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Original Research Article

Hsc70 interacts with KCNA5 to promote the proliferation of granulosa cells in polycystic ovarian syndrome

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Abstract

Purpose: *To investigate the function and interaction of heat-shock cognate protein 70 (Hsc70) and potassium voltage-gated channel subfamily A member 5 (KCNA5) in polycystic ovary syndrome (PCOS).*
Methods: *Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot were used to measure Hsc70 and KCNA5 expression in PCOS. The effect of Hsc70 on granulosa cell apoptosis was determined by flow cytometry. Cell counting kit-8 (CCK-8) and colony formation assay were used to assess the impact of Hsc70 on cell proliferation, while the interaction of Hsc70 with KCNA5 were evaluated by Western blot, CCK-8, and colony formation assay, respectively.*
Results: *Hsc70 expression was upregulated in PCOS compared to the control ($p < 0.05$). In granulosa cells, Hsc70 overexpression promoted cell proliferation but inhibited apoptosis ($p < 0.05$), whereas Hsc70 knockdown suppressed cell proliferation and promoted apoptosis ($p < 0.05$). Furthermore, KCNA5 expression was downregulated in PCOS ($p < 0.05$) and was negatively correlated with Hsc70 expression. KCNA5 knockdown promoted granulosa cell proliferation and also attenuated the Hsc70 knockdown-mediated inhibition of granulosa cell proliferation ($p < 0.05$).*
Conclusion: *Hsc70 promotes granulosa cell proliferation by interacting with KCNA5, further defining the molecular mechanism behind PCOS.*

Keywords: *Hsc70, KCNA5, Granulosa cells, Polycystic ovary syndrome, Apoptosis*

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 10 – 15 % of women worldwide. There are three major phenotypic characteristics of PCOS, including hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology [1]. PCOS patients have an increased risk of obstetrical complications, infertility, type two diabetes mellitus, cardiovascular disease, endometrial cancer, and psychiatry [1]. Lifestyle interventions as a first-line PCOS treatment improve fertility, ovulation, mood, and metabolic dysfunction, while medical interventions such as metformin and statins are used as a second-line treatment [2]. However, pharmacological interventions have side-effects such as ovarian hyperstimulation

syndrome [3]. Granulosa cells synthesize diverse peptides and sex steroids, and the ovaries of PCOS patients have a higher granulosa cell proliferation rate [4]. Hence, understanding the mechanisms underlying abnormal granulosa cell proliferation in PCOS is critical for developing effective treatments.

Heat-shock cognate protein 70 (Hsc70) is a constitutively and ubiquitously expressed molecular chaperone that maintains cell protein homeostasis under both stressful and normal conditions [5]. Hsc70 plays an important role in diverse cellular processes such as proliferation, differentiation, and apoptosis [6]. Thus, Hsc70 is regarded as a potential biomarker for the treatment of certain diseases, including cardiovascular, cancer, and neurological diseases [6,7]. Importantly, Hsc70 functions as a chaperone by cooperating with co-chaperones and many other partner molecules, such as Hsp70-binding protein 1 (HSPBP1) and c-terminus of Hsc70 interacting protein (CHIP) proteins [7]. For example, Hsc70 acts jointly with CHIP to regulate potassium voltage-gated channel subfamily A member 5 (KCNA5) protein function and expression in cells [8].

KCNA5, also known as *K_V1.5*, encodes the KCNA5/Kv1.5 potassium channel that mediates resting membrane potential [9]. KCNA5 is implicated in the proliferation and apoptosis of various cells, such as human mammary epithelial cells and cancer cells [9]. Moreover, KCNA5 is downregulated in PCOS, and KCNA5 overexpression suppresses granulosa cell proliferation in PCOS [10,11]. However, the interaction of Hsc70 with KCNA5 in PCOS has yet to be investigated.

In this study, we measured Hsc70 levels in PCOS, assessed the effects of Hsc70 on granulosa cell proliferation and apoptosis, and determined the interaction between Hsc70 and KCNA5 in granulosa cells.

METHODS

Patients and clinical sample collection

This study was approved by the Ethics Committee of Hubei Hospital of Integrated Traditional Chinese and Western Medicine (approval no. 2016-46). The participants recruited for this study included 15 healthy women and 15 PCOS patients. PCOS patients were diagnosed at the Hubei Hospital of Integrated Traditional Chinese and Western Medicine, where the inclusion criteria were as follows (two out of three): excess androgen, chronic oligo-ovulation or anovulation, and polycystic ovaries. All participants provided written informed consent. Peripheral blood samples were collected from healthy women and PCOS patients, which were subsequently transferred to EDTA-treated tubes. The collected samples were processed using the Ficoll density gradient centrifugation method and were then prepared for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. All procedures in this study were performed according to the World Medical Association Declaration of Helsinki [12].

Cell culture and transfection

Normal ovarian epithelial cells (IOSE80) and human granulosa-like tumor cells (KGN) were purchased from Hunan Fenghui Biotechnology (Changsha, China). The KGN cell line was selected to study the effect of Hsc70 on PCOS at the cellular level because this cell line has the physiological characteristics of ovarian cells and is the major producer in the ovary. All cell lines were incubated with DMEM/F-12 medium (Gibco, Carlsbad, CA) supplemented with 100 U/mL penicillin G, 0.1 mg/mL streptomycin sulfate, and 10 % fetal bovine serum (FBS, Gibco) and were cultured at 37 °C in a humidified incubator with 5 % CO_2.

Hsc70 overexpression plasmid, control plasmid, Hsc70 short hairpin RNA (shRNA) (shHsc70), and negative control shRNA (shNC) were purchased from Yunzhou Biotechnology Co., Ltd. (Guangzhou, China). KCNA5 siRNA (siKCNA5) and negative control siRNA (siNC) were obtained from Thermo Fisher Scientific (USA). Vectors were transfected into KGN cells for 48 hours using the Lipofectamine® 3000 (Invitrogen) kit.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from cells was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and the concentration was measured using a spectrophotometer (NanoDropND-2000, Thermo Fisher Scientific) at 260 nm. Complementary DNA (cDNA) was reverse transcribed from the isolated RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The - Prism 7500 fast real-time PCR system (Applied Biosystems) was used to perform qPCR, with the PCR reaction including the cDNA template, each primer, RNase-free water, and 1 × SYBR Master Mix (Applied Biosystems). The PCR primers, which are based on previous studies [8,13], are shown in Table 1.

Table 1: Primer sequences

Primer	Sequence (5'-3')
Hsc70 forward	AATGCTGACCTCTTCCGTGGCACC
Hsc70 reverse	TGGCATACCACCAGGCATGCCACCGGC
KCNA5 forward	AGGCTCCTCAGGATGCAG
KCNA5 reverse	GCGACCCGGAGATGTTTATG
β-actin forward	GCGCGGCTACAGCTTCAC
β-actin reverse	GGGGCCGGACTCGTCATA

Western blot assay

Total protein from cells was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime). Extracted protein concentration was quantified using the bicinchoninic acid (BCA) method. Samples were denatured at 100 °C, then proteins were separated on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequently electro-transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5 % non-fat milk then incubated with primary antibodies at 4 °C overnight. After washing with tris-buffered saline (TBS) with 0.05 % Tween-20, the membranes were incubated with the secondary antibody.

The antibodies used in this study were purchased from Abcam and include anti-Hsc70 (ab51052, dilution 1/1000), anti-Bax (ab182733, 1/2000 dilution), anti-Bcl-2 (ab182858, 1/2000 dilution), anti-Cleaved Caspase-3 (ab32042, 1/500 dilution), anti-KCNA5 (ab110469, 1/1000 dilution), anti-β-actin (ab179467, 1/5000 dilution), and goat anti-Rabbit IgG H&L (HRP) (ab205718, 1/2000 dilution). Membrane-bound antibodies were detected using chemiluminescence reagents (Sigma-Aldrich), and protein bands were quantified using ImageJ software. Protein expression was standardized using β-actin expression.

Cell counting kit-8 (CCK-8) assay

The CCK-8 assay was utilized to assess KGN cell proliferation. After transfection, KGN cells were harvested and seeded at a density of 4 × 10^3 cells per well into 96-well plates. The seeded cells were incubated for 24, 48, 72, and 96 hours. At each time point, CCK-8 reagent (10 µL) was added to each well, and the cells were incubated for an additional 1 hour. Sample absorbance was measured using the Biotek ELx800 microplate reader at 450 nm.

Colony formation assay

After transfection for 48 hours, KGN cells were harvested and resuspended in fresh medium at a density of 200 cells/mL, and 1 mL of cell suspension was added to 24-well culture plates. Cells were incubated at 37 °C for 2 weeks, with the culture medium changed every 2 or 3 days. After the two-week incubation, the culture medium was removed from the plates. Cells were fixed with 100 % methanol at room temperature for 20 minutes, stained with crystal violet for 5 minutes at room temperature, and then washed with phosphate buffered saline (PBS). Cell colonies were then viewed and counted using bright-field microscopy.

Flow cytometry

Following transfection for 48 hours, KGN cells were harvested, washed in cold PBS, and resuspended in 1 × annexin-binding buffer (1 × 10^5 cells/mL). Each 100 µL of cell suspension was stained with Annexin V-FITC (5 µL) and PI working solution (5 µL) for 15 minutes in the dark, followed by adding 400 µL 1 × annexin-binding buffer. Cell apoptosis was analyzed using flow cytometry (BD Biosciences, USA).

Statistical analysis

Each experiment was repeated at least three times. GraphPad Prism 7.0 software (GraphPad, USA) was used to analyze experimental data, which are expressed as mean ± standard deviation (SD). Differences between two groups were evaluated using Student's *t* test, and differences among multiple groups were assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. A p value of < 0.05 was considered statistically significant.

RESULTS

Aberrant high Hsc70 expression in PCOS

To determine PCOS patient Hsc70 expression levels, peripheral blood samples were collected from healthy women and PCOS patients, and qRT-PCR was used to measure the expression of *HSPA8* mRNA, which encodes Hsc70. *HSPA8* mRNA was upregulated in PCOS patients compared with healthy women (Figure 1 A). Next, Hsc70 expression in normal ovarian epithelial cells (IOSE80) and human granulosa-like tumor cells (KGN) was measured using qRT-PCR and western blot. KGN cells had higher expression of *HSPA8* mRNA (Figure 1 B) and Hsc70 protein (Figure 1

C) compared to IOSE80 cells, indicating that Hsc70 expression is upregulated in PCOS.

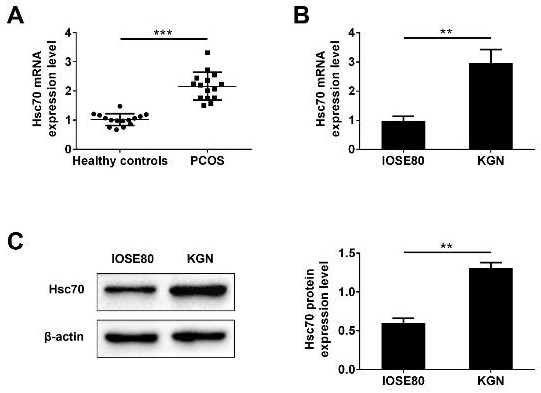

Figure 1: Aberrant high Hsc70 expression in PCOS. (A) *HSPA8* mRNA expression levels in peripheral blood samples from healthy women and patients were measured using qRT-PCR. (B, C) *HSPA8* mRNA and Hsc70 protein expression in normal ovarian epithelial cells (IOSE80) and human granulosa-like tumor cells (KGN) were assessed using qRT-PCR and western blot, respectively. $^{**}P < 0.01$, $^{***}p < 0.001$ versus healthy control or IOSE80 cells

Effect of Hsc70 on KGN cell proliferation

To investigate the effect of Hsc70 on PCOS cells, CCK-8 and colony formation assays were utilized to determine KGN cell proliferation after transfection with Hsc70 overexpression plasmid (Hsc70), Hsc70 shRNA (shHsc70), or their corresponding control vectors. Western blot showed that transfection with the Hsc70 plasmid increased Hsc70 protein expression compared to transfection with the control plasmid, while transfection with shHsc70 significantly decreased Hsc70 levels when compared to transfection with shNC (Figure 2 A). Furthermore, in KGN cells, Hsc70 overexpression enhanced cellular viability, and Hsc70 knockdown reduced cellular viability (Figure 2 B). Additionally, compared with the corresponding controls, Hsc70 overexpression increased and Hsc70 knockdown decreased KGN cell colony formation (Figure 2 C). Therefore, these data indicate that KGN cell proliferation is promoted by Hsc70 overexpression and inhibited by Hsc70 knockdown.

Effect of Hsc70 on KGN cell apoptosis

Hsc70 overexpression suppressed KGN cell apoptosis, whereas Hsc70 knockdown induced cell apoptosis (Figure 3 A). Additionally, Hsc70 overexpression decreased the pro-apoptotic proteins Bax and cleaved caspase-3 and increased the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) (Figure 3 B). Hsc70 knockdown increased the expression of Bax and cleaved caspase-3 and decreased Bcl-2 as compared to shNC (Figure 3 B). Therefore, these data indicate that in KGN cells, Hsc70 overexpression suppresses apoptosis while Hsc70 knockdown promotes apoptosis.

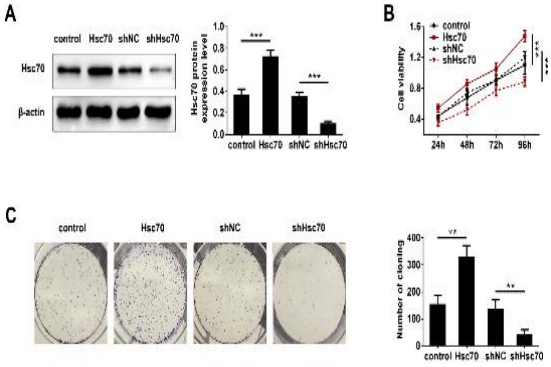

Figure 2: Effect of Hsc70 on KGN cell proliferation. KGN cells were transfected with Hsc70 overexpression plasmid (Hsc70), control plasmid (control), Hsc70 shRNA (shHsc70), or negative control shRNA (shNC), respectively. (A) After the transfection period, Hsc70 protein expression was determined using western blot. (B, C) CCK-8 and colony formation assays were used to measure KGN cell proliferation. $^{**}P < 0.01$, $^{***}p < 0.001$ versus control or shNC

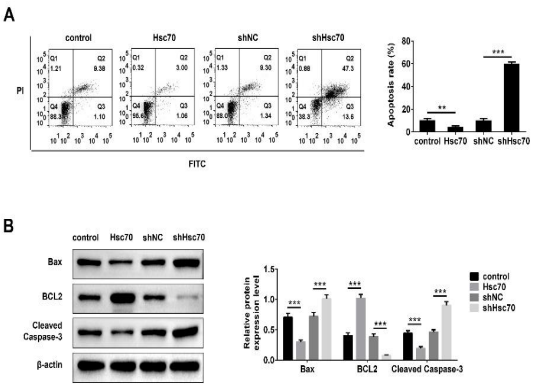

Figure 3: The effect of Hsc70 on KGN cell apoptosis. (A) Flow cytometry was used to measure KGN cell apoptosis after transfection with the indicated plasmids. (B) Western blot was used to determine the protein expression of Bax, Bcl-2, and cleaved caspase-3. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control or shNC

Negative correlation between Hsc70 and KCNA5 expression

KCNA5 mRNA expression in peripheral blood from healthy women and PCOS patients was assessed using qRT-PCR. *KCNA5* mRNA levels were lower in PCOS patients than in healthy women (Figure 4 A). Furthermore, KGN cells had

decreased *KCNA5* mRNA and KCNA5 protein expression compared to IOSE80 cells (Figure 4 B and C). Hence, our results suggest that KCNA5 expression is downregulated in PCOS. Next, to determine KCNA5 expression in KGN cells transfected with Hsc70 plasmid, control plasmid, shHsc70 or shNC, qRT-PCR and western blot were used. Compared to their respective controls, Hsc70 overexpression downregulated levels of *KCNA5* mRNA and KCNA5 protein, and Hsc70 knockdown upregulated KCNA5 expression (Figure 4D and 4E). Altogether, the data indicate that Hsc70 and KCNA5 expression are negatively correlated in PCOS.

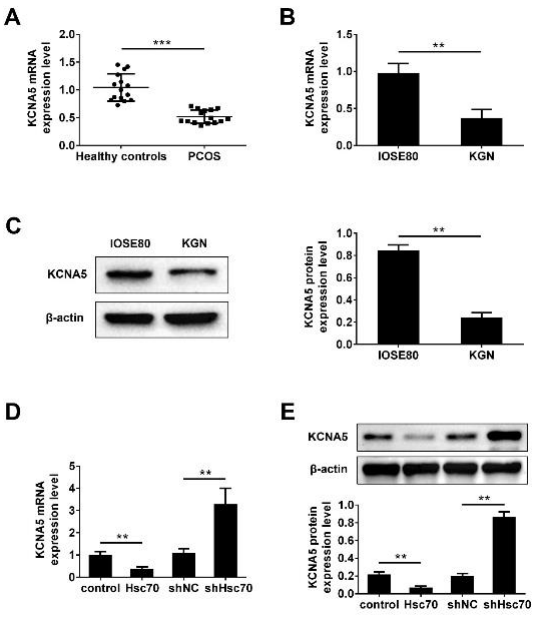

Figure 4: Negative correlation between Hsc70 and KCNA5 expression. (A) *KCNA5* mRNA expression in peripheral blood samples from healthy women and PCOS patients was assessed using qRT-PCR. (B, C) The expression levels of *KCNA5* mRNA and KCNA5 protein in IOSE80 and KGN cells were evaluated using qRT-PCR and western blot. (D, E) After KGN cells were transfected with Hsc70 plasmid, control plasmid, shHsc70, or shNC, *KCNA5* mRNA expression and KCNA5 protein expression were assessed using qRT-PCR and western blot, respectively. $^{**}p < 0.01$, $^{***}p < 0.001$ versus healthy controls, IOSE80, control or shNC

Interaction of Hsc70 with KCNA5 in KGN cells

To further investigate the interplay between Hsc70 and KCNA5 in KGN cells, CCK-8 and colony formation assays were used to assess transfected KGN cell proliferation. KCNA5 knockdown significantly decreased KCNA5 protein expression and weakened the previously observed increase in KCNA5 expression induced by Hsc70 knockdown (Figure 5 A). Furthermore, compared to shNC+siNC, KGN cell viability and colony formation were suppressed by Hsc70 knockdown, but were promoted by knocking down KCNA5 in addition to Hsc70 (Figure 5 B and C). Moreover, the Hsc70 knockdown-mediated inhibition of KGN cell proliferation was attenuated by additionally knocking down KCNA5 (Figure 5 B and C). Therefore, these results suggest that Hsc70 knockdown inhibits KGN cell proliferation by upregulating KCNA5 expression.

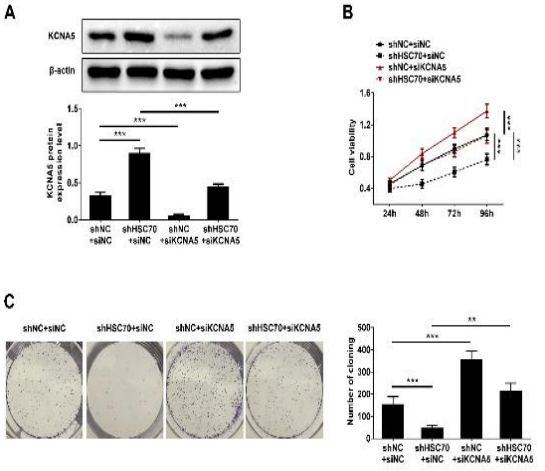

Figure 5: Interplay between Hsc70 and KCNA5 in KGN cells. KGN cells were transfected with shNC+siNC, shHsc70+siNC, shNC+siKCNA5 or shHsc70+siKCNA5. (A) KCNA5 protein expression was assessed using western blot. (B, C) KGN cell proliferation was determined using CCK-8 and colony formation assays, respectively. $^{**}p < 0.01$, $^{***}p < 0.001$ versus shNC+siNC or shHsc70+siNC

DISCUSSION

This study aimed to investigate the function and interactive mechanism of Hsc70 in PCOS. Our results showed that Hsc70 expression was upregulated in PCOS compared to the control, and Hsc70 overexpression contributed to KGN cell proliferation. Furthermore, KCNA5 was found to interact with Hsc70 to mediate KGN cell proliferation.

Hsc70 is a molecular chaperone that is involved in a wide range of biological processes such as protein homeostasis, signal transduction, cell growth, and apoptosis [5,6]. Consequently, Hsc70 plays an important role in many diseases [7]. For instance, Hsc70 protects against lipopolysaccharide-induced hypertrophic responses by reducing the levels of proinflammatory mediators [14]. Furthermore, human glioma cells overexpress Hsc70, which increases cellular proliferation and mobility [13].

Although Hsc70 is especially abundant in ovaries and embryos [15], the effect of Hsc70 on PCOS has yet to be reported.

Our work found that Hsc70 expression was higher in the peripheral blood of PCOS patients than in healthy women. Furthermore, KGN cells had higher Hsc70 expression compared to IOSE80 cells. Moreover, in KGN cells, Hsc70 overexpression promoted cellular proliferation and inhibited apoptosis, whereas Hsc70 knockdown suppressed cellular proliferation and induced apoptosis.

Mounting evidence demonstrates that Hsc70 functions by cooperating with co-chaperones and many other partner molecules [7]. Hsc70 plays an important part in cellular survival during stressful conditions by interacting with co-chaperone Bcl-2 associated athanogene (BAG) family molecular chaperone regulator 1 (BAG-1) [16]. Additionally, the interaction between Hsc70 and CHIP, another co-chaperone of Hsc70, regulates protein folding and degradation [17]. Furthermore, Kim *et al* showed that Hsc70 interacts with CHIP to suppress adipocyte differentiation by degrading peroxisome proliferator-activated receptor γ (PPARγ) [18]. Importantly, Hsc70 interacts with CHIP to facilitate KCNA5 degradation in mammalian cells [8]. KCNA5 is downregulated in PCOS, and KCNA5 upregulation inhibits granulosa cell proliferation in PCOS [10,11]. However, the interplay between Hsc70 and KCNA5 in PCOS has not been reported to date.

This work assessed the interplay between Hsc70 and KCNA5 in KGN cells using several biological experiments. Consistent with previously published studies [10,11], our data indicates that KCNA5 is downregulated in PCOS. Additionally, compared to the controls, Hsc70 overexpression decreased KCNA5 levels, and Hsc70 knockdown increased KCNA5 expression. Therefore, our data suggests that Hsc70 and KCNA5 expression are negatively correlated. Importantly, Li *et al* demonstrated that Hsc70 regulates KCNA5 degradation by complexing with CHIP and KCNA5, and Hsc70 knockdown increases KCNA5 protein expression through decreasing KCNA5 ubiquitination [8].

Additionally, in our study, KCNA5 knockdown promoted KGN cell proliferation and attenuated the inhibition of cell proliferation caused by Hsc70 knockdown. Gao *et al* revealed that the microRNA miR-3940-5p decreases KCNA5 expression to promote granulosa cell proliferation in PCOS [10]. Furthermore, Zhou *et al* showed that KCNA5 overexpression attenuates the promotive effect of miR-3188 on granulosa cell viability in PCOS [11]. Therefore, it is logical that knocking down Hsc70 inhibits KGN cell proliferation by upregulating KCNA5 expression.

CONCLUSION

The findings of this study suggest that Hsc70 overexpression promotes granulosa cell proliferation but inhibits apoptosis in PCOS, whereas Hsc70 promotes KGN cell proliferation by interacting with KCNA5. These findings further define the molecular mechanism behind PCOS and indicate that Hsc70 and KCNA5 are potential biomarkers for diagnosing and treating PCOS.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and that all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lu Deng and Jiaoqin Wang designed the study and supervised the data collection. Lu Deng analyzed the data and interpreted the data. Jiaoqin Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved this manuscript.

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