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Short communication: Upregulation of hypoxia/reoxygenation-induced Shc3 by downregulated miR-455-5p, suppresses trophoblast invasion and is associated with placental inflammation and angiogenesis in preeclampsia

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Abstract

Preeclampsia is characterized by insufficient invasion of extravillous trophoblasts and is a consequence of failed adaption of extravillous trophoblasts to changes in the intrauterine environment developing embryo. Specific miRNAs are implicated in the development of pre-eclampsia (PE). miR-455-5p is present at low levels in PE but its role is not known. Combining cell and molecular biology methods, we provide evidence of the function and mechanism of miR-455-5p action, and identify its potential target, Shc3, in PE. In vitro, when miR-455-5p was overexpressed in HTR-8/SVneo cells they migrated and invaded more rapidly under hypoxia/reoxygenation (H/R) than in either hypoxic or normoxic conditions. In contrast, apoptosis of HTR-8/SVneo was reduced in H/R. Shc3 was identified as a direct downstream target gene of miR-455-5p. Overexpression of Shc3 reversed the effect of miR-455-5p, promoting apoptosis and suppressing invasion and migration of HTR-8/SVneo under H/R. Shc3 was highly expressed in H/R, but its level was reduced in isolated hypoxic or normoxic environments. Furthermore, we showed Shc3 overexpression is involved in placental inflammation and angiogenesis inhibition. Finally, we showed that the downregulation of miR-455-5p in PE contributes to increased Shc3 in extravillous trophoblasts, thereby limiting extravillous trophoblast cell invasion. Elevated Shc3 is associated with placental inflammation and angiogenesis inhibition. Thus Shc3 serves as a potential biomarker for PE diagnosis and treatment.

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Abbreviations: PE, Preeclampsia; H/R, Hypoxia/reoxygenation; miR, microRNA; Shc3, Src homolog and collagen homolog 3; HRP, Horseradish Peroxidase; ECM, Endothelial Cell Medium; UTR, Untranslated region; GEO, Gene Expression Omnibus; GSE, Gene Expression Omnibus Series; ANOVA, Analysis of Variance.

Introduction

Pre-eclampsia (PE) is a pregnancy-specific disease with several complex syndromes characterised by high blood pressure, proteinuria, and oedema that usually occurs after 20 weeks of gestation [1, 2]. PE can cause multiple organ dysfunction, restrict foetal growth, perinatal death, and accounts for approximately 70% of all pregnancy-related hypertension disorders [3]. There is a widespread belief that PE is a placental disease [4] associated with apoptosis and inadequate invasion of the maternal uterine spiral arteries during early pregnancy, subsequent remodelling and narrowing of these arteries, placental ischemia/reperfusion and endothelial injury, oxidative stress, and inflammation [5].

Trophoblasts play a crucial role during pregnancy. Trophoblast adaptations to the developmental environment surrounding the embryo are vital for a healthy placental implantation. At the early stage of pregnancy, the embryo initially develops in a hypoxic environment, during which extravillous trophoblast cells exhibit robust anti-apoptosis, migration, and invasion capabilities [6]. In the later stages of pregnancy, extravillous trophoblast cells gradually transition from a hypoxic/reoxygenated environment to a normoxic environment at the maternal-foetal interface, during which their invasive capacity decreases [7, 8]. However, the mechanism by which extravillous trophoblasts change their apoptosis and invasive capacities in response to environmental variations and its relevance to PE is still poorly understood.

miRNAs are associated with the development of PE, including miR-210, miR-155, miR-335 and others [9]. However, fewer miRNAs have been reported with regard to the placental development environment in PE. Recent studies revealed that miR-455-5p is a hypoxia-related miRNA [10, 11] that can improve tissue ischemia/reperfusion injury [12, 13]. Interestingly, miR-455-5p was reported downregulated in PE placentas [14], but its pathological mechanism not well understood. In this study, we determined miR-455-5p was relevant in extravillous trophoblast invasion, inflammation and angiogenesis of preeclampsia in response to the intrauterine environment. The HTR-8/SVneo cell model was used because primary trophoblast cells derived from fresh placental tissues have limited lifespan and restricted to divide [15].

Materials and methods

Clinical specimens

PE patients and healthy, normotensive pregnant women (n = 15 each) in the Nanjing Maternity and Child Health Care Hospital donated their placenta tissues (From 2022.06.13~2024.06.12). The placental tissues used in the experiment were isolated from the midsection between the chorionic and maternal basal surfaces near the umbilical cord. The Declaration of Helsinki was followed during all progress. The study was approved by the Ethics Committee of Nanjing Maternity and Child Health Care Hospital (No. 2022-KY043). All study participants provided written informed consent.

Antibodies, RNAs and plasmids

SHC3, Beta Actin antibodies and HRP-conjugated Affinipure Goat Anti Rabbit IgG(H+L) were obtained from Proteintech (Wuhan, China). MiR-455-5p mimics, inhibitors, and their negative controls (NC) were purchased from RiboBio (Guangzhou, China). The SHC3 overexpression as well as control plasmids were constructed by Corues Biotechnology (Nanjing, China). RNA sequencing and analysis were performed by Nanjing High-Tech Precision Medicine Technology (Nanjing, China) Co., Ltd.

Cell culture and hypoxia treatment

HTR-8/SVneo cells were obtained from Ek-Bioscience (Shanghai, China) and cultured in RPMI-1640 medium (KeyGen, China) with 10% foetal bovine serum (Gibco, USA) and 1% (v/v) penicillin-streptomycin-glutamine mixed Solution, at 37˚C and 5% CO_2. EA.hy926 cells were obtained from Shanghai Cell Bank (Shanghai, China) and cultured in ECM medium (ScienCell, USA) with 5% foetal bovine serum (ScienCell, USA), 1% endothelial cell growth supplement (ECGS) and 1% (v/v) penicillin-streptomycin-glutamine mixed Solution, at 37˚C and 5% CO_2.

Logarithmic phase HTR8/SVneo cells (5×10^5 cell/well) were seeded in 6-well plates (2 mL 1% FBS medium/well) and incubated 24 h in hypoxic environments (94% N_2, 5% CO_2, 1% O_2) to simulate the ischaemic–hypoxic state in early pregnancy in vitro.

Cell transfection, hypoxia, normoxia and H/R treatment

HTR8/SVneo cells were transfected with equal amounts of miR-455-5p mimic, inhibitor and NC using Lipofectamine 3000 (Invitrogen, USA). The transfected cells were firstly incubated at 37˚C, 5% CO_2 for 6 h then replaced with fresh FBS medium. Transfected cells were categorized into three groups: hypoxia, normoxia, H/R. The hypoxia group was treated with 1% FBS medium in 1% O_2, 5% CO_2, 94% N_2. The normoxia group was treated with 10% FBS medium. The H/R group was first cultured in 1% FBS medium in 94% N_2, 5% CO_2, 1% O_2 for 8 h, then placed in 95% air, 5% CO_2 with 10% FBS medium for 4 h; this process was performed twice.

EA.hy926 cells (5×10^5 cell/well) were seeded in 6-well plates and treated with H/R before transfection. After transfected with equal amount of the Shc3 plasmid and NC using Lipofectamine 3000, cells were firstly incubated at 37˚C, 5% CO_2 for 6 h then replaced with fresh ECM medium and treated with H/R for 24h.

RNA isolation and qRT-PCR analysis

The total RNA of primary trophoblast tissue and cultured cells was isolated using TRIzol (Invitrogen, USA). Then 1 μg RNA was reverse transcribed in a final volume of 20 μL cDNA (miRNA or total) using miRNA qRT-PCR Kit (Ribobio) or All-in-one RT Perfect for qPCR (Vazyme). miRNA or mRNA levels was measured with LightCycler®480 (Roche). MiR-455-5p was amplified using the following PCR conditions: 95˚C (10 minutes); 95˚C for 2 s, 60˚C for 20 s, 70˚C for 10 s (45 cycles); 95˚C for 15 s; and 60˚C for 60 s. SHC3 mRNA was amplified using the following cycles: 95˚C (30 seconds); 95˚C for 10 s, 60˚C for 30 s, 95˚C for 15 s (45 cycles); 60˚C for 60 s. The $2^{-\Delta\Delta Ct}$ method was applied for relative expression calculation. The small nuclear/nucleolar RNA U6 was used to normalize the miR-455-5p levels. β-actin was used to normalize the Shc3 levels. All primer sequences are shown in **S1 Table**.

Transwell assay

24 hours after transfection, HTR-8/SVneo were resuspended at a density of 5×10^5 cells/mL in medium and added to the upper chamber of a 24-well Transwell plate (8.0-μm inserts). For the invasion experiment, Transwell inserts were pre-coated with 60 μL of prepared Matrigel (1:9 dilution in cold medium) on the upper and incubated overnight at 37˚C. Medium with 20% FBS was used in the lower. HTR-8/SVneo were incubated for 18 h or 60 h respectively for the migration and invasion experiments.

Inserts with HTR-8/SVneo cells were fixed with 4% paraformaldehyde for 20 min and stained with Crystal Violet for 10 min. After washing and air-drying, cell images were captured with a Nikon ECLIPSE Ti-U microscope (Japan).

Apoptosis

Apoptosis was measured using flow cytometry with Annexin V-PE/7-AAD Detection Kit (Vazyme). After washing with PBS, cells were resuspended in 1 × Binding Buffer, followed by incubation with 5 μl Annexin V-PE and 7-AAD in the dark at room temperature for 10 minutes. Apoptosis of each group cells was detected by flow cytometer. 10000 cells were collected and examined for each group.

Western blot analysis

48 hours after transfection, cells were lysed with RIPA lysis buffer (Beyotime, China). Total protein of each sample was measured with BCA kit (Beyotime). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Vazyme). The proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Merck). Membranes were blocked with 5% bovine serum albumin for 1 h at room temperature. After incubating with primary antibodies (anti-SHC3 or anti-β-actin) at 4˚C overnight, the cells were incubated with secondary antibody for 1h at room temperature, Target protein signals were detected using the SuperPico ECL Chemiluminescence Kit and normalized to β-actin.

Luciferase reporter assay

Luciferase reporter plasmids (pmiR-RB-Report™, RiboBio) including wild-type or mutated Shc3 3'UTR were constructed. To identify interaction relationships of miR-455-5p and Shc3, the plasmids, together with miR-455-5p or NC mimics (NC refers to scrambled sequences), were transfected into HTR-8/SVneo cells using Lipofectamine 3000. Following 24 h incubation, the co-transfected cells were collected, and the relevant luciferase activity of each sample was evaluated according the Dual-Luciferase Reporter Kit (Vazyme), and a luminometer.

Immunohistochemistry

Tissue sections were de-paraffinized in xylene before high-temperature antigen retrieval. 3% H_2O_2 treatment at room temperature for 10 minutes was used to block endogenous peroxidases. Sections were incubated with primary Shc3 antibody (1:100, 4˚C) and secondary antibody at room temperature. After rinsing with PBS, the signal was developed with diaminobenzidine, counterstained with haematoxylin, and mounted with neutral resin, images of each section were captured with an optical microscope.

Bioinformatics analysis

Predicted target genes of miR-455-5p using TargetScan (https://www.targetscan.org/) were subsequent compared with RNA sequencing results. The search terms "preeclampsia" and "placenta" were entered into the GEO database (https://www.ncbi.nlm.nih.gov/geo/). Then raw RNA-Seq data of placental tissues and trophoblast extracellular vesicles from 13 normotensive pregnant individuals and patients with PE was obtained from GEO database (GSE190971). DESeq2 was applied for differential analysis using the R programming language (version 4.3.0). The cutoff criteria were $|\log FC| \geq 2$ & P value <0.05. Shc3 was focused on and marked out. Graphical visualisation was generated by the Xiantao tool (https://www.xiantaozi.com/).

Tube formation

After Matrigel matrix was seeded into a 96-well plate at 37˚C for 1 h, transfected EA.hy926 cells (1×10^4 cell/well) were seeded in each well for 3h and then observed using a microscope.

Image J software was applied to quantify the number of master junctions and the master segment lengths of tubular networks.

Hematoxylin-eosin (HE) staining

Placental tissues were washed with PBS and fixed in 4% paraformaldehyde for 24 h, then dehydrated and embedded with paraffin before cutting into sections (5 μm). Sections were treated in 50˚C for 60 min and dewaxed with xylene, ethanol and water. After staining with hematoxylin and eosin, sections were sealed in neutral gum and observed with an optical microscope.

Statistical analysis

All experimental results were normally distributed using the Shapiro-Wilk test ($p > 0.05$) and analysed through GraphPad Prism 8. The outcomes are presented as the means ± standard error of the mean of at least three independently repeated experiments. The two independent-sample t-test (for two groups) and one-way ANOVA (for multiple groups) followed by Bonferroni's test were used to determine the differences. P-values (p) < 0.05 were considered statistically significant.

Results

MiR-455-5p is at low levels in the PE placenta. Increased miR-455-5p levels inhibit apoptosis and promote migration and invasion of HTR-8/SVneo cells in H/R

Significant blood pressure differences were observed between healthy pregnant women and PE patients. Although the birth weight (g) in the PE group appears to be lower than that in the normotensive group, there is no statistical difference in maternal age (years), delivery (weeks), and birth weight (g) between the two groups (S2 Table).

Lower levels of miR-455-5p were detected in PE placental tissues (Fig 1A). PCR analysis of miR-455-5p levels without transfection (Control) of HTR8/SVneo, or after transfection of these cells with miR-455-5p mimic-NC, were significantly lower than transfection with a miR-455-5p mimic (Fig 1B). Compared with the miR-455-5p-NC group, miR-455-5p-mimic transfected HTR8/SVneo cells did not show altered migration or invasion under hypoxic or normoxic conditions (Fig 1C, 1D). In the hypoxia/reoxygenation condition, transfected miR-455-5p-mimic stimulated both migration and invasion of HTR8/SVneo cells compared with the miR-455-5p-mimic-NC and both 455-5p-mimic-inhibitor and 455-5p-mimic-inhibitor-NC. (Fig 1E).

Apoptosis of transfected HTR8/SVneo cells in H/R conditions showed the ratio of apoptotic cells in the miR-455-5p overexpression group (8.77%) was the lowest compared with the other groups while the inhibition group (18.11%) showed the opposite effect (Fig 1F).

Shc3 is a direct target of miR-455-5p

To determine how miR-455-5p regulates apoptosis, migration and invasion, RNA-Seq was performed to identify differentially expressed genes between miR-455-5p and miR-455-5p-NC transfected HTR8/SVneo cells. (Fig 2A). By analysis of the predicted miR-455-5p target genes in the TargetScan database, we identified one potential target gene, Shc3 (Fig 2B). The sequence of miR-455-5p is shown as are the Shc3 3'UTR wild type (WT) miRNA binding site sequence and the mutated She3 3'UTR MUT sequence. (Fig 2C).

Downregulation of Shc3 mRNA and protein by miR-455-5p was confirmed using quantitative PCR and Western blotting (Fig 2E, 2F). The control and miR-455-5p types were as

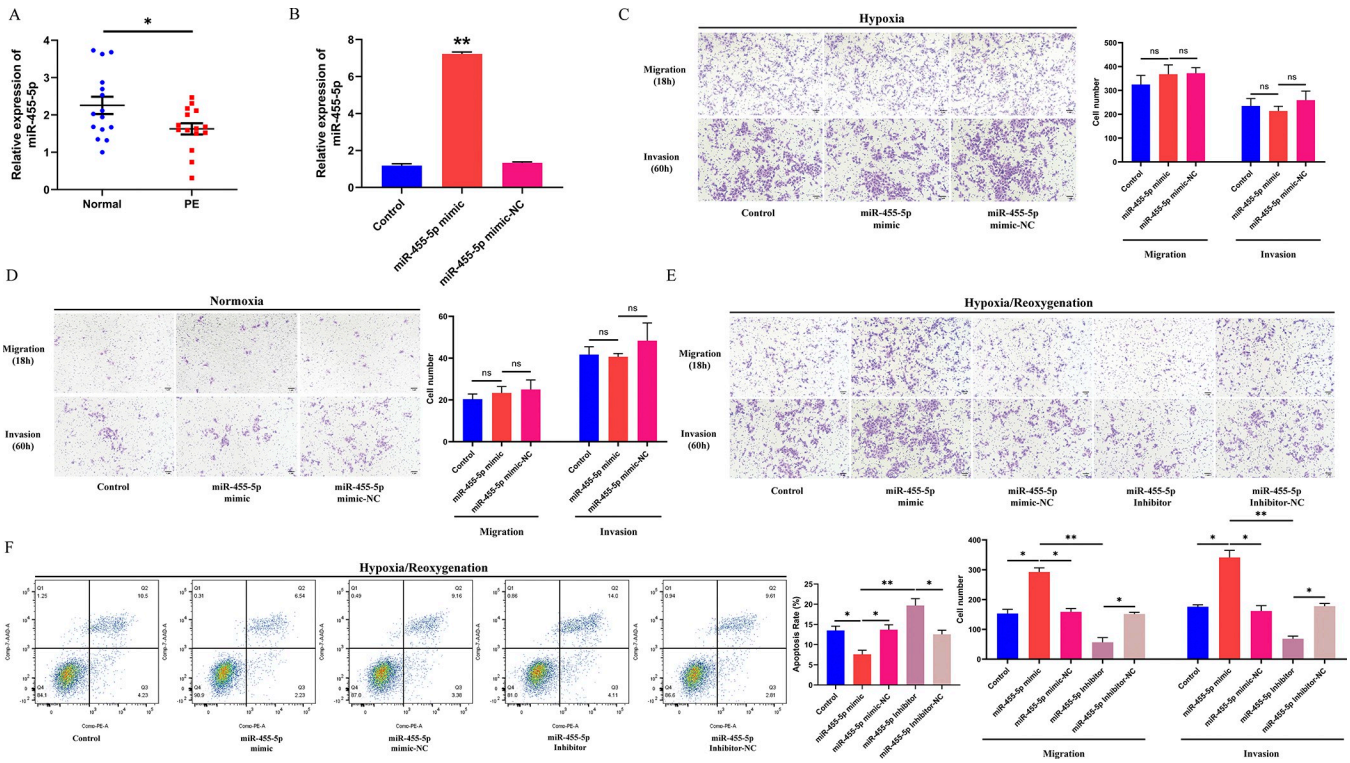

Fig 1. miR-455-5p increases HTR-8/SVneo migration and invasion and reduces apoptosis in H/R. (A) miR-455-5p levels in placental tissues of pregnant individuals both with and without PE. (B) miR-455-5p levels without transfection (Control) or after transfection of HTR8/SVneo cells with a miR-455-5p mimic and a miR-455-5p mimic-NC [miR-455-5p mimic with scrambled sequence (NC)]. (C) HTR8/SVneo migration and invasion without transfection (Control) or after transfection of HTR8/SVneo cells with a miR-455-5p mimic or a miR-455-5p mimic-NC (miR-455-5p mimic with scrambled sequence) in hypoxia. (D) As in C but in a normoxic environment. (E) as in (C) but in a hypoxic environment H/R. (F) Flow cytometry analysis of HTR8/SVneo cell without transfection (Control) or after transfection with a miR-455-5p mimic, a miR-455-5p mimic-NC, a miR-455-5p inhibitor, or a miR-455-5p inhibitor-NC in a H/R environment. Data presented indicate the average values ± standard deviation (**$p < 0.01$, *$p < 0.05$). The findings presented have been obtained through a minimum of three separate experiments.

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described in Fig 1F above. Subsequently, the luciferase reporter assay showed that luciferase activity was decreased by about 30% following miR-455-5p overexpression in HTR8/SVneo cells transfected with the reporter vector carrying wildtype Shc3 3'UTR. The mutated type Shc3 3'UTR showed no difference (Fig 2D). These data provide evidence that miR-455-5p regulates Shc3 mRNA expression by directly targeting its 3'UTR.

MiR-455-5p regulates HTR-8/SVneo cell migration, invasion and apoptosis via Shc3

To clarify whether miR-455-5p regulates the migration, invasion and apoptosis of HTR8/SVneo cells under H/R via Shc3, miR-455-5pmimic and miR-455-5p-mimic-Shc3, or miR-455-5p-mimic-Shc3NC overexpression plasmids were co-transfected into HTR-8/SVneo cells. The Transwell assay revealed that the co-transfected group (miR-455-5p-mimic-Shc3) exhibited significant inhibition of HTR8/SVneo cell migration and invasion compared with the miR-455-5p mimic group (Fig 3A). Meanwhile, the apoptosis ratio of co-culture group was the highest (23.73%) compared with other groups (Fig 3B). These results indicated that Shc3 suppressed migration and invasion, but promoted apoptosis of HTR8/SVneo cells. miR-455-5p may reverse this phenomenon by downregulating Shc3. Western blot results further

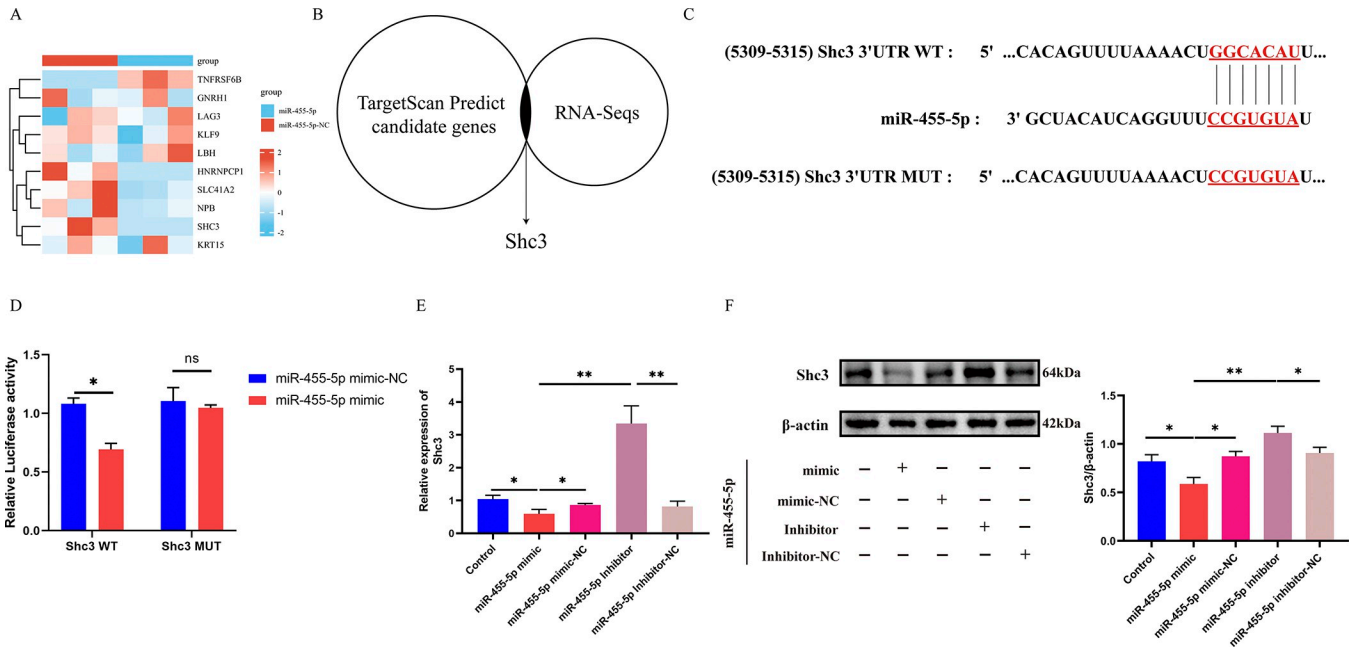

Fig 2. Shc3 is a direct target of miR-455-5p. (A) Heatmap of RNA-Seqs. (B) Selection of potential miR-455-5p downstream genes. (C) Schematic diagram of luciferase reporter-Shc3 3'UTR constructs. (D) The outcomes of miR-455-5p overexpression on the luciferase activity in HTR8/SVneo cells transfected with the WT or MUT luciferase reporter constructs. (E) The effect of miR-455-5p inhibition and overexpression on Shc3 mRNA expression in HTR8/SVneo cells. (F) The level of Shc3 protein expression after miR-455-5p inhibition and overexpression in HTR8/SVneo. Data presented shows the average values ± standard deviation (**$p < 0.01$, *$p < 0.05$). The findings presented were obtained from a minimum of three independent experiments.

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supported these results (Fig 3C). Higher than normotensive expression of Shc3 was detected in PE placental tissues by immunohistochemistry (Fig 3D).

H/R promotes Shc3 production and Shc3 may be transported outside the trophoblast cells via the extracellular vesicles

To determine how miR-455-5p regulates migration, invasion and apoptosis of HTR8/SVneo cells, specifically in H/R conditions rather than normoxic or hypoxic conditions, and whether Shc3 is involved in the process, HTR8/SVneo cells were cultured separately in normoxic, hypoxic, and H/R conditions. Western blot analysis, showed the highest of Shc3 expression in H/R conditions (Fig 4A), suggesting that the promotion of migration and invasion of HTR8/SVneo cells by miR-455-5p is effective under H/R conditions, but not under normoxic or hypoxic conditions.

In addition, analysis of the GSE190971 dataset revealed that Shc3 was highly expressed in the placental tissues and extracellular medium/large vesicles, with no low differential expression in extracellular small vesicles (Fig 4B).

Shc3 is involved in placental inflammation and inhibition of angiogenesis

Database analysis showed Shc3 was mainly enriched in placental tissue and extracellular medium/large vesicles. We found elevated Shc3 expression in PE placental tissues, which showed features of severe inflammation. Compared to the normotensive group, PE placental villi exhibited more fibrous exudates and blood vessel congestion. Also a higher number of neutrophils and lymphocytes were detected in the PE placental villi interstitium, which was not observed in normotensive placentas (Fig 4C). After successful transfection of Shc3 in the

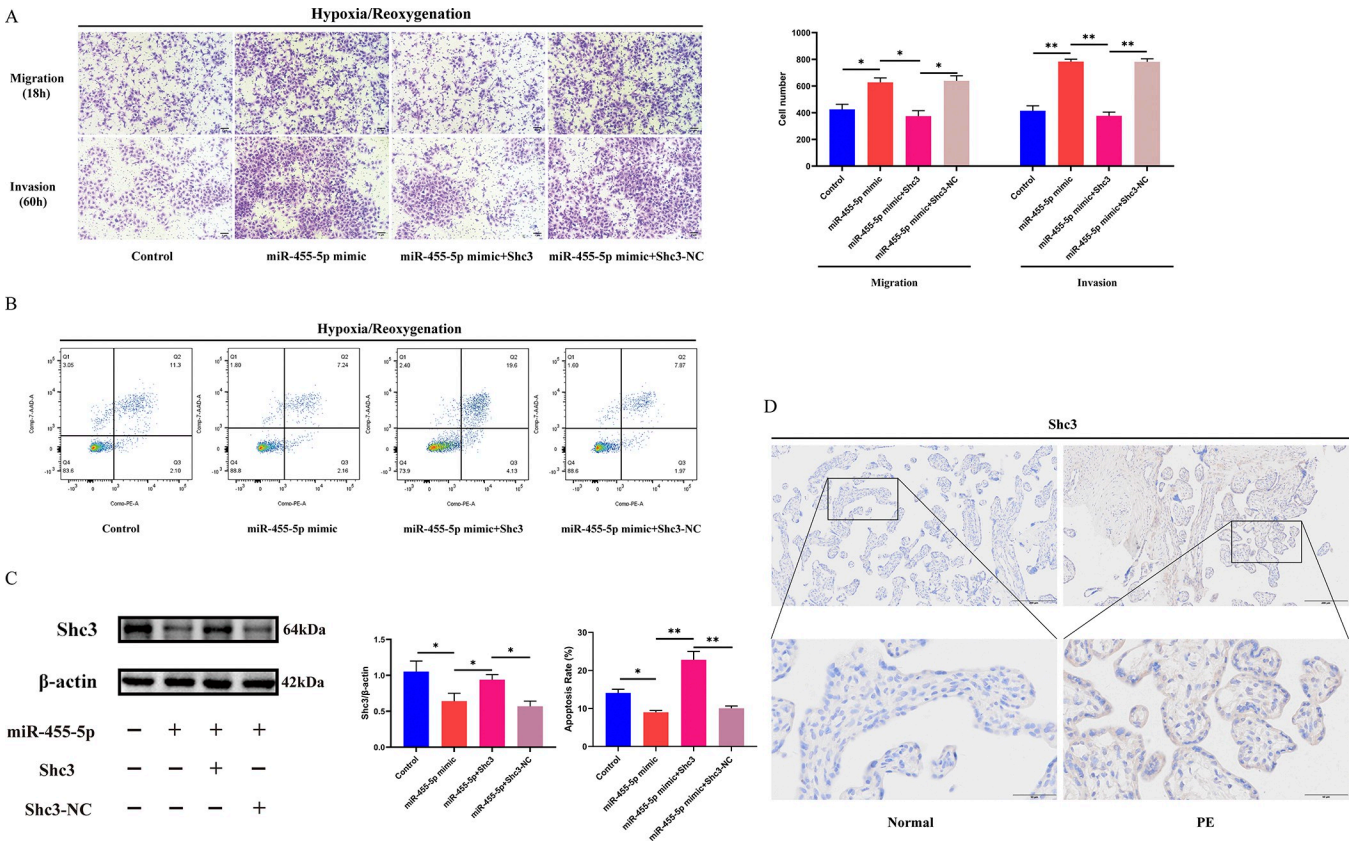

Fig 3. miR-455-5p regulates HTR-8/SVneo migration and invasion, and apoptosis by targeting Shc3. (A) The migration and invasion of HTR8/SVneo cells with miR-455-5p overexpression plasmids co-cultured with the plasmids of Shc3, or Shc3-NC in H/R. (B) Flow cytometry data of the apoptosis of HTR8/SVneo cells with miR-455-5p overexpression plasmids co-cultured with the plasmids expressing Shc3 or Shc3-NC in H/R. (C) Western blot results of Shc3 expression in HTR8/SVneo cells transfected with the miR-455-5p mimic, Shc3, or Shc3-NC. (D) The results of Shc3 expression examined by immunohistochemistry. Data presented indicate the average values ± standard deviation ($^{**}p < 0.01$, $^{*}p < 0.05$). The findings presented were obtained from a minimum of three independent experiments.

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human endothelial cell line EA.hy926 under the H/R condition (Fig 4D), angiogenesis inhibition was observed (Fig 4E).

Discussion

Placental implantation and development involve fluctuations in oxygen concentration. During early embryonic development (before the 10th week of pregnancy) [6], a low-oxygen environment helps maintain the pluripotency and differentiation potential of embryonic cells and protects against oxidative damage [16, 17]. However, as the embryo grows and undergoes organ differentiation, the maternal body must provide more oxygen for growth [18]. The counteraction of side effects caused by increased oxygen concentrations, such as an increase in reactive oxygen species (ROS), is a crucial issue that must be addressed during placental development.

In addition, during pregnancy, the foetus and placenta require a significant amount of oxygen. As blood vessels contract, placental tissues quickly experience localised hypoxia. However, once the maternal blood flow is re-established, tissue oxygenation rapidly increases [19]. These fluctuations in oxygen levels may provide the foundation for the H/R environment that affects placental trophoblasts. H/R is unavoidable during normal embryonic development and it is also a factor that contributes significantly to placental oxidative stress and pregnancy-

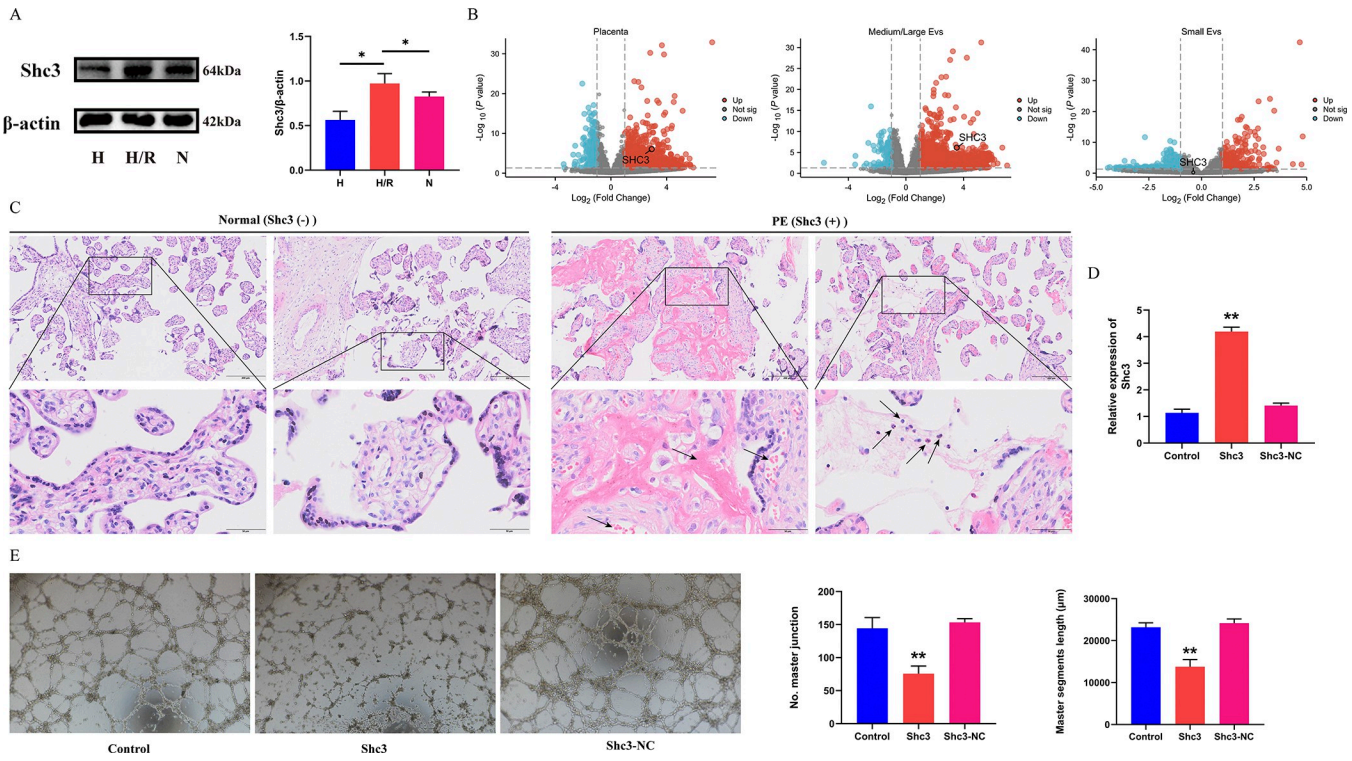

Fig 4. (A~B). H/R promotes the production of Shc3, which may be transported to the extracellular space through medium/large extracellular vesicles. (A) Western blot results of Shc3 expression in hypoxia, H/R, and normoxia, respectively. (B) Volcano plot of Shc3 mRNA expression in placental tissue, extracellular medium/large vesicles and small vesicles. (C~E). Shc3 is involved in placental inflammation and angiogenesis inhibition. (C) Hematoxylin-eosin staining results on normotensive placental tissues and high Shc3 expression PE placental tissues. (D) The level of Shc3 in EA.hy926 after transfection with the plasmids of Shc3, and Shc3-NC, in H/R compared with controls. (E) Angiogenesis of EA.hy926 after transfection with the plasmids of Shc3, and Shc3-NC in H/R, as determined by the number of master junction and master segment length.

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associated complications such as PE. How the placenta responds to H/R is not completely understood. Based on our results, we propose that the specific outcomes mentioned above depend on the duration of the H/R process. First, under normotensive physiological conditions during pregnancy, miR-455-5p is expressed at normal levels, which reduces levels of Shc3, which is a negative regulator of trophoblast cell apoptosis and invasion, in response to the H/R environment. This ensures proper placental invasion, and allows for proper progression through the H/R environment and into the next stage of pregnancy. Patients with decreased miR-455-5p in the extravillous trophoblast cells of the placenta, have insufficient miR-455-5p expression to adequately reduce Shc3 during H/R. Consequently, increased levels of Shc3 promote apoptosis and inhibits further invasion of extravillous trophoblast cells into the maternal decidua and underlying endometrium, resulting in inadequate placental invasion. This in turn, leads to subsequent adverse events such as shallow spiral artery remodelling, inadequate placental perfusion, and oxidative stress.

Shc3 (also known as N-SHC, SHCC, Rai) belongs to the Shc family (ShcA, ShcB, ShcC, and ShcD) [20], which are adaptor proteins mainly participating in various intracellular signalling pathways by binding to various receptors, including antigen receptors, G protein-coupled receptors, growth factor receptors, integrins, and cytokine receptors, particularly through the Ras/MAPK and PI3K/Akt pathways [20, 21]. Shc3 in mainly expressed in brain tissue [22], and is associated with nervous system development, brain tissue aging, cerebral ischaemia, oxidative stress, neurodegenerative diseases, and brain tumours [23]. Recent research shows that

in addition to neurological disorders, Shc3 upregulation deteriorates the progression of hepatocellular carcinoma and breast cancer [24–26]. This suggests that the high expression of Shc3 in tissues other than brain tissue in adults is likely linked to detrimental outcomes. Since PE features chronic placental ischaemia and oxidative stress at the maternal–foetal interface, similar to Shc3-mediated cerebral ischaemia and oxidative stress under pathological conditions, we believe that abnormal Shc3 elevation may be an important factor contributing to the onset of PE.

The lack of an animal model that accurately mimics all the characteristics of human PE, especially the inadequate invasion of extravillous trophoblast cells in the early developing placenta [27], means that the induction of this process in vivo is currently not feasible. Thus, in vitro human cell culture experimentation is utilized. However, there is controversy regarding the use of HTR-8/SVneo cells to conduct research into trophoblast-related diseases. For example, vimentin is not expressed in primary extravillous trophoblast cells but is expressed in HTR-8/SVneo cells [28]. However, in this study, the clinically relevant abnormal increased expression of Shc3, discovered through this cell line, mediates impaired trophoblast cell invasion in an H/R environment. This finding was supported by clinical specimens and the GSE190971 dataset, which identifies important genes in the pathogenesis of PE by combining bioinformatic analysis and experimental confirmation. Differential analysis of the GSE190971 dataset revealed that Shc3 is highly expressed in the placenta and extracellular vesicles of PE patients. These findings collectively indicate that miR-455-5p downregulation, discovered through experimental analysis of HTR-8/SVneo cells, reduces the migratory and invasive abilities of extravillous trophoblast cells, thus contributing to the onset of PE. Further support for the generality of our HTR-8/SVneo data would be to repeat the experiments using multiple extravillous trophoblast cell models such as Jar, JEG-3 and BeWo. We believed that this study is the first to investigate the mechanism of miR-455-5p downregulation in PE.

Furthermore, the analysis of dataset GSE190971 suggested a potential role for Shc3 through the medium/large extracellular vesicle pathway. Overexpressed Shc3 reduced the angiogenesis ability of EA.hy926 cells. Placentas with high Shc3 levels were associated with severe inflammatory responses, such as placental congestion and inflammatory cell infiltration, suggesting a potential association between Shc3, endothelial injury, and inflammation in PE. Notably, Shc3 is associated with the development of specific immune cells, namely T cell subsets Th1 and Th17 [29], which are closely related to PE [30–32].

The increased production of extracellular vesicles is feature of PE. Small extracellular vesicles (usually exosomes) function in both early-onset and late-onset PE, and are associated with abnormal angiogenesis, inflammation, and cell proliferation [33]. The medium/large subtype of extracellular vesicles includes microvesicles, shedding bodies, ectosomes, apoptotic bodies, and microparticles with diameters exceeding 200nm [34], are implicated in these processes. Given Shc3's role in mediating apoptosis of HTR8/SVneo under H/R, we speculate that the medium/large extracellular vesicles may correspond to apoptotic bodies. Confirmation studies on the nature of the medium/large extracellular vesicles and elucidation of the mechanisms underlying Shc3's involvement in abnormal angiogenesis and inflammation are planned for future research endeavors.

In conclusion, our findings indicate that under H/R conditions, miR-455-5p in placental extravillous trophoblast cells target the 3'UTR of Shc3, leading to its degradation. This degradation process helps maintain the normal migratory and invasive capacity of extravillous trophoblasts, ensuring proper placental implantation, invasion into the maternal decidua and underlying endometrium and spiral artery remodelling. However, if miR-455-5p expression is reduced in extravillous trophoblasts under H/R conditions, Shc3 is poorly degraded. As a result, Shc3 increases in extravillous trophoblast cells, promoting cellular apoptosis, which

suppresses their migration and invasion. This ultimately leads to shallow placental implantation and invasion and the onset of PE. In addition, Shc3 may be transported to the extracellular space by the medium/large extracellular vesicle, which may influence angiogenesis and inflammation in PE. Thus, Shc3 serves as a potential biomarker for PE. Considering the authenticity of biomarker status, the level of Shc3 may change with the gestational age, and it is particularly important to track the level of Shc3 in PE patients at different gestational ages, which will be a focus of future studies. Our findings provide new insights and potential biomarkers for the diagnosis and treatment of PE.

Supporting information

S1 Table. Primer sequences.
(DOCX)

S2 Table. Clinical information of the collected patients. Clinical information of the healthy pregnant women and PE patients, $^{**}p < 0.01$.
(DOCX)

S1 Graphical abstract.
(TIF)

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