

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

**Xiaoyu Long**

Peking University Third Hospital

**Honghao Wang**

Yantai University

**Xiaohui Zhu**

Peking University Third Hospital

**Rong Li**

Peking University Third Hospital

**Yan Yang** (✉ [sofoa@sina.com](mailto:sofoa@sina.com))

Peking University Third Hospital

**Jie Qiao**

Peking University Third Hospital <https://orcid.org/0000-0003-2126-1376>

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## Research article

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# Abstract

**Background**—Previous studies have shown that patients with polycystic ovary syndrome (PCOS) tend to suffer from low-grade chronic inflammation. Besides, our previous studies have confirmed that IL-18 is highly expressed in the serum and endometrium of patients with PCOS. Nevertheless, the mechanism underlying IL-18 elevation remains unclear. The aim of this study was to explore the signaling pathways that lead to the up-regulation of IL-18 in the endometrium of PCOS patients.

**Materials and Methods:** By using the TF-Search tool, we predicted that C/EBP- $\beta$  might be a transcription factor of IL-18, and deacetylase SIRT1 might be involved in its regulation. Consequently, SIRT1 and C/EBP- $\beta$  in proliferative endometrium of PCOS patients and control groups were analyzed using immunohistochemistry, real-time quantitative PCR and Western Blot; the diagnosis of PCOS was based on the 2003 Rotterdam ESHRE/ASRM criteria. The interaction between C/EBP- $\beta$  and IL-18 was verified by double luciferase assay.

**Results:** The gene and protein levels of SIRT1 and C/EBP- $\beta$  in proliferative endometrium of PCOS patients were significantly higher compared to the control group. Immunohistochemical experiments confirmed that SIRT1 was mainly expressed in the endometrial nucleus, while C/EBP- $\beta$  was mainly expressed in the endometrial nucleus and cytoplasm. The interaction between C/EBP- $\beta$  and IL-18 was confirmed by double luciferase assay.

**Conclusion:** SIRT1 and C/EBP- $\beta$  are highly expressed in the endometrium of PCOS patients, and may participate in the regulation of IL-18. These results further our understanding of the role of C/EBP- $\beta$  in PCOS and may be used as a basis for the development of targeted therapies for this disease.

## Background

Polycystic ovary syndrome 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 the increasing prevalence of obesity (2). While it is generally believed that PCOS is related to hypothalamic-pituitary-ovarian axis dysfunction, adrenal dysfunction, heredity, metabolism, and other factors, its exact pathogenesis remains unclear. Recent studies have indicated that patients with PCOS suffer from 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).

IL-18 is a member of the IL-1 family and its role in inflammation is similar to that of IL-1 $\beta$  in. IL-18 is a proinflammatory cytokine that is mainly produced by mononuclear macrophages. IL-18 participates in various biological activities and is a growth and differentiation factor of Th-1 cells. It can induce the

production of interferon-gamma (IFN-gamma) by B cells, T cells and NK cells, and participate in the body's anti-infective immunity, and inflammatory response as a pro-inflammatory cytokine.

Our previous studies have demonstrated that the inflammatory factor interleukin 18 (IL-18) is increased in the serum, and in the endometrium of patients with PCOS (7), both of which are correlated with obesity (8, 9). CCAAT enhancer-binding protein beta (C/EBP- $\beta$ ) is an important transcription factor involved in cell proliferation, differentiation, and other processes (10). Fields and Ghorpade (11) reported that C/EBP- $\beta$  regulates multiple IL-1 $\beta$ -induced human astrocyte inflammatory genes. Moreover, Kang *et al* (12) determined that loss of *P38 $\alpha$*  in macrophages results in decreased IL-18 expression and inhibition of the activation of C/EBP- $\beta$  induced by lipopolysaccharides. Accordingly, C/EBP- $\beta$  may regulate the expression of IL-18. Besides, C/EBP- $\beta$  exhibits polylysine acetylation (13), which may be related to SIRT1, a critical enzyme involved in acetylation. However, the regulatory effects of C/EBP- $\beta$  on IL-18 and the signaling pathway mediating these effects have not yet been examined. In the present study, we explored the expression of C/EBP- $\beta$  and SIRT1 in the proliferative phase of the endometrium in women with and without PCOS. We investigated whether C/EBP- $\beta$  may regulate IL-18 expression and explored the signaling mechanism underlying IL-18 up-regulation in the endometrium of PCOS patients.

## Materials And 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) Oligo- and/or anovulation (2) clinical hyperandrogenism, and (3) polycystic ovaries. Women who received any hormonal treatment within 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  $-80^{\circ}\text{C}$  for quantitative real-time polymerase chain reaction and western blot analyses. One-piece was used for histological and immunohistochemical examinations. Endometria 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) and 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- $\beta$  antibody (diluted 1:50, ab32358, Abcam) and anti-SIRT1 antibody (diluted 1:50, ab32441, Abcam) diluted in PBS 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 (Beijing qualityyard and biotechnology Co., Ltd, Peking, China) 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) in an incubator at 37°C and 5% CO<sub>2</sub> 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 transfection efficiency. As a control, equal amounts of GFP plasmid were separately transfected from the target plasmid. Luciferase was detected 48 hours after transfection. The culture medium in 24-well plates was sucked out and 300  $\mu$ l Passive Lysis Buffer was added to the plate. Reactions were continued 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  $\mu$ l Stop & Glo Reagent was added to each well. Renilla luminescence was detected by enzyme-labeled instrument after shaking and mixing for 3 minutes. Relative fluorescence values of fluorescence intensity/renilla luminescence intensity were used as indicators to determine the difference among groups.

### 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- $\beta$* ; 5'-AGAACCCATGGAGGATGAAAG -3' (forward) and 5'-TCATCTCCATCAGTCCCAAATC-3'(reverse) for *SIRT1*. The comparative  $\Delta\Delta C_t$  method was performed to measure relative gene expression (ABI User Bulletin 2).

### Western blot

Western blotting was performed to detect C/EBP- $\beta$  (14) according to the previously described method. Briefly, ten endometrium samples from PCOS group and ten endometrium samples from normal control groups, each containing 60  $\mu$ g of protein were electrophoresed on 10% polyacrylamide gels, after which they were denatured for five minutes in 100°C 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- $\beta$  (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  $\beta$ -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. P-value of < 0.05 was considered statistically significant.

## Results

### Clinical characteristics

The general conditions of eighteen PCOS patients and eighteen normal controls are described in **Table 1**. All women with PCOS displayed oligomenorrhea and polycystic ovaries. The PCOS and control groups were similar with respect to age and body mass index (BMI). There were no significant differences in basic follicle-stimulating hormone (FSH), estradiol (E2) and androgen (A) between the PCOS group and normal control group. Yet, the levels of luteinizing hormone (LH) and LH/FSH in PCOS patients were statistically higher than those in the control group.

### C/EBP- $\beta$ and SIRT1 staining in endometria samples

As assayed by immunochemistry, C/EBP- $\beta$  and SIRT1 were detected in the endometrial samples of both patients with PCOS and normal women. Strong, dense immunostaining of C/EBP- $\beta$  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 (**Figure 1**).

### C/EBP- $\beta$ enhances IL-18 mRNA transcription in the human endometrium

We next determined whether C/EBP- $\beta$  promotes IL-18 secretion. The recombinant plasmids pGL4.10-IL-18 and pEnter-C/EBP- $\beta$  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- $\beta$

were successfully cloned into the dual-luciferase reporter vector and could be used for luciferase detection. Based on a dual luciferase assay, C/EBP- $\beta$  significantly increased luciferase activity in pGL4.10-IL-18, and this effect was dependent on the IL-18 promoter sequence (**Figure 2**).

### **SIRT1 and C/EBP- $\beta$ are overexpressed in PCOS patients.**

We performed qPCR to further characterize C/EBP- $\beta$  and SIRT1 levels in the endometria of women with and without PCOS. C/EBP- $\beta$  mRNA expression was significantly higher in the endometria of PCOS patients compared to normal women ( $P=0.018$ ) (Table 2). SIRT1 mRNA expression was also significantly higher in the endometria of PCOS patients compared to normal women ( $P=0.024$ ; **Table 1**).

Western blots were performed to further verify the high expression of C/EBP- $\beta$  and SIRT1 in the endometrium of patients with PCOS. These results showed that the expression of SIRT1 was higher in the endometrial samples of PCOS compared to normal controls. Significantly higher C/EBP- $\beta$  expression was also detected 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, including 18 patients from PCOS group and another 18 patients from control groups, totally 36 patients enrolled in all experiment. All 36 samples were used in q-PCR experiments, and 20 of them were proceeded in Western Blots.

## **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 indicated that IL-18 was also overexpressed in the endometria of patients with PCOS compared to control subjects. We inferred that the overexpression of IL-18 in women with PCOS might 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 remains unknown.

Bioinformatics analysis indicated that C/EBP- $\beta$  was a target of the IL-18 promoter. C/EBPs encompasses a family of six proteins, of which the C/EBP- $\alpha$  and C/EBP- $\beta$  isoforms are the most widely expressed. C/EBP- $\beta$  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 processes (16). C/EBP- $\beta$  regulates multiple IL-1 $\beta$ -induced human astrocyte inflammatory genes. IL-18 and IL-1 $\beta$  function by similar mechanisms in the proinflammatory process (17). Previous studies have indicated similarities in the regulation of IL-18 and C/EBP- $\beta$  (18).

In our study, we found that the expression of C/EBP- $\beta$  mRNA in the endometria of patients with PCOS was 2.2 times higher than that of the control group, which was in accordance with changes in IL-18 mRNA levels. Similarly, we found that compared with the control group, C/EBP- $\beta$  protein levels was 1.2-fold in the proliferative endometria of patients with PCOS. These results indicate that C/EBP- $\beta$  may participate 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- $\beta$  and IL-18. C/EBP- $\beta$  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- $\beta$  may promote the transcription of pGL4.10-IL-18.

In the endometria of patients with PCOS, high expression of C/EBP- $\beta$  upregulated the expression of IL-18 by activating the IL-18 promoter. Recent studies have shown that C/EBP- $\beta$  has an important role in the regulation of reproductive functions in female mice (19). C/EBP- $\beta$  is directly involved in the ovulation process. The lack of C/EBP- $\beta$  may result in ovulation dysfunction (20). Mantena *et al*/established that C/EBP- $\beta$  is an essential mediator of steroid responsiveness of the epithelium and stroma in the mouse uterus that has an important role in the proliferation and differentiation of endometrial cells (21). The expression of C/EBP- $\beta$  is rapidly induced in the pregnant uterus at the time of blastocyst attachment. Plante *et al.* have reported that in the normal human menstrual cycle, C/EBP- $\beta$  mRNA and protein expression levels also change, with increased nuclear immunostaining in the mid-secretory phase, which highlights a role of C/EBP- $\beta$  in human endometrial receptivity (22).

Nevertheless, our results indicated that C/EBP- $\beta$  expression increases during the proliferative endometria phase in patients with PCOS, compared with normal patients, which is not consistent with a decline in endometrial receptivity in PCOS. Villavicencio *et al*/ (23) 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 the endometrium is moved forward in these PCOS patients. However, there is a 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 enzyme involved in acetylation, and a previous study showed that C/EBP- $\beta$  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. These results suggest that SIRT1 regulates C/EBP- $\beta$  expression through deacetylation.

## Conclusion

In this study, we detected the upregulation of C/EBP- $\beta$  and SIRT1 in the proliferative endometria of patients with PCOS. SIRT1 may activate the SIRT1/C/EBP- $\beta$ /IL-18 signaling pathway, thus leading to the upregulation of IL-18 expression in the endometria of patients with PCOS, which in turn may be related to the endometrial receptivity abnormality of PCOS patients. These results further our understanding of the role of C/EBP- $\beta$  in PCOS and may be used as a basis for the development of targeted therapies for this disease.

## Declaration Statements

## **Ethics approval 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).

## **Consent for publication**

Written informed consent for publication was obtained from all participants.

## **Availability of data and material**

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

## **Competing interests**

The authors declare that they have no competing interests

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## **Authors' contributions**

XL: Wrote the manuscript; contributed to experimental design and research plan; performed all experimental work; HW: Assisted with organization and collection of clinical specimens by gynecologists; contributed to experimental planning and design; critically reviewed manuscript proofs. XZ: provided feedback and assistance on experimental design and execution; critically revised manuscript and approved final version of manuscript; RL: assisted with organization and collection of clinical specimens by gynecologists; critically reviewed manuscript and approved final version of manuscript; YY: contributed to experimental planning and design; critically reviewed manuscript proofs, provided intellectual input on the manuscript and approved final version of manuscript; JQ: 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.

## **Acknowledgments**

Not applicable

## **Authors' information (Optional)**



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## Tables

**Table1. Clinical characteristic of women with and without PCOS**

	PCOS group(n=18)	Control group(n=18)	P
Age,years	30.6 $\pm$ 4.74	29.87 $\pm$ 4.79	0.677
BMI,kg/m <sup>2</sup>	23.47 $\pm$ 2.91	22.64 $\pm$ 3.76	0.503
FSH,mIU/ml	6.11 $\pm$ 1.82	6.67 $\pm$ 1.98	0.433
LH,mIU/ml	8.44 $\pm$ 4.84	2.91 $\pm$ 1.40	0.001*
E2,pmol/L	201.56 $\pm$ 77.22	184.47 $\pm$ 36.65	0.445
A,nmol/L	11.56 $\pm$ 4.98	8.04 $\pm$ 4.96	0.068
LH/FSH	1.48 $\pm$ 0.78	0.46 $\pm$ 0.25	0.001*

*Note: The data were analyzed by t-tests*

*BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone;*

*E2: estradiol; A: androgen*

*\*Significant difference between the control group and PCOS group (P <0.05)*

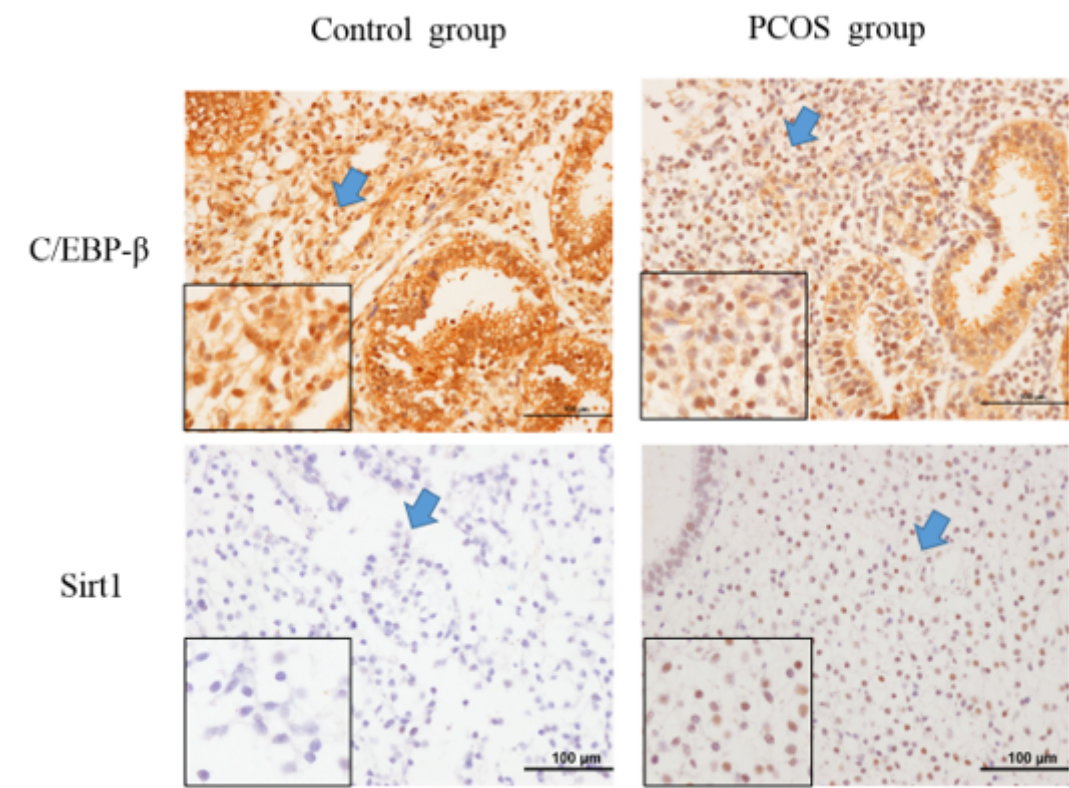
**Table2.** C/EBP-β and Sirt1 mRNA levels in endometria of patients with polycystic ovary syndrome (PCOS) and controls (median (p25, p75))

	Control group(n=10)	PCOS group(n=10)	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: The data were analyzed by Mann-Whitney U tests*

*\*Significant difference between the control group and PCOS group (P <0.05)*

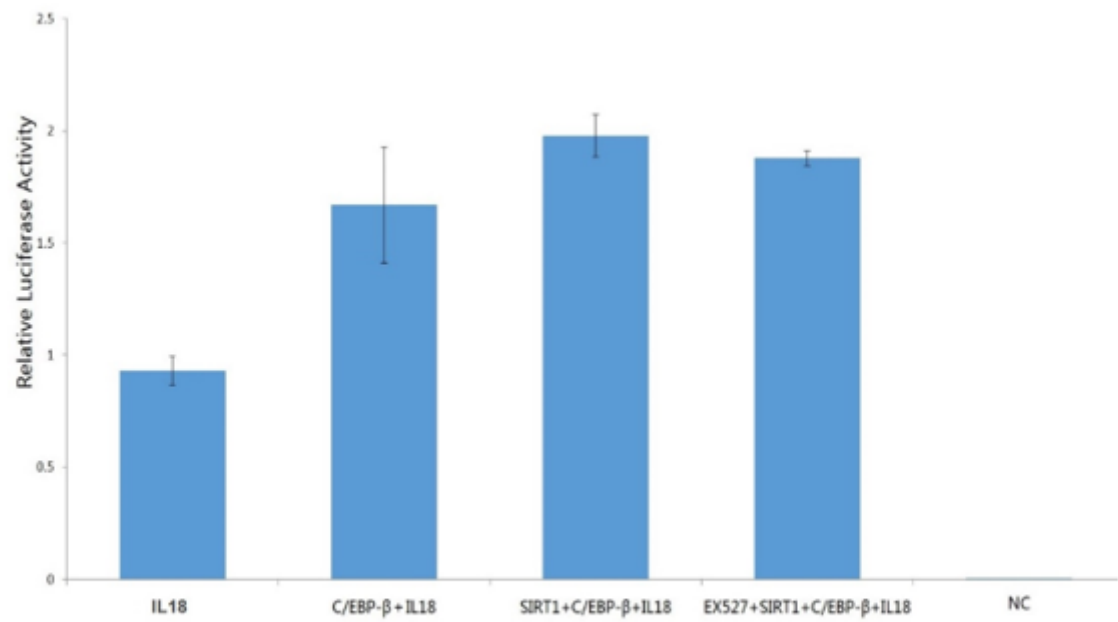
Figures



**Figure 1**

Immunohistochemistry analysis of C/EBP-β and SIRT1 protein expression in the proliferative endometria phase in 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

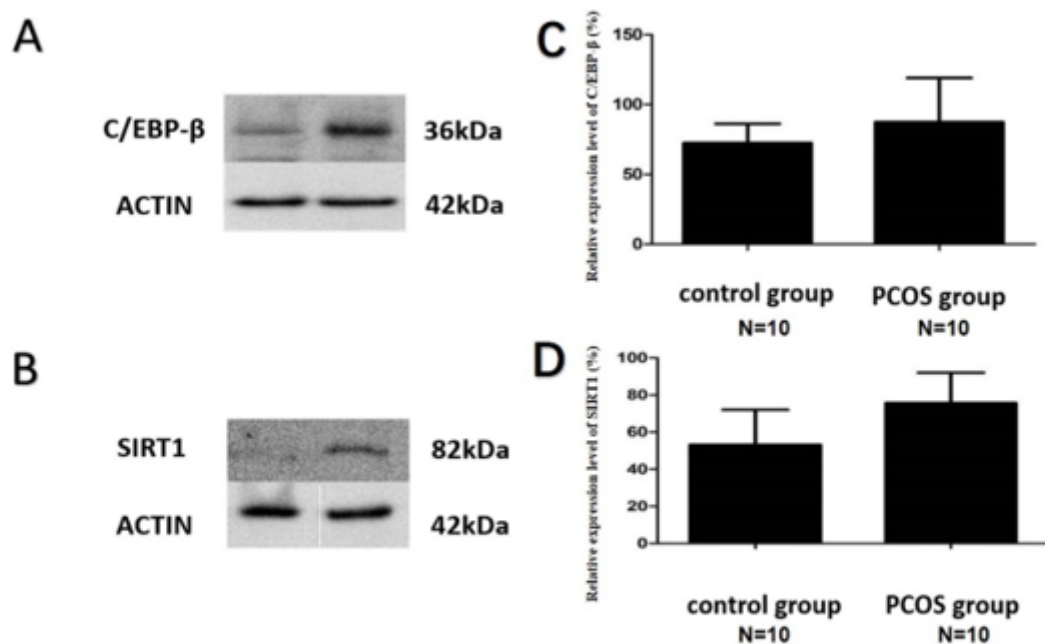
endometrial stroma cells. (The part of the figure was enlarged 4 times displayed in the lower left corner of this figure.)



*Note: NC: negative control only with culture medium. n=3*

**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. Note: NC: negative control only with culture medium. n=3



*Note: Results are expressed by relative value. Vertical axis represents relative gray value. Each band which measured by Image J and target protein bands are normalized by actin blotted from same electrophoresis Lane.*

**Figure 3**

C/EBP-β and SIRT1 protein expression were both increased in the endometria of PCOS patients compared to levels observed in normal women. Note: Results are expressed by relative value. Vertical axis represents relative gray value. Each band which measured by Image J and target protein bands are normalized by actin blotted from same electrophoresis Lane.