR-8/SVneo cells. On the contrary, compared with vector group cells, the overexpression of circ_0008726 was observed to significantly inhibit the proliferation ability of HTR-8/SVneo cells (Figure 2A). The results of scratch experiment and transwell invasion experiments showed that knocking down circ_0008726 can significantly promote cell migration and invasion. On the contrary, the overexpression of circ_0008726 can significantly inhibit the migration and invasion ability of HTR-8/SVneo cells (Figure 2B,C).

3.3 | miR-1290 can act as a potential target of circ_0008726

In recent years, with significant advances in research filed, the ceRNA hypothesis has been extensively applied to describe the mechanism of interactions between RNAs and can explain complicated biological functions.[20,21] In the study, the existence of ceRNA might also affect the biological functions of trophoblast cells. Through the comparison and analysis of circinteractome, circMIR, and circbank software, we found that circ_0008726 contained multiple miRNAs binding sites. The intersection of these 3 databases was obtained (Figure 3A), and the results showed that miR-1290 contains a potential binding site with circ_0008726. In order to further confirm the binding relationship between miR-1290 and circ_0008726, we cloned the wild-type and mutant circ_0008726 sequences into the luciferase reporter gene vector (Figure 3B) and verified the binding relationship through dual-luciferase reporter gene experiments. The results indicated that compared with the control group, the luciferase activity was significantly reduced after co-transfection with miR-1290 mimics and circ_0008726 wild-type. However, after co-transfection of

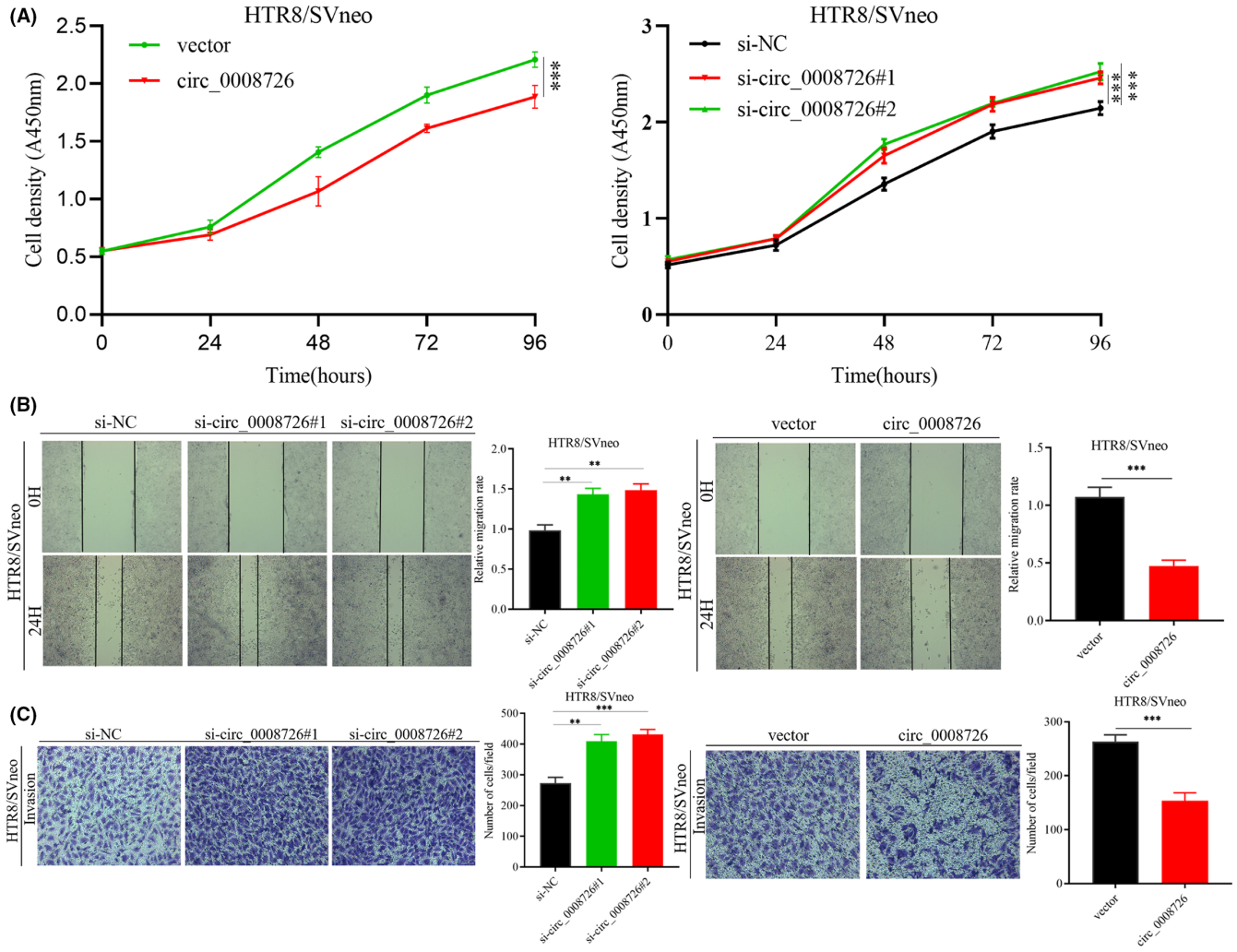

FIGURE 2 circ_0008726 inhibited the proliferation, migration, and invasion of trophoblast cells (A) The viability of HTR-8/SVneo cells was analyzed by CCK-8 assay. (B) The migration of HTR-8/SVneo cells was determined by wound healing test. (C) The invasion ability of HTR-8/SVneo cells was analyzed by Transwell invasion. $^{**}p<0.01$, $^{***}p<0.001$

miR-1290 mimics and circ_0008726 mutant, no significant change in luciferase activity was observed (Figure 3C,D). These results indicated that miR-1290 can effectively interact with circ_0008726. In addition, to further verify their mutual regulation relationship miRNA-NC and miR-1290 mimics were transfected in HTR-8/SVneo and HEK-293T cells, respectively, and qRT-PCR results showed that compared with miRNA-NC group, the expression level of circ_0008726 in the miR-1290 group was significantly reduced (Figure 3E). The above results indicated that circ_0008726 might regulate the occurrence and development of PE in combination with miR-1290.

3.4 | LHX6 can be targeted by miR-1290

Since miRNA can bind to the 3'UTR of target mRNA to regulate gene expression, we further explored the downstream target genes of miR-1290. Through file search and database analysis, it was found that LHX6 can serve as a potential target gene downstream of miR-1290. In addition, LHX6 has also been confirmed as a downstream target of miR-1290 in both endometrial and glioma cells.[22,23] Therefore, we verified whether there also existed a similar relationship in HTR8/SVneo cells. The qRT-PCR results showed that compared with the normal control group, the expression level of LHX6 increased significantly after transfection with miR-1290 inhibitor, while its expression level decreased significantly after transfection with miR-1290 mimics (Figure 4A). Interestingly, we found that knocking down the expression of circ_0008726 in HTR8/SVneo cells significantly reduced the expression level of LHX6 at both protein and mRNA level (Figure 4B,C). However, after overexpression of circ_0008726, the expression levels of LHX6 protein and mRNA were also significantly increased. In addition, after co-transfection of miR-1290 mimics and circ_0008726 overexpression plasmids in HTR8/SVneo cells, we found that compared with the control group, transfection of miR-1290 mimics alone can significantly inhibit the expression of LHX6 protein. However, the upregulation of miR-1290 and the upregulation of circ_0008726LHX6 protein expression level was partially restored (Figure 4D).

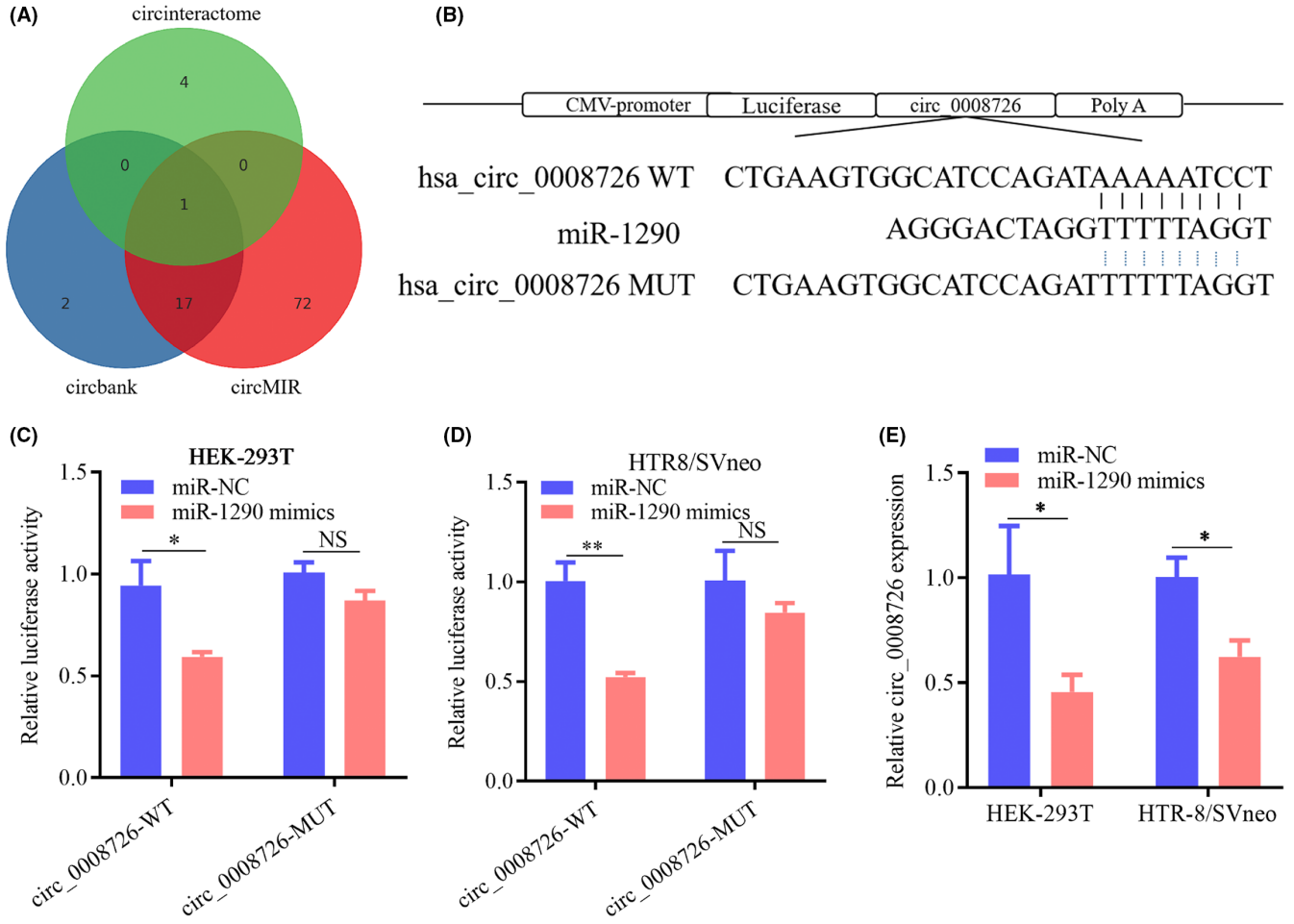

FIGURE 3 miR-1290 can serve as a potential target of circ_0008726. (A) The database predicted that there was a potential binding miRNA with circ_0008726. (B) The predictive analysis of the binding site of miR-1290 and circ_0008726; (C) luciferase reporter gene detection for HEK239T luciferase activity in cells; (D) luciferase reporter gene assay was carried out to detect luciferase activity in HTR-8/SVneo cells; (E) qRT-PCR was used to detect the expression level of circ_0008726 in HTR-8/SVneo cells. $^{*}p<0.05$, $^{**}p<0.01$

3.5 | circ_0008726 suppressed the growth, migration, and invasion by targeting miR-1290

In order to functionally explore whether circ_0008726 can also regulate the phenotype of HTR8/SVneo cells through sponge miR-1290. After co-transfection of miR-1290 mimics and circ_0008726 overexpression plasmids in HTR8/SVneo cells, we found that compared with the control group, transfection of miR-1290 mimics alone can significantly promote the cellular proliferation, migration, and invasion. However, when both miR-1290 and circ_0008726 were upregulated, the cellular proliferation, migration, and invasion ability was partially restored (Figure 5A–C).

4 | DISCUSSION

CircRNAs are a type of endogenous ncRNAs which are found in abundance and display stable expression. Due to their robust stability, high degree of conservation among species, and tissue specificity, they can serve as promising molecular markers and potential molecular targets for clinical diagnosis, treatment, and prognosis.[24,25] Although with the continuous development of sequencing technology, more and more differentially expressed circRNAs have been identified and their functional verifications have been done; however, still several important biological functions of circRNAs remain to be elucidated.[26] In this study, we have screened the differentially expressed hsa_circ_0008726 from the database. This gene was found to be highly expressed in PE tissue samples and cell lines, which suggested that hsa_circ_0008726 may play an important role in the occurrence and development of PE.

This study also revealed that the level of hsa_circ_0008726 was significantly upregulated in the placenta tissue of PE patients, and hsa_circ_0008726, as well as miR-1290, possess direct interaction binding sites. A number of previous studies have shown that miR-1290 plays an important role in regulating cell migration and invasion. For example, miR-1290 can promote the metastasis of oral squamous cell carcinoma by targeting CCNG2[27]; miR-1290 can drive colorectal cancer progression by targeting INPP4B cell proliferation[28]; neurons can also attenuate apoptosis by ingesting endothelial cell-derived exosomes miR-1290.[29] In addition, prior studies have also shown that miR-1290 can regulate cell proliferation, migration, and invasion by targeting LHX6 protein.[22] Moreover, a similar study in trophoblast cells also

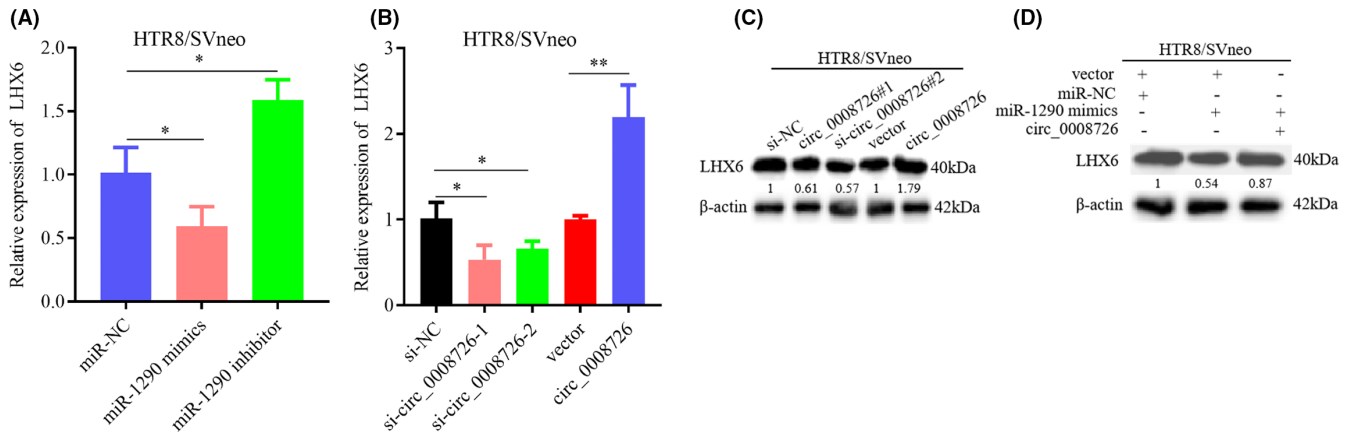

FIGURE 4 LHX6 can be targeted by miR-1290. (A and B) qRT-PCR was employed to detect the expression level of LHX6 in HTR-8/SVneo cells; (C and D) Western Blot was used to analyze the expression level of LHX6 protein in HTR-8/SVneo cells. $^{*}p<0.05$, $^{**}p<0.01$

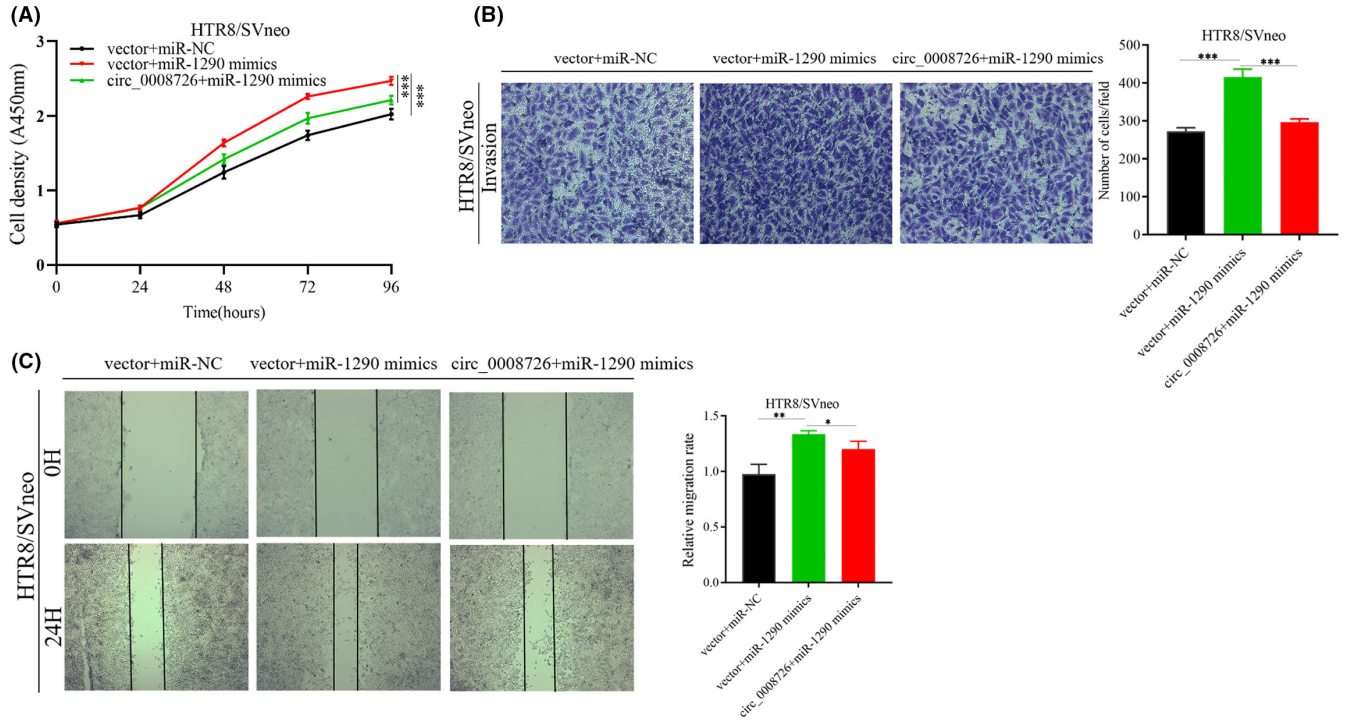

FIGURE 5 circ_0008726 suppressesd HTR8/SVneo cell growth, migration, and invasion by targeting miR-1290. (A) The viability of HTR-8/SVneo cells was analyzed by CCK-8 assay. (B) The migratory potential of HTR-8/SVneo cells was determined by wound healing test. (C) The invasion ability of HTR-8/SVneo cells was examined by Transwell assay. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

demonstrated that exosomal miR-1290 can promote the potential interaction between the endometrium and the embryo[23] by targeting LHX6, which was consistent with our findings. In the current study, we found that hsa_circ_0008726, miR-1290, and LHX6 can display an interaction relationship. It was observed that hsa_circ_0008726 can competitively bind miR-1290 to promote the expression of LHX6 and regulate the various biological functions of trophoblast cells. Trophoblast cells, as the main component cells of the placenta, can display substantial invasion ability similar to tumor invasion.

Although we have identified the mechanism by which hsa_circ_0008726 can inhibit the occurrence and development of PE by promoting the biological functions of trophoblast cells, there are still several aspects that need to be improved. First of all, due to the paucity of the precious clinical samples, our organization had too few samples for verification. In the future studies, we can consider using large samples of inflammation. Secondly, this study did not employ animal models to analyze the specific molecular mechanisms of action of hsa_circ_0008726. Finally, LHX6 can play an important role in regulating the biological functions of cells, but its role in PE is less well understood, and more profound mechanism exploration is needed to clarify its important role in PE regulation.

In conclusion, our results showed that the competitive combination of hsa_circ_0008726 and miR-1290 can functionally eliminate the inhibitory effect of miR-1290 on LHX6, thereby attenuating the occurrence and development of PE by significantly suppressing the proliferation, migration, and invasion of trophoblast cells. Overall,

our findings have established new pathogenesis for PE and are expected to provide new targets for PE treatment.

ACKNOWLEDGEMENT

This study was supported by the Ningbo Natural Science Foundation Project (No.2021J239) and Ningbo Public Welfare Technology Plan Project (No.2021S101). The authors are thankful to the patients who took part in this study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are included within the article and its additional files.

ORCID

Shuai Fang https://orcid.org/0000-0002-6633-755X

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How to cite this article: Zhang Y, Fang S, Wang J, Chen S, Xuan R. Hsa_circ_0008726 regulates the proliferation, migration, and invasion of trophoblast cells in preeclampsia through modulating the miR-1290-LHX6 signaling pathway. *J Clin Lab Anal*. 2022;36:e24540. doi: 10.1002/jcla.24540