RESEARCH

miR-199a-5p stimulates ovarian granulosa cell apoptosis in polycystic ovary syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder and one of the most common causes of infertility in women. PCOS patients have been found with dysregulated miRNA, which is indicative of their roles as noninvasive biomarkers and novel therapeutic targets in PCOS. Herein, this study sets out to explore the mechanism of action of miR-199a-5p in PCOS in relation to the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway via Wilms' tumor 1 (WT1) regulation in a rat model of PCOS. The expression of miR-199a-5p was highly expressed in ovarian cortical tissues and serum of PCOS patients, as examined by RT-qPCR. Ovarian granulosa cells (GCs) were harvested from PCOS rat model, followed by subsequent purification. Gain- and loss-of-function experiments of miR-199a-5p were performed to determine its functions in PCOS. Cell viability, cell apoptosis and serum hormone levels were assessed, the results of which showed that downregulation of miR-199a-5p contributed to the promotion of GC viability and inhibition of apoptosis, while simultaneously inducing the elevation of serum E2 level and reduction of serum AMH, PG, LH and FSH levels in the PCOS rat model. WT1 was identified as a target gene of miR-199a-5p by dual-luciferase reporter gene assay, and inhibition of miR-199a-5p resulted in the activation of WT1-mediated JAK/STAT3 pathway. The activated JAK/STAT3 pathway suppressed the development of PCOS by miR-199a-5p, indicating a mechanism by which miR-199a-5p could potentially prevent PCOS through the WT1-mediated JAK/STAT3 pathway.

Key Words

- ▶ microRNA-199a-5p
- ▶ polycystic ovary syndrome
- ▶ Wilms' tumor 1
- ▶ ovarian granulosa cells
- ▶ JAKSTAT3 pathway

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Introduction

Polycystic ovary syndrome (PCOS) has been regarded as the most prevalent endocrine disease in women of reproductive age (Fauser *et al.* 2012). PCOS is characterized by hyperandrogenism, oligoovulation, and polycystic ovaries (Jones *et al.* 2012), the symptoms of which include irregular menstrual cycles, infertility, increased androgen levels, and insulin resistance (Maleedhu *et al.* 2014). In addition, women diagnosed with PCOS have higher risks of developing endometrial cancer or ovarian

cancer (Galazis *et al.* 2012). Although the exact etiology of PCOS remains uncertain, potential contributing factors include abnormal folliculogenesis and gonadotropin production secondary to both genetic and environmental causes (Song *et al.* 2016). Currently available treatment options address individual symptoms of patients and may provoke untoward side effects, making them ineffective in treating the underlying pathology of PCOS (Arentz *et al.* 2014). Therefore, a more comprehensive and

deeper understanding regarding the potential molecular mechanisms of PCOS pathophysiology would benefit the development of novel diagnostic and therapeutic strategies for PCOS (Sorensen *et al.* 2014).

The granulosa cells (GCs) exert a vital function in follicular development, which is further indicative of their participation in PCOS pathology (Wang *et al.* 2018a); specifically, the dysfunction in GCs is strongly associated with the pathogenesis of PCOS (Yi *et al.* 2020). Emerging studies have reported that the aberrant expression of miRNA function as crucial regulators of various biological processes (Jiang *et al.* 2015), and contributes to the abnormality of folliculogenesis in PCOS via the regulation of proliferation of GCs (He *et al.* 2019). For instance, miR-19b reportedly affects the proliferation of GCs and participates in the development of PCOS (Zhong *et al.* 2018). miR-199a-5p is one of the most important miRNAs implicated in ovarian function. miR-199a-5p inhibits the epithelial–mesenchymal transition of ovarian ectopic endometrial stromal cells by targeting ZEB1, thus preventing endometriosis (Liu *et al.* 2020). Moreover, miR-199a-5p expression is a biomarker for the prognosis of stage I epithelial ovarian cancer (Marchini *et al.* 2011). As mentioned in a previous study, higher levels of miR-199a-5p in atretic follicles than healthy follicles (9–17 mm, classified based on steroidogenic capacity), and that the miRNA target interactions, namely miR-199a-5p/miR-155-HIF1A in GCs, are involved in follicle development (Donadeu *et al.* 2017). Thus, identifying a potential mechanism by which miRNAs result in the development of PCOS might be conducive to finding novel diagnostic and therapeutic targets for this syndrome (Sorensen *et al.* 2014, Huang *et al.* 2016). Wilms' tumor 1 (WT1), a zinc-finger protein, is known to modulate the growth of several organs and tissues, involved in various cellular biological activities, including proliferation, differentiation and apoptosis (Toska & Roberts 2014). The most important one in the present context is the finding that WT1 is expressed in ovarian GCs and plays a fundamental role in the progression of the ovarian follicle differentiation (Gao *et al.* 2014). Interestingly, WT1 knockdown can accelerate hepatocellular carcinoma cell apoptosis by blocking the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway (Lv *et al.* 2015), which is also one of the crucial pathways associated with the pathogenesis of PCOS (Maliqueo *et al.* 2015). In addition, the JAK/STAT pathway, specifically STAT3, is a critical tumor biomarker that participates in angiogenesis and tumor growth (Zhang *et al.* 2011). Once activated by JAK, STAT3 might result in tumor development through

its effect on various cellular processes, such as metastasis, proliferation, and cell survival (Gritsina *et al.* 2015). Based on these findings and our computer-aided analysis of target genes, we hypothesized a possible regulatory network of miR-199a-5p, WT1 and the JAK/STAT3 pathway in the pathogenesis of PCOS. Therefore, the current study involving a variety of biochemical and genetic techniques was carried out to thoroughly investigate the effects of miR-199a-5p on PCOS and establish its molecular mechanisms in relation to the WT1 gene and the JAK/STAT3 pathway.

Materials and methods

Ethics statement

The current study was approved by the Ethics Committee of Linyi Peoples Hospital and performed in strict accordance with the Declaration of Helsinki. All participants signed informed consent documentation (clinical trial: ChiCTR1900026839) prior to enrollment. Animal experiments were approved by the Animal Ethics Committee of Linyi Peoples Hospital (Approval No. 201711005) and carried out according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Extensive efforts were made to ensure minimal suffering of the animals used in this study.

Study subjects

Ovarian cortical tissues were collected from 35 infertile female patients diagnosed with PCOS who were subjected to ovarian wedge excision at the Linyi Peoples Hospital from April 2017 to December 2017. The diagnosis of PCOS was conducted based on the Rotterdam 2003 criteria. All enrolled patients had to meet at least two items in the following criteria: (1) infrequent ovulation and/or anovulation; (2) clinical features of hyperandrogenism or hyperandrogenemia; (3) underwent ultrasonic testing within 3–5 days after the withdrawal from the menstrual cycle and progesterone (PG) treatment, the findings of which displayed large (more than 12 mm) follicles in the unilateral or bilateral ovary. Meanwhile, normal ovarian tissues were harvested from 28 healthy women of reproductive age (age < 35 years) with a regular menstrual cycle and without PCOS. They underwent gynecological examination in the follicular phase, and histopathological examination confirmed a lack of evident morphological abnormalities. None of the enrolled subjects were receiving hormonal treatment such as contraceptives,

antiandrogens, and insulin sensitizers, which could influence sex hormone secretion and metabolism. None of the subjects reported pregnancy 6 months prior to their enrollment in the present study, and none were pregnant at enrollment (Fu *et al.* 2018).

Establishment of PCOS rat models

Fifty immature female Sprague Dawley rats weighing 80–120 g and aged 25 days were purchased from the Experimental Animal Center of Zhengzhou University (Zhengzhou, Henan, China). To develop a PCOS model, 26 randomly selected rats were subcutaneously injected with dehydroepiandrosterone (6 mg/100 g body weight on a daily basis; Sigma-Aldrich), dissolved in 0.2 mL oil for 21 consecutive days. All 26 rats were successfully induced with PCOS (Benrick *et al.* 2017). The remaining 24 rats were administered with subcutaneous injections of 0.2 mL oil daily for 21 days and served as controls (Zhang *et al.* 2007).

Isolation, purification and culture of ovarian GCs

Control rats ($n=6$) and rats with PCOS ($n=6$) were selected randomly and subcutaneously injected with 40 IU pregnant mare serum gonadotropin (HOR-272, Prospec Bio, East Brunswick, NJ, USA). After 48 h, the rats were killed by anesthetic overdose, followed by the extraction of ovarian tissues. The ovarian surface capsule and surrounding adipose tissues were removed under a microscope, and the red blood cells were washed with normal saline. Ovarian follicles placed in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco) were punctured using a 1 mL syringe needle to allow the release of follicular GCs. Furthermore, the follicular cells were dispersed with a Pasteur pipette. The single cell suspension obtained was filtered through a 200-mesh sieve and centrifuged at 503 g for about 8 min. The collected GCs were subsequently cultured with DMEM/F12 medium containing 15% fetal bovine serum (FBS; Gibco) at 37°C with 5% CO₂. After 24 h, the non-adherent cells were removed, and the remaining cells were cultured. Finally, upon the cell confluence reaching 80%, the cells were isolated, centrifuged, and passaged (Jiang & Ma 2018).

Cell transfection

The ovarian GCs isolated from the rats with PCOS were either left untreated or transfected with miR-199a-5p inhibitor (100 nmol/L), shRNA targeting WT1 (sh-WT1) or their controls inhibitor-negative control (NC) and

scrambled shRNA (sh-NC). Tofactitinib, a JAK/STAT3 inhibitor (CP-690550, Pfizer; 10 µM) was utilized to disrupt the JAK/STAT3 pathway in ovarian GCs, with dimethyl sulphoxide (DMSO; 3176, Tocris Bioscience, UK; 10 µM) treatment as the vehicle control (Fukuyama *et al.* 2015). Ovarian GCs from the control rats were either left untreated or transfected with miR-199a-5p mimic or mimic NC. All plasmids involved in the experiment were commercially available from RiboBio Co., Ltd. (Guangzhou, Guangdong, China). About 24 h prior to transfection, cells were seeded in a six-well plate with DMEM/F12 medium supplemented with 10% FBS. When the cell confluence reached about 70%, cells underwent re-suspension in serum-free DMEM/F12 and were seeded into a new six-well plate. Cell transfection was conducted using Lipofectamine 2000 reagents (Invitrogen) based on the manufacturer's directions. After transfection for 6 h, the medium was renewed, and upon 48 h of culture, the cells were harvested for subsequent analyses.

RNA isolation and quantitation

Serum was obtained from whole blood samples harvested from the anterior cubital region of female patients in the early follicular phase of their spontaneous menstrual cycle. In accordance with the instructions in the cDNA kit (K1622; Fermentas Inc., Ontario, CA, USA), the total RNA was isolated from cells, tissues and the serum of PCOS patients and healthy controls using TRIzol reagent (16096020, Thermo Fisher Scientific Inc.) and reversely transcribed into cDNA. Next, RT quantitative PCR (RT-qPCR) was conducted on an ABI 7500 instrument (Applied Biosystems) using the SYBR® Premix Ex Taq™ (Tli RNaseH Plus) kit (TaKaRa). The reaction cycle entailed pre-denaturation at 94°C for 5 min, and 30 cycles of denaturation at 94°C for 30 s, annealing at 54.5°C for 30 s, extension at 72°C for 30 s, and the final extension at 72°C for 10 min, with preservation at 4°C. The expression of miR-199a-5p was quantitated with the TaqMan miRNA assay (Ambion) with U6 serving as the internal control. The primer sequences were synthesized by Guangzhou RiboBio Biotechnology Co., Ltd. (Guangzhou, Guangdong, China) (Table 1). The expression of WT1 or miR-199a-5p was calculated and determined by the 2^{-ΔΔCt} method (Livak & Schmittgen 2001).

Western blot analysis

Total protein was extracted using radio-immunoprecipitation assay lysis buffer containing

Table 1 Primer sequences for RT-qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
miR-199a-5p	GTGCTCAGGCTGTTTCAGAC	TATGGTTGTTCTGCTCTCTGTCTC
WT1	ACAGAATACACACGCACGGT	GGCGTTTCTCACTGGTCTCA
β -actin	ACCACAGCTGAGAGGGAAATCG	AGAGGTCTTTACGGATGTCAACG
U6	CTCGCTTCGGCAGCAC	AACGCTTCACGAATTTGCGT

RT-qPCR, RT quantitative PCR; miR-199a-5p, microRNA-199a-5p; WT1, Wilms' tumor 1.

phenylmethylsulfonyl fluoride (Thermo Scientific). Next, the concentration of the total protein was measured using a bicinchoninic acid kit (CW0014, Bocai Biotechnology, Shanghai, China). Subsequently, 40 μ g of the protein was isolated using 10% sulfate PAGE and then transferred onto a polyvinylidene fluoride membrane (Bio-Rad), which was then blocked with 5% skim milk powder at room temperature for 1 h. The membrane underwent incubation overnight at 4°C with the following diluted primary rabbit antibodies to β -actin (ab8224, 1:10,000), anti-Müllerian hormone (AMH; ab103233, 1:500), cleaved caspase-3 (ab49822, 1:500), Bcl-2-associated X protein (Bax) (ab53154, 1:1000), B-cell lymphoma 2 (Bcl-2) (ab182858, 1:2000), STAT3 (ab119352, 1:5000), WT1 (ab224806, 1:2000) and mouse antibody to phosphorylated (p)-STAT3 (ab76315, 1:2000). Following incubation, the membrane was washed at least three times with Tris-buffered saline with Tween 20 (TBST). The membrane was then incubated with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin G (IgG; ab6728, 12,000) or goat anti-rabbit IgG (ab6721, 1:2000) for 1 h. All primary and secondary antibodies were purchased from Abcam Inc. Bands were then developed using enhanced chemiluminescence reagents (Millipore) and quantified with the Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

Cell counting kit-8 (CCK-8) assay

The viability of the ovarian GCs was measured using a CCK-8 kit (C0038, Beyotime Biotechnology Co., Ltd., Shanghai, China) at different time points in culture. The transfected ovarian GCs (100 μ L) were seeded into a 96-well plate at a density of 2×10^3 cells/well. The cells were incubated with fresh medium containing 20 μ L CCK-8 solution (5 g/L) reagent under dark conditions for 4 h. A microplate reader (Infinite 200, Tecan, Switzerland) was used to measure the absorbance value at 450 nm. The obtained results were then used to plot the growth curve with absorbance on the ordinate axis and incubation time as the abscissa (Wu *et al.* 2018).

Flow cytometry

Transfected ovarian GCs were seeded in a 6-well plate, and cultured for 48 h, followed by trypsinization and fixation with 75% ethanol at -20°C overnight. The fixed cells were incubated with 0.5 μ g/mL of RNase A (Thermo Fisher Scientific Inc.) and 100 μ g/mL of propidium iodide (PI) for 30 min. Finally, cell apoptosis was determined using the Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis kit (Abcam) and analyzed on a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA, USA) equipped with the CellQuest software (Becton Dickinson). The cells that did not stain with FITC/PI were regarded as the NC and FITC-stained or PI-stained cells were used as controls while the ovarian GCs exposed to hydrogen peroxide served as a positive control.

Dual-luciferase reporter gene assay

A prediction about the binding sites between miR-199a and WT1 was performed using the biological information analysis website (Targetscan: <http://www.targetscan.org/>). Dual-luciferase reporter gene assay was applied to verify experimentally that WT1 was a direct target of miR-199a-5p. With its DNA as the template, the WT1 sequence was amplified and purified. Next, WT1 WT (WT1-wt, containing miR-199a bindings sites) and WT1 mutation type (WT1-mut, with the miR-199a binding site mutated) fragments were inserted into the pMIR-reporter vector (Beijing Huayueyang Biotechnology Co., Ltd., Beijing, China) by endonuclease cleavage (SpeI and HindIII) and ligated using T4 DNA ligase at 4°C overnight. The products were then transferred into DH5 α competent cells for selection and identification, whereby the extracted plasmids were identified by restriction endonuclease cleavage and sequencing. The correctly sequenced luciferase reporter plasmids WT1-wt and WT1-mut were, respectively, co-transfected with miR-199a-5p mimic into the GCs. After 48 h of transfection, the luciferase activity was evaluated using a luciferase detection kit (RG005, Beyotime Biotechnology Co., Ltd.) according to the manufacturer's protocols on a Glomax20/20 luminometer (Promega).

Animal treatment

A total of 18 rats with PCOS and 18 control rats were randomly selected for *in vivo* experiments (both PCOS and control rats were assigned into scramble control+DMSO, antagomir (anta)-miR-199a-5p+DMSO, anta-miR-199a-5p+tofacitinib (an inhibitor of JAK/STAT3 signaling pathway) groups, $n=6$ in each group). Rats in the PCOS group were subcutaneously injected with DHEA every day for 21 days to establish the PCOS model, while rats in the control group were subcutaneously injected with 0.2 mL oil every day for 21 days. After daily injection of DHEA or oil, the scrambled control or anta-miR-199a-5p (Exiqon, Denmark) was immediately delivered, respectively, either to PCOS rats or to control rats via caudal vein injection at a dose of 0.75 nmol/rat (Heuslein *et al.* 2018). An equal volume of DMSO (3176, Tocris Bioscience, Bristol, UK) and tofacitinib (dissolved in DMSO; CP-690550, Pfizer) were intraperitoneally injected into the rats at a dosage of 30 mg/kg/time (Fukuyama *et al.* 2015). The rats' body weight was monitored and recorded daily. After 21 consecutive days, rats from each group were anesthetized by Avertin overdose to collect heart blood samples (Yuan *et al.* 2016). The blood was centrifuged at 503 *g* for 3 min, and the plasma was preserved at -40°C . The rat ovarian tissues were also extracted after euthanasia. After the removal of ovaries membrane and the surrounding adipose tissue, the ovarian tissues were fixed by immersion in 4% paraformaldehyde and preserved at -40°C .

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining

The fixed ovarian tissues were dehydrated in an ethanol series, cleared, paraffin-embedded and cut into 4- μm thick sections. The apoptosis of ovarian GCs was detected according to the conditions provided on a TUNEL kit (C1086, Beyotime Biotechnology Co., Ltd.) and observed under a microscope (XSP-2C, Shanghai Batuo Instrument Co., Ltd., Shanghai, China) (Shen *et al.* 2012).

Enzyme-linked immunosorbent assay (ELISA)

The serum of rats was collected for detection of AMH, PG, estradiol (E2), luteinizing hormone (LH), and follicular estrogen (FSH) levels using ELISA kits (Yan Hui Biological Technology Co., Ltd., Shanghai, China), according to the manufacturer's instructions. Three replicates were made for each sample.

Hematoxylin–eosin (HE) staining

The fixed ovarian tissues prepared as per the above description were mounted on polylysine-coated glass slides and heated at 60°C overnight. The staining procedures were conducted following the instructions in the HE Staining Kit (Beyotime Biotechnology Co., Ltd.) (Wang *et al.* 2018b). Under an optical microscope (Olympus), pathological changes were observed in the ovarian tissue sections. Under the microscope, the cell nucleus presented with blue color while the cytoplasm has a red appearance. The number of corpus luteum and cystic ovarian follicles, and the ratio of cystic expansive follicles were then calculated. Additionally, the thickness of cellular layers in the theca folliculi and in GCs was measured. These results were assessed by researchers with at least 5 years of experience in histology. Identical procedures were used for the clinical ovarian tissue samples.

Statistical analysis

All experimental data were analyzed using SPSS 21.0 software (IBM Corp.). Measurement data following normal distribution were presented as mean $\pm$ S.D. Data with skewed distribution and defect variances were expressed as interquartile range. Comparison between two groups was conducted using independent sample *t*-test. The skewed data were calculated using the Wilcoxon signed-rank test. Comparison among multiple groups was performed using one-way ANOVA with Tukey's *post hoc* test for pairwise comparisons. Data at different time points were compared using repeated-measures ANOVA with Tukey's *post hoc* test. Pearson's correlation coefficient was used for correlation analysis. Enumeration data were expressed as percentage, and compared using the Chi-square test. A *P* value less than 0.05 was considered to be statistically significant.

Results

miR-199a-5p is upregulated in patients with PCOS

The expression of miR-199a-5p was determined by RT-qPCR, which showed (Fig. 1A and B) that the expression of miR-199a-5p was higher in ovarian tissues (3.648 ± 0.952) and serum (2.496 ± 0.318) of PCOS patients than that in ovarian tissues (1.034 ± 0.192) and serum (1.016 ± 0.225) of healthy controls, indicating an upregulation of miR-199a-5p in PCOS. The clinical characteristics of PCOS patients and healthy controls are illustrated in Table 2.

Figure 1

miR-199a-5p expression is upregulated in ovarian cortical tissues and serum of PCOS patients. (A) miR-199a-5p expression was determined by RT-qPCR in ovarian cortical tissues of PCOS patients ($n = 35$) and healthy controls ($n = 28$), normalized to U6; (B) miR-199a-5p expression was determined by RT-qPCR in serum samples from PCOS patients ($n = 35$) and healthy controls ($n = 28$), normalized to U6; (C) HE staining analysis of ovarian tissues from PCOS patients ($n = 35$) and healthy controls ($n = 28$) (scale bar = 25 μm , 400 $\times$); ** $P < 0.01$ vs healthy controls by independent sample t -test.

Moreover, HE staining showed that in the healthy controls, the ovarian tissues presented with a smooth surface, multiple corpus luteum and follicles at multiple growth phases within cortex, tight fiber texture distributed among follicles, and neatly arranged granular cells. In contrast, the ovarian tissues in PCOS patients exhibited higher cortex fibrosis, lower quantity in cell number, presence of blood vessels, a surplus of underdeveloped and blocked follicles at various growth phases, theca cell hyperplasia, and a decrease in the quantity of corpus luteum (Fig. 1C).

Downregulation of miR-199a-5p promotes viability and inhibits apoptosis in ovarian GCs

The abnormal expression of miR-199a-5p in ovarian cortical tissues and serum from PCOS patients was suggested as a potential pathological mediator of PCOS. To test this hypothesis, ovarian GCs isolated in a rat model of PCOS were transfected with miR-199a-5p inhibitor, and ovarian GCs from the control rats were transfected with miR-199a-5p mimic. The results revealed elevated expression of miR-199a-5p in the ovarian GCs of PCOS rats in comparison with the control rats ($P < 0.05$). Transfection with the miR-199a-5p inhibitor reduced the expression of miR-199a-5p while miR-199a-5p mimic elevated its expression in GCs ($P < 0.05$; Fig. 2A). The results of CCK-8 assay showed that miR-199a-5p inhibitor led to increased viability of ovarian GCs from the PCOS rats, while miR-199a-5p mimic decreased viability of ovarian GCs from the control rats ($P < 0.05$; Fig. 2B). Furthermore, flow cytometric data showed that cell apoptosis was decreased in miR-199a-5p inhibitor-transfected ovarian GCs from the PCOS rats, while apoptosis was increased in miR-199a-5p mimic-transfected ovarian GCs from the control rats (Fig. 2C). Thus, inhibition of miR-199a-5p may promote cell viability and simultaneously suppress apoptosis of ovarian GCs.

Subsequent results of Western blot analysis showed that the inhibition of miR-199a-5p led to increased levels of the anti-apoptotic factor Bcl-2 and reduced levels of AMH and the pro-apoptotic factors cleaved-caspase3 and Bax in ovarian GCs from PCOS rats ($P < 0.05$). On the contrary, upregulation of miR-199a-5p decreased the level of Bcl-2 in ovarian GCs from the control rats, accompanied by increased levels of AMH, cleaved-caspase3 and Bax

Table 2 Clinical characteristics between PCOS patients and healthy controls.

Characteristics	Control ($n = 28$)	PCOS ($n = 35$)	P-value
Age (years)	27.96 $\pm$ 2.96	29.80 $\pm$ 4.23	0.056
Body mass index (kg/m ²)	19.49 $\pm$ 1.68	20.23 $\pm$ 1.96	0.118
Follicle stimulating hormone (IU/L)	7.39 $\pm$ 0.66	4.43 $\pm$ 0.53	0.001
Luteinizing hormone (IU/L)	21.78 $\pm$ 1.98	39.01 $\pm$ 3.25	0.008
Prolactin (mg/mL)	9.09 $\pm$ 0.86	14.53 $\pm$ 1.27	0.001
Estradiol (pg/mL)	31.22 $\pm$ 2.53	54.76 $\pm$ 5.89	0.001
Glucose (mmol/L)	5.98 $\pm$ 0.61	3.22 $\pm$ 0.31	0.001
Testosterone (nmol/L)	2.53 $\pm$ 0.22	5.08 $\pm$ 0.50	0.001
Hyperandrogenism % (n)	3 (10.71)	15 (42.86)	0.005
Normal androgen% (n)	20 (71.43)	13 (37.14)	0.007
Insulin (U/L)	5.62 $\pm$ 0.63	16.98 $\pm$ 1.79	0.001
Hyperinsulinemia % (n)	2 (7.14)	11 (31.43)	0.018
Number of mature oocytes	16.26 $\pm$ 1.65	9.39 $\pm$ 1.1	0.001
Number of good quality embryos	9.91 $\pm$ 1.17	5.71 $\pm$ 0.66	0.001

Measurement data were presented as mean $\pm$ s.d. and analyzed by independent sample t -test. Enumeration data were expressed as percentage and analyzed by chi-square test.

PCOS, polycystic ovary syndrome.

Figure 2

Inhibition of miR-199a-5p reduces apoptosis and suppresses viability of ovarian GCs from PCOS rats *in vitro*. GCs were isolated from PCOS rats and then transfected with miR-199a-5p inhibitor or inhibitor-NC, while GCs isolated from control rats were transfected with miR-199a-5p mimic or mimic-NC. (A) miR-199a-5p expression was determined by RT-qPCR, normalized to U6; (B) viability of rat ovarian GCs was assessed by CCK-8 assay; (C) quantitative analysis for apoptosis of ovarian GCs from control rats and PCOS rats in response to miR-199a-5p mimic or inhibitor was assessed by flow cytometry; (D and E) representative Western blots of AMH, cleaved caspase-3, Bax and Bcl-2 proteins and their quantitation in ovarian GCs from rats with PCOS, normalized to β -actin. * $P < 0.05$ vs the GCs from control rats or those transfected with mimic-NC; # $P < 0.05$ vs the GCs from PCOS rats or those transfected with inhibitor-NC. Independent sample *t*-test was used for data comparison in panel A, and C, D, E. Repeated measures analysis of variance with Tukey's *post hoc* test was conducted to compare data in panel B. Data are shown as mean $\pm$ s.d. of three technical replicates.

($P < 0.05$) (Fig. 2D and E). These results support the finding that inhibition of miR-199a-5p promotes cell viability while suppressing apoptosis in ovarian GCs.

Downregulation of miR-199a-5p induces activation of the JAK/STAT3 pathway in ovarian GCs

Western blot analysis revealed that transfection of miR-199a-5p inhibitor led to a higher ratio of p-STAT3/STAT3 in ovarian GCs from the PCOS rats ($P < 0.05$; Fig. 3A). In contrast to the GCs transfected with mimic NC, the ovarian GCs from the control rats were observed to have a lower ratio of p-STAT3/STAT3 upon transfection with miR-199a-5p mimic ($P < 0.05$; Fig. 3B). Thus, downregulation of miR-199a-5p might potentially mediate the activation of the JAK/STAT3 pathway in ovarian GCs.

miR-199a-5p inhibits activation of the JAK/STAT3 pathway by targeting WT1 in ovarian GCs

The bioinformatics database (Targetscan; <http://www.targetscan.org/>) predicted specific binding sites between

the WT1 gene sequence and the miR-199a-5p sequence, suggesting WT1 as a target gene of miR-199a-5p (Fig. 4A). The results of RT-qPCR evidently revealed downregulated expression of WT1 in ovarian cortical tissues from PCOS patients, and WT1 expression exhibited a negative correlation with miR-199a-5p expression in ovarian cortical tissues (Fig. 4B and C). There was no significant difference in relative luciferase activity in GCs after co-transfection with WT1-mut and miR-199a-5p mimic ($P > 0.05$), but co-transfection of miR-199a-5p mimic and WT1-wt decreased the luciferase activity in GCs ($P < 0.05$; Fig. 4D). These results indicate that miR-199a-5p directly targets WT1 gene.

To verify the hypothesis that miR-199a-5p might inhibit the JAK/STAT3 pathway by targeting WT1, WT1 was downregulated by transfection with sh-WT1, sh-WT1-1 and sh-WT1-2. The most effective silencing was obtained with sh-WT1, which was therefore used in subsequent experiments (Fig. 4E). The ovarian GCs from the PCOS rats were then transfected with sh-WT1 or sh-NC in the presence of miR-199a-5p inhibitor. The results of western

Figure 3

The JAK/STAT3 pathway is blocked by miR-199a-5p in ovarian GCs. (A) Representative Western blots of STAT3 and p-STAT3 proteins and their quantitation in ovarian GCs from PCOS rats after transfection of miR-199a-5p inhibitor, normalized to β -actin; (B) representative Western blots of STAT3 and p-STAT3 in ovarian GCs from control rats after transfection of miR-199a-5p mimic, normalized to β -actin; * $P < 0.05$ vs the GCs from control rats or those transfected with mimic-NC; # $P < 0.05$ vs the GCs from PCOS rats or those transfected with inhibitor-NC by independent sample *t*-test. Data are shown as mean $\pm$ s.d. of three technical replicates.

blot analysis showed that the protein level of WT1 and the p-STAT3/STAT3 ratio were elevated secondary to the inhibition of miR-199a-5p in ovarian GCs in the presence of sh-NC, while these effects were reversed following transfection with sh-WT1 ($P < 0.05$; Fig. 4F and G). Thus, miR-199a-5p specifically binds to WT1 and subsequently blocks the JAK/STAT3 pathway in ovarian GCs.

Downregulation of miR-199a-5p inhibits rat ovarian GC apoptosis by activating the JAK/STAT3 pathway *in vitro*

The results of CCK-8 assay showed that the transfection of miR-199a-5p inhibitor increased ovarian GC viability, while tofacitinib (an inhibitor of JAK/STAT3) treatment reversed this finding in cells ($P < 0.05$; Fig. 5A). Flow cytometry

revealed that the apoptosis of GCs was decreased upon inhibition of miR-199a-5p, which was rescued following the treatment of cells with tofacitinib ($P < 0.05$; Fig. 5B).

Western blot analysis showed that the inhibition of miR-199a-5p increased protein levels of Bcl-2 and reduced the protein levels of AMH, cleaved caspase-3 and Bax in cells. These changes were all reversed by tofacitinib treatment in cells ($P < 0.05$; Fig. 5C and D). Consistent with the aforementioned data, these findings demonstrate that inhibition of miR-199a-5p prevents apoptosis of rat ovarian GC through activation of the JAK/STAT3 pathway *in vitro*.

Downregulation of miR-199a-5p inhibits ovarian GC apoptosis by activating the JAK/STAT3 pathway *in vivo*

Finally, we attempted to substantiate the *in vivo* regulation of miR-199a-5p and the JAK/STAT3 pathway, rats were treated with anta-miR-199a-5p and/or tofacitinib (an inhibitor of the JAK/STAT3 pathway). Body weight was lower in PCOS rats following treatment with both anta-miR-199a-5p and DMSO than that of the PCOS rats treated with both scrambled control and DMSO. PCOS rats treated with both anta-miR-199a-5p and tofacitinib had higher body weight compared to PCOS rats treated with both anta-miR-199a-5p and DMSO ($P < 0.05$; Fig. 6A). Analysis using ELISA suggested that PCOS rats with anta-miR-199a-5p and DMSO had lower serum levels of AMH, PG, LH and FSH, and higher E2 serum levels in ovarian GCs relative to those in scrambled controls ($P < 0.05$). The combined treatment of anta-miR-199a-5p and tofacitinib elevated serum levels of AMH, PG, LH, and FSH, while reducing E2 serum levels in PCOS rats when compared to the combined treatment of anta-miR-199a-5p and DMSO ($P < 0.05$; Table 3). HE staining showed that delivery of anta-miR-199a-5p in DMSO reduced the ratio of vesicular ovarian follicles in PCOS rats, while thickness of granule cell layer was coherently higher. With the same treatment, the GCs were intact and arranged neatly, while thinning of the vacuolar membrane layer was observed along with increased number of corpus luteum tissues in rats ($P < 0.05$). However, compared with the group treated with anta-miR-199a-5p and DMSO, there were increased proportion of vesicular ovarian follicles, thin GC layer, atrophied and disarranged GCs, reduced corpus luteum tissues and thickened vacuolar membrane layer in the PCOS rats following dual treatment with anta-miR-199a-5p and tofacitinib ($P < 0.05$) (Fig. 6B and Table 4). anta-miR-199a-5p and/or tofacitinib treatment exerted no

Figure 4

miR-199a-5p inhibits activation of the JAK/STAT3 pathway by targeting WT1 in ovarian GCs. (A) Putative miR-199a-5p binding sites in the 3'untranslated region of WT1 mRNA in the Targetscan website (<http://www.targetscan.org/>). (B) The expression of WT1 was determined by RT-qPCR in ovarian cortical tissues (the left image) and serum (the right image) of PCOS patients ($n = 35$) and healthy controls ($n = 28$), normalized to β -actin; $**P < 0.01$ vs healthy controls by independent sample t -test; (C) Pearson correlation of miR-199a-5p expression with WT1 expression in ovarian cortical tissues of PCOS patients ($n = 35$); (D) the luciferase activity of WT1-wt and WT1-mut was measured by dual-luciferase reporter gene assay in GCs after co-transfection with mimic-NC or miR-199a-5p mimic; $**P < 0.01$ vs the GCs transfected with mimic-NC by independent sample t -test; (E) the expression of WT1 was determined by RT-qPCR in GCs from PCOS rats after transfection with sh-WT1, sh-WT1-1 and sh-WT1-2, normalized to β -actin; $**P < 0.01$ vs the GCs transfected with sh-NC (blank group) by one-way ANOVA with Tukey's *post hoc* test; (F and G) representative Western blots of WT1, p-STAT3 and STAT3 proteins and their quantitation in cells, normalized to β -actin; $**P < 0.01$ vs the GCs co-transfected with inhibitor-NC and sh-NC; $##P < 0.01$ vs the GCs co-transfected with miR-199a-5p inhibitor and sh-NC by one-way ANOVA with Tukey's *post hoc* test. Data are shown as mean $\pm$ s.d. of three technical replicates.

significant effects on body weight, serum hormone levels, or histopathological findings.

TUNEL assay showed lower cell apoptosis rate in the ovarian tissues from the PCOS rats treated with anta-miR-199a-5p in the presence of DMSO ($P < 0.05$), which was rescued by tofacitinib ($P < 0.05$; Fig. 6C and D). Western blot analysis revealed that treatment with anta-miR-199a-5p in DMSO increased levels of Bcl-2 in the ovarian tissues obtained from the PCOS rats, while the levels of AMH, cleaved caspase-3 and Bax were decreased ($P < 0.05$). In comparison to the combined treatment of anta-miR-199a-5p and DMSO, the combined treatment with anta-miR-199a-5p and tofacitinib presented a lower level of Bcl-2 and higher levels of AMH, cleaved caspase-3 and Bax in the ovarian tissues from the PCOS rats ($P < 0.05$; Fig. 6E and F). Nevertheless, inhibition of miR-199a-5p and/or disruption of the JAK/STAT3 pathway did not induce significant changes in cell apoptosis and the levels of apoptosis-related proteins in the ovarian tissues from the control rats, indicating that inhibition of miR-199a-5p

inhibits the apoptosis of rat ovarian GCs by activating the JAK/STAT3 pathway *in vivo*.

Discussion

Dysregulated miRNAs have been implicated in several metabolic disorders, including PCOS (Murri *et al.* 2013). The pathogenesis of PCOS involves insulin-induced apoptosis of GCs (Song *et al.* 2018). Therefore, the present study explored the effect of miR-199a-5p on the apoptosis in ovarian GCs, while monitoring its effects on hormones and functional molecules involved in PCOS progression. We found that forced reduction of miR-199a-5p upregulated WT1 and increased the activity of the JAK/STAT3 pathway, thus resulting in inhibited apoptosis in rat PCOS models.

In our initial studies using surgical samples, ovarian cortical tissues obtained from PCOS patients were observed to have high miR-199a-5p expression. Aberrant expression of miR-199a-5p has been reported in numerous kinds of tumors, such as breast cancer

Figure 5

Downregulation of miR-199a-5p inhibits the apoptosis of ovarian GCs through activation of the JAK/STAT3 pathway *in vitro*. GCs from PCOS rats were treated with tofacitinib or DMSO in the presence of miR-199a-5p inhibitor. (A) Viability of ovarian GCs was assessed by CCK-8 in cells; (B) quantitative analysis for apoptosis of ovarian GCs was examined by flow cytometry in cells; (C and D) representative Western blots of AMH, cleaved caspase-3, Bax and Bcl-2 proteins and their quantitation in cells, normalized to β -actin. ** $P < 0.01$ vs the treatment of inhibitor-NC and DMSO. ## $P < 0.01$ vs the treatment of miR-199a-5p inhibitor and DMSO. One-way ANOVA with Tukey's *post hoc* test was used for data comparison in panel B, C, D, E, and repeated measures ANOVA with Tukey's *post hoc* test was used to analyze data in panel A. Data are shown as mean $\pm$ s.d. of three technical replicates.

(Yi *et al.* 2013), and ovarian cancer (Liu *et al.* 2018). Interestingly, genome-wide circulating miRNA expression profiling identified upregulated levels of miR-122, miR-193b and miR-194 while miR-199b-5p was downregulated in serum obtained from a large cohort of women with PCOS (Jiang *et al.* 2016). However, the upregulation of miR-199a-5p expression in the present study was validated in both serum and ovarian cortical tissue samples of patients with PCOS. Our study further verified that the inhibition of miR-199a-5p repressed apoptosis of ovarian GCs in PCOS rats, as evidenced by increased levels of Bcl-2 and reduced Bax and cleaved-caspase 3 levels. Regarding the effect of miR-199a-5p on GC apoptosis, either inhibitor or mimic treatment only results in 5–10% alteration in apoptosis. According to a previous report (Eng *et al.* 2007), 5–10% of the apoptosis rate significantly improved the pregnancy outcome of the PCOS mouse model. Another study revealed that the proportion of other intervention methods on GC apoptosis rate was also about 10% (Fu *et al.* 2018). Therefore, we believe that 5–10% of the apoptosis rate has important biological significance for the apoptosis of ovarian GCs in PCOS rats.

In addition, miR-199a-5p was identified as the mature form of miR-199a (Lino Cardenas *et al.* 2013). Moreover, another study highlighted the positive correlation of uterine miR-199a levels with PG treatment and negative correlation with E2 treatment (Williams *et al.* 2012). GCs exert crucial functions in the release of sex steroids and various growth factors during the developmental process of the oocyte (Song *et al.* 2018). At the follicular stage of the menstrual cycle, FSH stimulates the conversion of androgens to estradiol in GCs. After ovulation, GCs can differentiate into granulosa lutein cells that produce PG, subsequently aiding maintenance in the presence of pregnancy (Amsterdam *et al.* 1998). In accordance with these aspects of endocrinology, we now find that inhibition of miR-199a-5p increased the serum E2 levels while reducing the serum levels of AMH, P, LH and FSH in the rat PCOS model.

In the current study, the bioinformatics database predicted that WT1 was a target gene of miR-199a-5p, which was further verified by dual-luciferase reporter gene assay. The expression of miR-199a-5p was negatively correlated with that of WT1 in clinical tissue samples.

Figure 6

Downregulation of miR-199a-5p suppresses ovarian GC apoptosis through activation of the JAK/STAT3 pathway in the PCOS rat model. PCOS and control rats were treated with tofacitinib or DMSO in the presence of anta-miR-199a-5p. (A) Body weight of PCOS ($n = 6$) and control rats ($n = 6$) at the 21st day after injection; (B) ovarian morphological changes in PCOS ($n = 6$) and control rats ($n = 6$) (scale bar = 250 μ m, 40 $\times$ at the top; scale bar = 25 μ m, 400 $\times$ at the bottom); (C) cell apoptosis was measured by TUNEL assay in the ovarian tissues from PCOS ($n = 6$) and control rats ($n = 6$) (scale bar = 50 μ m, 200 $\times$); (D) statistical analysis of panel C; (E and F) representative Western blots of AMH, Bcl-2, cleaved caspase-3 and Bax proteins and their quantitation in the ovarian tissues from PCOS ($n = 6$) and control rats ($n = 6$), normalized to β -actin. ** $P < 0.01$ vs the treatment of scramble control and DMSO. ## $P < 0.01$ vs the treatment of anti-miR-199a-5p and DMSO by one-way ANOVA with Tukey's *post hoc* test. The above results are measurement data expressed as mean $\pm$ s.d.

Table 3 Quantitative analysis for serum hormone levels in response to anta-miR-199a-5p and tofacitinib (an inhibitor of JAK/STAT3) alone or in combination.

Group	AMH (pg/mL)	PG (ng/mL)	E2 (pg/mL)	LH (mIU/mL)	FSH (mIU/mL)
Control + scramble control + DMSO	2.09 $\pm$ 0.25	54.27 $\pm$ 6.78	91.36 $\pm$ 5.21	1.43 $\pm$ 0.19	1.20 $\pm$ 0.17
Control + anta-miR-199a-5p + DMSO	2.32 $\pm$ 0.13	56.46 $\pm$ 6.88	97.54 $\pm$ 9.99	1.55 $\pm$ 0.09	1.25 $\pm$ 0.10
Control + anta-miR-199a-5p + tofacitinib	2.04 $\pm$ 0.21	52.51 $\pm$ 5.65	95.16 $\pm$ 8.53	1.62 $\pm$ 0.12	1.27 $\pm$ 4.95
PCOS + scramble control + DMSO	5.71 $\pm$ 0.87	144.71 $\pm$ 14.52	53.54 $\pm$ 6.33	6.32 $\pm$ 0.66	4.95 $\pm$ 0.51
PCOS + anta-miR-199a-5p + DMSO	3.69 $\pm$ 0.35 ^a	78.71 $\pm$ 7.98 ^a	77.63 $\pm$ 8.9 ^a	3.87 $\pm$ 0.34 ^a	2.97 $\pm$ 0.31 ^a
PCOS + anta-miR-199a-5p + tofacitinib	5.43 $\pm$ 0.72 ^b	145.62 $\pm$ 15.32 ^b	54.62 $\pm$ 5.56 ^b	5.87 $\pm$ 0.67 ^b	5.11 $\pm$ 0.52 ^b

One-way ANOVA with Tukey's *post hoc* test was used to compare the data among multiple groups, $n = 6$.

^a $P < 0.01$ compared with the PCOS + scramble control + DMSO group; ^b $P < 0.01$ compared with the PCOS + anta-miR-199a-5p + DMSO group; the above results were measurement data and expressed as mean $\pm$ s.d.

AMH, anti-Mullerian hormone; E2, estradiol hormone; FSH, follicular stimulating hormone; LH, luteinizing hormone; miR-199a-5p, microRNA-199a-5p; PCOS, polycystic ovary syndrome; PG, progesterone.

Table 4 Quantitative analysis for ovarian morphological parameters of rats in response to anta-miR-199a-5p and tofacitinib (an inhibitor of JAK/STAT3) alone or in combination.

Group	Follicles/total follicles (%)	Number of corpus luteum	Thickness of the granular cell layer/ μm	Thickness of the membrane layer/ μm
Control + scramble control + DMSO	12.21	17.17 $\pm$ 1.33	0.216 $\pm$ 0.020	0.011 $\pm$ 0.003
Control + anta-miR-199a-5p + DMSO	12.50	18.17 $\pm$ 1.17	0.220 $\pm$ 0.025	0.011 $\pm$ 0.001
Control + anta-miR-199a-5p + tofacitinib	11.07	16.33 $\pm$ 1.03	0.196 $\pm$ 0.031	0.009 $\pm$ 0.001
PCOS + scramble control + DMSO	40.43	5.67 $\pm$ 1.03	0.042 $\pm$ 0.007	0.051 $\pm$ 0.006
PCOS + anta-miR-199a-5p + DMSO	17.19 ^a	14.67 $\pm$ 1.86 ^a	0.114 $\pm$ 0.013 ^a	0.020 $\pm$ 0.002 ^a
PCOS + anta-miR-199a-5p + tofacitinib	41.75 ^b	6.50 $\pm$ 1.05 ^b	0.039 $\pm$ 0.005 ^b	0.049 $\pm$ 0.004 ^b

The vesicular follicles/total follicles were expressed as a percentage, and other data were measurement data expressed as mean $\pm$ s.d. One-way ANOVA with Tukey's *post hoc* test was used to compare the data among multiple groups, $n = 6$.

^a $P < 0.01$ compared with the PCOS + scramble control + DMSO group; ^b $P < 0.01$ compared with the PCOS + anta-miR-199a-5p + DMSO group. miR-199a-5p, microRNA-199a-5p; PCOS, polycystic ovary syndrome.

The expression of WT1 gene has been previously implicated in human infertility, contributing to malformations of the gonad, impairment in spermatogenesis, and premature ovarian failure (Jedidi *et al.* 2018). Several elements were proven to be involved in the regulation of the apoptosis of ovarian GCs, among which WT1 is an essential factor in maintaining the survival of GC by modulating Bax and caspase 3 in early folliculogenesis (Park *et al.* 2014). Furthermore, a mutation in WT1 (p. P126S and p. R370H) leads to impaired proliferation of GCs (Wang *et al.* 2015), in conjunction with upregulation of follicle-stimulating hormone receptor (FSHR), 3 β -hydroxysteroid dehydrogenase and aromatase as well as defects in polarity establishment in GCs, which is responsible for aberrant follicle development (Gao *et al.* 2014). Based on these findings, miR-199a-5p might potentially target WT1, influencing the functions of GC and follicle development in PCOS.

Furthermore, Li *et al.* provided evidence that the transfection of WT1 isoforms triggered the activation of chemokines and certain factors associated with the JAK/STAT pathway (Li *et al.* 2014). STAT3 is a member of the STAT family of transcription factors, whose activation can be mediated by the JAK in cancer cells (Wen *et al.* 2015). As STAT3 participated in cell proliferation, differentiation, and apoptosis in different cell types, it showed high levels in GCs of subordinate follicles after deviation, thus exerting functions in follicular atresia (Gasperin *et al.* 2015). Upregulation of miR-199a-5p reduced the extent of JAK1 and STAT3 phosphorylation, suggesting that disruption of the JAK/STAT pathway could be induced by miR-199a (Zhu *et al.* 2018). The present results evidently showed that miR-199a-5p blocked the activation of the JAK/STAT3 pathway by targeting WT1. Moreover, STAT3 has been previously shown to be involved in the regulation

of reproduction through the transduction of signals that react to growth factors and cytokines. Functionally, miR-125a-5p induced mouse GC apoptosis by targeting STAT3 (Wang *et al.* 2017). In the present study, treatment with tofacitinib, an inhibitor of the JAK/STAT3 pathway, rescued the ovarian GC apoptosis, which was inhibited following the loss of miR-199a-5p. Hence, we suggest that miR-199a-5p could mediate the development of PCOS through negative regulation of the JAK/STAT3 pathway.

The collective findings from this study were supportive of the notion that inhibition of miR-199a-5p can inhibit rat ovarian GCs apoptosis through the activation of the WT1-mediated JAK/STAT3 pathway. During the onset of PCOS, inhibition of miR-199a-5p triggered activation of the JAK/STAT3 pathway by targeting WT1, thereby inhibiting the apoptosis of ovarian GCs (Fig. 7).

Figure 7

The molecular mechanism involved miR-199a-5p affecting PCOS in relation to WT1 and the JAK/STAT3 pathway. miR-199a-5p disrupted the JAK/STAT3 pathway by targeting WT1, which further downregulated the Bcl-2 expression and upregulated the expression of AMH, cleaved caspase-3 and Bax, thereby enhancing the apoptosis of ovarian GCs and finally inducing the progression of PCOS.

Thus, targeting miR-199a-5p could potentially serve as a promising therapeutic target for PCOS. DHEA, one of most enriched circulating androgens in PCOS, is widely used in the development of a rodent model that displays the significant features commonly observed in PCOS patients (Motta 2010). However, an improved animal model of PCOS shall be taken into consideration since a DHEA-induced PCOS model is insufficient for the exploration of metabolic abnormalities such as insulin resistance (Zhu *et al.* 2018). Due to the complex microenvironments, there may be other molecules subject to the direct regulation by miR-199a-5p, which requires further investigations. Moreover, additional studies are also necessary for other animal models to validate those findings and clinical trials are warranted to test the potential for clinical application of miR-199a-5p-targeted therapy in human PCOS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Shuai Shao and Hui Wang participated in the conception and design of the study. Wei Shao and Na Liu performed the analysis and interpretation of data. Shuai Shao and Wei Shao contributed to drafting the article. All authors have read and approved the final submitted manuscript.

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