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Clinical significance and biological roles of lncRNA *CTBP1-AS* in polycystic ovary syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is among the most prevalent endocrine and metabolic disorders affecting women of reproductive age. Multiple factors, including genetic predisposition, environmental influences, and lifestyle choices, are considered significant contributors to the development of PCOS. A kind of long noncoding RNA—C-Terminal binding protein 1 antisense (lncRNA *CTBP1-AS*) has been proven to be a new androgen receptor regulator. Previous studies showed that the lncRNA *CTBP1-AS* gene was highly expressed in a small sample of PCOS patients and was associated with the risk of PCOS, but its specific function and mechanism have not been clearly reported. In this study, the expression of lncRNA *CTBP1-AS* was detected by real-time quantitative PCR (RT-qPCR) in PCOS patients. In addition, lncRNA *CTBP1-AS* was overexpressed in KGN cells to explore its effect on granulocyte function. The results showed that the expression levels of lncRNA *CTBP1-AS* were increased in peripheral blood mononuclear cells and follicular fluid granulosa cells of PCOS patients compared with controls, which correlated with androgen levels and sinus follicle number; overexpression of lncRNA *CTBP1-AS* increased apoptosis and decreased cell migration ability, thus promoting the progression of PCOS. This study explores new biomarkers and therapeutic targets for the clinical individualized diagnosis and treatment of PCOS.

Keywords Polycystic ovary syndrome, lncRNA, *CTBP1-AS*, Expression regulation, Granulosa cells

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Introduction

Polycystic ovary syndrome (PCOS), a common heterogeneous endocrine and metabolic disorder, is one of the most common reproductive endocrine disorders in women of childbearing age, affecting up to 20% of women worldwide [1, 2]. Nevertheless, the etiology and pathogenesis of PCOS are complex and influenced by environmental, psychological, and genetic factors, but the exact etiology has not yet been identified [3]. At the same time, the clinical manifestations of PCOS are highly heterogeneous, and there is no specific treatment in the clinic. For women of childbearing age, the main treatment is ovulation promotion and assisted reproduction techniques, and for women who have no requirement

for childbearing, the prevention and treatment of long-term complications and metabolic syndrome should also be carried out, and such metabolic and endocrine disorders may affect the whole life of a woman [4, 5]. Therefore, the diagnosis and treatment of PCOS still need to be further researched into its pathogenesis-related factors and progression mechanisms, to identify new diagnostic and therapeutic targets, provide early diagnosis of PCOS patients, and provide new and better individualized diagnostic and therapeutic methods.

Long noncoding RNA of C-Terminal binding protein 1 antisense (lncRNA *CTBP1-AS*) is a newly discovered long noncoding RNA [6]. It has been demonstrated that lncRNA *CTBP1-AS*, a potential androgen receptor regulator, functions in the androgen receptor (AR) signaling pathway [7, 8]. Notably, aberrant expression of AR can lead to enhanced activation of otherwise normal levels of androgens (testosterone and dihydrotestosterone) in the body, resulting in hyperandrogenemia [9]. The available evidence suggests that metabolic disturbances due to hyperandrogenemia are one of the important features of the pathological changes in PCOS [10]. Recently, lncRNA *CTBP1-AS* as a new androgen-regulated long noncoding RNA has gradually attracted the attention of many scholars [11]. Previous studies have found that lncRNA *CTBP1-AS* is associated with the pathogenesis of PCOS, but their samples are small and limited to the detection of a single type of cells in patients with PCOS. The influence of lncRNA *CTBP1-AS* on the occurrence and development of PCOS as well as a clear mechanism of action still requires further study [6, 12]. Therefore, the effect of lncRNA CTBP1-AS in the development of PCOS and its definitive mechanism remains to be further explored.

Our study aimed to further explore the correlation between lncRNA *CTBP1-AS* and the pathogenesis of PCOS by expanding the sample size and investigate the functional effects of lncRNA *CTBP1-AS* on ovarian GCs by cellular assays. This study confirmed the functional role of lncRNA *CTBP1-AS* in the occurrence and development of PCOS, and explored new targets for molecular diagnosis and gene therapy of PCOS.

Materials and methods
Study design
This study employed a case-control design and was conducted at the Reproductive Medicine Center of the Affiliated Hospital of Youjiang Medical University for Nationalities in Guangxi from January 2018 to March 2019.

Study population
Patients diagnosed with PCOS constituted the case group, while age-matched patients undergoing assisted reproductive technology for infertility due to tubal or male factors served as the control group.

Peripheral blood specimens
125 peripheral blood specimens were collected, comprising 85 cases from the PCOS group and 40 controls. The mean age of participants was 27.38±4.35 years.

Inclusion criteria of peripheral blood specimens
Inclusion criteria were as follows: ①Case group: The diagnostic criteria for PCOS were based on the Rotterdam criteria recommended by ESHRE/ASRM in 2003: (1) oligo-ovulation and/or anovulation (OA); (2) increased serum androgen levels and/or hyperandrogenism (HA); (3) polycystic ovarian changes. The presence of two of the above three items at the same time needs to rule out other diseases, such as Cushing syndrome, congenital adrenal hyperplasia, and other diseases causing high androgen, as well as hyperprolactinemia and other diseases causing abnormal ovulation. ②Control group: patients with infertility due to tubal or male factors, regular menstruation (menstrual cycle of 28±7 days), no clinical signs of hyperandrogenism, normal ovulation by ultrasound monitoring, and normal morphology of both ovaries during the same period.

Clinical indicators of peripheral blood specimens
Table 1 presents the clinical indicators for peripheral blood specimens from patients with PCOS and controls.

Follicular fluid specimens
A total of 30 follicular fluid specimens were collected, 13 in the case group and 17 in the control group; the mean age was 32.87±4.15 years.

Inclusion criteria of follicular fluid specimens
The inclusion criteria were as follows: ① Case group: patients with PCOS who underwent in vitro fertilization and embryo transfer (IVF-ET) for the first time due to female factor (diagnostic criteria as before). ②Control group: patients who underwent IVF-ET for the first time due to tubal factor infertility, with regular menstruation, no clinical manifestations of hyperandrogenism, and no abnormality in the morphology of bilateral ovaries under ultrasound.

Clinical indicators of follicular fluid specimens
Table 2 presents the clinical indicators for follicular fluid specimens from patients with PCOS and controls. At the same time, Table 3 presents the clinical indicators of IVF-ET for follicular fluid specimens from patients with PCOS and controls.

Table 1 Clinical indicators of peripheral blood samples

Clinical indicators	PCOS (*N* = 85)	Control(*N* = 40)	t / Z	*p* value
Age(years)	26(24,29)	28(24, 31)	-1.397	0.163
Duration of infertility (years)	2(1, 4)	2(1, 4)	-0.51	0.61
Menstrual cycles(days)	68(60, 90)	31(30, 40)	-7.907	< 0.001*
Antral follicle count	31(28, 40)	18(15, 20)	-8.358	< 0.001*
Basic sex hormone				
FSH (IU/L)	5.78 ± 1.84	5.81 ± 1.78	-0.095	0.925
LH (IU/L)	8.67(5.53, 12.60)	4.16(3.64, 5.39)	-5.547	< 0.001*
E2 (pg/mL)	56.0(45.0, 68.5)	47.0(38.0, 59.7)	-2.844	0.004*
P (ng/mL)	0.46(0.30, 0.70)	0.49(0.22, 0.60)	-0.506	0.613
T (ug/L)	0.76 ± 0.27	0.47 ± 0.19	6.253	< 0.001*
PRL (ug/L)	12.58 ± 5.74	10.80 ± 4.84	1.7	0.092
AMH (ng/mL)	7.55(5.00,10.62)	4.48(2.37, 6.48)	-4.701	< 0.001*
FPG (mmol/L)	4.87(4.60, 5.23)	4.75(4.52, 5.15)	-1.5	0.134
FINS (pmol/L)	75.0(58.1, 89.1)	52.0(40.5, 68.5)	-4.01	< 0.001*
HOMAIR	2.44(1.77, 2.91)	1.57(1.26, 2.08)	-4.319	< 0.001*
BMI (kg/m^2)	22.7(20.1, 24.9)	21.3(19.1, 24.9)	-1.527	0.127

Data are presented as the mean ± standard deviation, or median (interquartile range). FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; P, progestogens; T, testosterone; PRL, prolactin; AMH, anti-mullerian hormone; FPG, Fasting Plasma Glucose; FINS, Fasting Insulin; HOMAIR, homeostatic model assessment of insulin resistance; BMI, body mass index; *P*-value from Mann-Whitney U test or independent t-test for continuous variables. *$P < 0.05$ indicates a statistically significant association between the variables

Table 2 Clinical indicators of follicular fluid samples

Clinical indicators	PCOS (*N* = 13)	Control(*N* = 17)	t / Z	*p* value
Age(years)	31.92 ± 4.59	33.59 ± 3.76	1.093	0.284
Duration of infertility (years)	3(2, 5)	3(2,4)	-0.301	0.764
Menstrual cycles(days)	60(37, 90)	30(30, 33)	-3.656	< 0.001*
Antral follicle count	24(19,30)	14(11, 16)	-3.558	< 0.001*
Basic sex hormone				
FSH (IU/L)	5.70(5.1, 6.35)	6.68(5.09, 8.64)	-1.612	0.107
LH (IU/L)	5.43(2.75, 11.48)	3.60(2.71, 4.73)	-1.78	0.075
E2 (pg/mL)	43.31 ± 12.61	54.18 ± 22.97	1.535	0.136
P (ng/mL)	0.36 ± 0.19	0.45 ± 0.18	1.352	0.187
T (ug/L)	0.50 ± 0.17	0.43 ± 0.16	-1.192	0.243
PRL (ug/L)	11.37(8.63,13.59)	12.3(9.99,17.45)	-1.089	0.276
AMH (ng/mL)	7.12 ± 3.13	3.42 ± 1.77	-3.829	0.001*
FPG (mmol/L)	5.05(4.58, 5.36)	4.80(4.60, 5.15)	-1.219	0.223
FINS (pmol/L)	58.92 ± 10.78	51.01 ± 15.15	-1.596	0.122
HOMAIR	1.88 ± 0.32	1.59 ± 0.53	-1.749	0.091
BMI (kg/m^2)	21.44 ± 1.73	21.95 ± 2.36	0.66	0.515

P-value from Mann-Whitney U test or independent t-test for continuous variables. *$P < 0.05$ indicates a statistically significant association between the variables

Cell culture and cell transfection

Human ovarian granulosa cells KGN were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Ausgenex), penicillin (100 U/mL, Solarbio), and puromycin (100 μg/mL, Thermofisher).

293 A and 293T cells were purchased from the Cell Bank of the Chinese Academy of Sciences, and the lncRNA *CTBP1-AS* overexpression plasmid was purchased from Suzhou Ltd. Adenovirus after successful packaging were transfected into the cultured KGN cells at Polybrene 4 μg/ml, MOI = 5 conditions, and the

fluorescence expression was observed 48–72 h after infection.

CCK-8 assay

CCK-8 assay was used to measure cell proliferation. In the presence of 1-Methoxy PMS, WST-8 in the reagent was reduced by dehydrogenases in the cells to a highly water-soluble yellow formazan product (Formazan dye). The number of produced formazan products was proportional to the number of living cells. The OD value of the optical density at 450 nm, measured using an enzyme marker, reflected the change in the number of living cells,

Table 3 Clinical indicators of IVF-ET of follicular fluid samples

Clinical indicators	PCOS($N = 13$)	Control($N = 17$)	t / Z / χ^2	*p* value
Gn Days of use(d)	9(9, 10)	10(10,12)	2.329	0.022*
Total Gn(IU)	1550(1200, 1562)	2125(1775,2575)	3.440	<0.001*
HCG days				
Endometrial thickness(mm)	11.0(11.0, 12.5)	11.0(10.0, 12.5)	0.021	0.983
E2(pg/mL)	4082.46±1997.17	3379.39±1194.54	1.201	0.240
No. of oocytes retrieved(n)	20.15±4.93	12.18±4.49	4.623	<0.001*
No. of MII oocytes(n)	15.08±5.01	9.35±3.84	3.548	0.001*
Number of fertilized(n)	11(7, 19)	9(6, 11)	1.643	0.100
No. of cleavage embryos(n)	10(7, 18)	9(5, 11)	1.368	0.171
No. of good-quality embryos(n)	7(5, 8)	4(3, 5)	2.147	0.035*
OHSS occurrence rate (%)	46.2(6/13)	11.8(2/17)	4.455	0.045*
Transplant cancellation rate (%)	46.2(6/13)	29.4(5/17)	0.889	0.287
Pregnancy rate (%)	42.9(3/7)	41.7(5/12)	0.003	0.663

P-value from Mann-Whitney U test or independent t-test for continuous variables, and Chi-square test or Fisher's exact test for categorical variables. *P < 0.05 indicates a statistically significant association between the variables

thus detecting differences in proliferation produced by different cells over time.

Flow cytometry
The cells were digested and centrifuged at 200 g. The supernatant was discarded. The cells were resuspended with 50 μL 1× Binding Buffer, and the dyes were added according to the following groups: without any dye (negative control), 5 μL Annexin V-APC (single positive control), 10μL 7-AAD (single positive control), 5 μL Annexin V-FITC and 10 μL PI (sample tube). The mixture was incubated for 15 minutes in the dark. The apoptosis rate was analyzed by flow cytometer (BD Biosciences).

Cell scratching assay
Cells were seeded in transwell chambers, and the chambers were immersed in 2% serum medium outside the chambers. 24 h later, the chambers were removed, and the cells flanking the outside of the chambers were stained and photographed with a light microscope.

Primer information
For the RT-qPCR analysis of lncRNA CTBP1-AS expression, specific primers were designed as follows: lncRNA CTBP1-AS Forward Primer: 5'-ACAACACAAAGCCC CGGAA-3', lncRNA CTBP1-AS Reverse Primer: 5'-AGT GAAGAATGGTCTCGCCC-3', GAPDH Forward Primer (Housekeeping Gene): 5'-GGGAAACTGTGGCGTGA T-3', GAPDH Reverse Primer: 5'- GAGTGGGTGTCGC TGTTGA-3'. These primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)
Total RNA was extracted by using AxyPrep Total RNA Small Volume Preparation Kit, and the extracted RNA was detected by applying a nucleic acid quantifier, including the content and purity. The RNA with qualified purity and concentration was subjected to reverse transcription using the Reverse Transcription Reaction Kit (Fermentas Inc., USA). Then, lncRNA CTBP1-AS and the internal reference GAPDH were amplified by PCR, and a sample from the same normal group was added as a reference sample for each RT-qPCR. The reaction system for RT-qPCR was 20 μl, consisting of 3 μl F, 3 μl R, 3 μl DEPC, 1 μl cDNA and 10 μl SYBR. The reaction conditions were: 95 ℃ for 10 min pre-denaturation followed by 95 ℃ for 15 s and 60 ℃ for 1 min for 40 cycles. Three replicate measurements were taken for each sample to ensure accurate results. The Ct mean values of each sample lncRNA *CTBP1-AS* and GAPDH were first calculated separately, and then the data were used to calculate the relative quantity (RQ) of expression using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis
SPSS 27.0 software was used for statistical processing. Measurement data conforming to normal distribution were expressed as x̄±s, and comparison of data between groups should be made by t-test or ANOVA according to the nature of the data; if they did not conform to normal distribution they were expressed as median (M) and quartiles (P_{25}, P_{75}), and comparison between groups was changed to rank sum test. Count data were expressed as rates (%), and comparisons between groups were made using the four-cell χ^2 test or Fischer's exact test. Correlations between variables were analyzed by Spearman's rank correlation analysis. A value of *P* < 0.05 was considered statistically significant.

Results
General clinical information
In the clinical data of peripheral blood samples, age, duration of infertility, basal FSH, P, PRL, FPG, and BMI

levels of two study groups were not statistically differences. The menstrual cycle, antral follicle count, basal LH, E2, T, AMH, FINS, and HOMAIR levels were higher in the PCOS group than in the control group($p<0.05$) (Table 1). In the clinical data of granulocyte samples, The menstrual cycle, antral follicle count, and AMH were higher in the PCOS group than in the control group. After pretreatment of the study subjects who entered the IVF-ET cycle, the differences in basal FSH, LH, E2, PRL, P, and T were not statistically significant when comparing the pre-superovulation sex hormone levels between the two groups. It suggested the two groups were more balanced in terms of sex hormones and other indicators at the time of entry into the treatment cycle, and the follow-up studies were comparable (Table 2). When comparing the clinical indicators of IVF-ET between the PCOS group and the control group, Gn days of use (d) and total Gn (IU) in the PCOS group were lower than those in the control group ($P<0.05$), whereas the number of oocytes retrieved, the number of MII oocytes, the number of good-quality embryos, and OHSS occurrence rate in the PCOS group were higher ($P<0.05$) (Table 3).

The expression of lncRNA *CTBP1-AS* in peripheral blood mononuclear cells and follicular fluid GCs

In our study, we found that patients with PCOS had a higher lncRNA *CTBP1-AS* level in peripheral blood mononuclear cells, compared with controls(0.86 ± 0.43 vs. 0.71 ± 0.29, $P=0.034$) (Fig. 1A). To better study the clinical heterogeneity of PCOS, we further divided the PCOS group into several subgroups according to the Rotterdam criteria and the main clinical characteristics (hyperandrogenic and insulin resistance), respectively, and analyzed them statistically using the control group as a reference. They were categorized into 4 subtypes according to the Rotterdam criteria: complete OA+HA+PCO ($n=33$), ovulatory HA+PCO ($n=4$), non-hyperandrogenic OA+PCO ($n=39$), and classic OA+HA ($n=9$);
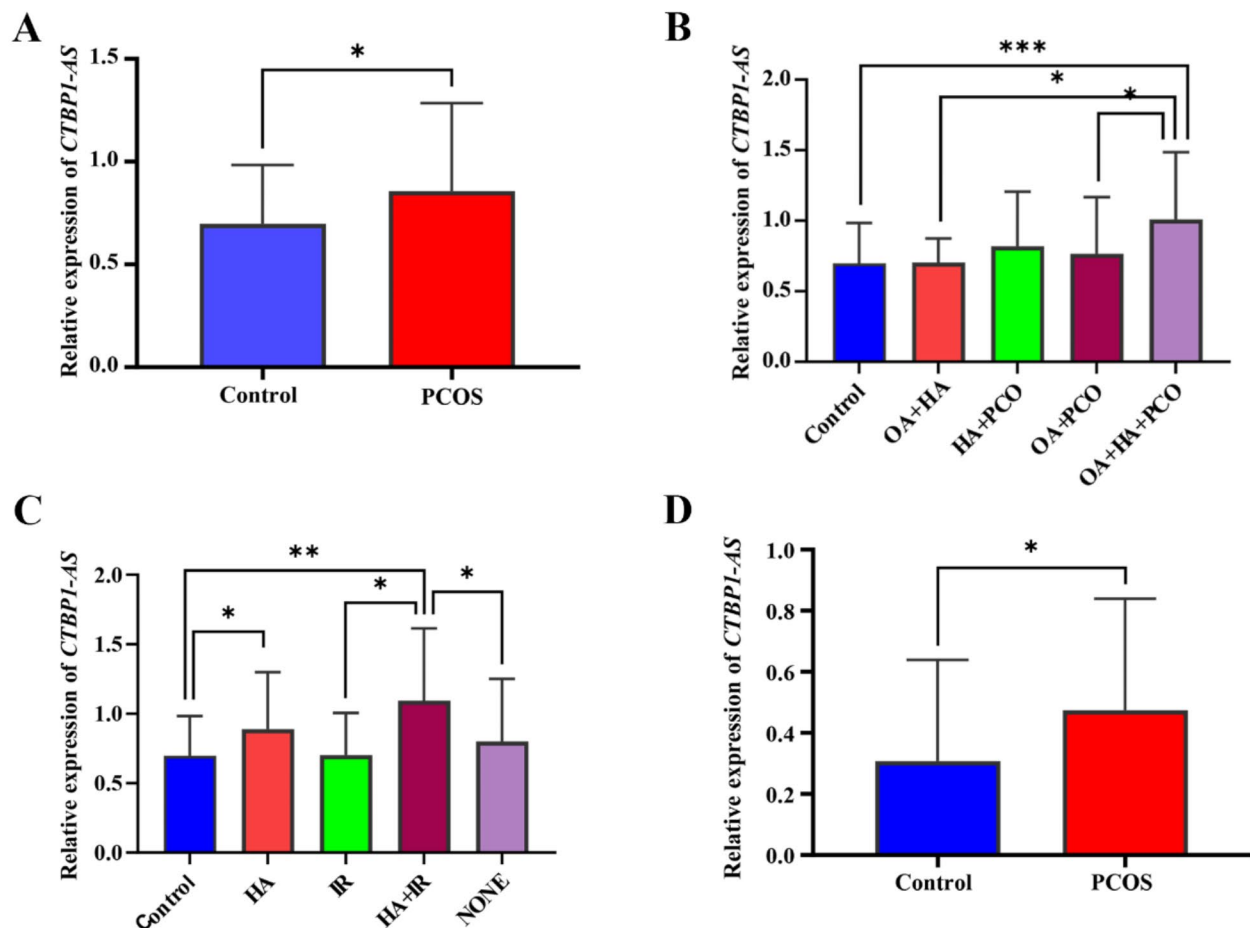

Fig. 1 The expression level of lncRNA CTBP1-AS significantly increased in patients with PCOS. (**A**) Relative expression levels of lncRNA CTBP1-AS in peripheral blood mononuclear cells of the control and PCOS groups. (**B**) Relative expression of lncRNA CTBP1-AS in peripheral blood mononuclear cells across PCOS subtypes. (**C**) Relative expression of lncRNA CTBP1-AS in peripheral blood mononuclear cells based on subgroup clinical characteristics. (**D**) Relative expression of lncRNA CTBP1-AS in granulosa cells from the control and PCOS groups

Table 4 Correlation between lncRNA CTBP1-AS expression levels in peripheral blood mononuclear cells and clinical parameters

Clinical indicators	Expression of lncRNA CTBP1-AS(N=125)	
	r	*p*
Age(years)	0.173	0.053
Duration of infertility (years)	0.088	0.328
Menstrual cycles(days)	0.134	0.136
Antral follicle count	0.194	0.030*
Basic sex hormone		
FSH (IU/L)	0.054	0.549
LH (IU/L)	0.083	0.357
E2 (pg/mL)	-0.056	0.537
P (ng/mL)	-0.032	0.726
T (ug/L)	0.202	0.024*
PRL (ug/L)	0.048	0.593
AMH (ng/mL)	0.149	0.098
FPG (mmol/L)	-0.019	0.835
FINS (pmol/L)	0.087	0.332
HOMAIR	0.065	0.473
BMI (kg/m2)	0.056	0.536

Statistical analysis of the data was performed using Spearman's test. *P<0.05 indicates a statistically significant association between the variables

Table 5 Correlation between lncRNA CTBP1-AS expression levels in GCs and clinical parameters

Clinical indicators	Expression of lncRNA CTBP1-AS(N=30)	
	r	*p*
Age(years)	-0.369	0.045*
Duration of infertility (years)	0.262	0.161
Menstrual cycles(days)	0.455	0.012*
Antral follicle count	0.312	0.093
Basic sex hormone		
FSH (IU/L)	-0.157	0.408
LH (IU/L)	0.005	0.98
E2 (pg/mL)	-0.09	0.636
P (ng/mL)	0.098	0.608
T (ug/L)	-0.056	0.771
PRL (ug/L)	0.101	0.595
AMH (ng/mL)	0.212	0.261
FPG (mmol/L)	0.305	0.102
FINS (pmol/L)	0.044	0.818
HOMAIR	0.122	0.521
BMI (kg/m2)	-0.294	0.114

Statistical analysis of the data was performed using Spearman's test. *P<0.05 indicates a statistically significant association between the variables

Four subgroups were categorized according to the two main clinical features of hyperandrogenic and insulin resistance: the hyperandrogenic group HA (n=36), the insulin-resistant group IR (n=14), the hyperandrogenic+insulin-resistant group HA+IR (n=10), and the no hyperandrogenic and insulin-resistant group NONE (n=25).

Statistical analysis of the four subtypes of the Rotterdam criteria for PCOS (Fig. 1B) revealed a statistically significant difference between the elevated expression of the lncRNA *CTBP1-AS* in OA+HA+PCO only compared with control group (1.01±0.47 vs. 0.71±0.29, P=0.001). In contrast, the expression of lncRNA *CTBP1-AS* in HA+PCO (0.81±0.38) and OA+PCO (0.76±0.40) was elevated compared with the control group, but the difference was not statistically significant; the expression of lncRNA *CTBP1-AS* in OA+HA (0.70±0.16) was not significantly different from the control group. Further two-by-two comparisons revealed that lncRNA *CTBP1-AS* expression in OA+HA+PCO also differed from OA+HA, OA+PCO and not from HA+PCO.

Statistical analysis of the four subgroups with the main clinical features of PCOS (hyperandrogenemia and insulin resistance) versus the control group (Fig. 1C) revealed that the expression of lncRNA *CTBP1-AS* was elevated in HA, and in HA+IR, and the difference was statistically significant (0.88±0.41 vs. 0.71±0.29, P=0.031; 1.09±0.51 vs. 0.71±0.29, P=0.004); the mean expression level of lncRNA *CTBP1-AS* was also elevated in NONE (0.80±0.44) compared to the control group, but none

of the differences were statistically significant (P>0.05); in IR (0.70±0.31) the mean expression level of lncRNA *CTBP1-AS* was not significantly different from the control group. Further two-by-two comparisons revealed that the lncRNA *CTBP1-AS* expression in HA+IR also differed from IR, the NONE group (P<0.05), and did not differ from the HA group. The mean expression level in GCs was higher in PCOS patients than in controls [0.39 (0.18, 0.66) vs. 0.17 (0.10, 0.34), P=0.043] (Fig. 1D).

The results in this part illustrate that the expression level of lncRNA CTBP1-AS is up-regulated in PCOS, especially elevated most significantly in OA+HA+PCO subtype and HA+IR subgroup.

The correlation between lncRNA *CTBP1-AS* transcript levels and clinical parameters

Spearman's rank correlation analysis was applied to analyze the correlation between lncRNA *CTBP1-AS* transcript levels and clinical parameters. The results showed that lncRNA *CTBP1-AS* in peripheral blood mononuclear cells was correlated with sinus follicle number and androgen levels (Table 4); lncRNA *CTBP1-AS* transcript levels in GCs were negatively correlated with the age of the subjects and positively correlated with menstrual cycle; lncRNA *CTBP1-AS* levels in GCs were negatively correlated with the total amount of Gn in the subjects (Table 5). The results of the correlation analysis between granulosa cells lncRNA *CTBP1-AS* transcript levels and IVF parameters showed a negative correlation between lncRNA *CTBP1-AS* levels and the total amount of Gn in the subjects (Table 6). Therefore, we hypothesized that

Table 6 Correlation between lncRNA CTBP1-AS expression levels in GCs and IVF-ET indicators

Clinical indicators	Expression of lncRNA CTBP1-AS($N=30$)	
	r	*p*
Gn Days of use (d)	-0.233	0.215
Total Gn (IU)	-0.399	0.029*
HCG day		
Endometrium(mm)	-0.211	0.262
E2 (pg/mL)	0.334	0.071
No. of oocytes retrieved	0.026	0.891
No. of MII oocytes	0.018	0.927
No.of fertilized oocytes	-0.065	0.734
No. of cleavage embryos	-0.118	0.534
No. of good-quality embryos	0.15	0.429

Statistical analysis of the data was performed using Spearman's test. *$P<0.05$ indicates a statistically significant association between the variables

lncRNA CTBP1-AS might be involved in the central part of PCOS pathogenesis through certain pathways.

Adenovirus of lncRNA *CTBP1-AS* overexpression infects ovarian granulosa cells

We hypothesized that lncRNA *CTBP1-AS* may be involved in the pathologic process of PCOS development by affecting the function of GCs and normal follicular development. To confirm the role of lncRNA *CTBP1-AS* in the pathogenesis of PCOS, lncRNA *CTBP1-AS* overexpression adenovirus was applied to infect KGN cells. According to the results of the pre-experiment, the optimal viral transfection conditions were obtained as

Polybrene 4 μg/ml, MOI=5, and the infection efficiency reached more than 90% as seen under the inverted fluorescence microscope after 48 h of transfection (Fig. 2A), which can be used for the subsequent cell function experiments. Total RNA was extracted from KGN cells after 48 h of adenovirus infection, and the overexpression and silencing efficiencies were detected by RT-qPCR using empty virus (Negative Control, NC) and wild type KGN cells without virus infection (Wild Type, WT) as controls, and the results showed that the expression of lncRNA CTBP1-AS was upregulated more than 3,000-fold in KGN cells overexpressing lncRNA CTBP1-AS (Over Expression, OE), whereas the difference in lncRNA *CTBP1-AS* expression between NC and WT was not statistically significant (Fig. 2B)

lncRNA *CTBP1-AS* overexpression affects follicular development by disturbing with the function of GCs

To investigate the effect of lncRNA *CTBP1-AS* on granulosa cell function, we overexpressed lncRNA *CTBP1-AS* in KGN cells and detected changes in cell growth, apoptosis, cell cycle, cell migration, and other functions. The proliferation of KGN cells was detected by CCK-8, and it was found that compared with WT and NC, the cell proliferation ability of KGN cells overexpressed lncRNA *CTBP1-AS* was reduced, but the difference was not yet statistically significant (Fig. 2C). The flow cytometry results indicated that there was no statistically significant difference in the proportion of cells in the G0-G1, S, and G2-M phases of KGN cells overexpressed lncRNA
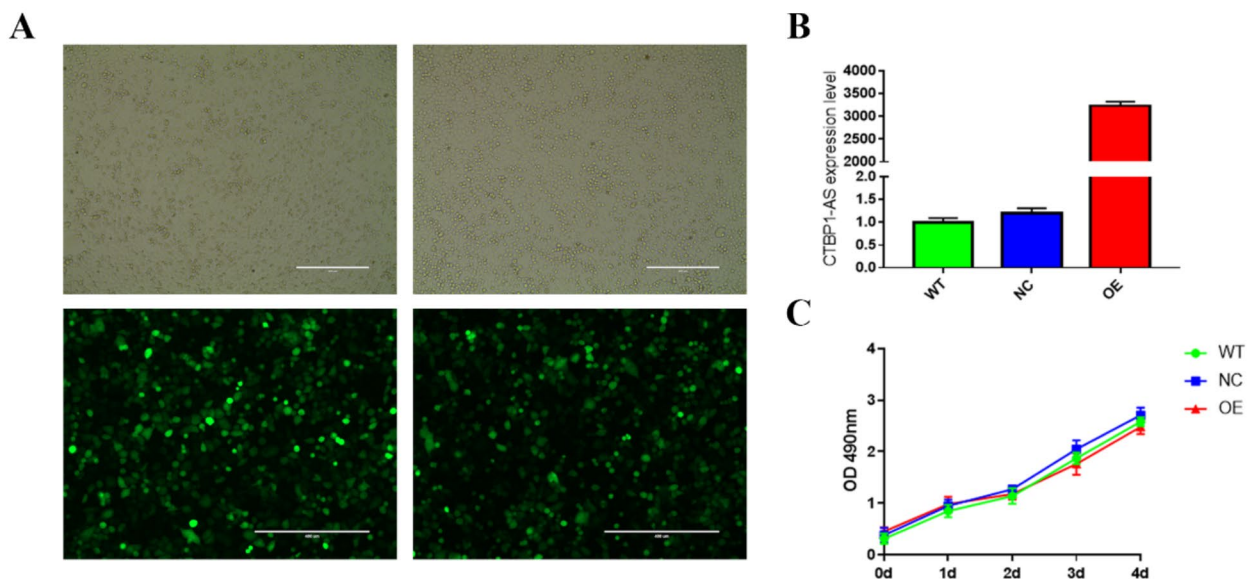

Fig. 2 The effect of overexpression of lncRNA CTBP1-AS on the proliferative function of KGN cells. (**A**) KGN cells were infected by adenovirus for 48 h. (**B**) The expression level of lncRNA CTBP1-AS in KGN cells were detected by RT-qPCR. (**C**) Cell viability was detected by CCK-8 assay in the Negative Control (NC), Wild Type (WT) without virus, and Overexpression of lncRNA CTBP1-AS (Over Expression (OE)) groups

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Table 7 The results of cell cycle

Cell Cycle(%)	WT	NC	OE
G0-G1	58.47 ± 3.25	61.65 ± 2.58	63.14 ± 3.84
S	31.64 ± 1.65	26.81 ± 1.84	27.25 ± 3.54
G2-M	9.89 ± 1.10	11.54 ± 0.98	9.62 ± 1.93

Data are presented as the mean ± standard deviation

CTBP1-AS (Table 7; Fig. 3A-B), suggesting that overexpression of lncRNA *CTBP1-AS* did not affect the cell cycle of KGN cells. In addition, flow cytometry was used to examine the effect of lncRNA *CTBP1-AS* overexpression on KGN cell apoptosis, and the results showed that the proportion of late apoptotic cells was increased in KGN cells overexpressed lncRNA *CTBP1-AS* as compared to WT and NC (Fig. 3C-D), which suggests that overexpression of lncRNA *CTBP1-AS* promotes apoptosis in KGN cells. Cell scratch wound assay and Transwell assay were used to investigate the effect of lncRNA *CTBP1-AS* on the cell migration profile. The results of cell scratch wound assay revealed that the migration ability of KGN cells was significantly decreased after overexpression of lncRNA *CTBP1-AS* (Fig. 4A-B); Transwell assay illustrated that overexpression of lncRNA CTBP1-AS was detrimental to cell migration (Fig. 5A-D). Both experimental results suggested that overexpression of lncRNA *CTBP1-AS* was unfavorable to cell migration. It can be seen that overexpression of lncRNA CTBP1-AS did cause corresponding functional changes in GCs, and the results of this part confirmed that lncRNA CTBP1-AS was involved in the pathological process of PCOS

development by affecting the function of ovarian granulosa cells.

Discussion

In this randomized clinical study, we analyzed the expression levels of lncRNA *CTBP1-AS* in peripheral blood mononuclear cells and ovarian granulosa cells of PCOS patients and controls who met the inclusion criteria, and found that the expression levels of lncRNA *CTBP1-AS* in peripheral blood mononuclear cells and ovarian granulosa cells were significantly higher in the PCOS group compared with that in the normal control group. This suggests that the high expression of lncRNA *CTBP1-AS* may be related to the pathogenesis of PCOS, and is a potential diagnostic marker and drug treatment target. Studies have been conducted to support this view, Liu [13] first reported that lncRNA*CTBP1-AS* expressed at elevated levels in peripheral blood mononuclear cells of PCOS patients and that abnormal expression of lncRNA *CTBP1-AS* was a risk factor for PCOS. In addition, a case-control study involving Kashmiri women also found that subjects with higher levels of lncRNA *CTBP1-AS* expression had a significantly higher risk of PCOS (OR = 11.36, 95% CI = 5.59–23.08, *P* < 0.001), further confirming the association between lncRNA CTBP1-AS and PCOS [6].

Hyperandrogenemia is one of the significant features of PCOS, which is often accompanied by elevated levels of androgens, including testosterone and androstenedione [14, 15]. At the same time, lncRNA CTBP1-AS was initially identified by researchers as a novel androgen
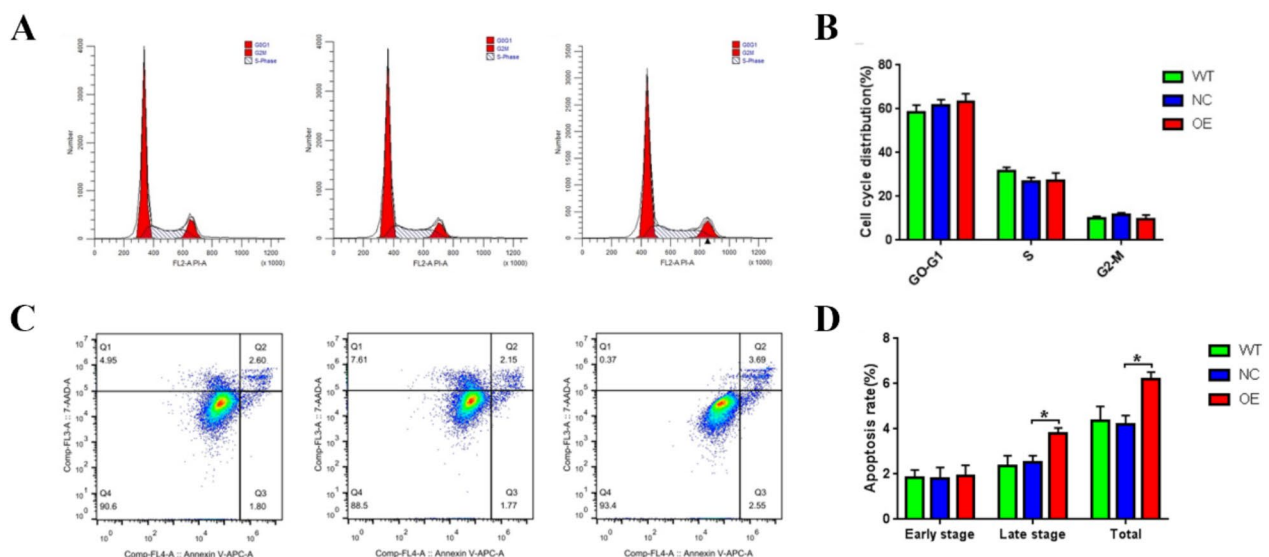

Fig. 3 The effect of overexpression of lncRNA CTBP1-AS on cell cycle and apoptosis in KGN cells. (**A**) Cell cycle of KGN cells in NC, WT and OE groups was detected by flow cytometry; (**B**) The results were visualised using bar graphs. (**C**) The apoptosis of KGN cells in NC, WT and OE groups was detected by flow cytometry; (**D**) The results were visualised using bar graphs
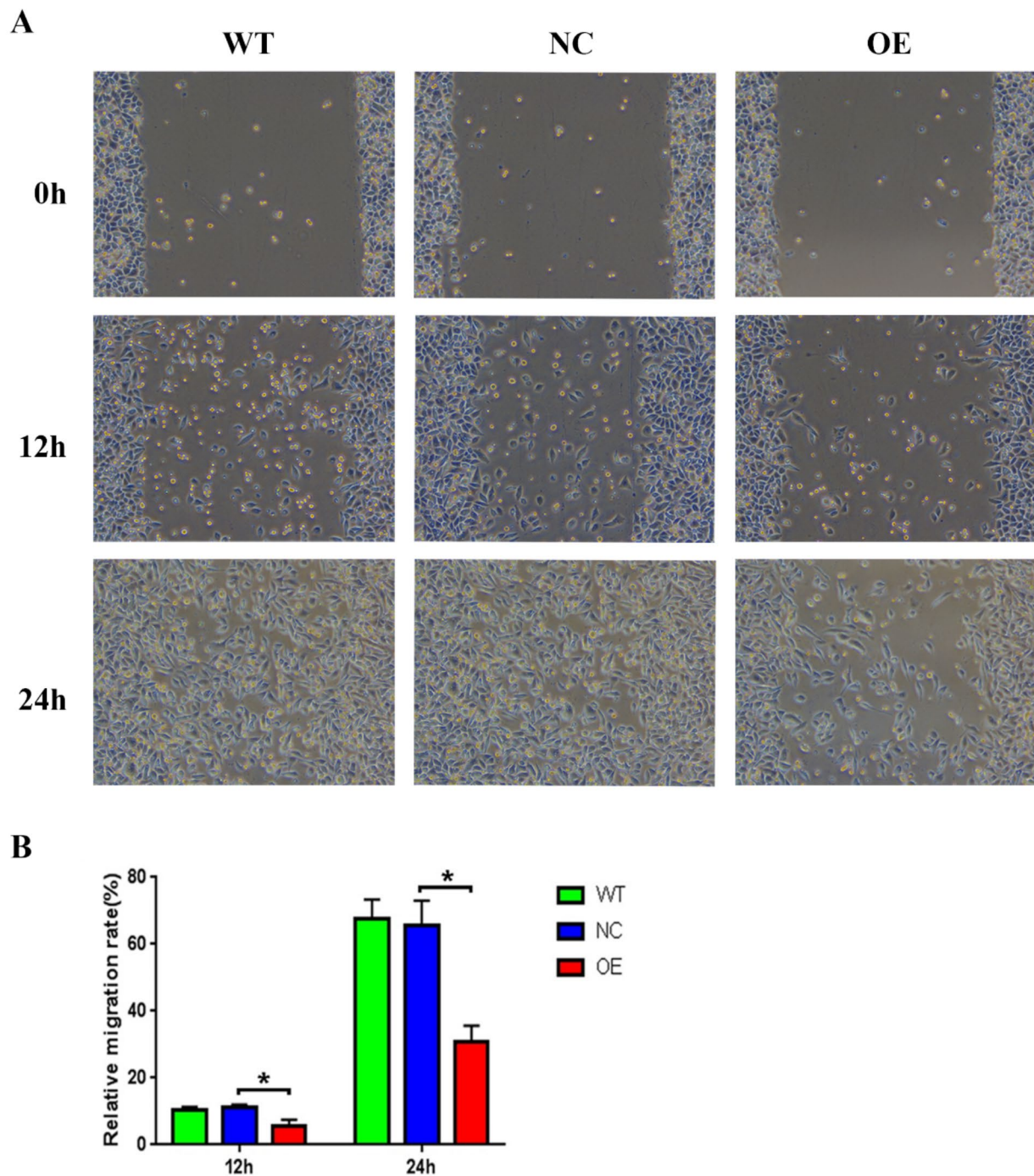
Fig. 4 The effect of overexpression of lncRNA CTBP1-AS on cell migration in KGN cells. (**A**) The cell migration of KGN cells in NC, WT and OE groups was detected by cell scratch wound assay. (**B**) The results were visualised using bar graphs

receptor modulator [16, 17], suggesting that lncRNA CTBP1-AS may play an important role in the pathogenesis of PCOS by regulating androgen levels. lncRNA *CTBP1-AS* expression was commonly upregulated in prostate cancer, and lncRNA *CTBP1-AS* promoted hormone-dependent and desmoplasia-resistant tumor growth. Mechanistically, lncRNA *CTBP1-AS* directly inhibited lncRNA CTBP1 expression by recruiting RNA-binding transcriptional repressors (PSFs) and histone deacetylases; lncRNA *CTBP1-AS* also suppressed tumor suppressor genes and promoted cell cycle progression through PSF-dependent mechanisms, thus exhibiting
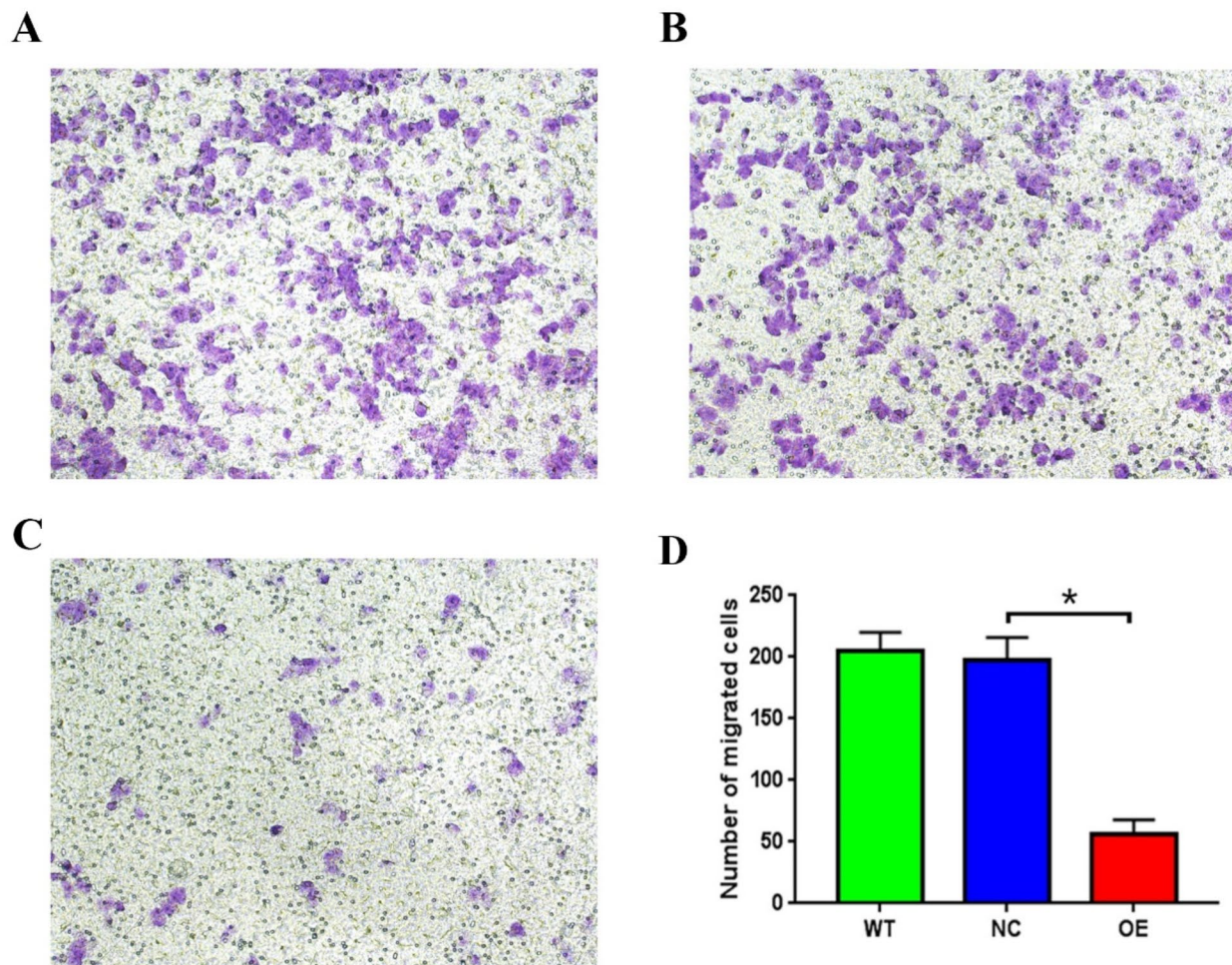
Fig. 5 The effect of overexpression of lncRNA CTBP1-AS on cell migration in KGN cells. Migratory capacity of WT (**A**), NC (**B**), OE (**C**) group was detected with Transwell cell migration; (**D**) Number of technically migrated cells in 100x field of view, the difference between groups was analysed by ANOVA, *P < 0.05

overall androgen-dependent functions [11, 18]. In our study, we found a close correlation between the expression level of lncRNA CTBP1-AS in peripheral blood mononuclear cells of PCOS patients and androgens, which indicates that lncRNA CTBP1-AS may be involved in the central part of the pathogenesis of PCOS through certain pathways.

Jin [19] applied high-density microarray technology to study differentially expressed lncRNAs in ovarian granulosa cells from PCOS patients (*n*=4), and the screening revealed that lncRNA *CTBP1-AS* expression was up-regulated in the PCOS-T group, by 1.3-fold and 1.5-fold, as respectively compared with the control and PCOS-N groups. A total of 30 follicular fluid specimens were collected in our study, and the average expression level of granulosa cells was higher in PCOS patients than in the control group, and the granulosa cell lncRNA *CTBP1-AS*

level was positively correlated with the menstrual cycle. The length of the menstrual cycle mainly depends on the time frame of the follicular phase. Ovarian granulosa cells (GCs) exist in a proliferative manner in developing follicles and undergo multiple biochemical processes during folliculogenesis. In developing follicles, GCs communicate bi-directionally with oocytes through gap junctions [20]. Numerous studies have reported that the survival and physiological status of granulosa cells can have an impact on follicular development [21, 22]. High levels of lncRNA *CTBP1-AS* may have affected the function of ovarian granulosa cells, leading to abnormal follicular development and prolonged follicular phase, which in turn caused menstrual abnormalities in PCOS.

The primary feature of PCOS is anovulatory infertility, the critical feature of which is impaired follicular development or ovulation, which is manifested by the

presence of many growing follicles at all stages of follicular development but ultimately the failure to form a mature dominant follicle [23, 24]. Numerous studies have revealed that the survival and physiological status of granulosa cells can affect follicular development and oocyte quality, and that rapid apoptosis of granulosa cells may lead to follicular dysfunction and follicular atresia, as well as a significant reduction in the maturation rate of the oocyte and oocyte developmental potential. On the other hand, enhancing granulosa cell activity and intracellular expression of related genes may improve oocyte quality to a certain extent and promote embryonic development potential [25–27]. Overexpression of the lncRNA CTBP1-AS may also affect ovarian granulosa cell function involved in the development of the pathological process of ovulatory disorders associated with PCOS. A study found that *CTBP1-AS* interacts with Polycomb histone (enhancer of zeste homolog 2 and embryonic (eZH2) and ectoderm development protein (eed)) in ovarian granulosa cells to regulate PCOS, and cryptotanshinone can reduce the expression level of *CTBP1-AS* in granulosa cells, which may be a new direction for PCOS treatment [12]. lncRNA *CTBP1-AS* overexpression may be involved in the pathogenesis of PCOS by affecting the function of ovarian granulosa cells. In our study, we found that the proportion of apoptotic cells in granulosa cells was increased after overexpression of lncRNA *CTBP1-AS*, especially the proportion of apoptotic cells in the late stage was significantly increased, which is consistent with the findings of increased apoptosis of granulosa cells in PCOS patients [28].

Furthermore, we found that the migratory ability of granulosa cells was significantly decreased after overexpression of the lncRNA *CTBP1-AS*, and hypothesized that it might be involved in the mechanism related to ovulation disorder in PCOS. Normally, ovulation is a process in which the oocyte is expelled together with its surrounding granulosa cells that form the oval corpuscle complex. Before ovulation, ovarian mound expansion occurs after mucoidization of the granulosa cells of the ovarian mound. If the expansion of the mound is impaired, it will lead to abnormal ovulation [29]. Consequently, the reduced migratory capacity of granulosa cells may cause abnormal mound expansion and abnormal follicular migration to the ovarian surface, which in turn leads to impaired ovulation in PCOS and increases the incidence of its unruptured follicular luteinization syndrome. In the microenvironment of the ovary, there are other complex mechanisms for the regulation of ovulation, such as the regulatory role between steroid hormones, prostaglandins, proteolytic enzymes, and other cytokines. We still need to conduct more exploratory studies to elucidate and validate this hypothesis. In conclusion, granulosa cells overexpressing lncRNA

CTBP1-AS exhibited an increased proportion of apoptotic cells and a significant decrease in migration ability, and the corresponding functional alterations in ovarian granulosa cells provide a positive experimental basis for elucidating the mechanism of action of PCOS development.

Conclusion

lncRNA *CTBP1-AS* may affect the function of human ovarian granulosa cells, resulting in an increase in apoptosis and a decrease in migration capacity, which causes impaired follicular development, ovulation disorders, and hyperandrogenemia in PCOS. However, the exact molecular genetic mechanism still needs to be further investigated.

Declarations

Ethics approval and consent to participate
The present study was approved by the ethics committee of The Affiliated Hospital of Youjiang Medical University for Nationalities (Guangxi, China; approval no. YYFY-LL-2022-61); 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.

Conflict of interest
The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Competing interests
The authors declare no competing interests.

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