# C/EBP-β and SIRT1 regulate IL-18 expression in the proliferative phase endometrium of patients with polycystic ovary syndrome (PCOS)

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**Research article**

**Keywords:** polycystic ovary syndrome (PCOS)

**Posted Date:** August 20th, 2019

**DOI:** https://doi.org/10.21203/rs.2.13176/v1



**Version of Record:** A version of this preprint was published at Gynecology and Obstetrics Clinical Medicine on June 1st, 2021. See the published version at https://doi.org/10.1016/j.gocm.2021.03.001.

# Abstract

Background Previous studies have shown that patients with polycystic ovary syndrome present with low-grade chronic inflammation. Our previous studies have confirmed that IL-18 is highly expressed in the serum and endometrium of patients with polycystic ovary syndrome. However, the mechanism of IL-18 elevation remains unclear. Therefore, this study aims to explore the signaling pathways that lead to the up-regulation of IL-18 in endometrium of PCOS patients. We predicted that C/EBP-β might be a transcription factor of IL-18 by using TF-Search tool, and deacetylase SIRT1 might be involved in its regulation. Method SIRT1 and C/EBP-β in proliferative endometrium of PCOS patients and control group by immunohistochemical method.The expression was localized. The genes and proteins of SIRT1 and C/EBP-β in endometrium of PCOS and control group were detected by real-time quantitative PCR and Western Blot respectively. The interaction between C/EBP-β and IL-18 was verified by double luciferase assay. Result(s): The gene and protein levels of SIRT1 and C/EBP-β in proliferative endometrium of PCOS patients were significantly higher than those of the control group. Immunohistochemical experiments confirmed that SIRT1 was mainly expressed in endometrial nucleus, while C/EBP-β was mainly expressed in endometrial nucleus and cytoplasm. The interaction between C/EBP-β and IL-18 was confirmed by double luciferase assay. Conclusion: SIRT1 and C/EBP-β are highly expressed in endometrium of PCOS patients, and may play a role in the regulation of IL-18.


# Background

Polycystic ovary syndrome (PCOS) is a multisystem, reproductive-metabolic disorder characterized by polycystic-appearing ovaries, hyperandrogenism, and irregular menstruation, which lead to infertility (1). The associated metabolic dysfunctions include insulin resistance, dyslipidemia, and an increasing prevalence of obesity (2). Recent studies have indicated that patients with PCOS exhibit chronic inflammation, which might be correlated with the pathogenesis of the disease (3,4). Although the main causes of infertility in patients with PCOS are anovulation and impaired oocyte maturation, endometrial dysfunction in the PCOS likely contributes to reduced endometrial receptivity, subfertility, and poor pregnancy outcomes in women (5,6).

Our previous studies demonstrated the inflammatory factor interleukin 18 (IL-18) is increased not only in the serum, but also in the endometrium of patients with PCOS (7), and both are correlated with obesity (8,9). CCAAT enhancer-binding protein beta (C/EBP-b) is an important transcription factor involved in cell proliferation, differentiation, and other processes (10). Fields and Ghorpade (11) reported that C/EBP-β regulates multiple IL-1β-induced human astrocyte inflammatory genes. IL-18 is a member of the IL-1 family and is similar to IL-1β in its role in inflammation. Kang et al. (12) determined that loss of *P38a* in macrophages results in decreased IL-18 expression and inhibition of the activation of C/EBP-β induced by lipopolysaccharides. Accordingly, C/EBP-β may regulate the expression of IL-18. In addition, C/EBP-β exhibits polylysine acetylation (13); this may be related to SIRT1, a critical enzyme involved in acetylation. However, the regulatory effects of C/EBP-β on IL-18 and the signaling pathway mediating these effects have not been examined. In the present study, we explored the expression of C/EBP-β and SIRT1 in the

proliferative phase of the endometrium in women with and without PCOS. We investigated whether C/EBP-β may regulate IL-18 expression and explored the underlying signaling mechanism by which IL-18 is up-regulated in the endometrium of PCOS patients.

# Methods

### Patients and endometrial sample collection

Tissues were obtained from the endometria of patients with PCOS (n=18) and healthy women (n=18) who underwent hysteroscopy at the Division of Reproductive Medicine Center, Peking University Third Hospital. The diagnosis of PCOS was based on the 2003 Rotterdam ESHRE/ASRM criteria, and was finalized if any two of the following three criteria were met and other causes were ruled out: (1) anovulatory dysmenorrheal, (2) clinical hyperandrogenism, and (3) polycystic ovaries. Women who received any hormonal treatment in the 3 months prior to the start of the study as well as patients with pelvic inflammatory disease, genital tract infection, chromosome abnormality, hysteromyoma, or endometriosis were excluded from the study. Written informed consent was obtained from each patient before study participation and ethics approval was obtained from the Research Ethics Committees of the Reproductive Center, Peking University Third Hospital.

The endometrial tissues were divided into three equal pieces. Two pieces of each sample were frozen in liquid nitrogen and maintained at – 80°C for quantitative real-time polymerase chain reaction and western blot analyses. One piece was used for histological and immunohistochemical examinations. Endometrias were obtained during the proliferative phase of the menstrual cycle (cycle days 5–11).

### Histology and immunohistochemistry

Five-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks, placed on coated slides, dewaxed in xylene, and then rehydrated in descending grades of ethanol (100–70%). Half of the sections were stained with hematoxylin and eosin. Antigen retrieval was performed using citric acid buffer (0.1 M, pH 6.0) by microwaving for 10 min on high power. After cooling to room temperature and washing three times in phosphate-buffered saline (PBS), endogenous peroxidase was quenched using 3% hydrogen peroxide for 10 min. After washing with PBS three times, the sections were incubated with anti-C/EBP-β antibody (diluted 1:50, ab32358, Abcam) and anti-SIRT1 antibody (diluted 1:50, ab32441, Abcam) diluted in PBS, and incubated for 2h at 37 °C in a humidified chamber. The negative controls were incubated with a solution devoid of any primary antibody. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:250; Beijing Zhongshan Biotechnology Co., Beijing, China). After incubation with the secondary antibody for 1h at 37 °C in a humidified chamber, the signals were viewed under an Axiokop2 microscope (Carl Zeiss, Thornwood, New York, NY, USA).

### Dual-luciferase assay

HEK293T cells in logarithmic phase were cultured in cell suspension, counted, and inoculated in 24-well plates (the number of cells was about 105, depending on the size of cells), and cultured in an incubator at 37 °C and 5% $CO_2$ until the degree of cell fusion reached about 60%. ROCHE:X-tremegene HP transfection reagent was used for plasmid transfection. The expression of fluorescently-labeled genes was observed 24-48 hours after transfection to determine the transfection efficiency. As a control, equal amounts of GFP plasmid were transfected separately from the target plasmid. Luciferase was detected 48 hours after transfection. The culture medium in 24-well plates was sucked out and 300 ml Passive Lysis Buffer was added to the plate. Reactions proceeded at 4°C for 20 minutes before cell lysis.

The cells were added into Lockwell maxisorp detection board, and Luciferase Assay Reagent was applied. Immediately after shaking and mixing, firefly luminescence was detected by enzyme-labeled instrument. After detecting firefly luminescence, 20 μl Stop & Glo Reagent was added to each well. Renilla luminescence was detected by enzyme-labeled instrument after shaking and mixing for 3 minutes.

### Quantitative RT-PCR

Quantitative RT-PCR was performed following a previously reported method (14). Dissociation curves for both target and housekeeping genes were utilized to ensure the absence of primer dimers and other non-specific amplification. PCR and real-time measurements of fluorescence were performed in the 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA), in at least triplicates, using SYBR Select Master Mix (Applied Biosystems). The primers were as follows: 5'-CCAAGAAGAC CGTGGACAAG-3' (forward) and 5'-TTGCGCATCTTGGCCTT-3'(reverse) for *C/EBP-β*; 5'-AGAACCCATGGAGGATGAAAG -3' (forward) and 5'-TCATCTCCATCAGTCCCAAATC-3'(reverse) for *SIRT1*. The comparative ΔΔCt method was performed to measure relative gene expression (ABI User Bulletin 2).

### Western blotting

Western blotting was performed, as described previously, to detect C/EBP-β (14). Briefly, ten endometrium samples of PCOS groups and ten endometrium samples of normal control groups each containing 60 μg of protein were electrophoresed on 10% polyacrylamide gels and transferred to PVDF (polyvinylidene fluoride) membranes. The membranes were blocked in Tris-buffered saline solution with 0.1% Tween 20 and 5% nonfat milk for 1 h at room temperature. The primary antibodies were anti-C/EBP-β (diluted 1:500, ab32358, Abcam) and anti-SIRT1 (diluted 1:50, ab32441, Abcam). Blots were incubated with primary antibodies overnight at 4 °C. After washing three times in Tris-buffered saline, the membranes were incubated for 1 h at room temperature with 1:500 horseradish peroxidase-conjugated secondary antibodies. Finally, the membranes were processed and visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA).The relative band density normalized to that of β-actin was determined from light scans of the resulting films.

### Statistical analysis

Statistical analyses were performed using SPSS 18.0 (Chicago, IL, USA). The Shapiro–Wilk test was performed to determine whether continuous variables were normally distributed. All error bars in figures indicate standard errors (SE). The data were analyzed by *t*-tests and Mann–Whitney U tests. Statistical significance was designated at $P < 0.05$.

# Results

### 1. C/EBP-β and SIRT1 staining in endometria samples

As assayed by immunochemistry, C/EBP-β and SIRT1 were detected in the endometrial samples of both patients with PCOS and normal women. Strong, dense immunostaining of C/EBP-β was observed in the nuclei of endometrial cells, and faint immunostaining was observed in the cytoplasm. Dense SIRT1 expression was observed in the nuclei of endometrial cells (Fig. 1).

### 2. C/EBP-β enhances IL-18 mRNA transcription in the human endometrium

We next determined whether C/EBP-β promotes IL-18 secretion. The recombinant plasmids pGL4.10-IL-18 and pEnter-C/EBP-β were sequenced and the relative luciferase activity was determined by a luciferase reporter assay. The results of a BLAST search indicated that the target sequences of IL-18 and C/EBP-β were successfully cloned into the dual luciferase reporter vector and could be used for luciferase detection. Based on a dual luciferase assay, C/EBP-β significantly increased luciferase activity in pGL4.10-IL-18, and this effect was dependent on the IL-18 promoter sequence (Fig. 2).

### 3. SIRT1 and C/EBP-β are overexpressed in PCOS patients.

We performed q-PCR to further characterize C/EBP-β and SIRT1 levels in the endometria of women with and without PCOS. C/EBP-β mRNA expression was significantly higher in the endometria of PCOS patients compared to that of normal women ($P=0.018$) (Table 1). SIRT1 mRNA expression was also significantly higher in the endometria of PCOS patients than in those of normal women ($P=0.024$) (Table 1).

To further verify the high expression of C/EBP-β and SIRT1 in the endometrium of patients with PCOS we performed western blots. These results showed that the expression of SIRT1 was higher in the endometrial samples of PCOS than normal controls. We also detected significantly higher C/EBP-β expression in endometrial samples obtained from women with PCOS as compared to expression levels in control samples (Fig. 3). All experiments were performed a minimum of three times with similar results obtained each time.

# Discussion

IL-18 is upregulated in the serum of patients with PCOS, and high IL-18 levels are correlated with insulin resistance, obesity, and hyperandrogenism (8,15). In addition, our results indicate IL-18 is also

overexpressed in the endometria of patients with PCOS relative to control subjects. We inferred that the overexpression of IL-18 in women with PCOS may result in reduced endometrial receptivity. However, it is not clear why the inflammatory factor IL-18 was upregulated in patients with PCOS. Furthermore, the regulatory pathway that mediates this relationship is unknown.

A bioinformatics analysis indicated that C/EBP-β is a target of the IL-18 promoter.

C/EBPs encompass a family of six proteins, of which the C/EBP-α and C/EBP-β isoforms are the most widely expressed. C/EBP-β was initially identified as a transcription factor that is highly expressed in the liver, adipose tissue, and lung tissue, and is involved in cell proliferation, differentiation, and other process (16). C/EBP-β regulates multiple IL-1β-induced human astrocyte inflammatory genes. IL-18 and IL-1β function by similar mechanisms in the proinflammatory process (17). Previous studies have indicated similarities in the regulation of IL-18 and C/EBP-β.

In our study, we found that C/EBP-β mRNA expression is significantly upregulated in the endometria of patients with PCOS, in accordance with changes in IL-18 mRNA levels. Similarly, we found that compared with the control group, C/EBP-β levels increased in the proliferative endometria of patients with PCOS. These results indicate that C/EBP-β may play a role in the regulation of IL-18 expression in the endometrium.

To further evaluate this hypothesis, we performed a dual luciferase assay to verify the relation between C/EBP-β and IL-18. C/EBP-β overexpression resulted in increased pGL4.10-IL-18 luciferase activity, and this effect depended on the IL-18 promoter sequence. These results implied that C/EBP-β may promote the transcription of pGL4.10-IL-18.

In the endometria of patients with PCOS, high expression of C/EBP-β upregulated the expression of IL-18 by activating the IL-18 promoter. Recent studies have shown that C/EBP-β plays an important role in the regulation of reproductive functions in female mice (18). C/EBP-β is directly involved in the ovulation process. The lack of C/EBP-β may result in ovulation dysfunction (19). Mantena SR et al. established that C/EBP-β is a key mediator of steroid responsiveness of the epithelium and stroma in the mouse uterus, and plays an important role in the proliferation and differentiation of endometrial cells (20). The expression of C/EBP-β is rapidly induced in the pregnant uterus at the time of blastocyst attachment. Plante et al. found that in the normal human menstrual cycle, C/EBP-β mRNA and protein expression levels also change, with increased nuclear immunostaining in the mid-secretory phase, indicating a role for C/EBP-β in human endometrial receptivity (21).

However, our research indicated that C/EBP-β expression increases during the proliferative phase of the endometria of patients with PCOS, compared with that in the normal control group, which is not consistent with a decline in endometrial receptivity in PCOS. Villavicencio et al. (22) described higher estrogen receptor expression during the proliferative phase. Chronic estrogen exposure or a lack of progesterone due to ovarian dysfunction can result in endometrial hyperplasia and carcinoma. We hypothesized that the implantation window of endometrium moved forward in these PCOS patients.

However, there is lack of evidence for this association. Thus, additional research is needed to characterize endometrial receptivity and to explain the poor reproductive performance associated with PCOS.

SIRT1 is an important enzymes involved in acetylation, and a previous study showed that C/EBP-β exhibits polylysine acetylation (13). In our study, we found that SIRT1 expression was upregulated in the endometria of patients with PCOS at both the gene and protein levels. This may suggest that SIRT1 regulates C/EBP-b expression through deacetylation.

# Conclusion

In conclusion, we detected the upregulation of C/EBP-β and SIRT1 in the proliferative endometria of patients with PCOS. SIRT1 may activate the SIRT1/C/EBP-β/IL-18 signaling pathway, resulting in the upregulation of IL-18 expression in the endometria of patients with PCOS, and which may be related to the endometrial receptivity abnormality of PCOS patients. These results improve our understanding of the role of C/EBP-β in PCOS and may provide a basis for the development of targeted therapies for this disease.

# List Of Abbreviations

PCOS: Polycystic ovary syndrome

C/EBP-b: CCAAT enhancer-binding protein beta

IL-18: interleukin 18

PVDF: polyvinylidene fluoride

# Declarations

### Ethics and consent to participate

This study was examined by the Peking University Third Hospital Medical Science Research Ethics Committee. The approval number of ethics examination and approval is: 2014 (083).

### Availability of data and material

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

### Competing interests

The authors declare that they have no competing interests

### Funding

This work was supported by the National Natural Science Foundation of China-Youth Foundation (grant number 81401169) and National Natural Science Foundation of China Emergency Management Project (grant number 81541015).

### Authors' contributions

Xiaoyu Long: Wrote the manuscript; contributed to experimental design and research plan; performed all experimental work; Xiaohui Zhu: Provided feedback and assistance on experimental design and execution; critically revised manuscript and approved final version of manuscript; Rong Li: Assisted with organization and collection of clinical specimens by gynecologists; critically reviewed manuscript and approved final version of manuscript. Yan Yang: Assisted with organization and collection of clinical specimens by gynecologists; contributed to experimental planning and design; critically reviewed manuscript proofs, provided intellectual input on the manuscript and approved final version of manuscript. Jie Qiao: Contributed to experimental planning and design; critically reviewed manuscript proofs, provided intellectual input on the manuscript and approved final version of manuscript

### Acknowledgements

Not applicable

### Authors' information (Optional)

Not applicable

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# Table 1

**Table 1.** C/EBP-β and Sirt1 mRNA levels in endometria of patients with polycystic ovary syndrome (PCOS) and controls (median (p25, p75)).

| | Control group | PCOS group | P |
|---|---|---|---|
| Sirt1 | 0.676(0.529,1.000) | 1.216(0.770,2.802) | 0.024* |
| C/EBP-β | 0.503(0.058,0.989) | 1.062(0.365,1.387) | 0.018* |

Note: *Significant difference between the control group and PCOS group (P <0.05).

# Figures

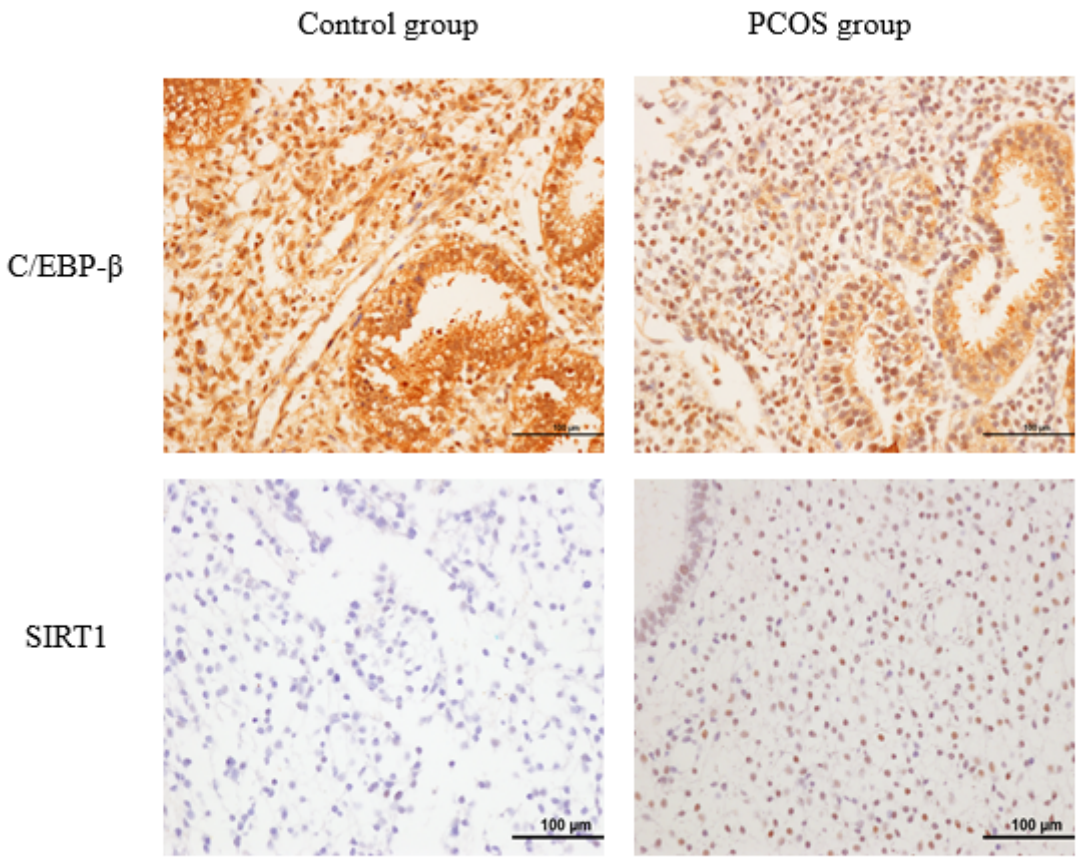
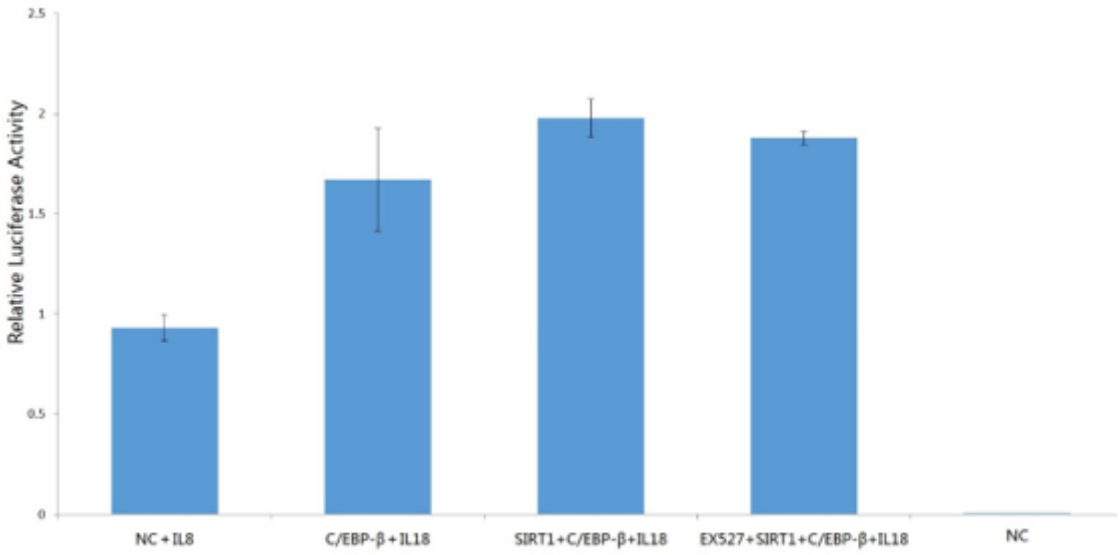


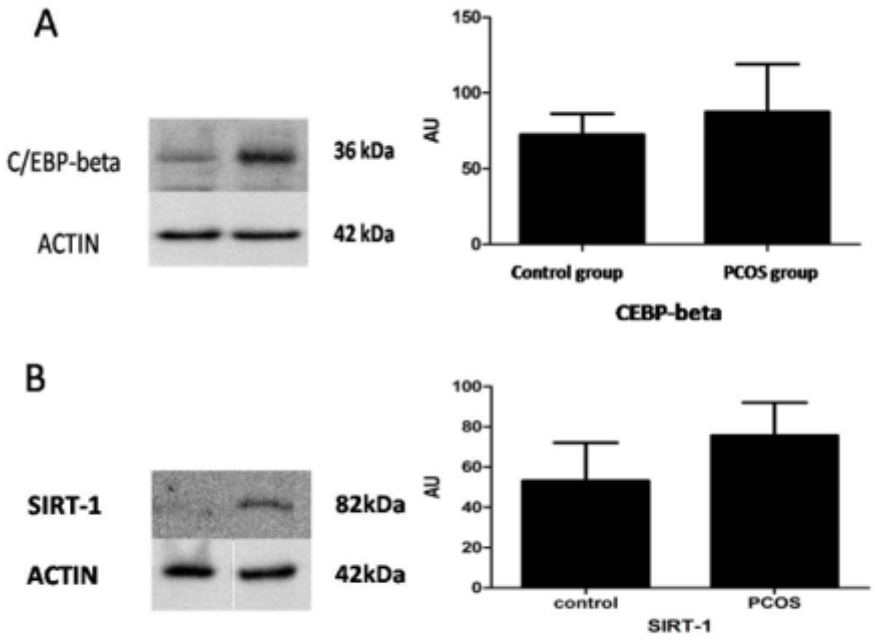

**Figure 1**

Immunohistochemistry analysis of C/EBP-β and SIRT1 protein expression in the proliferative phase of the endometria of patients with and without PCOS. C/EBP-β protein expression was dense in the nuclei of endometrial stroma cells and faint in the cytoplasm. SIRT1 protein was densely detected in the nuclei of endometrial stroma cells.




## Figure 2

C/EBP-β increased the luciferase activity of pGL4.10-IL18; SIRT1 increased the luciferase activity of C/EBP-β-IL18, EX527 decreased the luciferase activity of SIRT1- C/EBP-β-IL18.




## Figure 3

C/EBP-β and SIRT1 protein expression were both higher in the endometria of PCOS patients compared to levels in normal women.