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Original Article

Upregulated lncRNA XIST drives trophoblast dysfunction through targeting miR-545-5p/POU2F1 axis in recurrent spontaneous abortion

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Abstract: Objective: To investigate the specific role of long non-coding RNA (lncRNA) XIST in recurrent spontaneous abortion (RSA). Methods: Placental tissue samples from RSA patients and healthy controls were obtained to assess expression of the XIST/miR-545-5p/POU2F1 axis. Functional assays evaluating cell proliferation, apoptosis, and invasion, as well as nuclear factor kappa B (NF-κB) pathway activity, were performed in HTR-8/Svneo trophoblasts following transfection with short hairpin RNA (shRNA), miRNA mimics, or inhibitors. The targeting relationship among these molecules was verified through dual luciferase reporter gene assays. An RSA mouse model was established to study the effects of XIST knockdown on this molecular axis and pregnancy outcomes *in vivo*. Results: In clinical RSA samples, lncRNA XIST expression was significantly upregulated. Knockdown of XIST in trophoblasts led to enhanced cell proliferation and invasion while reducing cell apoptosis. In the RSA mouse model, suppression of XIST decreased the embryonic resorption rate and improved pregnancy outcomes. Mechanistic studies revealed that XIST acts as a competing endogenous RNA that sponges miR-545-5p, thereby relieving repression of its downstream target POU2F1. Dysregulation of the XIST/miR-545-5p/POU2F1 axis contributed to trophoblast dysfunction and RSA pathogenesis through activation of the pro-inflammatory NF-κB signaling pathway. Conclusion: These findings suggest that lncRNA XIST may serve as a potential prognostic marker for RSA and promote disease progression via the miR-545-5p/POU2F1 regulatory axis.

Keywords: lncRNA XIST, miR-545-5p, POU2F1, recurrent spontaneous abortion, trophoblast

Introduction

Recurrent spontaneous abortion (RSA), defined as the occurrence of two or more consecutive spontaneous abortions with the same partner, affects approximately 5% of pregnancies and significantly impairs reproductive outcomes [1]. Although its precise prevalence is difficult to determine, most studies indicate that RSA occurs in 1-3% of women of reproductive age [2, 3]. Established etiologies include antiphospholipid syndrome, infection, abnormal uterine anatomy, endocrine and metabolic disorders and chromosomal abnormalities [4]. However, nearly 50% of the cases remain unexplained [5]. Beyond its adverse effects on female reproductive health, RSA is associated with substantial psychological burden and cardiovascular risk [6]. Therefore, identifying novel therapeutic targets and clarifying their underlying molecular mechanisms is of vital importance for improving clinical management.

Placental trophoblast dysfunction represents a central pathological feature of RSA. Successful pregnancy relies on the precisely regulated functions of extravillous trophoblasts (EVTs), including controlled proliferation, differentiation, deep invasion into the decidua, and remodeling of uterine spiral arteries. Impaired tropho-

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blast invasion and defective vascular remodeling are hallmark features of abnormal placentation in RSA [7]. Pathological analyses consistently demonstrate excessive trophoblast apoptosis alongside reduced proliferative and migratory capacity in RSA villous tissues, directly linking these cellular defects to pregnancy loss [8-10]. Concurrently, immune dysregulation at the maternal-fetal interface, particularly a shift toward a pro-inflammatory state and aberrant activation of the nuclear factor kappa B (NF-κB) pathway, can directly compromise trophoblast viability and function, thereby exacerbating disease progression [11, 12]. Therefore, elucidating the upstream molecular mechanisms that co-regulate essential trophoblast processes (e.g., proliferation, invasion, apoptosis) and inflammatory microenvironment is crucial for understanding RSA pathogenesis.

Long non-coding RNAs (lncRNAs) participate in diverse biological processes, although they do not have protein-coding functions [13]. Increasing evidence indicates that lncRNAs are closely associated with pregnancy regulation, influencing miscarriage through modulation of endometrial tolerance, embryonic development, trophoblast differentiation, inflammatory signaling, and placental vascular formation [14], making lncRNAs as potential diagnostic biomarkers and therapeutic targets for RSA. MicroRNAs (miRNAs), typically 19-24 nucleotides in length, regulate gene expression post-transcriptionally by binding to complementary sequences within the 3'-untranslated region (3'UTR) of target messenger RNA (mRNA), leading to translational repression or degradation. Literately, miRNAs are abundantly expressed in human blood, placenta, meconium, and chorionic villi and play important roles in regulating cell proliferation, differentiation, and apoptosis, thereby influencing trophoblast invasion and implantation processes [15]. Importantly, lncRNAs regulate miRNAs activity through the competing endogenous RNAs (ceRNAs) mechanism, whereby lncRNAs and mRNAs share common miRNA-binding sites and competitively sequester miRNAs, ultimately modulating gene expression networks [16-18]. Thus, lncRNAs and miRNAs are crucial in epigenetically regulating trophoblast function and abortion concurrently [19-21].

Among lncRNAs implicated in reproductive processes, lncRNA XIST has garnered attention. Although classically recognized for its role in X-chromosome inactivation, emerging evidence suggests its dysregulation might be involved in pregnancy complications such as preeclampsia and fetal growth restriction, possibly through modulating trophoblast function and inflammation [22-24]. miR-545-5p has been reported to regulate cellular proliferation, apoptosis, and inflammatory responses in various biological contexts [25]. The transcription factor POU2F1 is a validated target of several miRNAs [26], and has been shown to modulate activity of the NF-κB pathway [27, 28], a key driving factor of inflammation and trophoblast dysfunction in RSA. Therefore, these findings suggest that the lncRNA XIST/miR-545-5p/POU2F1 axis may regulate the NF-κB signaling pathway and trophoblast fate, thereby contributing to RSA pathogenesis. However, the specific role and regulatory dynamics of this axis in RSA remain largely unexplored. Although altered expression of certain lncRNAs or miRNAs has been noticed in RSA chorionic tissues compared with elective abortion controls, their mechanistic roles and therapeutic potential remain incompletely defined [29]. Therefore, this study aimed to clarify the functional role of lncRNA XIST in the pathogenesis of RSA.

Materials and methods

Ethics and clinical subjects

Prior to study initiation, ethical approval was obtained from the Medical Ethics Committee of the First Affiliated Hospital of Yunnan University (Yunnan Provincial Hospital of Traditional Chinese Medicine) (Approval No. XW-2022-007). Placental chorionic tissues were obtained from two matched groups (n=10 each): patients diagnosed with RSA and healthy controls undergoing elective termination of pregnancy for non-medical reasons. The two groups were matched for gestational age (6-10 weeks), maternal age, and body mass index (BMI). All RSA patients underwent comprehensive etiological evaluation, including transvaginal ultrasonography to exclude uterine anomalies (e.g., septate or bicornuate uterus). Chromosomal microarray analysis (CMA) was performed on chorionic villus samples from both groups to exclude embryonic chromosomal abnormalities, and only euploid conceptuses were included. After collection, placental chorionic tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Cell culture and transfection

The HTR-8/Svneo cell line (Zhongqiao Xinzhou Biotechnology Co., Ltd., Shanghai, China) was cultured in RPMI-1640 supplemented with 10% FBS (Gibco, USA) under standard conditions (37°C, 5% CO_2). Cells in the logarithmic growth phase were seeded in 6-well plates at a density of 1×10^5 cells per well and cultured to 80-90% confluence prior to transfection. For gene modulation experiments, cells were transfected with either: 1) sh-XIST and pcDNA3.1-POU2F1 plasmids (GenePharma, Shanghai) along with their corresponding scrambled shRNA or empty vector controls; or 2) miR-545-5p mimics (50 nM), inhibitors (100 nM), or corresponding negative controls (NCs; Ribobio, China) using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol. Transfection efficacy exceeded 70% as verified by GFP co-expression.

Cell proliferation

HTR-8/Svneo cells were cultured and transfected as described above. After 24 h of transfection, cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. The absorbance value (OD value) at 450 nm was detected using a microplate reader. Each experimental condition was performed in triplicate, and the OD value was used for statistical analysis.

Apoptosis analysis

Transfected HTR-8/Svneo cells were collected and stained with 5 μL Annexin V-FITC (BioLegend; Cat. No. 640906) and 10 μL PI (Sigma; Cat. No. P4864) for 15 minutes at room temperature in the dark. Apoptosis was analyzed using a BD FACSCanto II flow cytometer, and 10,000 events were recorded for each sample.

Cell invasion assay (Transwell)

Transwell chambers (24-well) were precooled at 4°C. Matrigel (50 μL) was evenly applied to the upper surface of the membrane and allowed to polymerize at 37°C for 30 min. HTR-8/Svneo cells were digested with trypsin, resuspended in serum-free medium, and adjusted to a density of 1×10^5 cells/mL. A total of 100 μL of cell suspension was added to the upper chamber, while the lower chamber was filled with complete medium as a chemoattractant. After incubation for 48 h at 37°C, non-invading cells on the upper surface were gently removed with cotton swabs. Cells invading the lower surface were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min at room temperature. Stained cells were observed and photographed under an inverted microscope (Olympus CKX41). Invading cells were counted in five randomly selected fields per insert at ×100 magnification by two independent investigators blinded to group allocation. Inter-observer agreement was excellent (intraclass correlation coefficient >0.95).

Dual luciferase reporter assay

For the luciferase assay, HEK293T cells were seeded in 6-well plates (2×10^4 cells/well) and co-transfected using Lipofectamine 3000. The transfection mixture contained pmirGLO-POU2F1-wild-type (WT) or mutant (MUT) reporter plasmid, the miR-545-5p mimic or NC, and a Renilla luciferase plasmid (10:1) as an internal control. Luciferase activities were assessed 48 h post-transfection with the Dual-Glo system (Promega, USA), and results were presented as the Firefly/Renilla luminescence ratio.

RSA model establishment and lentiviral administration

Female CBA/J mice (n=40), male DBA/2 mice (n=15), and male BALB/c mice (n=5), weighing 20-25 g, were sourced from Hunan Slake Kingda Experimental Animal Co., Ltd. (Changsha, China; Animal License No. SCXK (Hunan) 2016-0002). All animal procedures were approved by the Laboratory Animal Ethics Committee of Nanjing University of Traditional Chinese Medicine (Approval No. DW2023-020). Mice were individually housed in plastic cages under controlled conditions (22±2°C, 40-55% humidity) with free access to food and water and a standard light-dark cycle. After one-week acclimatization, the normal pregnancy group comprised 10 female CBA/J mice mated with 5 male BALB/c mice (2:1). The RSA model was established by mating 30 female CBA/J mice with 15 male DBA/2 mice (2:1). Vaginal plugs were checked on the morning and evening of the next day. Detection of a plug was considered day zero of pregnancy, and then pregnant females were housed separately. To evaluate the in vivo role of XIST, systemic knockdown

Table 1. Primer sequence

Names	Forward (5'-3')	Reverse (5'-3')
XIST	CTTGGATGGGTTGCCAGCTA	TCATGCCCCATCTCCACCTA
miR-545-5p	ACACTCCAGCTGGGAGGGGCAG	CTCAACTGGTGTCGTGGAGTC
POU2F1	AATCCACTTTCCACCCTACGC	AAACAGGTAGCAGTTTGGGGT
IGFBP1	GCCAACGAGAACTCTATA	CACTGTTTGCTGTGATAA
PRL	TCTCACTACATCCATACC	CTTGTTCCTTGTCTTCAG

was performed using lentiviral vectors delivered via tail vein injection. Two days before mating, mice in the intervention groups received a single tail vein injection of lentiviral particles (sh-XIST or negative control (NC) sequence; 10 μL) diluted in 100 μL PBS [30]. RSA model mice were randomly assigned to three groups (n=10 per group): (1) RSA model group, (2) RSA + lentiviral NC group, (3) RSA + XIST lentiviral group.

Sample collection

On gestational day 14, pregnant mice were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg). Fetal resorption rate was calculated as the percentage of resorbed embryos relative to the total number of implantation sites. Placental tissues were carefully dissected; a portion of the tissues was snap-frozen in liquid nitrogen for molecular studies, while the remainder was fixed in 4% paraformaldehyde for histological evaluation.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of IL-1β and TNF-α were measured using commercially available mouse ELISA kits (mlbio; IL-1β: #ml301814, LLOD 10 pg/mL; TNF-α: #ml002095, LLOD 8 pg/mL) in strict accordance with the manufacturer's guidelines.

Quantitative real-time PCR (qRT-PCR)

Human chorionic villus tissues (experimental group and control group) as well as mouse chorionic tissues collected on gestational day 14 were used for RNA analysis. Total RNA was extracted using TRIzol reagent (15596-018, Thermo Fisher, Massachusetts, USA). Complementary DNA (cDNA) was synthesized by reverse transcription, and each sample was diluted 1:10 prior to amplification. Gene expression of IGFBP1, PRL, XIST and POU2F1 was quantified using the SCRIPT™ mRNA/lncRNA qRT-PCR Starter Kit. miR-545-5p expression was detected using the Bulge-Loop™ miRNA qRT-PCR Starter Kit (C10211-2, Ribobio, China). Primers for lncRNA XIST and miR-545-5p were purchased from Guangzhou Ribobio Co., and primer sequences are listed in **Table 1**.

Western blot analysis

Proteins from human and mouse placental tissues were extracted using 1% RIPA buffer (Beyotime, China) containing protease and phosphatase inhibitors. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto PVDF membranes. Membranes were blocked with 5% BSA prepared in TBST for 2 h at room temperature and incubated overnight at 4°C with primary antibodies diluted in TBST: anti-POU2F1 (1:2000; Proteintech, Cat# 10387-1-AP), anti-p65 (1:1000; Bioss, Cat# bs-23217R), anti-phospho-p65 (1:1000; Bioss, Cat# bs-0982R), and anti-GAPDH (1:4000; Abmart, Cat# TA-7021). After washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:2000; Cell Signaling Technology, Cat# 7074) for 2 h at room temperature. Protein bands were visualized using an ECL kit and quantified with ImageJ software.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptosis in mouse placental tissues was assessed using the TUNEL assay. Paraffin-embedded tissue sections were prepared and processed according to the manufacturer's instructions for the TUNEL detection kit (Beyotime, China). After staining, apoptotic cells were visualized and imaged under a light microscope.

Immunohistochemistry

Placental angiogenesis was evaluated by CD31 immunohistochemistry. Following deparaffinization and rehydration, tissue sections underwent antigen retrieval in EDTA buffer (pH 9.0) using microwave heating. After blocking endogenous peroxidase with 3% H_2O_2 and nonspecific binding with normal goat serum, sections were incubated overnight at 4°C with rabbit

anti-CD31 primary antibody (1:150, Bioss# bs-0195R). After incubation with secondary antibody, the signal was visualized using DAB chromogen, followed by hematoxylin counterstaining. Images were acquired using a Leica confocal microscope system.

Statistical analysis

All statistical analyses were performed using SPSS software (version 27.0). Data were presented as mean ± standard error of the mean (SEM) from at least three independent experiments. Prior to statistical comparison, data normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using the Brown-Forsythe test. For comparisons between two independent groups, an unpaired Student's t-test was used for normally distributed data, whereas the Mann-Whitney U test was applied when normality or variance homogeneity assumptions were not met. For comparisons among three or more independent groups, one-way analysis of variance (ANOVA) was performed for parametric data, followed by Tukey's honestly significant difference (HSD) post-hoc test. For non-parametric data, the Kruskal-Wallis test was applied, followed by Dunn's post-hoc test. Correlations between continuous variables were analyzed using Pearson's correlation coefficient for normally distributed data. A two-tailed *P* value <0.05 was considered statistically significant.

Result

Upregulation of lncRNA XIST regulated trophoblast proliferation, invasion, and apoptosis in RSA

Quantitative PCR analysis demonstrated that lncRNA XIST expression was significantly higher in villous tissues from RSA patients compared with those from normal early pregnancy termination controls (**Figure 1A**), suggesting potential involvement of lncRNA XIST in the pathogenesis of RSA. To elucidate the biological function of XIST, loss-of-function experiments were performed in HTR-8/Svneo cells. qPCR confirmed that transfection with shRNA specifically targeting XIST effectively reduced its expression (**Figure 1B**). CCK-8 assays indicated that XIST knockdown significantly promoted the proliferation of trophoblast cells (**Figure 1C**). Consistently, Transwell assays revealed that XIST knockdown significantly enhanced cell invasion ability (**Figure 1D**, **1E**). In contrast, flow cytometry analysis further demonstrated that XIST knockdown effectively inhibited cell apoptosis (**Figure 1F**, **1G**). Additionally, ELISA resluts revealed that the secretion of pro-inflammatory cytokines IL-1β and TNF-α was reduced after XIST knockdown (**Figure 1H**, **1I**). These results collectively indicate that elevated XIST expression suppresses trophoblast proliferation and invasion, while promoting apoptosis and inflammatory responses.

lncRNA XIST functioned as a ceRNA by sponging miR-545-5p to regulate POU2F1

To elucidate the molecular mechanism of XIST, the ENCORI (https://masysu.com/encori/index.php) database predicted that miR-545-5p harbors complementary binding sites for both XIST and the 3'UTR of POU2F1. Analysis of clinical samples supported this prediction. qPCR analysis revealed that miR-545-5p was notably decreased in the villous tissues of RSA patients compared to normal controls (P<0.0001; **Figure 2A**). Conversely, POU2F1 expression was significantly elevated in RSA tissues at both mRNA and protein levels (P<0.0001; **Figure 2B-D**).

This regulatory relationship was further confirmed *in vitro*. Knocking down XIST in HTR-8/Svneo cells resulted in increased miR-545-5p expression and a concomitant reduction of POU2F1 mRNA levels (P<0.0001, **P<0.01; **Figure 2E**, **2F**). Dual-luciferase reporter assays provided direct evidence of binding: miR-545-5p mimics potently inhibited the luciferase activity of vectors containing WT binding sites in XIST and POU2F1, whereas no inhibitory effect was observed in constructs harboring mutant sites (**Figure 2G-I**). Collectively, these results establish that XIST functions as a molecular sponge for miR-545-5p, thereby upregulating the expression of its target gene, POU2F1.

XIST inhibited trophoblast proliferation, invasion, and promoted apoptosis by regulating the miR-545-5p/POU2F1 axis

XIST-induced trophoblast dysfunction was mediated by its sponge effect on miR-545-5p: To ascertain whether the biological effects of XIST are mediated through the miR-545-5p/POU2F1 axis, a series of rescue experiments were per-

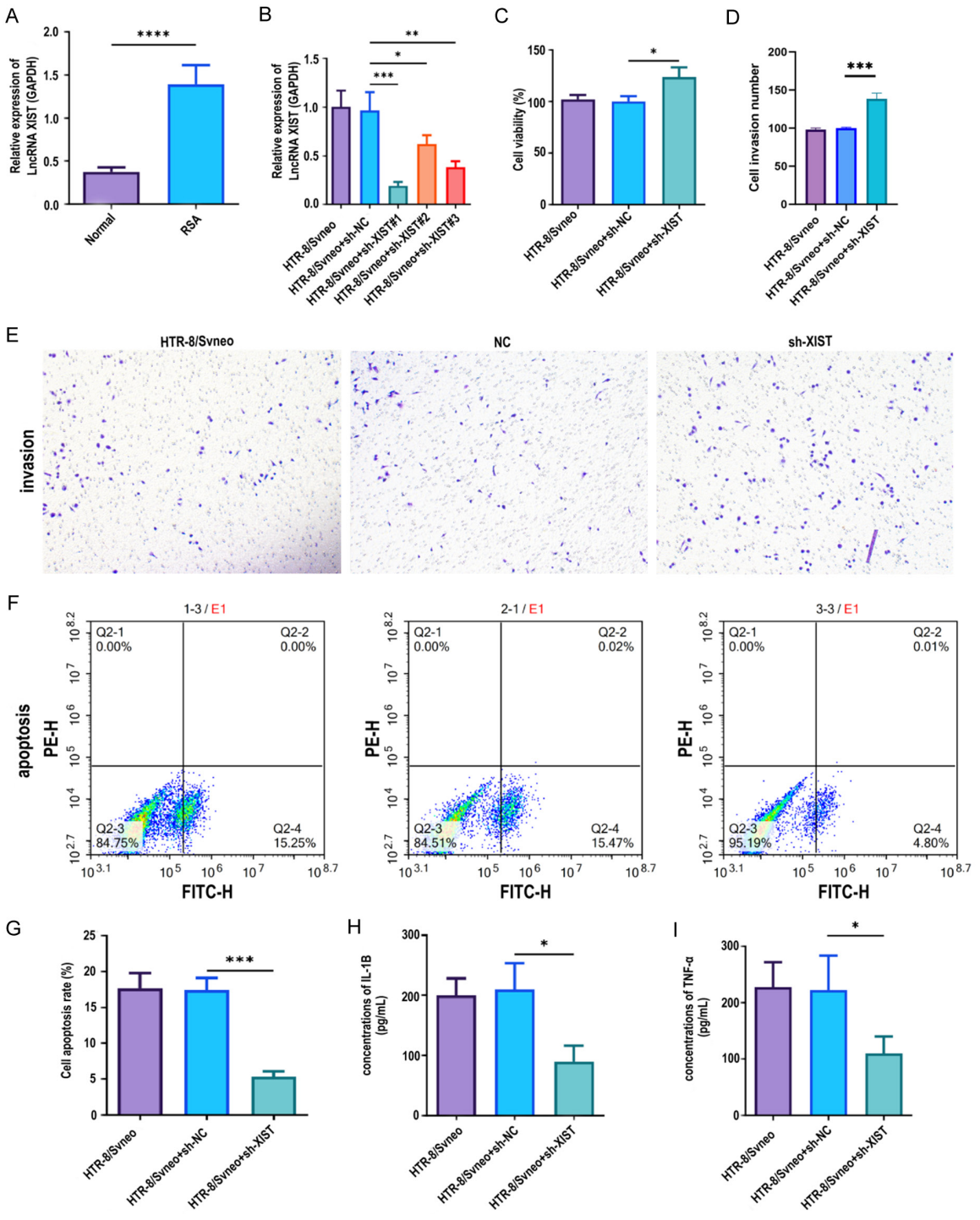

Figure 1. Effects of long non-coding RNA (lncRNA) XIST knockdown on trophoblast proliferation, invasion, and apoptosis in recurrent spontaneous abortion (RSA). (A) The expression of lncRNA XIST was detected by quantitative real-time PCR (qPCR) in placental chorionic tissues from RSA patients and healthy controls. (B) Transfection efficiency of short hairpin RNA (shRNA) #1, #2, and #3 targeting XIST (sh-XIST) in HTR-8/Svneo cells was detected by qPCR. (C) Cell viability was assessed by cell counting kit-8 (CCK-8) assay after XIST knockdown. (D, E) Cell invasion was evaluated by Transwell assay (scale bar = 100 μm). (F, G) Cell apoptosis was analyzed by flow cytometry. (H, I) The concentrations of inflammatory cytokines (H) IL-1β and (I) TNF-α in cell supernatant were measured using enzyme-linked immunosorbent assay (ELISA) after XIST knockdown. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

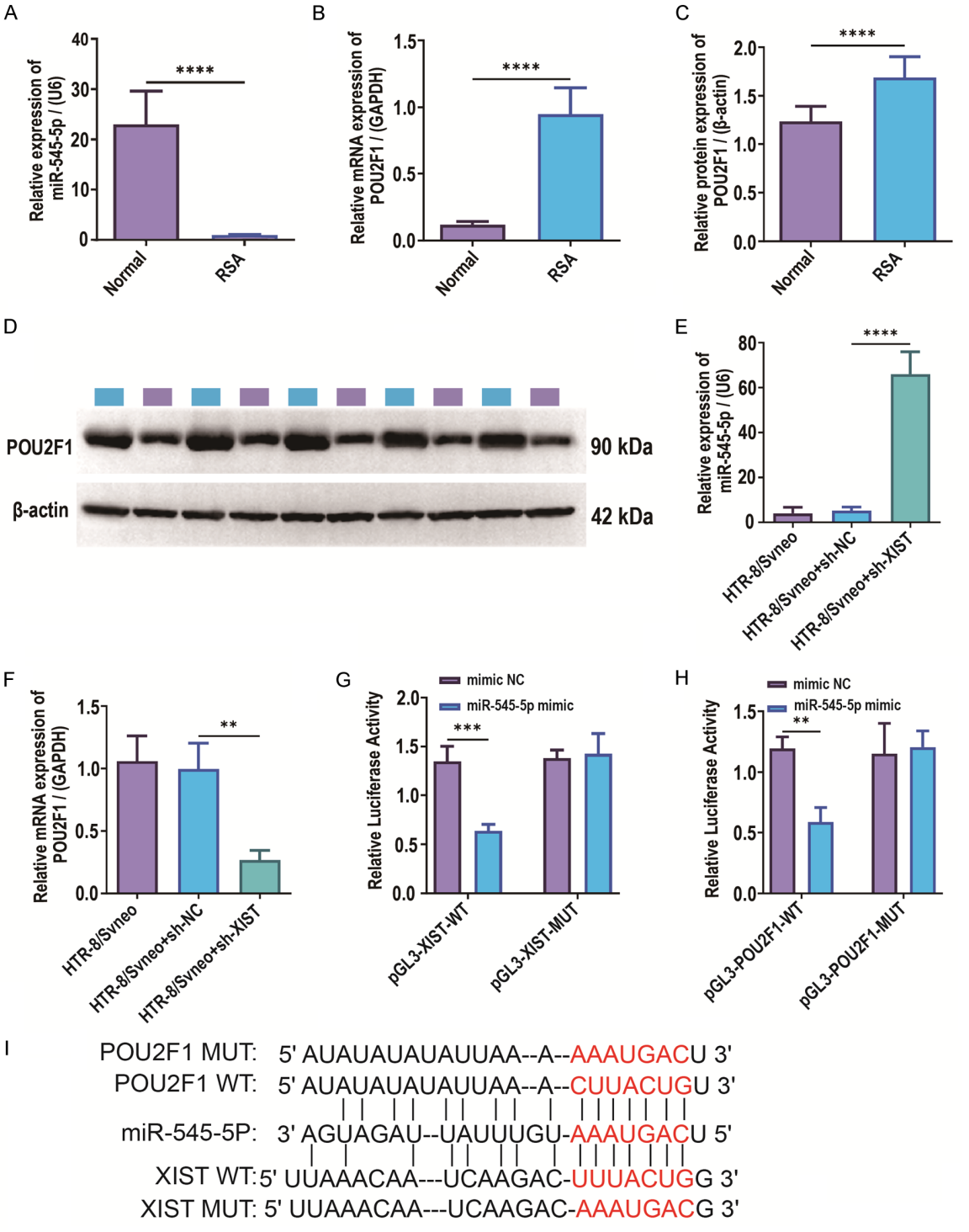

Figure 2. lncRNA XIST functioned as a competitive endogenous RNA (ceRNA) that sponges miR-545-5p to regulate POU2F1 expression. A, B. Relative expression levels of miR-545-5p and POU2F1 mRNA in clinical placental tissues were detected by qPCR. C, D. POU2F1 protein expression in clinical placental tissues was detected by western blot. E, F. Relative expression levels of miR-545-5p and POU2F1 mRNA in HTR-8/Svneo cells following XIST knockdown were detected by qPCR. G. Luciferase activity in HEK293T cells co-transfected with wild-type (WT) or mutant (MUT) XIST reporter and miR-545-5p mimic or NC. H. Luciferase activity in HEK293T cells co-transfected with WT or MUT POU2F1 3'UTR reporter and miR-545-5p mimic or NC. I. Predicted binding site within the lncRNA XIST/miR-545-5p/POU2F1 axis. **P<0.01, ***P<0.001, ****P<0.0001.

formed. First, we verified that miR-545-5p serves as a crucial downstream mediator of XIST. Knockdown of XIST significantly enhanced the viability (**Figure 3A**) and invasion capacity (**Figures 3F**, **3G**, S1, S2) of HTR-8/Svneo cells, while reducing the apoptosis rate and secretion of inflammatory cytokines (**Figures 3B-E**, S1, S2). However, simultaneous inhibition of miR-545-5p markedly attenuated the protective effects induced by XIST knockdown (**Figure 3A-E**). At the molecular level, XIST knockdown significantly reduced POU2F1 protein levels and suppressed the NF-κB pathway (p-p65/p65 ratio), whereas inhibition of miR-545-5p reversed these molecular changes (**Figure 3H**, **3I**). These findings indicate that the detrimental effects of XIST on trophoblast function depend on its negative regulation of miR-545-5p.

Overexpression of miR-545-5p preserved trophoblast function: Next, the independent biological role of miR-545-5p was examined. Transfection with a miR-545-5p mimic significantly enhanced cell viability (**Figure 4A**) and invasion capacity (**Figures 4F**, **4G**, S1, S2), while inhibiting apoptosis and inflammatory cytokine secretion (**Figures 4B-E**, S1, S2). Correspondingly, at the molecular level, miR-545-5p overexpression significantly reduced POU2F1 protein levels and inhibited the NF-κB pathway (**Figure 4H**, **4I**).

POU2F1 functioned as a key downstream target of miR-545-5p: To confirm that POU2F1 mediates the protective effects of miR-545-5p, we conducted a rescue experiment by co-transfecting a POU2F1 overexpression plasmid in cells overexpressing miR-545-5p. Forced restoration of POU2F1 effectively reversed the miR-545-5p-induced improvements in cell viability (**Figure 5A**), cytokine secretion (IL-1β and TNF-α, **Figure 5B**, **5C**), apoptosis (**Figures 5D**, **5E**, S1, S2), and invasion (**Figures 5F**, **5G**, S1, S2). Western blot analysis further confirmed that enforced POU2F1 expression counteracted the suppression of the NF-κB pathway caused by miR-545-5p overexpression (**Figure 5H**, **5I**). In summary, these experiments demonstrate that upregulated XIST acts as a competing endogenous RNA that sponges the protective miR-545-5p, thereby relieving its repression of POU2F1. This results in increased POU2F1 expression, activation of the NF-κB pathway, ultimately driving trophoblast dysfunction.

Role of the XIST/miR-545-5p/POU2F1 axis in RSA pathogenesis in vivo

After establishing the XIST/miR-545-5p/POU2F1 regulatory axis *in vitro*, we next evaluated its in vivo relevance and therapeutic potential using a well-characterized RSA mouse model. Consistent with the *in vitro* findings, the RSA model group exhibited a significantly elevated embryo resorption rate, which was effectively ameliorated after *in vivo* knockdown of XIST (**Figure 6A**, **6B**). Histological analysis demonstrated that XIST knockdown alleviated placental apoptosis (**Figure 6C**, **6D**), promoted angiogenesis (**Figure 6E**, **6F**), and restored the expression of decidualization markers (**Figure 6G**, **6H**). At the molecular level, placental tissues from RSA mice recapitulated the dysregulation observed *in vitro*, characterized by elevated XIST and POU2F1 levels, suppressed miR-545-5p expression (**Figure 6I-K**). XIST knockdown effectively normalized the expression of this axis and its downstream inflammatory markers, including IL-1β and TNF-α (**Figure 6L**, **6M**). WB analysis further confirmed that knockdown of XIST inhibited the activation of the NF-κB pathway in RSA mice (**Figure 6N**, **6O**). These findings conclusively demonstrate that the entire XIST/miR-545-5p/POU2F1/NF-κB signaling axis is activated in placental tissues during RSA pathogenesis.

Discussion

At present, the pathogenesis of RSA is multifactorial and remains incompletely understood. Current interventions mainly include progesterone supplementation and immunotherapy; however, their efficacy is not satisfactory and potential risks remain [31]. This study provides mechanistic evidence that the lncRNA XIST/miR-545-5p/POU2F1 regulatory axis contributes to the pathogenesis of RSA through a ceRNA mechanism, ultimately leading to aberrant activation of the NF-κB pathway and trophoblast dysfunction.

Trophoblasts are the key functional cells involved in embryo implantation and placental formation. After fertilization and implantation, trophoblasts located in the outer layer of placen-

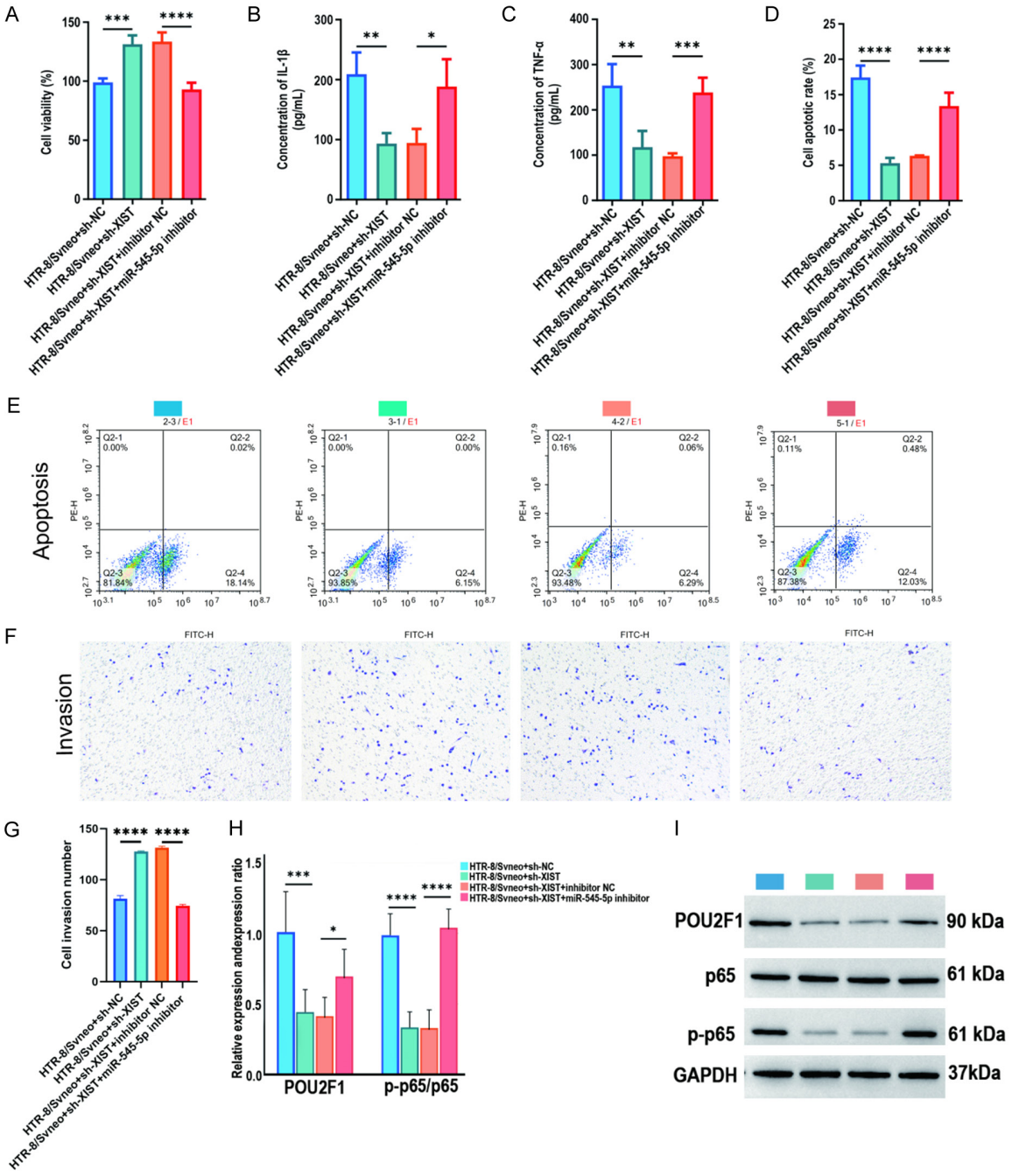

Figure 3. Knockdown of XIST promoted trophoblast function in a miR-545-5p-dependent manner. A. Cell viability was assessed by CCK-8 assay. B, C. Secretion levels of the pro-inflammatory cytokines IL-1β and TNF-α were measured by ELISA. D, E. Apoptosis rates were analyzed by flow cytometry. F, G. Cell invasion ability was evaluated by Transwell assay (scale bar = 100 μm). H, I. Western blot analysis of POU2F1 expression and NF-κB pathway activation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

tal villi rapidly proliferate, differentiate, and invade the maternal decidua, gradually invading the basal layer of the uterus and blood vessels. This process promotes spiral artery remodeling and neovascularization, laying the foundation for the development of the embryo and the placenta. The invasive function of the cells is an important basis for ensuring the reconstruction of the uterine spiral arteries and successful pregnancy [32, 33]. During early

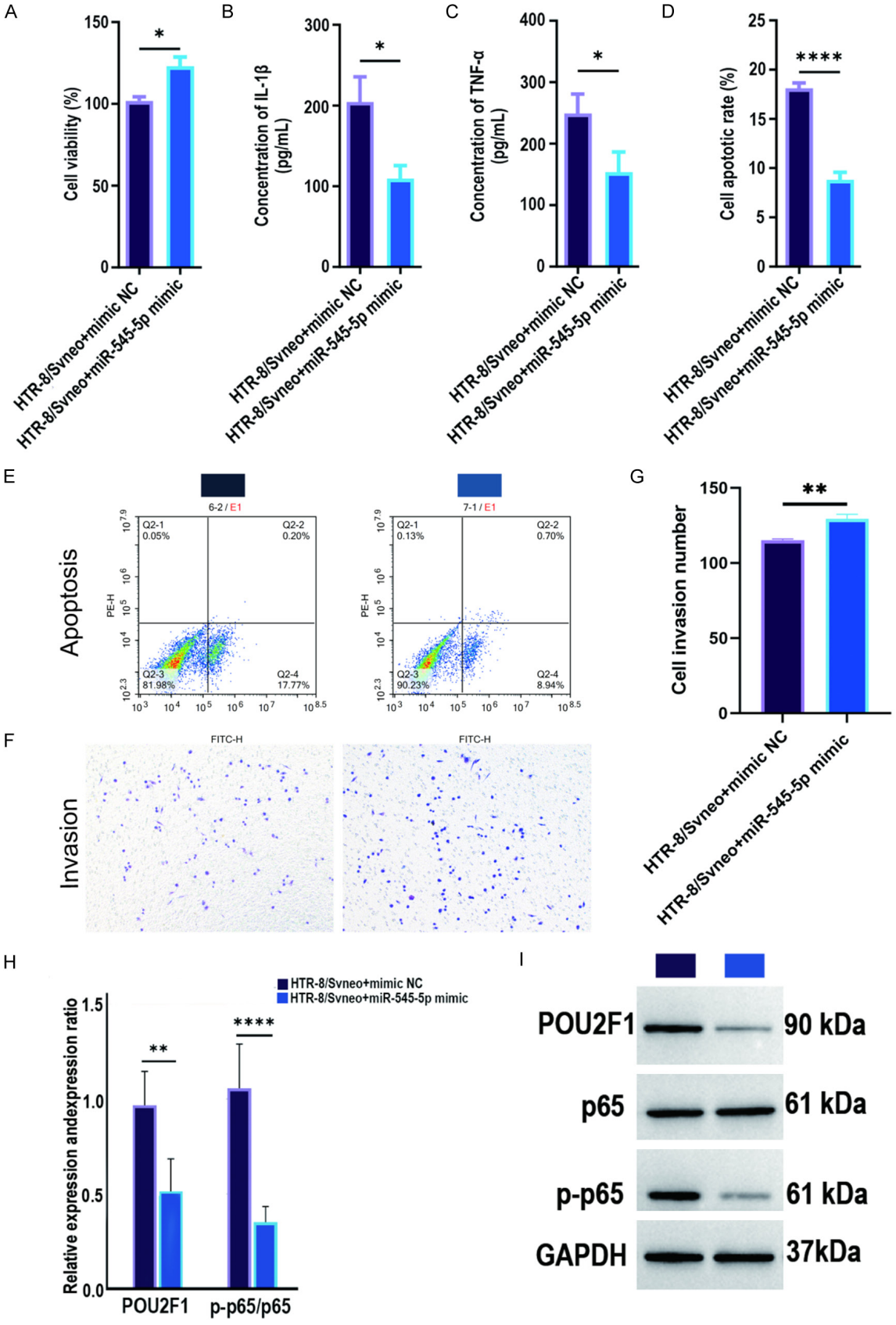
A
Cell viability (%)
150
100
50
0
*
HTR-8/Svneo+mimic NC
HTR-8/Svneo+miR-545-5p mimic
B
Concentration of IL-1β (pg/mL)
300
200
100
0
*
HTR-8/Svneo+mimic NC
HTR-8/Svneo+miR-545-5p mimic
C
Concentration of TNF-α (pg/mL)
400
300
200
100
0
*
HTR-8/Svneo+mimic NC
HTR-8/Svneo+miR-545-5p mimic
D
Cell apototic rate (%)
25
20
15
10
5
0
****
HTR-8/Svneo+mimic NC
HTR-8/Svneo+miR-545-5p mimic
E
Apoptosis
6-2 / E1
7-1 / E1
Q2-1
0.05%
Q2-2
0.20%
Q2-3
81.98%
Q2-4
17.77%
Q2-1
0.13%
Q2-2
0.70%
Q2-3
90.23%
Q2-4
8.94%
PE-H
G
Cell invasion number
150
100
50
0
**
HTR-8/Svneo+mimic NC
HTR-8/Svneo+miR-545-5p mimic
F
Invasion
FITC-H
FITC-H
H
HTR-8/Svneo+mimic NC
HTR-8/Svneo+miR-545-5p mimic
Relative expression andexpression ratio
1.5
1.0
0.5
0.0
**
****
POU2F1
p-p65/p65
I
POU2F1
90 kDa
p65
61 kDa
p-p65
61 kDa
GAPDH
37kDa

Figure 4. Overexpression of miR-545-5p enhanced trophoblast viability and invasion while suppressing apoptosis and inflammation. A. Cell viability was assessed by the CCK-8 assay. B, C. Secretion levels of the pro-inflammatory cytokines IL-1β and TNF-α were measured by ELISA. D, E. Apoptosis rates were analyzed by flow cytometry. F, G. Cell invasion ability was evaluated by Transwell assay (scale bar = 100 μm). H, I. Western blot analysis of POU2F1 expression and NF-κB pathway activation. *P<0.05, **P<0.01, ****P<0.0001.

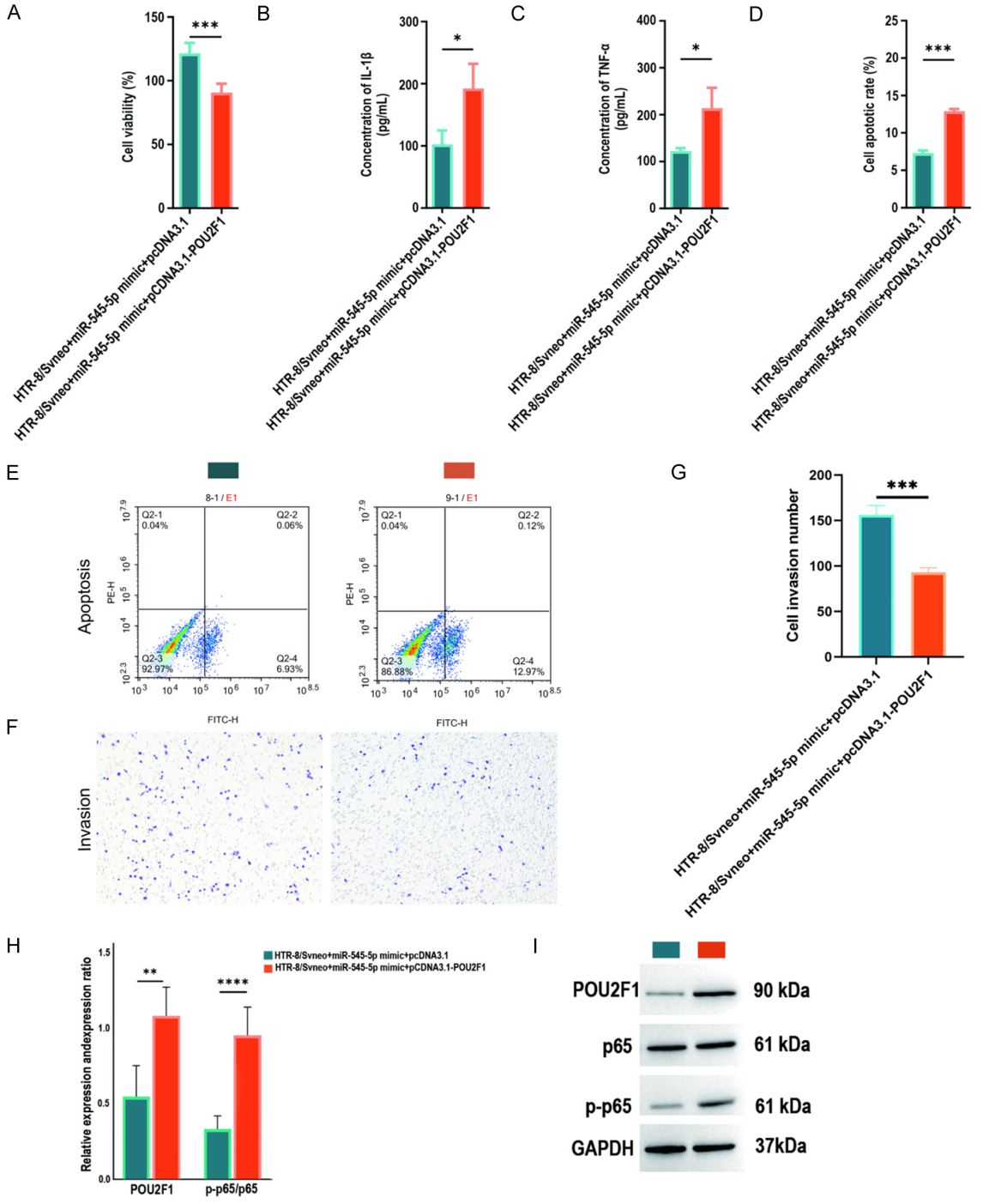

Figure 5. Restoration of POU2F1 reversed the protective effects induced by miR-545-5p overexpression. A. Cell viability was assessed using CCK-8 assay. B, C. Secretion levels of the pro-inflammatory cytokines IL-1β and TNF-α measured by ELISA. D, E. Apoptosis rates were analyzed by flow cytometry. F, G. Cell invasion ability was evaluated by Transwell assay (scale bar = 100 μm). H, I. Western blotting was used to detect POU2F1 protein levels and NF-κB pathway activation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

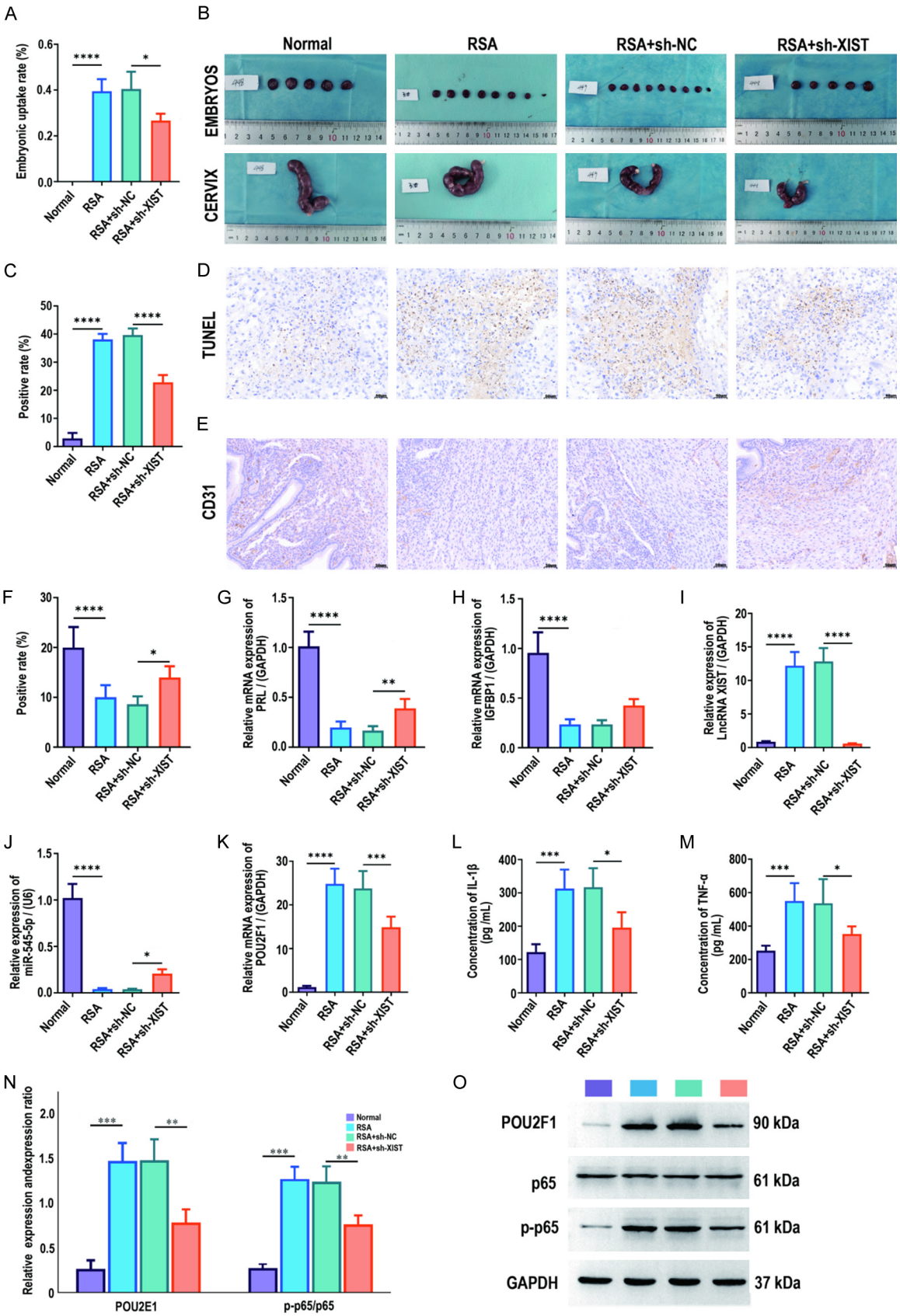
A
Embryonic uptake rate (%)
Normal
RSA
RSA+sh-NC
RSA+sh-XIST
B
Normal
RSA
RSA+sh-NC
RSA+sh-XIST
EMBRYOS
CERVIX
C
Positive rate (%)
D
TUNEL
E
CD31
F
Positive rate (%)
G
Relative mRNA expression of PRL / (GAPDH)
H
Relative mRNA expression of IGFBP1 / (GAPDH)
I
Relative expression of LncRNA XIST / (GAPDH)
J
Relative expression of miR-545-5p / (U6)
K
Relative mRNA expression of POU2F1 / (GAPDH)
L
Concentration of IL-1β (pg /mL)
M
Concentration of TNF-α (pg /mL)
N
Relative expression andexpression ratio
POU2E1
p-p65/p65
O
POU2F1
90 kDa
p65
61 kDa
p-p65
61 kDa
GAPDH
37 kDa

Figure 6. The XIST/miR-545-5p/POU2F1 axis is critically involved in RSA pathogenesis *in vivo.* A, B. Representative anatomical images of uterine horns from different groups and quantitative analysis of the embryo resorption rate. C, D. Apoptosis in placental tissues was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (scale bar = 50 μm). E, F. Placental angiogenesis was assessed by immunohistochemical staining for CD31 (scale bar = 50 μm). G, H. The mRNA expression levels of PRL, and IGFBP1 in placental tissues were detected by qRT-PCR. I-K. The mRNA expression levels of lncRNA XIST, miR-545-5p and POU2F1 in placental tissues were detected by detected by qRT-PCR. L, M. The serum concentrations of IL-1β and TNF-α were measured by ELISA. N, O. The protein expression of POU2F1 and NF-κB pathway-related proteins in placental tissues was detected by western blot. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

pregnancy, trophoblast adhesion to endometrial cells is also crucial. The strong adhesive properties of trophoblasts facilitate stable embryo implantation and contribute to functional placental formation [34]. During normal pregnancy, the proliferation and apoptosis of trophoblasts are in a dynamic balance. Disruption of this balance, manifested as insufficient proliferation or excessive apoptosis, can affect placental function, leading to abnormal embryonic development and even miscarriage [35, 36].

As a major class of epigenetic regulators, lncRNA modulate various physiological processes at the cellular level, such as X chromosome inactivation [37], genomic imprinting, chromatin modification, transcriptional regulation, and nuclear transport. Additionally, lncRNA impacts embryonic stem cell differentiation, pluripotency, cell cycle regulation, and cell apoptosis, thereby playing critical roles in development and disease [38, 39]. Transcriptomic analyses have identified 1,449 lncRNAs with dysregulated expression in villous tissue from RSA patients [40]. Further research revealed that lncRNAs regulate trophoblast invasion by modulating transcription of target genes, such as plasma cell tumor variant translocation gene 1, thereby affecting placental development and pregnancy outcomes [41]. In this study, lncRNA XIST was found to be significantly upregulated in RSA placental villous tissue samples. Previous reports indicate that lncRNA XIST suppresses trophoblast proliferation and invasion, leading to diseases such as miscarriage and eclampsia. These findings support the hypothesis that aberrant XIST expression may contribute to RSA. Further detection confirmed that lncRNA XIST is highly expressed in RSA placental villous tissue, suggesting its involvement in disease progression.

The targeted relationship of lncRNA XIST with miR-545-5p was verified through Starbase database prediction, qRT-PCR, and dual luciferase reporter assays. In RSA villous tissues, XIST and POU2F1 expression levels were significantly elevated, along with a considerable downregulation in the expression of miR-545-5p, supporting a regulatory role of miR-545-5p in modulating POU2F1 expression. These findings suggest that the XIST/miR-545-5p/POU2F1 axis may participate in multiple aspects of trophoblast biology. Evidence has demonstrated that aberrant expression of lncRNA and miRNAs is associated with adverse pregnancy outcomes and pregnancy-related disorders [42]. In this study, knockdown of XIST significantly reduced the expression of XIST and POU2F1 and upregulated the expression level of miR-545-5p, further supporting the existence of this regulatory axis.

Previous studies have shown that transient activation of the NF-κB pathway during early pregnancy is beneficial for embryo implantation process in mice [43]. However, excessive activation of NF-κB can promote trophoblast apoptosis and contribute to placental hemorrhage and necrosis, potentially triggering abnormal maternal-fetal immune responses [44]. Furthermore, NF-κB, as a crucial transcription factor, plays a central role in various cellular processes such as inflammation and cell apoptosis.

Our data demonstrate that phosphorylation of p65 is influenced by the XIST/miR-545-5p/POU2F1 axis, suggesting that XIST may promote RSA progression through POU2F1-dependent activation of NF-κB pathway. The key role of NF-κB in the trophoblast and placenta prompted us to explore the role of lncRNA XIST in activating the NF-κB pathway in RSA. In this study, functional assays, including CCK-8, western blot, flow cytometry, Transwell, and ELISA were used to detect the effects of lncRNA XIST on the proliferation, apoptosis, and invasion of human trophoblast cell lines (HTR-8/Svneo) and its correlation with the NF-κB pathway,

indicating that silencing XIST reduced trophoblast apoptosis, enhanced invasion ability, suppressd NF-κB activation, and decreased the expression of TNF-α and IL-1β. Collectively, these findings indicate that inhibition of XIST expression may attenuate trophoblast inflammatory responses and improve trophoblast function [45-47].

LncRNA XIST, a ~17 kb-long RNA transcribed from the inactive X chromosome in female mammals, is implicated in regulating X chromosome inactivation, leading to the genetic silencing of one X chromosome during development [48]. In the biological context, its functions are primarily associated with diverse pathological processes, including the modulation of cell proliferation, invasion, migration, differentiation, drug resistance, and apoptosis. Depending on the cellular context, XIST can function either as an oncogenic suppressor or driver in various cancer types [49].

miR-545 has been reported to be down-regulated in several cancers, including oral squamous cell carcinoma, hepatocellular carcinoma, and cervical cancer, affecting apoptosis, proliferation, invasion, and migration [50-52]. Previous studies demonstrated that miR-545-5p directly targets c-Met, a key player in epithelial-mesenchymal transition and modulates tumor cell proliferation, migration, and invasion [53]. POU2F1, also known as OCT1 (octamer binding transcription factor 1), is a member of the POU domain transcription factor family and is widely expressed across tissues [54]. POU2F1 plays a role as a proto-oncogene in most cases, and has prognostic and diagnostic value in multiple malignant tumors. Miao ZH et al., reported that LINC00342 promotes tumor cell proliferation, migration, and invasion through the miR-545-5p/MDM2 axis in vitro, while suppressing cell apoptosis. LINC00342 knockdown inhibits the growth of colon adenocarcinoma (COAD) in vivo by regulating miR-545-5p/MDM2 [55]. In summary, these studies have provided a new perspective for exploring the functions of lncRNA and regulating miRNA so as to understand their role in disease development [56].

Conclusion

lncRNA XIST is upregulated in RSA and contributes to trophoblast dysfunction by sponging and inactivating the protective miR-545-5p, leading to the derepression of POU2F1 and subsequent activation of the NF-κB pathway. This promotes inflammation, inhibits trophoblast invasion and proliferation, and induces excessive apoptosis. Therefore, targeting the XIST/miR-545-5p/POU2F1 axis may represent a promising therapeutic strategy for RSA, although further studies are needed to explore its clinical application value.

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Disclosure of conflict of interest

None.

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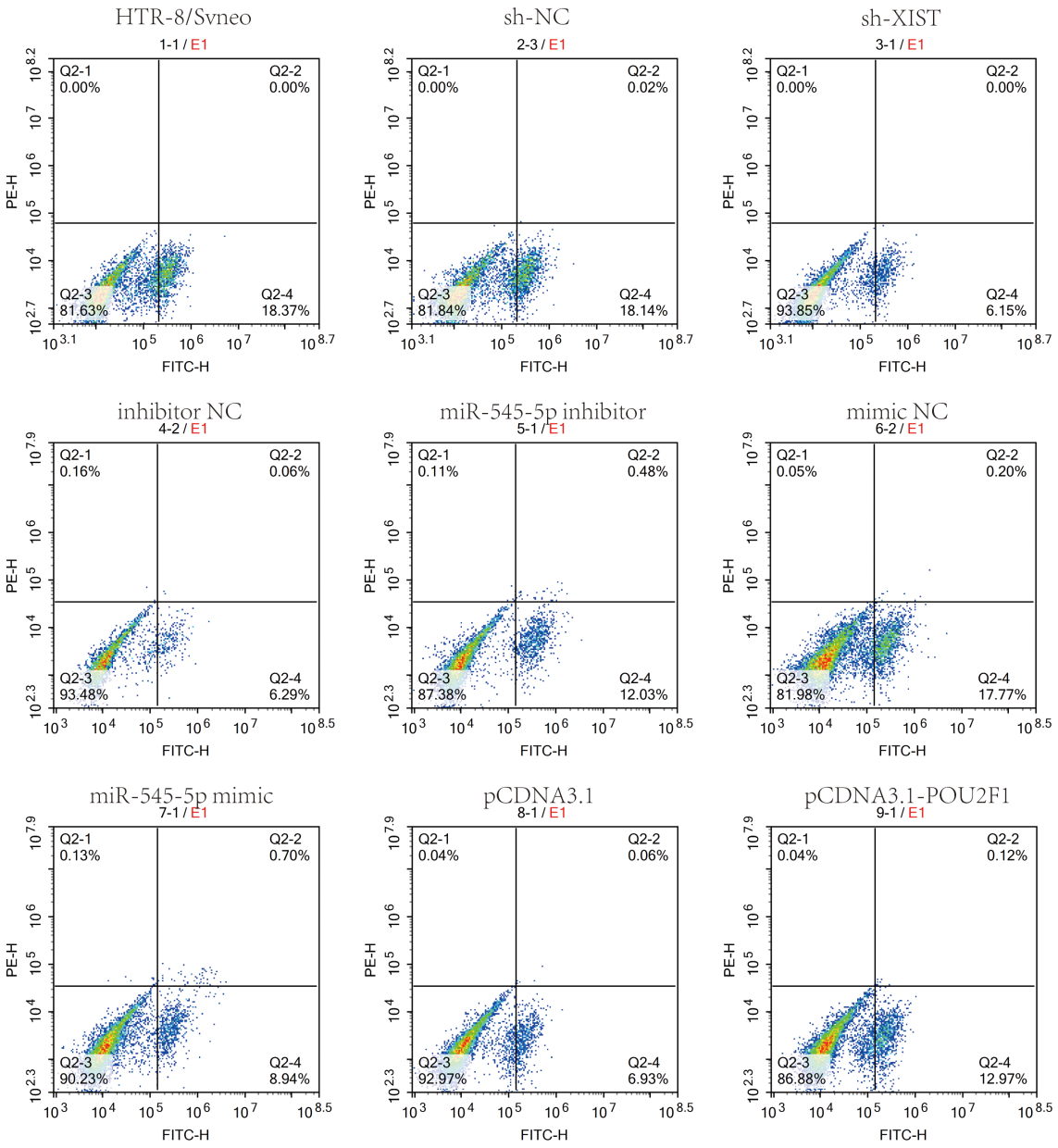

Figure S1. Cell apoptosis was assessed by flow cytometry using Annexin V-FITC/propidium iodide (PI) double staining. Representative dot plots showing apoptosis in each group are presented.

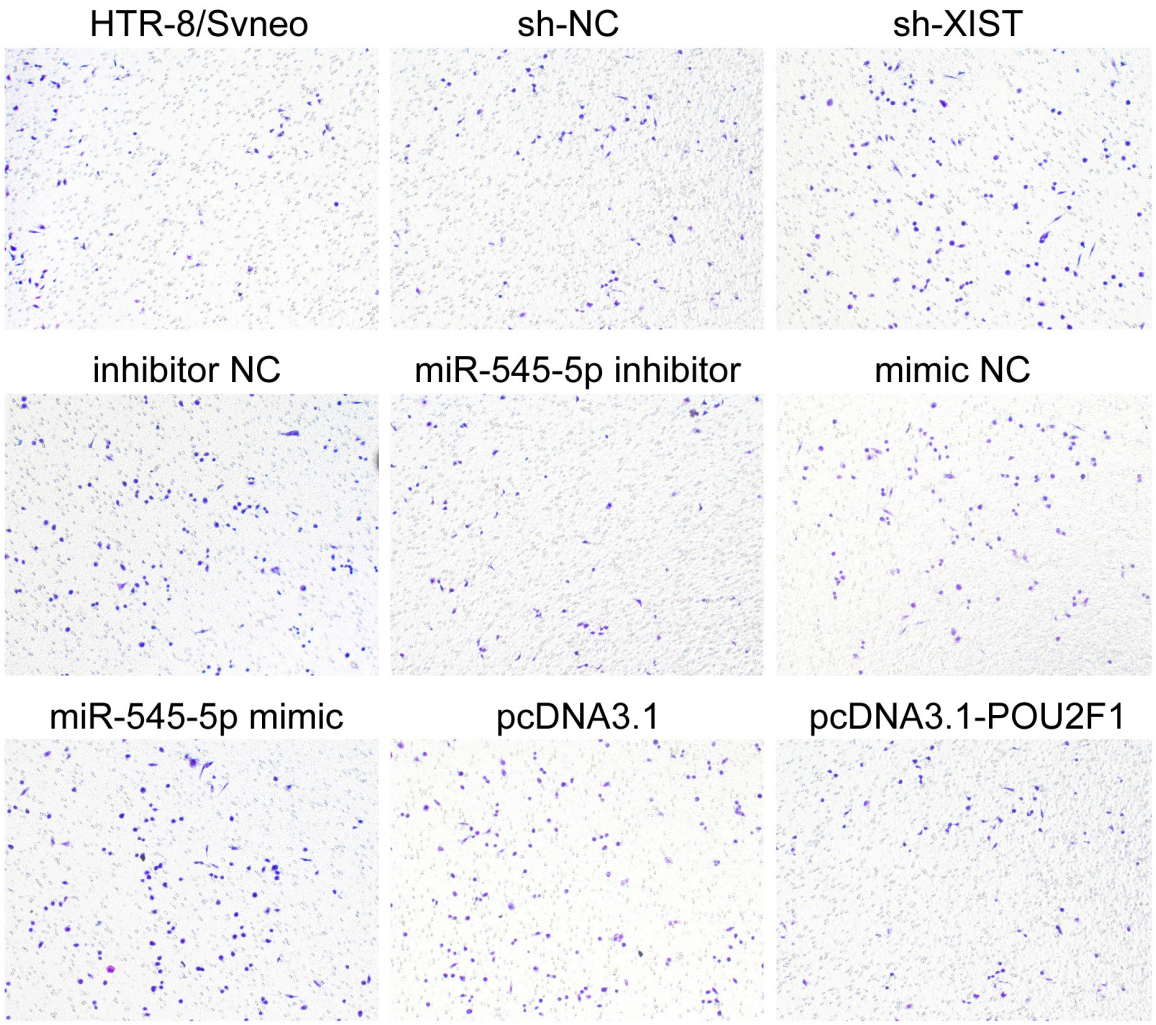

Figure S2. Cell invasion ability was evaluated using a Matrigel-coated Transwell assay. Representative images of invaded cells stained with crystal violet are shown (scale bar = 100 μm).