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Reduced expression of SCF in serum and follicle from patients with polycystic ovary syndrome

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Abstract. – OBJECTIVE: In humans, stem cell factor (SCF), produced by cumulus granulosa cells during the follicular phase, plays a crucial role in follicular development. Remarkably, polycystic ovary syndrome (PCOS), one of the main reasons affecting women fertility, is accompanied by some abnormal follicles. Is there a relationship between SCF and PCOS? This study aimed to compare the expression of SCF in follicle and serum from patients with and without PCOS undergoing *in vitro* fertilization (IVF) treatment and to investigate the potential relationship between aberrant SCF expression and PCOS.

PATIENTS AND METHODS: Serum, follicular fluid (FF) samples and granulosa cells (GCs) from 48 patients with PCOS (PCOS group) and 62 normal ovulatory patients (control group) were collected. SCF was evaluated in FF, serum, and GCs by using enzyme-linked immunosorbent assay, immunofluorescence staining, Western blot and real-time PCR. The rates of metaphase II (MII) oocyte, fertilization, embryo cleavage and high-quality embryo between PCOS group and control group were also analyzed.

RESULTS: The rates of MII oocyte and fertilization were significantly lower in PCOS group than those in control group ($p < 0.05$). No difference was observed for the rate of embryo cleavage and high-quality embryo in these two groups. The concentrations of SCF in serum and FF from PCOS patients were remarkably lower than those in the controls ($p < 0.05$). Moreover, the expressions of SCF protein and SCF mRNA in GCs from PCOS patients were also decreased compared with the controls ($p < 0.05$).

CONCLUSIONS: PCOS patients showed a reduced SCF expression in serum and follicle, which might be associated with oocyte dysmaturity and low fertilization rate.

Key Words:
SCF, Serum, Follicular fluid, GCs, Oocyte, PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common of female reproductive endocrine disorders affecting about 10% of women of childbearing age[1]. It is characterized by two of the three following criteria: clinical or biochemical hyperandrogenism, oligo- or amenorrhea, and polycystic ovaries (PCO)[2]. Patients with PCOS often suffer from metabolic disturbances such as diabetes, abdominal adiposity, high cholesterol and high blood pressure, which are contributed to the development of cardiovascular diseases and even gynecological cancers[3-6]. As a consequence, these patients are accompanied with long-term health risks and low quality of life. However, its etiology and pathogenesis are still unclear.

Clinical investigations showed that PCOS is associated with disordered follicular development[7]. Normally, the process of follicular development can be divided into four phases: primordial follicle, primary follicle, secondary follicle and mature follicle, and are tightly regulated by hormones, intraovarian regulators, as well as cell-cell interactions[8,9]. Therefore, the balance of inhibitory signals and stimulatory growth factors determine whether follicle maturation will proceed.

Stem cell factor (SCF) is a pleiotropic cytokine with a molecular weight of 25 to 36 kD that exerts its influence on target cells through binding the c-kit receptor, a tyrosine kinase receptor. Data from several researches[10] suggested that SCF and c-kit might play a key role in the regulation of cell proliferation, differentiation, migration, and apoptosis[10]. Studies conducted[10] in the last decades have found an extensive distribution of SCF and c-kit in several kinds of cells, including melanin cells, hematopoietic stem

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cells, gastrointestinal Cajal interstitial cells, mast cells and reproductive cells. Aberrant expression of SCF and/or c-kit may result in the abnormal development of these cells, even lead to the related tumorigenesis[11]. Previous researchers[12-15] have observed the expression of SCF and c-kit on granulose cells (GCs) and oocytes in mammalian and human ovaries, respectively. Subsequent animal experiments[16] have found that the interaction between SCF and the c-kit was relevant to the initiation of follicular transition from primordial into primary follicles. It is worth noting that recombinant mouse SCF promoted an increase in the diameter of oocytes from primordial to early primary follicles in the mouse, and these effects were inhibited by co-culture with SCF-neutralizing antibody[17]. These data suggest that SCF might be a critical element in the regulation of follicular development.

All above results in reminder us to hypothesize that maybe there is a relationship between aberrant SCF expression and PCOS related abnormal follicles. Hence, we measured the expression of SCF in serum and follicle from PCOS patients undergoing IVF treatment to explore the potential role of SCF in PCOS-related abnormal follicles.

Patients and Methods

Patients Selection

From October 2015 to July 2016, 48 patients with PCOS (PCOS group) and 62 patients with normal ovulatory cycles (control group) who received intracytoplasmic sperm injection (ICSI) were enrolled. They were in good physical and mental condition.

The selection criteria are that the study group patients undertook ICSI because of severe asthenospermia or azoospermia. PCOS patients were diagnosed based on the Rotterdam criteria[2]. In the control group, patients received ICSI during the same period for causes other than the ones in the study group. All the patients were otherwise healthy. Patients with a history of the following procedures or disorders were excluded: ovarian surgery, radiotherapy or chemotherapy, premature ovarian failure, ovarian dysfunction, endometriosis, hyperprolactinemia, thyroid dysfunction, or ovulation induction within 3 months.

Controlled Ovarian Stimulation

Follicular aspirates were collected during oocyte retrieval following published procedures[18] and ovarian stimulation was performed with the use of a prolonged protocol. Briefly, gonadotropin-releasing hormone agonist (GnRH-a, Ipsen, Boulogne-Billancourt, France) was used in the second day of menstrual cycle for pituitary down-regulation. According to the patient's age, body mass index, serum basal FSH levels, LH levels, estradiol levels and antral follicle count, initial doses of 75-112.5 IU/d of recombinant human FSH (Merck-Serono, Darmstadt, Germany) were used. The time and dose of recombinant human FSH were adjusted according to ovarian response as monitored by serum estradiol levels and vaginal ultrasound. When the dominant follicle was ≥ 19 mm in diameter or at least 3 follicles were ≥ 17.5 mm in diameter, recombinant human FSH was stopped, and a single injection of 6000-8000 IU of hCG (Merck-Serono, Darmstadt, Germany) was administered. Oocyte retrieval was performed 36-40 hours later under transvaginal ultrasound guidance.

Serum and FF Collection

Serum and FF samples were collected during oocyte retrieval. The follicular fluids were pooled in sterile tubes for each patient and immediately centrifuged at 3000 rpm for 10 min. Supernatants were aspirated, divided into aliquots, and frozen at -80°C for future analysis.

ELISA for SCF Measurements

Concentrations of SCF in FF and serum were determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). All of the procedures were performed according to the manufacturers' instructions.

Human GCs Collection

Follicular fluids were centrifuged at 2000 rpm for 5 min. The cells were resuspended with DMEM/F12 (Life Technologies, Carlsbad, CA, USA) medium and transferred to a 50% (volume fraction) Percoll gradient (Sigma-Aldrich, St. Louis, MO, USA); they were centrifuged at 4000 rpm for 20 min to purify human GCs from any red blood cells. After washing and recentrifugation, sheets of human GCs were digested with hyaluronidase at a 1:1 ratio for 2 min to separate them. The GCs were removed using a pipette and washed with phosphate buffered saline (PBS). The cells were stored at -80°C for future analysis. For immunofluorescence staining, the cells were smeared on polyL-lysine processing slides and

kept at 37°C for 10 min. After the cells were dried, they were fixed with 4% paraformaldehyde for 20 min and stored at -80°C.

Immunofluorescence Staining of GCs

The cells were fixed with 4% paraformaldehyde for 20 min at 25°C. GCs were rinsed with 1% Triton X-100 for 30 min; washed with PBS for three times (5 min each time). Non-specific binding sites were blocked with 5% goat serum albumin (Zhongshanjinqiao Biotechnology, Beijing, China) for 60 min. Then, the samples were incubated with mouse anti-SCF (Santa Cruz, Dallas, TX, USA) antibodies at 4°C overnight; primary antibody was replaced with PBS in negative controls. The samples were taken out and kept at room temperature for 30 min, washed with PBS for three times (5 min each time), incubated with Cy3-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) for 1 hours at 25°C in the dark, washed with PBS for three times (5 min each time). The samples were stained with DAPI (Zhongshanjinqiao Biotechnology, Beijing, China) for identifying nuclei, and observed with a fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot

Total proteins were extracted from cells using the RIPA lysis buffer containing protease inhibitors (Applygen, Beijing, China) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). The protein concentrations were determined by NanoDrop 2000c spectrophotometer using BCA protein assay kit (Applygen, Beijing, China). After loading equal amount of protein samples, SDS-PAGE (12% sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed. The proteins were then transferred to a PVDF membrane (Merck-Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% non-fat dry milk or 5% BSA for 1 hour, the membrane was incubated with anti-SCF antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight, washed in TBST, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour. Visualization of the proteins was detected with ECL chemiluminescence. Beta-actin (Santa Cruz Biotechnology, Dallas, TX, USA) was used as a loading control. The intensity values were assessed and analyzed with Image J software.

RNA Extraction and Real-time PCR

Total RNA was extracted from cells with RNeasy kit (Qiagen, Beijing, China) according to the manufacturers' instructions. Reverse transcription reactions were performed using Super cDNA First-Strand Synthesis Kit (CWBiotech, Beijing, China). Real-time PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using Ultra SYBR Mixture with ROX (CWBiotech, Beijing, China). The following primers were used: SCF (Forward: CAGAGTCAGTGTCACAAAACCATT, Reverse: TTGGCCTTCCTATTACTGCTACTG); GAPDH (Forward: AGAAGGCTGGGGCTCATTTG, Reverse: AGGGGCCATCCACAGTCTTC). The reactions were incubated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. All reverse transcription reactions included no-template controls, and all PCR reactions were run in triplicate.

Oocyte and Embryo Assessment

Oocyte was regarded as being at the metaphase II (MII) stage when the first polar body was observed in the oocyte cytoplasm. All the MII stage oocytes were treated with ICSI. The oocyte maturation rate refers to the number of MII oocytes divided by the total number of all retrieved oocytes. Oocyte fertilization was assessed 16-20 hours after ICSI. Normal fertilization was confirmed when two pronuclei (2PN) were found in the cytoplasm. The normal fertilization rate refers to the number of fertilized oocytes divided by the total number of all retrieved oocytes. Embryo cleavage was examined 40-44 hours after ICSI. Normal embryo cleavage was defined when the fertilized egg developed into an embryo with 4-6 blastomeres. The cleavage rate refers to the number of cleaved zygotes divided by the total number of all zygotes. Embryo evaluation was conducted 64-68 hours after oocyte fertilization. A high-quality embryo should consist of 7-9 blastomeres with a uniform size, and the fragment proportion should be less than 20%. The high-quality embryo rate refers to the number of high-quality embryos divided by the total number of all embryos.

Statistical Analysis

The software package SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for all data analysis. In general, results among experimental groups

Table I. Comparison of general conditions of PCOS group and control group.

General indicators	Control	PCOS	*p* value
No. of cases	62	48	
No. of ova	13.28 ± 7.36	17.50 ± 6.01	> .05
Age (y)	31.20 ± 5.13	29.35 ± 5.15	> .05
FSH (IU/L)	6.36 ± 2.67	5.52 ± 1.36	> .05
LH (IU/L)	4.13 ± 2.21	8.47 ± 4.35	< .05
E_2 (ng/mL)	44.79 ± 51.62	50.07 ± 47.78	> .05
PRL (μg/L)	16.62 ± 7.79	15.87 ± 7.89	> .05
T (nmol/L)	37.27 ± 47.10	758.41 ± 1561.22	< .05
Gn dosage (IU)	2472.97 ± 1152.97	2091 ± 934	> .05

Note: The data were expressed as mean ± SD.

were analyzed by student's *t*-test or one-way ANOVA. For all tests, *p*-value < 0.05 was considered statistically significant.

Results

General Conditions

The general characteristics of the control group and PCOS group were summarized in Table I. The levels of LH and T were significantly higher in PCOS group than those in control group.

Comparison of the Rates of MII Oocyte, Fertilization, Embryo Cleavage and High-Quality Embryo between PCOS Group and Control Group

Previous studies[7] have found that PCOS patients were often accompanied with aberrant follicles and unsatisfactory fertilization rate. In our study, we compared the clinical outcomes between the PCOS group and control group. As shown in Table II, the MII oocyte rate and normal fertilization rate were remarkably reduced in PCOS group when compared with the control group (71.04 ± 10.13 *vs.* 84.07 ± 13.11, $p < 0.05$; 54.94 ± 6.01 *vs.* 77.29 ± 8.23, $p < 0.05$). No difference was observed for the cleavage rate and high-quality embryo rate between these two groups (97.02 ± 4.36 *vs.* 96.70 ± 5.70, $p > 0.05$; 40.13 ± 1.79 *vs.* 42.81 ± 2.89, $p > 0.05$). In line with previous studies, our data also showed a high rate of immature oocyte and low rate of fertilization in PCOS patients.

Expressions of SCF in Serum and FF from PCOS Group and Control Group

It is reported that SCF/c-kit system played an important role in the process of follicular development[19]. Then, we evaluated the SCF concentration in serum and FF samples from these two groups. The mean SCF concentrations in sera from PCOS group and control group were 1.74 ± 0.19 ng/mL and 2.27 ± 0.21 ng/mL, respectively (Table III). The mean SCF concentration in FFs from PCOS group and control group were 0.28 ± 0.06 ng/mL and 0.83 ± 0.04 ng/mL, respectively (Table III). Compared with the controls, the concentrations of SCF in serum and FF from PCOS group were significantly lower than those from the control group ($p < 0.05$) (Figure 1), implying a potential correlation between the reduced SCF expression and PCOS.

Table II. Comparison of the rates of MII oocyte, normal fertilization, cleavage and high-quality embryo between PCOS group and control group.

Variable	Control	PCOS	*p* value
MII oocyte rate (%)	84.07 ± 13.11	71.04 ± 10.13	< .05
Normal fertilization rate (%)	77.29 ± 8.23	54.94 ± 6.01	< .05
Cleavage rate (%)	96.70 ± 5.70	97.02 ± 4.36	> .05
High-quality embryo rate (%)	42.81 ± 2.89	40.13 ± 1.79	> .05

The data were expressed as mean ± SD.

Table III. Comparison of SCF levels in serum and FF from PCOS group and control group.

Group	The level of SCF in serum (ng/mL)	The level of SCF in FF (ng/mL)
PCOS (n = 48)	1.74 ± 0.19	0.28 ± 0.06
Control (n = 62)	2.27 ± 0.21	0.83 ± 0.04
p value	< 0.05	< 0.05

The data were expressed as mean ± SD.

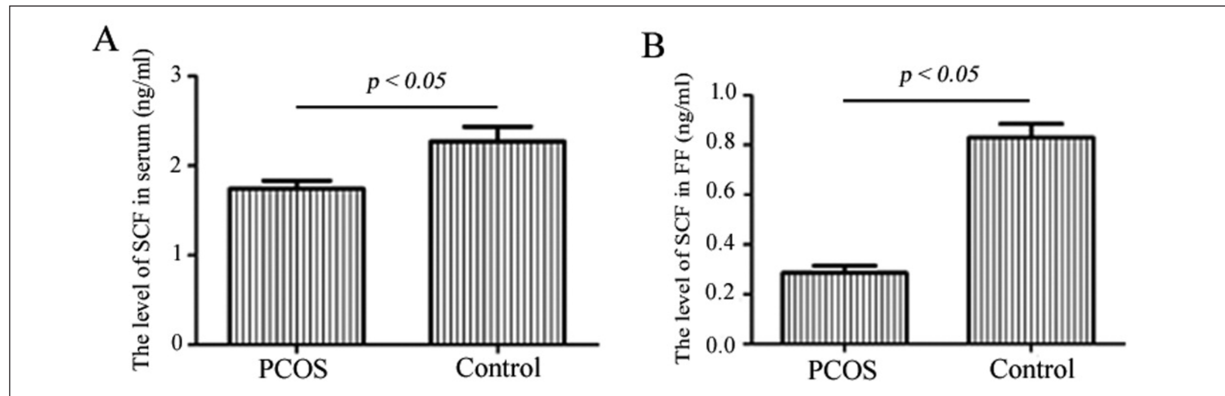

Figure 1. Levels of SCF in serum *(A)* and FF *(B)* from PCOS patients and the controls were investigated by ELISA Kit. Both in serum and FF, the levels of SCF from patients with PCOS were significantly lower than those in the controls ($p < 0.05$).

Expression of SCF in GCs from PCOS Group and Control Group

The reduced SCF concentration in FF from PCOS patients promoted us to the hypothesis that whether the main cause of this reduction is attributed to the GCs in PCOS. Therefore, we next compared the SCF expression in GCs from PCOS group and control group. Firstly, we investigated the expression of SCF in GCs by immunofluorescence staining and found that SCF was expressed in the cytoplasm and membrane of human GCs (Figure 2), suggesting that SCF was produced and secreted by GCs. Notably, compared with the control group, the expression of SCF was obviously weaken in GCs from PCOS group (Figure 2). To further confirm our findings, we detected the expression of SCF protein by

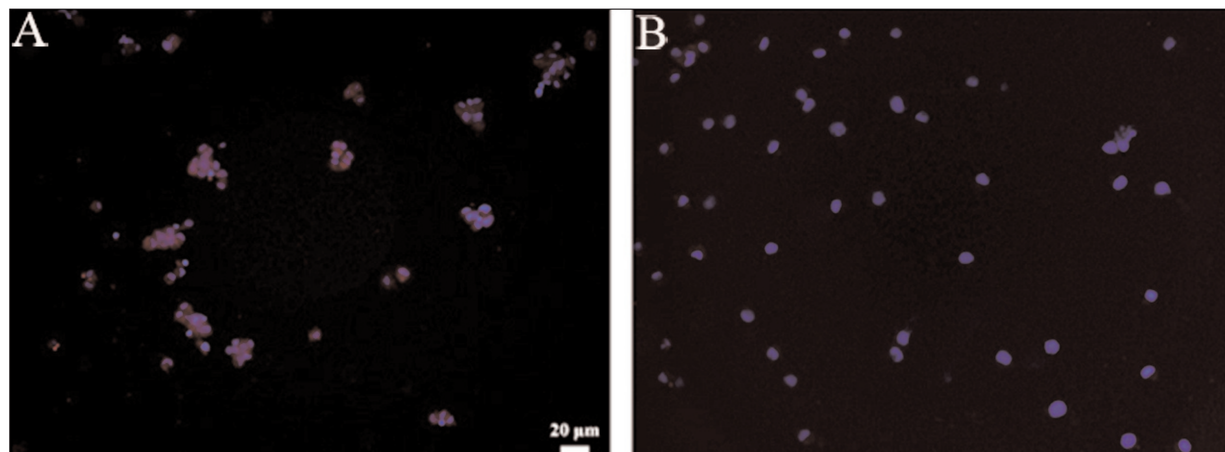

Figure 2. Immunofluorescence of SCF (*red*) in human GCs from the controls *(A)* and PCOS patients *(B)*. SCF was expressed in the cytoplasm and membrane of human GCs. Compared with the controls, SCF was apparently reduced in GCs from PCOS patients.

Western blot. As we expected, PCOS patients expressed a lower level of SCF than the controls ($p < 0.05$) (Figure 3A). Furthermore, real time-PCR also showed a decreased SCF mRNA in GCs from PCOS group when compared with the control group ($p < 0.05$) (Figure 3B). These findings indicated that the production of SCF was reduced in GCs from PCOS patients.

Discussion

SCF and its receptor, c-kit, are proved to play an essential role in mammalian and human reproduction[20]. It is reported that SCF and c-kit are expressed on the surface of cumulus GCs and oocytes at all stages of follicular development in postnatal ovaries of human, respectively[15]. Inhibition of SCF/c-kit system disrupts the primordial follicular development, primary follicle growth, the follicular fluid formation of preantral follicles, and the ovulation of mature follicles *in vivo*[21]. These researches suggested a sequential requirement for SCF/c-kit in ovarian follicular development. Previous studies have found that patients with PCOS were often accompanied with follicular dysplasia in their ovary[7]. Therefore, we speculate that maybe there is a relationship between aberrant SCF expression and PCOS?

Firstly, we analyzed and compared the clinical outcomes of patients with and without PCOS. Our results showed that the rates of MII oocyte and fertilization were remarkably lower in PCOS group than those in control group. This might be due to some oocytes collecting from PCOS patients were immature, though they retrieved more

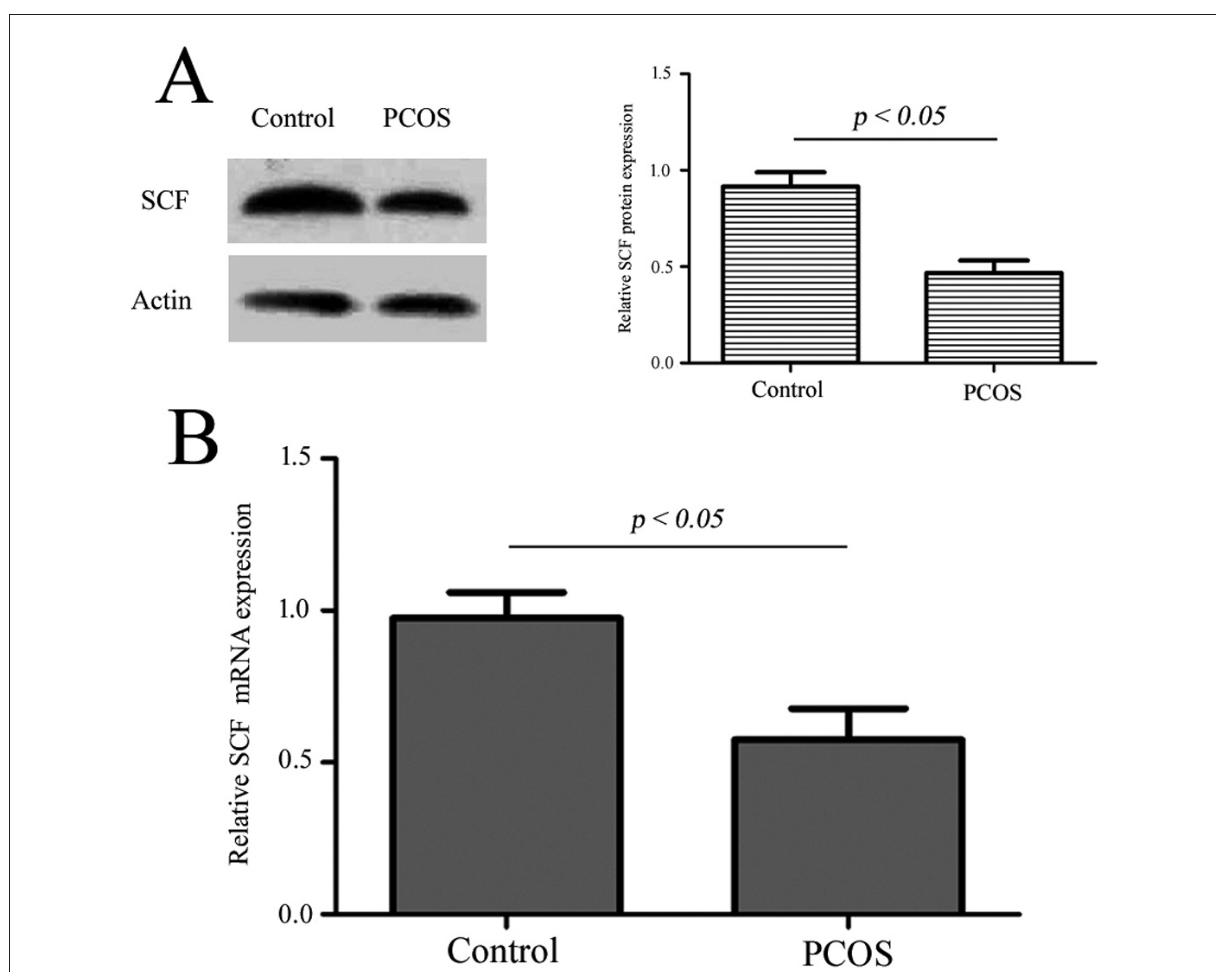

Figure 3. Expressions of SCF protein and mRNA in human GCs from PCOS patients and the controls. Compared with the controls, both SCF protein ***(A)*** and SCF mRNA ***(B)*** were significantly decreased in GCs from PCOS patients ($p < 0.05$).

oocytes. However, no difference was observed for the cleavage rate and high-quality embryo rate between these two groups, implying that once the fertilization was completed, there was no distinction in embryo developmental potential between PCOS patients and the controls undergoing *in vitro* fertilization-embryo transfer (IVF-ET). Yin et al[22] have investigated the relationship between embryo arrest and PCOS during IVF-ET, and demonstrated that embryo arrest rate was not affected by PCOS in the process of IVF-ET. These results indicated that the leading cause of sterility in PCOS patients was oocyte dysmaturity, possibly resulting from the abnormal follicular development in ovaries of PCOS patients.

Due to the key role of SCF in follicular development[23], we wonder that whether the immature oocytes in ovaries of PCOS patients are associated with the aberrant SCF expression. For this reason, we measured the SCF concentrations in serum and FF samples from patients with and without PCOS, respectively. The results showed that both in serum and FF, the concentrations of SCF were significantly lower in PCOS patients than those in controls, indicating a close relation between aberrant SCF production and oocyte dysmaturity. By our results, Gizzo et al[24] have analyzed the correlation between the serum concentration of SCF and the number of MII oocytes retrieved from elderly poor responder patients undergoing IVF, and proved that the SCF concentration in serum was positively associated with MII oocytes rate. Smikle et al[25] investigated the correlation between SCF concentration in FF and clinical outcomes in patients undergoing IVF. The results showed that increased SCF concentration in FF might improve pregnancy rates after oocyte retrieval, fertilization, and embryo transfer. Additionally, *in vitro* co-culture mouse oocytes with SCF[26] further confirmed this positive correlation between SCF and oocyte maturation. The authors found that increased SCF protein significantly promoted first polar body extrusion of preovulatory mouse oocytes and facilitated the development of follicles in mammalian. A possible explanation for this promotion might be the PI3K/Akt/Foxo3a signaling pathway. Zhang et al[27] presented that activation of PI3K/Akt signaling pathway by SCF/c-kit interaction could inhibit the apoptosis of mouse primordial follicle oocytes and promoted the development of oocytes. Whereas, the detailed mechanism need to be further investigated.

Indeed, the communication between oocytes and GCs is essential for the development of normal follicular and oocytes, in which GCs secrete various kinds of nutritional factors to promote oocyte growth[28], simultaneously, oocytes product several factors to regulate GCs development[29]. Therefore, the function status of GCs is often considered as the mirror of oocyte quality[30-32]. It is well known that PCOS patients are often accompanied with aberrant GCs in follicles, displaying with the decreased ability of proliferation[33] and the functional abnormality[34]. As we know, SCF is produced by GCs, and exert its regulatory function by binding to c-kit receptor. Consequently, dysfunction of GCs in ovaries of PCOS patients is likely to influence the normal production of SCF. Therefore, we compared the SCF expression in GCs from these two groups. The immunofluorescence staining showed that SCF was mainly expressed in the cytoplasm and membrane of human GCs. Evidently, a weaken SCF expression was observed in GCs from PCOS patients compared with the controls. This finding was subsequently confirmed by Western blot and RT-PCR, showing an apparently decreased SCF protein and mRNA expressions in GCs from PCOS patients compared with the controls. These data suggested that the dysfunction of GCs on SCF production might disturb the normal follicular development, sequentially caused the occurrence of immature oocytes in ovaries of PCOS patients, and ultimately resulted in reproductive disorder in these patients.

Conclusions

We suggest new insights into the relationship between the serum and follicular levels of SCF and PCOS. Reduced expressions of SCF were found in serum, follicular fluid as well as GCs in ovaries of PCOS patients, and might be associated with oocyte dysmaturity, which finally leading to sterility in women with PCOS. Therefore, targeting SCF might provide the potential strategies for treatment of patients suffering from PCOS in the future.

Acknowledgements

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Ethical Approval and Consent to Participate

The present study was approved by the Clinical Ethical Committee of Jiangxi Provincial Maternal and Child Health Hospital, and informed patients consents were obtained before the initiation of the study.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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