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LncRNA NEAT1 participates in diminished ovarian reserve by affecting granulosa cell apoptosis and estradiol synthesis via the miR-204-5p/ESR1 axis

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Abstract

Long non-coding RNAs (lncRNAs) affect the biological functions of granulosa cells (GCs) through multiple mechanisms, including epigenetic regulation, transcriptional regulation, post-translational modification, and cell signaling. Our previous study found that lncRNA NEAT1 expression is significantly downregulated in the GCs of patients with diminished ovarian reserve (DOR); however, its exact regulatory mechanism remains unclear. This study aimed to investigate the role of NEAT1 in GC function and DOR pathogenesis. We determined that the downregulated NEAT1 expression in the GCs of patients with DOR is closely associated with ovarian reserve function and assisted reproductive outcomes. Functional assays revealed that NEAT1 promotes KGN cell proliferation by increasing the proportion of S-phase cells and inhibiting apoptosis. Bioinformatics analysis combined with dual-luciferase reporter assays confirmed that NEAT1 acts as a molecular sponge for miR-204-5p, thereby upregulating ESR1, a direct target gene of miR-204-5p. Additionally, both NEAT1 and ESR1 exhibited significantly different. Mechanistic experiments demonstrated that NEAT1 acts as a competitive endogenous RNA and adsorbs miR-204-5p through molecular sponging, thereby promoting the expression of ESR1 and upregulating the expression of key enzymes (steroidogenic acute regulatory protein and cytochrome P450 family 19 subfamily A member 1) involved in the synthesis of steroid hormones. This induces estradiol biosynthesis and activates the downstream mitogen-activated protein kinase (MAPK) signaling pathway, increasing the phosphorylation of extracellular signal-related kinase and cyclic adenosine monophosphate response element-binding protein, which collectively drives cell cycle progression, enhances proliferation, and inhibits apoptosis of KGN cells. This suggests that NEAT1 regulates GC proliferation, apoptosis, and steroidogenesis via the miR-204-5p/ESR1/MAPK axis, providing novel insights into the epigenetic mechanisms underlying DOR pathogenesis.

Keywords LncRNA NEAT1, miR-204-5p/ESR1 axis, Diminished ovarian reserve, Apoptosis, Estradiol synthesis

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Introduction

Diminished ovarian reserve (DOR) is characterized by a decrease in the number of recruitable follicles retained in the ovaries and a decline in oocyte quality. DOR results from a combination of risk factors, including age, genetics, iatrogenic, immune, and environmental factors [1]. The clinical manifestations of DOR include menstrual disorders, symptoms associated with sex hormone deficiency or fluctuation, and reduced fertility, which seriously affects women's reproductive health and quality of life. Regarding treatment strategies for DOR, hormone replacement therapy alleviates the symptoms associated with estrogen deficiency; however, it fails to restore ovarian function and has little effect on fertility [2]. Therefore, an in-depth study of DOR pathogenesis and exploration of effective interventions are essential for the protection of women's reproductive health.

Granulosa cells (GCs), the key components of follicles, are the primary sites of estrogen synthesis. Communication and interaction between GCs and oocytes are instrumental in oocyte development, maturation, and fertilization and are bidirectional [3]. Evidence suggests that the apoptotic, proliferative, and steroid hormone-synthesizing capacities of GCs are essential in folliculogenesis [4, 5]. GC dysfunction induces follicular atresia, leading to a decrease in the number and quality of oocytes and estrogen levels, which are key factors in DOR pathogenesis [6]. Investigating the mechanisms that regulate the biological functions of GCs is crucial for a deeper understanding of the molecular basis of DOR and identifying potential targets for the development of targeted therapeutic approaches.

Competing endogenous RNAs (ceRNAs) constitute a complex regulatory network in which non-coding RNAs, such as long non-coding RNAs (lncRNAs) or circular RNAs, and mRNAs share the same miRNA response elements, thus forming a competitive relationship to regulate each other's expression levels [7, 8]. Thus, ceRNAs play an important role in regulating gene expression. ceRNA-mediated regulation is involved in cell proliferation, differentiation, apoptosis, and other biological processes [9] and plays an important role in the development of tumors and female reproductive endocrine diseases [10, 11]. As important transcriptional regulatory molecules in the ceRNA network, lncRNAs, such as H19, HOTAIR, and MALAT1, affect biological functions, hormone synthesis, and secretion in GCs by regulating key signaling pathways, such as the PI3K/Akt and TGFβ/SMAD [12, 13] pathways. A previous study [14] showed that lncRNA NEAT1 expression is significantly reduced in the GCs of women with DOR and is used as a biomarker of DOR; however, the specific molecular mechanism remains poorly elucidated.

In the current study, we collected clinical GC samples from patients with DOR and constructed a ceRNA regulatory axis using bioinformatics to investigate the function and potential molecular mechanisms of NEAT1 in DOR. The results showed that NEAT1 acts as a molecular sponge to regulate the mitogen-activated protein kinase (MAPK) signaling pathway by adsorbing miR-204-5p and upregulating estrogen receptor 1 (ESR1), thereby inhibiting apoptosis, promoting cell proliferation, and affecting the cell cycle and steroid biosynthetic functions in GCs. This study explored the potential role of ceRNAs in DOR pathogenesis from a novel perspective. The discovery of a therapeutic target may provide a new scientific basis for protecting women's reproductive health, and allow for future clinical applications.

Materials and methods

Ethical approval of the study protocol

This study was approved by the Reproductive Medicine Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (Ref: 2021-103-KY); written informed consent was obtained from all participants and the study was performed in strict compliance with the Declaration of Helsinki.

Clinical sample collection

Forty patients who underwent assisted reproductive technology (ART)-based assisted conception using an antagonist regimen at the Department of Reproduction and Genetics (Affiliated Hospital of Shandong University of Traditional Chinese Medicine) were included in this study. The study included 20 patients with DOR and 20 women with normal ovarian reserve (NOR). The diagnosis of DOR was based on the fulfillment of at least two of the following criteria [15]: (i) bilateral anterior follicle count (AFC) < 6, (ii) anti-*Müllerian* hormone (AMH) < 1.10 ng/mL, and (iii) basal follicle-stimulating hormone (FSH) of 10–40 mIU/mL. The NOR group included women with normal ovarian reserve but infertility due to male or tubal factors. Patients with structural malformations of the reproductive organs, endometriosis, other endocrine disorders (such as thyroid dysfunction and hyperprolactinemia), or chromosomal abnormalities were excluded to avoid confounding effects on the ovarian reserve parameters and hormone levels. Follicular fluid discarded after oocyte retrieval was collected, and GCs were extracted, purified via density gradient centrifugation [16], and immediately stored in a refrigerator at -80 °C.

Cell culture

The human ovarian GC tumor cell line KGN was purchased from Procell Life Science and Technology Co. (Wuhan, China). The cells were cultured in Dulbecco's

modified Eagle's medium/F-12 medium (BasalMedia, Shanghai, China) supplemented with 10% fetal bovine serum (BasalMedia) and 1% penicillin-streptomycin solution (Biosharp, Hefei, China). Cells were routinely passaged in a 37 °C, 5% CO_2 incubator, and passaged with 0.25% trypsin-EDTA (Biosharp) digestion, while the cell density was maintained at 80–90%.

Cell transfection

NEAT1 overexpression plasmid (OE-NEAT1), NEAT1 small interfering RNA (siRNA; si-NEAT1), ESR1 siRNA (si-ESR1), miR-204-5p mimics (mimics-miR-204-5p), miR-204-5p inhibitor (inhibitor-miR-204-5p), and the corresponding negative controls were obtained from Tsingke Biotech Co. Ltd. (Beijing, China). KGN cells were inoculated in six-well plates (2×10^5 cells/well) 24 h before transfection, and cell fusion reached 70–80% at transfection. Plasmids, siRNAs, and miR-204-5p mimics/inhibitors were transfected into KGN cells using Lipofectamine® 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 6 h of transfection, the medium was changed to complete medium, and incubation was continued for 48 h. Transfection efficiency was determined using quantitative reverse transcription PCR (RT-qPCR).

Fluorescence in situ hybridization (FISH)

A FISH Kit (RiboBio, Guangzhou, China) was used to analyze the subcellular localization of NEAT1. KGN cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 10 min. Prehybridization buffer (containing 50% formamide and 2× SSC buffer) was added to the cells that were incubated for 30 min, followed by overnight incubation in a hybridization buffer containing a fluorescein isothiocyanate (FITC)-labeled NEAT1 probe (Sangon Biotech, Shanghai, China). The following day, the cells were washed with a gradient of 2×, 1×, and 0.5× SSC buffer to remove unbound probes, while the nuclei were counterstained with DAPI for 8 min under dark conditions, blocked, and imaged under a laser confocal microscope (Leica, Germany).

Dual-luciferase reporter assay

Bioinformatic analysis predicted the downstream targets and complementary pairing sites of NEAT1 and miR-204-5p. Wild-type (NEAT1-WT or ESR1-WT) and mutant (NEAT1-MUT or ESR1-MUT) plasmids containing miR-204-5p binding sites were constructed and cloned into a pGL4 vector (Promega, Madison, WI, USA). KGN cells were inoculated in 24-well plates (1×10^5 cells/well) and co-transfected with luciferase reporter plasmids, mimics-miR-204-5p, inhibitor-miR-204-5p, and the corresponding negative controls using Lipofectamine® 3000. After 48 h, luciferase activity in the cells was measured using a Dual-Luciferase Reporter Gene Assay Kit (Promega).

Cell counting kit-8 (CCK-8) assay

A CCK-8 Kit (Vazyme Biotech Co. Ltd., Nanjing, China) was used to analyze cellular activity. KGN cells were plated in 96-well plates at a density of 4×10^3 cells/well (100 μL/well). After cell adherence, 10 μL CCK-8 was added to each well and incubated at 37 °C for 2 h in the incubator. Absorbance was measured at 450 nm using a multifunctional enzyme marker (Bio-Rad, Hercules, CA, USA).

EdU proliferation assay

Cell proliferation was assessed by measuring DNA synthesis using the EdU Cell Proliferation Kit (Beyotime, Shanghai, China). KGN cells were incubated with 500 μL of EdU working solution for 2 h. The cells were then fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton-100 for 10 min, and the nuclei were stained with Hoechst 33,342 for 10 min in the dark. Images were captured using a fluorescence microscope (Nikon, Tokyo, Japan).

Flow cytometric assay

Apoptosis was detected using an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Vazyme). KGN cells were collected, washed with phosphate-buffered saline (PBS), and resuspended in binding buffer. Subsequently, the cells were stained with Annexin V-FITC and PI for 15 min in the dark. The cells were analyzed using a flow cytometer (BD, Franklin Lakes, NJ, USA).

The cell cycle was analyzed using a Cell Cycle and Apoptosis Detection Kit (Beyotime). KGN cells were fixed with pre-cooled PBS and 70% ethanol at 4 °C overnight. The cells were then resuspended in PI staining solution for 30 min at 37 °C in the dark, and detected using a flow cytometer.

RT-qPCR

Total RNA was extracted from clinical GC samples and KGN cells using the Total RNA Extraction Reagent RNA Isolator (Vazyme). The concentration and purity of the extracted RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For miRNA quantification, reverse transcription was performed using miRNA-specific stem-loop primers (Sangon Biotech) and HiScript II Q RT SuperMix (Vazyme), which is optimized for small RNA analysis. For mRNA analysis, total RNA was reverse-transcribed using random hexamer primers provided in the same kit. RT-qPCR was performed using a fluorescent quantitative

PCR instrument (Bio-Rad) and ChamQ SYBR® qPCR Master Mix (Vazyme). GAPDH or U6 was used as the reference gene for mRNA and miRNA quantification, respectively, and the $^{2-\Delta\Delta}$Ct method was used to quantify the expression levels of RNAs. The specific primers used in this study were obtained from Sangon Biotech, and their sequences are presented in Table 1.

Western blotting

Proteins were extracted from cells using radioimmunoprecipitation assay lysis buffer (Servicebio, Wuhan, China), and the protein concentration was determined using a Bicinconinic acid Protein Assay Kit (CWBIO, Taizhou, China). Equal amounts of protein were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels and subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad). Non-specific sites on the membranes were blocked by incubation with 5% skimmed milk for 2 h at 22 °C. The membranes were then incubated with primary antibodies overnight at 4 °C. The membranes were washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at 22 °C. After washing with TBST buffer, the protein bands on the membrane were visualized using an enhanced chemiluminescence Detection Kit (Vazyme) and scanned using a gel imaging system. GAPDH was used as the internal reference. All the antibodies used in western blotting, including ESR1 (20698-1-AP), phospho-ERK1/2 (80031-1-RR), ERK1/2 (11257-1-AP), phospho-CREB1 (28792-1-AP), CREB1 (12208-1-AP), StAR (12225-1-AP), CYP19A1 (16554-1-AP), and GAPDH (10494-1-AP) were purchased from Proteintech Group Inc. (Wuhan, China).

Table 1 Primer sequences for gene expression

Gene	Primer sequence (5′-3′)
NEAT1	Forward: AGTGTGAGTCCTAGCATTG
	Reverse: GAACTTCCTCCTCCTAAGC
hsa-miR-204-5p	Forward: CGCGTTCCCTTTGTCATCCT
	Reverse: AGTGCAGGGTCCGAGGTATT
hsa-miR-125a-5p	Forward: TCGGCAGGTCCCTGAGACCCTT
	Reverse: CTCAACTGGTGTCGTGGA
StAR	Forward: GCCACATTTGCCAGGAAACAATG
	Reverse: CTCCTGGTCACTGTAGAGAGTCT
CYP19A1	Forward: GCAAAGCACCCTAATGTTGAAGA
	Reverse: CGAGTCTGTGCATCCTTCCAATA
ESR1	Forward: CTCTAACCTCGGGCTGTGC
	Reverse: AGATGCTTTGGTGTGGAGGG
NOTCH2	Forward: ATCCCACAAAGCCTAGCACC
	Reverse: CCTTGTCCCTGAGCAACCAT
IGFBP5	Forward: GAAGCAGATGTGTCTCTGCCC
	Reverse: TTCCCTCTGCTTCCTGGTTC
GAPDH	Forward: TGCACCACCAACTGCTTAGC
	Reverse: GGCATGGACTGTGGTCATGAG
U6	Forward: CAGCACATATACTAAAATTGGAACG
	Reverse: ACGAATTTGCGTGTCATCC

CYP19A1, cytochrome P450 family 19 subfamily A member 1; ESR1, estrogen receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP5, insulin-like growth factor binding protein 5; NEAT1, nucleo-enriched abundant transcript 1; NOTCH2, recombinant notch homolog 2; StAR, steroidogenic acute regulatory protein; U6, RNU6

Enzyme-linked immunosorbent assay (ELISA)

KGN cells were routinely cultured, and androstenedione (10 μg/mL) was added to the culture medium to induce the synthesis of estrogen. After 24 h of culture, the supernatant was collected and centrifuged at 3000× *g* for 10 min at 4° C to remove cell debris. Subsequently, a Human Estrogen E ELISA Kit (Cusabio, Wuhan, China) was used according to the manufacturer's instructions, and the optical density was measured at 450 nm using an enzyme marker. The concentration of estrogen in the samples was calculated using a standard curve.

Statistical analysis

The experimental data were analyzed using SPSS software (version 23.0; IBM, Armonk, NY, USA). All results are expressed as the mean ± standard deviation. The normality of the data distribution was assessed using the Shapiro–Wilk test. Student's *t*-test or Wilcoxon test was used to assess the significance of the differences between the two groups of data. Pearson's correlation test was used for the correlation analysis between variables. Graphs were generated using GraphPad Prism (version 8.0; San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Baseline characteristics

The baseline characteristics of the patients in the two groups are listed in Table 2. Serum AMH and AFC levels were significantly lower ($p < 0.001$), and basal FSH level was significantly higher ($p < 0.001$) in patients with DOR compared with that in the NOR group. Baseline profiles, such as age, infertility duration, body mass index (BMI), basal luteinizing hormone (LH), basal estradiol (E_2), and basal progesterone (P) levels, were not significantly different between the two groups of patients ($p > 0.05$).

Downregulation of NEAT1 is associated with DOR occurrence and ART outcomes

First, we analyzed the expression levels of NEAT1 in GC samples using RT-qPCR. The results showed that NEAT1 expression was significantly downregulated in the GCs of patients with DOR compared to those in the NOR group ($p < 0.001$; Fig. 1a). Pearson correlation analysis showed that NEAT1 expression levels had a significantly negative correlation with basal FSH ($p < 0.001$; Fig. 1e) and significantly positive correlation with AFC, AMH, basal E_2, E_2

Table 2 Comparison of baseline characteristics between the NOR and DOR groups

	NOR ($n=20$)	DOR ($n=20$)	*p*-value
Patient age (years)	34.20 ± 3.62	33.65 ± 4.39	0.668
Infertility duration (years)	3.75 ± 2.59	4.10 ± 2.61	0.673
BMI (kg/m^2)	21.42 ± 2.05	21.80 ± 1.85	0.524
AFC (n)	15.35 ± 3.92	5.60 ± 1.96	< 0.001
AMH (ng/mL)	3.71 ± 1.31	0.69 ± 0.30	< 0.001
Basal FSH (mIU/mL)	7.01 ± 0.83	13.21 ± 3.02	< 0.001
Basal LH (mIU/mL)	5.20 ± 2.84	4.32 ± 1.66	0.240
Basal E_2 (pg/mL)	49.07 ± 18.89	37.79 ± 17.24	0.056
Basal P (ng/mL)	0.39 ± 0.21	0.54 ± 0.36	0.112
Duration of stimulation (d)	9.15 ± 1.46	9.30 ± 2.30	0.807
Dosage of gonadotrophin (IU)	2135.63 ± 503.36	2562.50 ± 804.33	0.051

Data are shown as mean ± standard deviation

AMH, anti-Müllerian hormone; AFC, antral follicle count; BMI, body mass index; E_2, estrogen; FSH, follicle-stimulating hormone; LH, luteinizing hormone; P, progesterone

on trigger day, number of oocytes retrieved, number of two-pronuclear zygotes, number of embryos available, and the number of superior embryos ($p<0.05$; Fig. 1c, d, g, l, n, o, p, and q). In addition, no significant correlation was observed between NEAT1 expression level and patient age, basal LH, basal P, gonadotropin duration, gonadotropin dosage, LH, or P on trigger day ($p>0.05$; Fig. 1b, f, h-k, and m). These results suggest that the downregulation of NEAT1 in the GCs of patients with DOR may be associated with ovarian reserve function and ART outcomes.

NEAT1 inhibits apoptosis and promotes proliferation of KGN cells

To confirm the subcellular localization of NEAT1 in KGN cells, a FISH assay was performed. The assay showed that NEAT1 was mainly localized in the cytoplasm (Fig. 2a), suggesting that NEAT1 acts as a molecular sponge for miRNAs to exert post-transcriptional regulation. To further investigate the biological functions of NEAT1, KGN cells were transfected with si-NEAT1 or OE-NEAT1 plasmids. RT-qPCR results revealed significant changes in NEAT1 expression in the cells after transfection ($p<0.05$; Fig. 2b). CCK-8 and EdU assays revealed that NEAT1 overexpression significantly increased cell viability and proliferation, whereas NEAT1 knockdown had the opposite effect ($p<0.01$; Fig. 2c, d). Flow cytometry results showed a significant decrease in apoptosis in the OE-NEAT1 group and significant increase in apoptosis in the si-NEAT1 group ($p<0.001$; Fig. 2e). Additionally, cell cycle analysis showed that NEAT1 overexpression led to a significant decrease in the proportion of cells in the G0/G1 phase ($p<0.01$) and significant increase in the proportion of cells in the S phase ($p<0.001$), indicative of an increase in cell proliferation. In contrast, NEAT1 knockdown significantly increased the proportion of cells in the G0/G1 phase ($p<0.05$) and decreased the proportion of cells in the S phase ($p<0.01$), indicating that si-NEAT1 blocked cells in the G1/S phase, thereby inhibiting cell proliferation (Fig. 2f).

miR-204-5p is a target of NEAT1

To predict miRNAs that potentially interact with NEAT1, we used starBase, miRcode, and LncBase databases (Fig. 3a). We screened miR-125a-5p and miR-204-5p as miRNAs common to the three databases, verified their expression levels in GCs using RT-qPCR, and determined that miR-204-5p was significantly upregulated in the GCs of patients with DOR ($p<0.001$; Fig. 3b). Figure 3c shows the complementary base sequences of NEAT1 and miR-204-5p. Based on the predicted binding sites, dual-luciferase reporter assays were performed, which confirmed that mimics-miR-204-5p significantly reduced the luciferase activity of the NEAT1-WT vector ($p<0.01$), whereas it did not affect the luciferase activity of the NEAT1-MUT vector ($p>0.05$). In contrast, the inhibitor-miR-204-5p significantly increased luciferase activity of the NEAT1-WT vector ($p<0.001$), but not that of NEAT1-MUT ($p>0.05$) (Fig. 3d). In addition, transfection with OE-NEAT1 significantly decreased miR-204-5p expression in KGN cells ($p<0.001$), whereas transfection with si-NEAT1 significantly increased miR-204-5p expression ($p<0.01$) (Fig. 3e). These data suggest that NEAT1 directly binds to miR-204-5p and inhibits its expression.

NEAT1 overexpression negatively regulates miR-204-5p to inhibit apoptosis and promote proliferation of KGN cells

We transfected KGN with mimics-miR-204-5p, inhibitor-miR-204-5p, and the corresponding negative controls to investigate the effects of miR-204-5p on cell proliferation and apoptosis. RT-qPCR was used to confirm transfection efficiency ($p<0.05$; Fig. 4a). The CCK-8 and EdU assays revealed that miR-204-5p overexpression significantly inhibited cell viability and proliferation, whereas miR-204-5p inhibition had the opposite effect ($p<0.05$; Fig. 4b, c). Flow cytometry showed that transfection with mimics-miR-204-5p induced apoptosis in KGN cells, whereas transfection with inhibitor-miR-204-5p significantly inhibited apoptosis ($p<0.001$; Fig. 4d). In addition, cell cycle analysis revealed that the downregulation of miR-204-5p promoted cell proliferation by increasing the proportion of KGN cells in the S phase ($p<0.001$). In contrast, miR-204-5p overexpression significantly decreased the proportion of S-phase cells ($p<0.01$) and increased the proportion of G0/G1-phase cells ($p<0.001$). Thus, the low expression of miR-204-5p promoted KGN cell

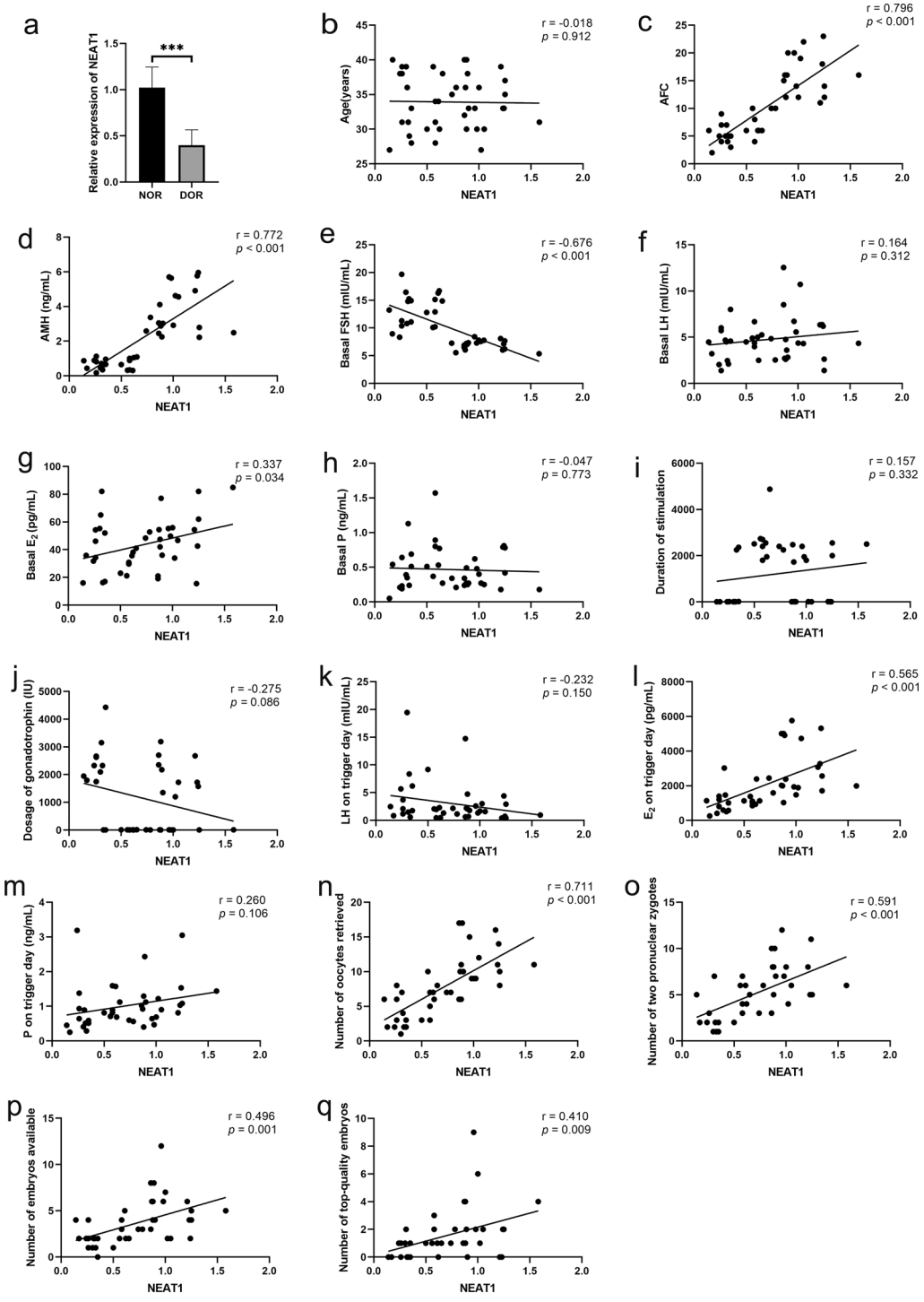

Fig. 1 NEAT1 expression levels in human GCs correlate with ovarian reserve and ART outcomes. (**a**) NEAT1 expression levels in GCs of the NOR and DOR groups. (**b**–**h**) Correlation of NEAT1 expression levels with age, AFC, AMH, basal FSH, basal LH, basal E_2, and basal P. (**i**-**q**) Correlation between NEAT1 expression levels and gonadotropin duration, gonadotropin dosage, LH on trigger day, E_2 on trigger day, P on trigger day, number of oocytes retrieved, number of two-pronuclear zygotes, number of embryos available, and number of superior embryos. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

proliferation by promoting the G1/S transition of the cells (Fig. 4e).

To further examine the correlation between NEAT1 and miR-204-5p in the regulation of KGN cell function, we co-transfected KGN cells with OE-NEAT1 and mimics-miR-204-5p. NEAT1 overexpression increased KGN cell viability and proliferation ($p < 0.01$), and this effect was reversed by transfection with miR-204-5p mimics

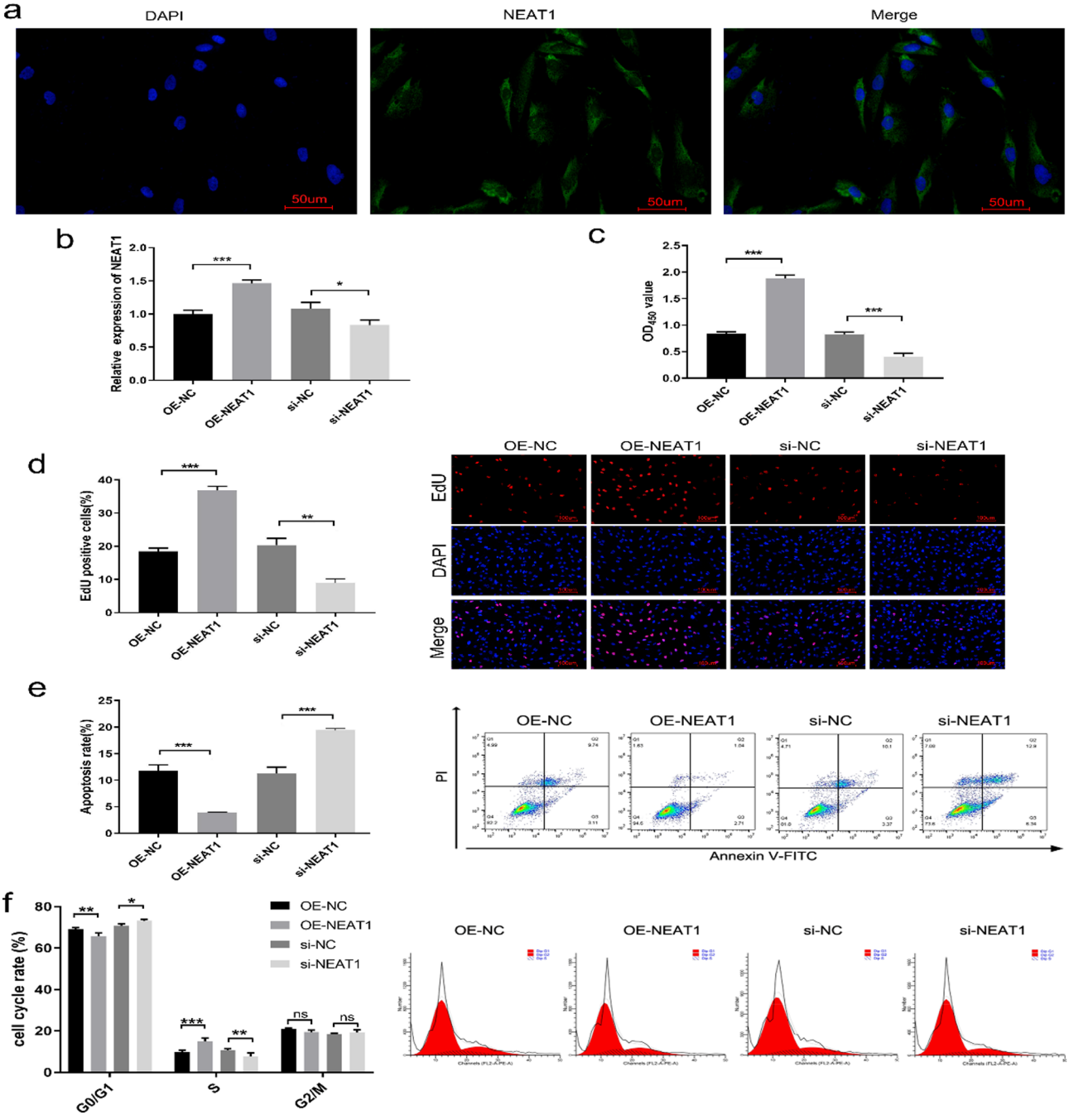

Fig. 2 Effects of NEAT1 on proliferation, apoptosis, and cell cycle of KGN cells. (**a**) FISH assay was used to determine the subcellular localization of NEAT1 in KGN cells. (**b**) RT-qPCR was performed to detect the transfection efficiency of the OE-NEAT1 and si-NEAT1 plasmids. (**c**–**d**) CCK-8 and EdU assays were used to detect the effect of NEAT1 on the viability and proliferative capacity of KGN cells. (**e**–**f**) Flow cytometry was used to assess the effect of NEAT1 on apoptosis and cell cycle of KGN cells. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

($p < 0.05$; Fig. 5a, b). In addition, the overexpression of miR-204-5p reversed the NEAT1-mediated effects on the apoptotic rate and G1/S transition of KGN cells ($p < 0.05$; Fig. 5c, d). These results suggest that NEAT1 affects KGN cell proliferation and apoptosis by targeting miR-204-5p.

ESR1 is a downstream target of miR-204-5p

We used multiple bioinformatics databases (starBase, miRcode, TargetScan, miRDB, and miRWalk) to predict the potential target genes of miR-204-5p (Fig. 6a). Three potential targets were identified at the intersection of the five databases. The expression levels of these potential targets were evaluated in clinical GC samples using

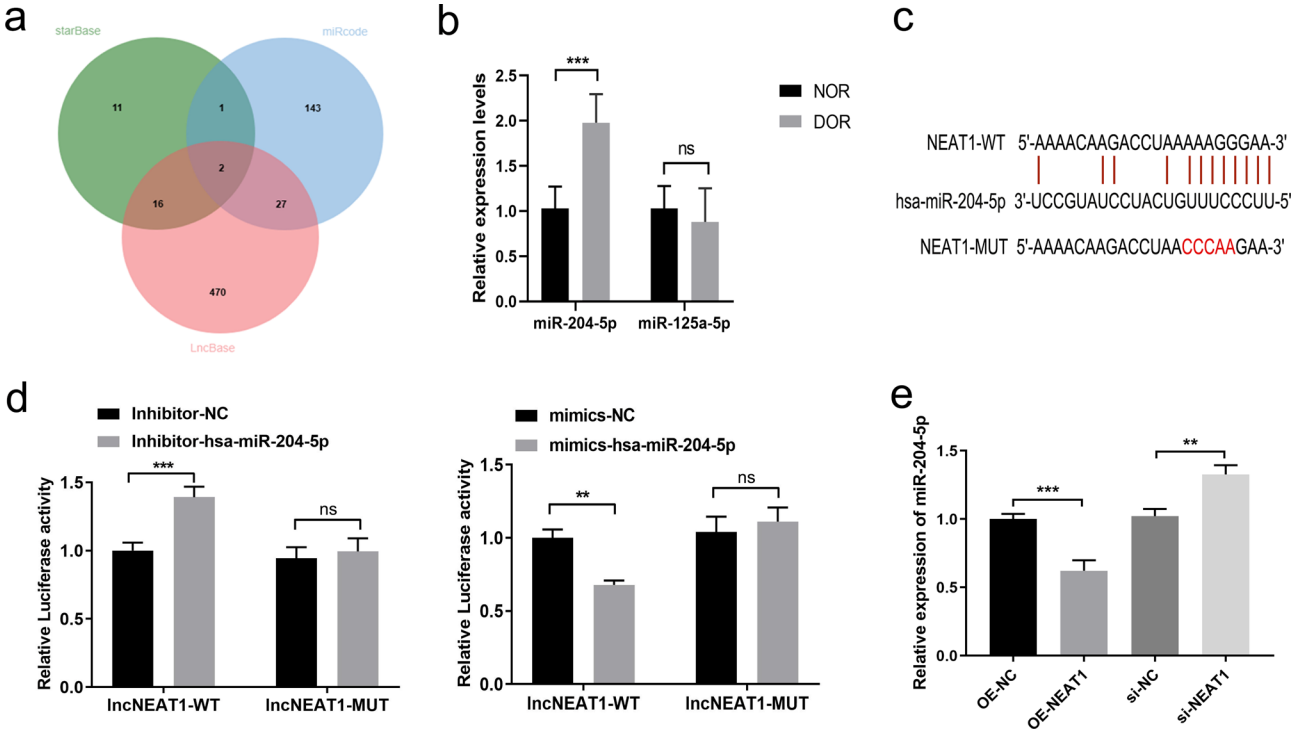

Fig. 3 miR-204-5p is a target of lncRNA NEAT1. (**a**) Downstream targets of NEAT1 were predicted using the starBase, miRcode, and LncBase databases. (**b**) RT-qPCR was used to detect the expression levels of miR-125a-5p and miR-204-5p in human GCs. (**c**) Bioinformatics software was used to predict the binding sites of NEAT1 and miR-204-5p. (**d**) A dual-luciferase reporter assay was used to verify the target-binding relationship between NEAT1 and miR-204-5p. (**e**) RT-qPCR was used to detect miR-204-5p expression levels following NEAT1 overexpression or silencing. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

RT-qPCR. ESR1 showed the most significant difference in expression between the two patient groups ($p < 0.001$; Fig. 6b). We predicted the potential binding sites of miR-204-5p on ESR1 (Fig. 6c). Dual-luciferase reporter assays were performed to verify the targeted regulatory relationship between the two molecules ($p < 0.001$; Fig. 6d). Transfection of KGN cells with miR-204-5p mimics decreased ESR1 mRNA and protein expression levels ($p < 0.05$), whereas transfection with miR-204-5p inhibitors increased ESR1 mRNA and protein expression ($p < 0.01$) (Fig. 6e, f). These data suggest that miR-204-5p may negatively regulate ESR1 expression.

Silencing of ESR1 reverses the effects of inhibitor-miR-204-5p on proliferation, apoptosis, and steroid biosynthesis in KGN cells

After transfecting KGN cells with inhibitor-miR-204-5p and si-ESR1, we performed a series of experiments to clarify the effect of ESR1 on miR-204-5p-mediated biological functions. CCK-8 and EdU assays showed that the inhibition of miR-204-5p significantly promoted the viability and proliferative capacity of KGN cells, whereas this effect was effectively reversed by silencing ESR1 ($p < 0.001$; Fig. 7a, b). Flow cytometric analysis showed that the miR-204-5p inhibitor significantly reduced the apoptotic rate ($p < 0.05$), increased the proportion of S-phase cells ($p < 0.001$), and reduced the proportion of G2/M-phase cells ($p < 0.001$). However, si-ESR1 transfection partially restored the apoptotic rate and cell cycle transition ($p < 0.05$; Fig. 7c, d). These results indicate that miR-204-5p targets the negative regulation of ESR1 to regulate the proliferation, cell cycle, and apoptosis of KGN cells.

ESR1 encodes estrogen receptor-α, which participates in estrogen synthesis by mediating estrogen signaling. Moreover, it regulates the MAPK signaling pathway [17], affecting cell proliferation, differentiation, and apoptosis. We further examined the expression levels of key steroidogenic genes, including steroidogenic acute regulatory protein (StAR) and cytochrome P450 family 19 subfamily A member 1 (CYP19A1), and important proteins in the MAPK signaling pathway. The results showed that transfection of KGN cells with inhibitor-miR-204-5p significantly upregulated the mRNA and protein expression of ESR1, StAR, and CYP19A1 and promoted E_2 biosynthesis ($p < 0.01$), whereas ESR1 silencing reversed these changes ($p < 0.05$; Fig. 7e-g). In addition, the miR-204-5p inhibitor increased the levels of phosphorylated extracellular signaling-related kinase (ERK) and cyclic adenosine monophosphate response element binding protein (CREB), which were reversed by si-ESR1 ($p < 0.001$; Fig. 7f).

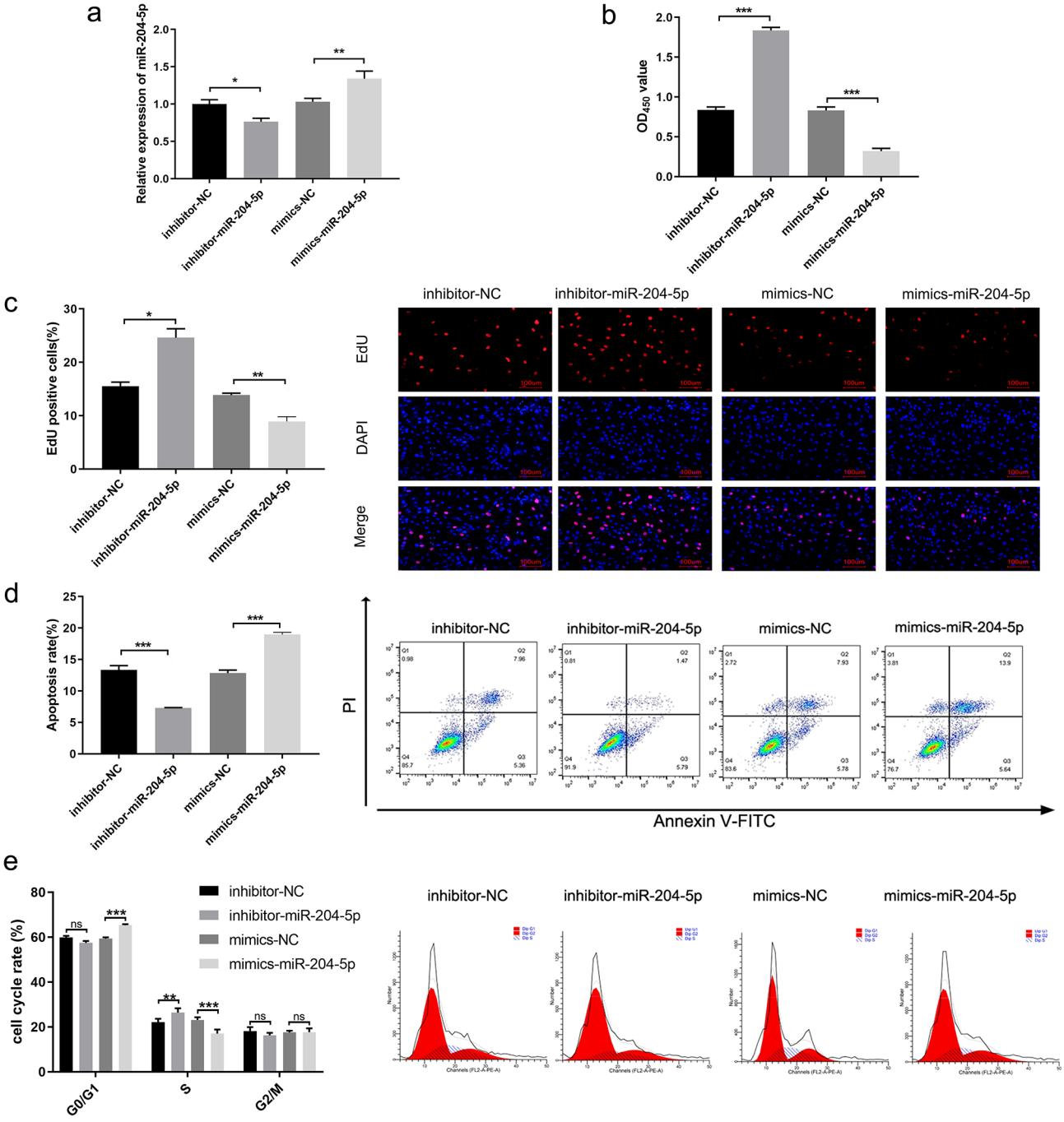

Fig. 4 Effects of miR-204-5p on proliferation, apoptosis, and cell cycle of KGN cells. (**a**) RT-qPCR was used to analyze miR-204-5p expression levels following transfection with mimics-miR-204-5p or inhibitor-miR-204-5p. (**b**–**c**) CCK-8 and EdU assays were performed to detect the effects of miR-204-5p on KGN cell viability and proliferation. (**d**–**e**) Flow cytometry was performed to assess the effects of miR-204-5p on KGN cell apoptosis and cell cycle. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

NEAT1 mediates the MAPK signaling pathway through the miR-204-5p/ESR1 axis to affect the biological functions in KGN cells

Then, we investigated whether NEAT1 affects the phenotype of KGN cells by regulating ESR1. KGN cells were transfected with si-NEAT1 or OE-NEAT1, and the mRNA and protein expression levels of ESR1 were analyzed using RT-PCR and western blotting, respectively. The results showed that ESR1 levels decreased significantly following si-NEAT1 transfection ($p < 0.01$), whereas they markedly increased following OE-NEAT1 transfection ($p < 0.05$) (Fig. 8a, b). To investigate whether NEAT1 is involved in functional alterations in GCs during DOR by regulating ESR1 expression and downstream

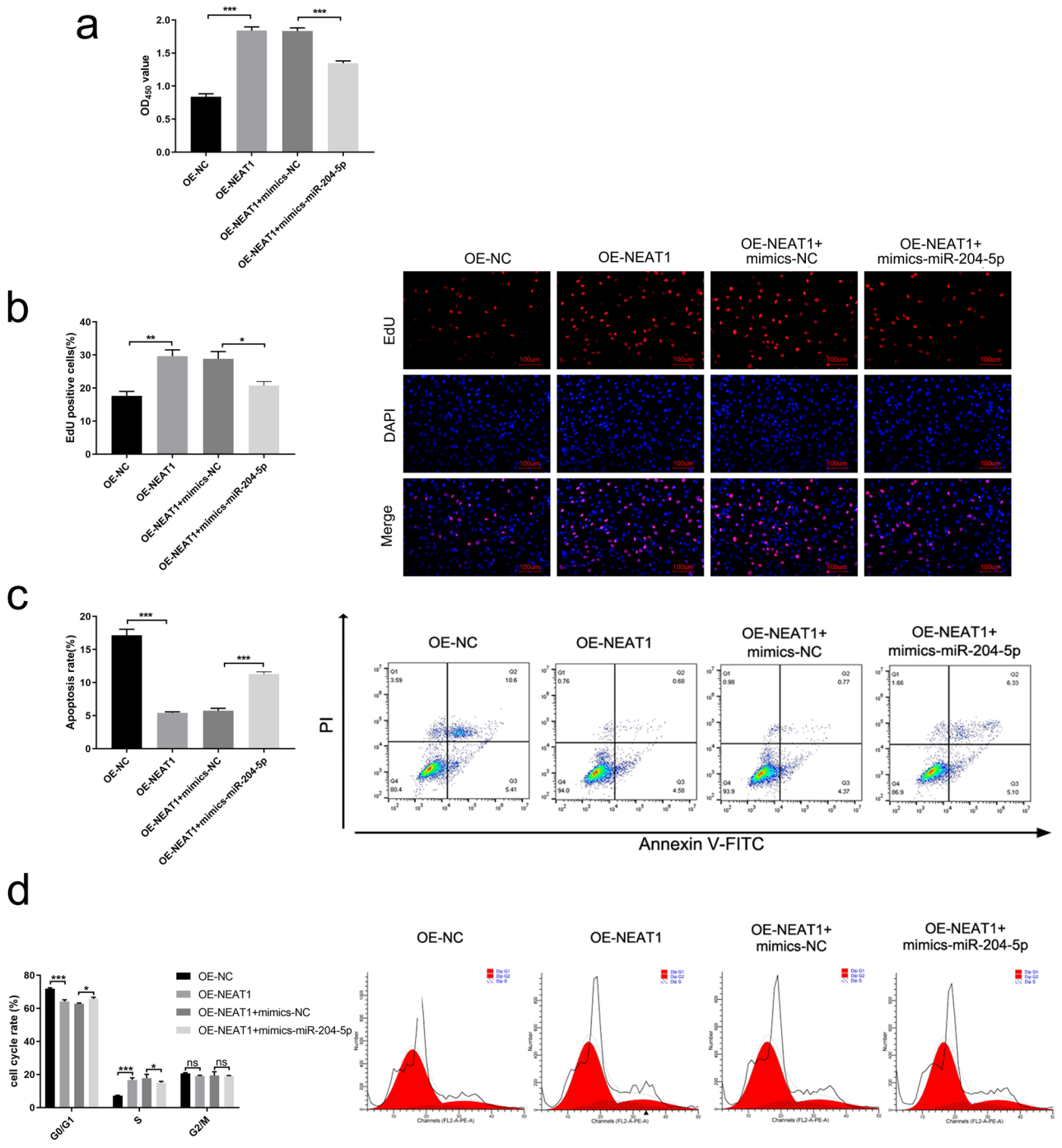

Fig. 5 miR-204-5p reversed the NEAT1-mediated biological effects in KGN cells. (**a**–**b**) KGN cell viability and proliferation were assessed using CCK-8 and EdU assays. (**c**–**d**) Flow cytometric analysis of KGN cell apoptosis and cell cycle. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

signaling pathways through sponging of miR-204-5p, the levels of ESR1, StAR, CYP19A1, and E_2 were analyzed in KGN cells overexpressing NEAT1 in the absence of mimics-miR-204-5p. The results showed that NEAT1 overexpression in KGN cells significantly increased the mRNA and protein expression of ESR1, StAR, and CYP19A1 and augmentated E_2 biosynthesis ($p < 0.001$), which was reversed by co-transfection with mimics-miR-204-5p ($p < 0.05$) (Fig. 8c-e). In addition, NEAT1 overexpression increased the phosphorylation of ERK and CREB ($p < 0.01$), which was reversed by mimics-miR-204-5p ($p < 0.001$) (Fig. 8e). These results suggest that NEAT1 acts as a miR-204-5p sponge to regulate ESR1 expression, which regulates biological functions, such as

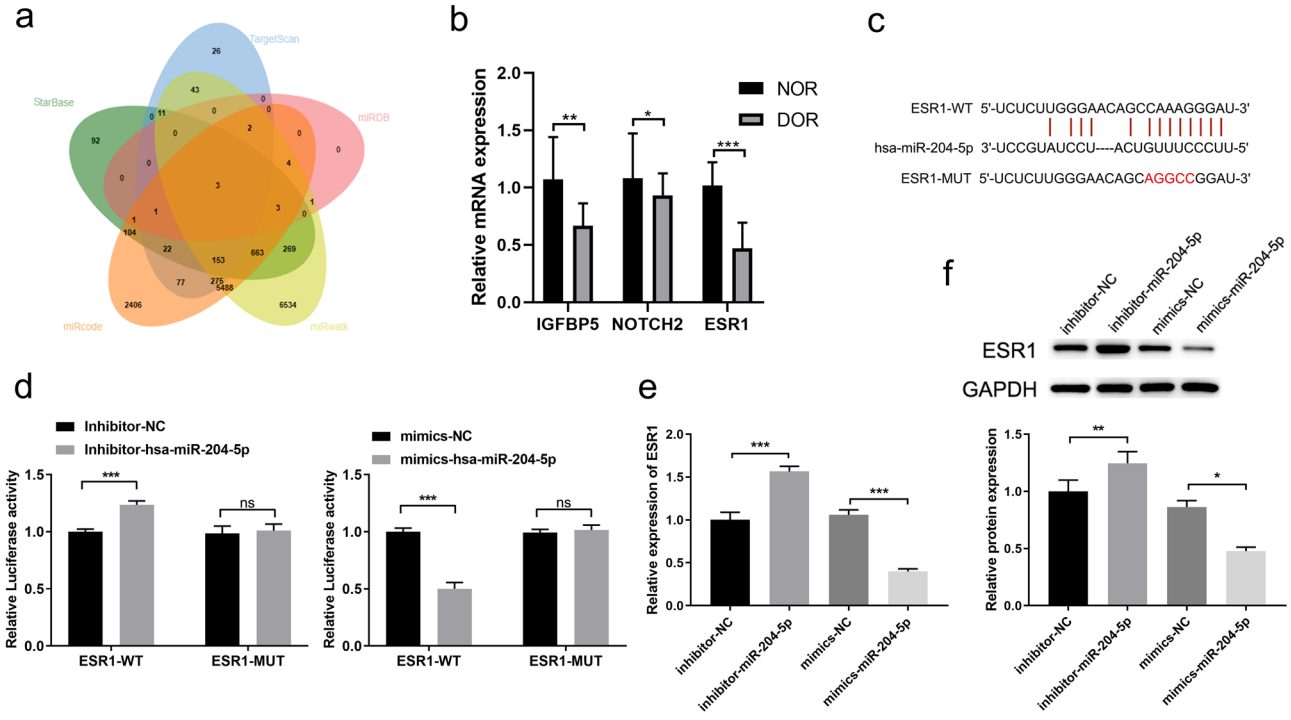

Fig. 6 ESR1 is a downstream target gene of miR-204-5p. (**a**) Downstream target genes of miR-204-5p were predicted using starBase, miRcode, TargetScan, miRDB, and miRWalk. (**b**) RT-qPCR was used to analyze the mRNA expression levels of IGFBP5, NOTCH2, and ESR1 in human GCs. (**c**) Bioinformatics software was used to predict the binding sites of miR-204-5p and ESR1. (**d**) Dual-luciferase reporter assay was performed to verify the target-binding relationship between miR-204-5p and ESR1. (**e**–**f**) Expression levels of ESR1 mRNA and protein following transfection with miR-204-5p mimics or inhibitors were detected using RT-qPCR and western blotting, respectively. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

proliferation, apoptosis, and steroid biosynthesis in KGN cells by activating the MAPK signaling pathway. In addition, we examined the level of steroid synthase expression and MAPK pathway activity in GCs from patients with DOR, which exhibited the same phenotypic characteristics as KGN cells. Specifically, ESR1, StAR, and CYP19A1 expression was downregulated ($p < 0.01$; Fig. 8f-g), and ERK and CREB phosphorylation levels were reduced in GCs derived from patients with DOR compared to that of the NOR group ($p < 0.001$; Fig. 8g).

Discussion

Reduced follicular quality and quantity are the main manifestations of DOR, leading to decreased fertility and reproductive endocrine dysfunction in females. Impaired GC function induces follicular atresia, which is the core pathogenesis of DOR. Therefore, elucidating the underlying molecular mechanisms is of great significance for maintaining ovarian function and promoting women's health. lncRNAs are involved in ovarian hypoplasia-like disorders by regulating several biological processes, including oocyte development, maturation, and luteinization [18–20]. Our previous study explored the expression profiles of lncRNAs in GCs derived from patients with DOR and identified the significantly downregulated lncRNA NEAT1 [14]. In the current study, based on bioinformatics predictions and the results of dual-luciferase reporter gene assays, we observed that lncRNA NEAT1 upregulated ESR1 expression through the adsorption of miR-204-5p, mediating the MAPK signaling pathway and affected proliferation, apoptosis, and steroidogenesis in KGN cells. Our findings elucidate the previously unrecognized epigenetic mechanism of DOR and potential therapeutic targets for ovarian dysfunction.

NEAT1 constitutes the skeletal structure of the subnucleosomal parafollicular patch and regulates gene expression by maintaining mRNA stability in the nucleus [21]. NEAT1 is involved in the cyclic recruitment and differentiation of follicles [22] and is closely associated with corpus luteum formation, pregnancy establishment, and maintenance [23]. NEAT1 is significantly downregulated in the ovarian tissues of patients with premature ovarian failure (POF), and NEAT1 overexpression inhibits p53 expression by affecting its transcription and stability, thereby ameliorating POF by inhibiting apoptosis [24]. Several recent studies have demonstrated that NEAT1 acts as a ceRNA to regulate the expression of downstream target genes involved in the development of reproductive endocrine diseases at the post-transcriptional level through the competitive adsorption of miRNAs. For instance, NEAT1 overexpression increases the expression of androgen receptors, follistatin, and insulin

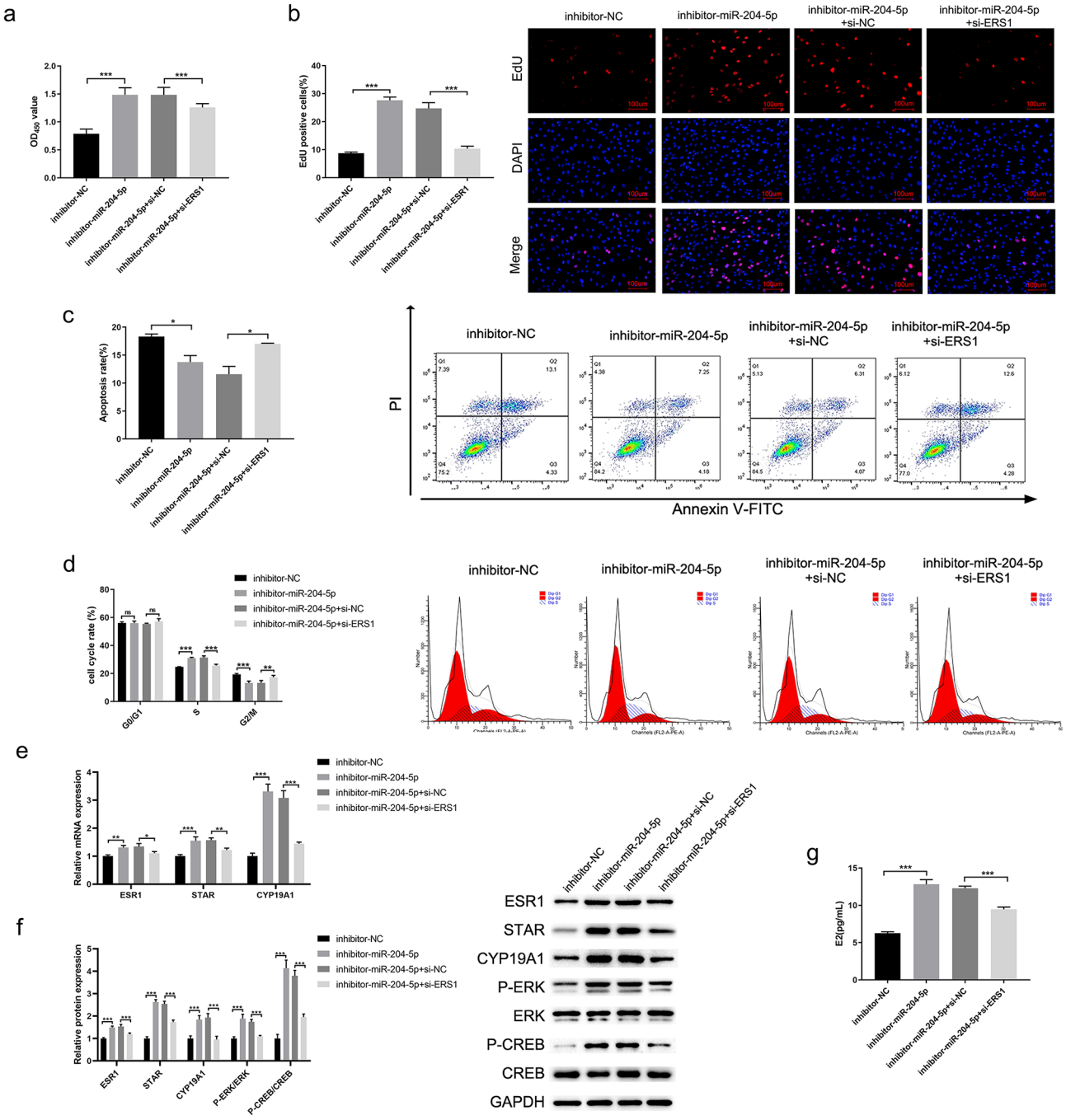

Fig. 7 Silencing of ESR1 reversed the inhibitor-miR-204-5p-mediated biological effects on KGN cells. (**a**–**b**) KGN cell viability and proliferation were assessed using CCK-8 and EdU assays. (**c**–**d**) Flow cytometric analysis of KGN cell apoptosis and cell cycle. (**e**) RT-qPCR was performed to detect the expression levels of key genes involved in steroid hormone synthesis. (**f**) Western blotting was performed to detect the expression levels of proteins related to steroid hormone synthesis and the MAPK signaling pathway. (**g**) ELISA was used to detect E_2 levels. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

receptor substrate 2 by sponging miR-30d-5p, which is involved in the development of polycystic ovarian syndrome [25]. Additionally, NEAT1 competitively binds to miR-874-3p, thereby regulating the proliferation of mouse GCs and producing E_2 and progesterone [26]. Furthermore, NEAT1 reduces miR-654 expression and regulates the STC2/MAPK pathway to reduce apoptosis and autophagy, making it a potential therapeutic target for POF [27]. Similarly, we observed significant downregulation of NEAT1 in the GCs of patients with DOR, which correlated with a reduced ovarian reserve and poor ART outcomes. FISH assay showed that NEAT1 was mainly localized in the cytoplasm, and its overexpression promoted proliferation and inhibited apoptosis of KGN

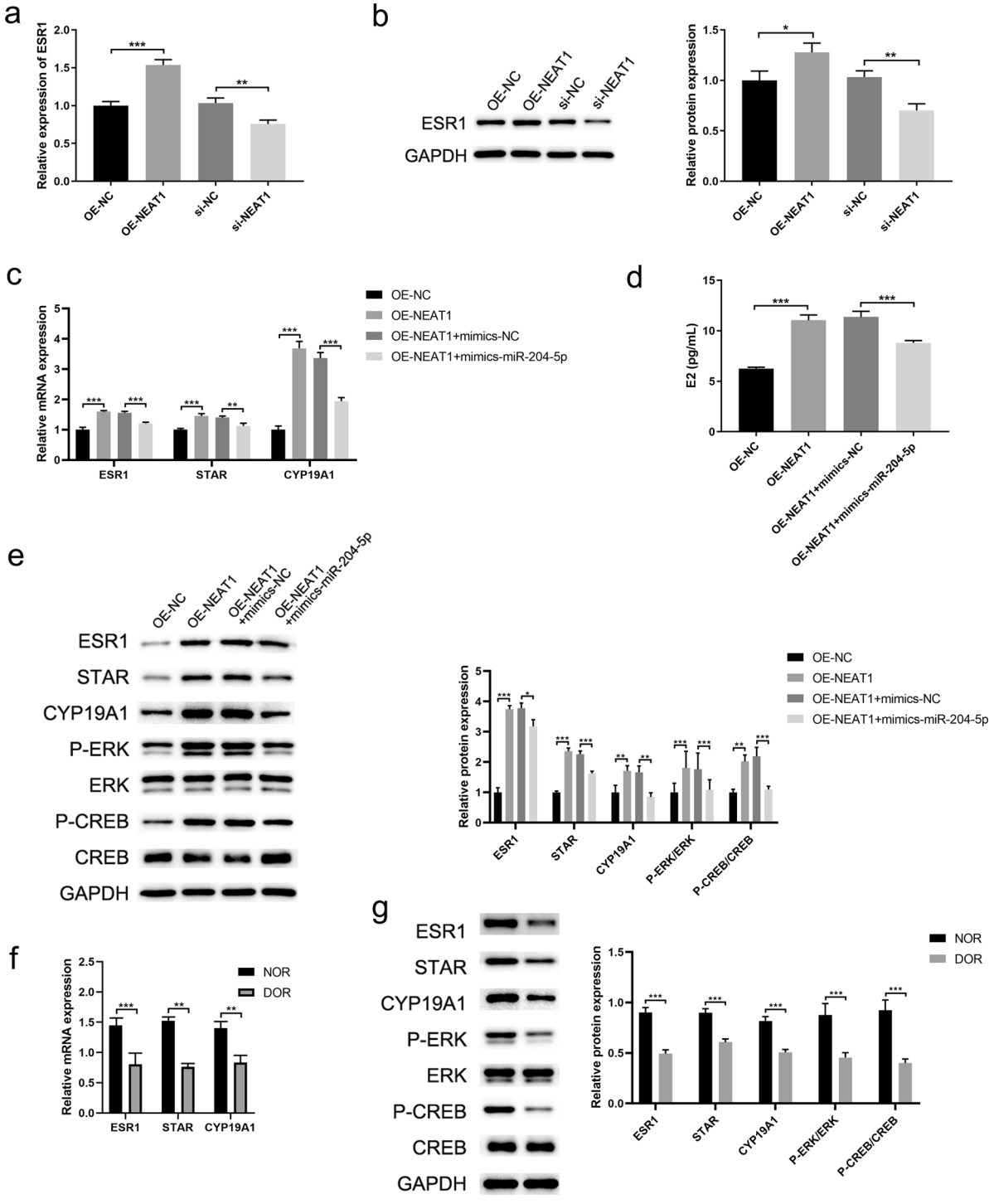

Fig. 8 NEAT1 regulates steroid hormone biosynthesis and the MAPK signaling pathway in KGN cells via the miR-204-5p/ESR1 axis. (**a**-**b**) RT-qPCR and western blotting were performed to evaluate the effect of NEAT1 on ESR1 mRNA and protein expression levels, respectively. (**c**) RT-qPCR was performed to analyze the expression of key genes involved in steroid hormone synthesis. (**d**) ELISA was performed to determine E_2 levels. (**e**) western blotting was performed to determine the levels of proteins related to steroid hormone synthesis and MAPK signaling pathway. (**f**-**g**) RT-qPCR and western blotting were used to assess the expression levels of steroid synthase and key molecules of the MAPK pathway in human GCs, respectively. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

cells, a function that may be mediated through the role of NEAT1 as a ceRNA. This suggests that NEAT1 is closely related to ovarian function by affecting multiple biological functions of GCs, warranting further investigation of its role in DOR.

We predicted the potential targets of NEAT1 based on data from multiple bioinformatics databases and observed that miR-204-5p was upregulated in GC samples from patients with DOR. In addition, dual-luciferase reporter assays confirmed that miR-204-5p binds to NEAT1. Subsequent functional and salvage assays showed that NEAT1 overexpression reversed the regulation of cell proliferation and apoptosis by miR-204-5p, suggesting that NEAT1 plays a protective role in DOR by adsorbing miR-204-5p. Notably, the NEAT1/miR-204-5p axis exhibits functional heterogeneity across different diseases. In rheumatoid arthritis, it promotes inflammatory responses and increases the proliferation of fibroblast-like synoviocytes through the nuclear factor kappa-B pathway [28], whereas in obesity-associated endometrial cancer, it mediates carcinogenesis through insulin-like growth factors-1 [29]. Although miR-204-5p mostly exhibits pro-carcinogenic properties in cancer by regulating cell proliferation, apoptosis, autophagy, and the immune microenvironment [30], its function in ovarian diseases remain unclear. miR-204-5p was determined to regulate the targeting of specific genes, such as ubiquitin-specific peptidase 47 and thrombospondin-1, to promote ovarian cancer progression [31, 32]. In this study, we found that low NEAT1 expression in DOR attenuated the adsorption of miR-204-5p, leading to GC dysfunction, functionally echoing the pro-apoptotic mechanism of the miR-204-5p/BCL2 axis in Cd toxicity [33].

miRNAs negatively regulate the expression of target genes by binding to the 3'-UTR of the target gene mRNA, promoting mRNA degradation, or inhibiting protein translation [34]. We used bioinformatics to predict the potential targets of miR-204-5p and confirmed its negative regulatory effect on ESR1 expression. ESR1 is an estrogen receptor α that mediates estrogen signaling, affecting follicle development, maturation, and steroid hormone synthesis, and is vital to maintaining ovarian function [35, 36]. StAR and CYP19A1, key enzymes involved in estrogen biosynthesis [37], regulate steroid synthesis by transporting free cholesterol and catalyzing the conversion of androgen. We observed a significant reduction in ESR1 and steroid synthase expression in GCs derived from patients with DOR, confirming that ESR1 regulates estrogen synthesis. In the present study, we found that inhibition of miR-204-5p significantly activated the phosphorylation of ERK and CREB, key molecules of the MAPK pathway, through upregulation of ESR1, and silencing of ESR1 reversed this effect, directly demonstrating that ESR1 is an upstream regulator of the MAPK pathway. This finding is consistent with that of Liu et al., who's showed that ESR1 promotes breast cancer progression through the MAPK/ERK pathway [38]. The MAPK pathway is known to coordinate cell proliferation and apoptosis through the regulation of cyclin D1 and Bcl-2 family proteins [39, 40], which is highly consistent with the results of the present study. Notably, the MAPK pathway has multiple roles in the regulation of female reproduction, and its reduced activity is closely associated with ovarian function decline [41, 42]. The most representative member of the ERK pathway in MAPK signaling, ERK, becomes phosphorylated and activated, and then translocates from the cytoplasm to the nucleus, where it directly or indirectly regulates the expression of a variety of target genes and affects a variety of cellular processes, such as proliferation, differentiation, and cell death [43]. CREB is a key downstream signaling molecule of the ERK pathway. Once activated, ERK phosphorylates downstream CREB and affects the proliferation, apoptosis, and steroidogenesis of GCs [44, 45]. Our findings showed that silencing ESR1 inhibited the MAPK signaling pathway and effectively counteracted the effects of transfection with the inhibitor-miR-204-5p on proliferation, apoptosis, and steroid biosynthesis in KGN cells.

Finally, we examined the relationships between NEAT1, miR-204-5p, and ESR1. We observed that NEAT1 overexpression promoted ESR1 expression, which activated ERK and CREB phosphorylation in the MAPK signaling pathway and upregulated the expression of StAR and CYP19A1 to increase estradiol production. However, these effects were eliminated by miR-204-5p. These findings suggest that the NEAT1/miR-204-5p/ESR1 axis influences GC function through the MAPK pathway, offering novel insights into the molecular mechanisms underlying DOR. However, this study has several limitations: (i) although the KGN cell line can mimic some of the functions of GCs (e.g., steroid synthesis), its tumorigenicity may lead to differences in the mechanisms of proliferation and apoptosis regulation from primary granulosa cells; (ii) the conclusions drawn are mainly based on in vitro experiments, and in vivo correlations of this axis need to be verified in a DOR animal model. In future studies, we intend to conduct in vivo and in vitro experiments using larger clinical sample sizes and DOR animal models to investigate the pathogenic mechanisms regulating ovarian decline in greater detail.

Conclusion

Our study revealed that NEAT1 expression was downregulated in the GCs of patients with DOR. NEAT1 regulates apoptosis, proliferation, and steroid biosynthesis via the miR-204-5p/ESR1 axis-mediated MAPK signaling pathway. This novel ceRNA regulatory axis contributes to our understanding of DOR pathogenesis.

Abbreviations

AFC	Anterior follicle counts
AMH	Anti-Müllerian hormone
ART	Assisted Reproductive Technology
BMI	Body mass index
ceRNA	Competing endogenous RNA
CREB	Cyclic adenosine monophosphate response element binding protein
CYP19A1	Cytochrome P450 family 19 subfamily A member 1
DOR	Diminished ovarian reserve
E_2	Estradiol
ERK	Extracellular signaling-related kinase
ESR1	Estrogen receptor 1
FSH	Follicle-stimulating hormone
GCs	Granulosa cells
LH	Luteinizing hormone
lncRNA	Long non-coding RNA
MAPK	Mitogen-activated protein kinase
NOR	Normal ovarian reserve
P	Progesterone
POF	Premature ovarian failure
StAR	Steroidogenic acute regulatory protein
siRNA	Small interfering RNA

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-025-01683-6.

Supplementary Material 1

Supplementary Material 2

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Author contributions

LD was a major contributor in writing the manuscript. HW and LD performed the experiments and analyzed the data. FQ and WC did bioinformatics analysis. YX and ML contributed to manuscript revision. YW and RY was responsible for sample collection. PC conceived and designed this study. All authors read and approved the final submitted version of this manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Reproductive Medicine of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (Ref: 2021-103-KY). Written informed consent was obtained from all participants prior to oocyte retrieval, and the protocol strictly adhered to the Declaration of Helsinki. As this study was not a clinical trial, Clinical trial number: not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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