MiRNA-335-5p negatively regulates granulosa cell proliferation via SGK3 in PCOS

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Abstract

Polycystic ovary syndrome (PCOS) is a major cause of infertility in women of reproductive age. However, its exact etiology remains unknown. In this study, we sequenced miRNAs in human follicular fluid and identified 16 downregulated and 3 upregulated miRNAs in PCOS group compared with non-PCOS group. Among the differential expressed miRNAs, miR-335-5p was verified lower abundance in PCOS than non-PCOS group using quantitative real-time PCR. Besides, miR-335-5p negatively correlated with antral follicle count, anti-Müllerian hormone and total testosterone. Bioinformatics analysis identified serum/glucocorticoid-regulated kinase family member 3 (*SGK3*) as a potential target gene of miR-335-5p. *SGK3* is involved in protein kinase B-mammalian target of rapamycin kinase (AKT-mTOR) signaling pathway and cell proliferation. Western blotting and cell counting kit-8 assays demonstrated that miR-335-5p mimic reduced, while miR-335-5p inhibitor increased, *SGK3* abundance, AKT-mTOR pathway and cell proliferation in human granulosa-like tumor KGN cells. Dual-luciferase reporter assays showed that miR-335-5p binds to the 3' untranslated region of *SGK3* mRNA. Furthermore, miR-335-5p was decreased and *SGK3* was elevated in human granulosa cells obtained from PCOS patients as compared with non-PCOS controls. These findings suggested that miR-335-5p is involved in granulosa cells proliferation by reducing *SGK3* expression, which might provide a molecular target to improve dysfunctional granulosa cells in patients with PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is a clinical syndrome involving metabolic abnormalities and reproductive disorders in reproductive aged women. It is one of the most common endocrine disorder, with an approximate incidence of 5–10% (Goodarzi *et al.* 2011). The characteristics of PCOS are hyperandrogenemia, polycystic ovarian morphology, menstrual dysfunction, chronic oligo-anovulation and metabolic complications (Moran & Teede 2009, Goodarzi *et al.* 2011). Many studies have suggested that the etiology of PCOS is related to genetic and environmental factors; however, little is known about its exact etiology.

Dysfunction of folliculogenesis is a leading cause of infertility in PCOS (Das *et al.* 2008, Franks *et al.* 2008, Patel & Carr 2008). Human follicular fluid not only provides a life-sustaining microenvironment for oocytes and surrounding granulosa cells but also contains many important regulatory molecules that are important for the maturation and quality of oocytes (Hanrieder *et al.* 2008, Revelli *et al.* 2009). Therefore, follicular fluid plays a crucial role in folliculogenesis. In addition to secretions of granulosa and thecal cells, follicular fluid also contains many plasma components that cross the

blood–follicular barrier via follicular basal lamina and thecal capillaries (Rodgers & Irving-Rodgers 2010), including proteins, metabolites and miRNAs (Hanrieder *et al.* 2008, 2009, Revelli *et al.* 2009, Sang *et al.* 2013).

miRNAs are single-stranded non-coding endogenous RNAs (approximately 20–24 nucleotides in length), which can bind to target gene mRNA, particularly at the 3' untranslated region (3' UTR), thereby regulating its expression (Bartel 2009, Shukla *et al.* 2011). miRNAs are shown to play vital roles in a wide range of physiological processes at the post-transcriptional level (Ying *et al.* 2006). Aberrant expression of miRNAs has been found to accelerate cell proliferation in tumorigenesis and to regulate pathological pathways (Johannidis *et al.* 2008, Ganepola *et al.* 2014, Huan *et al.* 2016). miRNAs can play important roles in the female reproductive system and folliculogenesis (Carletti & Christenson 2009). Sang *et al.* first reported that the supernatant, microvesicles or exosomes from cell-free human follicular fluid contain miRNAs, which are important in metabolic and reproductive signaling pathways (Sang *et al.* 2013). Moreover, the miRNAs in human follicular fluid are powerful tools in the *in vitro* fertilization (IVF) process, which can predict the development of expanded blastocysts and clinical pregnancy outcome (Scalici

et al. 2016). However, there have been few studies concerning the relationship between miRNAs in human follicular fluid and the pathogenesis of PCOS. The aims of our study were (1) to explore the differential expression of miRNAs in follicular fluid of women with PCOS compared with non-PCOS controls and (2) to investigate the role of the differential expressed miRNAs in pathological defects in PCOS, such as dysfunction of granulosa cells or abnormal folliculogenesis.

Based on miRNA-sequencing results, we found that the expression of miR-335-5p was significantly decreased in PCOS patients as compared with non-PCOS women, which suggested that miR-335-5p might be associated with PCOS. Subsequently, we studied the downstream targets of miR-335-5p and its possible mechanism in PCOS, which might provide a promising molecular target for PCOS treatment in the future.

Materials and methods

Patients

In this study, a total of 106 patients were recruited from the Center of Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, between April 2015 and April 2016. The Ethics Committee of Ren Ji Hospital approved the study and all women gave their written informed consent before participation. Fifty-five of them were PCOS patients. The diagnosis of PCOS was performed according to the [Rotterdam Consensus \(2004\)](#). Fifty-one non-PCOS patients underwent IVF or intracytoplasmic sperm injection (ICSI) because of tubal factor or male factor infertility. In this study, participants with genetic diseases, thyroid diseases, a family history of diabetes and serious medical and surgical disease complications were excluded.

Human follicular fluid collection

Patients received human chorionic gonadotrophin during IVF/ICSI when the diameter of the follicle was >18 mm. At the time of oocyte retrieval, the fluid from the first aspirated dominant follicle was collected. The fluid was centrifuged for 10 min at 2500 *g* to remove blood and debris. The supernatant was then collected and cryopreserved at -80°C.

Library construction and miRNA sequencing

The follicular fluids from three PCOS patients and three non-PCOS patients were used for miRNA sequencing. According to the manufacturer's instructions, Ion Total RNA-Seq Kit, v2.0 (Life Technologies) was used to construct cDNA libraries for single-end sequencing. PAGE was applied for size selection of the miRNA-sequencing cDNA library, which was then processed for the proton sequencing process. The mixture of samples that were diluted and mixed was processed on a OneTouch 2 instrument (Life Technologies) and enriched on a OneTouch 2 ES station (Life Technologies) for preparing the template-positive Ion PI Ion Sphere Particles (Life Technologies)

according to Ion PI Hi-Q OT2 200 Kit (Life Technologies). After enrichment, according to Ion PI Hi-Q Sequencing 200 Kit (Life Technologies), the mixed samples were loaded on to 1 v3 Proton Chip (Life Technologies) and sequenced on Proton Sequencers. Furthermore, the EB-Seq algorithm was used to identify differential expressed miRNAs using the following criteria: false discovery rate (FDR) <0.05.

Bioinformatics analysis

Potential miRNA targets were identified in the miRanda database (<http://www.microrna.org/>). Gene ontology (GO) analysis was used to study biological processes for the target genes of differential expressed miRNAs. The GO annotations from NCBI (<http://www.ncbi.nlm.nih.gov/>), UniProt (<http://www.uniprot.org/>) and the Gene Ontology (<http://www.geneontology.org/>) were downloaded. To identify the significant GO categories, Fisher's exact test was applied to calculate the *P* values and the result of multiple hypothesis was used to correct the FDR ([Zeeberg et al. 2003](#), [Al-Shahrour et al. 2004](#)). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was used to identify significant signaling pathways involving the differential expressed miRNA. Fisher's exact test was applied again to identify the significant pathways. In addition, we performed miRNA-mRNA network analysis according to the significant GO analysis and KEGG pathways.

MicroRNA extraction and purification

Additional dominant follicular fluid from 14 PCOS and 16 non-PCOS patients were used to validate the miRNA-sequencing results. According to manufacturer's instructions, RNA from follicular fluid was extracted using the TRIzol LS Reagent (Invitrogen). Firstly, 0.25 mL of follicular fluid was added gently to 0.75 mL of TRIzol reagent. After incubating the mixture for 5 min, 0.2 mL of chloroform was added. The samples were then mixed vigorously for 15 s and incubated for another 10 min at room temperature. The samples were centrifuged at 12,000 *g* for 15 min at 4°C, and the upper clarified phase was slowly transferred to a new tube. Afterward, 0.5 mL of 100% isopropanol was added. The samples were centrifuged at 12,000 *g* for 20 min at 4°C after incubating at -20°C for 3 h. The mixture was washed with 1 mL of 75% ethanol and centrifuged at 12,000 *g* for 5 min at 4°C. The RNA quality was then determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific) after adding 20 µL of RNase-free water. Finally, 250 ng of total RNA was reverse-transcribed to cDNA using an miRNeasy Mini Kit (Qiagen).

Isolation of human granulosa cells

After oocyte retrieval, human granulosa cells from 38 PCOS and 32 non-PCOS patients were used to verify target genes. The granulosa cells were collected and purified as described previously ([Iwase et al. 2009](#)). In brief, the pellets were purified by density centrifugation with Ficoll-Paque (GE-Health Care Bio-Science) and then digested in hyaluronidase (Sigma) at

37°C for 7 min. The granulosa cells were also stored at -80°C until RNA or protein extraction.

Quantitative real-time PCR

Total RNA from granulosa cells and the human granulosa-like tumor cell line, KGN, were extracted using an RNA isolation kit (Foregene), according to the manufacturer's instructions and quantified with NanoDrop ND-2000 spectrophotometer. RNA (300 ng) was reverse-transcribed into cDNA using the PrimeScript RT Master Mix Perfect Real-Time kit (TaKaRa). The miRNA and mRNA expression levels were then assessed using quantitative real-time polymerase chain reaction (qRT-PCR) with a SYBR RT-PCR kit (TaKaRa). The primer sequences for miRNAs and mRNAs were hsa-miR-335-5p, TCA AGA GCA ATA ACG AAA AAT GT; hsa-miR-27b-3p, TTC ACA GTG GCT AAG TTC TGC; hsa-miR-660-5p, TAC CCA TTG CAT ATC GGA GTT G; *U6*, forward: GGA ACG ATA CAG AGA AGA TTA GC, reverse: TGG AAC GCT TCA CGA ATT TGC G; *SGK3*, forward: AAA GAC GAG CAG GAC TAA ACG, reverse: GGA CTG TCC ATT TGA AGG AAT GC and *ACTB*, forward: GGA CTT CGA GCA AGA GAT GG, reverse: AGC ACT GTG TTG GCG TAC AG. The reactions were performed using the following program in an ABI PRISM7700 sequence detector system (Life Technologies, Inc.): 5 min at 95°C; followed by 40 cycles of 10 s at 95°C, 20 s at 59°C and 20 s at 72°C; ending with a 5 min extension at 72°C. Relative expression was normalized to the internal control (*ACTB* for mRNA or *U6* small nuclear RNA for miRNA). Currently, there is no standard for an internal control for miRNAs. *U6* RNA was used as a reference for miRNA analysis in follicular fluid in previous studies (Sang *et al.* 2013, Zhang *et al.* 2017a). The fold changes were analyzed using the $2^{-\Delta\Delta CT}$ (cycle threshold) method (Livak & Schmittgen 2001).

Western blotting analysis

Total protein was extracted using ice cold radioimmunoprecipitation assay (RIPA) lysis buffer (Cwbio), containing a phosphatase inhibitor (Roche) and a protease inhibitor cocktail (Roche). Then, 25 µg of protein from each sample was electrophoresed in SDS polyacrylamide gels and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated with antibodies against SGK3 (serum/glucocorticoid-regulated kinase family member 3; 1:500 dilution; Proteintech), mTOR (mammalian target of rapamycin kinase; 1:1000 dilution; Cell Signaling), Phospho-mTOR (1:1000 dilution; Ser2448, Cell Signaling), AKT (protein kinase B; 1:1000 dilution; Cell Signaling), Phospho-AKT (1:1000 dilution; Ser473, Cell Signaling) overnight at 4°C. After washing four times with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with the appropriated secondary antibody, conjugated with horseradish peroxidase (Proteintech) for 1 h at room temperature. Bands with peroxidase activity were detected by an enhanced chemiluminescent detection system (Merck Millipore) and visualized using a G-Box chemiluminescence image capture system (Syngene). GAPDH (glyceraldehyde-3-phosphate

dehydrogenase; 1:5000 dilution; Proteintech) as a protein loading control.

Cell culture and transfection

The KGN cell line is undifferentiated and possesses the physiological characteristics of ovarian cells. Thus, KGN cells were used to reveal the roles of miR-335-5p in this study. The cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Blood Bioind Stem Origin, Israel), 0.1 mg/mL streptomycin sulfate and 100 U/mL penicillin G (Invitrogen) in a humidified incubator at 37°C with 5% CO₂. Cells were seeded in six-well dishes at 1×10^5 cells per well. The miR-335-5p mimics (sense: UCA AGA GCA AUA ACG AAA AAU GU, antisense: CAU UUU UCG UUA UUG CUC UUG A), corresponding scrambled control (sense: UUU GUA CUA CAC AAA AGU ACU G, antisense: CAG UAC UUU UGU GUA GUA CAA A), miR-335-5p inhibitor (sense: ACA UUU UUC GUU AUU GCU CUU GA) and corresponding scrambled control (sense: CAG UAC UUU UGU GUA GUA CAA A) were purchased from Ribobio (Guangzhou, China). According to the manufacturer's protocol, either miRNA mimics (50 nM/well) or inhibitor (100 nM/well) and their corresponding scrambled controls were transfected into KGN cells using Lipofectamine 3000 (Invitrogen) (7.5 µL/well) diluted in 125 µL of opti-MEM Medium (Gibco). The medium was replaced by fresh medium 24 h after transfection, and cells were cultured for another 24 h.

Dual-luciferase reporter assay

The *SGK3*-3' UTR and the *SGK3*-mutated-3' UTR fragment were amplified by qRT-PCR. The fragment was then cloned into the Psicheck luciferase promoter vector (Promega) to develop the Luc-Psicheck-*SGK3*-3' UTR and Luc-Psicheck-*SGK3*-mut-3' UTR vectors. KGN cells in 24-well plates were co-transfected with Luc-Psicheck-*SGK3*-3' UTR, Luc-Psicheck-*SGK3*-mut-3' UTR and miR-335-5p mimics or its scrambled control using Lipofectamine 3000 reagent (Invitrogen) according to the instructions of the manufacturer. In addition, cells were transfected with the pRL-SV40 vector as an internal standard. After transfection for 48 h, Renilla and firefly luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Cat. No. E1910). The Renilla luciferase activity was normalized to the firefly luciferase activity.

Cell counting Kit-8 assay

Cell proliferation was assessed using the cell counting kit-8 assay (CCK-8) method. To assess the effect of miR-335-5p on proliferation, cells were transfected with miRNAs for 24 h and then reseeded in 96-well plates at 5×10^3 cells/100 µL/well. The cells were cultured for 24, 48 and 72 h. According to the protocol of the manufacturer, CCK-8 solution was added to each well (MCE, USA). The absorbance was read at 450 nm

after incubating for 3 h. The assay was set in sextuplicate and repeated three times.

Statistical analysis

All the results were presented as mean $\pm$ standard error of the mean (S.E.M.). All data were checked for their normal distribution by submission to the Kolmogorov–Smirnov test. After normal distribution test, we used the paired or unpaired Student's *t*-test to determine the significance of the observed differences. We applied Spearman rank correlations to compare and correlate quantitative variables. All statistical analyses were performed using SPSS 22 for Windows (IBM). $P < 0.05$ was considered statistically significant.

Results

Basic characteristics of the human subjects

The clinical characteristics of all participants (55 PCOS and 51 non-PCOS) are summarized in Table 1. No significant differences were observed in terms of age, duration of infertility and levels of follicle-stimulating hormone (FSH), basic estradiol (E2) and prolactin between the PCOS and non-PCOS groups. However, BMI, and the level of total testosterone (TT), luteinizing hormone (LH) and anti-Müllerian hormone (AMH), the antral follicle count (AFC) and the LH/FSH ratio were significantly increased in the patients with PCOS compared with those in the non-PCOS group ($P < 0.05$). Furthermore, the average number of retrieved oocytes in the patients with PCOS was higher than that in the non-PCOS group ($P < 0.05$).

Differential miRNA expression in human follicular fluid samples

Based on the deep miRNA-sequencing results, we obtained a total of 75,942,083 sequences reads, among

which 16,523,445 sequences reads were derived from 2376 miRNAs, which were perfectly matched in the human miRNAs database (miRBase v21). Moreover, the EBSeq algorithm was used to identify miRNAs that were differentially expressed between the PCOS and non-PCOS group. Using our designated criteria of an FDR < 0.05 and a fold change > 1.5 , a total of 19 differential expressed miRNAs were identified in the PCOS group compared with the non-PCOS group, including 3 upregulated and 16 downregulated miRNAs (Table 2), which were shown in the heatmap (Fig. 1A).

The role of miRNAs in targeting essential genes

GO analysis and KEGG pathway enrichment analysis were used to identify significantly enriched biological processes associated with the differential expressed miRNAs between PCOS and non-PCOS patients (shown in Fig. 1B and C). Many biological processes, including the FoxO signaling pathway, NF-kappa B signaling pathway, MAPKs signaling pathway, Wnt signaling pathway and PI3K-AKT signaling pathway were shown to be enriched. All these signaling pathways are correlated to cell proliferation, differentiation and apoptosis (Chang *et al.* 2017, Ma *et al.* 2017, Lu *et al.* 2018). These results suggest that the differential expressed miRNA in human follicular fluid were potentially related to cell proliferation and differentiation processes during folliculogenesis and oocyte maturation.

Verification of miRNAs expression

To validate the reliability of the miRNA-sequencing data, we detected the abundance of hsa-miR-335-5p, hsa-miR-27b-3p, and hsa-miR-660-5p, which were overtly different between the PCOS and non-PCOS groups, using qRT-PCR. We chose miR-335-5p, miR-27b-3p and miR-660-5p due to their high abundance in follicular fluid. Furthermore, the heatmap figure showed these three miRNA samples were homogenous (Fig. 1A). The results showed that the abundance of miR-335-5p was significantly decreased in the follicular fluid from the patients with PCOS compared with that in the follicular fluid from the non-PCOS patients ($P < 0.01$), which was in consistent with the sequencing results (Fig. 2A). The levels of hsa-miR-27b-3p and hsa-miR-660-5p were decreased in the PCOS group; however, the differences were not significant (Fig. 2B and C).

Correlation of basic clinical features with the expression of miR-335-5p

Many clinical features were correlated with the abundance of miR-335-5p in follicular fluid. In our studied subjects, the abundance of miR-335-5p in human follicular fluid was significantly negatively correlated with AFC ($\rho = -0.623$, $P = 0.001$), AMH ($\rho = -0.570$, $P = 0.01$) and TT ($\rho = -0.707$, $P = 0.001$).

Table 1 Basic clinical characteristic of the participants.

	PCOS ($n = 55$)	NC ($n = 51$)	<i>P</i> value
Age (years)	28.13 $\pm$ 0.41	27.37 $\pm$ 0.46	0.225
Duration of infertility (year)	3.47 $\pm$ 0.23	2.88 $\pm$ 0.23	0.075
BMI (kg/m ²)	23.25 $\pm$ 0.45	21.62 $\pm$ 0.38	0.007
FSH (mIU/mL)	6.00 $\pm$ 0.20	6.23 $\pm$ 0.16	0.362
LH (mIU/mL)	9.16 $\pm$ 0.81	4.56 $\pm$ 0.28	< 0.001
LH/FSH ratio	1.51 $\pm$ 0.11	0.74 $\pm$ 0.04	< 0.001
Basic E2 (pg/mL)	37.06 $\pm$ 2.46	39.73 $\pm$ 4.25	0.581
TT (nmol/L)	1.33 $\pm$ 0.09	0.83 $\pm$ 0.06	< 0.001
Prolactin (ng/mL)	16.93 $\pm$ 1.03	19.66 $\pm$ 1.40	0.116
AMH (ng/mL)	12.31 $\pm$ 0.77	5.09 $\pm$ 0.23	< 0.001
AFC (<i>n</i>)	22.78 $\pm$ 1.02	14.76 $\pm$ 0.52	< 0.001
Retrieved oocytes (<i>n</i>)	18.58 $\pm$ 0.99	12.45 $\pm$ 0.65	< 0.001

Values are presented as mean $\pm$ S.E.M. Significant statistical difference ($P < 0.05$).

AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NC, negative control; TT, total testosterone.

Table 2 Differentially expressed miRNAs of PCOS group compared with non-PCOS group (FDR <0.05).

miRNA	PCOS (n=3)	NC (n=3)	Log2FC	FDR	Trend
hsa-miR-664a-3p	212.97	102.33	1.06	8.46759E-08	Up
hsa-miR-200c-3p	2238.49	67.33	5.06	0.016756936	Up
hsa-miR-449b-5p	97.53	24.30	2.01	0.036780595	Up
hsa-miR-202-5p	36372.76	75295.85	-1.05	0	Down
hsa-miR-335-5p	1974.33	5536.41	-1.49	2.4869E-14	Down
hsa-miR-660-5p	2329.12	3916.78	-0.75	1.07248E-13	Down
hsa-miR-185-5p	3837.98	5791.81	-0.60	2.81261E-08	Down
hsa-miR-188-5p	188.50	378.05	-1.000	6.75422E-05	Down
hsa-miR-507	42.85	128.51	-1.580	0.000296398	Down
hsa-miR-513c-3p	10.05	59.57	-2.57	0.000308073	Down
hsa-miR-509-5p	245.28	806.62	-1.72	0.000317965	Down
hsa-miR-501-3p	339.47	512.14	-0.59	0.000679795	Down
hsa-miR-30a-3p	1647.71	2749.27	-0.74	0.001498431	Down
hsa-miR-513a-3p	17.15	72.55	-2.08	0.001823906	Down
hsa-miR-22-5p	1031.50	2094.05	-1.02	0.006488394	Down
hsa-miR-27b-3p	7842.16	13488.83	-0.78	0.009647922	Down
hsa-miR-5196-3p	10.98	38.47	-1.81	0.010461877	Down
hsa-miR-1224-5p	63.68	129.00	-1.02	0.021641476	Down
hsa-miR-548ak	10.02	28.69	-1.52	0.02263186	Down

FDR, false discovery rate; NC, negative control; PCOS, polycystic ovarian syndrome.

However, the miR-335-5p level in human follicular fluid was not associated with BMI ($\rho = -0.256$, $P = 0.172$), the number of retrieved oocytes ($\rho = -0.323$, $P = 0.082$), FSH and LH ($\rho = 0.022$, $P = 0.911$; $\rho = 0.202$, $P = 0.283$, respectively). These results indicate that a low abundance of miR-335-5p might be correlated with abnormal folliculogenesis in PCOS.

Network analysis of miR-335-5p

The results of miR-335-5p-mRNA network were shown in Fig. 3. KEGG pathway enrichment analysis revealed important roles of miR-335-5p in the folliculogenesis process and the normal function of ovaries, including the Wnt, MAPK, TGFB and PI3K-AKT signaling pathways. GO analysis demonstrated certain biological functions associated with miR-335-5p, such as embryo development, antral ovarian follicle growth and regulation of cell proliferation. This figure also showed

certain potential target genes of miR-335-5p, such as *SGK3*, *FOXO3*, *TGFBR1* and *CD47*. Up to now, little is known about the role of *SGK3* in granulosa cells. Thus, *SGK3* was selected as target gene in our study.

miR-335-5p directly inhibits SGK3 expression

To predict the target genes of miR-335-5p, we used bioinformatics prediction software to search for its direct mRNA targets. According to KEGG and GO analysis results, *SGK3* caught our attention among the predicted target genes of miR-335-5p. To determine whether *SGK3* is a direct target gene of miR-335-5p, we cloned the *SGK3* wild-type 3' UTR (wt 3' UTR) or a deletion mutated sequence (mut 3' UTR) into a reporter plasmid, downstream of the luciferase gene. The dual-luciferase plasmid construction model was shown in Fig. 4A. The results showed that miR-335-5p mimics reduced the fluorescence of wt 3' UTR compared with

Figure 1 (A) Heatmap of the 19 miRNAs that were differentially expressed in the follicular fluid between PCOS ($n = 3$) and non-PCOS groups ($n = 3$). The left three columns are the non-PCOS group and the right three columns are the PCOS group. Green to red equates to an increase in miRNA expression. (B) Gene ontology (GO) analysis of the biological processes of the target genes of the differential expressed miRNAs in follicular fluid. (C) KEGG pathway analysis of the target genes of the differential expressed miRNAs in the follicular fluid.

Figure 2 Verification of the miRNA-sequencing results by qRT-PCR in human follicular fluid. Differential abundance of miR-335-5p, miR-27b-3p and miR-660-5p by qRT-PCR in additional follicular fluid samples from PCOS ($n = 14$) and non-PCOS ($n = 16$) patients are shown in (A), (B) and (C), respectively. Significant statistical difference ($P < 0.05$). Student's t -test was used and all data are reported as mean $\pm$ S.E.M. ** $P < 0.01$.

the control group (Fig. 4B, lanes 1, 2, 3 and 4) while had no effect on the fluorescence of mut 3' UTR (Fig. 4B, lanes 5 and 6). Furthermore, the mimics or inhibitor of miR-335-5p were transfected into KGN cells to observe the mRNA and protein expression levels of SGK3. The results showed that miR-335-5p mimics reduced, while miR-335-5p inhibitor increased, the mRNA and protein expression levels of SGK3 in KGN cells. These results suggest that SGK3 is a direct target gene of miR-335-5p.

miR-335-5p inhibited KGN cells proliferation

Since SGK3 was a target gene regulated by miR-335-5p, and it was shown to affect the cell proliferation in many types of cells (Sun *et al.* 2016, Chen *et al.* 2018), we hypothesized that miR-335-5p might be associated with the growth and proliferation in granulosa cells. CCK-8

assay revealed that the miR-335-5p mimics significantly decreased cell proliferation, whereas the miR-335-5p inhibitor promoted the proliferation of KGN cells (Fig. 5A and B), suggesting that miR-335-5p may be a cell proliferation suppressor in granulosa cells.

Effects of miR-335-5p on the PI3K-AKT signaling pathway

In order to identify the signaling pathway that was involved in the regulation of cell proliferation by miR-335-5p, we measured the total protein levels of AKT and mTOR, as well as the phosphorylation of AKT and mTOR, which was screened by KEGG pathway enrichment analysis (Fig. 1C) and was shown to affect the cell proliferation (Liu *et al.* 2018, Wang *et al.* 2018). The results showed that miR-335-5p mimics obviously

Figure 3 miRNA-mRNA network analysis of miR-335-5p according to the significant GO and KEGG pathways. The purple cycle nodes on the left represent genes identified by KEGG pathway analysis, and the orange cycle nodes represent genes identified by GO analysis.

Figure 4 miR-335-5p directly inhibited *SGK3* expression by binding to its 3'-untranslated region (UTR). (A) The dual-luciferase plasmid model of *SGK3*. (B) Validation of miR-335-5p binding to the *SGK3* 3'-UTR using the dual-luciferase reporter assay. (C) and (F) miR-335-5p mimics or inhibitor transfection efficiencies. (D) and (E) mRNA and protein levels of *SGK3* after miR-335-5p mimics transfection. (G) and (H) mRNA and protein levels of *SGK3* after miR-335-5p inhibitor transfection. The relative protein level was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Significant statistical difference ($P < 0.05$). Student's *t*-test was used and all data are reported as mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$.

inhibited the activation of AKT and mTOR (Fig. 5C), as shown by the reduced the phosphorylation of AKT and mTOR. In contrast, the miR-335-5p inhibitor promoted

the activation of AKT and mTOR. These results indicate that miR-335-5p might exert its effect through the PI3K-AKT-mTOR signaling pathway.

Figure 5 The effect of overexpression of miR-335-5p on the proliferation of KGN cells. (A) CCK-8 assay revealing that miR-335-5p mimics led to the suppression in proliferation. (B) The miR-335-5p inhibitor promoted the proliferation of KGN cells. (C) and (D) The protein expression levels of AKT, p-AKT, mTOR and p-mTOR in KGN cells after miR-335-5p mimics or inhibitor transfection. The relative protein level was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Significant statistical difference ($P < 0.05$). Student's *t*-test was used and all data are reported as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$.

SGK3 expression increased in PCOS granulosa cells

In order to verify the association of SGK3 and miR-335-5p with PCOS development, we further tested the abundance of miR-335-5p and SGK3 in human granulosa cells obtained from PCOS and non-PCOS patients. The results show that the abundance of miR-335-5p was decreased in human granulosa cells from the PCOS group (Fig. 6A), which was consistent with the results in follicular fluid, while the abundance of SGK3 mRNA and protein were significantly increased in the PCOS group (Fig. 6B and C). These observations may indicate a crucial role of miR-335-5p and SGK3 in the development of PCOS.

Discussion

In this study, we used high-throughput sequencing to identify differential expressed miRNAs in human follicular fluid of PCOS and non-PCOS patients.

We observed a low abundance of miR-335-5p in the follicular fluid of patients with PCOS and explored its possible role in the pathogenesis of PCOS.

Our sequencing results demonstrated that 19 miRNAs were differentially expressed between the PCOS and non-PCOS groups. Among these differential expressed miRNAs, miR-335-5p was further verified to present a lower level in the follicular fluid of PCOS patients compared with that in non-PCOS patients by qRT-PCR. Furthermore, correlation analysis of miR-335-5p and clinical characteristics showed that miR-335-5p was negatively correlated with AFC and AMH, which were proposed as predictors of ovarian reserve and clinical outcome in different infertility states (Broekmans *et al.* 2006). AMH prevents premature depletion of ovarian follicles and regulates steroidogenesis in granulosa cells (La Marca *et al.* 2009), and plasma AMH levels and AFC were significantly higher in patients with PCOS than in non-PCOS patients. Therefore, we believed that the abundance of miR-335-5p was related to

Figure 6 (A) and (B) The abundance of miR-335-5p and the mRNA level of *SGK3* in the PCOS ($n=32$) and non-PCOS ($n=25$) groups. (C) The *SGK3* protein level in the PCOS ($n=6$) and non-PCOS ($n=7$) group. The relative protein level was normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Significant statistical difference ($P<0.05$). Student's *t*-test was used and all data are reported as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$.

endocrine abnormality and an increased number of growing follicles.

miR-335 was reported to be an anticancer agent in various organs (Scarola *et al.* 2010, Tome *et al.* 2011, Cao *et al.* 2013, Zhang *et al.* 2017b) and participate in the process of bone formation and differentiation (Zhang *et al.* 2011, Wang *et al.* 2017), which suggest that miR-335-5p may play an important role in cell proliferation and migration. However, there has been no report implying an effect of miR-335-5p in human follicular fluid and granulosa cells. Using GO and KEGG pathway analysis, as well as bioinformatics software, we revealed that *SGK3* was a potential target gene for miR-335-5p, which were confirmed by the dual-luciferase reporter assay and transfection of miR-335-5p mimics and inhibitors into KGN cells. KGN cell line was a valid model for granulosa cells. One study reported the developmental stage of KGN cells may be close to that of immature granulosa cells (Nishi *et al.* 2001).

The *SGK* family, including *SGK1*, *SGK2* and *SGK3*, dominates a wide range of biological responses, including control of cell growth, proliferation, metabolism and intracellular material exchange possesses (Pearce *et al.* 2010). Alliston *et al.* demonstrated that *SGK* is located in the nucleus and regulates the proliferation and differentiation of granulosa cells (Alliston *et al.* 2000). Given the *SGK3* is the direct inhibited by miR-335-5p and miR-335-5p was involved in the proliferation of

granulosa cells, we believed that the effect of miR-335-5p on granulosa cells proliferation might be mediated by downregulation of *SGK3* expression, which is further supposed by the findings that the abundance of *SGK3* is higher, whereas the abundance of miR-335-5p is lower in granulosa cells in PCOS patients compared with those in non-PCOS subjects. Furthermore, GO and KEGG pathway analysis results also revealed that miR-335-5p is involved in PI3K-AKT-mTOR signaling pathway, a critical regulator of proliferation and differentiation of granulosa cells (Makker *et al.* 2014), which further confirmed by western blotting. Since *SGK3* could activate mTORC1 by phosphorylating TSC complex subunit 2 (TSC2) (Bago *et al.* 2016), we suspected that the effect of miR-335-5p on granulosa cells proliferation might be mediated by activation of mTOR pathway through induction of *SGK3* expression.

In conclusion, we confirmed that human follicular fluid contained large amounts of miRNAs, some of which were differentially expressed between non-PCOS and PCOS patients. Among these differential expressed miRNAs, miR-335-5p abundance was observed lower both in follicular fluid and granulosa cells obtained from PCOS patients as compared with non-PCOS patients. We further revealed that miR-335-5p suppressed granulosa cell proliferation, which might be mediated by mTOR pathway via targeting and inhibiting *SGK3* expression. These observations provided a new perspective on the dysfunction of granulosa cells in PCOS and indicated that miR-335-5p could be a new molecular target to improve dysfunctional granulosa cells in 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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