

Long Noncoding RNA CTBP1-AS Regulates Ovarian Granulosa Cells Proliferation and Autophagy and Participates in Polycystic Ovary Syndrome

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Research

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Abstract

Objective

This study aims to investigate the expression of long noncoding RNA CTBP1-AS in patients with polycystic ovarian syndrome (PCOS) and its effects on the proliferation and autophagy of ovarian granulosa cells.

Methods

Real-time polymerase chain reaction assay was used to analyze the expression levels of CTBP1-AS in peripheral blood leukocytes of 40 PCOS patients and 40 non-PCOS women and the CTBP1-AS expression in ovarian granulosa cells and transfect ovarian granulosa cells with pcDNA3.1-CTBP1-AS and si-CTBP1-AS, respectively. Consequently, the CCK-8 kit was used to analyze the effect of CTBP1-AS on the proliferation of ovarian granulosa cells. Moreover, Western blotting was used to detect the expression levels of autophagy-related proteins LC3II/I and P62.

Result

The CTBP1-AS expression in the peripheral blood of PCOS patients was higher compared with non-PCOS patients ($P < 0.05$). Furthermore, the CTBP1-AS expression of ovarian granulosa cells in PCOS patients was higher compared with non-PCOS patients ($P < 0.05$). Consequently, CTBP1-AS overexpression in ovarian granulosa cells promotes the proliferation of ovarian granulosa cells and autophagy levels ($P < 0.05$). The CTBP1-AS expression interference in ovarian granulosa cells can inhibit the proliferation of ovarian granulosa cells and autophagy levels ($P < 0.05$).

Conclusion

The CTBP1-AS expression in peripheral blood and ovarian granulosa cells of PCOS patients significantly increased, and CTBP1-AS could promote the proliferation of ovarian granulosa cells and the level of autophagy.

Background

Polycystic ovary syndrome (PCOS) is a common gynecological endocrine disease. It is characterized by clinical and biochemical manifestations of excessive androgen, persistent anovulation, and polycystic changes of the ovary often accompanied by insulin resistance and obesity. The disease has a worldwide incidence of about 5%–20% among women of childbearing age [1-3]. However, the etiology of PCOS is not yet clear. Although a lot of research and treatment has been done on the clinical symptoms caused by PCOS, the overall effect of comprehensive treatment and management is still not satisfactory to understand PCOS and its long-term complications. Therefore, revealing the etiology of PCOS, exploring the mechanism of the occurrence and development of the disease, and finding effective therapeutic targets are keys to the current PCOS research field.

Autophagy is an intracellular self-eating phenomenon, which is an important process of evolutionarily conserving the turnover of intracellular substances in eukaryotes. Autophagosome formation plays a major role in the autophagy process. Moreover, autophagosomes act as *scavengers* in cells by fusing with lysosomes to degrade some damaged organelles and proteins. Thus, autophagy is an effective cell response to environmental changes [4]. Continuous studies have shown that autophagy, like apoptosis and senescence, is a very important biological phenomenon, which participates in biological development and growth, affects cell transfer, and assists information exchange among microenvironments [5]. Consequently, continuous clinical studies are centered on deepening research on autophagy [6].

Long noncoding RNA (LncRNA) is a kind of noncoding RNA with a length >200 nt, which can play an important role in transcriptional silencing, transcriptional activation, and chromosome modification. It can also regulate gene expression by affecting transcription and translation levels, and then regulate cell biological function [7]. According to research reports, the occurrence and development of PCOS are accompanied by some changes in the expression level of LncRNAs, indicating that LncRNAs may be involved in the disease [8]. A recent study showed that the LncRNA CTBP1-AS expression level in peripheral blood leukocytes of PCOS patients was significantly higher compared with the control group, and the risk of disease in individuals with high expression was significantly higher compared with individuals with low expression [9]. Moreover, Takayama et al. [10] have shown that LncRNA CTBP1-AS can promote androgen receptor transcriptional activity and cell cycle in prostate cancer. Dalton et al. found that CTBP1 activity and/or expression will increase with the development of prostate cancer cells in the metabolic syndrome environment, resulting in differential regulation of mRNA and miRNA, inhibition of cell adhesion molecules and miR-205-5p, and changes in cytoskeleton leading to cohesion, mesenchymal orientation, and invasiveness enabling the tumor to easily metastasize. At present, the role of LncRNA CTBP1-AS in PCOS is not very clear. Thus, this study aims to analyze the LncRNA CTBP1-AS expression in peripheral blood leukocytes and ovarian granulosa cells in PCOS patients and further verify its effects on ovarian granulosa cell proliferation and autophagy to provide potential therapeutic targets for targeted therapy in PCOS patients.

Materials And Methods

Sample sources and main reagents

Peripheral blood leukocytes and ovarian granulosa cells were collected from 40 PCOS patients and 40 non-PCOS women. The patients were treated with assisted reproductive in vitro fertilization/intracytoplasmic sperm injection in the reproductive medicine center of the hospital of this study because of various infertility or infertility reasons. All participating patients have signed the informed consent form for the experiment. The transfection reagent Lipofectamine®3000 was purchased from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA, USA). Trizol kit was purchased from Nanjing Nuoweizan Biological Technology Co., Ltd. (Nanjing, China). Moreover, P62 and LC3 antibodies were purchased from Shanghai Saixin Biological Company (Shanghai, China). Overexpression plasmids

pcDNA3.1 and siRNA were purchased from Jikai Equipment Manufacturing Co., Ltd. (Shijiangzhuang, China).

Cell culture

PCOS and normal ovarian granulosa cell lines were purchased from the American Type Culture Association cell bank. The two kinds of cells were cultured in Dulbecco's modified eagle medium containing 10% fetal bovine serum. Moreover, penicillin (100 mg/L) and streptomycin (100 mg/L) were added to the culture medium. The cells were subcultured in incubators with saturated humidity, 37°C, and 5% CO₂. Furthermore, the culture medium was changed every 3 days. When the cell coverage rate reached ~80%, the cells were digested with 0.25% trypsin (including ethylenediaminetetraacetic acid) and observed under an inverted microscope. An appropriate amount of culture medium was added to stop digestion after the cells contracted and became round. The cells were suspended by gently blowing the culture surface with a graduated eyedropper and diluted with the culture medium.

Cell transfection

According to the requirements of the reagent manual, the logarithmic growth phase cells growing to 70% confluence degree were inoculated in a six well culture plate of $0.8-4 \times 10^5$ cells per well. Moreover, 2 mL corresponding culture medium was added to each well, and transfection was carried out the next day. The cells were then collected for follow-up experiments after being routinely cultured in the incubator for 48 h.

Real-time quantitative PCR

Following the instructions, the Trizol kit was used to extract the total RNA of the cells. The reverse transcription reaction system was 20 µL, and the reaction conditions were 25°C, 5 min; 42°C, 30 min; 85°C, 5 min; and 4°C. With 18S as the internal reference, the real-time PCR reaction system was 20 µL, and the reaction conditions were 95°C, 10 min; 95°C, 15 s; 60°C; and 1 min at 40 cycles. The 2-Ct method was used to analyze the data. Three multiple holes were noted in all reactions, and the experiment was repeated thrice.

Cell activity assay

Cell viability was detected by the CCK8 kit. Furthermore, 3×10^3 cells were inoculated into the 96-well plate with five multiple holes in each group. On the second day, the culture medium was changed into the serum-free medium (total volume, 100 µL/well) and incubated for 24 h. The cells were then transfected into CTBP1-AS, and the transfection reagent and blank control groups were setup. Moreover, 10 µL CCK-8 solution was added to each well at 24 and 48 h and placed in the incubator for 2 h. The absorbance of each well was measured by the enzyme labeling instrument.

Western blotting

Ovarian granulosa cell lysates were extracted by the radio-immune precipitation assay, proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride membrane. In addition, 5% of fetal bovine serum was used to seal the membrane overnight at 4°C. LC3 and p62 were the first (concentration, 1: 1,000) and second (1:1,000) antibodies incubated, respectively. Finally, they were detected with the enhanced chemiluminescence substrate kit, and the experiment was repeated thrice.

Statistical analyses

The data were processed by the Statistical Package for the Social Sciences, version 18.0 (SPSS Inc., Chicago, IL, USA). The measurement data were expressed by mean \pm standard deviation ($\bar{x} \pm s$). Furthermore, the data were compared by *t*-test, one-way analysis of variance, or analysis of variance of repeated measurement design. The least significant difference *t*-test was used for pairwise comparison.

Results

Patients' characteristics

Analyzing the basic data of 40 PCOS patients and 40 normal patients, it was found that there was no significant difference in age, BMI, basic FSH, and type of infertility between the two groups. The other indicators based on LH, LH/FSH, T and AMH were higher in the PCOS patient group than in the normal patient group, and the difference was statistically significant (Table. 1, $P < 0.05$).

CTBP1 expression in peripheral blood and ovarian granulosa cells of PCOS patients

Total RNA was extracted from the peripheral blood of PCOS patients, and the total RNA in the peripheral blood of paired non-PCOS women was used as the control group. The relative CTBP1-AS expression level was detected by real-time quantitative PCR assay. The results showed that the CTBP1-AS mRNA expressions in the peripheral blood of PCOS patients and non-PCOS women were 2.68 ± 0.17 and 1.04 ± 0.09 , respectively. Moreover, the difference was statistically significant by *t*-test. The CTBP1-AS mRNA expression in the peripheral blood of PCOS patients was higher compared with non-PCOS women (Fig. 1A, $P < 0.05$). The CTBP1-AS expression in ovarian granulosa cells of PCOS patients was analyzed by real-time PCR assay with the ovarian granulosa cells of non-PCOS women as the control group. The results showed that CTBP1-AS mRNA expressions in ovarian granulosa cells of PCOS patients and non-PCOS women were 2.88 ± 0.29 and 1.08 ± 0.04 , respectively. The difference was statistically significant by *t*-test. The expression of CTBP1-AS mRNA in ovarian granulosa cells of PCOS patients was higher than that of non-PCOS patients (Fig. 1B, $P < 0.05$).

Effects of overexpression and RNA interference on CTBP1-AS expression

To examine the CTBP1-AS function in ovarian granulosa cells in follow-up experiments, the pcDNA3.1-CTBP1-AS and si-CTBP1-AS overexpression were used to interfere with the CTBP1-AS expression in ovarian granulosa cells. Real-time quantitative PCR assay was used to detect CTBP1-AS expression in

transfected ovarian granulosa cells. The results showed that the CTBP1-AS mRNA expression in the overexpression and control groups were 1 ± 0.13 and 14.32 ± 1.45 , respectively, after pcDNA3.1-CTBP1-AS transfection. The difference was statistically significant by *t*-test, and the expression in the overexpression group was significantly higher compared with the control group (Fig. 2A, $P < 0.05$). After si-CTBP1-AS transfection, the CTBP1-AS mRNA expression in the interference and the control groups were 0.24 ± 0.03 and 1 ± 0.07 , respectively, and the difference was statistically significant by *t*-test. However, the expression in the interference group was lower compared with the control group ($P < 0.05$, Figure 2). The above results suggest that overexpression and RNA interference can be used to improve and knock down the CTBP1-AS expression level in follow-up experiments.

Effect of CTBP1-AS on the proliferation of ovarian granulosa cells

The CCK-8 assay was used to analyze the effect of CTBP1-AS on the proliferation of ovarian granulosa cells. However, the proliferation of ovarian granulosa cells was different between the overexpression and control groups with time ($P = 0.006$). Moreover, the CTBP1-AS overexpression could promote the proliferation of ovarian granulosa cells as shown in Figure 3A. The proliferation of ovarian granulosa cells was significantly different between the interference and control groups with time ($P = 0.000$). Consequently, the interference with CTBP1-AS could inhibit the proliferation of ovarian granulosa cells as shown in Figure 3B. These results suggest that CTBP1-AS can promote the proliferation of ovarian granulosa cells.

Effect of CTBP1-AS on autophagy of ovarian granulosa cells

The Western blotting assay was used to detect the CTBP1-AS effect on the expression of autophagy-related proteins LC3 and p62 to study the CTBP1-AS effect on autophagy of ovarian granulosa cells. The results showed that LC3-II/I and p62 expressions significantly increased (1 ± 0.13 vs. 18.01 ± 1.865) and decreased (1 ± 0.18 vs. 0.23 ± 0.06), respectively, after CTBP1-AS overexpression in ovarian granulosa cells. After knocking down the CTBP1-AS expression in ovarian granulosa cells, LC3-II/I and p62 expressions significantly decreased (1 ± 0.16 vs. 0.33 ± 0.04) and increased (1 ± 0.06 vs. 3.82 ± 0.32), respectively. Moreover, the difference was statistically significant compared with the control group ($P < 0.05$, Figure 4). These results suggest that CTBP1-AS can promote autophagy of ovarian granulosa cells.

Table 1. Basic information of the experimental group and the control group

	PCOS	non-PCOS	<i>P</i> -value
average age(years)	32.67±1.75	32.50±2.18	0.886
Patient BMI(kg/m ²)	23.07±3.65	23.15±3.72	0.620
Baseline FSH(IU/L)	5.92±0.54	5.85±0.63	0.813
Baseline LH(IU/L)	15.31±1.70	6.72±0.98	<0.01
LH/FSH	2.45±0.78	1.15±0.35	<0.01
Baseline T	0.53±0.25	0.41±0.24	<0.01
AMH	5.88±0.31	3.45±0.33	<0.01
INS			
Type of infertility			
Primary infertility	23	14	0.07
Secondary infertility	17	26	

Discussion

Continuous studies have confirmed that LncRNA can play an important role in the occurrence and development of PCOS. Researchers have found that many LncRNA is differentially expressed in PCOS, and many LncRNA expressions are closely related to PCOS prognosis [12]. Liu [13] and other studies showed that LncRNA plasmacytoma variant translocation 1 (PVT1) and phosphatase and tensin homolog (PTEN) in follicular PCOS fluid increased while miR-17-5p decreased. The PVT1 inhibition could increase miR-17-5p, thus reduce PTEN expression, promote cell proliferation, and reduce the apoptosis rate of ovarian granulosa cells in PCOS. Moreover, Huang et al. found that LncRNA PWRN2 as a ceRNA can reduce the availability of miR-92b-3p for TMEM120B target binding during the nuclear maturation of PCOS oocytes. This ceRNA network provides new information and helps clarify the metabolic abnormalities that lead to abnormal oocyte development in PCOS. In a recent study, Takayama et al. found that CTBP1-AS is a novel androgen-regulated LncRNA, associated with the androgen receptor (AR) signal pathway and can be used as an AR regulator. CTBP1-AS is located in the antisense region of CTBP1, and CTBP1 is the core AR inhibitor. Furthermore, LncRNA CTBP1-AS directly inhibits the CTBP1 expression by recruiting RNA binding to transcriptional repressor polypyrimidine tract-binding protein-related splicing factor and histone deacetylase, thus promoting the AR transcriptional activity. In addition, the upregulated CTBP1-AS suppresses other endogenous tumor suppressor genes in prostate cancer and promotes the growth of hormone-dependent and castrated-resistant tumors [16]. Therefore, CTBP1-AS-repeat polymorphism like CAG may be involved in the pathogenesis of hyperandrogenic diseases, such

as PCOS. These studies show that CTBP1-AS is differentially expressed in PCOS and is closely related to PCOS development. This study confirmed that CTBP1-AS has a tendency of differential high expression in PCOS and can promote the proliferation of ovarian granulosa cells.

The influence of LncRNA on the occurrence and development of various diseases is a wide concern. Current studies have shown that LncRNA can participate in cell proliferation, invasion, metastasis, metabolism, immunity, and other processes [17]. Autophagy, as a conservative and lysosome-dependent degradation process, is regulated by LncRNA as confirmed by many researchers [18]. Moreover, autophagy is a lysosome-mediated degradation pathway that participates in the physiological and pathological processes of reproduction by regulating gamete formation and follicular growth, atresia, and differentiation. Studies have shown that autophagy plays an important role in metabolic disorders associated with PCOS. Furthermore, autophagy is related to fat dysfunction and plays an important role in the regulation of systemic insulin sensitivity in obesity [21-22], which suggests that autophagy may play an important role in PCOS development. However, it is not clear whether autophagy is increased in granulosa cells from PCOS patients. Li [23] and other studies have shown that excessive androgen contributes to the autophagy activation of granulosa cells in PCOS patients, which provides new insights into the effect of autophagy on the function of granulosa cells in PCOS. Thus, ovarian granulosa cell autophagy plays an important role in the occurrence and development of PCOS.

The effect of CTBP1-AS on the autophagy process is not clear. High-throughput analysis of LncRNA and autophagy shows that >600 LncRNA may be involved in the autophagy process [24]. This study confirmed that CTBP1-AS could significantly promote the autophagy marker protein LC3 II/I in ovarian granulosa cells. Thus, CTBP1-AS had a high expression trend. In cytological experiments, CTBP1-AS could promote the proliferation of ovarian granulosa cells. Moreover, through autophagy detection, CTBP1-AS significantly promoted the occurrence of autophagy in ovarian granulosa cells, which provided a new clue for exploring the regulatory mechanism of autophagy in ovarian granulosa cells.

Conclusions

The CTBP1-AS expression in peripheral blood and ovarian granulosa cells of PCOS patients was significantly increased. Thus, CTBP1-AS could promote the proliferation of ovarian granulosa cells and the autophagy level.

Abbreviations

PCOS: polycystic ovarian syndrome; LncRNA: Long noncoding RNA; BMI: Body Mass Index; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; AMH: Anti-mullerian hormone; PVT1: plasmacytoma variant translocation 1; PTEN: phosphatase and tensin homolog

Declarations

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Availability of data and materials

All data supporting the conclusion of this article are included in this published article.

Authors' contributions

KXS, YLX and YXY have been contributed to the initial literature search, acquisition, analysis and design the first draft of article. KXS is the main performer of the experiment, JBS helps to complete part of the data analysis. KXS and YXY proofread the final manuscript before submission. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiment was reviewed and approved by the Ethics Committee of the General Hospital of Northern Theater Command, and all participating patients gave informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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Figures

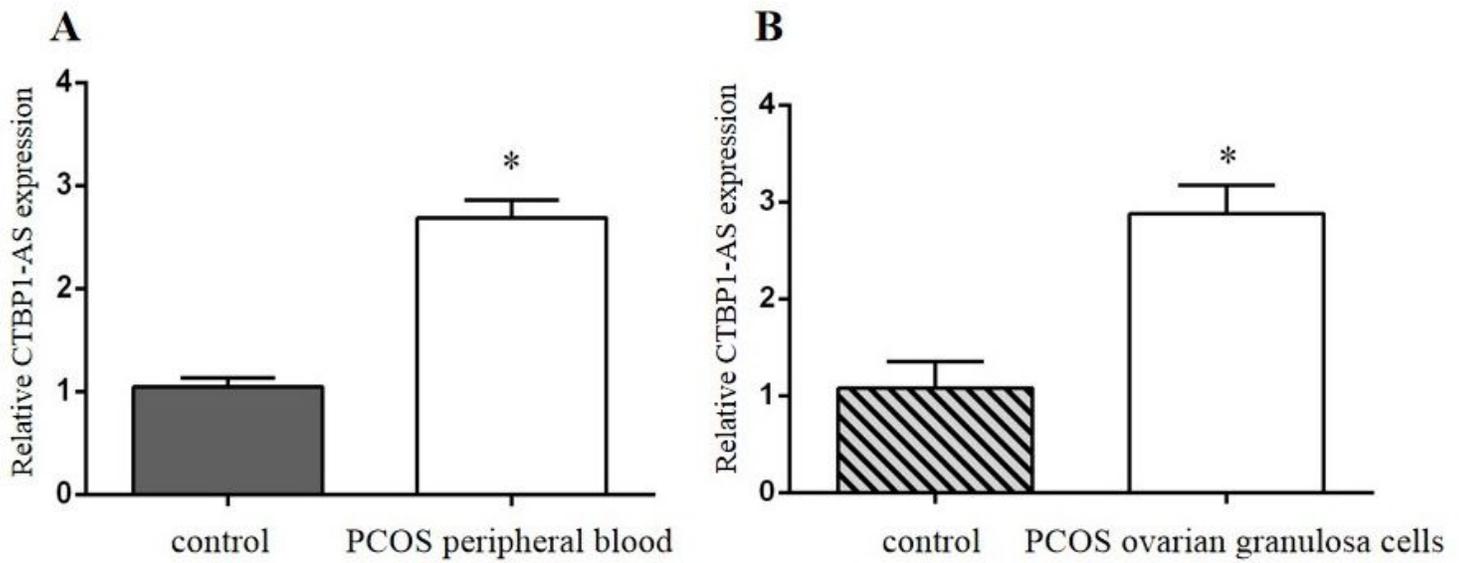


Figure 1

A CTBP1-AS expression in the peripheral blood of PCOS and non-PCOS patients. B The CTBP1-AS expression in ovarian granulosa cells of PCOS and non-PCOS patients. *P < 0.05.

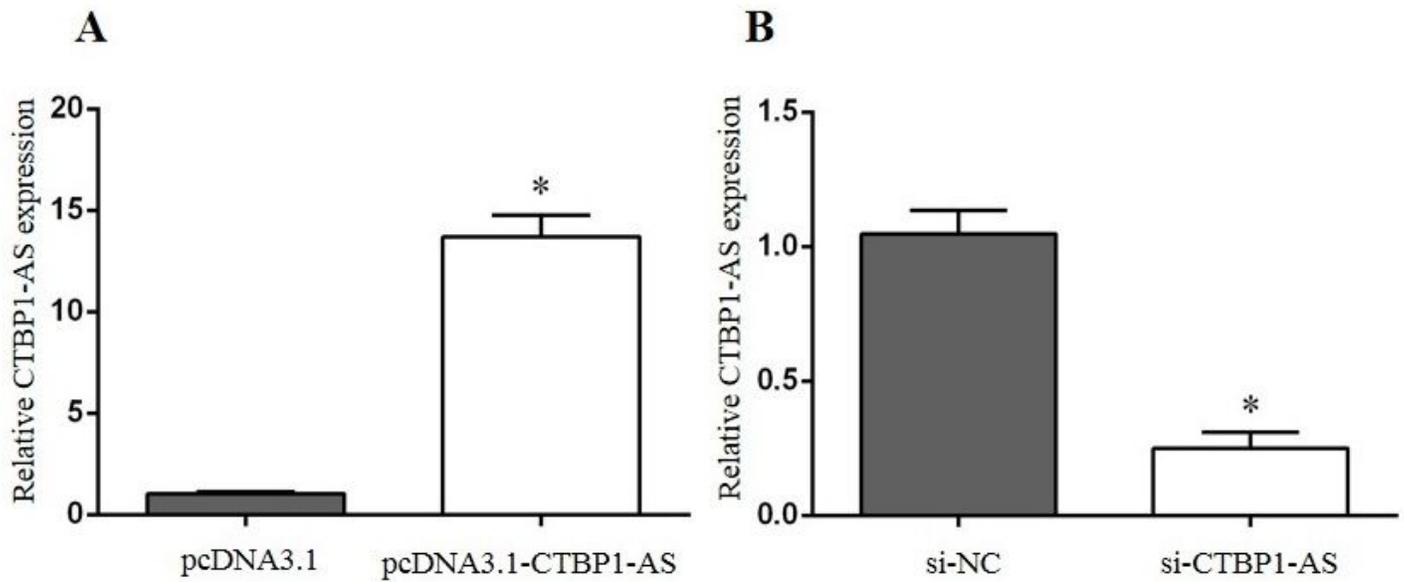


Figure 2

A Determination of transfection efficiency after CTBP1-AS overexpression. B Determination of transfection efficiency after knocking down CTBP1-AS; *P < 0.05.

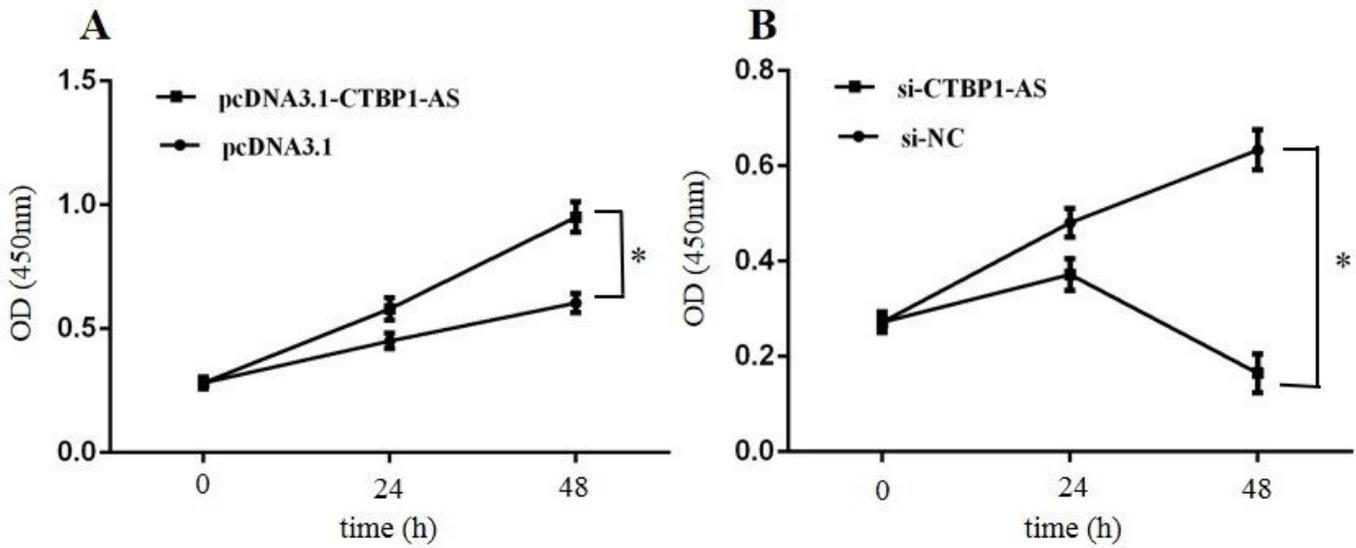


Figure 3

Effect of CTBP1-AS on the proliferation of ovarian granulosa cells.

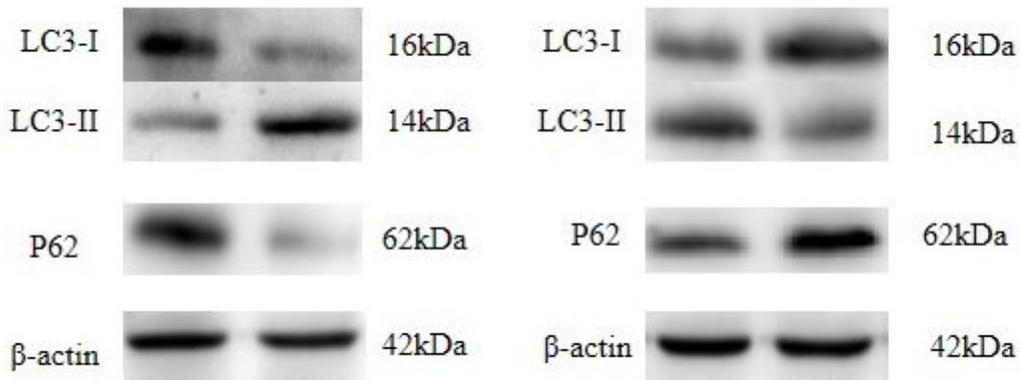


Figure 4

Effect of CTBP1-AS on autophagy of ovarian granulosa cancer cells.