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Original Article

Effect of rNA interference on Oatp3a1 gene expression on biological characteristics and immune factors of ovarian granulosa cells in rats with PCOS

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Abstract: Polycystic ovary syndrome (PCOS) is a common endocrinal metabolic disease, and its pathogenesis has not yet been thoroughly studied. The purpose of this experiment was to investigate the effect of RNA interference on Oatp3a1 gene expression on the biological viability and immune factors of ovarian granulosa cells in rats with PCOS. First, rats were intragastrically administered 1 mg/kg letrozole to successfully construct PCOS model. Western blot, qRT-PCR, CCK8 and flow cytometry were used to detect the gene expression, immune factor protein expression, cell proliferation and apoptosis in ovarian granulosa cells transfected with siRNA Oatp3a1 in rats with PCOS, respectively. The results showed that follicle stimulating hormone receptor (FSHR) was located on the cell membrane of rat ovarian granulosa cells, and letrozole successfully induced PCOS rat model. In PCOS rat ovarian granulosa cells, the mRNA expression level of Oapta1 was higher than that in normal rat ovarian granulosa cells. At the same time, compared with the sham group, the protein expression of NF-κB, TGF-β1 and VEGF in si-Oatp3a1 group was significantly down-regulated ($P < 0.05$), and the cell proliferation rate was significantly decreased in si-Oatp3a1 group ($P < 0.05$) in comparison with the sham group. The apoptotic rate was increased obviously ($P < 0.05$), which was about 2.5 times that of the sham group. This indicates that in the ovarian granulosa cells of rats with PCOS, the interference of Oatp3a1 gene expression can significantly inhibit cell proliferation and promote apoptosis, while inhibiting the expression of immune factors TGF-β1 and VEGF can reduce the expression of NF-κB protein, thereby suppressing the activation of the NF-κB signaling pathway.

Keywords: Oatp3a1, polycystic ovary syndrome, ovarian granulosa cells in rats, NF-κB

Introduction

Polycystic ovary syndrome (PCOS) is considered to be the most frequent female endocrine disease and is closely related to heredity and environment [1, 2]. This problem affects 5-20% of women of reproductive age worldwide [3]. The disease was first defined by Stein and Leventhal in 1935 and is also known as Stein-Leventhal syndrome [4]. Excessive androgen, abnormal ovarian morphology, atypical gonadotropin secretion, hyperinsulinemia, peripheral insulin resistance and irregular anovulatory hemorrhage are the main features of this disease [5]. Although many scientists have invested a great deal of research into PCOS, the pathogenesis has not been clearly studied. Moreover, the incidence has increased over the past decade, and the adverse effects on women's health have become more and more serious [6, 7]. Therefore, the pathogenesis, prevention and treatment of PCOS still require further research.

The organic anion transit peptide (OATP) belongs to the solute carrier superfamily. It is a membrane transport protein widely distributed in gastrointestinal, liver, kidney, blood-brain barrier, etc., and mediates the transcellular transport of various internal and external substances [8]. OATP can be divided into six families according to sequence similarity, in which human Oatp3a1 is a 710 amino acid protein and has 97% sequence homology with the rat Oatp3a1 gene [9-11]. Several studies had shown that Oatp3a1 is highly expressed as a carcinogenic factor in liver cancer, breast cancer and pancreatic cancer, which promotes

cell migration [12-14]. As for PCOS, Plaza-Parrochia F et al. detected the protein expression of Oatp3a1, and found that the protein expression of Oatp3a1 was increased in endometria from women with PCOS [15]. However, the expression of Oatp3a1 and its function have rarely been reported on the biological characteristics of rat ovarian granulosa cells. Therefore, we selected the Oatp3a1 gene as our interested gene in PCOS.

In this study, we used letrozole to construct rat PCOS model. Western blot, CCK8 and flow cytometry were further used to study the effects of Oatp3a1 gene expression on immune factors, cell proliferation, and apoptosis of PCOS rats ovarian granulosa cells. These provide a new idea for the diagnosis and treatment of PCOS.

Materials and method

Experimental materials

The experimental animals in this study were selected from female Sprague-Dawley (SD) rats weighing 50 g-60 g and aged 30 days, provided by Guangdong Medical Animal Center. The experiment was approved by the Experimental Animal Ethics Committee of Sun Yat-sen Memory Hospital of Sun Yat-sen University. 6-well, 12-well, 24-well, 48-well and 96-well cell culture plates were provided by Corning, USA. DMEM-high glucose medium, fetal bovine serum, penicillin-streptomycin solution, and PBS potassium phosphate buffer were purchased from Hyclone, USA.

Construction of PCOS model and isolation and culture of primary rat ovarian granulosa cells

The SD rats were randomly divided to control, normal and PCOS groups as 5 rats per group. The rats were gavaged with letrozole at 1 mg/kg once a day for 21 days to construct the PCOS model accroding to the previous study [16]. The rat in the control group were intragastrically fed with 1% carboxymethyl cellulose (CMC) by gavage once a day for continuous gavage for 21 days. The rat in the normal group were fed as normal. Weigh and record the rats, and then take the serum of rats in the PCOS group, control group and normal group to detect the level of sex hormones: testosterone (T), luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E2) and progesterone (P). Then rats were injected with pentobarbital sodium for euthanasia, and then the ovaries of each group were taken to measure the ovarian volume.

Then bisect the ovaries of each group of rats, puncture the follicles with a needle, gently blow several times to disperse the cells, filter and centrifuge, isolate PCOS rat ovarian granulosa cells (PCOS-GC), and carry out primary culture. Normal ovarian granulosa cells (normal-GC) were isolated from the normal group of rats and subjected to primary culture. The cells were filtered through a 200-mesh nylon mesh, and centrifuged at 800 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in fresh DMEM (high glucose) medium. The cells were cultured in an incubator at 37°C under 5% CO_2 using DMEM high glucose medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution.

Identification of rat ovarian granulosa cells by follicle stimulating hormone receptor (FSHR) immunohistochemical staining

The cell climbing slices were cut to the appropriate size, soaked in concentrated sulfuric acid for overnight, then rinsed with tap water for 5 times and sterilized for use. The cell slices were placed in a 24-well culture plate under sterile condition, and the cell suspension was added for culture. When the cells were grown to a confluency of 70%, the slices were taken out, fixed with 4% paraformaldehyde for 20 min, washed three times with PBS, added with 0.3% H_2O_2, and incubated at room temperature for 30 min to remove endogenous peroxide. The slices were washed 3 times with PBS for 5 min each time and 0.2% Triton X-100 was added for 5 min. Next, after blocking with 10% goat serum for 30 min, the cells were incubated with the primary antibody against anti-FSHR (N-20) (sc-7798, Santa Cruz, USA) overnight at 4°C. After washing three times with PBS, the secondary anti-HRP-labeled IgG was added to the cell slices and incubated at room temperature for 1 hour in the dark. The cell slices were washed with PBS 3 times with 5 minutes each time, and hematoxylin was added for staining for 10 min. 50%, 75%, 85%, 95% and 100% ethanol were used for gradient dehydration for 1 time respectively, and dimethylbenzene for vitrification for 2 times with 10 minutes each time, Finally, the

cell slices were sealed with neutral gum, examined with fluorescence microscope and photographed (Olympus, Japan) at 200 ×.

Cell transfection

PCOS rat ovarian granulosa cells were divided into sham group, si-NC group and si-Oatp3a1 group. siRNA Oatp3a1 and siRNA NC were designed and supplied by Guangzhou MssBio Co., Ltd. (China). The primers sequences of siRNA1 were F: 5'-UUCACGCCAGUGUGCGGGGCCGdTdT-3' and R: 5'-AAGUGCGGUCACACGCCCCGGCTdTd-3'. The primers sequences of siRNA2 were F: 5'-CUGCUACGUCUCCUUCCUCUUCdTdT-3' and R: 5'-GACGAUGCAGAGGAAGGAGAAGTdTd-3'. Rat ovarian granulosa cells were transfected with non-interfering siRNA (si-NC group) and siRNA inhibiting Oatp3a1 expression (si-Oatp3a1 group), respectively. Rat ovarian granulosa cells were cultured without any transfection as sham group. 24 h before the transfection, the cells grown in the logarithmic phase were seeded in a 6-well culture plate at a seeding density of 2×10^5 cells/well, and transfected in strict accordance with the instructions of lipofectamine 2000 (Invitrogen, USA).

RT-qPCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). AMV Reverse Transcriptase (Promega, USA) was used for cDNA synthesis. 1 μg of total RNA and 0.5 μL oligo dT, 0.5 μL primers were added to the reaction system. The primers sequences of Oatp3a1 were F: 5'-TCGTTTGTTGGGTTTCATCC-3' and R: 5'-GTCTAGGGTCAGAGTAGAGG-3'. The primers sequences of β-actin were F: 5'-AGGGAAATCGTGCGTGACAT-3' and R: 5'-GAACCGCTCATTGCCGATAG-3'. qRT-PCR was performed using SYBR Green qPCR SuperMix (Invitrogen, USA). The amplification reaction system included 40 thermo cycles of 5 min at 95°C, 15 s at 95°C, and 32 s at 60°C. The expression levels were calculated by the $2^{-\Delta\Delta CT}$ method.

CCK-8 experiment

The cells were seeded in a 96-well plate at a density of 1×10^5/ml, and transfected after the cells were attached. After 0, 1, 2, and 3 days of cell transfection, 10 μl of CCK-8 solution (Beyotime, China) was added, and after 4 hours of incubation, the absorbance of each sample was read by a microplate reader (Thermo Fisher Scientific, USA) at a wavelength of 450 nm.

Cell apoptosis assay

Flow cytometry was performed to assess the ability of cell apoptosis. After the transfected cells were discarded, the medium was added to a PBS solution, and then trypsin containing no EDTA was added. After the cells were detached, the cells were resuspended by adding 0.5 ml of the medium, transferred to a 15 ml conical tube and placed on ice. Next, 1.25 μl of Annexin V-FITC and 10 μl of propidium iodide were added to each conical tube for 15 min in the dark, and immediately detected by flow cytometry (BD, USA).

Western blot

RIPA extraction buffer was added to the transfected cells, and the cells were lysed by shaking for 15 min, centrifuged at 14,000 rpm for 15 min. The supernatant was collected, and the protein concentration was determined by BCA. 2 volumes of gel loading buffer were added and boiled for 8 min to denature the protein. 20 ug of protein was subjected to SDS-PAGE electrophoresis and subsequently transferred to a PVDF (Millipore Co., USA) membrane. The PVDF membrane was fixed with TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20) containing 5% skim milk for 2 h at room temperature. After fixation, the PVDF membrane was washed three times with TBST, and then incubated overnight with antibody GAPDH (Abeam, UK), Oatp3a1 (Abeam, UK), VEGF (Abeam, UK), TGF-β1 (Abeam, UK) and NF-κB (Abcam, UK) at 4°C, respectively. Subsequently, after washing the membrane with TBST, the PVDF membrane was incubated at room temperature for 2 h in a TBST solution containing the secondary antibody corresponding to the primary antibody. The ECL reagent was used for fluorescent color development, and the gel image processing system was used to detect and analyze the molecular weight of the strip and the gray value of the strip.

Statistical analysis

All results in this study were expressed in mean ± SD using SPSS 22.0 analysis, and all experiments were repeated in three independent experiments. Differences between groups were analyzed using t-test or one-way analysis of

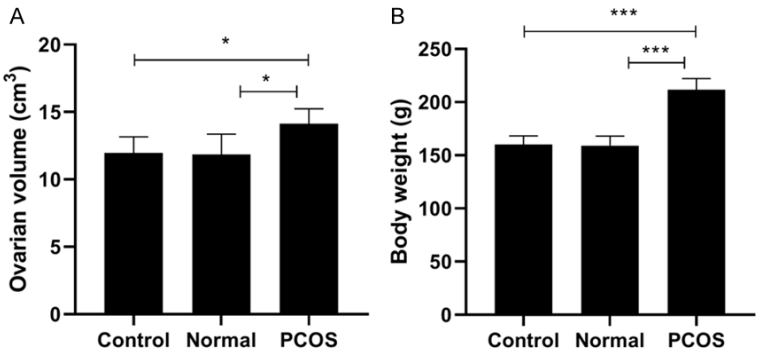

Figure 1. Ovarian volume and body weight of rats in PCOS group, control group and normal group. A. Ovarian volume of rats in PCOS group, control group and normal group. B. Body weight of rats in PCOS group, control group and normal group. *P < 0.05, ***P < 0.001.

variance. P < 0.05 is considered to have significant statistical differences.

Result

The effects of PCOS on ovarian volume, body weight and sex hormones

We used letrozole to construct the rat PCOS model. The rat ovarian volume in PCOS group increased by 1.2-fold compared with control group (**Figure 1A**), as well as the rat weight in PCOS group also increased by 1.3-fold compared with control group (**Figure 1B**). Then, we also detected the levels of sex hormones (P, T, E2, FSH, and LH) in rat serum. The results displayed that the levels of P, E2 and FSH were significantly decreased in PCOS group, while the levels of T and LH were significantly increased in PCOS group (**Figure 2A-E**). These data proved that PCOS could increase the ovarian volume and body weight of rat, and led to the imbalance of sex hormones in rat serum.

Purity identification of primary rat ovarian granulosa cells

Immunofluorescence detection results showed that FSHR showed red coloration on the cell membrane and not coloration in the nucleus (**Figure 3A**). It is proved that the isolated primary rat ovary granule cells have high purity and can be used in the following experiments. In addition, compared with the ovarian granulosa cells in the normal-GC group, the nucleus of the ovarian granulosa cells in the PCOS-GC group was large and round, the number of cells increased and the cells grew together (**Figure 3B**). Therefore, the PCOS rat model was successfully constructed.

MRNA expression of Oatp3a1 in granulosa ovarian cells of PCOS rats

RT-qPCR was used to detect the mRNA expression of Oatp3a1 in ovarian granulosa cells of PCOS rats. The results showed that compared with normal rat ovarian granulosa cells, the mRNA level of Oatp3a1 in PCOS rat ovarian granulosa cells was up-regulated (P < 0.001) (**Figure 4A**). The results showed that the gene expression of Oatp3a1 was lower in si-RNA1 transfected PCOS rat ovarian granulosa cells (**Figure 4B**). Therefore, siRNA-1 was chosen in the following experiments. Then, we detected the transfection efficiency of si-Oatp3a1 in rat ovarian granulosa cells. The results showed that the si-Oatp3a1 let to more than 70% decrease of Oatp3a1 expression in rat ovarian granulosa cells (**Figure 4C**). These data proved that si-Oatp3a1 could significant reduce the Oatp3a1 expression so that it could be used to the following experiments.

Effect of si-Oatp3a1 on proliferation of rat ovarian granulosa cells

Rat ovarian granulosa cells were transfected with si-Oatp3a1, and CCK-8 was used to detect the effect of interfering Oatp3a1 gene expression on cell proliferation. The results showed that the OD value was significantly lower than that of the control group at 2 and 3 days after transfection of si-Oatp3a1 (P < 0.05) (**Figure 5A**), and the cell proliferation rate of the si-Oatp3a1 group was calculated to be 131.09% and 182.7%, respectively, both were lower than the control group (P < 0.05) (**Figure 5B**). However, no significant difference was observed between sham group and si-NC group. This indicated that down-regulation of Oatp3a1 gene expression inhibited proliferation of rat ovarian granulosa cells.

Effect of Oatp3a1 gene expression interference on apoptosis of rat ovarian granulosa cells

In this experiment, the apoptosis of rat ovarian granulosa cells transfected with si-Oatp3a1 was detected by flow cytometry. As shown in

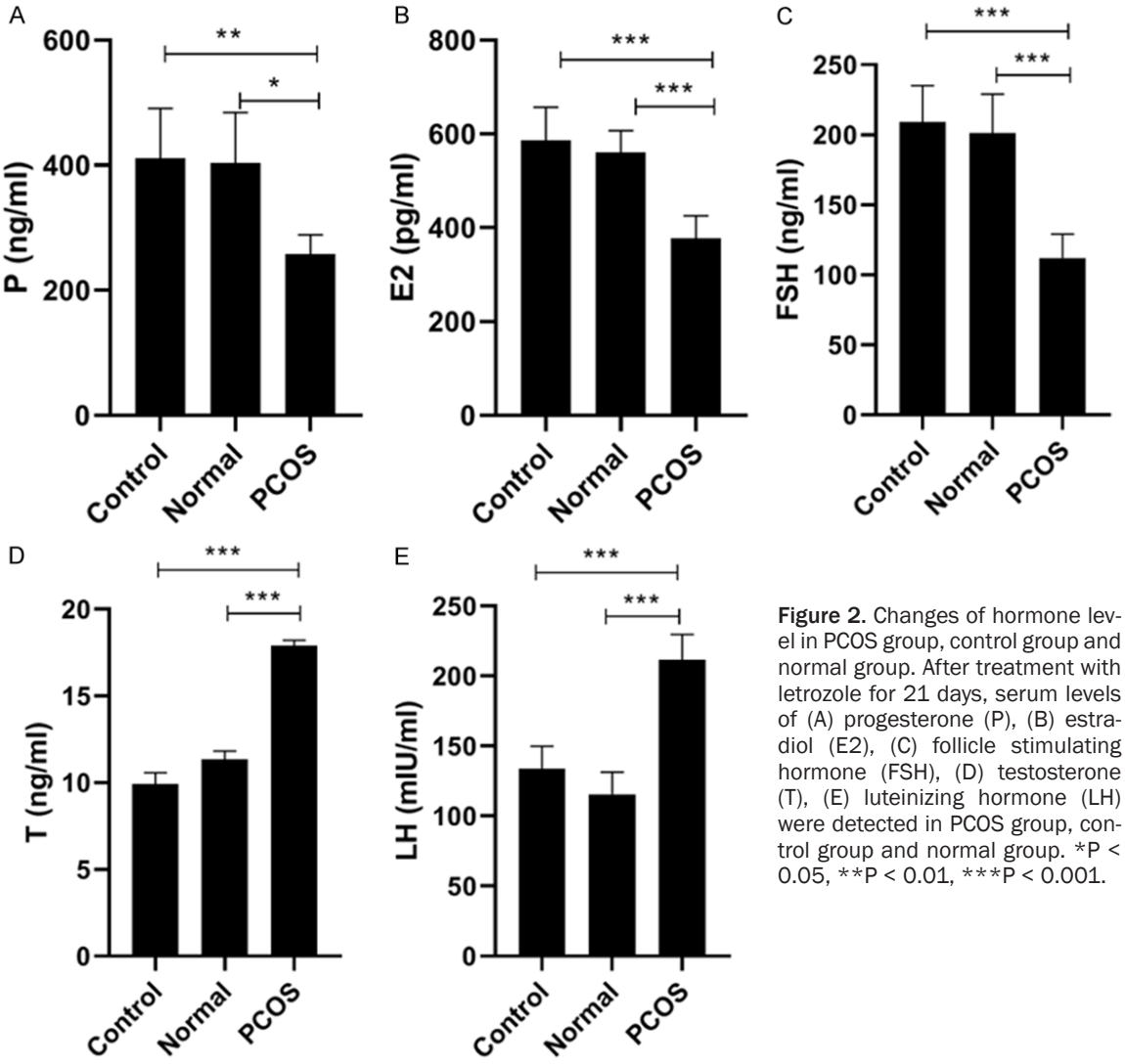

Figure 2. Changes of hormone level in PCOS group, control group and normal group. After treatment with letrozole for 21 days, serum levels of (A) progesterone (P), (B) estradiol (E2), (C) follicle stimulating hormone (FSH), (D) testosterone (T), (E) luteinizing hormone (LH) were detected in PCOS group, control group and normal group. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6, the apoptotic rate of the si-Oatp3a1 group was significantly increased ($P < 0.05$), which was 2.5 times that of the control group. The above experimental results indicate that inhibition of Oatp3a1 expression can promote apoptosis of rat ovarian granulosa cells.

Protein expression levels of NF-κB, VEGF and TGF-β1 in rat ovarian granulosa cells in each group

The effects of ovarian granulosa cells transfected with si-Oatp3a1 on the expression of NF-κB, VEGF and TGF-β1 protein were detected by Western blot. As shown in **Figure 7**, interference with Oatp3a1 gene expression significantly reduced NF-κB, VEGF, and TGF-β1 ($P < 0.05$) protein expression, while there was no significant difference between the sham group compared with the si-NC group. Western blot results indicated that decreasing the expression of si-Oatp3a1 inhibited the expression of immune factors VEGF and TGF-β1, which may be related to the decreased expression of NF-κB protein.

Discussion

PCOS threatens the health of women around the world, and the study of its pathogenesis can provide a new direction for the diagnosis and treatment of this disease. In this paper, the ovarian granulosa cell model of PCOS rats was successfully constructed by letrozole, and it was found that PCOS could increase the ovarian volume and body weight of rat, and led to the imbalance of sex hormones level in rat

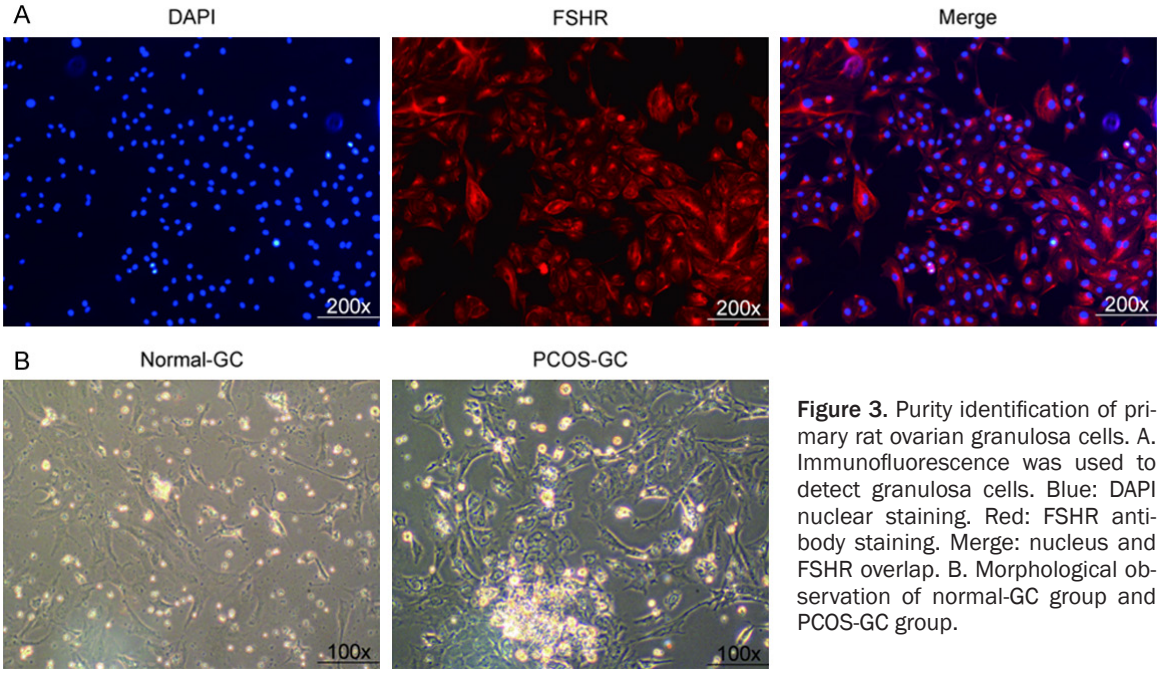

Figure 3. Purity identification of primary rat ovarian granulosa cells. A. Immunofluorescence was used to detect granulosa cells. Blue: DAPI nuclear staining. Red: FSHR antibody staining. Merge: nucleus and FSHR overlap. B. Morphological observation of normal-GC group and PCOS-GC group.

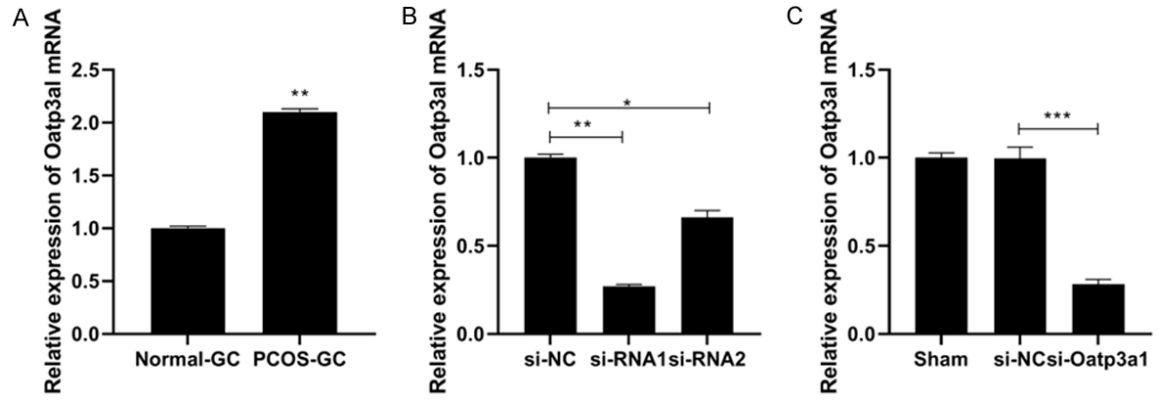

Figure 4. Oatp3a1 mRNA levels are up-regulated in PCOS rat ovarian granulosa cells. A. RT-qPCR was used to detect the mRNA expression of Oatp3a1 in normal-GC group and PCOS-GC group. B. Validation of the transfection efficiency of Oatp3a1 siRNA. C. The transfection efficiency of si-Oatp3a was detected by RT-qPCR. *P < 0.05, **P < 0.01, ***P < 0.001.

serum. Meanwhile, the expression of Oatp3a1 was up-regulated in the granulosa cells of PCOS rats. Therefore, by using siRNA to reduce the expression of Oatp3a1 gene in PCOS rat granulosa cells. The low expression of Oatp3a1 gene in PCOS rat ovarian granulosa cells can effectively inhibit the expression of TGF-β1 and VEGF protein, reduce the proliferation ability of PCOS rat ovarian granulosa cells, and promote the proliferation of PCOS rat ovarian granulosa cells. It also reduced the expression of NF-κB protein and inhibited the activation of signal pathway.

PCOS is a common reproductive endocrine disease in women of childbearing age. Stewart et al. studied 465 PCOS families and found that PCOS is related to insulin receptor [17]. Azziz et al. detected a significant increase in sex hormone levels in the blood of 80%-90% of PCOS patients [18]. At the same time, Fetemeh et al. successfully induced PCOS model to collect blastocysts by stimulating follicular development with Letrozole [19]. Therefore, we used letrozole subcutaneously injected into rats to construct PCOS model, so as to separate the ovarian granulosa cells of PCOS rats for the

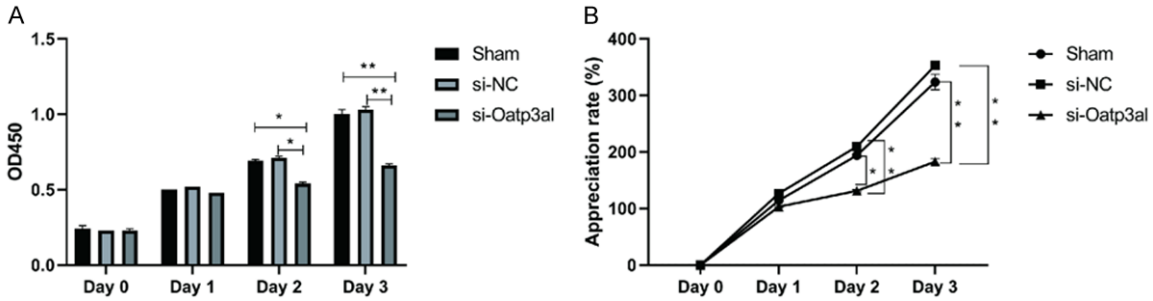

Figure 5. si-Oatp3a1 inhibits proliferation of rat ovarian granulosa cells. A. Rat ovarian granulosa cells were transfected with Oatp3a1 interfering RNA, and the OD values from 0 to 3 days of cells in each group were detected by CCK-8. B. The appreciation rate was calculated from the OD values of ovarian granulosa cells in each group from 0 to 3 days. The data represents mean ± SD and each set of experiments was repeated at least three times. *P < 0.05, **P < 0.01.

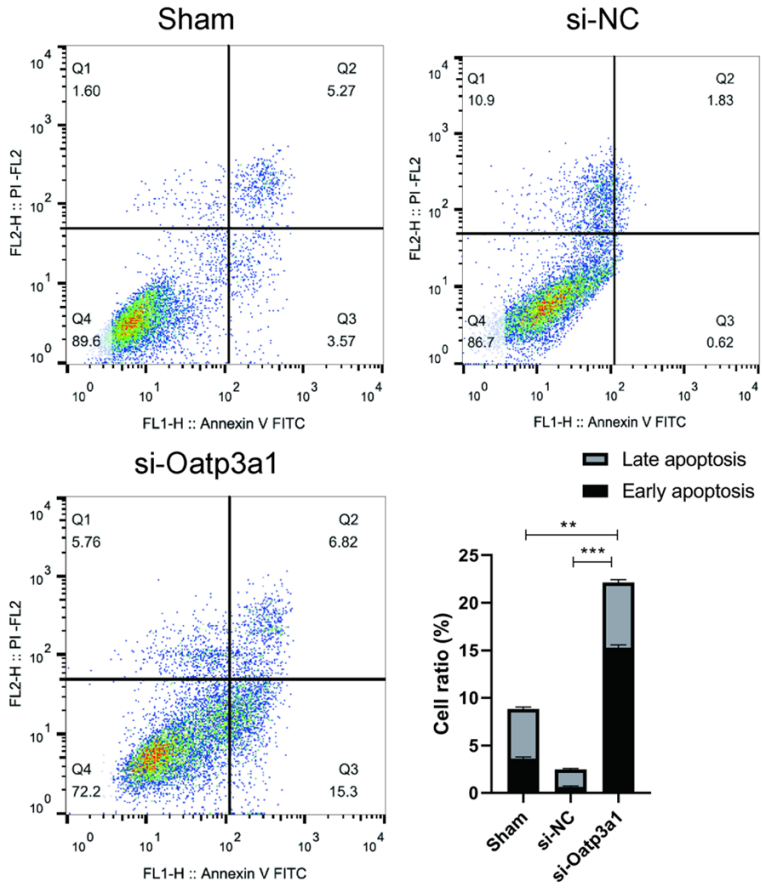

Figure 6. si-Oatp3a1 promotes the cell apoptosis of rat ovarian granulosa cells. The data represents mean ± SD and each set of experiments was repeated at least three times. **P < 0.01, ***P < 0.001.

subsequent test of cell viability. Follicle stimulating hormone receptor (FSHR) is a kind of transmembrane glycoprotein, which is expressed in ovarian granulosa cells of adult animals [20], so it can be used as a marker protein of ovarian granulosa cells. We used immunohistochemistry and immunofluorescence to detect the purity of FSHR protein. As a result, more than 98% of the cells were stained and FSHR was located on the cell membrane, which indicated that the purity of the primary rat ovarian granulosa cells was high.

Organic anion transporters (OATPs), an important solute carrier superfamily, mediate the transport of a variety of endogenous and exogenous biological compounds, such as bile acids, prostaglandins, cyclic nucleotides, steroid hormones, etc. [21-25]. At present, the exploration of the function and role of Oatp3a1 are more common in cancer research. For example, Liedauer et al. [26] detected 21 human bone tumor samples by RT-PCR and found that the mRNA level of Oatp3a1 was significantly increased. In breast cancer, Kindla J et al. [27] found that Oatp3a1 localizes to the plasma membrane of epithelial cells of the mammary duct and up-regulates expression. However, there was little research about Oatp3a1 in PCOS. Only Plazaparrochia et al. [15] detected the expression of Oatp3a1 in female endo-

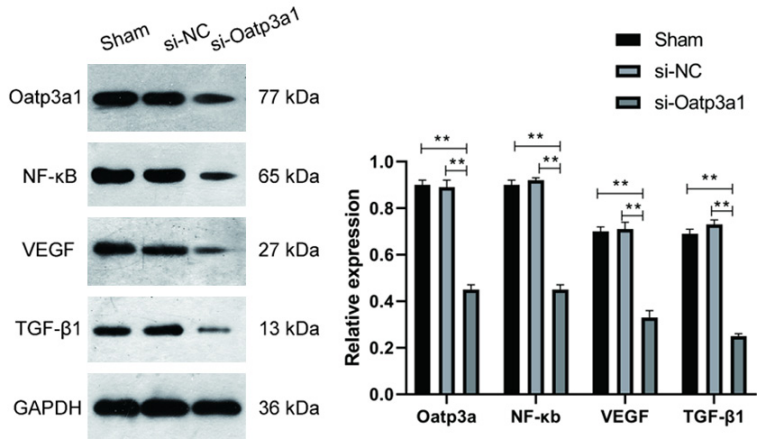

Figure 7. Effects of Oatp3a1 on the expression of NF-κB, VEGF and TGF-β1. Western blot was used to detect the protein expression of NF-κB, VEGF and TGF-β1 in rat ovarian granulosa cells transfected with Oatp3a1 siRNA (si-Oatp3a1), siRNA NC (si-NC). The protein expression level is calculated as the gray value of each protein band, and the data represents mean ± SD. Each set of experiments was repeated at least three times. **P < 0.01.

metrium of PCOS by RT-PCR and western blot. The results showed that the expression of Oatp3a1 in female endometrium of PCOS increased, but there was no statistical difference (P > 0.05). Das et al. [28] measured the expression of Ki-67 (the cell proliferation marker) in the granulosa cells from PCOS patients, and found Ki-67 was higher in the PCOS group. Meanwhile, they also found the apoptosis rate was raised in PCOS group by Cell apoptosis assay. This study investigated the effect of interference with Oatp3a1 gene expression on ovarian granulosa cell viability in PCOS rats. It was found that interference with Oatp3a1 gene expression significantly inhibited the proliferation of ovarian granulosa cells and promoted apoptosis in PCOS rats.

Vascular endothelial growth factor (VEGF) is a regulate ovarian steroidogenesis whose expression in follicular fluid increases with the development of follicles [29, 30]. VEGF with high level in PCOS was associated with increased ovarian stromal blood flow [31], which might be the reason of the loss of intraovarian autoregulatory mechanism seen in PCOS patients. Transforming growth factor-β1 (TGF-β1) plays an important role in follicular development and granulosa cell growth, and its expression in PCOS is closely related to the expression of NF-κB [32, 33]. In the serum of patients with PCOS, elevated TGF-β1 leads to increased consumption of TSP-1 and elevated levels of NF-κB [34]. PCOS patients are usually associated with cryptorrhea and inflammation [35, 36], because NF-κB acts as an important inflammatory signaling pathway that promotes endometrial inflammation, limiting the chances of pregnancy [37]. Therefore, we examined the expression of the immune factors TGF-β1, VEGF and NF-κB after interference with Oatp3a1 gene expression in PCOS rat ovarian granulosa cells. The results showed that the expression of the immune factors TGF-β1 and VEGF protein was significantly decreased in the ovarian granulosa cells of PCOS rats after interfering with the expression of Oatp3a1 gene. The decrease of VEGF could reduce the ovarian stromal blood flow, which played a negative role in PCOS. At the same time, the expression of NF-κB protein was down-regulated after interference with Oatp3a1 gene expression. This indicated that the interference of Oatp3a1 gene expression inhibited TGF-β1, thereby inhibiting the expression of NF-κB, which may help to alleviate the inflammatory response in patients with PCOS.

Conclusion

This paper demonstrates for the first time that in the ovarian granulosa cells of PCOS rats, the interference of Oatp3a1 gene expression inhibits the proliferation of ovarian granulosa cells in PCOS rats and promotes apoptosis. In addition, our results also indicate that interference with Oatp3a1 gene expression can inhibit the expression of immune factors TGF-β1 and VEGF, which may be related to the decreased expression of NF-κB protein and provide new ideas and drug targets for the treatment and diagnosis of PCOS.

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Disclosure of conflict of interest

None.

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