Novel lncRNA-HZ04 Promotes BPDE-Induced Human Trophoblast Cell Apoptosis and Miscarriage by Up-Regulating IP3R1/CaMKII/SGCB Pathway by Competitively Binding with miR-hz04

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Research

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Abstract

Background

Miscarriage seriously hinders human reproduction. BaP (benzo(a)pyrene) and its metabolite BPDE (benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide) could cause trophoblast cell dysfunctions and might further induce human miscarriage. However, the underlying mechanisms remain largely unknown.

Results

Herein, we identified a novel lnc-HZ04 up-regulated and a novel miR-hz04 down-regulated in the unexplained recurrent miscarriage (RM) villous tissues relative to the healthy control tissues and also in the BPDE-exposed trophoblast cells. lnc-HZ04 served as competing endogenous RNA (ceRNA), directly and specifically bound with miR-hz04 on its target site, and diminished the inhibition effects of miR-hz04 on IP_3R_1 mRNA expression level and its mRNA stability, thus activated Ca^{2+} -mediated IP_3R_1 /p-CaMKII/SGCB apoptosis pathway, which further promoted trophoblast cell apoptosis. The miR-hz04 target site, but not its mutant, on lnc-HZ04 played important roles in these regulations. In normal trophoblast, relatively less lnc-HZ04 and more miR-hz04 inhibited this apoptosis pathway and gave normal pregnancy. After exposure to BPDE or in the RM tissues, relatively more lnc-HZ04 and less miR-hz04 activated this apoptosis pathway and induced miscarriage. BaP could also induce mice miscarriage by up-regulating this corresponding murine apoptosis pathway.

Conclusions

This work discovered that lnc-HZ04 served as a ceRNA for miR-hz04 and up-regulated IP_3R_1 /CaMKII/SGCB pathway, which promoted the BPDE-induced human trophoblast cell apoptosis and the occurrence of miscarriage, providing novel scientific and clinical understanding in the occurrence of unexplained miscarriage.

Background

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in our daily environment. It was reported that the total PAHs on atmospheric fine particulate matter (PM) samples were 5.60-16.3, 3.02-7.75 and 0.658-3.44 ng m⁻³ in Oporto, Florence, and Athens in Southern European, respectively [Science of the Total Environment 595 (2017) 494–504], and up to 174 ng m⁻³ in a suburban area of Zhengzhou, China [Atmospheric Environment 104 (2015) 228e236]. High levels of benzo(a)pyrene (BaP), a representative of PAHs, have been detected out on environmental PM and also in fried or smoked food. The total BaP intake was estimated to be 125 ng/person/day based on the daily food consumption and BaP level [1]. Moreover, the lipophilicity and long half-life of BaP further increase its accumulation in body.

Approximately 15%-25% of total pregnant women experience miscarriage [2], and roughly 3% suffer from recurrent miscarriage (RM, two or more consecutive miscarriages) [3]. In general, chromosomal

abnormalities, hormonal abnormalities, uterine deformation, psychological trauma, infection, stressful life events, and immune disorders are considered as the known causes for RM [4, 5]. However, there are still approximately 50% RM unexplained [6]. Thus, it is urgent to identify the causes for the unexplained RM and explore their pathogenesis and regulation mechanisms.

Increasing epidemiological studies have indicated that exposure of PAHs is associated with miscarriage [7, 8, 9]. Female smokers have significantly higher levels of BaP (1.32 ± 0.68 ng/ml) in their follicular fluid compared with their non-smoking (0.03 ± 0.01 ng/ml) counterparts [10]. Higher levels of BaP were found in the follicular fluid of women who were not pregnant (1.79 ± 0.03 ng/ml) compared with those that achieved a pregnancy (0.08 ± 0.03 ng/ml) [11]. BaP can be metabolized to BPDE (benzo(a)pyren-7,8-dihydrodiol-9,10-epoxide), which further form BPDE-DNA adducts [12]. A case-control study of miscarriage has shown that high levels of BPDE-DNA adducts in human maternal blood is correlated with the high risk of miscarriage during early pregnancy [13]. In animal studies, after treatment with BaP, the uterus morphology becomes abnormal, the number of germ cells and the fertility rates decrease, and the miscarriage rates increase [14] [15]. All these evidences show that BaP could induce miscarriage. However, the underlying mechanisms remain largely unknown.

Extravillous trophoblast cells take important roles in embryo implantation and the formation of functional placenta [16]. Trophoblast cell apoptosis results in abnormal placental development and miscarriage [17] [18]. A higher degree of trophoblast cell apoptosis has been found in the tissues of the miscarriage patients [19]. On the other hand, human trophoblast cells are very sensitive to environmental carcinogens. Recently, we have found that BPDE could result in human trophoblast Swan 71 cell apoptosis due to the disorders of mitochondrial fission/fusion [20]. However, the detailed regulation mechanisms are still poorly understood.

Intracellular Ca^{2+} plays important roles in the regulation of a variety of cellular processes, such as cell cycle, apoptosis, and migration [21] [22]. Recent studies have shown that inositol trisphosphate receptor type 1 (IP_3R_1)-mediated Ca^{2+} release increases the cytosolic free Ca^{2+} level, which induces miscarriage in rat [23]. Ca^{2+} binds to calmodulin, which activates the calmodulin-dependent protein kinase (CaMK), increases the CaMKII phosphorylation [24], and then activates guanylyl cyclases (SGC), which triggers a wide range of signaling pathways, including mitochondrial damage and cell apoptosis [25]. Thus, $\text{IP}_3\text{R}/\text{Ca}^{2+}/\text{CaMKII}/\text{SGCB}$ pathway could regulate cell apoptosis. However, whether this pathway is also involved in the regulation of human trophoblast cell apoptosis is completely unknown.

Long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) are emerging to play significant roles in the epigenetic regulation of trophoblasts cell functions and miscarriage [26, 27, 28, 29, 30, 31]. These lncRNAs or miRNAs are differentially expressed in the RM villous tissues relative to those in the health control tissues, and they regulate the signaling pathways that are related with the occurrence of miscarriage [32, 33]. lncRNAs generally regulate miRNAs by taking the roles of competing endogenous RNAs (ceRNAs) [34]. In this mechanism, lncRNA and mRNA, which harbor the same miRNA binding site, could mutually and negatively regulate each other's expression levels by competitively binding with this

miRNA [35]. Reversely, miRNAs could also bind to their target lncRNAs to promote their degradation [34]. However, there are still a large number of unidentified non-coding RNAs. The combined roles of lncRNA and miRNA in the regulation of miscarriage were poorly reported. Moreover, the non-coding RNAs that could simultaneously regulate the occurrence of miscarriage and the BPDE-induced human trophoblast cell apoptosis have never been reported.

In this work, we expected to correlate the occurrence of miscarriage and the BPDE-induced human trophoblast cell apoptosis, to find out novel non-coding RNAs and signaling pathway that are simultaneously differentially regulated in both the RM tissues and the BPDE-treated trophoblast cells, to explore the roles of these non-coding RNAs in the regulation of this pathway in both the RM tissues and the BPDE-treated trophoblast cells, to discover new mechanisms underlying BPDE-induced miscarriage due to human trophoblast cell apoptosis, and to provide novel scientific and clinical understanding in the occurrence of miscarriage.

Materials And Methods

Chemicals

Benzo[a]pyren-7, 8-dihydrodiol-9, 10-epoxide (BPDE) was from MRIGlobal (purity 99.9%, MRIGlobal, Kansas City, Missouri, USA). Anhydrous DMSO, benzo[a]pyrene (BaP, purity 99.9%), corn oil, and actinomycin D were from Sigma-Aldrich (St. Louis, MO, USA). BPDE was dissolved in DMSO and stored at -80 °C. All the vehicle control and the treated cultures contained the identical amount of DMSO (0.03%, v/v).

Cell culture

Human trophoblast Swan 71 cells are immortalized by human telomerase, which were constructed by Gil Mor group at Yale University [36]. Human trophoblast Swan 71 and HTR-8/SVneo cells were cultured in DMEM/F12 medium (GIBCO, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, GIBCO), MEM Non-Essential Amino Acids Solution (GIBCO), and 100 mM Sodium Pyruvate Solution (GIBCO) at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO₂.

Tissues collection and statement

Fifteen patients with RM (the RM group) in the age between 25 and 30 were recruited, who were treated at the West China Fourth Hospital (Sichuan Province, China) between March and December in 2019. All patients had two or more consecutive unexplained miscarriage. Any patient with one of the following features was excluded [37]: (1) autoimmune abnormality, luteal phase defects, hyperprolactinemia, antiphospholipid antibody syndrome, or hyperandrogenemia; (2) uterine abnormalities or cervical incompetence; (3) abnormal karyotype of the parents or abortus; (4) polycystic ovarian syndrome; (5) the symptoms of endocrine or metabolic diseases (e.g., diabetes, hyperthyroidism, and hypothyroidism); (6) viral infectious disease (e.g., syphilis, gonorrhea, AIDS, tuberculosis); (7) tuberculosis, HBV, HCV, HIV, or with positive results from γ -interferon release tests; and (8) eclampsia or pre-eclampsia. Additionally,

fifteen women in the matched age between 25 and 30 were recruited as the healthy control (the HC group), who had undergone artificial miscarriages to terminate unwanted pregnancies at 6-10 weeks of gestation. All of the HC group have previous pregnancies and lacked any of the eight features as described above. The villous tissue samples were dissected from other abortus materials from these two groups at 6-10 weeks of gestation, serially washed, and immediately frozen in liquid nitrogen. RNA and proteins were extracted from these tissue samples and used for RT-qPCR (15 pairs) and western blot (10 random pairs) analysis, respectively. The relative levels of two biomolecules in each RM or HC sample were linearly correlated using SPSS 24.0 software (SPSS Inc, Chicago, IL, USA). The research protocols were approved by the Medical Ethics Committee of the West China Fourth Hospital, Sichuan University. Written informed consents were obtained from all the participants.

High-throughput RNA sequencing

Total RNA was extracted from the Swan 71 cells treated with 0, 0.5, or 1.5 μ M BPDE for 24 h. High-throughput RNA sequencing was conducted on the HiSeq 2000 sequencing platform (BGI-Shenzhen, Shenzhen, China) according to BGI commercial standard process (<https://www.bgi.com/>). The raw sequencing data are analyzed by bioinformatics developed by BGI. Differentially expressed miRNAs, lncRNAs, and mRNAs were searched in the NCBI database (<http://www.ncbi.nlm.nih.gov/>) to determine their genome loci. The target genes of miR-hz04 were predicted by RNA22 (<https://cm.jefferson.edu/rna22/Interactive/>) [38].

5' and 3' rapid amplification of cDNA ends (RACE) assays

The 5'- and 3'-RACE assays were performed using the SMARTer RACE 5'/3' Kit (Takara Bio, Tokyo, Japan). Total RNAs were extracted using the RNeasy Micro Kit (Bio-Rad, Hercules, CA, USA) and treated with DNase. RNA integrity was assessed using the Agilent 4200 TapeStation system. After reverse transcription of total RNA to cDNA, 5'- and 3'-RACE PCRs were performed using the universal and gene-specific primers listed in Table S3. The resulting PCR products were cloned into linearized pRACE vector and were sequenced. The full-length nucleotide sequence of lnc-HZ04 was shown in Fig. S1C.

Cell transfection

SiRNAs of lncRNA-HZ04 (si-HZ04), siRNAs of IP₃R₁ (si-IP₃R₁) [39], miRNA-hz04 mimics (miR-hz04 mimics), miRNA-hz04 inhibitor (miR-hz04 inhibitor), and their negative control (NC) were synthesized by Thermo Fisher Scientific Company (Waltham, MA, USA) and their sequences were shown in Tables S5. The siRNA sequences are specific to the target sequence and the NC sequences do not recognize any other sequences by searching against the human genome using BLAST. Plasmid with overexpression of lnc-HZ04 (pcDNA3.1-HZ04-wt/mut) or IP₃R₁ mRNA (pcDNA3.1-IP₃R₁-wt/mut) containing wild-type (wt) or mutant (mut) target site for miR-hz04 was synthesized (Addgene, Watertown, MA, USA). Empty pcDNA3.1 was used as negative control (Vector). Before transfection, trophoblast cells were seeded in 6-well plates and incubated in starvation medium (0.1% or 1% FBS/DMEM) for 24 h. Then, cells were transiently transfected with 50 nM miRNA (siRNA, mimics, inhibitor, or its NC) or 2.0 μ g plasmid (pcDNA3.1-HZ04,

pcDNA3.1-IP₃R₁, or Vector) per well and incubated for 24 h in Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. After 24 h, cells were harvested and the knockdown or overexpression efficiencies were validated by RT-qPCR analysis.

Cell apoptosis analysis

Swan 71 cells, which have been exposed to BPDE (0, 0.25, 0.5, 1.0, or 1.5 μ M) for 24 h, or transfected with various miRNA or plasmid for 24 h, were harvested and treated with a commercial AnnexinV-FITC/Propidium Iodide (PI) Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The cells were immediately detected by flow cytometry (BD Accuri C6, Becton-Dickinson, Franklin Lakes, NJ, USA) and the data was analyzed by FlowJo software (TreeStar, San Carlos, CA, USA).

Measurement of the intracellular Ca²⁺ concentrations

After exposure to BPDE (0, 0.25, 0.5, 1.0, or 1.5 μ M) or transfection with various miRNA or plasmid for 24 h, Swan 71 cells were harvested and their intracellular Ca²⁺ concentrations were detected using Cell Ca²⁺ Kit (Invitrogen) according to the manufacturer's instructions. The fluorescence intensity corresponding to Ca²⁺ concentration in Swan 71 cells was detected on ECLIPSE Ti fluorescence microscope (Nikon), and the data was analyzed by Image J software. Ca²⁺ concentrations were expressed as the change folds of the fluorescence intensity of the treated cells relative to that of the control cells.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen). RNA quality and concentration were measured with a NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, mRNA and lncRNA were reversely transcribed into cDNA using the Prime Script RT Reagent Kit with gDNA Eraser (Takara). MiRNA was transcribed into cDNA using microRNA First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative detection was performed using an iQ5 real-time detection system (Bio-Rad Laboratories) and SYBRVR Premix Ex TaqTM II (Bio-Rad Laboratories). The reaction conditions were 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 65 °C. The expression levels of RNA were calculated using the 2^{- $\Delta\Delta$ Ct} method. GAPDH mRNA was used as the normalization internal standard for lncRNA and mRNA; and U6 was used for miRNA. The primers used in these RT-qPCR assays were listed in Table S6.

Western blot analysis

Total proteins were extracted from cells or tissues using RIPA lysis buffer (Thermo Fisher Scientific) according to the manufacturer's instructions. The protein concentrations in lysates were detected by BCA Kit (Thermo Fisher Scientific). The equal weight of proteins were separated by 6%-12% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories). After blocking with 5% BSA in TBST buffer containing 20 mM Tris-HCl (pH = 7.5 at 25 °C), 150 mM NaCl, and 0.1% Tween-20 for 1 h at 25 °C, the membranes were incubated in the primary antibody in blocking solution overnight at 4 °C. After wash

thrice in TBST for 15 min, the membranes were incubated with the secondary antibody for 1 h at 25 °C. Immunoblots were visualized using enhanced chemiluminescence (Thermo Fisher Scientific) by Molecular Imager ChemiDoc XRS+ imaging system (Bio-Rad Laboratories) and the integrated density values were analyzed with Image J software with GAPDH or β -Tublin as the internal standard. The primary antibodies contained anti-IP₃R₁ (dilution 1:1000, ab264281, Abcam, Cambridge, UK), anti-CaMKII (dilution 1:1000, 4436S, Cell Signaling Technology, Boston, USA), anti-p-CaMKII (dilution 1:1000, 3356S, Cell Signaling Technology), anti-SGCB (dilution 1:100, sc-5306, SANTA Cruz, Texas, USA), anti-GAPDH (dilution 1:10000, ab8245, Abcam). The secondary antibodies included goat anti-rabbit IgG (dilution 1:5000, ab205718, Abcam) and goat anti-mouse IgG (dilution 1:5000, ab205719, Abcam). Blotting for GAPDH was used throughout as a loading control. The intensity of the western blot bands was quantified by Image J.

Fluorescence in situ hybridization (FISH)

FISH assays were performed using Invitrogen™ FISH Tag™ Detection Kits (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, Swan 71 cells were rinsed with PBS and then fixed in 4% formaldehyde for 10 min at 25 °C. The cells were permeabilized in PBS containing 0.5% Triton X-100 on ice for 10 min, and then washed with PBS. Subsequently, Inc-HZ04 was hybridized with Cy5-labelled ssDNA probe (5'-Cy5-GGCTXTTGCCTGTGGTCTCTTCTTCG-3', X means locked nucleotide acid modification) (Empire Genomics, Williamsville, NK, USA) at 37 °C for 12 h. After staining nucleus with DAPI, images were digitally captured by confocal microscopy (N-STORM+A1R, Nikon, Japan) at 600 × magnification. Nuclei with ≥ 5 foci was considered positive [40] and the relative levels of Inc-hz04 in the nucleus and cytoplasm were calculated from 20 random cells.

mRNA stability assays

The *de novo* RNA synthesis was blocked by addition of 5 μ g/mL ActD (Apexbio, Madison, WI, USA) to Swan 71 or HTR-8/SVneo cells with overexpression or knockdown of Inc-HZ04 and/or miR-hz04. The total RNA was extracted after 0, 1, 2, or 3 h, and the mRNA levels of Inc-HZ04 or IP₃R₁ were detected by RT-qPCR. GAPDH was used as the normalization internal standard. Expression levels were calculated using the Δ Ct method by normalizing to the 0 h time point.

Dual-Luciferase reporter assays

Lnc-HZ04 and IP₃R₁ mRNA containing wild-type (wt) or mutant (mut) binding site for miR-hz04 were generated and fused into the luciferase pGL3-basic reporter vector (Promega, Madison, USA). Swan 71 cells were seeded into 6-well plates and then co-transfected with 100 ng pmirGLO-HZ04-wt/-mut, or pmirGLO-IP₃R₁-wt/-mut and 200 nM miR-hz04 mimics or control in Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After transfection, the firefly luciferase activity in each well was measured using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Ago2 RNA immunoprecipitation (RIP) assays

Ago2 RIP assays were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, cell lysates of Swan 71 cells or Swan 71 cells transfected with pcDNA3.1-HZ04, si-HZ04, pcDNA3.1-IP₃R₁, or si-IP₃R₁ were incubated with magnetic beads conjugated with antibody of human Ago2 (dilution 1:50, ab186733, Abcam) or mouse IgG (dilution 1:5000, ab190475, Abcam) as negative control at 4 °C. The immunoprecipitated RNAs were extracted and their enrichments on Ago2 or IgG were detected by RT-qPCR.

MS2-RIP assays

We co-transfected pcDNA3.1-MS2, pcDNA3.1-MS2-HZ04-wt/-mut, or pcDNA3.1-MS2-IP₃R₁-wt/-mut along with pMS2-GFP (Addgene) into Swan 71 cells or Swan 71 cells with overexpression or knockdown of wt IP₃R₁ mRNA using Viafect reagent. After 48 h, cell lysate was obtained and used for RNA immunoprecipitation (RIP) experiments using GFP antibody (dilution 1:100, ab290, Abcam) and the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) at 4 °C according to the manufacturer's instructions. The immunoprecipitated RNAs were extracted and the enrichments of miR-hz04 and miR-502-3p, which was used as negative control, by lnc-HZ04 or IP₃R₁ mRNA were detected by RT-qPCR. The primers used for detection of miR-hz04 or miR-502-3p were listed in Table S6.

RNA immunoprecipitation (RIP) assays

RNA immunoprecipitation (RIP) experiments were carried out using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Briefly, cells lysates were incubated with protein A+G magnetic beads at 4 °C overnight. The beads conjugated antibody of IP₃R₁ (dilution 1:100, ab264281, Abcam), CaMKII (dilution 1:100, sc-5306, SANTA Cruz), p-CaMKII (dilution 1:100, sc-32289, SANTA Cruz), SGCB (dilution 1:100, sc-393679, SANTA Cruz), or non-immune species matched IgG (Abcam). The lysate was also used as pre-immunoprecipitation input. After extensive wash, the immunoprecipitated RNA was extracted. lnc-HZ04 and negative control GAPDH mRNA were quantified by RT-qPCR, with the pre-immunoprecipitation as input and the IgG as control. The primers used in these assays were listed in Table S6.

In vitro RNA pull-down assays

lnc-HZ04 or IP₃R₁ mRNA containing wild-type (wt) or mutant (mut) target site for miR-hz04 was transcribed *in vitro* from pGEM-T-HZ04-wt/-mut or pGEM-T-IP₃R₁-wt/-mut, respectively. The vector pGEM-T was used as the negative control. All the transcripts were biotin-labeled by T7 RNA polymerase with the Biotin RNA Labeling Mix (Roche), treated with RNase-free DNase I (Roche), and then purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). These transcripts were then incubated with the lysates of Swan 71 cells or Swan 71 cells with overexpression or knockdown of wild-type lnc-HZ04 or IP₃R₁ mRNA

overnight at 4 °C. The complexes were isolated by streptavidin agarose beads (Invitrogen) and RNA in the pull-down material was detected by RT-qPCR. The relative retention was calculated against the input RNA level.

Mice and experimental design

C57BL/6 mice with age between 6 and 8 weeks (Charles River, Beijing, China) were raised in a filter-top cages with a 12 h light/dark cycle, autoclaved bedding, food, and water. Female mice were mated with male mice overnight. The appearance of vaginal plug was defined as day 1 (D1). Pregnant mice were randomly divided into three groups. Each group (n = 9) were dosed daily with corn oil (control) or 0.05 or 0.2 mg/kg BaP in corn oil by oral gavage from D1 to D13 [41] [42]. The pregnant mice were euthanized on D14. The embryo resorption was identified by smaller or darker appearance than the viable and pink healthy embryos [43]. The embryo resorption rate for each mouse was calculated as the number of the embryo resorption divided by the total number of embryos (the resorption and healthy embryos). The average resorption rate in each group was calculated. A random placenta was collected from each of nine mice in each group. RNA and proteins were extracted from each placenta tissue for RT-qPCR (each n = 9 mice) and western blot (each n = 6 random mice) analysis, respectively. The relative levels of two biomolecules in each mouse placenta tissue were linearly correlated using SPSS 20.0 software. The research protocols were approved by the Medical Ethics Committee of the West China Fourth Hospital, Sichuan University.

Statistical analysis

Each experiment was repeated independently thrice with similar results and the data were presented as mean $\pm$ SD (standard deviation). Statistical analysis was performed using SPSS 24.0 software (SPSS Inc, Chicago, IL, USA). Data are analyzed using independent-samples Student's test between two groups and one-way analysis of variance for more groups with Dunnett's or LSD post hoc test. The correlation was analyzed by pearson analysis. All graphs were prepared using GraphPad Prism version 8.0. Differences were considered significant when $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

Results

A novel lnc-HZ04 and IP₃R₁/p-CaMKII/SGCB pathway were up-regulated and a novel miR-hz04 was down-regulated in both trophoblast cells treated with BPDE and human RM tissues

In our recent work, we have found that BPDE exposure could induce human trophoblast Swan 71 cell apoptosis [20]. Then, we further explored the roles of novel non-coding RNAs in the regulation of trophoblast cell apoptosis. Based on the occupational exposure, diffusion of BaP from environment into cells, and high accumulation of BaP metabolite in follicular fluid and serum, we still used 0-1.5 μ M BPDE to treat human trophoblast Swan 71 or HTR-8/SVneo cells [20] [44]. Swan 71 cells treated with 0, 0.5, or 1.5 μ M BPDE were used for transcriptome sequencing to identify the differentially expressed mRNAs, novel lncRNAs, and novel miRNAs. In the lncRNA sequencing data, total 223 novel differentially

expressed lncRNAs were identified, all of which were not annotated in NCBI database. After selecting lncRNAs with expression level differences > 2-fold and p values < 0.05 in both 0 vs 0.5 and 0.5 vs 1.5 groups, 22 were monotonously increased and 6 were monotonously decreased with BPDE concentrations. Then, these 28 novel lncRNAs were validated by RT-qPCR in the untreated or 0.5 μ M BPDE-treated Swan 71 cells (Fig. S1A). In this work, we selected the novel lnc-14628 and investigated its roles in the regulation of trophoblast cell functions in details.

This lncRNA was further identified to be up-regulated in the BPDE-treated Swan 71 or HTR-8/SVneo cells by RT-qPCR analysis (Fig. 1A, B). It was verified as a sense transcript with a full length of 318 nucleotides (nt) by rapid amplification of cDNA ends (RACE) assays (Fig. S1B, Table S1) and resided at chromosome 16 (chr 16: 3,170,699 - 3,171,016, NC_000016.10). Thus, this lncRNA was also termed as lnc-HZ04 and submitted to NCBI with No. MT821846. The protein-coding potential of lnc-HZ04 was very weak, as predicted by Coding Potential Assessment Tool (<https://lilab.research.bcm.edu/cpat/index.php>) and NCBI ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) [45]. Additionally, lnc-HZ04 was predicted to have no conserved domains using Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/cdd/>) and Pfam (<http://pfam.xfam.org/>) [46, 47]. The data supported that lnc-HZ04 could not encode a protein. lnc-HZ04 was distributed to both the cytoplasm and nucleus in Swan 71 cells, as determined by fluorescent in situ hybridization (FISH) (Fig. S1C, D).

BPDE could induce trophoblast cell apoptosis [20]. The apoptosis of Swan 71 cells was significantly increased after BPDE treatment (Fig. S1E), similar to our previous results [20]. It has been reported that IP_3R_1 is an important protein that regulates apoptosis of ovarian carcinoma cells [48] and also regulates calcium homeostasis in Purkinje cells [49]. In our mRNA sequencing data, we also found that the mRNA level of IP_3R_1 was significantly monotonously increased with BPDE concentrations, with expression level differences > 2-fold and p values < 0.05 in both 0 vs 0.5 and 0.5 vs 1.5 groups (Fig. S1F). This up-regulation was further confirmed by RT-qPCR in the BPDE-treated Swan 71 and HTR-8/SVneo cells (Fig. 1C,D). In signaling pathway, the mRNA and protein levels of CaMKII, p-CaMKII, and SGCB, which were located downstream of IP_3R_1 , were all up-regulated in the BPDE-treated Swan 71 and HTR-8/SVneo cells (Fig. S1G, H; 1E). Since Ca^{2+} is required to activate this pathway [24, 25], the cellular Ca^{2+} level in Swan 71 cells was determined. It was found that the Ca^{2+} level was increased with BPDE concentrations (Fig. S1I). Therefore, BPDE may induce trophoblast cell apoptosis by activating the cellular Ca^{2+} -mediated IP_3R_1 /p-CaMKII/SGCB signaling pathway.

Sequence alignment using NCBI Blast showed that no regions in lnc-HZ04 sequence could be complementary with the sense or antisense strand mRNA of IP_3R_1 , CaMKII, or SGCB. Additionally, lnc-HZ04 is located on chr16, distinct from that of IP_3R_1 in chr3, CaMKII in chr10, and SGCB in chr4, ruling out the possible *cis* regulation of IP_3R_1 , CaMKII, or SGCB expression by lnc-HZ04. Moreover, the interactions of lnc-HZ04 with the mRNA or protein of IP_3R_1 , CaMKII, or SGCB were very weak, as indicated by NCBI Blast and *catRAPID* (http://service.tartagialab.com/page/catrapid_group) [50, 51]. Furthermore, RIP assays also confirmed that none of IP_3R_1 , CaMKII, p-CaMKII, or SGCB protein could interact with lnc-

HZ04 (Fig. S1J). Therefore, these results suggested that lnc-HZ04 seemed not to directly interact with the mRNA or protein of IP₃R₁/p-CaMKII/SGCB signaling pathway. Since IP₃R₁ was located upstream of this signaling pathway, we tried to seek for some novel miRNAs that might interact with both lnc-HZ04 and IP₃R₁ mRNA.

In the miRNA sequencing data, total 44 novel differentially expressed miRNAs were identified, all of which were not annotated in NCBI database. After selecting the miRNAs with expression level differences > 2-fold and p values < 0.05 in both 0 vs 0.5 and 0.5 vs 1.5 groups, four were monotonously increased and nine were monotonously decreased with BPDE concentrations. Then, these 13 miRNAs were validated by RT-qPCR in the untreated or 0.5 µM BPDE-treated Swan 71 cells (Fig. S1K). Among these candidates, a novel miR-hz04, which has been submitted to NCBI with No. MW192196, might interact with both lnc-HZ04 and IP₃R₁ mRNA because both of which harbored the binding site for miR-hz04 (Fig. 1F), as predicted by RNA22 (<https://cm.jefferson.edu/rna22/Interactive/>) [38]. MiR-hz04 was further confirmed to be down-regulated in the BPDE-treated Swan 71 and HTR-8/SVneo cells (Fig. 1G, H). Thus, lnc-HZ04 and miR-hz04 might be involved in the up-regulation of IP₃R₁/p-CaMKII/SGCB apoptosis pathway in the BPDE-treated trophoblast cells.

It has been reported that the apoptosis of human trophoblast cells could lead to miscarriage [18]. In order to correlate the occurrence of unexplained miscarriage with the BPDE-induced human trophoblast cell apoptosis, and also to verify whether the regulation mechanisms identified in trophoblast cells were also consistent with those in the villous tissues, we collected the villous tissue samples from the unexplained RM and healthy control (HC) groups (each n = 15). The RM group had experienced two or more consecutive unexplained miscarriage, and the known causes, such as chromosomal abnormalities, hormonal abnormalities, uterine deformation, have been excluded. Additionally, the parameters of the RM and HC groups, such as age, body mass index, menopause days, had no significant differences (Table S2). Then, the expression levels of lnc-HZ04 and this IP₃R₁/p-CaMKII/SGCB signaling pathway were detected in the HC and RM tissues. RT-qPCR and western blot analysis showed that lnc-HZ04, IP₃R₁, CaMKII, p-CaMKII, and SGCB were all significantly up-regulated but miR-hz04 was down-regulated in the RM tissues relative to the healthy control tissues (Fig. 1I-N). These changes were completely agreed with those found in the BPDE-treated trophoblast cells, suggesting that both the RM tissues and the BPDE-treated trophoblast cells may share the similar regulation mechanism, in which lnc-HZ04 and miR-hz04 might be involved in the regulation of IP₃R₁/p-CaMKII/SGCB apoptosis pathway in both the RM tissues and BPDE-treated trophoblast cells.

Up-regulated lnc-HZ04 activated Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB pathway to induce cell apoptosis in the BPDE-treated trophoblast cells and the human RM tissues

It has been identified that lnc-HZ04 was up-regulated in both the RM tissues and the BPDE-treated trophoblast cells (Fig. 1A, B, I). Then, the roles of lnc-HZ04 in the regulation of trophoblast cell apoptosis were explored. lnc-HZ04 was overexpressed by transfecting pcDNA3.1-HZ04 and silenced by

transfecting siRNA-HZ04 (si-HZ04) in the untreated or 0.5 μ M BPDE-treated Swan 71 or HTR-8/SVneo cells, and the efficiencies were validated by RT-qPCR analysis (Fig. S2A, B). Overexpression of Inc-HZ04 increased, whereas knockdown of Inc-HZ04 inhibited, the apoptosis of the untreated or BPDE-treated Swan 71 cells (Fig. 2A, B; Fig. S2C, D), showing that Inc-HZ04 promoted trophoblast cell apoptosis. In pathway, overexpression of Inc-HZ04 up-regulated, whereas knockdown of Inc-HZ04 down-regulated, the mRNA and protein levels of IP₃R₁, CaMKII, p-CaMKII, and SGCB in the untreated or BPDE-treated Swan 71 or HTR-8/SVneo cells (Fig. 2C-J; S2E, F). Moreover, overexpression of Inc-HZ04 also increased, whereas knockdown of Inc-HZ04 decreased, the cellular Ca²⁺ level in both the untreated and BPDE-treated Swan 71 cells (Fig. 2K, L; Fig. S2G, H). Thus, the up-regulated Inc-HZ04 activated Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB signaling pathway and promoted cell apoptosis of the untreated or BPDE-treated trophoblast cells.

The apoptosis of human trophoblast cells could lead to miscarriage [18]. Then, Inc-HZ04 and this IP₃R₁/p-CaMKII/SGCB pathway were detected in the villous tissues. It was found that the RNA and protein levels of Inc-HZ04, IP₃R₁, CaMKII, p-CaMKII, and SGCB were all up-regulated in the RM tissues relative to those in the HC tissues (Fig. 1I-N). After normalization of their relative levels, IP₃R₁, CaMKII, p-CaMKII, and SGCB were all positively and linearly correlated with Inc-HZ04 in the RM tissues (Fig. 2M, N; S2I-M). The data points were highly dispersed in the RM group but were relatively compressed in the HC group. The locations of data points in both groups were obviously separated, manifesting that these molecules were differentially regulated. The correlations among Inc-HZ04, IP₃R₁, p-CaMKII, and SGCB in the RM tissues were similar to those found in the BPDE-treated trophoblast cells, suggesting that both cases may share the similar regulation mechanism in which the up-regulated Inc-HZ04 activated the IP₃R₁/p-CaMKII/SGCB pathway.

Down-regulated miR-hz04 activated Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB pathway to induce trophoblast cell apoptosis in the BPDE-treated trophoblast cells and the human RM tissues

Since miR-hz04 was down-regulated in both the RM tissues and the BPDE-treated trophoblast cells (Fig. 1G, H, M), the roles of miR-hz04 in the regulation of trophoblast cell apoptosis were explored. MiR-hz04 was overexpressed by transfecting its mimics and silenced by transfecting its inhibitor in both the untreated and 0.5 μ M BPDE-treated Swan 71 or HTR-8/SVneo cells, and their efficiencies were validated by RT-qPCR analysis (Fig. S3A, B). Overexpression of miR-hz04 inhibited, whereas knockdown of miR-hz04 increased, cell apoptosis in either the untreated or BPDE-treated Swan 71 cells (Fig. 3A, B; S3C, D). For the signaling pathway, overexpression of miR-hz04 reduced, whereas knockdown of miR-hz04 increased, the mRNA and protein levels of IP₃R₁, CaMKII, p-CaMKII, and SGCB in the untreated or BPDE-treated Swan 71 or HTR-8/SVneo cells (Fig. 3C-F; S3E-H). Overexpression of miR-hz04 also decreased the cellular Ca²⁺ level; and vice versa (Fig. 3G, H; S3I, J). Taken together, the down-regulated miR-hz04 activated Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB pathway and induced cell apoptosis in the untreated and BPDE-treated trophoblast cells.

The similar regulations were also observed in the RM tissues. MiR-hz04 was down-regulated but the IP₃R₁/p-CaMKII/SGCB pathway was up-regulated in the RM tissues relative to those in the HC tissues (Fig. 1J-N). After normalization of their relative levels, IP₃R₁, CaMKII, p-CaMKII, and SGCB were all negatively and linearly correlated with miR-hz04 in the RM tissues but were almost constant and independent of miR-hz04 levels in the HC tissues (Fig. 3I, J; S3K-O). The locations of data points in both groups were relatively separated. These results were similar to those found in the BPDE-treated trophoblast cells. Therefore, the RM tissues and the BPDE-treated trophoblast cells may share the similar mechanism in which the down-regulated miR-hz04 activated Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB pathway and induced trophoblast cell apoptosis.

Lnc-HZ04 acted as a ceRNA for miR-hz04 and up-regulated IP₃R₁ expression level to inhibit trophoblast cell apoptosis

Since miR-hz04 might base pair with lnc-HZ04 or IP₃R₁ mRNA, we hypothesized that lnc-HZ04 in the cytoplasm might function as a ceRNA for miR-hz04 and abolishes miR-hz04-inhibited IP₃R₁ expression level. Firstly, overexpression of lnc-HZ04 reduced, whereas knockdown of lnc-HZ04 increased, the expression level of miR-hz04 in Swan 71 and HTR-8/SVneo cells (Fig. 4A, B; S4A), supporting that lnc-HZ04 negatively regulated miR-hz04 level in human trophoblast cells. lnc-HZ04 overexpression up-regulated the mRNA and protein levels of IP₃R₁, an effect which could be diminished by overexpressing miR-hz04 in either Swan 71 or HTR-8/SVneo cells (Fig. 4C-F; S4B). In contrast, knockdown of miR-hz04 abolished the reduction in the mRNA and protein levels of IP₃R₁ caused by knockdown of lnc-HZ04 (Fig. 4C-F; S4B). All these results supported that lnc-HZ04 functioned as a ceRNA for miR-hz04 and diminished the inhibition effects of miR-hz04 on IP₃R₁ expression level.

To further explore how lnc-HZ04 and miR-hz04 regulated IP₃R₁ mRNA level, the mRNA stability of IP₃R₁ was determined after blockage of mRNA transcription by adding actinomycin D. Overexpression of lnc-HZ04 enhanced IP₃R₁ mRNA stability in Swan 71 and HTR-8/SVneo cells; and overexpression of miR-hz04 diminished this enhancement effect (Fig. 4G, H). Knockdown of lnc-HZ04 reduced IP₃R₁ mRNA stability, which could be abolished by silencing miR-hz04 in both cells (Fig. 4J, K). As control, the stability of GAPDH mRNA was not changed after alteration of lnc-HZ04 and/or miR-hz04 (Fig. S4C-F). For cell phenotype, the ability of lnc-HZ04 to increase cell apoptosis was abolished by overexpressing miR-hz04 (Fig. 4I; S4G). Knockdown of lnc-HZ04 reduced cell apoptosis, which was diminished by silencing miR-hz04 (Fig. 4L; S4H). Taken together, lnc-HZ04 acted as a ceRNA for miR-hz04 and diminished the inhibitory effects of miR-hz04 on IP₃R₁ expression level and mRNA stability, and finally induced trophoblast cell apoptosis.

Lnc-HZ04 up-regulated IP₃R₁ level by direct and specific binding of miR-hz04 on its binding site on lnc-HZ04

The potential interactions of miR-hz04 with lnc-HZ04 or IP₃R₁ mRNA were directly determined using lnc-HZ04 or IP₃R₁ mRNA containing wild-type (wt) or mutant (mut) target site for miR-hz04 (Fig. 5A). Dual luciferase assays showed that miR-hz04 could directly interact with its target site on lnc-HZ04 or IP₃R₁ mRNA, but not with its mutant (Fig. 5B, C), confirming that miR-hz04 could interact with lnc-HZ04 or IP₃R₁ mRNA by direct complementary binding onto its target site.

Next, Ago2 RIP assays showed that both lnc-HZ04 and IP₃R₁ mRNA could bind with Ago2 protein but not with IgG control (Fig. S5A, B), showing that both RNAs could interact with miRNA. MS2-RIP assays further confirmed that the endogenous miR-hz04 was enriched by lnc-HZ04 or IP₃R₁ mRNA containing wt target site for miR-hz04 but not by its mutant or the empty vector (Fig. 5D-F). MiR-502-3p [52], which lacks the sequence that might be complementary with lnc-HZ04 or IP₃R₁ mRNA, could not be pull down by either wt or mut lnc-HZ04 or IP₃R₁ mRNA. Subsequently, RNA pull-down experiments provided more solid evidence. MiR-hz04 could be pull down by biotin-labelled wt lnc-HZ04 or IP₃R₁ mRNA, but not by their mutant or the bead with empty vector (Fig. 5G-I). MiR-502-3p as negative control could not be pull down by wt or mut lnc-HZ04 or IP₃R₁ mRNA. Taken together, these results confirmed that miR-hz04 could directly interact with lnc-HZ04 or IP₃R₁ mRNA by directly binding onto its target site.

Furthermore, the competitive binding of miR-hz04 with lnc-HZ04 or IP₃R₁ mRNA was determined. Ago2 RIP assays showed that overexpression of lnc-HZ04 increased its enrichment by Ago2 protein but decreased the enrichment of IP₃R₁ mRNA (Fig. 5J). In contrast, knockdown of lnc-HZ04 reduced its enrichment by Ago2 but increased the enrichment of IP₃R₁ mRNA (Fig. 5K). Similarly, overexpression of IP₃R₁ decreased, where knockdown of IP₃R₁ increased, the enrichment of lnc-HZ04 by Ago2 protein (Fig. 5L, M). Moreover, RNA pull-down assays further showed that miR-hz04, which was enriched by wt lnc-HZ04 but not by its mutant, was reduced after overexpression of IP₃R₁ mRNA and increased after knockdown of IP₃R₁ mRNA (Fig. 5N, O). Similarly, miR-hz04, enriched by wt IP₃R₁ mRNA, was reduced after overexpression of lnc-HZ04 and increased after knockdown of lnc-HZ04 (Fig. 5P, Q). Taken together, all these results supported that lnc-HZ04 and IP₃R₁ mRNA competitively bound with miR-hz04 by direct binding miR-hz04 on its target site.

Finally, the effects of miR-hz04 binding site at lnc-HZ04 on the mRNA and protein levels of IP₃R₁ were further explored. Overexpression of wt lnc-HZ04, but not its mutant or empty vector, could up-regulate the mRNA and protein levels of IP₃R₁ in both Swan 71 and HTR-8/SVneo cells (Fig. 5R-T). Moreover, co-transfection of mutant lnc-HZ04 and miR-hz04 abolished the promotion effects contributed by wt lnc-HZ04 and showed only the inhibitory effects of miR-hz04 on the expression levels of IP₃R₁ (Fig. 5R-T). Taken together, these results showed that this miR-hz04 target site at lnc-HZ04 played crucial roles in the regulation of IP₃R₁ expression level in human trophoblast cells.

MiR-hz04 down-regulated the level of lnc-HZ04 in human trophoblast cells

Unexpectedly, we also found that miR-hz04 could regulate lnc-HZ04 level. lnc-HZ04 was markedly down-regulated in miR-hz04-overexpressed Swan 71 cells and up-regulated in miR-hz04-silenced cells (Fig. S5C, D). To further explore this inhibitory effects, the RNA stability of lnc-HZ04 was determined after blockage of RNA transcription. Overexpression of miR-hz04 reduced lnc-HZ04 RNA stability (Fig. S5E); whereas knockdown of miR-hz04 enhanced its stability (Fig. S5F). As control, GAPDH mRNA stability was not changed by miR-hz04 (Fig. S5G, H). Taken together, the results demonstrated that miR-hz04 down-regulated lnc-HZ04 level by reducing its RNA stability.

In tissues, lnc-HZ04 was negatively and linearly correlated with miR-hz04 in the RM tissues, similar to that found in the untreated or BPDE-treated trophoblast cells (Fig. S5I). However, the level of lnc-HZ04 was relatively constant and independent of miR-hz04 level in the HC tissues. The locations of data points in both groups were obviously separated, manifesting that these non-coding RNAs were differently regulated in both groups and they could be considered as miscarriage biomarkers. Taken together, the RM tissues and trophoblast cells may share the similar regulation mechanism in which lnc-HZ04 acted as a ceRNA for miR-hz04 and diminished the miR-hz04-inhibited IP_3R_1 /p-CaMKII/SGCB pathway.

Down-regulated miR-hz04 activated murine IP_3R_1 /Camk2g/Sgcb pathway in pregnant mice with BaP-induced miscarriage

Environmental BaP exposure might be one of the causes for the unexplained miscarriage. Then, a mouse model was made in which pregnant mice were treated with 0, 0.05, or 0.2 mg/kg BaP to induce miscarriage [41] [42]. BaP could be metabolized to ultimately carcinogenic BPDE in mice. The uteri were collected from every mouse in each group. The obvious embryo resorption [43] was observed in the BaP-treated mice (Fig. 6A) and the embryo resorption rates were increased in the BaP-treated mice group (Fig. 6B), supporting that exposure of BaP to pregnant mice resulted in miscarriage [53] [54]. IP_3R_1 , CaMKII, and SGCB are conserved in human, rhesus, elephants, dogs, and mice (Fig. S6A-C, Table S4), showing that this apoptosis pathway is evolutionarily conserved among these species. However, lnc-HZ04 sequence is conserved in human and rhesus, but not in mice (Fig. S6D), indicating its specific regulation roles in human. The levels of miR-hz04 were reduced (Fig. 6C), whereas those of murine IP_3R_1 , Camk2g, and Sgcb were all increased (Fig. 6D-G), in the BaP-treated mice group. After normalization of their relative levels, IP_3R_1 , Camk2g, p-Camk2g, and Sgcb were all negatively and linearly correlated with miR-hz04 in the placenta tissues in the BaP-treated mice (Fig. 6H, I; S6E-I). The locations of data points in both groups were obviously separated, showing that this pathway was differentially regulated in both groups and these molecules could be considered as miscarriage biomarkers. All these correlations in the BaP-treated mice were similar to those found in human trophoblast cells and the human RM tissues, indicating that the three cases may share the similar pathway and regulation mechanism: the down-regulated miR-hz04 activated IP_3R_1 /p-CaMKII/SGCB pathway and increased trophoblast cell apoptosis, finally induced miscarriage after BPDE/BaP exposure.

Discussion

It has been reported that some lncRNAs or miRNAs could regulate the occurrence of miscarriage, such as lncRNA MEG8 [27], HOTAIR [26], lnc-SLC4A1-1 [29], miR-98 [31]. However, all these non-coding RNAs were identified from the tissues of RM patients who have already miscarried. The dysfunctions of BPDE-exposed human trophoblast cells and the occurrence of unexplained miscarriage have never been correlated and their underlying mechanisms have never been explored. In this study, we identified a novel up-regulated lnc-HZ04 and a novel down-regulated miR-hz04 in both the BPDE-exposed trophoblast cells and the RM tissues, providing new explanation for the unexplained miscarriage: environmental BaP could induce miscarriage by increasing trophoblast cell apoptosis by up-regulating IP₃R₁/p-CaMKII/SGCB pathway mediated by a novel lnc-HZ04 and a novel miR-hz04.

The detailed regulation mechanisms of lnc-HZ04 and miR-hz04 were proposed (Fig. 6J). MiR-hz04 directly binds to its target site at IP₃R₁ mRNA and promotes IP₃R₁ mRNA degradation. lnc-HZ04 acts as a ceRNA for miR-hz04, directly and competitively binds with miR-hz04, and diminishes the miR-hz04-inhibited IP₃R₁ RNA stability and expression level, thus up-regulating Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB apoptosis pathway, which promotes trophoblast cell apoptosis. In normal trophoblast cells, lnc-HZ04 is down-regulated and miR-hz04 is up-regulated, which inactivates IP₃R₁/p-CaMKII/SGCB pathway and inhibits trophoblast cell apoptosis, giving normal pregnancy. If trophoblast cells were exposed to BPDE or in the RM villous tissues, lnc-HZ04 is up-regulated and miR-hz04 is down-regulated, and thus IP₃R₁/p-CaMKII/SGCB pathway is activated to result in trophoblast apoptosis and then induce miscarriage. In pregnant mice with BaP-induced miscarriage, the changes in murine miR-hz04 and IP₃R₁/p-CaMKII/SGCB are consistent with those found in the BPDE-treated trophoblast cells and the RM tissues, suggesting that environmental BaP could induce miscarriage by up-regulating the Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB apoptosis pathway. In human system, an extra lnc-HZ04 is involved in the epigenetic regulation of this pathway, showing more complicated regulation network in human system than that in mice.

It has been demonstrated that lncRNAs generally act as ceRNAs to rescue the miRNAs-inhibited target mRNA levels and up-regulate the mRNA-related cellular functions [55, 56]. For example, by acting as an endogenous "sponge", lncRNA-HULC down-regulates miR-hz0472 to reduce the translational repression of its target gene PRKACB, which induces phosphorylation of CREB and modulates self-regulation in hepatocellular cancer [57]. Additionally, lncRNA-ATB up-regulates ZEB1 and ZEB2 by competitively binding to the miR-200 family and then induces EMT and invasion in hepatocellular carcinoma [14, 58]. On the contrary, miRNAs can also regulate the expression levels of lncRNAs [59]. MiR-150 negatively modulates the expression of lncRNA-MIAT to suppress the proliferation, migration, and tube formation of human microvascular endothelial cells [60]. In this work, we revealed that lnc-HZ04 functions as a ceRNA for miR-hz04, directly interacts with miR-hz04, and further reduces miR-hz04 level. At the same time, miR-hz04 also down-regulates lnc-HZ04 level by promoting lnc-HZ04 degradation. Finally, lnc-HZ04 diminishes the miR-hz04-inhibited the mRNA expression level of IP₃R₁ and promotes trophoblast cell apoptosis.

IP₃/Ca²⁺/CaMK signaling pathway has been reported to modulate cell proliferation of cholangiocytes [61]. Ca²⁺/CaMK signaling cascade could modulate the expression and exon selection of IP₃R₁ and contributes the migration and synaptic refinement in granule cells from rat cerebellar cortex [62]. The downstream CaMKII and sGC were also reported to inhibit cell cycle progression in the prefrontal cortex of mice [63] and to participate in spinal reflex potentiation in anesthetized rats [64]. In this work, we revealed that the up-regulated IP₃R₁ could activate cellular Ca²⁺-mediated CaMKII/SGCB pathway to induce trophoblast apoptosis in the BPDE-treated trophoblast cells and in human RM villous tissues. The biomolecules in this pathway, as well as the intracellular free Ca²⁺ ion, might serve as miscarriage biomarkers.

It should be stressed that it is the trophoblast cell apoptosis but not BPDE that directly induces miscarriage. Once the IP₃R₁/p-CaMKII/SGCB apoptosis pathway is activated, the upstream BPDE would become less important for the downstream miscarriage. More broadly, not only BaP or BPDE, but also other factors or pathways, such as cadmium [65] or hexavalent chromium (Cr^{VI}) [66], could also induce human trophoblast apoptosis, and possibly induce miscarriage. Therefore, BPDE is only one of the upstream environmental factors that could induce trophoblast cell apoptosis, and it is the trophoblast cell apoptosis that directly induces miscarriage.

Conclusions

In this work, we have identified novel lnc-HZ04 which acts as a ceRNA for novel miR-hz0 and promotes trophoblast cell apoptosis by up-regulating Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB signaling pathway, finally resulting in miscarriage. This pathway serves as a bridge to connect the BPDE-induced trophoblast cell apoptosis and the occurrence of miscarriage, providing novel scientific and clinical understanding in the occurrence of miscarriage.

Declarations

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Authors' contributions

W.H., M.D., and H.Z. designed the study. W.H. and M.D. performed most of the experiments. W.H., M.D., and H.Z. wrote the draft manuscript. C.M. and T.L. were responsible for animal experiments. T.Q. and Z.X. contributed to the tissue preparation. J.X., W.C. and P.T. conducted the remaining experiments.

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Availability of data and materials

All data and materials are included in the manuscript, Figures, and the supplementary materials.

Ethical Approval and Consent to participate

The research protocols about villous tissue assays were approved by the Medical Ethics Committee of the West China Fourth Hospital, Sichuan University. Written informed consents were obtained from all the participants. The research protocols about mice assays were approved by the Medical Ethics Committee of the West China Fourth Hospital, Sichuan University.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Fig.1

Figure 1

Lnc-HZ04 and IP₃R₁/p-CaMKII/SGCB pathway were up-regulated and miR-hz04 was down-regulated in both the BPDE-exposed human trophoblast cells and the human RM tissues (A and B) RT-qPCR analysis of the level of Lnc-HZ04 in the BPDE-treated Swan 71 (A) or HTR-8/SVneo (B) cells. (C and D) RT-qPCR analysis of the mRNA level of IP₃R₁ in the BPDE-treated Swan 71 (C) or HTR-8/SVneo (D) cells. (E) Western blot analysis of the protein levels of IP₃R₁, CaMKII, p-CaMKII, and SGCB in the BPDE-treated

Swan 71 or HTR-8/SVneo cells, with GAPDH as internal standard. The relative intensity of each band was quantified. (F) The complementary sequences of miR-hz04 and its binding site on Inc-HZ04 and IP3R1 mRNA. (G and H) RT-qPCR analysis of the level of miR-hz04 in the BPDE-treated Swan 71 (G) or HTR-8/SVneo (H) cells. (I-M) RT-qPCR analysis of the RNA levels of Inc-HZ04 (I), IP3R1 (J), CaMKII (K), SGCB (L), and miR-hz04 (M) in the HC and RM tissues (each n = 15). (N) Western blot analysis of the protein levels of IP3R1, CaMKII, p-CaMKII, and SGCB in the HC and RM tissues (each n = 10). The relative intensity of each band was quantified. Representative data in (E, I-N) represent three independent experiments. Data in (A-D, G-H) show mean $\pm$ SD of three independent experiments. Two-tailed Student's t-test for (I-M); one-way ANOVA analysis for (A-D, G-H); *p < 0.05, **p < 0.01, and ***p < 0.001. RM, recurrent miscarriage group; HC, healthy control group; n, the number of biologically independent samples.

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Fig.2

Figure 2

Up-regulated Inc-HZ04 activated Ca^{2+} -mediated IP_3R_1 /p-CaMKII/SGCB pathway and induced cell apoptosis in the BPDE-exposed trophoblast cells and the human RM tissues. (A and B) Flow cytometry analysis of the apoptosis rate of the untreated or 0.5 μ M BPDE-treated Swan 71 cells with overexpression (A) or knockdown (B) of Inc-HZ04. (C-H) RT-qPCR analysis of the mRNA levels of IP_3R_1 (C, F), CaMKII (D, G), and SGCB (E, H) in the untreated or 0.5 μ M BPDE-treated Swan 71 cells with overexpression (C-E) or

knockdown (F-H) of lnc-HZ04. (I and J) Western blot analysis of the protein levels of IP3R1, CaMKII, p-CaMKII, and SGCB in the untreated or 0.5 μ M BPDE-treated Swan 71 (I) or HTR-8/SVneo (J) cells with overexpression or knockdown of lnc-HZ04. (K and L) The intracellular Ca²⁺ level in the untreated or 0.5 μ M BPDE-treated Swan 71 cells with overexpression (K) or knockdown (L) of lnc-HZ04. (M and N) The correlation between the mRNA (M, each n = 15) or protein (N, each n = 10) levels of IP3R1 and the levels of lnc-HZ04 in the HC (blue) and RM (red) groups. Representative data in (I-J, M-N) represent three independent experiments. Data in (A-H, K-L) show mean $\pm$ SD of three independent experiments. One-way ANOVA analysis for (A-H, K-L); pearson analysis for (M-N); *p < 0.05, **p < 0.01, and ***p < 0.001. RM, recurrent miscarriage group; HC, healthy control group; NC, negative control of siRNA; Vector, empty vector of pcDNA3.1; pcDNA3.1-HZ04, overexpression of lnc-HZ04; si-HZ04, knockdown of lnc-HZ04; n, the number of biologically independent samples.

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Fig.3

Figure 3

Down-regulated miR-hz04 activated Ca²⁺-mediated IP₃R₁/p-CaMKII/SGCB pathway and induced cell apoptosis in the BPDE-treated trophoblast cells and the human RM tissues. (A and B) Flow cytometry analysis of the apoptosis of the untreated or 0.5 μ M BPDE-treated Swan 71 cells transfected with miR-hz04 mimics (A) or miR-hz04 inhibitor (B). (C and D) RT-qPCR analysis of the mRNA level of IP₃R₁ in the untreated or 0.5 μ M BPDE-treated Swan 71 (C) or HTR-8/SVneo (D) cells transfected with miR-hz04

mimics or inhibitor. (E and F) Western blot analysis of the protein levels of IP3R1, CaMKII, p-CaMKII, and SGCB in the untreated or 0.5 μ M BPDE-treated Swan 71 (E) or HTR-8/SVneo (F) cells transfected with miR-hz04 mimics or inhibitor. (G and H) The intracellular Ca²⁺ level in the untreated or 0.5 μ M BPDE-treated Swan 71 cells with overexpression (G) or knockdown (H) of miR-hz04. (I and J) The correlation between the mRNA (I, each n = 15) or protein (J, each n = 10) levels of IP3R1 and the levels of miR-hz04 in the HC (blue) and RM (red) tissues. Representative data in (E-F, I-J) represent three independent experiments. Data in (A-D, G-H) show mean $\pm$ SD of three independent experiments. One-way ANOVA analysis for (A-D, G-H); pearson analysis for (I-J); *p < 0.05, **p < 0.01, and ***p < 0.001. RM, recurrent miscarriage group; HC, healthy control group; mimics NC, negative control of mimics; inhibitor NC, negative control of inhibitor; miR-hz04 mimics, overexpression of miR-hz04; miR-hz04 inhibitor, knockdown of miR-hz04; n, the number of biologically independent samples.

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Fig.4

Figure 4

Lnc-HZ04 functioned as a ceRNA for miR-hz04, up-regulated IP3R1 expression level, and promoted trophoblast cell apoptosis (A and B) RT-qPCR analysis of miR-hz04 expression level in Swan 71 cells with overexpression (A) or knockdown (B) of Lnc-HZ04. (C and D) The mRNA level of IP3R1 in Swan 71 cells with overexpression (C) or knockdown (D) of Lnc-HZ04 and/or miR-hz04. (E and F) The protein level of IP3R1 in Swan 71 (E) and HTR-8/SVneo (F) cells with overexpression or knockdown of Lnc-HZ04 and/or

miR-hz04. (G and H, J and K) The stability of IP3R1 mRNA in Swan 71 (G and J) and HTR-8/SVneo (H and K) cells with overexpression or knockdown of lnc-HZ04 and/or miR-hz04. (I and L) Flow cytometry analysis of the apoptosis of Swan 71 cells with overexpression (I) or knockdown (L) of lnc-HZ04 and/or miR-hz04. Representative data in (E-F) represent three independent experiments. Data in (A-D, G-L) show mean $\pm$ SD of three independent experiments. One-way ANOVA analysis for (A-D, I, L); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NC, negative control of siRNA; Vector or Vec, empty vector of pcDNA3.1; mimics NC or mim-NC, negative control of mimics; inhibitor NC or inh-NC, negative control of inhibitor; pcDNA3.1-HZ04 or OE-HZ04, overexpression of lnc-HZ04; miR-hz04 mimics or mim-hz04, overexpression of miR-hz04; si-HZ04, knockdown of lnc-HZ04; miR-hz04 inhibitor or inh-hz04, knockdown of miR-hz04.

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miR-hz04. (G and H, J and K) The stability of IP3R1 mRNA in Swan 71 (G and J) and HTR-8/SVneo (H and K) cells with overexpression or knockdown of lnc-HZ04 and/or miR-hz04. (I and L) Flow cytometry analysis of the apoptosis of Swan 71 cells with overexpression (I) or knockdown (L) of lnc-HZ04 and/or miR-hz04. Representative data in (E-F) represent three independent experiments. Data in (A-D, G-L) show mean $\pm$ SD of three independent experiments. One-way ANOVA analysis for (A-D, I, L); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NC, negative control of siRNA; Vector or Vec, empty vector of pcDNA3.1; mimics NC or mim-NC, negative control of mimics; inhibitor NC or inh-NC, negative control of inhibitor; pcDNA3.1-HZ04 or OE-HZ04, overexpression of lnc-HZ04; miR-hz04 mimics or mim-hz04, overexpression of miR-hz04; si-HZ04, knockdown of lnc-HZ04; miR-hz04 inhibitor or inh-hz04, knockdown of miR-hz04.

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miR-hz04. (G and H, J and K) The stability of IP3R1 mRNA in Swan 71 (G and J) and HTR-8/SVneo (H and K) cells with overexpression or knockdown of lnc-HZ04 and/or miR-hz04. (I and L) Flow cytometry analysis of the apoptosis of Swan 71 cells with overexpression (I) or knockdown (L) of lnc-HZ04 and/or miR-hz04. Representative data in (E-F) represent three independent experiments. Data in (A-D, G-L) show mean $\pm$ SD of three independent experiments. One-way ANOVA analysis for (A-D, I, L); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NC, negative control of siRNA; Vector or Vec, empty vector of pcDNA3.1; mimics NC or mim-NC, negative control of mimics; inhibitor NC or inh-NC, negative control of inhibitor; pcDNA3.1-HZ04 or OE-HZ04, overexpression of lnc-HZ04; miR-hz04 mimics or mim-hz04, overexpression of miR-hz04; si-HZ04, knockdown of lnc-HZ04; miR-hz04 inhibitor or inh-hz04, knockdown of miR-hz04.

Fig.5

Figure 5

Lnc-HZ04 up-regulated IP3R1 level by direct and specific binding of miR-hz04 on its binding site on Lnc-HZ04 (A-C) PmirGLO vector containing Lnc-HZ04 (B) or IP3R1 mRNA (C) sequence with wild-type (wt) or mutant (mut) binding site for miR-hz04 together with miR-hz04 were co-transfected into Swan 71 cells for dual luciferase detection. (D-F) RT-qPCR analysis of the level of miR-hz04 that was associated with Lnc-HZ04 or IP3R1 mRNA containing wt or mut miR-hz04 binding site in MS2-RIP assays using Swan 71

cells, with miR-502-3p as negative control. (G-I) RT-qPCR analysis of the level of miR-hz04 pull down by biotinylated lnc-HZ04 and IP3R1 mRNA containing wt or mut miR-hz04 binding site in RNA pull-down assays using Swan 71 cells, with miR-502-3p as negative control. (J to M) RT-qPCR analysis of the level of lnc-HZ04 or IP3R1 mRNA enriched by Ago2 protein using Swan 71 cells with overexpression or knockdown of lnc-HZ04 (J and K) or IP3R1 mRNA (L and M) in Ago2 RIP assays. (N-Q) RT-qPCR analysis of the level of miR-hz04 enriched by wt or mut lnc-HZ04 (N and O) or IP3R1 mRNA (P and Q) in Swan 71 cells with overexpression or knockdown of IP3R1 mRNA (N and O) or lnc-HZ04 (P and Q), respectively. (R-S) RT-qPCR analysis of IP3R1 mRNA level in Swan 71 (R) or HTR-8/SVneo (H) cells with overexpression of wt or mut lnc-HZ04 and miR-hz04 mimics or mimics NC. (T) Western blot analysis of the protein level of IP3R1 in Swan 71 or HTR-8/SVneo cells with overexpression of wt or mut lnc-HZ04 and miR-hz04 mimics or mimics NC. Representative data in (T) represent three independent experiments. Data in (B-C, E-F, H-S) show mean $\pm$ SD of three independent experiments. Two-tailed Student's t-test for (B-C, J-M); one-way ANOVA analysis for (E-F, H-I, N-S); ns, non-significance; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NC, negative control of siRNA; Vector, empty vector of pcDNA3.1; mimics NC, negative control of mimics; inhibitor NC, negative control of inhibitor; pcDNA3.1-HZ04, overexpression of lnc-HZ04; pcDNA3.1-IP3R1, overexpression of IP3R1 mRNA; si-HZ04, knockdown of lnc-HZ04; si-IP3R1, knockdown of IP3R1 mRNA; miR-hz04 mimics, overexpression of miR-hz04; miR-hz04 inhibitor, knockdown of miR-hz04.

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Lnc-HZ04 up-regulated IP3R1 level by direct and specific binding of miR-hz04 on its binding site on Inc-HZ04 (A-C) PmirGLO vector containing Inc-HZ04 (B) or IP3R1 mRNA (C) sequence with wild-type (wt) or mutant (mut) binding site for miR-hz04 together with miR-hz04 were co-transfected into Swan 71 cells for dual luciferase detection. (D-F) RT-qPCR analysis of the level of miR-hz04 that was associated with Inc-HZ04 or IP3R1 mRNA containing wt or mut miR-hz04 binding site in MS2-RIP assays using Swan 71

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Fig.6

Figure 6

Murine miR-hz04 was down-regulated and IP3R1/p-Camk2g /Sgcb pathway was up-regulated in pregnant mice model with BaP-induced miscarriage (A) Embryo resorption (red arrows) in pregnant mice model with BaP-induced miscarriage. (B) The average rates of embryo resorption in each group of BaP-treated mice (n = 9 mice). (C to F) RT-qPCR analysis of the RNA levels of miR-hz04 (C), IP3R1 (D), Camk2g (E), and Sgcb (F) in each group (each n = 9 mice). (G) Western blot analysis of the protein levels of IP3R1,

Camk2g, p-Camk2g, and Sgcb in each group (each n = 6 random mice) and their relative intensities were quantified. (H and I) The correlation between the mRNA (H, each n = 9 mice) and protein (I, each n = 6 random mice) levels of IP3R1 and the levels of miR-hz04 in the control mice group (0, blue) and 0.2 mg/kg BaP-treated mice group (0.2, red). (J) The regulation mechanism of lnc-HZ04/miR-hz04. Lnc-HZ04 acts as a ceRNA for miR-hz04 and diminishes the inhibition effects of miR-hz04 on IP3R1 mRNA expression, thus activates Ca²⁺-mediated IP3R1/p-CaMKII/SGCB pathway to promote trophoblast cell apoptosis. In normal trophoblast cells, relatively less lnc-HZ04 and more miR-hz04 inhibit this apoptosis pathway and lead to normal pregnancy. After exposure to BPDE, relatively more lnc-HZ04 and less miR-hz04 activate this apoptosis pathway and induce miscarriage. Representative data in (A and C-I) represent three independent experiments. Data in (B) show mean $\pm$ SD of nine mice in each group. One-way ANOVA analysis for (B-F); pearson analysis for (H-I); *p < 0.05, **p < 0.01, and ***p < 0.001. The number n, the number of biologically independent mice.

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