MiR-17-5P regulates autophagy of ovarian granulosa cells in patients with polycystic ovary syndrome via targeting ATG7

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Abstract

Background: Abnormal activation of autophagy is considered to be an important mechanism of polycystic ovary syndrome (PCOS). In our earlier study, we found that the expression of autophagy-related protein 7 (ATG7) was increased in ovarian granulosa cells (GCs) of patients with PCOS. The purpose of this study was to investigate the effect of ATG7 on autophagy of ovarian GCs and further enrich the pathogenesis of PCOS.

Methods: The associated microRNA of ATG7 was predicted by bioinformatics software, and the targeting relationship between them was verified by dual-luciferase reporter assay. In addition, the expressions of miR-17-5P and ATG7 in ovarian GCs were measured by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis (WB).

Results: It was found that miR-17-5P was a microRNA associated with ATG7. MiR-17-5P could directly bind to the 3′untranslated region of ATG7 and inhibit its expression. At mRNA level, the expression of miR-17-5P in GCs of PCOS women was significantly lower than that of normal women (P<0.01). At both mRNA and protein levels, the expression of ATG7 in GCs of PCOS women was significantly higher than that of normal women (P<0.01).

Conclusion: Downregulation of miR-17-5P expression promotes autophagy of ovarian GCs by upregulating ATG7 expression, thus serving an important role in PCOS pathogenesis.

Plain Language Summary

Polycystic ovary syndrome (PCOS) is a common disease caused by endocrine and metabolic disorders in women of childbearing age. Autophagy is the process of self-phagocytosis of cells, which realizes the metabolic needs of cells and the renewal of some organelles by degrading their own cytoplasmic proteins or organelles. Recent studies have found that autophagy plays an important role in the pathogenesis of PCOS. In our earlier study, we found that the expression of autophagy-related protein 7 (ATG7) was increased in ovarian granulosa cells of PCOS patients. This study was designed to investigate the effect of ATG7 on autophagy of ovarian granulosa cells and further enrich the pathogenesis of PCOS. In this study, we predicted the associated microRNAs of ATG7 using bioinformatics software. MiR-17-5P closely related to autophagy was selected. And the targeted regulatory relationship between miR-17-5P and ATG7 was verified by dual-luciferase reporter assay. Collected the ovarian GCs from 38 PCOS patients and 38 normal women (as control) on the day of oocytes retrieval, and detected the expression of miR-17-5P and ATG7 in ovarian granulosa cells by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis. The results showed that miR-17-5P was a microRNA associated with ATG7. MiR-17-5P could directly bind to the 3′untranslated region of ATG7 and inhibit its expression. Compared with normal women, the expression of miR-17-5P was down-regulated and ATG7 was up-regulated in PCOS patients. Findings of this study showed that downregulation of miR-17-5P expression promotes

autophagy of ovarian granulosa cells by upregulating ATG7 expression, thus serving an important role in PCOS pathogenesis.

Background

Polycystic ovary syndrome (PCOS) is one of the common causes of female infertility, with a prevalence of 6–20% among women of childbearing age worldwide [1]. The main clinical manifestations are ovulation disorder, hyperandrogenemia, ovarian polycystic changes, and so on. PCOS associated infertility, menstrual disorders, endocrine and metabolic abnormalities and other problems bring great psychological burden to patients. At present, the pathogenesis of PCOS has not been fully clarified, and the treatment of PCOS mainly focuses on adjusting lifestyle and ovulation induction. With the progress of assisted reproductive technology, pregnancy opportunities of PCOS patients have been significantly improved, but its expensive cost has also increased the economic burden of low-income families. It is of great clinical significance to explore the pathogenesis of PCOS, search cost-effective treatment methods, and finally improve the pregnancy outcome.

Autophagy, that is, the "self phagocytosis" of cells, plays an important role in maintaining the stability of the intracellular environment by decomposing unnecessary or dysfunctional cellular components. Autophagy is a double-edged sword. On the one hand, it improves the adaptability of cells to adverse environments and plays an important role in resisting pressure, energy regulation, immune regulation, reproductive regulation, cell differentiation, proliferation and death, and maintaining cell homeostasis [2–5]; on the other hand, it also promotes cell death and disease. Multiple studies have found that autophagy is an important pathogenesis of PCOS. Autophagy defects exist in GCs of follicles at different developmental stages in PCOS patients [6, 7]. Autophagy can not only affect the recruitment, development, and discharge of follicles but also participate in the occurrence of endocrine and metabolic disorders. Therefore, exploring the mechanism of autophagy in the pathogenesis of PCOS will assist to alleviate the clinical symptoms of PCOS patients and improve the pregnancy outcome through the intervention of autophagy. Our previous study found that the expression of ATG7 was up-regulated in GCs of PCOS patients. However, the specific mechanism of ATG7 involved in PCOS is still unclear. In this study, we found microRNAs that regulated the expression of ATG7 and explored whether they were involved in the pathogenesis of PCOS by regulating the autophagy of GCs.

Methods

Research subjects

The selected cases came from the patients who received in vitro fertilization / intracytoplasmic sperm injection(IVF / ICSI) in the Reproductive and Genetic Center in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine from January 2020 to May 2021. 38 patients who met the diagnostic criteria of PCOS and 38 patients who underwent assisted reproduction due to male factors were selected. All patients were fully informed of the purpose and process of the study, voluntarily

participated in the study, and signed written informed consent. This study was approved by the ethics committee of the Reproductive and Genetic Center in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (ethical approval No. SDSZYYSZ20191220). The diagnostic criteria of PCOS were formulated according to the 2003 Rotterdam diagnostic criteria[8]: ① Rare ovulation and/or anovulation; ② Having clinical manifestations of hyperandrogenism and/or hyperandrogenemia, and excluding other diseases that might lead to elevated androgen; ③ Ultrasound showed that there were 12 or more follicles with a diameter of 2 ~ 9mm on the same section of one or both ovaries, and/or the ovarian volume was more than 10ml. PCOS could be diagnosed if two of the above three items were met.

Inclusion criteria: (1) Patients who met the diagnostic criteria of PCOS; (2) Patients who needed assisted reproductive technology to get pregnant; (3) Patients in the normal group needed to choose patients who were infertile simply because of malefactors. And the woman had regular menstruation and normal endocrine and ovulation functions; (4) Those who did not participate in other scientific research projects or took other drugs during the same period.

Exclusion criteria: (1) Patients who had taken hormone drugs 3 months before enrollment; (2) Patients suffering from serious cardiovascular and cerebrovascular diseases, blood diseases, infectious diseases, mental diseases, and sexually transmitted diseases; (3) Patients suffering from endocrine dysfunction diseases related to thyroid, adrenal gland, and pituitary gland; (4) Patients with endometrial polyps, uterine fibroids and other reproductive system organic lesions; (5) Chromosome abnormality.

Controlled Ovarian Stimulation Protocol

All patients included in the study were treated with GnRH antagonist (GnRH-ant) regimen for controlled ovarian hyperstimulation. basal follicle-stimulating hormone (bFSH), basal luteinizing hormone (bLH), basal estradiol (bE_2), basal progesterone (bP_4), and transvaginal ultrasound were measured on the third day of menstruation or the third day after withdrawal bleeding to evaluate the basic state of ovary. Those who could enter the cycle started the controlled ovarian hyperstimulation program with gonadotropin (Gn) on the same day. The types of Gn included human recombinant folicle-stimulating hormone (rFSH, gonalf), human recombinant luteinizing hormone (rLH, Luveris, Merck Serono), human menopausal gondotropin (HMG). The type and dose of Gn were determined according to the level of basic endocrine hormone and the number of basal sinus follicles. Closely monitored the changes in blood hormone levels of patients. Transvaginal ultrasound was taken to monitor the development of follicles. When the diameter of dominant follicles reached 12mm and the level of serum E_2 exceeded 300pg/ml, GnRH antagonist (cetrotide 0.25 mg, Merck Serono) 0.25mg/day would be injected until the trigger day. When at least three follicles had a diameter greater than or equal to 17 mm or two follicles had a diameter greater than or equal to 18 mm, human chorionic gondotrop (hCG) 4000 ~ 10000 IU or recombinant human chorionic gondotrophin (rhCG, Ovidrel) ™) 250ug were given to induce follicular maturation. The dosage of these hormones was determined based on the number of follicles and the serum hormone levels. Transvaginal oocytes retrieval was performed under ultrasound guidance at 36 hours after ovulation

triggering. The follicular fluid of mature follicles with a diameter of 18 ~ 20 mm was collected after oocytes collection.

Extraction of granulosa cells

The collected follicular fluid was centrifuged at 2000 rpm for 5 min, and the waste liquid was discarded and mixed with PBS. Added an appropriate amount of lymphocyte separation solution into a 15ml centrifuge tube, slowly added the mixed solution to the upper layer of lymphocyte separation solution, and centrifuged at 1500rpm for 20 min. After centrifugation, the mixture was divided into three layers, and the middle layer was flocculent white GCs. Carefully absorbed the GCs, transferred them to the centrifugal tube containing PBS, mixed them evenly, and centrifuged at 2000rpm for 5 min. Discarded the supernatant and washed it again. Slowly discarded the supernatant and transferred the sediment to the EP tube. Filled in the record, marked the specimen information, and stored it in the refrigerator at -80 ℃.

Cell culture

HEK-293T cells were thawed in a 37℃ water bath. After thawing, they were centrifuged at 1300rpm for 3 min. Sucked the supernatant of the cryopreservation solution, added the serum cell culture medium to resuspend the cells, inoculated the cell suspension into the culture dish containing the serum cell culture medium, shook it evenly and placed it in the 37℃ and 5% CO_2 incubator. The culture medium was changed after 24 hours, and the cells were subcultured when the cell confluency reached about 80%.

Prediction of associated microRNA

The microRNAs associated with ATG7 were predicted through TargetScan (http://www.targetscan.org/), starBase (https://starbase.sysu.edu.cn/), and miRWalk (http://mirwalk.umm.uni-heidelberg.de/). Autophagy-related miR-17-5P was selected, and then its binding sites with target genes were predicted and the targeting relationship was verified.

Plasmid transfection

HEK-293T cells were inoculated into 24 well culture plate and cultured in 37℃ and 5% CO_2 incubator until the cell confluency reached more than 60%. Transfection of 1μg plasmid required 2μL X-tremegeneHP, and they were dissolved in 100μl opti-MEM according to this ratio. After mixing, the mixture was left at room temperature for 20 min; 293T cells were added to the mixture, cultured at 37℃ and 5% CO_2 for 5–6 hours, and then supplemented with 200μl complete medium containing 10% fetal bovine serum. The transfection efficiency was judged 24–48 hours after transfection. When transfecting the target plasmid, 0.5μg green fluorescent protein particles would be transfected separately to judge whether the transfection system was normal. Luciferase was detected 48 hours after transfection.

Dual-luciferase reporter assay

 Added luciferaseassaybufferII into the luciferaseassaysubstrate bottle and completely dissolved the substrate to form luciferaseassayreagent. Before cell lysis, PassiveLysisBuffer5× was diluted with D-Hanks and prepared as 1×; Sucked the culture medium from the 24 well plates and added 300μl

PassiveLysisBuffer1×, put it in the refrigerator at 4℃ for 20 min. After the cells were fully lysed, shook with a vibrating plate machine for 3–5 min (not too violent) and mixed evenly. ② Added Stop&Glo®Substrate50× to Stop&Glo®Buffer, dissolved it fully, and diluted it to 1× Reagent. ③ Dissolved the cell lysate in step② at room temperature, sucked 40μl into the Lockwellmaxisorp detection plate, added 20μL LuciferaseAssayReagent, shook and mixed, and immediately used the enzyme labeling instrument to detect the fluorescence of F-Luciferase. ④ After that, added 20μL Stop&Glo®Reagent into each well, shook and mixed, stood for 3min, and then used the enzyme labeling instrument to detect the fluorescence value of R-Luciferase.

Quantitative real-time polymerase chain reaction

The primer sequences of ACTB and ATG7 shown in Table 1 were synthesized by Shanghai Genechem Co., LTD. U6 and miR-17-5P primer sequences were purchased from Guangzhou Ruibo Biotechnology Co., Ltd. Total RNA was isolated from ovarian GCs samples collected clinically using Trizol Kit (SuperfecTRI™), according to the manufacturer's instructions. cDNA was synthesized using universal RT-PCR Kit (Promega). Quantitative PCR was carried out using SYBR Green qPCR Master Mix according to the manufacturer's instructions. ACTB and U6 were used as internal reference genes. All reactions were carried out in triplicate and repeated three times. The relative gene expression of miR-17-5P and ATG7 was calculated using $2^{-\Delta\Delta Ct}$ method for ovarian GCs samples[9].

Table 1
Primers used for quantitative real-time polymerase chain reaction

Gene	Primer	Primer sequence
U6	Primer F	Article number: SSD089261711
	Primer R	Article number: ssD0904071007
ACTB	Primer F	5'-GCGTGACATTAAGGAGAAGC-3'
	Primer R	5'-CCACGTCACACTTCATGATGG-3'
ATG7	Primer F	5'-CTGCCAGCTCGCTTAACATTG-3'
	Primer R	5'-CTTGTTGAGGAGTACAGGGTTTT-3'
miR-17-5p	Primer F	Article number: SSD809230874
	Primer R	Article number: SSD089261711

Western blot

RIPA Lysis Buffer (Beyotime) was used to extract total protein from ovarian GCs collected clinically, according to the manufacturer's instructions. Protein concentration was determined using Enhanced BCA Protein Assay Kit (Beyotime). Added new lysate and adjusted the protein concentration of each sample to 2 μg/μL. Then added 1/5 volume of 6x dropping buffer, mixed well, boiled in 100℃ metal bath for 10min, and stored at -80 ℃ for standby after brief centrifugation. Equal amount of protein (50μg) was

electrophoresed by SDS-PAGE. Then, using the transfer electrophoresis device, the protein was transferred to the PVDF membrane under the condition of 4°C and 300 mA constant current for 150 min. The PVDF membrane was sealed at room temperature with TBST solution containing 5% skimmed milk for 1 hours. The blocking solution diluted the primary antibody against ATG7 and incubated with the blocked PVDF membrane at 4°C overnight. Horseradish peroxide secondary antibody was diluted with blocking solution, and PVDF membrane was incubated at room temperature for 1.5 hours. Finally, Western blotting was detected by chemiluminescence (lumiglo®).

Statistical analysis

SPSS statistics 21.0 software was used for data analysis and statistical calculation, and GraphPad Prism 8.0.1 software was used for drawing. The measurement data was expressed as mean ± standard deviation (SD). A student's t-test was used to compare the data between two groups. If the data did not meet the normality and/or homogeneity of variance, nonparametric test was used. Qualitative variables were expressed as frequencies and percentages and were analyzed using theχ^2-test. $P < 0.05$ means the difference was statistically significant.

Results

Basic characteristics of the two groups

In the normal group, one patient was pregnant during the test, and a total of 75 patients were finally included in the study. No obvious adverse reactions were found in all patients. The results in Table 2 showed the age, infertility duration, infertility type, body mass index (BMI), bFSH, bLH, bE_2, bP_4, and antral follicle count (AFC) of 38 PCOS women and 37 normal women. Obviously, there was no significant difference in bFSH, bE_2, and bP_4 between the two groups. However, the BMI (27.23 ± 4.26 vs. 23.33 ± 4.34, $P < 0.01$), bLH (9.38 ± 0.90 vs. 6.30 ± 1.18, $P < 0.05$) and AFC (24.97 ± 8.69 vs. 18.76 ± 6.73, $P < 0.01$) of patients with PCOS were significantly higher than those of normal women.

Table 2
The characteristics of PCOS women and normal women

Characteristics		PCOS(n = 38)	NORMAL(n = 37)	*P*-value
Age(year)		32.26 ± 3.72	33.84 ± 4.84	0.10[a]
Infertility duration (years)		3.13 ± 1.82	2.65 ± 1.96	0.27[a]
Infertility type (n, %)	Primary infertility	22(57.89%)	21(56.76%)	0.92[b]
	Secondary infertility	16(42.11%)	16(43.24%)	
BMI(kg/m^2)		27.23 ± 4.26	23.33 ± 4.34	□0.01[a]
Basal FSH(mIU/ml)		6.46 ± 1.57	6.59 ± 1.41	0.71[a]
Basal LH(mIU/ml)		9.38 ± 0.90	6.30 ± 1.18	0.02[a]
Basal E_2(pg/ml)		51.21 ± 39.93	48.81 ± 33.45	0.78[a]
Basal P_4(pg/ml)		0.63 ± 0.35	0.66 ± 0.34	0.71[a]
AFC(n)		24.97 ± 8.69	18.76 ± 6.73	□0.01[a]

Notes: Data are presented as mean ± SD or n (%). a: student's t-test. b: χ2 -test.

MiR-17-5p is the associated microRNA of ATG7

The microRNAs associated with ATG7 were predicted through TargetScan, starBase, and miRWalk. The prediction results of the three databases were taken as the intersection, and a total of 51 microRNAs that may be associated with ATG7 were screened (Fig. 1, Table 3). In this study, miR-17-5P related to autophagy was selected for validation.

Table 3
microRNAs associated with ATG7

MicroRNA name			
hsa-miR-17-5p	hsa-miR-146a-5p	hsa-miR-582-5p	hsa-miR-761
hsa-miR-22-3p	hsa-miR-185-5p	hsa-miR-588	hsa-miR-3184-5p
hsa-miR-93-5p	hsa-miR-186-5p	hsa-miR-654-5p	hsa-miR-3619-5p
hsa-miR-106a-5p	hsa-miR-193a-3p	hsa-miR-330-5p	hsa-miR-3918
hsa-miR-129-5p	hsa-miR-200a-3p	hsa-miR-423-5p	hsa-miR-378g
hsa-miR-7-5p	hsa-miR-296-5p	hsa-miR-582-3p	hsa-miR-3194-3p
hsa-miR-204-5p	hsa-miR-370-3p	hsa-miR-589-5p	hsa-miR-1343-3p
hsa-miR-210-3p	hsa-miR-372-3p	hsa-miR-541-3p	hsa-miR-2467-3p
hsa-miR-214-3p	hsa-miR-20b-5p	hsa-miR-320b	hsa-miR-664b-3p
hsa-miR-221-3p	hsa-miR-146b-5p	hsa-miR-320c	hsa-miR-766-5p
hsa-miR-222-3p	hsa-miR-193b-3p	hsa-miR-1271-5p	hsa-miR-6807-3p
hsa-miR-223-3p	hsa-miR-520a-3p	hsa-miR-320d	hsa-miR-7153-5p
hsa-miR-141-3p	hsa-miR-526b-3p	hsa-miR-1914-3p	

There is a negative targeted regulation relationship between miR-17-5p and ATG7

Using bioinformatics software, we predicted the specific binding sites between miR-17-5P and ATG7. The schematic diagram of the combination sequence was shown in Fig. 2a. Dual-luciferase reporter assay was conducted to verify whether there was a targeted regulation relationship between miR-17-5P and ATG7 (Table 4 showed the experimental grouping). The picture after cell transfection is shown in Fig. 2b,c. The results of dual-luciferase reporter assay showed that compared with group 7, the expression of luciferase in group 8 decreased after transfection of hsa-mir-146b overexpression plasmid ($P < 0.05$), indicating that the whole transfection assay system worked normally. The expression of luciferase in the wild-type target genome (group 4) was lower than that in the mutant target genome (group 6) ($P < 0.05$). The expression of luciferase in the 3'UTR empty plasmid group (group 2) was higher than that in the target gene 3'UTR plasmid group (group 4) ($P < 0.05$). Taken together, these results suggested that ATG7 was a target gene of miR-17-5P. MiR-17-5P could bind to the 3'UTR of ATG7 and inhibit its expression (Fig. 2d).

Table 4
dual-luciferase reporter assay group

Group	Plasmid combinations
1	Luc-(ATG7)-3'UTR-NC+(hsa-mir-17)-NC
2	Luc-(ATG7)-3'UTR-NC+(hsa-mir-17)
3	Luc-(ATG7)-3'UTR+(hsa-mir-17)-NC
4	Luc-(ATG7)-3'UTR+(hsa-mir-17)
5	Luc-(ATG7)-3'UTRMut+(hsa-mir-17)-NC
6	Luc-(ATG7)-3'UTRMut+(hsa-mir-17)
7	3'UTR-TRAF6 + miRNA-NC
8	3'UTR-TRAF6 + miRNA-(hsa-mir-146b)

MiR-17-5p was highly expressed in PCOS patients while ATG7 was low expressed

After oocytes retrieval, GCs were extracted from the follicular fluid. The relative expressions of miR-17-5P and ATG7 in GCs were measured by qRT-PCR and western blot assay. Results showed that at mRNA level, the expression of miR-17-5P in GCs of PCOS women was significantly lower than that of normal women (Fig. 3a). At both mRNA and protein levels, the expression of ATG7 in GCs of PCOS women was significantly higher than that of normal women (Fig. 3b, c, d).

Discussion

Abnormal GCs autophagy is associated with the pathogenesis of PCOS. In our previous study, we have found that the expression of ATG7 is up-regulated in ovarian GCs of PCOS patients. However, the precise mechanism of ATG7 participating in the pathogenesis of PCOS by regulating autophagy has not been clarified. In this study, we found the microRNA associated with ATG7 through bioinformatics software and dual-luciferase reporter assay. MiR-17-5P could negatively regulate the expression of ATG7. The detection of clinical GCs samples showed that compared with normal women, the expression of miR-17-5P in ovarian GCs of PCOS patients decreased, while the expression of ATG7 mRNA and protein increased. This proved that miR-17-5P regulates autophagy of GCs by targeting ATG7 and played an important role in PCOS.

Autophagy, as an evolutionarily conserved and highly regulated catabolic process, can affect follicular growth and is closely related to the clinical symptoms of PCOS, such as insulin resistance, hyperandrogenemia, obesity, chronic low-grade inflammation, and so on[10–12]. At present, a large number of studies have confirmed that autophagy is involved in different stages of follicular

development. ATG7 influenced the survival of germ cells and primordial cells. The number of germ cells and primordial follicles in the ovaries of ATG7 deficient mice was small, and many follicles changed in structure or lose function [6]. The loss of ATG7 or the dose-dependent reduction of autophagy-specific gene becn1 would not only lead to the significant reduction of germ cells in perinatal female mice[13] but also lead to the excessive loss of primordial follicles in newborn mice[14]. Sun et al. [15]found that overexpression of miR-378-3P induced autophagy and increased the number of primordial follicles. After the miR-378-3P knockdown, autophagy was inhibited and the number of primordial follicles decreased. During the development from primordial follicles to mature follicles, the vast majority of follicles died due to follicular atresia, and only a small part (less than 1%) of follicles could mature and ovulate. Autophagy played an important role in follicular atresia and the selection of dominant follicles. By immunofluorescence staining of rat ovarian autophagy markers (MAP1LC3, LC3), it was found that LC3 showed a high expression level in GCs at all developmental stages. Cell death in the form of autophagy might mainly participate in follicular atresia by affecting GCs [7]. Nicole et al. [16] found that after stimulating human ovarian GCs with oxidized low-density lipoprotein (oxLDL), autophagy was over-activated and mortality increased. Oxidative stress caused GCs death by initiating programmed cell death in the form of autophagy, which was a common cause of follicular atresia.

Autophagy is involved in the regulation of endometrial function and periodic remodeling of human endometrium. Basal level autophagy is essential for maintaining endometrial homeostasis and mediating endometrial specific functions, including menstrual cycle, embryo implantation, and decidualization[17]. The imbalance of autophagy regulation was also associated with the disorder of the endometrial hyperplasia-secretion cycle, which might directly lead to the decrease in endometrial receptivity. The down-regulation of autophagy gene expression in the endometrium of PCOS patients might lead to a decrease in endometrial receptivity [18].

Autophagy is associated with a variety of endocrine and metabolic disorders in PCOS patients. First of all, autophagy is associated with elevated androgen levels. The mRNA abundance of autophagy-specific gene becn1, ATG5, and ATG7 in ovarian GCs of PCOS patients was significantly increased. The mRNA abundance of becn1 was positively correlated with the level of serum basal total testosterone. Dihydrotestosterone (DHT) increased the ratio of LC3-II/LC3-I in GCs in a dose-dependent manner[10]. Li et al. [19] found that androgen inhibited GCs autophagy and proliferation by activating the PI3K / Akt signaling pathway. Secondly, autophagy is linked to insulin resistance. Autophagy has been shown to play an important role in regulating the normal function of pancreatic β-cells and insulin target tissues. Rapamycin, an autophagy inducer, could activate the inhibited autophagy, enhance insulin sensitivity and improve insulin resistance in T2DM rats [20]. High mobility group box-1 (HMGB1) led to insulin resistance in GCs of PCOS patients by inducing abnormal autophagy. While uncontrolled autophagy led to the further release of HMGB1 and reduced insulin sensitivity. Thirdly, autophagy is associated with chronic low-grade inflammation. The expression of inflammatory transcriptome in ovarian GCs was up-regulated in patients with PCOS[21]. The imbalance of inflammation would break the normal pattern of follicular development, impair the quality of follicles, and lead to anovulatory infertility. Autophagy proteins affected almost all cell types involved in the pathogenesis of inflammation, such as macrophages, T

cells, lymphocytes, and dendritic cells[22]. Nuclear factor kappa-B (NF-κB) was an important factor regulating the cellular inflammatory response. Early studies have shown that autophagy induces NF-κB activation and enhances the association between NF-κB and Becn1, thereby regulating the inflammatory pathway [23]. Last but not least, autophagy is associated with obesity. Adipose tissue has important endocrine functions. Autophagy is a key regulator of white and brown adipose tissue production. Dysregulated autophagy would damage fat accumulation in vitro and in vivo [24]. Luo et al. [25] found that Chemerin and its receptor CMKLR1, which were closely related to obesity and metabolic syndrome, were overexpressed in PCOS rats. Chemerin promoted autophagy by inhibiting PI3K / Akt / mTOR and MAPK signaling pathways. In addition, ATG7 played an important role in normal adipogenesis. Inhibiting autophagy by destroying ATG7 had anti-obesity and insulin sensitization effects [26]. These studies suggested that a variety of autophagy genes played a role in the pathogenesis of PCOS. Autophagy excess and autophagy deficiency would affect the normal physiological activities of the body. Maintaining a coordinated and balanced autophagy state was of great significance for the prevention and treatment of PCOS.

As an important autophagy-related protein, ATG7 is involved in two important ubiquitin-binding systems in the autophagy process. One is involved in the ubiquitin-like reaction between LC3-□ and phosphatidylethanolamine (PE), which is transformed into fat-soluble LC3-□ and adsorbed on the autophagosome membrane. The other is involved in the ubiquitin-like reaction between ATG12 and ATG5, forming the ATG12-ATG5 linker and binding with ATG16, which is involved in the formation of preautophagosome structure [27]. It can be seen that ATG7 is an essential molecule in the process of autophagy and plays a major role in the process of autophagy. ATG7 can be used as a marker to reflect the level of autophagy. And autophagy can be regulated by interfering with the expression of ATG7. Previous studies have found that the expression of ATG7 is up-regulated in ovarian GCs of PCOS patients. The purpose of this study was to explore the specific mechanism of ATG7 participating in the pathogenesis of PCOS by regulating autophagy.

In this study, we predicted the microRNA associated with ATG7 by bioinformatics software to further explore the pathogenesis of PCOS. Among the 51 associated microRNAs, miR-17-5P associated with autophagy was selected. MiR-17-5P belongs to the miR-17-92 family and is located on human chromosome 13. It can regulate a variety of autophagy-related genes such as ATG7 and ATMN1, and play a regulatory role in different stages of autophagy. At present, research on miR-17-5P regulating autophagy is mainly concentrated in the field of tumor[28]. A study by Zhang et al. [29] found that miR-17-5P down-regulated the expression of DEAD-Box Helicase 5(DDX5) and inhibited autophagy. In the liver cancer transplantation model, miR-17-5P inhibitor could promote autophagy flux and inhibited tumor growth. Hou et al.[30] found that miR-17-5P targeted beclin-1 to inhibit autophagy. Increasing the expression of miR-17-5P could reduce autophagy activity and play an important role in the radiosensitivity of glioma cells. Targeting miR-17-5P / beclin-1 autophagy pathway might be an effective way to improve the efficacy of glioma radiotherapy. Knockout of miR-17-5P up-regulated the expression of PTEN and inhibited the proliferation and autophagy of thyroid cancer cells. Finally, it could reduce the

malignancy of thyroid cancer and inhibit the inactivation of Akt / mTOR pathway, which provided a new way for the treatment of thyroid cancer [31].

Dual-luciferase reporter assay confirmed that miR-17-5P was the associated microRNA of ATG7, and miR-17-5P could negatively regulate the expression of ATG7. There was still a lack of research on miR-17-5P / ATG7 mediated autophagy in human ovarian tissue. By detecting the expression levels of miR-17-5P and ATG7 in human ovarian GCs, we found that compared with normal women, the expression of miR-17-5P was lower and the expression of ATG7 was higher in ovarian GCs of PCOS patients. This suggested that miR-17-5P might target ATG7 mediated GCs autophagy and played a role in the pathogenesis of PCOS.

The disorder of GCs autophagy could affect the connection and communication between GCs themselves and between GCs and oocytes. Therefore, maintaining the coordinated balance of GCs autophagy could indirectly affect the growth, development, and maturation of oocytes. In previous studies, it was found that autophagy balance was the key link to promote follicular development, improve endocrine and metabolic disorders and improve endometrial receptivity. Our study found that the autophagy pathway miR-17-5P / ATG7 might be involved in the pathogenesis of PCOS. This discovery provided a new idea for the treatment of PCOS by developing miR-17-5P analogs or short peptides that specifically inhibited the activity of ATG7.

The same miRNA can regulate the expression of multiple genes at the same time. Slight changes in miRNA pattern may have a significant impact on cell function. Constantly enriches the theoretical research of miRNA pathway, which is helpful to explore more therapeutic methods. Therefore, compared with the traditional single-target therapies, miRNA-based therapy may be a promising treatment.

In this study, we found a new possible pathogenesis of PCOS. In the future, this mechanism may be used to guide the exploration of reliable and efficient treatment methods for infertile patients with PCOS. However, this study has some limitations. Only in vitro experiments were carried out, and in vivo animal experiments are needed to further confirm these findings.

Conclusion

In conclusion, this study showed that there was a targeted regulatory relationship between miR-17-5P and ATG7. Low expression of miR-17-5P could up-regulate the expression of ATG7, induce autophagy of GCs, and lead to the occurrence of PCOS. In addition, we also revealed that the low expression of miR-17-5P and the high expression of ATG7 in patients with PCOS could be regarded as potential diagnostic markers and therapeutic targets of PCOS.

Abbreviations

PCOS: polycystic ovary syndrome; GCs: granulosa cells; qRT-PCR: quantitative real-time polymerase chain reaction; WB: western blot analysis; IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; E_2: estrogen; P_4:

progesterone; AFC: antral follicle count; Gn: gonadotropin; HMG: human menopausal gondotropin; hCG: human chorionic gondotrop; DHT: Dihydrotestosterone; HMGB1: High mobility group box-1; NF-κB: Nuclear factor kappa-B; PE: phosphatidylethanolamine.

Declarations

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Authors' contributions

Study design was performed by Xiaona YU, Fang LIAN, and Shan XIANG. Clinical data collection was completed by Yi YU and Conghui PANG. Clinical sample collection was completed by Lu GUAN and Li DONG. Data analysis were done by Xin XIN. Manuscript writing was performed by Xiaona YU. All authors have read and approved the manuscript.

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Conflict of interests

The authors declare that there are no conflict of interests.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author upon request.

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Figures

Figure 1

Venn diagram of microRNA associated with ATG7

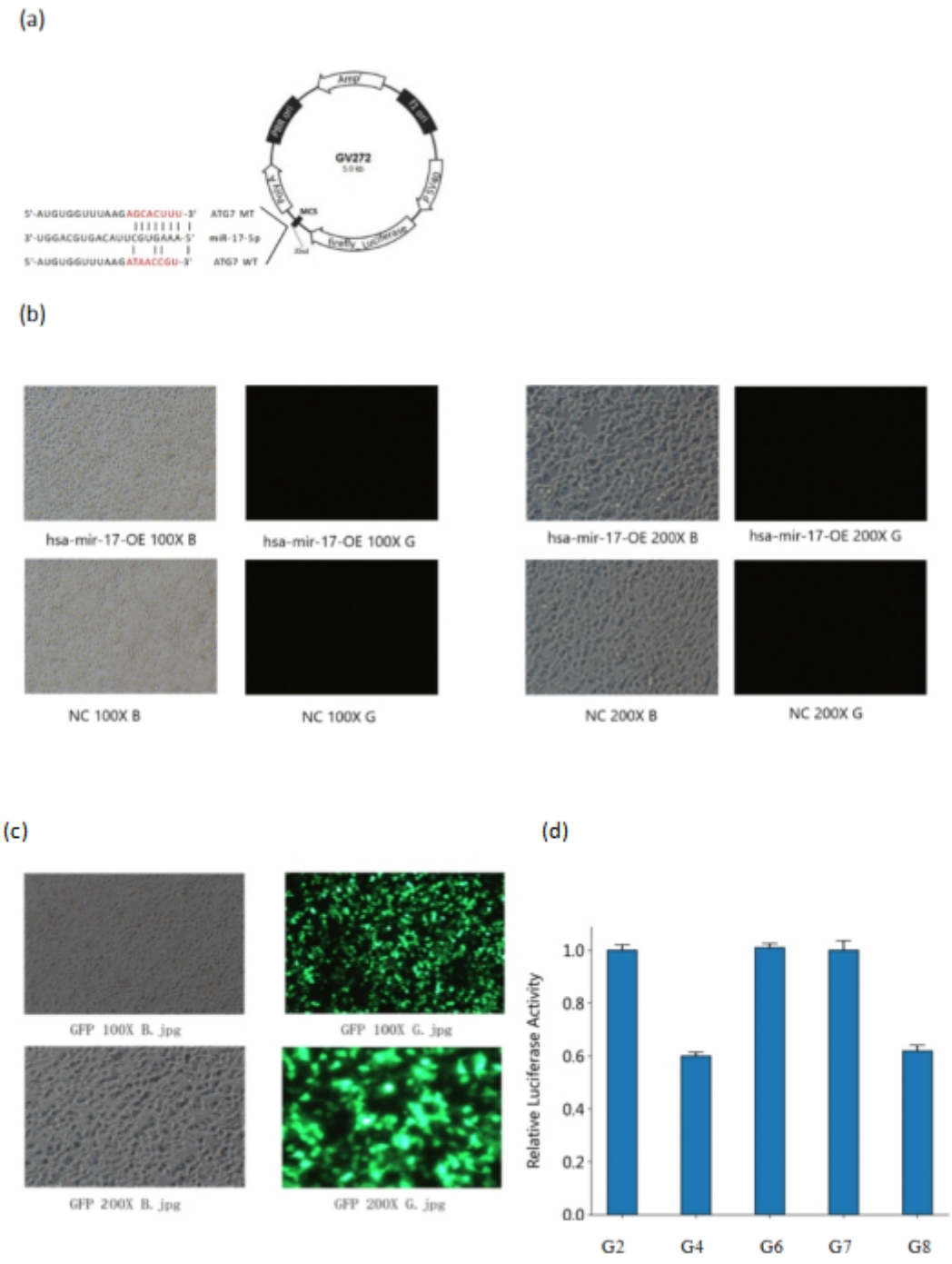

Figure 2

The predicted binding sites and mutations in the binding sites of miR-17-5p in the 3′UTR of ATG7 **a)**; Picture after cell transfection **b)**; Picture of green fluorescent protein transfected in the same batch **c)**; Experimental results of dual-luciferase reporter assay **d)**.

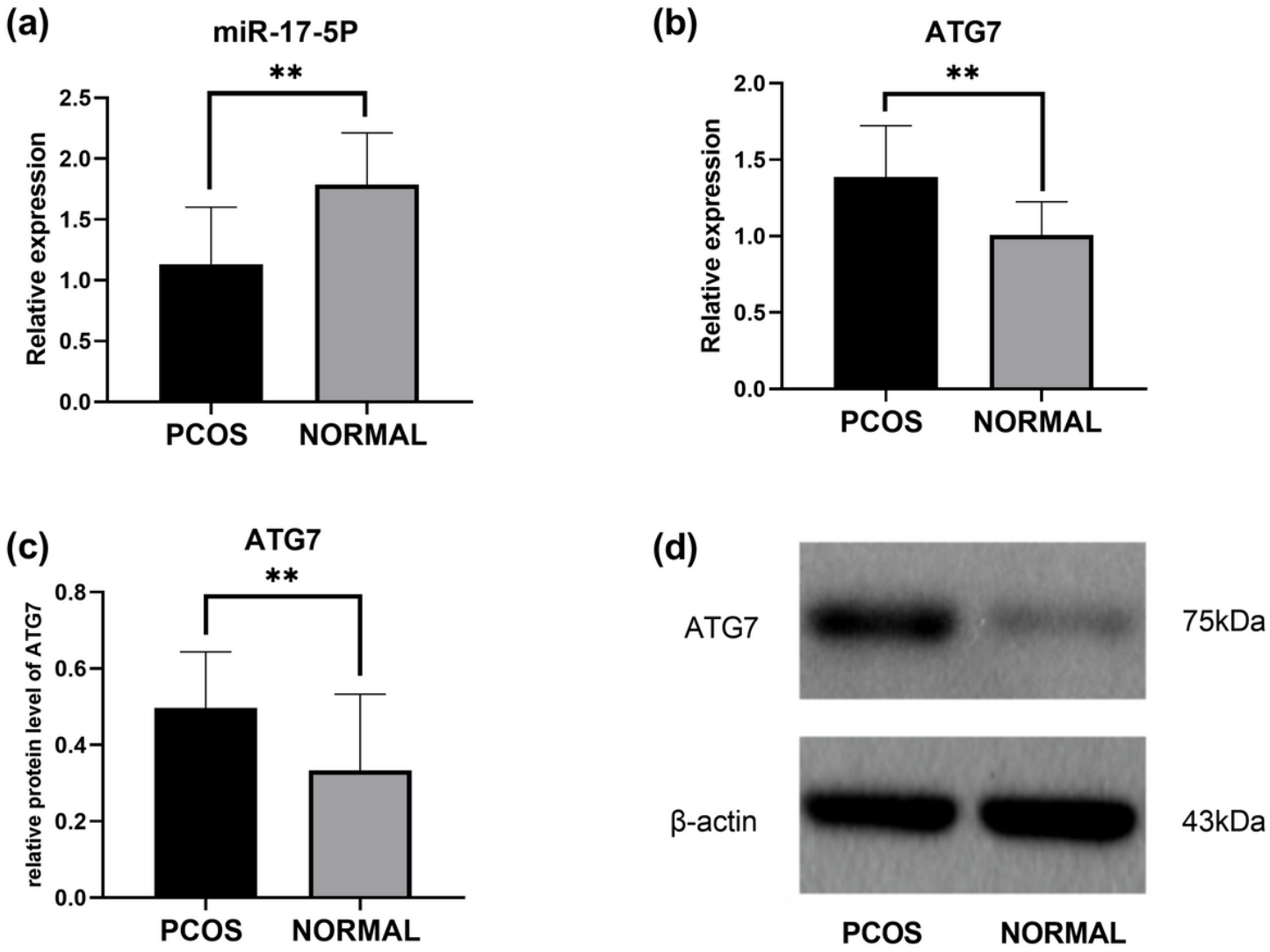

Figure 3

Relative expression of miR-17-5P as determined by qRT-PCR in GCs from PCOS and NORMAL group **a)**. Relative ATG7 mRNA expression as determined by qRT-PCR in GCs from PCOS and NORMAL group **b)**. Quantification of ATG7 protein expression after normalization with β-actin **c)**. Representative bands of ATG7 proteins as determined by western blot assay in GCs from PCOS and NORMAL group **d)**. $^{**}P < 0.01$.