

## Lower expression of ER- $\alpha$ 36 is associated with the development of endometrial hyperplasia in PCOS patients

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**Summary.** Objective: To explore the expression change of ER- $\alpha$ 36 in endometria of PCOS patients. Methods: Proliferative endometria were collected and divided into three groups: CE group (n=30), proliferative endometria from control women; PCOSE group (n=30), proliferative endometria from PCOS patients; HPCOSE group (n=15), hyperplastic endometria from PCOS patients. The cellular localization of ER- $\alpha$ 36 and ER- $\alpha$ 66 was analyzed by immunohistochemistry, and expression of ER- $\alpha$ 36 and ER- $\alpha$ 66 was determined by immunohistochemistry and Real-Time Quantitative PCR. The correlation between serum hormone concentration and expression of ER- $\alpha$ 36 and ER- $\alpha$ 66 was analyzed. Results: ER- $\alpha$ 36 was localized on the cell surface, cytoplasm and nucleus of glandular epithelial cells of both CE and PCOSE groups, mainly on the cell surface, and ER- $\alpha$ 66 was localized on the nucleus. The protein and mRNA expression of ER- $\alpha$ 36 was decreased from CE, PCOSE to HPCOSE group, while expression of ER- $\alpha$ 66 showed no obvious difference among groups. The expression ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 of CE, PCOSE and HPCOSE group showed a decreasing tendency. The expression of ER- $\alpha$ 36 and its expressive ratio to ER- $\alpha$ 66 was negatively correlated with serum concentration of LH, LH/FSH, testosterone and androstenedione. Conclusions: Lower expression of ER- $\alpha$ 36 is associated with the development of endometrial hyperplasia in PCOS patients.

**Key words:** Polycystic ovary syndrome, Estrogen receptor- $\alpha$ 36, Endometrial hyperplasia

### Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age, which is characterized by oligomenorrhea or amenorrhea, hyperandrogenemia or hyperandrogenism and polycystic ovaries (Carmina, 2004). The prevalence of PCOS among women of reproductive age is 6.7% (Chang, 2004). The association between PCOS and endometrial hyperplasia has been reported for many years (Coulam et al., 1983; Dahlgren et al., 1991, 1992; Escobedo et al., 1991; Wild et al., 2000; Schmeler et al., 2005; Navaratnarajah et al., 2008), indicating higher incidence of endometrial hyperplasia in PCOS patients.

However, the molecular mechanism underlying higher prevalence of endometrial hyperplasia in PCOS patients is not totally understood. The multi-factorial and heterogenous natures of PCOS make it difficult to explore the risk factors associated with development of endometrial hyperplasia in PCOS patients. It may be attributed to persistent stimulation of endometrial tissue by estrogen (mainly estrone) without progesterone-induced inhibition of proliferative endometria and differentiation to secretory endometria (Balen, 2001; Hardiman et al., 2003). Some researchers found that a change in expression of ER- $\alpha$ 66 (the classic estrogen receptor  $\alpha$ ) was observed in endometria from PCOS patients, and that might lead to alteration of transcription

of genes involved in the cell cycle and induce development of endometrial hyperplasia in PCOS patients (Maliqueo et al., 2003; Quezada et al., 2006). However, the exact role of ER- $\alpha$ 66 in endometria remains unclear as different investigations reported discrepant changes of expression of ER- $\alpha$ 66.

Recently, ER- $\alpha$ 36, a novel variant of estrogen receptor  $\alpha$ , was identified and cloned with a molecular weight of 36 kDa. It was transcribed from previously unidentified promoter located in the first intron of the original classic estrogen receptor  $\alpha$  66 (ER- $\alpha$ 66) gene (Wang et al., 2005). ER- $\alpha$ 36 differs from ER- $\alpha$ 66 by lacking both transcriptional activation domains (AF-1 and AF-2), but it retains DNA-binding domain and partial ligand-binding domains. It possesses a unique 27 amino acid domain that replaces the last 138 amino acids encoded by exons 7 and 8 of the ER- $\alpha$ 66 gene. However, there is no clinical research on ER- $\alpha$ 36 expression and its relationship with ER- $\alpha$ 66 in endometrial hyperplasia in PCOS patients.

Therefore, the present research explored the role of ER- $\alpha$ 36 and ER- $\alpha$ 66 in the endometria of PCOS through examining the protein and mRNA expression of ER- $\alpha$ 36 and ER- $\alpha$ 66 in proliferative and hyperplastic endometria of PCOS women. These results will be useful for a better understanding of the high prevalence of endometrial hyperplasia in PCOS patients.

## Materials and methods

### *Collection of samples*

Women who received infertility treatment from Nov 2009 to Oct 2010 in Reproductive Medical Centre of Peking University Third Hospital were recruited. None of the women had received hormonal therapy or other medications for 3 months prior to recruitment into the study. PCOS was diagnosed on the basis of Rotterdam Consensus: oligo- or anovulation, clinical or biochemical evidence of hyperandrogenism and the presence of polycystic ovaries (Carmina, 2004). Women with hysteromyoma, endometriosis, endometrial polyps, endometritis and appendagitis were excluded. The control group was defined as non-PCOS women with tubal or male factor infertility.

The endometrial specimens were obtained with a pipelle suction curette from the corpus of the uterus. All the endometria were obtained in the follicular phase of the menstrual cycle and verified as proliferative phase by an experienced histopathologist according to the histopathological criteria of Noyes (Nejatbakhsh et al., 2012). The hyperplasia endometria of PCOS patients were diagnosed using the 2003 WHO classification (Steinbakk et al., 2011).

Endometria were classified as follows: CE group (n=30), proliferative endometria without hyperplasia from non-PCOS women with tubal or male factor infertility; PCOSE group (n=30), proliferative endometria without hyperplasia from PCOS patients;

and HPCOSE group (n=15), hyperplasia endometria from PCOS patients. The diagnostic of endometrial hyperplasia was confirmed by histological studies by an experienced histopathologist.

Blood samples were collected on the 2nd to 5th day of menstrual cycle or when there was no follicle larger than 10 mm detected by transvaginal ultrasound. Total testosterone (TT), androstenedione (A) and estradiol were tested according to the Immulite 1000 assay based on chemiluminescence (DPC, USA).

The study was approved by the Ethics Committee of Peking University Third Hospital and all patients signed written informed consents.

### *Materials and reagents*

ER- $\alpha$ 36 specific antibody against the 20 unique amino acids at the C-terminal of ER- $\alpha$ 36 was described before (Wang et al., 2005). The primers for specific amplification of cDNA of human ER- $\alpha$ 36 (BX640939, 1145–1434 bp) were: forward, 5'-CAAGTGGTTT CCTCGTGTCTAAAGC-3' and reverse, 5'-TGTTG AGTGTTGG TTGCCAGG-3' (Invitrogen, USA); The primers for amplification of cDNA of human ER- $\alpha$ 66 were: forward, 5'-AAGTTCAGGCACAATTGGATG-3' and reverse, 5'-CCCTGCATGACACTGATTACA-3' (Invitrogen, USA); Human glyceraldehyde -3-phosphate dehydrogenase (GAPDH) cDNA was amplified by the forward primer 5'-ACGGATTTGGTTCGTATTGGG-3' and the reverse primer 5'-TGATTTTGGG GGGATC TCGC-3'.

### *Immunofluorescence and confocal microscopy*

The cellular localization of ER- $\alpha$ 36 was determined by indirect immunofluorescence, according to a previous method (Lin et al., 2009). Frozen sections of endometrial biopsies of 5  $\mu$ m were fixed for 10 minutes in acetone stored overnight at 4°C and then rinsed with PBS for 10 min. After being permeabilized with 0.5% Triton X-100 for 60 min at room temperature, tissues were blocked in 10% BSA-supplemented PBS for 1.5 hours and incubated overnight at 4°C with anti-ER- $\alpha$ 36-specific antibody or normal rabbit IgG. After three washes in PBS, the tissues were labeled with FITC-conjugated secondary antibody for 2 hours protected from light. After three washes in PBS, the DNA dye PI was used for nuclear staining. Microscopic analysis was performed using a Confocal Laser-Scanning Microscope (Zeiss LSM 510 META, Germany).

### *Immunohistochemistry*

Tissue slides were deparaffinized with xylene and rehydrated through a graded alcohol series. Antigen retrieval was carried out by immersing the slides in sodium citrate buffer and boiling in a waterbath at 100°C for 30 minutes. The endogenous peroxidase activity was blocked by incubation in a 3% hydrogen

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peroxide/methanol/methanol buffer for 10 minutes. The slides were rinsed in PBS and incubated with normal goat serum to block nonspecific staining. The slides were then incubated with ER- $\alpha$ 36 specific antibody or normal rabbit IgG overnight at 4°C in a humidified chamber. The sections were incubated with the second antibody for 30 minutes. Diaminobenzidine was used as a chromogen, and sections were counterstained with hematoxylin. Before nuclear counterstaining, pictures were taken by Leica Q 550cw Optical microscope with a Leica DC 300F camera (Leica Company, Germany) and the staining intensity of glandular cells of endometria was evaluated by using Leica Qwin Standard V2.8 image software for the average optical density. As a negative control, duplicate sections were immunostained without exposure to primary antibodies, which was replaced by PBS.

### RNA extractions and cDNA synthesis

Total RNA was extracted from tissues frozen in liquid nitrogen using TRIzol reagent according to the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed into single-strand complementary DNA (cDNA) using moloney-murine leukemia virus (M-MLV) reverse transcriptase (TianGen, Beijing, China). Briefly, the RNA was denatured by heating for 5 min at 70°C followed by rapid cooling on ice, and then used for reverse transcription (5  $\mu$ g of total RNA, 20 U of RNase inhibitor, 2.5 mmol/L of each dNTP, 10  $\mu$ mol/L reverse primer and 200 U of M-MLV reverse transcriptase in a total volume of 20  $\mu$ l). For reverse transcription, tubes were incubated at 42°C for 50 min.

### Real-Time Quantitative PCR

SYBR Green Real-Time Quantitative PCR was

performed using iQ5 Real-Time PCR Detection System (Bio-Rad, USA). The Real-Time Quantitative PCR reaction was carried out in triplicate for each sample. Briefly, 20  $\mu$ l of reaction mixture containing 2  $\mu$ l of cDNA template was amplified as follows: ER- $\alpha$ 36 denaturation at 95°C for 15 min and 40 cycles at 95°C for 15 s, 68°C for 60 s; ER- $\alpha$ 66, denaturation at 95°C for 15 min and 40 cycles at 95°C for 15 s, 63°C for 60 s. Quantitative analysis was performed using the comparative threshold cycle (CT) method (Livak and Schmittgen, 2001).

### Statistical analysis

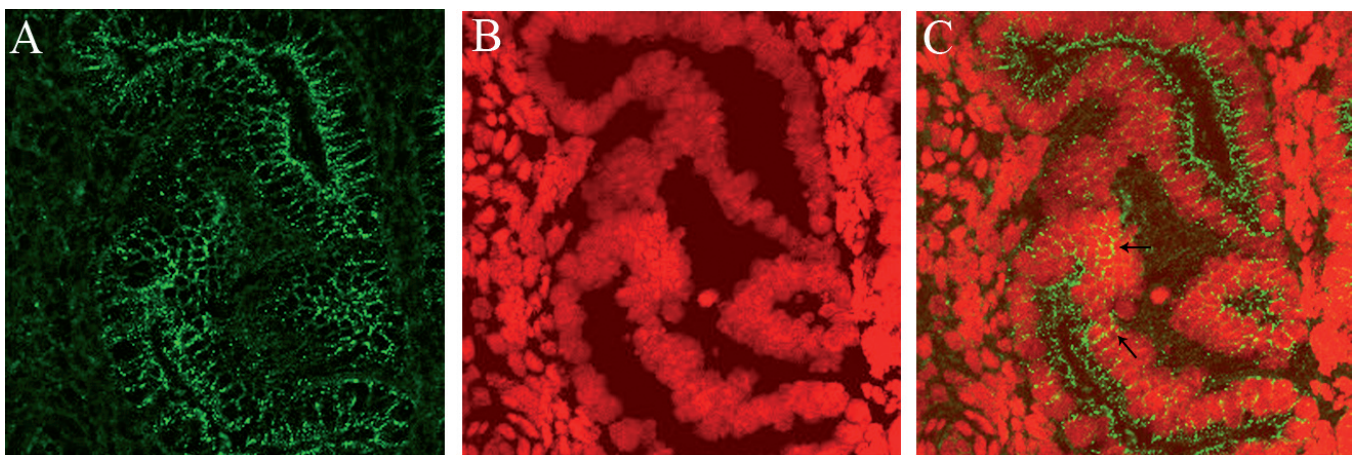
All data was analyzed using SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). The difference in expression of ER- $\alpha$ 36 and ER- $\alpha$ 66 among three groups was compared using the one-way ANOVA test or nonparametric Kruskal-Wallis H test. Correlation analysis was done using Pearson or Spearman correlation coefficient.  $p < 0.05$  was considered statistically significant.

## Results

### Location of ER- $\alpha$ 36 and ER- $\alpha$ 66 in glandular epithelial cells of endometrium

ER- $\alpha$ 36 is a novel variant of ER- $\alpha$ 66 generated by alternative promoter usage and alternative splicing. To examine the cellular localization of ER- $\alpha$ 36, immunofluorescence assay was performed using ER- $\alpha$ 36 specific antibody against the unique 20 amino acids at the C-terminal of ER- $\alpha$ 36. As shown in Fig. 1, ER- $\alpha$ 36 protein (green) was localized on the cell surface, cytoplasm and nucleus of glandular epithelial cells of endometria, mainly on the cell surface.

Moreover, the results of immunohistochemistry also



**Fig. 1.** The localization of ER- $\alpha$ 36 identified by indirect immunofluorescence. **A.** The signal of ER- $\alpha$ 36 protein (green) on endometria. **B.** The signal of DNA (red) staining by PI. **C.** Merged image of **A** and **B** showed that ER- $\alpha$ 36 was localized on cell surface, cytoplasm and nucleus of glandular epithelial cells of endometria, mainly on the cell surface; yellow, co-localization signals (arrow indication).



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affirmed the localization of ER- $\alpha$ 36. The expression of ER- $\alpha$ 36 is mainly localized on the cell surface in the epithelial cells of CE, PCOSE and HPCOSE (Fig. 2A-C).

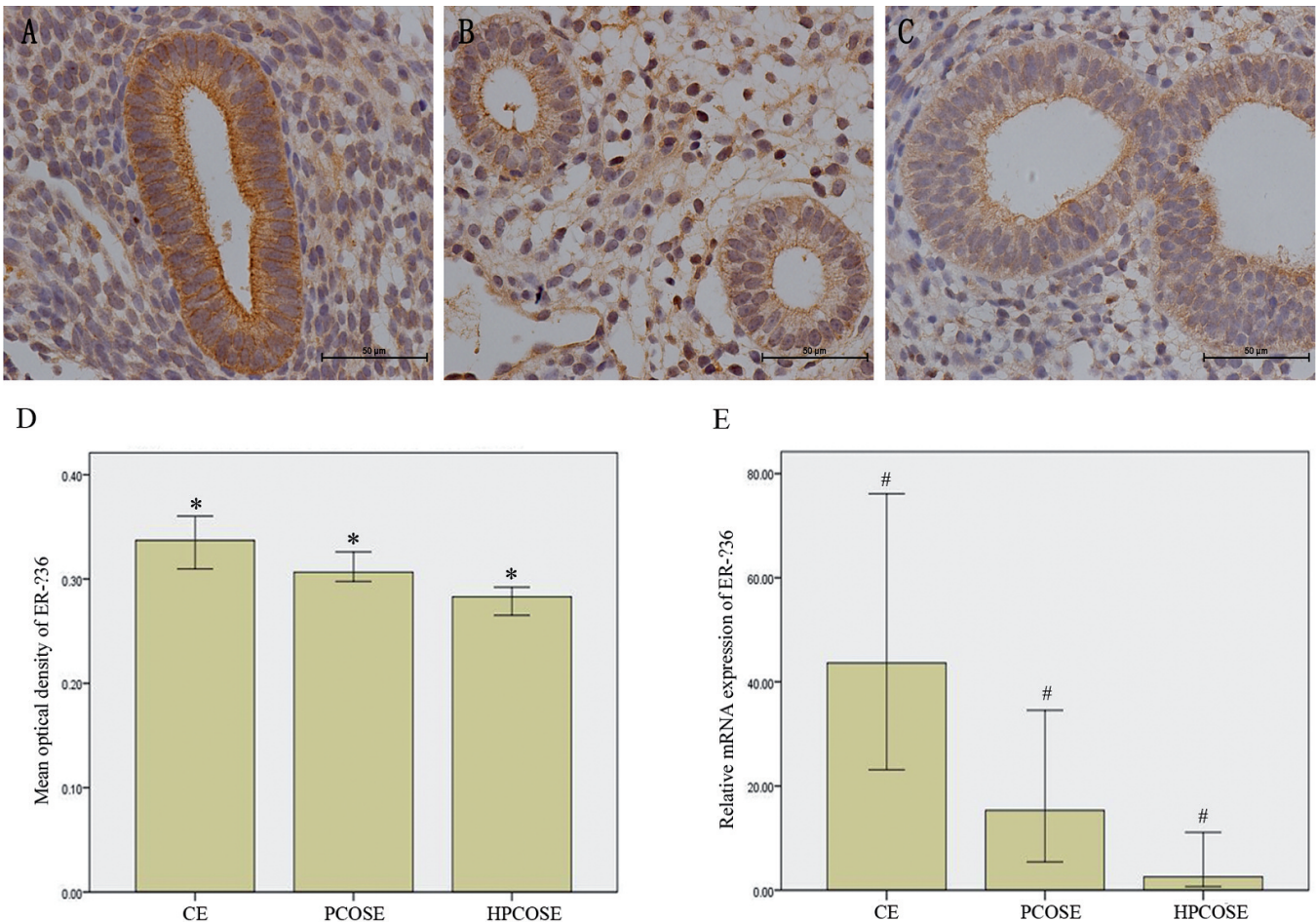
Immunohistochemistry showed that ER- $\alpha$ 66 was localized on the nucleus of glandular epithelial cells of CE, PCOSE and HPCOSE (Fig. 3A-C).

### Expression change of ER- $\alpha$ 36 and ER- $\alpha$ 66 in endometria

The staining of ER- $\alpha$ 36 gradually decreased from CE, PCOSE to HPCOSE group according to immunohistochemistry results. Furthermore, the staining intensity of ER- $\alpha$ 36 of CE, PCOSE and HPCOSE groups was analyzed using mean optical density (OD) value. The mean OD value of ER- $\alpha$ 36 of the three groups was  $0.342\pm 0.045$ ,  $0.311\pm 0.027$  and  $0.282\pm 0.022$  (Fig. 2D) respectively, and had significant differences

among groups ( $p < 0.001$ ). The mRNA expression of ER- $\alpha$ 36 of the three groups is shown in Fig. 2E. The relative mRNA level of ER- $\alpha$ 36 of CE, PCOSE and HPCOSE group was 43.612 (18.008-86.149), 15.324 (3.637-50.992) and 2.533 (0.758-10.289) respectively, and had significant differences among groups ( $p < 0.001$ ).

Analysis of staining intensity of ER- $\alpha$ 66 in CE, PCOSE and HPCOSE group by immunohistochemistry showed that the mean OD value of ER- $\alpha$ 66 was  $0.398\pm 0.058$ ,  $0.361\pm 0.030$  and  $0.413\pm 0.030$  (Fig. 3D) respectively. The staining intensity between PCOSE group and HPCOSE group had a significant difference ( $p < 0.001$ ). The mRNA expression of ER- $\alpha$ 66 of three groups is shown in Fig. 3E. The relative mRNA level of ER- $\alpha$ 66 in CE, PCOSE and HPCOSE group was 8.021 (2.402-19.627), 6.571 (4.074-14.448) and 9.883 (5.756-25.107), and had no significant difference among groups ( $p = 0.272$ ).



**Fig. 2.** The expression change of ER- $\alpha$ 36. The localization of ER- $\alpha$ 36 was examined in proliferative endometria of control group (CE, **A**), proliferative endometria of PCOS group (PCOSE, **B**) and hyperplasia endometria of PCOS group (HPCOSE, **C**). **D.** The mean optical density (OD) value of ER- $\alpha$ 36 in CE, PCOSE and HPCOSE groups. The mean OD value of the three groups was  $0.342\pm 0.045$ ,  $0.311\pm 0.027$  and  $0.282\pm 0.022$  respectively. \*: difference between groups were significant ( $p < 0.001$ ). **E.** The expression level of ER- $\alpha$ 36 mRNA in CE, PCOSE and HPCOSE groups. The relative mRNA level of the three groups was 43.612 (18.008-86.149), 15.324 (3.637-50.992) and 2.533 (0.758-10.289) respectively, shown as median (25% quartile-75% quartile). #: difference between groups was significant ( $p < 0.001$ ).

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### Relative expression of ER- $\alpha$ 36 to ER- $\alpha$ 66 in endometria

We further evaluated the expression ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 in endometria. As shown in Fig. 4A, the mean OD ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 was 0.926 (0.854-0.975) in CE, 0.879 (0.812-0.965) in PCOSE and 0.709 (0.696-0.738) in HPCOSE respectively, and there was a significant difference between CE and HPCOSE, PCOSE and HPCOSE ( $p < 0.001$ ). The mRNA expression ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 was 5.764 (1.761-13.866) in CE, 1.733 (0.726-5.902) in PCOSE and 0.591 (0.067-0.771) in HPCOSE respectively, and there was a significant difference among groups ( $p < 0.05$ ) (Fig. 4B).

### Correlation between expression of ERs and serum hormone level

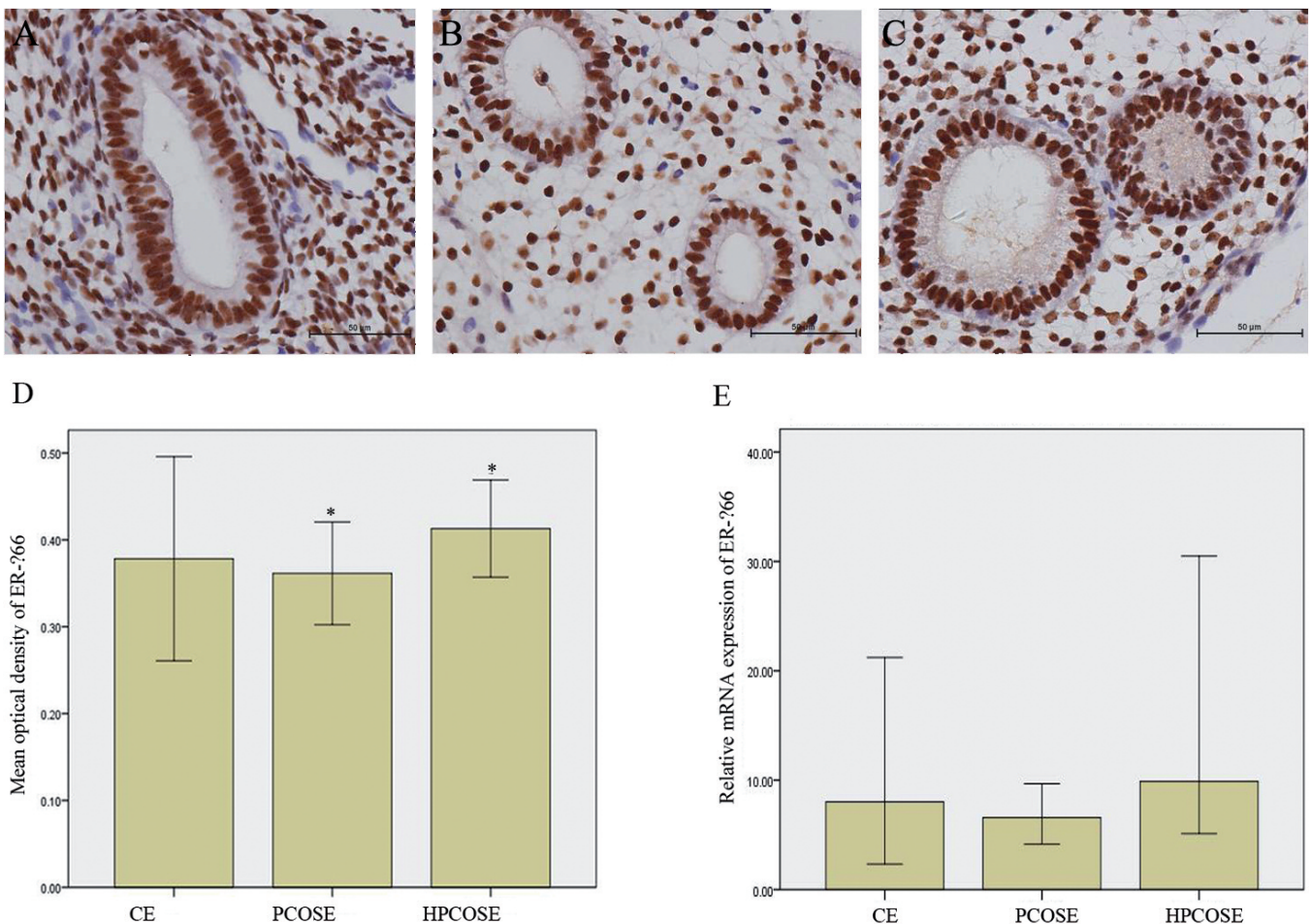
We further analyzed the effect of serum hormone

level on expression of ERs among three groups, including luteinizing hormone (LH), follicle stimulating hormone (FSH), LH/FSH, estradiol, testosterone and androstenedione.

We found no significant correlation between serum hormone level and expression of ER- $\alpha$ 66, neither at

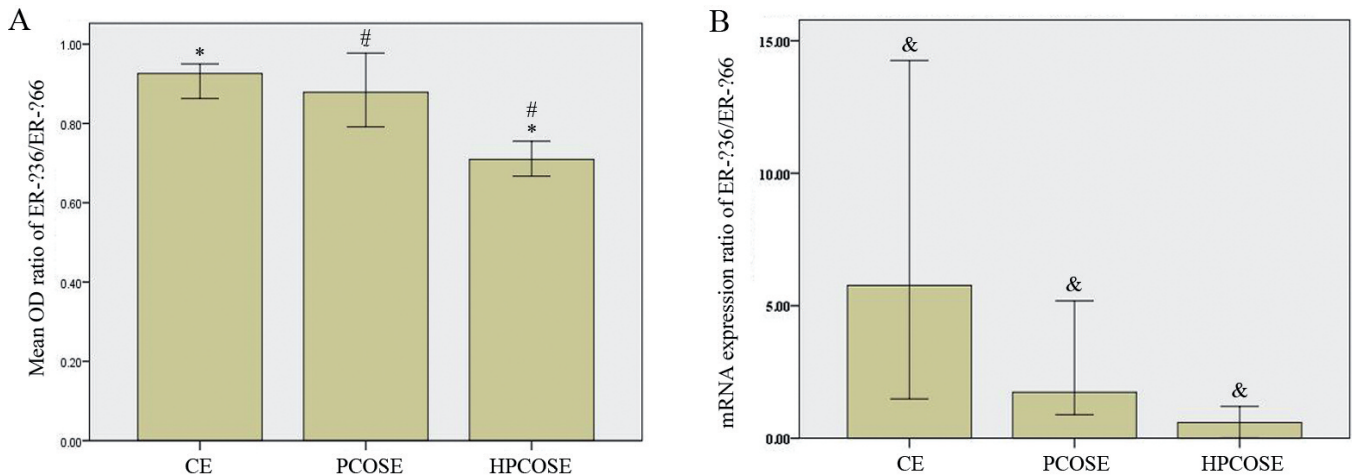
**Table 1.** Correlation between expression level of ER- $\alpha$ 36 and serum hormone level.

	LH	LH/FSH	Estradiol	Testosterone	Androstenedione
Mean OD value of ER- $\alpha$ 36					
r	-0.48	-0.57	-0.334	-0.52	-0.469
p	<0.001	<0.001	0.02	<0.001	<0.001
Relative mRNA expression of ER- $\alpha$ 36					
r	-0.347	-0.603	-0.403	-0.233	-0.254
p	0.01	<0.001	<0.001	0.042	0.028



**Fig. 3.** The expression change of ER- $\alpha$ 66. The localization of ER- $\alpha$ 66 was examined in proliferative endometria of control group (CE, **A**), proliferative endometria of PCOS group (PCOSE, **B**) and hyperplasia endometria of PCOS group (HPCOSE, **C**). **D.** The mean optical density (OD) value of ER- $\alpha$ 66 in CE, PCOSE and HPCOSE groups. The mean OD value of the three groups was  $0.398 \pm 0.058$ ,  $0.361 \pm 0.030$  and  $0.413 \pm 0.030$ . \*:  $p < 0.001$  compared to PCOSE vs. HPCOSE group. **E.** The expression level of ER- $\alpha$ 66 mRNA in CE, PCOSE and HPCOSE groups. The relative mRNA level of the three groups was 8.021 (2.402-19.627), 6.571 (4.074-14.448) and 9.883 (5.756-25.107) respectively, shown as median (25% quartile-75% quartile), and had no significant difference among groups ( $p = 0.272$ ).





**Fig. 4. A.** The mean optical density (OD) ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 in CE, PCOSE and HPCOSE groups. The mean OD ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 of the three groups was 0.926 (0.854-0.975), 0.879 (0.812-0.965) and 0.709 (0.696-0.738) respectively, shown as median (25% quartile-75% quartile). \*: between CE and HPCOSE difference was significant ( $p < 0.001$ ); #, between PCOSE and HPCOSE difference was significant ( $p < 0.001$ ). **B.** The mRNA expression ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 in CE, PCOSE and HPCOSE groups. The mRNA expression ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 of the three groups was 5.764 (1.761-13.866), 1.733 (0.726-5.902) and 0.591 (0.067-0.771) respectively shown as median (25% quartile-75% quartile). &: among groups differences were significant ( $p < 0.05$ ).

protein level nor at mRNA level. However, the mean OD value of ER- $\alpha$ 36 of the three groups was negatively correlated with serum level of LH ( $r = -0.48$ ,  $p < 0.001$ ), LH/FSH ( $r = -0.57$ ,  $p < 0.001$ ), estradiol ( $r = -0.334$ ,  $p = 0.02$ ), testosterone ( $r = -0.520$ ,  $p < 0.001$ ) and androstenedione ( $r = -0.469$ ,  $p < 0.001$ ). The relative mRNA level of ER- $\alpha$ 36 of the three groups was also negatively correlated with serum level of LH ( $r = -0.347$ ,  $p = 0.01$ ), LH/FSH ( $r = -0.603$ ,  $p < 0.001$ ), estradiol ( $r = -0.403$ ,  $p < 0.001$ ), testosterone ( $r = -0.233$ ,  $p = 0.042$ ) and androstenedione ( $r = -0.254$ ,  $p = 0.028$ ) (table 1).

## Discussion

Recently, ER- $\alpha$ 36, a novel variant of estrogen receptor  $\alpha$  was identified and cloned. Increasing research focused on its expression and functions. Our present study first explored the role of ER- $\alpha$ 36 and its relative expression level to classic estrogen receptor, ER- $\alpha$ 66 in endometrial hyperplasia in PCOS patients. The present study found that ER- $\alpha$ 36 was mainly localized on the cell surface of glandular epithelial cells of endometria in both control and PCOS groups, and ER- $\alpha$ 66 was localized on the nucleus. The protein and mRNA level of ER- $\alpha$ 36 was gradually decreased from CE, PCOSE to HPCOSE group. However, there was no obvious difference in expression of ER- $\alpha$ 66 among the three groups. The expression ratio of ER- $\alpha$ 36 to ER- $\alpha$ 66 presented a decreasing tendency from CE, PCOSE to HPCOSE group. Furthermore, the expression of ER- $\alpha$ 36 was negatively correlated with serum concentration of LH, LH/FSH, estradiol and testosterone.

The immunofluorescence and immunohistochemistry results have shown that ER- $\alpha$ 36 was localized

on the cell surface, cytoplasm and nucleus of glandular epithelial cells of endometria, mainly on the cell surface, which is consistent with recent findings (Lin et al., 2010). ER- $\alpha$ 66, the classic estrogen receptor, belongs to the nuclear receptor family of transcription factors and its transcriptional activation is mediated by two activation function domains, AF-1 and AF-2. However, ER- $\alpha$ 36, the new variant of estrogen receptor, is generated from a promoter located in the first intron of the ER- $\alpha$ 66 gene. ER- $\alpha$ 36 differs from ER- $\alpha$ 66 by lacking both AF-1 and AF-2, but it retains the DNA-binding domain, partial dimerization and ligand-binding domains. Several clinical investigations suggested that ER- $\alpha$ 36 played negative role in cancers, such as colorectal cancer and breast cancer (Jiang et al., 2008; Zheng et al., 2010). Present immunohistochemistry and Real-Time PCR analysis demonstrated that the expression of ER- $\alpha$ 36 and its relative expression level to ER- $\alpha$ 66 was lower in HPCOSE group than in CE and PCOSE groups. Wang found that ER- $\alpha$ 36 inhibits the transcription activities mediated by AF-1 and AF-2 domains of ER- $\alpha$ 66 (Wang et al., 2006). It is possible that lower expression of ER- $\alpha$ 36 induced ER- $\alpha$ 66 transcription activity and then led to endometrial hyperplasia. However, the exact role of ER- $\alpha$ 36 needs further research.

Our research also found that the expression level of ER- $\alpha$ 36 was negatively correlated with serum level of LH, LH/FSH, estradiol, testosterone and androstenedione. Previous studies suggested that serum level of LH, LH/FSH, testosterone and androstenedione was elevated in PCOS patients (Skampardonis et al., 2011; Ma et al., 2011). High tissue concentration of androstenediol is generated in the PCOS endometrium,

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which possesses estrogenic activity (Wood and Strauss, 2002; Jamnongjit and Hammes, 2006; Plaza et al., 2010). It is possible that the elevated molecules with estrogenic activity in endometrium influence the expression of ER- $\alpha$ 36 and further weaken its dominant-negative effect in the ER- $\alpha$ 66, which induces endometrial hyperplasia in PCOS patients. However, the mechanism should be explored in the future.

In summary, we report here that ER- $\alpha$ 36 plays a negative role in endometrial hyperplasia of PCOS patients. Our present results give important information to further understand the mechanism of higher prevalence of endometrial hyperplasia in PCOS patients. The role of ER- $\alpha$ 36 in endometria of PCOS patients will provide useful information for designing new drugs for the treatment of endometrial problems of PCOS patients.

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