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Comprehensive assessment the expression of core elements related to IGFIR/PI3K pathway in granulosa cells of women with polycystic ovary syndrome

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ABSTRACT

Objective: Polycystic ovary syndrome (PCOS) is the most common multisystem endocrinopathy in women, characterized by chronic hyperandrogenism, ovulatory dysfunction, and polycystic ovaries. But its etiology remains elusive. A plethora of information suggests phosphatidylinositol-3-kinase (PI3K) pathway is key to the pathogenesis of PCOS but little is known about the expression pattern and possible role of insulin like growth factor 1 receptor (IGFIR)/PI3K pathway in PCOS. The goal of this study was to determine whether the core elements of the IGF1R/PI3K pathway were differentially expressed in GCs isolated from PCOS.

Study design: Western blot (WB) and reverse transcription-polymerase chain reaction (RT-PCR) for IGF1R, insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2) and phosphatase and tensin homolog (PTEN) related to IGFIR/PI3K pathway were performed in GCs isolated from 60 PCOS patients and 60 controls.

Results: Compared to controls, body mass index (BMI), the levels of fasting plasma glucose (FPG), fasting insulin (FINS), anti-Mullerian hormone (AMH), testosterone (T), luteotropic hormone (LH), homeostasis model assessment of insulin resistance (HOMA-IR), antral follicle count (AFC) were markedly elevated while follicle stimulating hormone (FSH) decreased ($p < 0.05$). Furthermore, at both mRNA and protein levels, the expression of IGF1R, IRS1, IRS2 were significantly increased whereas PTEN was dramatically decreased in PCOS patients ($p < 0.05$).

Conclusion: Our findings indicate that IGFIR/PI3K pathway is differently expressed in PCOS GCs compared with controls, with IGFIR, IRS1, IRS2 significantly increased while PTEN decreased. Thus, our study probably provides new evidences about the pathogenesis of PCOS in term of molecular mechanism.

Introduction

Polycystic ovary syndrome (PCOS) is the most common multisystem endocrinopathy affecting 6–10% women of reproductive age, characterized by hyperandrogenism, polycystic ovaries, ovulatory dysfunction [1]. Although, genetic and environmental factors play a significant role in the pathogenesis PCOS, the etiology of PCOS remains unclear [2]. The phenotype of mice with phosphatidylinositol-3-kinase (PI3K) pathway up-regulation is reminiscent of human PCOS and suggests that dysregulated PI3K pathway may be involved in the pathogenesis of PCOS [3,4]. In addition, previous studies have reported that PI3K pathway plays vital roles in the etiology of PCOS, ranging from insulin resistance and hyperandrogenism to follicular development, as well as granulosa cells (GCs) proliferation [5–7].

In both ovary and endometrial of PCOS patients, insulin like growth factor 1 (IGF1) level is markedly elevated compared to that in control patients and it is well established requirement for

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stimulating PI3K pathway [8–11]. The activation process of insulin like growth 1 receptor (IGFIR)/PI3K signaling pathway is showed in Fig. 1. An essential step to activate IGFIR is bound by IGF1 mainly, followed by insulin receptor substrates 1 (IRS1) and insulin receptor substrates 2 (IRS2) phosphorylation [12]. Recruitment of these molecules activates multiple elements of PI3K pathway. Phosphatase and tensin homolog (PTEN) is a tumor suppressor and negative regulator of PI3K pathway through converting PIP3 back to PIP2 [13]. A plethora of information suggests PI3K pathway is key to the pathogenesis of PCOS but little is known about the expression pattern and possible role of IGFIR/PI3K pathway in PCOS. The IGFIR/PI3K signaling pathway plays its role mainly in the ovarian GCs [14]. Therefore, in the current study, we aim to investigate whether the core elements of the IGF1R/PI3K pathway are differentially expressed in GCs isolated from PCOS patients and controls.

Material and methods

Subjects

For mRNA analysis, we recruited patients from the Center for Reproductive Medicine, Shandong University between 2015 and 2016. A total of 130 (67 PCOS patients and 63 controls) participants were recruited, who were undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) for infertility. Diagnosis of PCOS was carried out according to the revised Rotterdam consensus(2003), satisfied at least two of the following criteria: oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, polycystic ovaries after exclusion of other etiologies (eg: congenital adrenal hyperplasia, androgen-secreting tumors, cushing's syndrome) [15]. Controls were 63 females with regular menstrual cycles, normal ovulatory function, who underwent IVF or ICSI because of tube and/or male factors. All participants were younger than 35 years old. Formal written consent was obtained from each patient, and this research was under a protocol approved by the Ethics Committee of Shandong University.

Clinical and biochemical measurements

The physical examinations were retrospectively analyzed, including age and body mass index (BMI). After 12 h overnight fasting, venous blood was collected to measure fasting insulin (FINS) and fasting plasma glucose (FPG). The homeostasis model assessment of insulin resistance(HOMA-IR) was calculated by the following equation: HOMA-IR = FPG (mM) × FINS (mIU/L)/22.5 [16]. On day 2–5 of the menstrual cycle, venous blood was used to determine the levels of anti-Mullerian hormone(AMH), testosterone(T), follicle stimulating hormone(FSH), luteotropic hormone (LH). Transvaginal ultrasonography was performed to assess the antral follicles count (AFC) of right and left ovaries.

Follicular fluid collection and retrieval of ovarian GCs

Ovarian stimulation and oocyte retrieval were performed using a previously described protocol [17]. After adequate follicle development, human chorionic gonadotropin (hCG) was administered to trigger ovulation. 36 h after hCG trigger, transvaginal ultrasound-guided aspiration was used to retrieve oocytes and collect follicular fluid. Then, GCs were purified with Ficoll-Paque (Solarbio, Beijing, China). The protocol isolating GCs from follicular fluid was modified slightly from the method as described previously [18]. Both GCs and follicular fluid samples were stored at −80 °C.

Total RNA extraction and RT-PCR

Total RNA was isolated from GCs after rapid thaw with Trizol Reagent (Life Technologies, Shanghai) following the manufacturer's instructions. Firstly, 0.2 mL of chloroform was added to per 1 mL of Trizol used for lysis and then incubated for 2–3 min. Secondly, after centrifuging the samples for 15 min at 12,000 × *g* at 4 °C, the aqueous phase containing the RNA was transferred to a new tube and the remnant was used to extract protein. The quality of RNA was checked at the absorbance of 260 nm/280 nm by a Nanodrop-2000 (ThermoFisher Scientific, American). According to the manufacturer's protocol, total RNA (1 μg) was reverse transcribed to cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, China). Then, cDNA amplification was performed by RT-PCR in triplicate using Quanti Nova SYBR Green PCR Kit (QIAGEN, Germany) following the manufacturer's instructions. The PCR primers for RT-PCR were all listed in Table 1. Melting curve analyses was used to confirm amplification specificity and

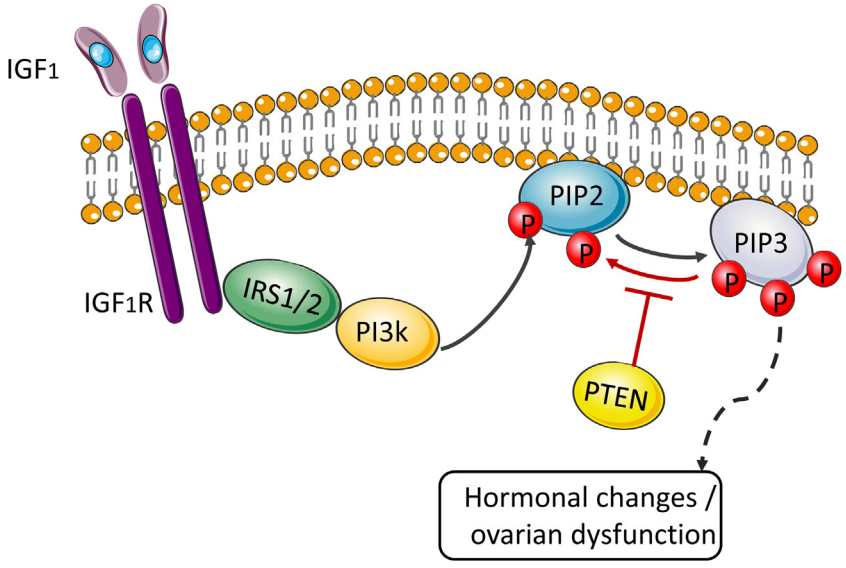

Fig. 1. The activation process of insulin like growth 1 receptor (IGFIR)/PI3K signaling pathway is showed in Fig. 1. An essential step to activate IGFIR is bound by IGF1 mainly, followed by insulin receptor substrates 1 (IRS1) and insulin receptor substrates 2 (IRS2) phosphorylation. Recruitment of these molecules activates multiple elements of PI3K pathway. Phosphatase and tensin homolog ten (PTEN) is a tumor suppressor and negative regulator of PI3K pathway through converting PIP3 back to PIP2.

Table 1
Sequences of primers for RT-PCR.

Gene	Sense(5'-3')	Anti-sense(5'-3')
IGF1R	ATGCTGACCTCTGTTACCTCT	GGCTTATTCCCCACAATGTAGTT
IRS1	CTGCACAACCGTGCTAAGG	CGTCACCGTAGCTCAAGTCC
IRS2	CGGTGAGTTCTACGGGTACAT	TCAGGGTGTATTCATCCAGCG
PTEN	CCAGGAGTTACTTCTATGCCTGA	CCAGGAGTTACTTCTATGCCTGA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
ACTIN	TTCGAGCAAGAGATGGCCA	CGTACAGGTCTTTGCGGAT

Abbreviations: IGF1R, insulin like growth factor 1 receptor; IRS1, insulin receptor substrate 1; IRS2, insulin receptor substrate 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PTEN, phosphatase and tensin homolog.

Table 2
Clinical and endocrine parameters in PCOS patients and in healthy controls.

Basic parameters	PCOS($n=60$)	Control($n=60$)	p value
Age(years)	28.44 ± 3.00	28.60 ± 2.86	NS
Body mass index(kg/m [2])	24.47 ± 3.80	21.77 ± 3.03	p < 0.001
FPG(mmol/L)	5.41 ± 0.46	5.19 ± 0.42	p = 0.005
FINS(mIU/L)	15.01 ± 8.64	7.83 ± 1.97	p < 0.001
HOMA-IR	3.59 ± 2.04	1.79 ± 0.50	p < 0.001
AMH(ng/ml)	8.39 ± 3.78	4.09 ± 1.91	p < 0.001
AFC	24.00 ± 6.44	13.56 ± 2.98	p < 0.001
T(ng/dl)	36.69 ± 15.85	23.31 ± 6.99	p < 0.001
FSH (U/L)	5.69 ± 0.90	6.55 ± 0.99	p < 0.001
LH (IU/L)	7.37 ± 3.61	5.26 ± 1.73	p < 0.001

Abbreviations: FPG: fasting plasma glucose; FINS: fasting insulin; HOMA-IR: homeostasis model assessment of insulin resistance; AMH: anti-Mullerian hormone; AFC: antral follicle count; T: testosterone; FSH: follicle stimulating hormone; LH: luteotropic hormone; E_2: estradiol; P: progesterone; PRL: prolactin. NS: not statistically significant. Values represented as mean ± standard deviation (SD).

the housekeeping gene ACTIN/GAPDH were used to normalize the expression of target genes. The relative expression of genes was calculated using the $2^{-\Delta CT}$ method and expressed as a fold change relative to that of the controls.

Protein extraction and western blot

After transferring the aqueous phase containing the RNA to a new tube, the remnant was used to extract protein. The proteins were precipitated, washed and then solubilized in 200 μL of 1% SDS according the manufacturer's instructions. Equal amounts of protein were separated by sodium dodecylsulfate polyacrylamide gel (SDS-PAGE), and the bands were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA).

The membranes were blocked with 5% milk, and then incubated with primary antibodies at 4°C overnight. After the membranes were incubated with peroxidase-conjugated secondary antibodies (Zhongshan, Beijing, China) for 1 h at room temperature, ChemiDoc MP Imaging System (BIO-RAD, Richmond, CA) and Image Lab Sofware were used to detect and analyze immunoreactive bands. Relative protein levels in each sample were normalized to ACTIN to standardize the loading variations. The primary antibodies for immunoblotting included anti-IGFIR(Proteintech, 66283-I-Ig), anti-IRS1(Cell Signaling Technology, 2390s), anti-IRS2(Cell Signaling Technology, 4502), anti-PTEN(Proteintech, 60300-1-Ig), anti-ACTIN(Cell Signaling Technology, 4970 s).

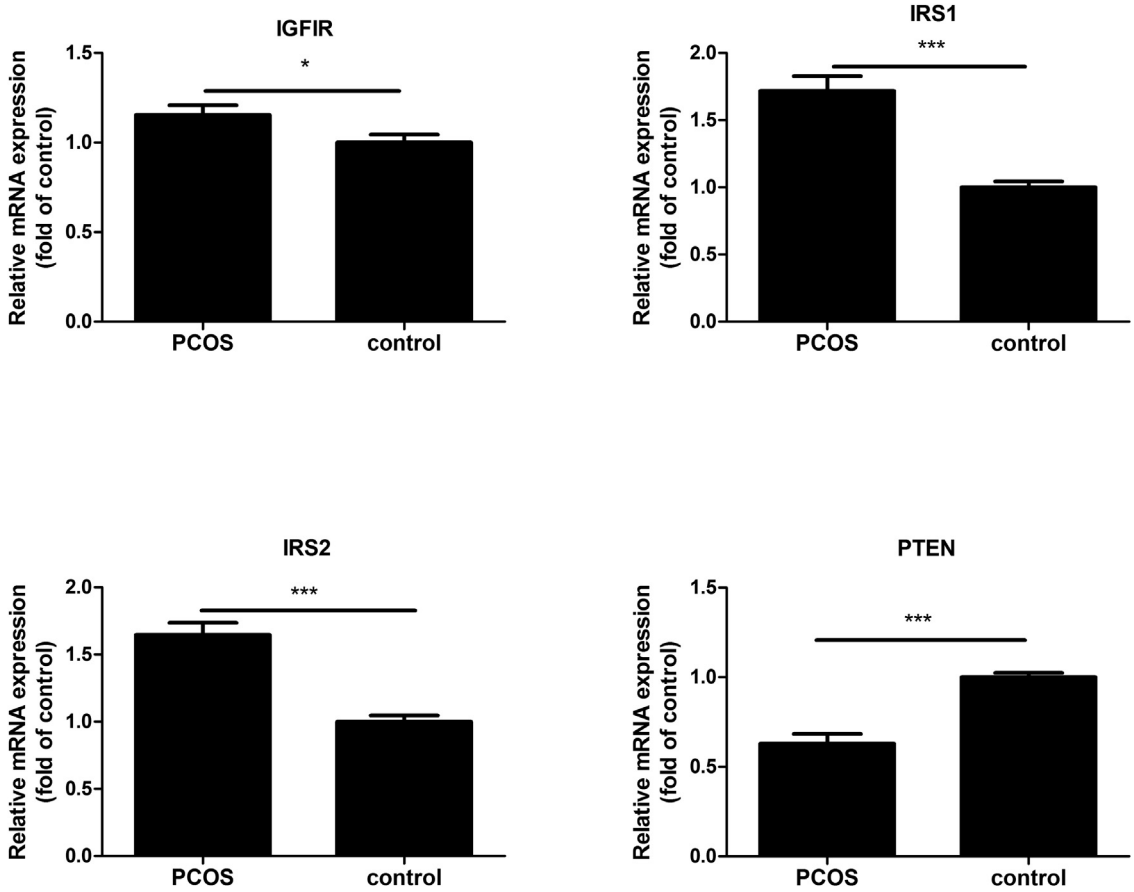

Fig. 2. Expression levels of core elements related to IGFIR/PI3K pathway. GAPDH was used as inner control. Compared to controls, the expression of IRS1, IRS2, IGFIR was significantly increased in PCOS group ($p < 0.001$, $p < 0.001$ and $p = 0.0313$ respectively), with PTEN dramatically declined ($p < 0.001$). Data were presented as mean and statistical analysis of the data was performed using the Student t-test. $^{***}p < 0.001$,$^{*}p = 0.0313$.

Statistical analysis

Kolmogorov–Smirnov was used to assess whether continuous variables were normally distributed. Normally distributed variables were presented as mean ± standard deviation (SD) and analyzed using the Student *t*-test to determine statistical significance. Logistic regression was used to adjust for age and BMI, to avoid their potential effects on expression of genes related to the IGFIR/PI3K pathway. Data were analyzed using SPSS 21.0 (SPSS, Chicago, IL, USA), and a value of $p< 0.05$ was considered statistically significant.

Results

Clinical characteristics of PCOS patients and controls

The clinical characteristics of the PCOS patients and controls were shown in Table 2. Compared to controls, BMI, FINS, FPG, HOMA-IR, AMH, AFC, T, LH were significantly increased in PCOS group ($p < 0.05$, for all) while FSH dramatically decreased ($p < 0.05$). Age was similar between PCOS patients and controls ($p > 0.05$).

Genes expression in GCs of PCOS patients and controls

Expression levels of core components in the IGFIR/PI3K pathway were examined by RT-PCR and data were analyzed after adjustment age and BMI. Using GAPDH as housekeeping gene, the expression of IRS1, IRS2, IGFIR was significantly increased in PCOS group ($p < 0.001$, $p < 0.001$ and $p = 0.0313$ respectively), with PTEN dramatically declined ($p < 0.001$). When ACTIN was used to normalize the expression of target genes, the levels of IRS1, IRS2, IGFIR were significantly increased, with PTEN decreased in PCOS group ($p < 0.001$for all). The above results showed in Figs. 2 and 3 respectively.

Protein expression in GCs of PCOS patients and controls

We had demonstrated that, in term of mRNA levels, IGFIR/PI3K pathway was differently expressed in PCOS GCs; therefore, we further used the GCs of 6 PCOS patients and 6 controls for protein assay and the results showed in Fig. 4. Compared to controls, the levels of IRS1, IRS2, IGFIR were increased in PCOS group, with PTEN decreased.

Discussion

In the present study, in order to investigate whether IGFIR/PI3K pathway plays a crucial role in the pathological mechanisms of PCOS, we analyzed several genes expression. Our findings indicated that IGFIR/PI3K pathway were up-regulated, with IGFIR, IRS1, IRS2 significantly increased while PTEN decreased in PCOS GCs.

IGF1R, which is a transmembrane tyrosine kinase receptor, is autophosphorylation following combining by its ligands IGF-I, IGF-II and insulin. In accordance with our research, previous study reveals that in mice with similar PCOS features including ovarian dysfunction, infertility, obesity and hyperglycemia, the expression of IGF1R is increased compared to controls [19]. Interesting, enhanced IGF1R expression is noted in PCOS follicles during early preantral development. Moreover, it is involved in regulation of initiation of human follicle growth, and also accelerated preantral follicle growth [20]. Furthermore, IGF1R,

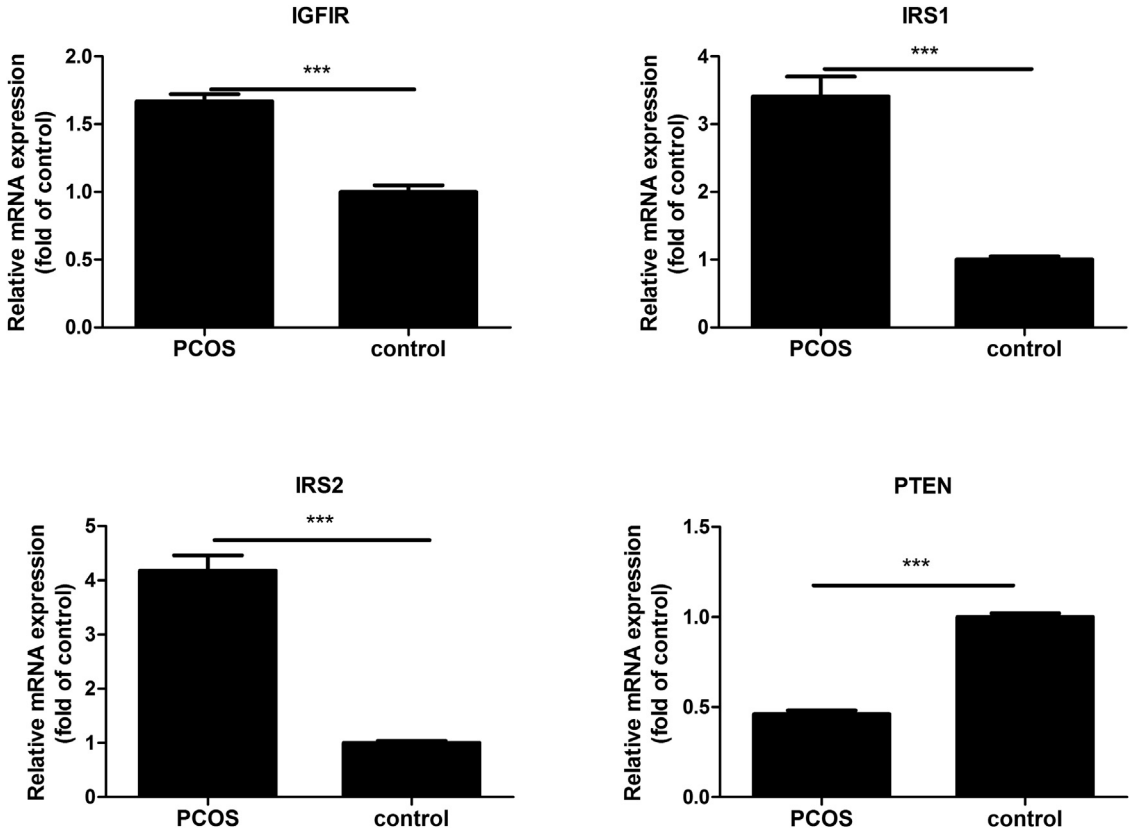

Fig. 3. Expression levels of core elements related to IGFIR/PI3K pathway. ACTIN was used as inner control. Compared to controls, the expression of IRS1, IRS2, IGFIR was significantly increased in PCOS group with PTEN dramatically declined ($p < 0.001$ for all). Data were presented as mean and statistical analysis of the data was performed using the Student *t*-test. $^{***}p < 0.001$.

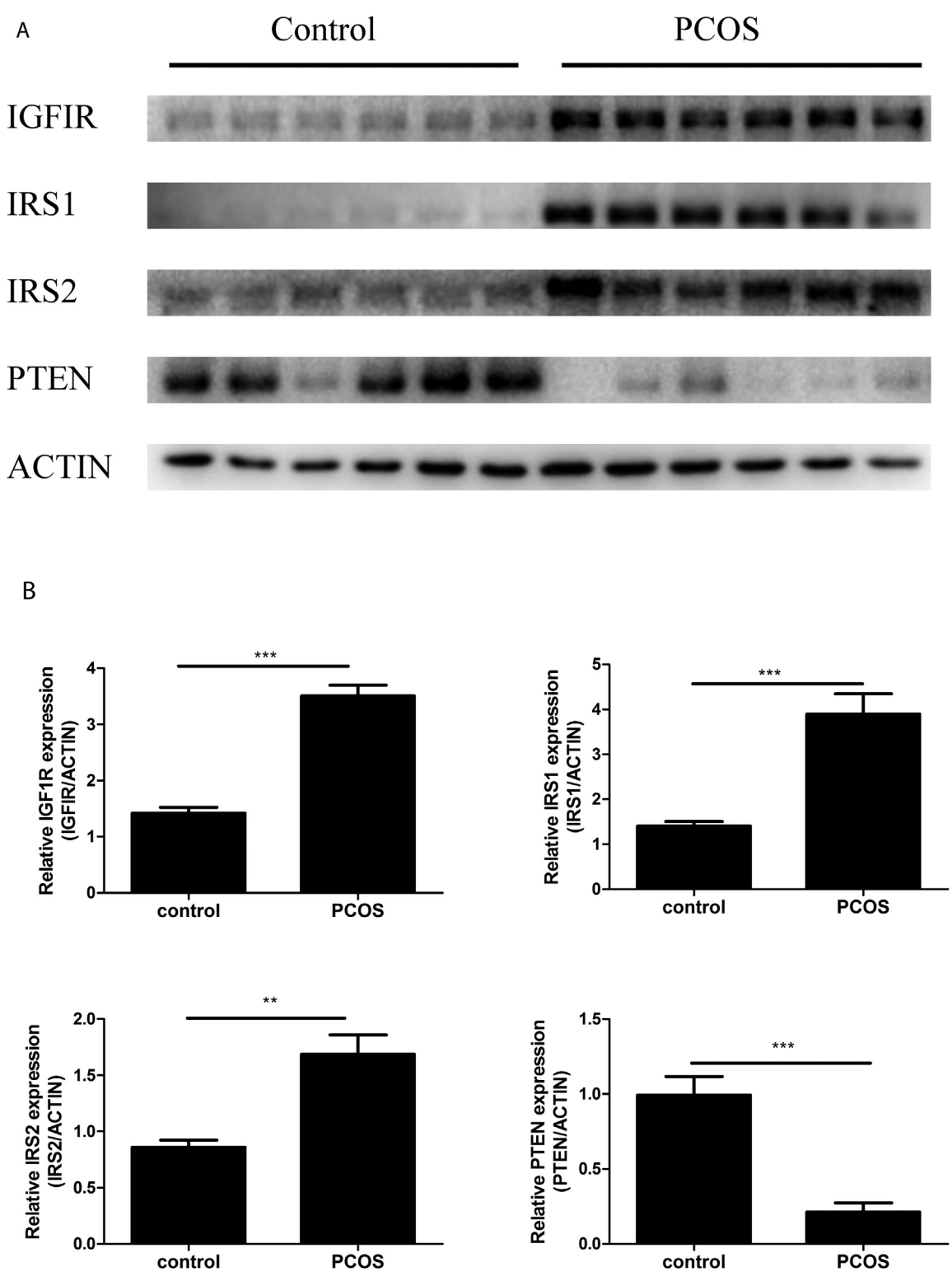

Fig. 4. **A**. Western blot analysis of IGFIR, IRS1, IRS2, PTEN in granulosa cells of PCOS and controls ($n = 6$). **B**. Quantitative expression of proteins. Values are expressed as mean ± SD $^{***}p < 0.001$, $^{**}p = 0.0011$.

activating by its ligands IGF-I, plays an vital role in stimulating proliferation and steroidogenesis of GCs during follicular development [21–23]. Our results showed that the expression of IGFIR was significantly increased in PCOS compared to controls, suggesting that increased IGF1R level has the potential to enhance GCs proliferation, follicle activation and hormone synthesis in PCOS. Hyperandrogenism is a characteristic feature of PCOS and often involves in disturbed reproductive performance. Findings have observed that androgen can increase IGFI and IGFIR gene expression in the GCs of follicles, thus, it is possible that hyperandrogenism facilitates IGFIR actions in both GCs proliferation and follicular development in PCOS [24,25].

Phosphorylated IGFIR initiates signal transduction through activation of intracellular substrates IRS1 and IRS2. IRS2

preferentially mediates mitogenic pathways, whereas IRS1 appears to contribute mainly to metabolic pathways [26]. H-W Yen, who compared the content of IRS1 and IRS2 in GCs isolated from 3 to 7 mm follicles, demonstrated that there were no changes in the expression of IRS1 and IRS2 in GCs from normal and PCOS women [27]. However, we in fact observed that the levels of IRS1 and IRS2 were significantly increased in PCOS GCs and was consistent with the results of earlier reports [28]. The differences were likely due to our GCs obtained from pre-ovulation follicles and the expression of IRS1 and IRS2 in GCs changed during follicular development in PCOS patients. The underlying mechanism for the observed increased expression of IRS1 and IRS2 was speculated to be related to the abnormal increase of LH in PCOS patients. Crucially, our data confirmed IRS1 and IRS2 were differently expressed in PCOS GCs, which may be the cause of both theca hyperplasia and hyperandrogenism leading to infertility in PCOS.

To investigate whether IGFIR/PI3K pathway is differently expressed in PCOS GCs, we also assessed the expression of PTEN, as it directly stimulates PI3K pathway by converting PIP3 to PIP2. Our study showed the expression of PTEN was significantly decreased in GCs of PCOS, while A Iwase [29] demonstrated the level of PTEN tended to be higher in luteinized GCs of PCOS patients. However, to the best of our knowledge, It is still unclear how PTEN expression is regulated during terminal follicular growth. On the one hand, studies reported that the ovaries of insulin-treated mice showed decreased expression of PTEN, while others indicated that the expression of PTEN induced with insulin in human luteinizing granulosa cells [29,30]. Therefore, further studies are needed to investigate about it. Down-regulation of PTEN is to be involved in the promoting of proliferation and differentiation of GCs in human ovary via the PI3K pathway [31]. Furthermore, another research found that selective deletion of PTEN in mice caused elevated androgen levels, ovary enlargement, antral follicle accumulation, early fertility loss, which was reminiscent of human PCOS, suggesting that dysregulated PTEN may be involved in PCOS pathogenesis [32]. These findings, along with our aforementioned observations of marked decreased expression of PTEN in GCs of PCOS, suggest that PTEN may be responsible for various immature follicles and abnormal GCs proliferation and, thus, may play a crucial role in the development of ovarian dysfunction in PCOS.

Our study has several strengths. First, our sample size was relatively larger than many previous expression studies in this fields. Second, age and BMI were adjusted during data analysis, in order to preclude their potential effects on the expression of genes related to the IGFIR/PI3K pathway. Moreover, this was the first time to detect the expression of IGFIR/PI3K pathway signaling molecules in GCs of PCOS. However, we only investigated several core elements not full components of IGFIR/PI3K pathway. Another limitation of our study is that, to estimate the expression level of IGFIR/PI3K pathway more precisely, GCs obtained before LH/hCG stimulation would be more appropriate. However, it is very difficult to collect abundant immature GCs at gynecologic surgery.

In summary, our findings indicated that IGFIR/PI3K pathway was differently expressed, with IGFIR, IRS1, IRS2 significantly increased while PTEN decreased in PCOS GCs, which might be dominantly involved in hormonal changes and ovarian dysfunction in PCOS. This suggests that dysregulation expression of core IGFIR/PI3K pathway components in GCs contributes to the pathogenesis of PCOS. However, further studies are required to investigate function and regulation of IGFIR/PI3K pathway in human ovaries.

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