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LncRNA PSMA3-AS1 promotes preterm delivery by inducing ferroptosis via miR-224-3p/Nrf2 axis

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ABSTRACT

Long non-coding RNAs (lncRNAs) have a vital potential in premature delivery. This research was intended to explore PSMA3-AS1's role in premature delivery as well as its possible molecular mechanism. We enrolled 100 premature delivery patients and 100 term patients. Fetal membranes were collected. RT-qPCR was adopted for evaluating PSMA3-AS1, miRNA-224-3p, along with Nrf2 expression. Cell function experiments were implemented to clarify PSMA3-AS1 functions in human trophoblast HTR-8/SVneo cells. Rescue together with mechanistic experiments were implemented for assessing the regulatory function and interaction between miR-224-3p and PSMA3-AS1 or Nrf2 axis in human trophoblast cells. The results uncovered that PSMA3-AS1 level presented downregulation in the fetal membrane tissues and human trophoblast cells. Overexpressed PSMA3-AS1 enhanced cell proliferation but suppressed ferroptosis in human trophoblast cells. Besides, PSMA3-AS1 elevation also attenuated the LPS-induced inflammatory response and restored the LPS-induced upregulation of 20α-HSD and downregulation of progesterone (P4). Mechanistically, miR-224-3p could bind to PSMA3-AS1 and present upregulation in fetal membranes and human trophoblast cells. Notably, overexpressed miR-224-3p offset the influences of PSMA3-AS1 on human trophoblast cell proliferation and ferroptosis. Furthermore, Nrf2 was targeted by miR-224-3p. Downregulated Nrf2 offset the influences of the miR-224-3p inhibitor and induced HTR-8/SVneo dysfunction. Additionally, Nrf2 transcriptionally activated PSMA3-AS1 and GPX4. In conclusion, PSMA3-AS1 expression is low during premature delivery and overexpressing PSMA3-AS1 promotes proliferation and suppresses ferroptosis of human trophoblast cells by interacting with miR-224-3p to downregulate Nrf2. Therefore, enhancing PSMA3-AS1 expression may be a promising therapeutic strategy to prevent premature delivery.

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Introduction

Premature delivery is associated with adverse pregnancy outcomes, which play important roles in neonatal diseases and neonatal death (1). Potential risk factors for premature delivery include but are not limited to, spontaneous preterm labor, preterm premature rupture of membranes (PROM), along with therapeutic preterm labor (2). The pathogenesis of premature delivery mainly includes abnormal structure and function of membranes, intra-amniotic infection, and preterm birth history of previous pregnancy (3). Various pathological mechanisms complicate the initiation and progression of premature delivery (4). It is particularly important to identify the risk factors for premature delivery to prevent preterm birth. Human trophoblast cells that can develop into the placenta and fetal membranes have therapeutic potential for the treatment of various diseases, including preterm birth, and can repair the uterus after injury (5). Death of trophoblast cells is associated with the rupture of fetal membranes (6).

Ferroptosis belongs to an iron-dependent type of non-apoptotic cell death induced by unrestrained lipid peroxidation-mediated membrane damage. It differs from other types of regulated cell death in the fields of morphology, genetics, as well as biochemistry (7). Ferroptosis is featured by the loss of the activity of a lipid repair enzyme GPx4, along with aggregated lethal reactive lipid oxygen species (lipid ROS) produced by the oxidation of polyunsaturated fatty acids (PUFAs) in mitochondria, endoplasmic reticulum, and lysosomes. Increasing literatures have revealed that ferroptosis may have a central potential in various pathological processes, such as cancer, digestive system, and reproductive system diseases (8). Previous studies also indicate that ferroptosis is involved in major placenta-related obstetric diseases (9, 10). During pregnancy, the maternal demand for iron elevates substantially to support fetoplacental development, while the iron load of trophoblasts is at high levels. Moreover, it has also been proposed that the elevation in hemoglobin and serum ferritin levels is linked to the elevated risks of premature delivery, which is suggested to be caused by iron excess-induced oxidative stress (11). Additionally, the hallmark of premature delivery, which is uterine contractions, is associated with placental hypoxia/reoxygenation, which is a risk factor for ferroptosis (12). These shreds of evidence suggest the potential of inhibiting ferroptosis for the prevention and management of preterm delivery. Therefore, the mechanism underlying ferroptosis in trophoblast cells

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was investigated in this study.

Long non-coding RNAs (lncRNAs) belong to the non-coding RNA family (13). LncRNAs act to be sponges and affect various diseases through competitively combining with microRNAs (miRNAs), miRNAs bind to their targets (14). LncRNAs are differentially expressed in human placentas obtained via premature delivery and regulate the biological functions in premature delivery (15). Zhao et al. found that lncRNA proteasome 20S subunit alpha 3-antisense RNA 1 (PSMA3-AS1) was differentially expressed in premature delivery and spontaneous preterm birth (16). PSMA3-AS1 serves to be a biomarker in multiple myeloma (17), lung cancer (18), colorectal cancer (19), and esophageal cancer (20). PSMA3-AS1 is also reported to regulate oxidative stress and GSH-Px activities to promote gastric cancer progression (21). Based on the transcriptomic analysis, a previous study has revealed that PSMA3-AS1 is identified as one of the hub genes implicated in co-regulatory networks that drive a myometrial transformation and thus produce an estrogen-sensitive phenotype in pregnant women (22). However, the mechanisms underlying the potential of PSMA3-AS1 in premature delivery have not yet been clarified. Hence, in the current research, we investigated the potential of PSMA3-AS1 in premature delivery and its potential underlying mechanism.

Materials and Methods

Patients

Clinical samples were collected from premature delivery patients (n = 100) and term patients (n = 100) hospitalized at the Fujian Provincial Maternity and Children's Hospital, affiliated with the Hospital of Fujian Medical University of Fuzhou City, from September 2019 to September 2020. Inclusion criteria: 1) premature delivery patients with PROM and gestational age of <37 weeks and term patients delivered at term; 2) primipara with singleton birth; 3) with favorable pregnancy outcome. Exclusion criteria: 1) patients had acute inflammatory diseases; 2) Patients with serious pregnancy complications; 3) Pathologic pregnancy. This study was approved by the Ethics Committee of Fujian Provincial Maternity and Children's Hospital. All patients provided written informed consent prior to their participation in the study. Fetal membrane tissues were randomly gathered from the maternal surface of the placenta, rapidly frozen in liquid nitrogen, and preserved at –80 °C for later use. The clinical information of the patients is listed in Table 1.

Cell culture and transfection

Cell Bank (Shanghai, China) offered human trophoblast cells (HTR-8/SVneo). Human myometrial cells were obtained from Procell (Wuhan, China). Cells were cultivated in Dulbecco's modified Eagle medium (DMEM) together with Ham's Nutrient Mixture F12 medium (HAM's F12, Sigma-Aldrich, USA) in 1:1 ratio containing 10% FBS (Gibco, NY, USA), 100 units/mL penicillin, 100 μg/mL streptomycin along with 0.25 μg/mL amphotericin B (MP Biomedicals, Illkrich, France) at 37 °C. For LPS treatment, cells were cultured in the culture medium which contained 100 ng/mL LPS (lipopolysaccharide, Sigma-Aldrich, USA) for 48 h with same volume of PBS used as the negative control.

For the cell transfection, oe-PSMA3-AS1/si-PSMA3-AS1, miR-224-3p mimic/inhibitor, oe-Nrf2/si-Nrf2, along with their negative controls (Abiocenter Biotech, USA) were transfected into the cells using the Lipofectamine 3000 reagent (Invitrogen, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

HTR-8/SVneo cells were mixed with the TRIzol reagent (Thermo Fisher Scientific) for extracting the total RNA. Reverse transcription and qPCR could be performed with a BlazeTaq One-Step SYBR Green RT-qPCR Kit (with ROX) (GeneCopoeia, USA) on a SEDI Thermo Cycler controlled by the Control Bus Net software package (Wealtec Bioscience, China). Nanjing Genscript Biotech Co., Ltd. designed and synthesized all primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and uracil 6 (U6) were adopted to be internal references for mRNA and miRNA, respectively. Fold changes in gene expression were calculated with the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows.

PSMA3-AS1, F: 5′-TTCCTCCAGGACAGCACCTAGT-3′ and R: 5′-CGTCTCTGATGTGGCTTATACGA-3′; *miR-224-3p*, F: 5′-TGATGTGGGTGCTGGTGTC-3′ and R: 5′-TTGTGTTGGGGCAGTACTG-3′; *GAPDH*, F: 5′-AGGTGAAGGTCGGAGTCAACG-3′ and R: 5′-AGGGGTCATTGATGGCAACA-3′; nuclear factor-erythroid 2-related factor 2 (*Nrf2*), F: 5′-GGCGTTAGAAAGCATCCTTCC-3' and R: 5'-GCAGAGGGCACACTCAAAGT-3′; and *U6*, F: 5′-CTCGCTTCGGCAGCACATATACT-3′ and R: 5′-ACGCTTCACGAATTTGCGTGTC-3′.

Enzyme-linked immunosorbent assay

HTR-8/SVneo cells received lysis and addition to an iron assay buffer (Iron Colorimetric Assay Kit; Leagene, China), followed by centrifugation to acquire the supernatant for the assay. After incubation with the assay buffer in a 96-well plate for 0.5 h at room temperature, HTR-8/SVneo was added to the reagent mix for 15 min. Absorbance at 562 nm was examined using a microplate reader (Thermo Fisher, USA).

The levels of glutathione (GSH) and malondialdehyde (MDA) in HTR-8/SVneo cells and progesterone (P4) levels in myometrial cells were measured using commercial kits (Sigma-Aldrich, USA).

Table 1. Clinical information of the patients and healthy controls.

	Premature delivery (n=100)	Healthy control (n=100)	P-value
Age (years)	29.36±3.92	29.45±3.64	0.8666
Gestational weeks	34.35±2.92	39.30 ± 1.00	<0.001
Birthweight (g)	2467.95±612.59	3379.03±328.52	<0.001
BMI (kg/m²)	20.83 ± 2.75	21.13 ± 2.97	0.4595

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The transfected HTR-8/SVneo cells were plated in a 96-well plate and permitted to adhere overnight. Subsequently, the MTT cell viability assay kit (Thermo Fisher Scientific, USA) was adopted following the manufacturer's advices, and the optical density was measured at 490 nm.

5-ethynyl-2'-deoxyuridine (EdU) assay

HTR-8/SVneo cells were planted in a 24-well plate with 200 μL of diluted EdU (BeyoClick EdU Cell Proliferation Kit; Beyotime, China). Followed by washing, cells were added with 200 μL of Hoechst for 30 min. Images of EdU-labeled and Hoechst-stained cells were captured by an Olympus light microscope.

Terminal deoxynucleotidyl transferase (TDT)-dUTP nick and labeling (TUNEL) assay

The cell death of HTR-8/SVneo cells was determined using the In Situ Cell Death Detection Kit (Roche, Switzerland). Briefly, the cells were deparaffinized, and permeabilized with 0.1% Triton X-100 (Beyotime, China), followed by incubation with 0.3% H_2O_2. After rinsing, cells were treated with the TUNEL reaction mixture at 37 °C for 1 h and the TdT reaction cocktail at 37 °C for half an hour. After washing, cells were dyed with hematoxylin for three min, and then imaged with a fluorescence microscope.

Western blotting assay

Radioimmunoprecipitation assay (Sigma-Aldrich, USA) buffer was adopted for extracting the proteins from HTR-8/SVneo cells. After determining protein concentrations with a BCA kit (Sigma-Aldrich, USA), proteins were separated via 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by shifting onto polyvinylidene fluoride membranes (Bio-Rad, USA). Followed by sealing with 5% skim milk for 2 h, the membranes were treated with the following primary antibodies: glutathione peroxidase 4 (GPX4; ab125066, 1:1000; Abcam, USA), heat shock protein family B member 1 (HSPB1; ab261738, 1: 1000, Abcam, USA), telomeric repeat binding factor 1 (TERF1/TRF1; ab129177, 1:1000; Abcam, USA), Ras (ab52939, 1:5000; Abcam, USA), Nrf2 (ab62352, 1:1000; Abcam, USA), TGF-α (ab208156, 1:1000; Abcam, USA), IL-6 (ab233706, 1:1000, Abcam, USA), IL-1β (ab254360, 1:1000; Abcam, USA), MCP-1 (ab214819, 1:1000, Abcam, USA), anti-20α-HSD (ab192785, 1:1000; Abcam, USA) and GAPDH (ab9485, 1:500; Abcam, USA) antibodies at 4 °C overnight, followed by incubation with secondary goat anti-mouse IgG (ab205719, 1:2000; Abcam, USA) and goat anti-rabbit IgG (ab6721, 1:2000; Abcam, USA) for 1 h. Finally, the protein bands were visualized with an ECL system (Thermo Fisher Scientific, USA).

Luciferase reporter assay

The wild-type and mutant Nrf2 3′-untranslated regions (UTRs) and PSMA3-AS1 luciferase reporter vectors (Guangzhou RiboBio Co., Ltd.) were transfected with the miR-224-3p mimic or miR-NC mimic into cells for 48 h. The luciferase activity was detected with a luciferase reporter assay kit (K801-200; BioVision Tech, USA).

Chromatin immunoprecipitation (ChIP) assay

Cells were processed with 1% formaldehyde (Sigma-Aldrich, USA) for 10 min, allowing intracellular DNA and protein to crosslink. After sonication for 4 min using a sonicator, protein A/G beads were added into the cell lysates and cultured with anti-Nrf2 antibody and IgG at 4 °C overnight. Next, the complex was rinsed thrice with elution buffer and DNA was purified and subjected to qRT-PCR analysis.

Statistical analysis

All experiments were implemented in triplicate. All data were analyzed with be help of GraphPad 6.0 and presented as the mean ± standard deviation. Student's *t*-test and one-way analysis of variance were adopted for statistical analysis. Pearson correlation analysis was adopted to explore the correlation between the expression of PSMA3-AS1 and Nrf2 in the fetal membranes of preterm delivery women. $P < 0.05$ meant statistical significance.

Results

Expression characteristic and cellular functions of PSMA3-AS1 in premature delivery

We first evaluated PSMA3-AS1 expression in premature delivery women and healthy delivery control women. It was discovered that PSMA3-AS1 expression presented reduction in the fetal membranes of women who underwent premature delivery in comparison with the normal controls (Fig. 1A). Table 1 revealed the clinical data of the patients. While age and body mass index exhibited no difference between 2 groups, gestational weeks and birth weights were markedly decreased in the premature deli-

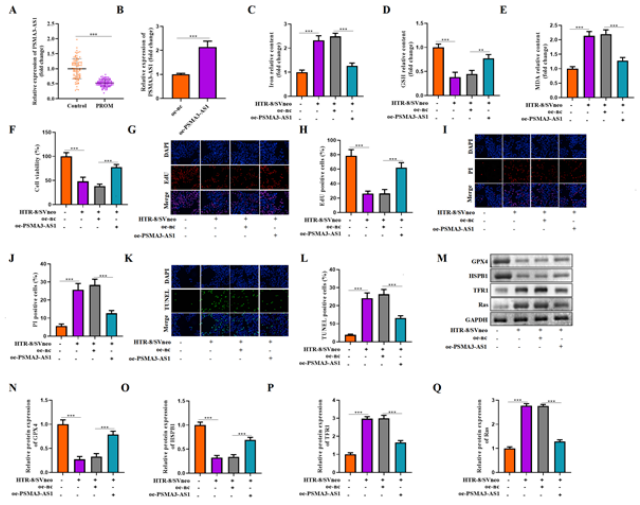


Figure 1. PSMA3-AS1 promoted cell proliferation and inhibited apoptosis in preterm delivery via ferroptosis. (A) RT-qPCR was used to measure the expression of PSMA3-AS1 in premature delivery fetal membranes. (B) PSMA3-AS1 expression levels in transfected HTR-8/SVneo cells were detected using qRT-PCR. (C-E) The levels of Iron, GSH, and MDA in transfected HTR-8/SVneo cells. (F) Cell viability was detected by MTT assay. (G-H) EdU staining was used to detect cell proliferation. (I-J) PI staining was used to detect cell death. (K-L) TUNEL staining was used to detect cell death. (M) The protein expression of GPX4, HSPB1, TFR1, and Ras were detected by western blot. (N-Q) The protein expression levels of GPX4, HSPB1, TFR1, and Ras were quantified using ImageJ software. **p<0.01, ***p<0.001.

very group than in the healthy controls. This data suggests that PSMA3-AS1 expression is low in premature delivery.

Next, we evaluated the role of PSMA3-AS1 in premature delivery processes. For this purpose, HTR-8/SVneo cells received transfection with the PSMA3-AS1 overexpression plasmids (oe-PSMA3-AS1) and negative control (oe-nc). As displayed in Fig. 1B, PSMA3-AS1 expression presented elevation in the oe-PSMA3-AS1 group in comparison with the control group, suggesting successful transfection. Overexpressed PSMA3-AS1 apparently increased the release of GSH, while decreasing the iron as well as MDA levels (Fig. 1C–E), facilitated the cell viability (Fig. 1F) and proliferation (Fig. 1G–H), and significantly suppressed HTR-8/SVneo cell death (Fig. 1I–L), highlighting the important role of PSMA3-AS1 in premature delivery.

To investigate whether PSMA3-AS1 was associated with ferroptosis, protein levels of ferroptosis-linked genes were examined. As displayed in Fig. 1M–Q, the protein levels of GPX4 and HSPB1 presented reduction whereas TFR1 and Ras presented upregulation in HTR-8/SVneo cells. Interestingly, overexpressed PSMA3-AS1 significantly elevated the GPX4 and HSPB1 expression while downregulated TFR1 and Ras expression. Collectively, these results indicate that PSMA3-AS1 enhances cell proliferation while represses apoptosis in premature delivery through regulating ferroptosis.

PSMA3-AS1 elevation attenuates the LPS-induced inflammatory response and upregulation of 20α-HSD in HTR-8/SVneo cells

Increasing evidence has demonstrated that the increased release of placental proinflammatory cytokines is closely related to miscarriage, preterm labor and preeclampsia (23-26). Therefore, LPS was adopted to stimulate the HTR-8/SVneo cells to induce an inflammatory response. It was found that TGF-α, IL-6, IL-1β as well as MCP-1 protein levels were definitely elevated in the LPS-treated human trophoblast cells (Fig. 2A-D). Moreover, PSMA3-AS1 overexpression was revealed to reverse the LPS-induced inflammatory response in human trophoblast cells (Fig. 2A-D), implying that PSMA3-AS1 serves to be a critical regulator that prevents preterm delivery by mediating the inflammation response. Additionally, LPS treatment is reported to induce the upregulation of 20α-hydroxysteroid dehydrogenase (20 α-HSD), an enzyme mediating progesterone (P4) in myometrial cells (27, 28). 20α-HSD is revealed to be closely associated with labor, and its deficiency in rodents is indicated to increase P4 levels and delayed parturition (29). Western blot displayed that the upregulation of 20α-HSD protein levels in myometrial cells caused by LPS stimulation was significantly reversed after overexpressing PSMA3-AS1 (Fig. 2E). Furthermore, ELISA results demonstrated that the levels of P4 were reduced after LPS treatment, which was demonstrated to be restored after PSMA3-AS1 overexpression in myometrial cells (Fig. 2F). Overall, these findings suggest that PSMA3-AS1 overexpression alleviates the inflammation and 20α-HSD upregulation in response to LPS treatment in HTR-8/SVneo cells.

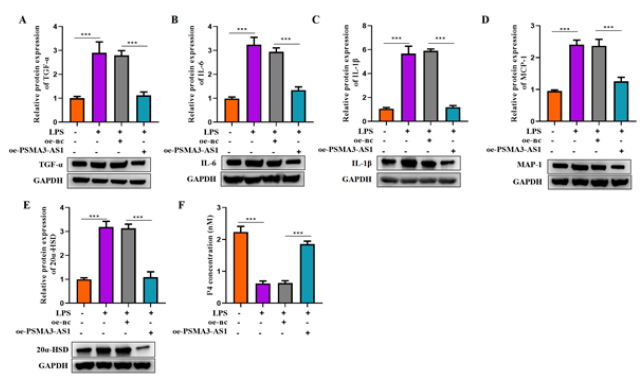


Figure 2. PSMA3-AS1 attenuates the LPS-induced inflammatory response and upregulation of 20α-HSD in HTR-8/SVneo cells. Western blot was used to measure the protein expression level of (A) TGF-α, (B) IL-6, (C) IL-1β and (D) MCP-1 in transfected HTR-8/SVneo cells with or without LPS (100 ng/mL) treatment for 48 h. (E) Western blot was conducted to examine the protein levels of 20α-HSD in transfected myometrial cells with or without LPS (100 ng/mL) treatment. (F) ELISA was used to detect the concentration of P4 in the culture medium of transfected myometrial cells with or without LPS (100 ng/mL) treatment for 48 h. ***p<0.001.

PSMA3-AS1 targets miR-224-3p

Since lncRNAs can repress miRNAs from their target genes through competing with mRNAs for binding sites, we conjectured that PSMA3-AS1 may exert its function in preterm delivery in such a way. StarBase 3.0 (http://starbase.sysu.edu.cn/) was adopted to discover the target of PSMA3-AS1. It was discovered a binding site of miR-224-3p and PSMA3-AS1. Fig. 3A shows the predicted and mutated binding sequences. Luciferase reporter experiments further confirmed that the luciferase activity of PSMA3-AS1-WT exhibited a reduction in HTR-8/SVneo cells under miR-224-3p elevation, while both displayed no marked alterations in the mutant groups (Fig. 3B). For further verifying whether PSMA3-AS1 could interact with miR-224-3p, HTR-8/SVneo cells were transfected with either si-PSMA3-AS1 or oe-PSMA3-AS1, followed by evaluating miR-224-3p expression. RT-qPCR data showed

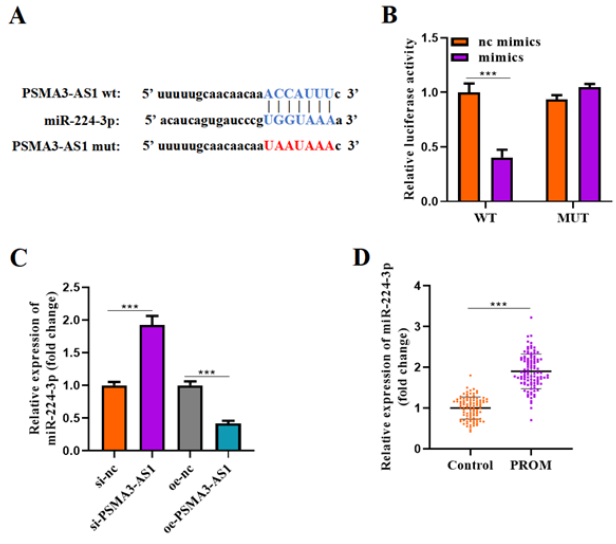


Figure 3. PSMA3-AS1 acted as a sponge for miR-224-3p in HTR-8/SVneo cells. (A) Bioinformatics predicted the binding sites between miR-224-3p and PSMA3-AS1. (B) Dual-luciferase reporter assay was conducted to confirm the interaction between miR-224-3p and PSMA3-AS1 in HTR-8/SVneo cells. (C-D) RT-qPCR was used to evaluate the gene expression level of miR-224-3p in transfected HTR-8/SVneo cells and fetal membrane samples of preterm delivery in indicated groups. ***p<0.001.

that miR-224-3p expression presented elevation in HTR-8/SVneo cells in which PSMA3-AS1 was silenced in comparison with the control group (Fig. 3C). Inversely, PSMA3-AS1 overexpression could downregulate miR-224-3p expression. In addition, miR-224-3p expression presented elevation in HTR-8/SVneo cells (Fig. 3D). Collectively, PSMA3-AS1 targets and regulates miR-224-3p.

Upregulated miR-224-3p reverses cellular functions of PSMA3-AS1 in HTR-8/SVneo cells

For clarifying the modulatory pattern of PSMA3-AS1-miR-224-3p in human trophoblast cells, we carried out rescue experiments. As revealed in Fig. 4A, miR-224-3p expression presented elevation in miR-224-3p mimic group in comparison with the control group. Then, it was demonstrated that upregulated miR-106-5p expression counteracted the increased release of iron, GSH, and MAD induced by oe-PSMA3-AS1 in human trophoblast cells (Fig. 4B–D). Subsequently, co-transfection of miR-224-3p mimic into the oe-PSMA3-AS1 transfected HTR-8/SVneo cells significantly reversed oe-PSMA3-AS1 induced increased cell viability (Fig. 4E) and proliferation (Fig. 4F–G), and reduced HTR-8/SVneo cell death (Fig. 4H–K). Moreover, the miR-224-3p mimic also alleviated the PSMA3-AS1-induced elevated ferroptosis (Fig. 4L–P). These results indicate that PSMA3-AS1 regulates cellular processes in human trophoblast cells by downregulating miR-224-3p.

Nrf2 is targeted by miR-224-3p

We then wanted to investigate the downstream targets of miR-224-3p. TargetScan 7.2 (http://www.targetscan.org/) unveiled that *Nrf2* was targeted by miR-224-3p (Fig. 5A). Thereafter, luciferase reporter experiments verified that the luciferase activity could be lessened in cells transfected with the miR-224-3p mimic together with Nrf2 wild-type 3'-UTR (Fig. 5B). Additionally, Nrf2 mRNA level was apparently lessened by miR-224-3p mimic while promoted upon miR-224-3p inhibitor (Fig. 5C). Moreover, Nrf2 mRNA level was significantly declined during the premature delivery of fetal membranes (Fig. 5D). These outcomes imply that miR-224-3p directly target *Nrf2* in human trophoblast cells.

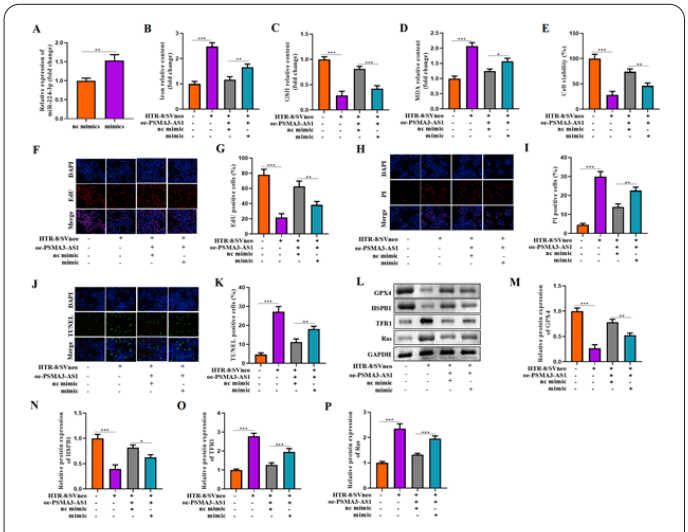


Figure 4. Up-regulation of miR-224-3p reversed the effects of PSMA3-AS1 on HTR-8/SVneo cellular function. The miR-224-3p expression level was detected using qRT-PCR. (B-D) The levels of Iron, GSH, and MDA in transfected HTR-8/SVneo cells. (E) Cell viability was detected by MTT assay. (F-G) EdU staining was used to detect cell proliferation. (H-I) PI staining was used to detect cell death. (J-K) TUNEL staining was used to detect cell death. (L) The protein expression of GPX4, HSPB1, TFR1, and Ras were detected by western blot. (M-P) The protein expression levels of GPX4, HSPB1, TFR1, and Ras were quantified using ImageJ software. *p<0.05, **p<0.01, ***p<0.001.

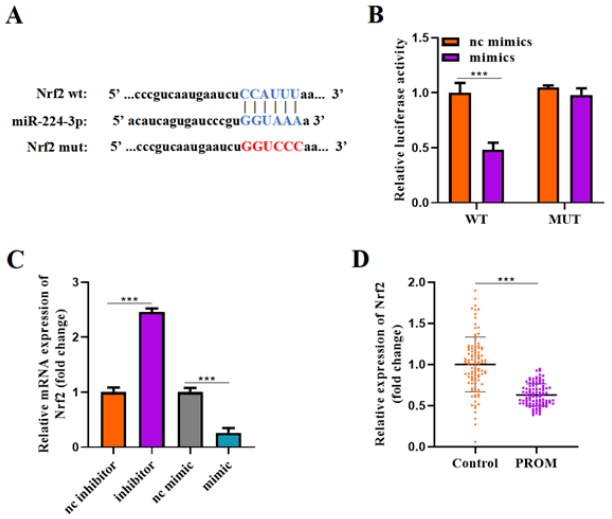


Figure 5. Nrf2 was a target gene of miR-224-3p. (A) Bioinformatics predicted the binding sites between miR-224-3p and Nrf2. (B) Dual-luciferase reporter assay confirmed that Nrf2 was a target of miR-224-3p in HTR-8/SVneo cells. (C-D) The expression of Nrf2 in premature delivery fetal membranes was determined by qRT-PCR. ***p<0.001.

Nrf2 regulates the cellular effects of the miR-224-3p in human trophoblast cells

Afterwards, the modulatory pattern of Nrf2-miR-224-3p in human trophoblast cells was clarified through rescue experiments. Through qRT-PCR, it was revealed that the Nrf2 expression presented reduction in the cells transfected with si-Nrf2 when compared with the control group (Fig. 6A). It was discovered that Nrf2 reversed the modulatory function of miR-224-3p on the release of iron, GSH, as well as MAD (Fig. 6B–D). In comparison with miR-224-3p inhibitor group, si-Nrf2 significantly lessened the cell viability (Fig. 6E) along with proliferation (Fig. 6F–G) while facilitating cell death in HTR-8/SVneo cells (Fig. 6H–K). Moreover, down-regulation of Nrf2 expression alleviated the impacts of the miR-224-3p inhibitor on GPX4, HSPB1, TFR1, and Ras protein levels (Fig. 6L–P). These outcomes demonstrate that Nrf2 influences the cellular functions of the miR-224-3p.

Nrf2 regulates ferroptosis in human trophoblast cells

Lastly, the regulatory effect of Nrf2 on human trophoblast cells ferroptosis was disclosed. qRT-PCR together with western blot analysis unveiled that GPX4 mRNA and protein levels presented elevation in human trophoblast cells in which Nrf2 was overexpressed and significantly downregulated when Nrf2 was silenced (Fig. 7A–B). We further verified the interaction between Nrf2 and GPX4 using an online tool and discovered a binding site of Nrf2 and GPX4, implying a direct interaction between Nrf2 and GPX4. Fig. 7C shows the binding motif of Nrf2 (Fig.

7C). Similarly, Nrf2 was found to bind to the promoter of GPX4 (Fig. 7D). Subsequent luciferase reporter experiments confirmed that the luciferase activity of the luciferase-labeled Nrf2 and wild-type GPX4 co-transfection group was significantly increased which was prominently reduced when co-transfected with si-Nrf2 (Fig. 7E–F). Collectively, these findings suggest that Nrf2 directly regulates HTR-8/SVneo cells ferroptosis.

PSMA3-AS1 is transcriptionally activated by Nrf2 in human trophoblast cells HTR-8/SVneo cells

Through catRAPID database, Nrf2 was predicted to be a transcription factor of PSMA3-AS1 (Fig. 8A). qRT-PCR revealed PSMA3-AS1 expression presented elevation after Nrf2 overexpression and was downregulated after Nrf2 knockdown (Fig. 8B). Consistently, the ChIP assay

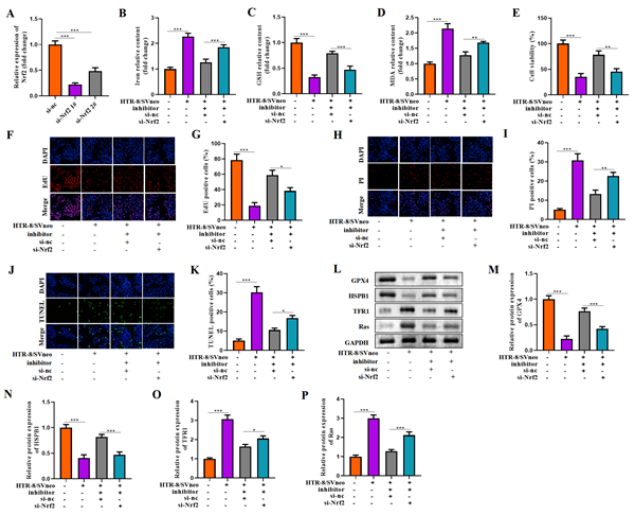


Figure 6. Nrf2 regulated the cellular function of the miR-224-3p in HTR-8/SVneo cells. (A) Nrf2 expression levels were detected using qRT-PCR. (B-D) The levels of Iron, GSH, and MDA in transfected HTR-8/SVneo cells. (E) Cell viability was detected by MTT assay. (F-G) EdU staining was used to detect cell proliferation. (H-I) PI staining was used to detect cell death. (J-K) TUNEL staining was used to detect cell death. (L) The protein expression of GPX4, HSPB1, TFR1, and Ras were detected by western blot. (M-P) The protein expression levels of GPX4, HSPB1, TFR1, and Ras were quantified using ImageJ software. *p<0.05, **p<0.01, ***p<0.001.

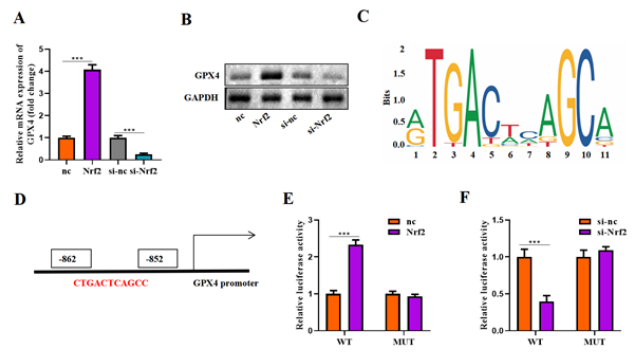
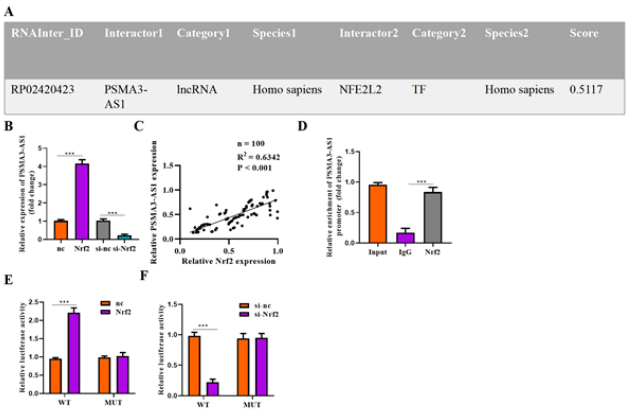


Figure 7. GPX4 was transcriptionally activated by Nrf2. The mRNA expression level of GPX4 was detected by qRT-PCR. (B) The protein expression of GPX4 was measured by western blot. (C) The binding motif of Nrf2. (D) The binding sequence between Nrf2 and GPX4 promoter. (E-F) Dual-luciferase reporter assay confirmed that GPX4 was transcriptionally activated by Nrf2. ***p<0.001.



RNAInter_ID	Interactor1	Category1	Species1	Interactor2	Category2	Species2	Score
RP02420423	PSMA3-AS1	lncRNA	Homo sapiens	NFE2L2	TF	Homo sapiens	0.5117

Figure 8. PSMA3-AS1 is transcriptionally activated by Nrf2 in HTR-8/SVneo cells. (A) Nrf2 was predicted as a transcription factor of PSMA3-AS1 on the catRAPID database (http://service.tartaglialab.com/page/catrapid_group). (B) qRT-PCR was used to detect the expression of PSMA3-AS1 in HTR-8/SVneo cells with Nrf2 overexpression or knockdown. (C) Pearson correlation analysis was used to explore the expression correlation between PSMA3-AS1 and Nrf2 in the fetal membranes of preterm delivery women (n=100). (D) ChIP was performed to detect the enrichment of PSMA3-AS1 promoter fragments in the immunoprecipitated complex of anti-IgG and anti-Nrf2. (E) Dual-luciferase reporter assays confirmed that PSMA3-AS1 was transcriptionally activated by Nrf2. ***p<0.001.

demonstrated that the PSMA3-AS1 promoter was abundantly enriched in the precipitates of anti-Nrf2, suggesting that Nrf2 interacted with PSMA3-AS1 promoter in HTR-8/SVneo cells (Fig. 8C). Moreover, PSMA3-AS1 and Nrf2 levels were positively correlated in the fetal membranes of preterm delivery women (R^2=0.6342, P<0.001) (Fig. 8D). Furthermore, luciferase reporter assay displayed that the luciferase activity of wild-type PSMA3-AS1 was elevated with Nrf2 overexpression and showed significant reduction after Nrf2 silencing in human trophoblast cells, which further certified that PSMA3-AS1 bound with Nrf2 in human trophoblast cells (Fig. 8E-F). In a word, this data indicates that Nrf2 serves as the transcription factor of PSMA3-AS1 that forms a positive feedback loop with PSMA3-AS1 in human trophoblast cells.

Discussion

Premature delivery is a predominant pregnancy complication that can result in adverse outcomes (30, 31). Deepening the understanding of the molecular mechanisms responsible for premature delivery contributes to the identification of novel approaches for the prevention of premature delivery (32). In this research, we clarified that PSMA3-AS1 facilitated HTR-8/SVneo cell proliferation and suppressed ferroptosis during premature delivery by regulating the miR-224-3p/Nrf2 axis.

LncRNAs are master regulators that play critical roles in various diseases (33). Accumulating evidence has demonstrated that lncRNAs regulate the cellular processes containing cell proliferation, differentiation, along with apoptosis (34). Placental lncRNAs have a clear relationship with the pathogenesis of preeclampsia that causes preterm birth and are revealed to regulate the proliferation, invasion, as well as migration of placental trophoblast cells. LncRNA SNHG29 enhances oxidative stress-

induced senescence in HTR8/SVneo cells via the p53/p21 signaling (35). PSMA3-AS1 has been reported to facilitate cholangiocarcinoma cell proliferation and migration along with invasion by modulating the miR-376a-3p and LAMC1 (36). A study also reveals that PSMA3-AS1 enhances bladder cancer cell viability and represses apoptosis by interacting with miR-214-5p to upregulate PD-L1 (37). Zhao et al. identified the altered expression of lncRNAs related to theubiquitin–proteasome system during premature delivery and found that lncRNA PSMA3-AS1 levels were downregulated in human placentas and fetal amniochorionic membranes during premature delivery (16). In this research, PSMA3-AS1 was downregulated in prematurely delivered fetal membranes, which was consistent with the previous findings. Mechanistically, overexpressed PSMA3-AS1 elevated HTR-8/SVneo cells viability, and proliferation while hindered ferroptosis, indicating that decreased PSMA3-AS1 expression may be a promising biomarker of premature delivery.

LncRNAs serve as sponges and modulate various cellular processes through binding to miRNAs (38). MiRNAs are reported to be critically involved in the pathological progress of preterm birth and are suggested as promising biomarkers for the risk prediction of preterm birth (39, 40). MiR-199a-3p is indicated to suppress the inflammation of cervical epithelial cells by suppressing the HMGB1/TLR4/NF-κB pathway in preterm birth (41). MiR-21 is revealed to be downregulated in placental tissues of lipopolysaccharides-stimulated infectious preterm birth mice and is suggested to promote the infection-stimulated preterm birth via targeting NF-κB (42). Our outcomes suggested that miR-224-3p, the target of PSMA3-AS1, can reverse the effects of PSMA3-AS1 in human trophoblast cells. This research is the first to investigate miR-224-3p's function during premature delivery.

Nrf2 was confirmed to be the downstream target of miR-224-3p, which can combine with the promoter of GPX4 and is associated with ferroptosis (43, 44). As an important transcription factor, Nrf2 modulates cell defense mechanisms against endogenous and exogenous stress. Increasing literatures have also unveiled that Nrf2 has a critical role in preterm birth. For example, Nrf2 activation in mesenchymal cells might prevent preterm birth or premature rupture of the membranes (45). A study further reveals that Nrf2 shows protective effects against ROS-induced preterm premature rupture of membranes by regulating the mitochondrial metabolic process (46). Consistently, in this research, downregulated Nrf2 offset the impacts of downregulated miR-224-3p on human trophoblast cells and resulted in lessened GPX4 expression, suggesting that ferroptosis is modulated by the miR-224-3p/Nrf2 axis in HTR-8/SVneo cells. Recently, trophoblasts are revealed to be susceptible to ferroptosis, and the ferroptosis of trophoblasts is closely linked to various placenta-related obstetric diseases (10). For example, the Nrf2/GPX4 pathway upregulated by DJ-1 suppresses ferroptosis of trophoblasts in preeclampsia (47). SIRT3 silencing is resistant to autophagy-dependent ferroptosis in gestational diabetes mellitus by inactivating the AMPK/mTOR signaling along with elevating GPX4 levels (48). Ferroptosis has been implicated in cell death and is induced by GPX4 inhibition.

Conclusion

In summary, our findings revealed that overexpressed lncRNA PSMA3-AS1 ameliorated premature delivery through interacting with miR-224-3p to downregulate Nrf2 expression; hence, it may be adopted as a promising therapeutic target to prevent premature delivery.

Declarations

Funding

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Competing interests

The authors declare that they have no conflicts of interest.

Ethics statement

All patients provided their written, voluntarily informed consent. All procedures were carried out in accordance with the guidelines outlined in the Helsinki Declaration and this study was approved by the Ethics Committee of the Affiliated Hospital of Fujian Medical University.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors contributions

JY conceived and designed the experiments. LQ, XL, and RC contributed significantly to the experiments and arranging data. YW performed data analyses. LQ wrote the draft manuscript. JY revised the manuscript. All authors read and approved the final manuscript.

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