

CTBP1-AS upregulation is associated with polycystic ovary syndrome and can be effectively downregulated by cryptotanshinone

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Abstract. Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies and primarily presents with hyperandrogenism. Although environmental factors and genetic factors are thought to be the major reason, there still exists a lot of questions need to be answered. High expression of C-terminal-binding protein 1 antisense (CTBP1-AS) was identified as an independent risk factor for PCOS; however, the molecular mechanism of CTBP1-AS in PCOS regulation is unknown. In the present study, the expression level of CTBP1-AS was found to be significantly upregulated in patients with PCOS compared with healthy control patients. CTBP1-AS knockdown was demonstrated to reduce the proliferation and promote the apoptosis of granulosa tumor cells *in vitro*. It was also identified that the two core catalytic subunits of Polycomb repressive complex 2 (enhancer of zeste homolog 2 and embryonic and ectoderm development protein) interacted with CTBP1-AS in primary granulosa cells and KGN cells. In addition, cryptotanshinone treatment was demonstrated to effectively downregulate CTBP1-AS expression level. Data from the present study suggested a pathophysiological role of CTBP1-AS in PCOS and may provide a new potential target for PCOS treatment.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies; it is characterized by a number of clinical

symptoms, including menstrual disorders, hyperandrogenism, infertility, insulin resistance, hirsutism, obesity and cystic ovaries (1-3). The worldwide incidence of PCOS is 5-10% among women of reproductive age, and it accounted for 70% of ovulation barrier sterility (4-6). Early diagnosis and timely treatment can contribute to the mitigation of the PCOS and may help in avoiding associated metabolic complications (7). However, due to the fact that many of the symptoms and signs of PCOS are also normal in puberty, PCOS diagnose criteria needs to be improved to prevent overdiagnosis of the syndrome (3,8). The Rotterdam diagnostic criteria for PCOS is the most widely used standard and includes hyperandrogenism, chronic anovulation (oligomenorrhoea or amenorrhoea) and polycystic ovary morphology (volume of the ovary >10 cm³ or at least 12 follicles measuring 2-9 mm) (9-11). Ruling out hyperandrogenism caused by other endocrinopathies, a patient with any two of above three criteria can be diagnosed with PCOS (12).

Confirmation of the etiology is key to determining the treatment of PCOS. To date, environmental factors and genetic factors are thought to be involved (13), including diet, unhealthy lifestyle and certain diseases (such as thyroid dysfunction, hyperprolactinemia and androgen-secreting tumors). Apart from these, a large number of genes have been identified as serving a role in the development of PCOS, such as GDF9 and androgen receptor (AR). Androgenic effect, exerted through the AR, is regarded as one of the most predominant mechanisms responsible for PCOS (14,15). As a steroid receptor and nuclear transcription factor, AR can control the expression of downstream target genes (16-18). In theory, hyperandrogenism is influenced by the changes of testosterone (T) level and AR activity. Previous studies have demonstrated that AR functions as a dynamic heterocomplex with a number of coactivators and corepressors (19-23). C-terminal-binding protein 1 antisense (CTBP1-AS) is a long non-coding RNA (lncRNA) that can directly suppress CTBP1 expression through sense-antisense binding, which subsequently promotes the transcriptional activity of AR (24,25). In addition to promoting prostate cancer progression (24), CTBP1-AS was also positively correlated with PCOS (12). However, the molecular mechanism of CTBP1-AS in PCOS remains largely unknown.

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In the present study, the levels of CTBP1-AS were compared between patients with PCOS and healthy controls. The effects of CTBP1-AS on the proliferation and apoptosis of granulosa cells was explored, and enhancer of zeste homolog 2 and embryonic (EZH2) and ectoderm development protein (EED) were indicated to interact with CTBP1-AS in primary granulosa cells and KGN cells. Notably, it was demonstrated that the level of CTBP1-AS could be significantly reduced by treatment of cryptotanshinone. The present findings may reveal a new pathophysiological relevance of CTBP1-AS in PCOS and may provide new potential target for PCOS treatment.

Materials and methods

Isolation and culture of ovarian granulosa cells. A total of 60 patients with PCOS and 60 healthy controls were recruited to the present study. After ruling out hyperandrogenism caused by other endocrinopathy, the Rotterdam diagnostic criteria was used to diagnose PCOS, particularly those with any two of the following features: Hyperandrogenism, chronic anovulation (oligomenorrhoea or amenorrhoea) and polycystic ovary morphology. All women with PCOS included in the study fulfilled all three criteria aforementioned; none of the healthy control women recruited in this study met any of the Rotterdam diagnostic criteria. All 120 patients were non-smokers, had no concurrent illness and took no regular medications prior to the study; a pregnancy test was performed prior to their inclusion in the study. The average age of patient group and control group were 29.21 ± 4.78 and 29.43 ± 3.82 years, respectively. No significant differences were identified for age or body mass index (BMI) between the two groups (Table I). The present study was approved by the ethics committee of The First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China; approval no. 2020-K-060-02); all subjects provided signed consent forms prior to recruitment to the study.

Both healthy controls and patients with PCOS were given clomiphene citrate at a dose of 50 mg/day on cycle days 5-9 to induce ovulation. Following ovulation induction, and 36 h after human chorionic gonadotropin (10,000 U) injection, two eggs were collected from each candidate using transvaginal ultrasound-guided retrieval; eggs from patients with PCOS were aspirated by an experienced operator before laparoscopic ovarian drilling (LOD) surgery, whereas those from the healthy control patients were immediately collected through 19-gauge single-lumen aspiration needles (K-OPS-7035-REH-ET; Cook) to aspirate under a suction pressure of 80 mmHg. Eggs from patients and healthy control were both performed transvaginally under ultrasound guidance in conscious sedation with fentanyl 1 $\mu\text{g}/\text{kg}$ IV (Fentanyl[®], B. Braun Medical AB) and propofol 30-40 mg IV (Propofol[®]-Lipuro, B. Braun Medical AB) was given when needed. The granulosa cells were detached under a compact cell culture light microscope (Olympus CKX53, Olympus) and kept in follicular fluids. Granulosa cells were centrifuged at 395 x g for 15 min at 4°C, the precipitate was harvested and mixed with 50% v/v Percoll solution (cat. no. 65455-52-9; Sigma-Aldrich; Merck KGaA) and centrifuged again at 395 x g for 10 min to remove red blood cells. Then, 0.2 g/l collagenase I solution (cat. no. 9001-12-1; Sigma-Aldrich; Merck KGaA) was

used to resuspend and digest the granulosa cells for 30 min at 37°C followed by centrifugation as aforementioned. Cells were harvested and seeded into a 12-well cell culture plate at 1×10^5 cells/ml. Each well contained 1 ml RPMI-1640 medium (cat. no. SH30809.01; HyClone; Cytiva) with 10% fetal bovine serum (cat. no. FB25015; Clark Bioscience) and 1% penicillin-streptomycin (PS; cat. no. SV30010; Hyclone; Cytiva). The cells were cultured in an incubator at 37°C with an atmosphere of 5% CO₂.

KGN cell culture. The KGN human ovarian granulosa tumor cell line (cat. no. CL0544) was purchased from Hunan Fenghui Biotechnology Co., Ltd. and cultured in DMEM/F-12 at 37°C with 5% CO₂ (cat. no. SH30023.01; HyClone; Cytiva) supplemented with 10% FBS and 1% PS.

RNA fluorescent in situ hybridization (FISH). To verify the subcellular localization of CTBP1-AS, a DIG RNA Labeling Kit (cat. no. 11175025910; Roche Diagnostics) was used to label CTBP1-AS probe (synthesized by RiboBio) according to the manufacturer's protocol. The sequence of the CTBP1-AS probe, complementary to CTBP1-AS, was 5'-TCATCATAA TTTCTTATCCTAAG-3'. A total of 50,000 primary granulosa cells were cultured on cover glasses in 24-well culture dishes for 24 h at 37°C with 5% CO₂. The cells were subsequently rinsed with PBS, fixed at room temperature in a solution of 3% formaldehyde and 10% acetic acid in PBS for 30 min, and then permeabilized with 0.5% Triton X-100 for 5 min at room temperature. After three washes with PBS, hybridization was performed in a moist chamber at 42°C overnight. Then, cells were washed by 4X saline sodium citrate (SSC) buffer with 0.1% Tween-20, and followed by 2X SSC, 1X SSC and PBS for 10 min at 42°C in dark. Cells were then blocked with 20% goat serum (cat. no. SL038; Beijing Solarbio) for 1 h at room temperature and followed by incubation with anti-DIG antibody (cat. no. 14682; Cell Signaling Technology) which diluted with 10% goat serum at 1:200 overnight at 4°C. After three washes with PBS, FITC-conjugated goat anti-rabbit secondary antibody (diluted with 10% goat serum at 1:1,000, cat. no. ab6717; Abcam) was added and incubated for 1 h at room temperature. Finally, the nucleus was counterstained with DAPI (cat. no. C0065; Beijing Solarbio Science and Biotechnology Co., Ltd.) for 20 min at room temperature. Results were visualized at 400 magnification using a Leica DMI4000B inverted fluorescence microscope (Leica Microsystems GmbH).

CTBP1-AS knockdown. For CTBP1-AS knockdown, two small interfering (si)RNAs were purchased from Sigma Genosys (Sigma-Aldrich; Merck KGaA) and their sequences are as follows: siCTBP1-AS #1, 5'-CCAAUUAUUAGACCACA AAA-3'; siCTBP1-AS#2, 5'-CAACUGUCAAGAAACAAU UAG-3'. Scrambled Stealth RNAi[™] Med GC (Thermo Fisher Scientific, Inc.) was used as a negative control (siControl). Cells were seeded in 6-well plate and grown to ~40% confluence at the time of transfection. 200 pmol (20 μM) siCTBP1-AS #1, siCTBP1-AS #1 or siControl was respectively transfected at room temperature by using the Lipofectamine RNAiMAX (cat. no. 13778-150; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The transfected cells

Table I. Clinicopathological characteristics of the patients with PCOS and the healthy control patients.

Clinicopathological feature	Control (n=60) ^a	PCOS (n=60) ^a	P-value
Age, years	29.43±3.82	29.21±4.78	NS
BMI, kg/m ²	23.12±2.13	24.35±3.32	NS
Total T, nmol/l	1.43±0.23	3.17±0.31	<0.001
Free T, pmol/l	19.32±5.65	25.32±5.21	0.038
E2, nmol/l	0.31±0.021	0.26±0.029	NS
SHBG, nmol/l	65.21±6.57	62.56±4.28	NS
DHEAS, nmol/l	5,119.45±666.21	6,351.32±996.67	0.025
FSH, IU/l	3.92±0.63	6.13±0.32	0.031
LH, IU/l	4.99±.34	9.32±1.85	0.015
Prolactin, ng/ml	11.27±1.29	13.98±3.65	NS
Fasting insulin, mIU/ml	5.52± 0.41	10.07±0.82	0.01
Fasting glucose, mmol/l	5.27±0.63	6.78±1.93	NS

^aData are presented as the mean ± SEM. BMI, body mass index; DHEAS, dehydroepiandrosterone-sulfate; E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; NS, not statistically significant; SHBG, sex hormone-binding globulin; T, testosterone.

were grown in antibiotic free DMEM/F-12 (8119239, Gibco; Thermo Fisher Scientific, Inc.) for 24 h and then re-seeded and cultured for an additional 24 h for further experiments with the density of 200 cells/well for colony formation assay and 30,000 for Cryptotanshinone treatment and immunofluorescence detection assay.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated with the High Purity Total RNA Extraction kit (cat. no. RP5611; BioTeke Corporation) and reverse-transcribed into cDNA with the super M-MLV reverse transcriptase (cat. no. PR6502; BioTeke Corporation) according to the manufacturer's instructions. qPCR was conducted to analyze CTBP1-AS and β -actin expression levels. qPCR was performed on an Applied Biosystems 7300 cycler using SYBR Green PCR MasterMix (Invitrogen; Thermo Fisher Scientific, Inc.). Thermocycling parameters are as follows: 95°C for 3 min, 40 cycles of 95°C for 20 sec and 60°C for 30 sec with FAM acquisition. The primer sequences used were as follows: CTBP1-AS forward, 5'-AACCTGGCA GCACGGAAGT-3'; CTBP1-AS reverse, 5'-GAGCACAAC CACCACCTCATC-3'; β -actin forward, 5'-CTGTGCCCATCT ACGAGGGCTAT-3'; β -actin reverse, 5'-TTTGATGTCACG CACGATTTCC-3'. Relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (26) and normalized to β -actin.

Preparation of nuclear and cytoplasmic RNA. A total of 5×10^6 cells were harvested, washed with cold PBS and centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was removed and the pellet was resuspended with cold nuclear fractionation buffer [140 mM NaCl, 10 mM Tris-HCl (pH 7.8), 1.5 mM MgCl₂, 0.5% NP-40 and 3 U/ml RNaseOUT (Thermo Fisher Scientific)]. After 5 min, the suspension was centrifuged 500 x g for 5 min at 4°C. The supernatant was collected as the cytoplasmic fraction, whereas the pellet was further washed with fractionation buffer three additional time, centrifuged at 2,000 x g for 5 min at 4°C and used as the

nuclear fraction. Total cytoplasmic and nuclear RNAs were extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and subsequently reverse transcribed to cDNA using super M-MLV reverse transcriptase (cat. no. PR6502; BioTeke Corporation) according to the manufacturer's instructions. The primer sequences used were as follows: U48 forward, 5'-AGTGATGATGACCCCAGGTA-3'; U48 reverse, 5'-GGT CAGAGCGCTGCGGTGAT-3'; 7SL forward, 5'-GTAGCT TTTCGCAGCGTCTC-3'; 7SL reverse, 5'-GCACTACAG CCCAGAACTCC-3'. The relative expression of CTBP1-AS was detected as aforementioned and calculated using the $2^{-\Delta\Delta C_q}$ method (26) and normalized to U48 and 7SL for nuclear and cytoplasmic fractions, respectively.

RNA pulldown and mass spectrometry analysis. Pierce Magnetic RNA-Protein Pull-Down Kit (cat. no. 20164; Thermo Fisher Scientific, Inc.) was used to perform RNA pulldown assay according to the manufacturer's instructions. Briefly, CTBP1-AS was synthesized by RiboBio Co. Ltd. and labeled using desthiobiotinylated cytidine bisphosphate and T4 RNA ligase. After labeling, CTBP1-AS was captured by streptavidin magnetic beads (50 μ l), which was contained in the kit, in RNA capture buffer at room temperature for 30 min. 5×10^7 KGN cells in logarithmic growth phase were harvested and lysed with Pierce IP Lysis Buffer (cat. no. 87787, Thermo Fisher Scientific, Inc.) with RNase inhibitor and protease/phosphatase inhibitor (cat. no. P0013; Beyotime). Subsequently, 1,000 μ g protein lysate were added to Protein-RNA Binding Buffer equilibrated RNA-bound beads. The mixture was incubated overnight with rotation at 4°C. The beads were then rinsed with wash buffer three times followed by vortexing and separation using a magnetic stand. 50 μ l Elution Buffer was added and incubated for 15-30 min at 37°C with agitation to elute the RNA-binding protein complex. After separation by the magnetic stand, the eluted samples were then harvested denatured for 10 min at 100°C in loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS (w/v), 0.1% BPB (w/v), 10% glycerol (v/v),

1% β -mercaptoethanol (v/v)] for SDS-PAGE electrophoresis followed closely by Coomassie brilliant blue staining and mass spectrometry analysis or western blotting detection. Mass spectrometry data was acquired by sending sample to Shanghai Bioprofile Ltd for mass spectrometry assay.

Western blotting. Protein concentrations from RNA pulldown assays were determined with a BCA Protein Assay Kit (cat. no. P0011; Beyotime Institute of Biotechnology). Proteins were denatured for 10 min at 100°C in loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS (w/v), 0.1% BPB (w/v), 10% glycerol (v/v), 1% β -mercaptoethanol (v/v)], separated by SDS-PAGE (10%), transferred into PVDF membranes and blocked with 5% skim milk for 2 h at room temperature. Membranes were incubated with anti-EZH2 (cat. no. 5246; Cell Signaling Technology, Inc.) or anti-EED (cat. no. 85322; Cell Signaling Technology, Inc.) antibodies, followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody (cat. no. 7074S, Cell Signaling Technology). All antibodies were diluted with 5% skim milk at 1:1,000 and incubated with the membranes for 2 h at room temperature. Protein bands were visualized with the Tanon High-sig ECL Western Blotting Substrate (cat. no. 180-501; Tanon Science and Technology Co., Ltd.).

Cell Counting Kit-8 (CCK-8) assay. Cell viability was measured using CCK-8 assay (cat. no. CK04; Dojindo Molecular Technologies, Inc.). Briefly, 5,000 primary granulosa cells were seeded into 96-well plate and transfected for 24 h with 10 pmol siCTBP1-AS #1, siCTBP1-AS #2 or siControl at room temperature. Then, transfected cells were cultured at 37°C with 5% CO₂ for ~24 h until the confluence reached 30-40%. 0, 24, 48 and 72 h later, CCK-8 solution was added and incubated for 2 h at 37°C. Absorbance at 450 nm was measured using CMax Plus (Molecular Devices, LLC).

ELISA. Blood samples (5 ml) were collected from all participants. The serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, estradiol (E2), total T, free T, sex hormone-binding globulin (SHBG) and dehydroepiandrosterone-sulfate (DHEAS), as well as fasting glucose and fasting insulin were determined by commercial ELISA kits for Testosterone (cat. no. ab108666) and DHEAS (cat. no. ab108669) purchased from Abcam. All detection were performed according to the manufacturer's protocol.

Flow cytometry detection of apoptosis. Cell apoptosis were detected using a FITC Annexin V Apoptosis Detection Kit I (cat. no. 556547; BD Biosciences). Primary granulosa cells were cultured to 30-50% confluence in 6-well plate, and then transfected with siRNAs for 48 h. Cells were harvested, washed with cold PBS and stained with propidium iodide (PI) alone or PI and Annexin V-FITC for 20 min at room temperature. Data were obtained using CytoFLEX (Beckman Coulter, Inc.) and analyzed by CytExpert software (Beckman Coulter, Inc. version 2.3).

Colony formation assay. SiCTBP1-AS #1, siCTBP1-AS #2 or siControl transfected primary granulosa cells were plated at 200 cells/well in 6-well dishes and cultured for 2-3 weeks

in DMEM/F-12 (8119239, Gibco) at 37°C with 5% CO₂, supplemented with 10% FBS (fetal bovine serum, FB25015, Clark) and 1% PS (penicillin-streptomycin, SV30010, Hyclone) and washed with PBS. The cells were fixed in cold methanol for 30 min. Fixed colonies were visualized by incubating the cells with 0.5% (w/v) crystal violet for 30 min. Residual crystal violet was rinsed away with three washes with PBS. Visible colonies, consisting of ≥ 50 cells, were counted. Each experiment was repeated three times.

Cryptotanshinone treatment and immunofluorescence. A total of 30,000 KGN cells were cultured on cover glasses in 24-well culture dishes. After 24 h incubation, different concentrations cryptotanshinone (5 μ M in Fig. 4 and 0, 2.5, 5, 10 μ M for Fig. S3) was added and the cells were incubated for another 24 h. The cells were rinsed with PBS, fixed in a solution of 3% formaldehyde and 10% acetic acid in PBS for 30 min at room temperature and then permeabilized with 0.5% Triton X-100 for 5 min at room temperature. After three washes with PBS, cells were incubated with diluted (1:500) anti-5-methylcytosine (5mC) antibody (cat. no. ab10805; Abcam) to label methylated DNA overnight at 4°C and the nuclei were counterstained with DAPI (5 μ g/ml) for 20 min at room temperature. Results were visualized and images captured under a Leica DMI4000B inverted fluorescence microscope and analyzed by Leica Application Suite V4 software (Leica Microsystems GmbH).

Analyzing the promoter region of CTBP1-AS. To analyze the promoter region of CTBP1-AS, we recruited the UCSC Genome Browser database (genome.ucsc.edu). CTBP1-AS (Homo sapiens, NR_104331) was used to analyze the CpG island existed in the promoter region.

Statistical analysis. Data are presented as the mean \pm SEM. Comparisons of non-normally distributed variables between clinicopathological characteristics of healthy control and patients with PCOS (Table I) and the comparisons of CTBP1-AS level in healthy controls and patients with PCOS (Fig. 1) were determined using Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's post hoc test. were performed using the Mann-Whitney U test. Student's t test was used to determine the statistical significance when only two groups were compared, and when three groups or more were compared, ANOVA followed by Tukey's post hoc test was used. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed using IBM SPSS Statistics 25.0 (IBM Corp.). All the experiments were repeated three times.

Results

CTBP1-AS is upregulated in PCOS. To investigate whether CTBP1-AS served a role in PCOS, 60 patients with PCOS and 60 healthy controls were recruited to the present study; there were no significant differences in age and BMI (Table I). ELISA serum analysis results demonstrated that patients with PCOS had higher levels of total T, free T, DHEAS, FSH, LH and fasting insulin than controls (Table I). However, no significant differences were identified for the

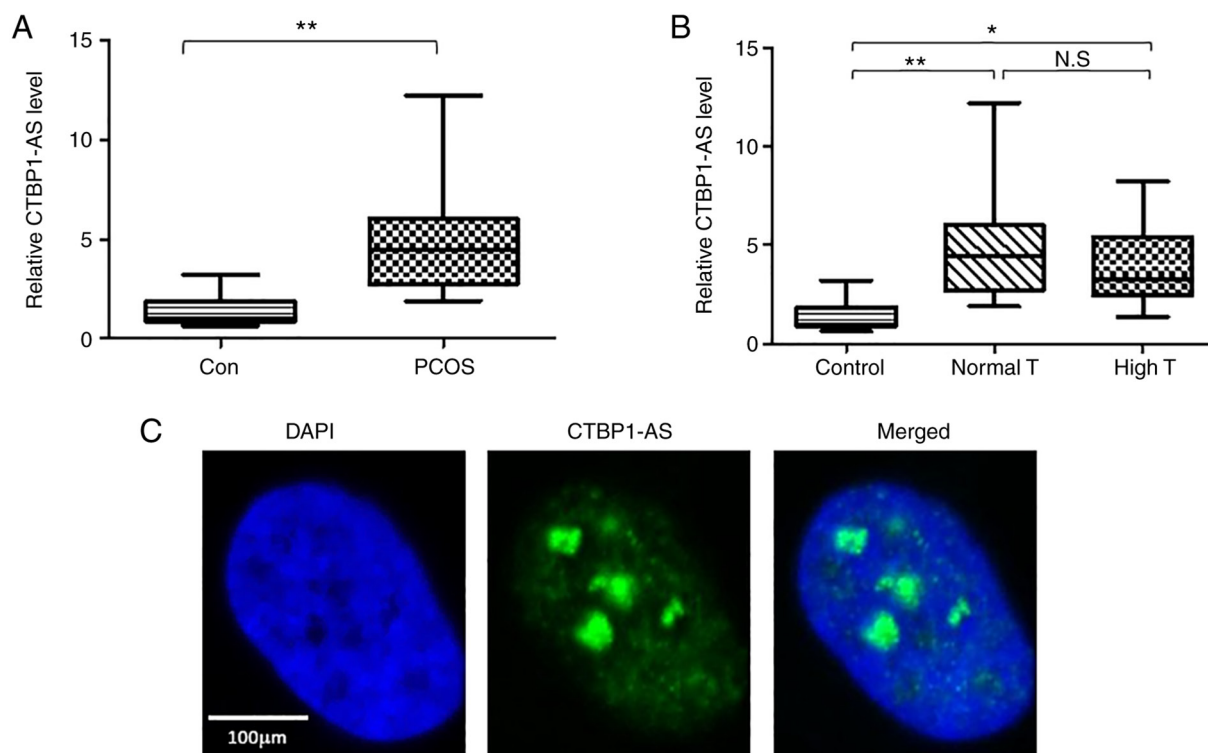


Figure 1. CTBP1-AS is upregulated in PCOS. RT-qPCR analysis of CTBP1-AS levels (A) in healthy controls and patients with PCOS and (B) in healthy controls and in patients with PCOS with normal or high T levels. Data are presented as median and interquartile ranges. * $P < 0.05$, ** $P < 0.01$. (C) RNA fluorescence *in situ* hybridization of CTBP1-AS in primary granulosa cells; scale bar, 100 μm . Con, control; CTBP1-AS, C-terminal-binding protein 1 antisense; PCOS, polycystic ovary syndrome; RT-qPCR, reverse transcription-quantitative PCR; T, testosterone.

concentrations E2, SHBG, prolactin and fasting glucose between the two groups.

Two oocytes were harvested from each patient and the ovarian granulosa cells were separated, cultured and subsequently used to detect the expression level of CTBP1-AS by RT-qPCR. Compared with healthy controls, the level of CTBP1-AS was significantly higher in patients with PCOS (Fig. 1A). Total T levels were notably different among patients with PCOS; therefore, the patients with PCOS were divided into high T and normal T group. Both groups exhibited higher levels of CTBP1-AS compared with the control group (Fig. 1B). These data suggested that CTBP1-AS may participate in PCOS process. Next, the localization of CTBP1-AS in granulosa cells was examined using RNA FISH. Consistent with a previous report (24), CTBP1-AS was expressed throughout the nucleus (Fig. 1C). To further evaluate the distribution of CTBP1-AS in granulosa cells, the expression levels in cytoplasmic and nuclear fractions were analyzed by RT-qPCR. The results demonstrated that the CTBP1-AS levels in the nucleus were ~150 times than that of the expression in cytoplasm (Fig. S1).

CTBP1-AS balances proliferation and apoptosis of granulosa cells. To further investigate the biological significance of CTBP1-AS in PCOS, CTBP1-AS expression was knocked down using siRNA. As shown in Fig. 2A, the expression levels of CTBP1-AS were significantly decreased in primary granulosa cells transfected with siCTBP1-AS #1 and siCTBP1 #2 compared with siControl transfected cells. The proliferative ability of granulosa cells was examined through CCK-8

assays, and the results demonstrated that proliferation was significantly inhibited by the siCTBP1-AS transfections compared with the control (Fig. 2B). Colony formation assays further demonstrated that CTBP1-AS knockdown suppressed the colony formation ability of primary granulosa cells compared with the control group (Fig. S2). In addition, the effect of CTBP1-AS on granulosa cell apoptosis was examined by flow cytometry. The results showed that the number of both apoptotic and necrotic cells significantly increased following CTBP1-AS knockdown (Fig. 2C and D). Taken together, these results indicated that CTBP1-AS may facilitate PCOS through balancing the proliferative and apoptotic rates of granulosa cells.

CTBP1 directly interacts with EZH2 and EED in granulosa cells. As CTBP1-AS could recruit and bind PTB-associated splicing factor in prostate cancer cells (24), the present study attempted to identify the interacting proteins of CTBP1-AS in granulosa cells using RNA pulldown and mass spectrometry. As shown in Fig. 3A, the enrichment efficiency of CTBP1-AS was ~9 times higher compared with the control group. After incubation with cell lysate, the mixture was collected and examined by SDS-PAGE, followed closely by Coomassie brilliant blue staining. The specific bands were further analyzed by mass spectrometry, and two Polycomb-group proteins, EZH2 and EED, were of particular interest (Fig. 3B). Both proteins make up the core catalytic subunits of Polycomb repressive complex 2 (PRC2) and PRC2 is responsible for catalyzing histone 3, lysine 27 trimethylation (H3K27me3) on chromatin (26,27). The interaction was further confirmed

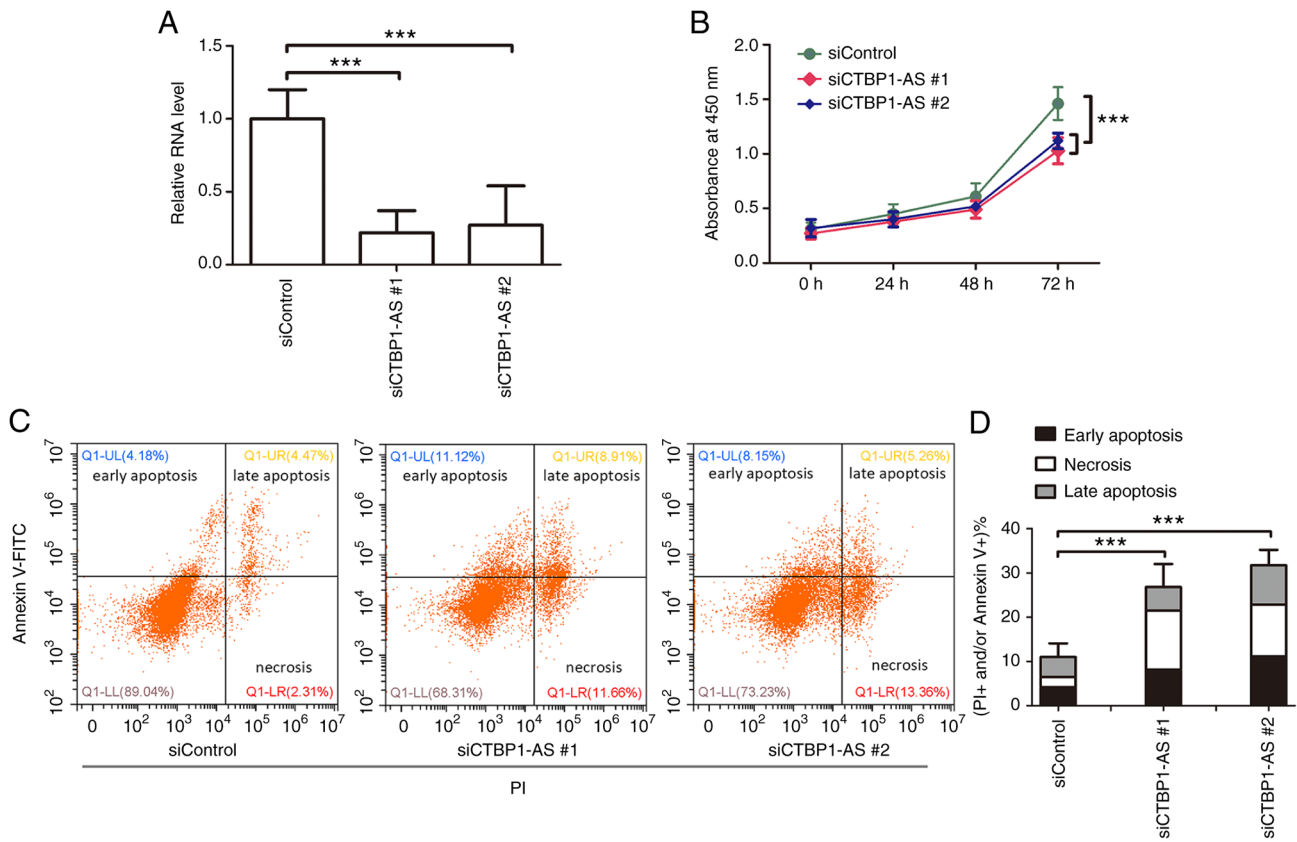


Figure 2. CTBPI-AS knockdown reduces proliferation and promotes the apoptosis of primary granulosa cells. (A) Reverse transcription-quantitative PCR analysis of the knockdown efficiency of siCTBPI-AS transfections. (B) Cell proliferative ability was determined by Cell Counting Kit-8 assay. Apoptosis induced by CTBPI-AS knockdown was detected by (C) flow cytometry, and (D) the percentages of early apoptosis (UL, Annexin V-FITC+/PI-), late apoptosis (UR, Annexin V-FITC+/PI+) and necrosis (LR, Annexin V-FITC-/PI+), single /PI negative and Annexin V-FITC/PI double-positive cells. Data are presented as the mean \pm SEM. *** $P < 0.001$. CTBPI-AS, C-terminal-binding protein 1 antisense; si, small interfering RNA.

in primary granulosa cells and KGN cells by RNA pulldown (Fig. 3C). It was inferred from these results that CTBPI-AS may regulate PCOS through EZH2- and EED-dependent H3K27me3 methylation.

Cryptotanshinone effectively reduces the level of CTBPI-AS in KGN cells. Cryptotanshinone can ameliorate insulin resistance and androgen excess in a PCOS rat model (28,29), but the molecular mechanism is still not fully understood. To better elucidate this mechanism, KGN cells were treated with 5 μ M cryptotanshinone for 24 h (Fig. 4A). The results of 5mC immunofluorescence staining suggested that the methylation level of the whole genome had a positive association with the concentration of cryptotanshinone (Fig. S3). Immunofluorescence intensity induced by 10 μ M cryptotanshinone was weaker compared with that induced by 5 μ M (Fig. S3); it was speculated that this may be caused by the cytotoxicity of cryptotanshinone. Furthermore, UCSC Genome Browser analysis showed that there existed CpG island in the promoter region of CTBPI-AS (Fig. 4B); therefore, we hypothesized that cryptotanshinone may regulate the level of CTBPI-AS through methylation. Consistent with this, cryptotanshinone treatment significantly inhibited the level of CTBPI-AS (Fig. 4C). These results indicated that CTBPI-AS may be a potential target of cryptotanshinone for PCOS treatment.

Discussion

As the main functional cells in the ovaries, granulosa cells participate in the development of follicles and steroid secretion (30). Atresia is usually triggered by oocyte apoptosis followed by granulosa cell apoptosis in the early stage of follicular development; however, granulosa cell apoptosis initiates atresia in mature follicles (31). Communication between oocytes and granulosa cells lasts throughout the oocyte cell development process. Granulosa cells can provide nutrients and growth factors for oocyte cells, and oocytes promote the growth and differentiation of granulosa cells (32). Previous reports have demonstrated that dysfunction of these cells induces abnormal follicle and related to PCOS (33,34); however, the molecular mechanism is still unknown. AR is generally thought to be closely related to PCOS, and lncRNA CTBPI-AS has been demonstrated to serve a role in PCOS regulation through AR (12). In the present study, the expression levels of CTBPI-AS were compared in the granulosa cells from 60 patients with PCOS and 60 healthy control patients. The data revealed that CTBPI-AS is upregulated in patients with PCOS, and knockdown of CTBPI-AS could significantly inhibit the proliferation and promote the apoptosis of granulosa cells *in vitro*.

Polycomb group proteins are considered paradigmatic epigenetic modulators that regulate cell proliferation and

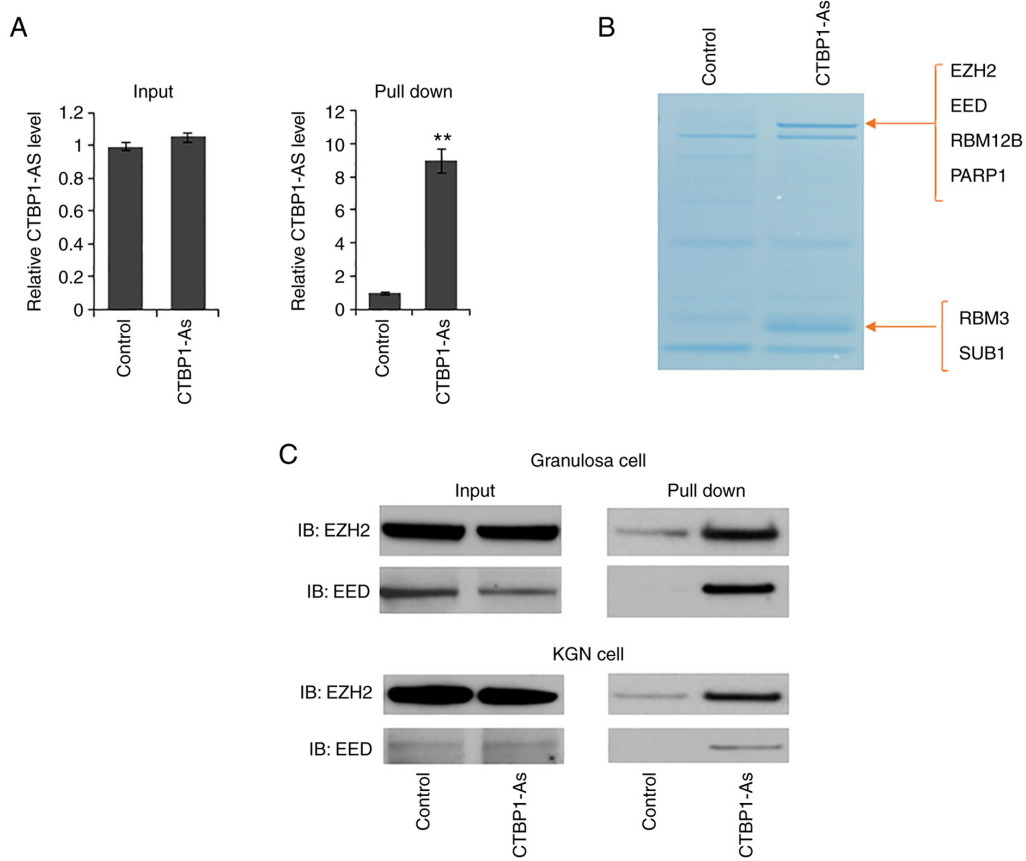


Figure 3. CTBP1 directly interacts with EZH2 and EED in granulosa cells. (A) The efficiency of RNA pull-down was detected by reverse transcription-quantitative PCR. Data are presented as the mean \pm SEM. ** $P < 0.01$ vs. control. (B) Samples from RNA pull-down assays were collected for SDS-PAGE and Coomassie brilliant blue staining. Arrows indicated the proteins identified by mass spectrometry. (C) Interaction between CTBP1-AS and EZH2 or EED were identified in granulosa cells and KGN cells by RNA pull-down and immunoblotting analysis. CTBP1-AS, C-terminal-binding protein 1 antisense; EED, embryonic ectoderm development protein; EZH2, enhancer of zeste homolog 2; PARP, poly-ADP-ribose polymerase; RBM12B, RNA binding motif protein 12B; RBM3, RNA binding motif protein 3.

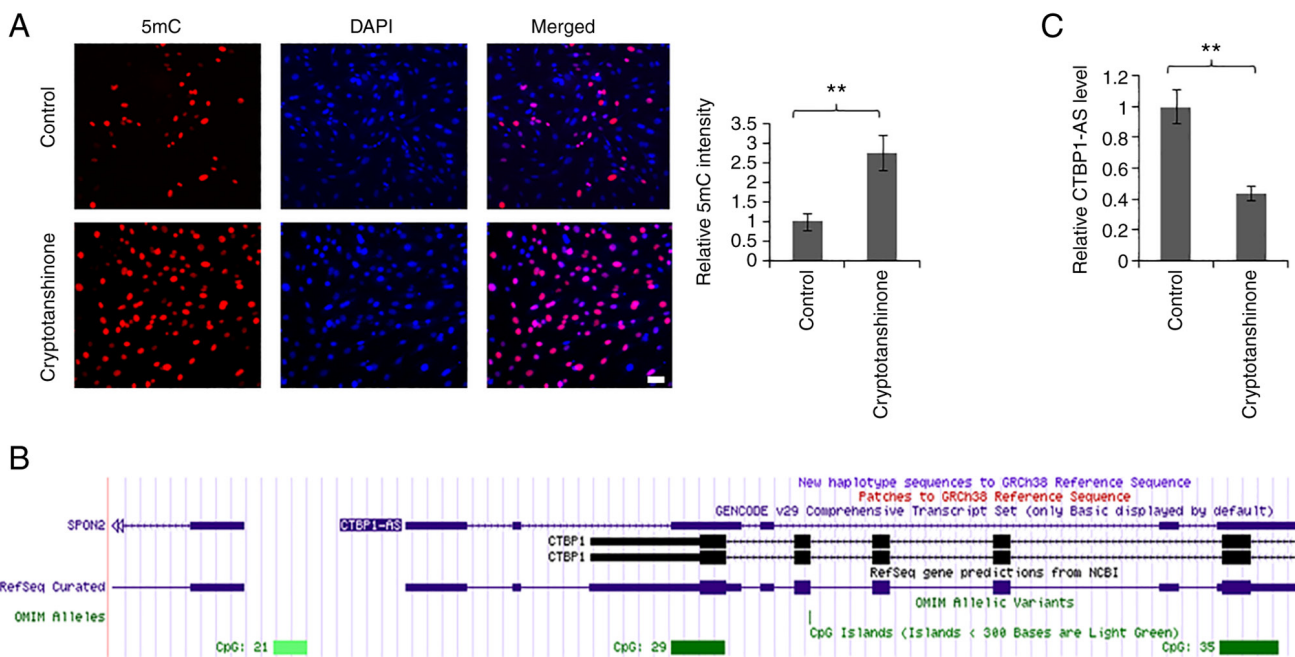


Figure 4. Cryptotanshinone effectively reduces the level of CTBP1-AS in KGN cells. (A) 5mC immunofluorescence of KGN cells treated with 5 μ M cryptotanshinone for 24 h; nuclei were stained with DAPI. The intensity of 5mC-labeled cells is shown on right. Scale bar, 100 μ m. (B) CpG islands in the promoter region of CTBP1-AS were analyzed by UCSC Genome Browser. (C) Reverse transcription-quantitative PCR analysis of CTBP1-AS expression levels in KGN cells treated with or without cryptotanshinone. Data are presented as the mean \pm SEM. ** $P < 0.01$. 5mC, 5-methylcytosine; CTBP1-AS, C-terminal-binding protein 1 antisense

differentiation, and organ development, as well as initiation and progression of certain diseases, such as prostate cancer and diffuse large B-cell lymphoma (DLBCL), through remodeling the chromatin structure and subsequent transcriptional repression (35). EZH2 and EED are the two core catalytic subunits of PRC2 and are required for histone H3 methylation (35). In prostate cancer, AR directly binds to the promoter and enhancer of EZH2 to regulate its expression (36). However, in castration-resistant prostate cancer cells, EZH2 can directly bind to and regulate AR (37). A recent study showed that EZH2 and EED could bind AR to regulate its expression level and the expression of downstream target genes (35). A previous study by Takayama *et al* (24) demonstrated that CTBP1-AS could promote AR transcriptional activity in prostate cancer. However, whether there exists interaction between EZH2, EED and CTBP1-AS is still not clear. The present study identified an interaction between these components in granulosa cells in patients with PCOS, which may provide a potential target for PCOS treatment.

Results from the present study demonstrated that the traditional Chinese medicine, cryptotanshinone, could effectively decrease the expression level of CTBP1-AS. CpG islands can be found in the promoter region of CTBP1-AS, and the methylation of this region could inhibit its transcription. Therefore, drugs that target the methylation of CTBP1-AS CpG islands may provide a new direction for PCOS treatment.

In conclusion, the present study results demonstrated an upregulated expression level of CTBP1-AS in PCOS granulosa cells and identified the interaction between CTBP1-AS and the Polycomb group proteins, EZH2 and EED. Most importantly, results demonstrated the efficiency of cryptotanshinone in reducing the level of CTBP1-AS expression. However, there are limitations to the present study. As PCOS is a complex pathological process caused by many factors, the effects of up- or downregulation of CTBP1-AS in a PCOS model was not comprehensively verified by *in vitro* and *in vivo* experiments. Therefore, additional studies are needed to further clarify the role of CTBP1-AS in PCOS.

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Availability of data and materials

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

Authors' contributions

MW contributed to experimental data analyses and wrote the manuscript. SZ, BW, JX, WZ and FW contributed experimental data. XD designed the experiments and supervised the research. MW and XD confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of The First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China; approval no. 2020-K-060-02); all subjects provided signed consent forms prior to recruitment to the study.

Patient consent for publication

All the patients in this project signed the consent forms.

Competing interests

The authors declare that they have no competing interests.

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