Identification of altered microRNAs and mRNAs in the cumulus cells of PCOS patients: miRNA-509-3p promotes oestradiol secretion by targeting *MAP3K8*

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Abstract

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder in women and is characterised by polycystic ovaries, hyperandrogenism and chronic anovulation. Although the clinical and biochemical signs of PCOS are typically heterogeneous, abnormal folliculogenesis is considered a common characteristic of PCOS. Our aim is to identify the altered miRNA and mRNA expression profiles in the cumulus cells of PCOS patients to investigate their molecular function in the aetiology and pathophysiology of PCOS. In this study, the miRNA expression profiles of the cumulus cell samples isolated from five PCOS and five control patients were determined by an miRNA microarray. At the same time, the altered mRNA profiles of the same cumulus cell samples were also identified by a cDNA microarray. From the microarray data, 17 miRNAs and 1263 mRNAs showed significantly different expression in the PCOS cumulus cells. The differentially expressed miRNA-509-3p and its potential target gene (*MAP3K8*) were identified from the miRNA and mRNA microarrays respectively. The expression of miRNA-509-3p was up-regulated and *MAP3K8* was down-regulated in the PCOS cumulus cells. The direct interaction between miRNA-509-3p and *MAP3K8* was confirmed by a luciferase activity assay in KGN cells. In addition, miRNA-509-3p mimics or inhibitor transfection tests in KGN cells further confirmed that miRNA-509-3p improved oestradiol (E_2) secretion by inhibiting the expression of *MAP3K8*. These results help to characterise the pathogenesis of anovulation in PCOS, especially the regulation of E_2 production.

Reproduction (2016) **151** 643–655

Introduction

Polycystic ovary syndrome (PCOS) is the most common and complex endocrinopathy. It affects 6–8% of reproductive-aged women (Shi *et al.* 2012) and accounts for approximately 75% of anovulatory infertility (Diao *et al.* 2004). It is a multi-factorial and heterogeneous syndrome with variable phenotypes, including hyperandrogenism, menstrual irregularity and polycystic ovarian morphology (Group REA-SPCW 2004). In addition to infertility, patients with PCOS have an increased risk of developing metabolic disorders, such as type 2 diabetes (T2D), insulin resistance, obesity (Kandaraki *et al.* 2009) and cardiovascular disease (Wild *et al.* 2000, Carmina 2009).

Although the clinical and biochemical signs of PCOS are typically heterogeneous, abnormal folliculogenesis is considered a common characteristic of PCOS

(Fux Otta *et al.* 2013, Christakou & Diamanti-Kandarakis 2014). In fact, there is an intrinsic difference between the polycystic ovary (PCO) and the normal ovary during the process of folliculogenesis (Franks *et al.* 1998). Anovulation is often induced by abnormal folliculogenesis in PCOS, which is characterised by the apparent arrest of antral follicle development at the 5–10 mm stage and, consequently, the failure to enter the preovulatory phase of the cycle (Franks *et al.* 1988). Although the exact pathogenesis of anovulation in PCOS is unclear, possible mechanisms have been proposed. Specifically, high levels of oestradiol in PCOS patients prevent an increase in follicle-stimulating hormone (FSH) level, which is an essential factor for follicular growth and ovulation induction. A low level of FSH consequently leads to anovulation, a major feature of PCOS (Kenigsberg *et al.* 2009).

To elucidate the pathophysiology of abnormal folliculogenesis in PCOS, many genes were identified using cDNA microarrays in theca cells (Wood *et al.* 2004), whole ovaries (Jansen *et al.* 2004, Oksjoki *et al.* 2005), oocytes (Wood *et al.* 2007), cumulus cells (Kenigsberg *et al.* 2009, Haouzi *et al.* 2012) and adipose tissue (Manneras-Holm *et al.* 2014). These studies revealed several ontologies and genes that are primarily involved in the insulin receptor signalling pathway, steroid biosynthesis and regulation of gonadotropin secretion (Mohamed-Hussein & Harun 2009). However, how these genes are post-transcriptionally regulated is poorly understood.

MicroRNA is a single-stranded and small non-coding RNA sequence (19–25 nucleotides in length) that negatively regulates the expression of target genes in a post-transcriptional manner, either by inhibiting translation or by degrading the target mRNA (Yates *et al.* 2013). It is notable that miRNAs were related to several reproductive disorders, such as endometriosis (Carletti & Christenson 2009), poor ovarian response (Donadeu *et al.* 2012) and, very recently, PCOS (Carletti & Christenson 2009, Hawkins & Matzuk 2010, Chen *et al.* 2013, Hossain *et al.* 2013, Murri *et al.* 2013, Roth *et al.* 2014, Liu *et al.* 2015, Shi *et al.* 2015, Xu *et al.* 2015). Regarding PCOS, microarray profiling of human follicular fluid revealed that 29 miRNAs were differentially expressed between PCOS and fertile oocyte donors. Pathway analysis revealed that target genes were involved in insulin regulation and inflammation (Roth *et al.* 2014). miRNA-21, miRNA-27b, miRNA-103 and miRNA-155 were differentially expressed in the serum between PCOS and normal women (Murri *et al.* 2013). It has been shown that miRNAs that play important roles in the metabolic and immune system processes are influenced by obesity and circulating androgen concentrations in PCOS patients. In addition, miRNAs are involved in regulating blastocyst development. The experiment showed that morphologically similar blastocysts derived from patients with polycystic ovaries exhibited a significant decrease in the expression of six miRNAs (let-7a, miRNA-19a, miRNA-19b, miRNA-24, miRNA-92 and miRNA-93) compared with donor-fertile control blastocysts (McCallie *et al.* 2010).

The aim of this study is to establish the miRNA and mRNA expression profiles of the cumulus cells isolated from PCOS or control patients who were under controlled ovarian stimulation (COS) cycles using a miRNA or a cDNA microarray analysis. The molecular functions of the differentially expressed miRNAs and the predictive target genes were further investigated. The results help to characterise the pathogenesis of abnormal folliculogenesis in PCOS.

Materials and methods

Participants

This study was approved by the Institutional Ethics Review Board of Affiliated Hospital of Qingdao Medical University

(Yuhuangding Hospital of Yantai, Shandong, People's Republic of China). A total of 36 participants (18 PCOS and 18 controls), who referred to our centre for IVF, were included in this study after obtaining written informed consent. All of the patients had no history of drugs affecting glucose and lipid metabolism and were without any known medical conditions or diseases, including Cushing's syndrome, congenital adrenal hyperplasia, androgen-secreting tumours and endometriosis. We measured the levels of FSH, luteotrophic hormone (LH), oestradiol (E₂), testosterone (T) and progesterone (P) on day 3 of the menstrual cycle before the ovarian stimulation for all of the patients by radioimmunoassay (RIA). The PCOS patients were diagnosed according to the revised Rotterdam European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine Criteria (2004). The PCOS patients were required to present at least two of the following criteria: chronic oligo-ovulation or anovulation, androgen excess and polycystic ovaries. The inclusion criteria of the recruited PCOS patients in this study were as follows: age <36 years, body mass index (BMI) ranging between 20 and 26 kg/m², basal serum LH/FSH levels more than 2.0 mIU/mL, serum testosterone levels more than 0.5 ng/mL, antral follicle count ranging between 18 and 35 and number of obtained oocytes ranging between 12 and 28 per cycle. The control patients had regular menstrual cycles, normal ovary sonographs and normal ovulating with bilateral tube occlusion; were non-diabetic; and showed no clinical signs of hyperandrogenism and anovulation. The clinical characteristics of the PCOS and control patients are summarised in Table 1.

Ovarian stimulation

Patients in both the groups received an agonist protocol as described previously (Huang *et al.* 2013a). Specifically, all of the patients subcutaneously received the GnRH agonist triptorelin acetate (0.05 mg/day, diphereline; Ipsen Pharma Biotech, Paris, France) starting at the mid-luteal phase. Once adequate pituitary down-regulation was confirmed (serum LH levels <3.0 ng/mL and serum E₂ levels <30 pg/mL), the patients

Table 1 Clinical characteristics of patients.

	Normal (n=18)	PCOS (n=18)	P value
Age (years)	34.6±2.2	32.6±3.1	NS
BMI (kg/m ²)	21.4±1.8	21.6±1.5	NS
FSH (mIU/mL)	6.51±1.2	5.41±1.3	<0.05
LH (mIU/mL)	4.30±1.4	11.91±2.6	<0.001
Basal LH/FSH	0.65±0.2	2.20±0.7	<0.001
E ₂ (pg/mL)	38.5±4.3	41.2±9.8	NS
Testosterone (ng/mL)	0.13±0.05	0.64±0.05	<0.001
Progesterone (ng/mL)	0.55±0.22	0.70±0.23	NS
Antral follicle count	10.2±1.1	24.1±3.7	<0.001
Oocytes obtained	8.5±3.0	17.8±5.2	<0.001
No. of MII oocytes	6.3±1.8	14.5±4.6	<0.001
No. of 2PN oocytes	5.7±1.4	13.8±3.9	<0.001
No. of transferable embryos (D3)	4.1±1.9	8.23±3.2	<0.001

Data are presented as the mean ± S.E.M.

NS, not statistically significant; BMI, body mass index; E₂, oestradiol; FSH, follicle-stimulating hormone; LH, luteotrophic hormone; PCOS, polycystic ovary syndrome; 2PN, two pronuclei in oocytes with normal fertilisation.

subcutaneously received recombinant FSH (150–187.5 IU; Gonaf, Follitropin Alfa, Serono) for COS. When two or more follicles were at least 18 mm in diameter and the serum E_2 levels were at least 300 pg/mL per dominant follicle, all patients received 250 µg hCG (Profasi; Serono).

Cumulus cell collection

The cumulus cells were collected as previously described (Huang *et al.* 2013a,b). Briefly, cumulus–oocyte complex (COC) retrieval was performed by vaginal puncture under ultrasound echo-guidance 36 h after hCG administration. After COC retrieval, a portion of the CCs surrounding a single oocyte was removed using a sharp needle, lysed in 80 µL lysis buffer (mirVana miRNA Isolation Kit; Ambion) and stored at –80°C until RNA extraction. The cumulus cells isolated from the five PCOS and five control patients were used in miRNA and mRNA microarrays. The cumulus cells isolated from others patients (13 PCOS and 13 control patients) were used in qRT-PCR and western blotting analyses.

KGN cell culture

KGN cells were selected and used for a luciferase activity assay and miRNA mimics/inhibitor transfection experiments. KGN (RCB1154; RIKEN, Wako, Japan) is a steroidogenic human ovarian granulosa tumour cell line. The characteristics of the KGN cells are as follows: (1) they showed detectable levels of basal secretion of pregnenolone and progesterone (P), both of which increased after stimulation with $(Bu)_2$ cAMP; (2) they showed basal secretion of the 17 α -hydroxylated steroids, dehydroepiandrosterone androstenedione, and the E_2 levels were very low or undetectable. All of the above steroids showed no significant change after stimulation with $(Bu)_2$ cAMP; and (3) the KGN cells exhibited a relatively high aromatase activity. When the cells were exogenously provided with enough androstenedione or testosterone, the secretion of oestrone and E_2 by the KGN cells was detectable (Nishi *et al.* 2001).

In this study, the KGN cells were cultured in 1:1 Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin). On the day before the luciferase activity assay or the miRNA transfection, the KGN cells were placed into the medium without serum and incubated overnight.

RNA extraction, labelling and hybridisation, and data analysis

The cumulus cells isolated from one patient (PCOS or control) were pooled together as one sample to extract the total RNA and were analysed individually. Total RNA containing small RNA was extracted from the cumulus cells using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocols. The purity and concentration of RNA were determined from OD_{260/280} readings using a spectrophotometer (NanoDrop ND-1000). RNA integrity was determined by capillary electrophoresis using the RNA

6000 Nano Lab-on-a-Chip kit and Bioanalyser 2100 (Agilent Technologies). Only RNA extracts with RNA integrity number values >6 underwent further analysis.

miRNA profiling was performed using an Agilent miRNA array. The Agilent array was designed with eight identical arrays per slide (8 × 60 K format), with each array containing probes interrogating 1887 human mature miRNAs from miRBase R18.0. Each miRNA was detected by probes repeated 30 times. The array also contained 2164 Agilent control probes. Microarray experiments were conducted according to the manufacturer's instructions. Briefly, the miRNAs were labelled using the Agilent miRNA labelling reagent. Total RNA (100 ng) was dephosphorylated and ligated with pCp-Cy3, and the labelled RNA was purified and hybridised to miRNA arrays.

mRNA profiling was performed using the Agilent human lncRNA+mRNA Array v2.0 (4 × 180 K format). Each array contains probes that interrogate approximately 39,000 human lncRNAs and approximately 32,000 mRNAs. The array also contained 4974 Agilent control probes. The microarray experiments were conducted according to the manufacturer's instructions as described by our recently published reports (Huang *et al.* 2016). Briefly, cDNA was labelled with a fluorescent dye (Cy3-dCTP) using the Eberwine's linear RNA amplification method and a subsequent enzymatic reaction. This procedure was improved using the CapitalBio cRNA Amplification and Labelling Kit (CapitalBio Corporation, Beijing, People's Republic of China). The labelled controls and the test samples, labelled with Cy3-dCTP, were dissolved in hybridisation solution. The arrays were hybridised in an Agilent hybridisation oven overnight.

The images were scanned using the Agilent microarray scanner (Agilent), gridded and analysed using Agilent feature extraction software version 10.10. The miRNA or mRNA array data were analysed for data summarisation, normalisation and quality control using GeneSpring software V12 (Agilent) separately. The default 90th (or 75th) percentile normalisation method was performed for the miRNA (or mRNA) array data preprocessing. To select the differentially expressed genes, we used threshold values with ≥ 1.5 - and ≤ -1.5 -fold changes and a *t*-test with a corrected *P* value of 0.05. The data were Log₂ transformed, and the median was centred by genes using the Adjust Data function of CLUSTER 3.0 software. Next, the data were further analysed using hierarchical clustering with average linkage. Finally, a tree visualisation was performed using Java Treeview (Stanford University School of Medicine, Stanford, CA, USA). The microarray data were analysed by CapitalBio Corporation, Beijing, People's Republic of China.

Target prediction and bioinformatics analysis of miRNAs

The targeted mRNA of the differentially expressed miRNAs was predicted by miRWalk 2.0 (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>) (Dweep *et al.* 2014). The database included information on miRNA–target interaction produced by several established miRNA prediction programs (i.e. RNA22, miRanda, miRDB, TargetScan, RNAhybrid, PITA, miRWalk) on 3'-UTRs of all known human, mouse and rat

genes (P value ≤ 0.05) (Dweep *et al.* 2011). Next, the miRNA-predicted target genes were analysed using a free web-based Molecular Annotation System 3.0 (MAS 3.0; www.capitalbio.com), which integrates three different open-source pathway resources: KEGG, BioCarta and GenMAPP. The significantly represented pathway was chosen by the threshold of the P value and FDR (corrected P value) < 0.05 derived from the hypergeometric test.

Candidate miRNAs and target gene expressions detected by quantitative real-time PCR

In this study, the expression levels of candidate miRNAs and target genes were tested by quantitative real-time PCR in additional ten PCOS and ten control samples respectively. To detect the expression of candidate miRNAs, 1 μ g total RNA was reverse transcribed into cDNA using a miScript Reverse Transcription Kit (Qiagen), and qRT-PCR was performed using the SYBR Green Assay (Takara Bio) according to the manufacturer's protocols. U6 was used as an internal control. The 20 μ L reaction mixture contained 10 μ L of 2 $\times$ QuantiTest SYBR Green PCR Master mix, 2 μ L of 10 $\times$ miScript Universal Primer, 0.5 μ L specific miRNA primer, 1 μ L cDNA template and RNase-free water. The PCR program was as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

To determine the mRNA expression level of predictive target genes, the first-strand complementary synthesis reaction was performed using a PrimeScript RT reagent kit (Perfect Real Time; TaKaRa Bio). Amplification reactions were conducted using SYBR Premix Ex Taq (Perfect Real Time; TaKaRa Bio) and an ABI PRISM 7300 system. *GAPDH* was used as an internal reference. The 20 μ L PCR mixture contained 10 μ L of 2 $\times$ QuantiTest SYBR Green PCR Master mix, 1 μ L cDNA template, 1 μ L each primer and RNase-free water. The PCR reactions were performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 60 s.

Each set of qRT-PCRs was repeated at least three times, and the fold change in the expression of each gene was analysed using the DDCT method (Livak & Schmittgen 2001). qRT-PCR primers of miRNAs and the target genes are listed in Table S1, see section on supplementary data given at the end of this article.

Western blotting analysis

The protein levels of miRNA target gene (*MAP3K8*) in the cumulus cells of PCOS and control patients were analysed using western blotting. Briefly, the protein lysates from cumulus cells were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Amersham Biosciences). After blocking in TBST (0.5% Tween 20 in TBS) containing 5% non-fat milk powder for 1 h, the membranes were incubated overnight at 4°C with an Anti-MAP3K8 antibody (Abcam; ab137589) and a monoclonal anti-GAPDH antibody (Sigma-Aldrich). The blots were then incubated with alkaline phosphatase (AP)-conjugated anti-mouse/rabbit IgG antibody (Promega). AP activity was recorded using the Lumi-Phos Kit (Pierce).

Luciferase reporter constructs and the luciferase activity assay

Molecular constructs were derived from the pmirGLO vector (Promega) by cloning of the DNA sequences encoding the 3'-UTR of miRNA-509-3p target gene (*MAP3K8*) downstream of the Renilla luciferase ORF. To construct luciferase reporters with mutations, PCR was performed using two sets of primers to generate two fragments of the 3-UTRs overlapping at the mutation sites. Next, these two fragments were annealed and used as templates to amplify mutated 3'-UTRs. For partial length 3'-UTR cloning, sequences encompassing the putative miRNA binding sites were amplified and cloned into the SacI and XhoI restrictions sites.

KGN cells were plated onto 24-well plates and allowed to grow for ~24 h before transfection. The constructed reporter vectors (300 ng) were transfected into cells together with the miRNA mimics and control mimics (100 nM) in Lipofectamine 2000 (2 μ L). Cells were lysed after ~24 h of transfection, and the luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalised to Renilla luciferase activities. The experiments were performed independently in triplicate.

MicroRNA mimics or inhibitor transfections in the KGN cells

To investigate the relationship between the candidate miRNA (miRNA-509-3p) and the target gene (*MAP3K8*), miRNA-509-3p mimics or an inhibitor was transfected into the KGN cells. The miRNA-509-3p mimics (forward: 5'-UGAUUGGUACGUCUGUGGGUAG-3'; reverse: 5'-ACCCACAGACGUACCAUCAUU-3') and the miRNA-509-3p inhibitor (5'-CUACCCACAGACGUACCAUCA-3') were commercially synthesised by Gene Pharma (Shanghai, China). A stable negative control (nc mimic) (forward: 5'-UUCUCCGAACGUGUCACGUTT-3'; reverse: 5'-ACGUGACACGUUCGGAGAATT-3') and a nc inhibitor (5'-CAGUACUUUUGUGUAGUACAA-3') were used as the negative controls. The mimics and inhibitors of miRNA-509-3p and their scrambled controls were transfected into the KGN cells with Lipofectamine 2000 (Invitrogen). After a 48-h transfection, the expressions of *MAP3K8* (mRNA or protein levels) and miRNA-509-3p in the corresponding transfected KGN cells were detected by qRT-PCR and western blotting analyses. Each experiment was performed six times.

Assay for E_2 secretion in the transfected KGN cells

Oestradiol secretion in the transfected KGN cells was determined by testing E_2 accumulation in the culture medium. The transfected KGN cells were plated onto 24-well plates at a density of 1×10^5 viable cells per well and were incubated with 10^{-5} M of T (testosterone, substrate for E_2) and hMG (500 mIU/mL). Next, the media were collected after 48 h, and the E_2 concentrations were measured by means of the enzyme immunoassay (EIA) Kit (Amerlite P, Amersham Pharmacia, Tokyo, Japan). The intra-assay and inter-assay coefficients of variation were $< 10\%$. Each experiment was performed six times independently.

Statistical analysis

In this study, all experiments were repeated at least three times. All data were analysed using Student's *t* test. The values were presented as the mean $\pm$ S.E.M. Statistical analysis was performed using SPSS 13.0 (SPSS). A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Identification of differentially expressed miRNAs and mRNAs between the PCOS and control cumulus cells

For the miRNA and mRNA expression profiles, the cumulus cells isolated from the five PCOS and five normal patients were analysed using microarrays. The raw microarray data were deposited in NCBI's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and can be accessed through the GEO series accession numbers GSE72274 and GSE65746. In total, 17 miRNAs were identified, which were differentially expressed between the PCOS and control cumulus cells (fold change >1.5 or <-1.5 ; $P < 0.05$). As given in Table 2, 16 miRNAs showed an increased expression and one miRNA showed a decreased expression in the PCOS cumulus cells. Among these miRNAs, hsa-miRNA-135b-5p (log 2 fold change $P/N=21.27$) was the most up-regulated miRNA, whereas hsa-miRNA-3940-5p (log 2 fold change $P/N=-1.53$) was the only down-regulated one. Meanwhile, among the 1263 differentially expressed mRNAs, 1213 mRNAs were up-regulated, whereas 50 mRNAs were down-regulated in the PCOS cumulus cells (Table S2). Of these mRNAs, SGCZ (NM_139167) (log 2 fold change $P/N=4.719$) was the most up-regulated mRNA, and HOXA3 (NM_153631) (log 2 fold change $P/N=-3.943$) was the most down-regulated one. A clustering analysis based on the differentially expressed mRNAs perfectly clustered the PCOS and the normal groups (Fig. 1).

Differentially expressed miRNA-targeted genes and pathways

The prediction of miRNA-targeted genes was performed to demonstrate altered gene expression in association with aberrant miRNA profiles in PCOS cumulus cells. Thus, target gene analysis was performed using miRWalk 2.0 (Table S3). As the miRNAs (hsa-miRNA-1273e, hsa-miRNA-4659a-3p and hsa-miRNA-652-5p) had no target gene predicted by miRWalk 2.0, we did not further analyse them. For the other miRNAs, the top 100 potential target genes for each miRNA were selected to identify the associated signalling pathways and biological functions. Regarding GO annotation, the associated biological processes, cellular components and molecular function in which these target genes were involved are presented in Tables S4, S5 and S6. We found that the putative target genes of altered miRNAs were involved in a wide range of biological processes, such as the regulation of transcription (GO: 0006355), development (GO: 0007275), protein amino phosphorylation (GO: 0006468) and signal transduction (GO:0007165). Moreover, we enriched the signalling pathways regulated by the predicted genes of altered miRNAs. As shown in Table S7, the pathway with the maximum enriched genes is 'Focal adhesion ($n=27$)', followed by (in descending order) 'Regulation of actin cytoskeleton' ($n=25$), 'ECM-receptor interaction' ($n=15$), 'MAPK signalling pathway' ($n=23$) and 'calcium signalling pathway' ($n=19$).

Comparison of the miRNA-targeted genes and the differentially expressed genes (DEGs) isolated by the cDNA microarray

Comparing the list of the DEGs isolated from the cDNA microarray (Table S2) and the list of predicted miRNA-targeted genes (Table S3), we found that several miRNA-targeted genes were also identified by the

Table 2 Differentially expressed miRNAs in cumulus cells of the PCOS patients.

miRNA name	Fold change	chr	Start	Stop	Strand	Active sequence
hsa-miRNA-135b-5p	21.26811	chr1	205417467	205417445	+	TCACATAGGAATGAAAAGCCATA
hsa-miRNA-152	5.63396	chr17	46114600	46114586	+	CCAAGTTCTGTCATGC
hsa-miRNA-193a-3p	5.529378	chr17	29887075	29887090	-	ACTGGGACTTTGTAGGC
hsa-miRNA-194-5p	6.040723	chr1	220291534	220291519	+	TCCACATGGAGTTGCT
hsa-miRNA-196a-5p	8.043263	chr12	54385548	54385567	-	CCCAACAACATGAACTACC
hsa-miRNA-200b-3p	5.285276	chr1	1102545	1102561	-	TCATCATTACCAGGCAG
hsa-miRNA-423-3p	4.024403	chr17	28444159	28444171	-	ACTGAGGGGCCTCA
hsa-miRNA-454-3p	5.431713	chr17	57215204	57215184	+	ACCCTATAAGCAATATTGCAC
hsa-miRNA-455-5p	7.912167	chr9	116011554	116011571	-	CGATGTAGTCCAAAGGCA
hsa-miRNA-4659a-3p	7.284604	chr8	6602739	6602754	-	CGTTGCCATGTCTAAGA
hsa-miRNA-509-3-5p	2.741761	chrX	146341200	146341185	+	CATGATTGCCACGCTCTG
hsa-miRNA-509-3p	2.128628	chrX	146340353	146340338	+	CTACCCACAGACGTAC
hsa-miRNA-513b-5p	2.300253	chrX	146280596	146280579	+	ATAAATGACACCTCCTTGT
hsa-miRNA-652-5p	5.063606	chrX	109298587	109298601	-	TGAATGGCACCTCTC
hsa-miRNA-95	6.591886	chr4	8007097	8007079	+	TGCTCAATAAATACCCGTTG
hsa-miRNA-1273e	4.261593	chr17	64425136	64425157	-	TCCACTTCTGGGTTTC
hsa-miRNA-3940-5p	-1.533231	chr19	6416462	6416452	+	CAGAGCCCCGCC

Figure 1 Cluster of mRNAs overexpressed in the cumulus cells from the PCOS patients. This figure shows the supervised hierarchical clustering of the mRNAs overexpressed in the cumulus cells (CCs) from the PCOS patients compared with controls. Distinct signatures in the PCOS and control groups were observed. The value of each mRNA was adjusted by a median-centring algorithm in log scale, and the colours indicated the relative mRNA expression in the red–green heat map. A value of 0 is indicated by pure black colour and represents no change from the median gene expression level in all of the samples. A value of -3 is indicated by pure green colour and represents a relative lower expression. A value of $+3$ is indicated by pure red colour and represents a relative higher expression.

mRNA microarray (Table 3). For example, *MAP3K8* (mitogen-activated protein kinase kinase 8, a predictive miRNA-509-3p target gene), *RND3* (Rho family GTPase 3, a predictive miRNA-200b-3p target gene) and *MEIS2* (Meis homeobox 2, a predictive miRNA-194-5p target gene) were down-regulated in the PCOS cumulus cells respectively. In addition, three miRNA-3940-5p-predictive target genes (adducin 2 (beta), *ADD2*; nucleosome assembly protein 1-like 2, *NAP1L2*; and sodium channel, voltage-gated, type IV, beta, *SCN4B*) were up-regulated in the PCOS cumulus cells.

Validation of the microarray results by quantitative RT-PCR

To validate the differentially expressed miRNA and mRNA profiles, the cumulus cell samples isolated from 20 additional samples (ten PCOS patients and ten controls), respectively, were used to extract the RNA for the qRT-PCR. Seven altered miRNAs, including six up-regulated miRNAs (miRNA-509-3p, miRNA-513b-5p, miRNA-423-3p, miRNA-200b-3p, miRNA-194-5p and miRNA-135b-5p) and one down-regulated miRNA (miRNA-3940-5p), were selected to validate the microarray data. Among these miRNAs, three (miRNA-509-3p, miRNA-513b-5p and miRNA-423-3p) were identified in the PCOS cumulus cells by next-generation sequencing (Liu *et al.* 2015, Xu *et al.* 2015). miRNA-135b-5p is the miRNA with the highest FC value in our microarray data. In addition, four miRNAs (miRNA-509-3p, miRNA-200b-3p, miRNA-194-5p and miRNA-3940-5p) were selected because their predictive target genes were also identified in our cDNA microarray data. At the same time, four predictive miRNAs target genes (*MAP3K8*, *RND3*, *MEIS2* and *NAP1L2*) were also selected to confirm the cDNA microarray data by qRT-PCR. The results showed that all the seven miRNAs and four mRNAs were detected in all of the samples, and the expression patterns of these genes (miRNAs or mRNAs) measured by qRT-PCR were in concordance with the miRNA or cDNA microarray data (Fig. 2), indicating that our miRNA and mRNA microarray data were reliable.

Confirmation of the direct interaction between miRNA-509-3p and its predictive target gene (*MAP3K8*)

Using the GO annotation and pathway analysis, we found that two predictive miRNA-509-3p target genes, *MAP3K8* and *IL1A* (interleukin 1, alpha), were involved in the MAPK signalling pathway (Tables S2 and S4). According to our cDNA microarray data, *MAP3K8* was down-regulated in the PCOS cumulus cells, whereas *IL1A* showed no significant changes in the PCOS cumulus cells compared with the control patients (Table S2). Thus, we hypothesised that it was more possible that miRNA-509-3p regulated *MAP3K8* directly in the PCOS cumulus cells.

Table 3 Differentially expressed miRNAs in the PCOS cumulus cells and their predicted target genes that also identified in cDNA microarray.

miRNA	Mirbase accession no.	FC (P/N)	miRNA regulation	Target gene symbol	Genbank accession	FC (P/N)	mRNA regulation
hsa-miRNA-509-3p	MIMAT0002881	2.129	Up	MAP3K8	NM_005204	1.615	Down
hsa-miRNA-200b-3p	MIMAT0000318	5.285	Up	RND3	NM_005168	2.713	Down
hsa-miRNA-194-5p	MIMAT0000460	6.041	Up	MEIS2	NM_170677	1.535	Down
hsa-miRNA-3940-5p	MIMAT0019229	1.533	down	ADD2	NM_017488	1.777	Up
				NAP1L2	NM_021963	2.227	Up
				SCN4B	NM_174934	1.621	Up

FC, fold change in microarray; P/N, PCOS vs normal.

To confirm the negative correlation between miRNA-509-3p and *MAP3K8*, we again evaluated the mRNA and protein expression levels of *MAP3K8* in three additional PCOS and three additional control cumulus cells. We found that both the mRNA and protein levels of *MAP3K8* were markedly decreased, whereas the expression level of miRNA-509-3p was significantly increased in the PCOS cumulus cells (Fig. 3A and B). These results proved that a negative correlation between miRNA-509-3p and *MAP3K8* in the PCOS cumulus cells really exists.

Next, to determine whether *MAP3K8* mRNA is a direct target of miRNA-509-3p, we constructed luciferase reporter constructs by cloning the DNA segment-encoding part of the WT or mutant (which cannot bind miRNA-509-3p) 3'-UTR of *MAP3K8* mRNA downstream of the Renilla luciferase gene (Fig. 4A) and transfected them with miRNA-509-3p mimics into the KGN cells. As shown in Fig. 4B, a significant decrease in the relative luciferase activity was detected in the KGN cells co-transfected with the *MAP3K8* WT constructs and the miRNA-509-3p mimics but not in the cells co-transfected with the *MAP3K8* mutant constructs and the miRNA-509-3p mimics. Thus, the above findings demonstrated that miRNA-509-3p suppresses the expression of *MAP3K8* in the cumulus cells by directly binding to the 3'-UTR of *MAP3K8* mRNA.

In addition, to further determine the regulation of miRNA-509-3p on *MAP3K8*, the KGN cells were transfected with the miRNA-509-3p mimics or a miRNA-509-3p inhibitor or their controls. The results showed that the miRNA-509-3p mimics dramatically up-regulated the endogenous miRNA-509-3p (Fig. 5A), and the miRNA-509-3p inhibitor significantly down-regulated the expression of miRNA-509-3p (Fig. 5B). Next, we assessed the effects of the miRNA-509-3p mimics and the inhibitor on *MAP3K8* expression in the KGN cells. These data showed that the mRNA and protein expression levels of *MAP3K8* were decreased by approximately two-fold (Fig. 5C) and 2.04-fold (Fig. 5E and G) in the miRNA-509-3p mimic-transfected KGN cells respectively. In addition, in the miRNA-509-3p inhibitor-transfected KGN cells, the mRNA and protein expression levels of *MAP3K8* were increased by approximately 2.84-fold (Fig. 5D) and 1.63-fold (Fig. 5F and H) respectively. In combination with the results from

the 3'-UTR luciferase reporter study, we conclude that miRNA-509-3p directly targets and inhibits *MAP3K8*.

miRNA-509-3p promotes E_2 production in the KGN cells

To further determine the molecular role of miRNA-509-3p in regulating *MAP3K8*, we determined the E_2 production in the medium of the KGN cells transfected

Figure 2 Differentially expressed miRNAs (hsa-miRNA-509-3p, hsa-miRNA-513b-5p, hsa-miRNA-423-3p, hsa-miRNA-200b-3p, hsa-miRNA-194-5p, hsa-miRNA-135b-5p and hsa-miRNA-3940-5p) and their potential target genes (*MAP3K8*, *RND3*, *MEIS2* and *NAP1L2*) studied through microarray experiments and validated by qRT-PCR. The qRT-PCR results were in line with the microarray data sets. The grey bars show the relative gene expression measured using qRT-PCR. The white bars show the relative gene expression measured using the microarrays. The same cumulus cells isolated from 20 additional samples (ten from PCOS patients and ten from controls) were used for the qRT-PCR. The selected genes are shown on the x-axis and the y-axis represents the fold change $CC_{PCOS}/CC_{control}$.

Figure 3 The expression levels of has-miRNA-509-3p and its predictive target gene (*MAP3K8*) in the cumulus cells of PCOS patients. (A) The mRNA expression levels of miRNA-509-3p and *MAP3K8* detected by qRT-PCR. The expression level of *MAP3K8* was markedly decreased, whereas the expression level of miRNA-509-3p was significantly increased in the PCOS cumulus cells. The signal intensity of the genes is shown on the y-axis in arbitrary units determined by qRT-PCR. *GAPDH* and *U6* were used as internal controls for *MAP3K8* or miRNA-509-3p respectively. The asterisk (*) indicates a significant difference in the gene expression between the CC categories ($P < 0.05$). The results are presented as the mean $\pm$ S.E.M. (B) The protein levels of the miRNA target gene (*MAP3K8*) in the cumulus cells of the PCOS and control patients were analysed by western blot. The protein level of *MAP3K8* was markedly decreased in the PCOS CCs compared with the control CC samples.

with the miRNA-509-3p mimics or the inhibitor. E_2 secretion in the medium of the KGN cells increased (2.79-fold) by hMG after adding T for 48 h. Interestingly, after adding T and hMG into the medium for 48 h, the medium from the KGN cells transfected with the miRNA-509-3p mimics showed an increased E_2 secretion (2.36-fold) compared with the KGN cells without transfection. Meanwhile, compared with the KGN cells without transfection, the E_2 secretion was decreased (1.69-fold) in the medium from the KGN cells transfected with the miRNA-509-3p inhibitor (Fig. 6).

Discussion

Folliculogenesis is a complex process that involves endocrine and intra-ovarian paracrine interactions to provide the most appropriate microenvironment for

Figure 4 Validation that miRNA-509-3p directly targets *MAP3K8* by the luciferase activity assay. (A) Illustration of the construction of the pmirGLO-*MAP3K8* UTR vectors. The DNA fragment encoding the 3'-UTR of the *MAP3K8* mRNA that contains the predicted wild-type or mutant miRNA-509-3p binding site was cloned downstream of the Renilla luciferase ORF in the pmirGLO vector. (B) The relative luciferase activity was assayed following co-transfection of miRNA-509-3p mimics with the constructs encoding the wild-type or mutant miRNA-509-3p binding site of *MAP3K8* 3'-UTR into the KGN cells. The constructed reporter vectors (*MAP3K8*-3'-UTR and *MAP3K8*-mut-3'-UTR) are shown on the x-axis, and the y-axis represents the normalised luciferase activity (firefly luciferase activities were normalised to Renilla luciferase activities). The experiments were performed independently in triplicate. * $P < 0.05$. NS, no significant difference. The results are presented as the mean $\pm$ S.E.M.

oocyte development. The cross talk between the oocyte and the cumulus cells in the microenvironment plays an important role in the acquisition of the development competency of the oocyte (Haouzi *et al.* 2012). In this study, to elucidate the mechanism of the abnormal folliculogenesis of PCOS, we reported the miRNA and mRNA expression profiles using the same cumulus cell samples isolated from PCOS and control patients. To the best of our knowledge, there are only three recently published reports (Liu *et al.* 2015, Shi *et al.* 2015, Xu *et al.* 2015), similar to ours, that identified the altered

Figure 5 The expression levels of miRNA-509-3p and its target gene (*MAP3K8*) in the KGN cells transfected with the miRNA-509-3p mimics or inhibitor. After transfection for 48 h, the expression of *MAP3K8* (mRNA or protein levels) and miRNA-509-3p in the transfected KGN cells was detected by qRT-PCR and western blotting analyses. The mRNA expression levels of miRNA-509-3p in the KGN cells transfected with the miRNA-509-3p mimics (A) or the inhibitor (B) are shown. The mRNA or protein expression levels of *MAP3K8* in the KGN cells transfected with the miRNA-509-3p mimics (C, E and G) or the inhibitor (D, F and H) are also shown. The relative expression of mRNA or protein is shown on the y-axis. *GAPDH* and *U6* were used as the internal controls for *MAP3K8* or miRNA-509-3p respectively. ** $P < 0.01$. NS, no significant difference. The results are presented as the mean $\pm$ S.E.M. Each experiment was performed six times. B, blank; M, scramble controls; N1, stable negative control for miRNA-509-3p mimics; N2, stable negative control for miRNA-509-3p inhibitor; mi, miRNA-509-3p mimics; in, miRNA-509-3p inhibitor.

miRNA profiles of PCOS patients using cumulus cells as the study material. By comparing the lists of the differentially expressed miRNAs in our data to that of the previous reports (Liu *et al.* 2015, Shi *et al.* 2015, Xu *et al.* 2015), we found that the miRNA profiles were not exactly the same. This difference may be due to two reasons. First, different molecular technology (miRNA microarray or next-generation sequencing) was used in these studies. Specifically, miRNA microarray was used in our study and in Shi's study (Shi *et al.* 2015),

Figure 6 Effect of miRNA-509-3p on E_2 production in the KGN cells. The transfected KGN cells were plated onto 24-well plates at a density of 1×10^5 viable cells per well and incubated with 10^{-5} M of T (substrate for E_2) and hMG (500 mIU/mL). Then, the media were collected after 48 h, and the concentration of E_2 in the corresponding medium was measured by EIA. The intra-assay and inter-assay coefficients of variation were $< 10\%$. Each experiment was performed six times independently. The data are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$. N, negative control (KGN cells that are not transfected); T, testosterone; hMG, human menopausal gonadotropin; mi, miRNA-509-3p mimics; in, miRNA-509-3p inhibitor.

whereas next-generation sequencing was used in Xu's (Xu *et al.* 2015) and Liu's (Liu *et al.* 2015) studies. Generally, many novel miRNAs are found by next-generation sequencing; however, only the miRNAs with a response to the probes that are included in the microarray can be detected using a miRNA microarray. In fact, in Xu's report (Xu *et al.* 2015), which isolated 59 differentially expressed miRNAs in the cumulus cells, more than ten miRNAs were novel. Second, we found that in other research (Liu *et al.* 2015, Shi *et al.* 2015, Xu *et al.* 2015), only cumulus-oocyte complexes with mature oocytes (MII) were included. However, in our study, cumulus-oocyte complexes with mature (MII) and immature (MI or GV) oocytes were all selected. Notably, despite receiving significantly less FSH, there were more follicles on the day of oocyte retrieval from the PCOS patients during IVF. Moreover, the ratio of immature oocytes of the PCOS patients was always higher than that of the normal patients. In our previous report (Huang *et al.* 2013a), we found that the gene expression profiles from cumulus cells isolated from MI and MII oocytes of PCOS were significantly different. In other words, oocyte nuclear maturation in PCOS is regulated by different genes. These differentially expressed genes may be regulated by different miRNAs in a post-transcriptional manner. The altered miRNAs play different roles during oocyte maturation in PCOS.

Although there are differences between our study and previous reports, we are excited to find that several miRNAs in our research were also identified in previous reports (Liu *et al.* 2015, Shi *et al.* 2015, Xu *et al.* 2015). For example, the expression of miRNA-509-3p was increased in the PCOS cumulus cells, according to Liu's (Liu *et al.* 2015), Shi's (Shi *et al.* 2015) and our data, with almost the same fold changes (2.2-fold in Liu's, 1.8-fold in Shi's and 2.13-fold in ours). miRNA-513b-5p, a miRNA isolated in Xu's (Xu *et al.* 2015), Liu's (Liu *et al.* 2015) and our research, also showed an up-regulated expression in the PCOS cumulus cells. In addition, in both Xu's (Xu *et al.* 2015) and our data, miRNA-423-3p showed an increased expression in the PCOS cumulus cells. These data indicate that when the same miRNAs (miRNA-509-3p, miRNA-513b-5p and miRNA-423-3p) are identified by different groups, they are more credible and attract greater research interest in their important roles in abnormal folliculogenesis of PCOS.

It is worth mentioning that another feature of our study is that we identified the miRNA and mRNA expression profiles together by using the same cumulus cell samples of the PCOS and control patients, whereas only the altered miRNAs from the PCOS cumulus cells were isolated in other reports (Liu *et al.* 2015, Shi *et al.* 2015, Xu *et al.* 2015). In addition, we compared the list of the DEGs from the mRNA microarray to the list of potential target genes of each altered miRNA identified by our miRNA microarray to find the potential associated miRNA-mRNA pairs in PCOS. Further research examining the functions of the associated miRNAs and their potential target mRNAs facilitates in the understanding of their roles in the pathogenetic development of PCOS. Coincidentally, we found that *MAP3K8*, the predicted target gene of miRNA-509-3p, was also identified from our mRNA microarray data. In our experiments, the expression level of miRNA-509-3p was increased, whereas the mRNA and protein expression levels of *MAP3K8* were decreased in the PCOS cumulus cells. Moreover, we demonstrated that miRNA-509-3p regulated *MAP3K8* expression by directly binding the 3'-UTR of mRNA based on bioinformatics and experimental validation.

According to the GO and pathway analysis, *MAP3K8* was predicted to be involved in the MAPK signalling pathway. The MAPK signalling pathway participates in LH- and FSH-induced steroidogenesis in granulosa cells (Seeger *et al.* 2001). Actually, granulosa cells from PCOS produce more E_2 under basal FSH and insulin stimulation, and the enzymes for steroids synthesis in the granulosa cells from PCOS may be active *in vivo* (Wang *et al.* 1998). Moreover, the follicular fluid steroid levels and *in vitro* studies of oestradiol production by granulosa cells isolated from PCOS clearly demonstrated that the granulosa cells of follicles from anovulatory women with PCOS remain steroidogenically active. In addition, abnormal (increased) oestradiol production in

the follicles from PCOS appears to be a function of the ovulatory status of the patient rather than the polycystic morphology *per se* (Franks *et al.* 1998). To investigate whether miRNA-509-3p and *MAP3K8* regulate E_2 production, miRNA-509-3p mimics and an inhibitor were transfected into the KGN cells, respectively, to determine the E_2 accumulation in the corresponding culture medium. The results indicate that miRNA-509-3p improves E_2 secretion by inhibiting the expression of *MAP3K8*.

MAP3K8 is a serine-threonine kinase with crucial physiological roles in G protein-coupled receptor-mediated extracellular signal regulated kinase (ERK), tumour necrosis factor, interleukin-1, CD40 and Toll-like receptor signal transduction (Hatziaepostolou *et al.* 2008). A previous report demonstrated that *MAP3K8* enhances ERK1/2 phosphorylation (Ceci *et al.* 1997), and it had been confirmed that ERK1/2 phosphorylation inhibits E_2 production via the MAPK signalling pathway in human granulosa cells (Deura *et al.* 2005). In addition, it has been verified that (ERK1/2) phosphorylation was decreased in PCOS theca cells compared with normal cells (Nelson-Degrave *et al.* 2005). This finding is consistent with the phenomenon that there is a high oestrogen production from the granulosa cells in PCOS patients under basal FSH stimulation (Wang *et al.* 1998). Thus, we speculated that ERK1/2 was the downstream molecular target of miRNA-509-3p and *MAP3K8*. miRNA-509-3p mediates the MAPK signalling pathway to regulate E_2 production by targeting *MAP3K8*.

Although miRNA-513b-5p and miRNA-423-3p were also regarded as altered miRNAs in the PCOS cumulus cells in our research and previous research (Liu *et al.* 2015, Shi *et al.* 2015, Xu *et al.* 2015), the potential target genes of miRNA-513b-5p or miRNA-423-3p were not included in the list of the DEGs in the PCOS cumulus cells that were identified in our mRNA microarray data.

miRNA-423-3p was up-regulated in the PCOS cumulus cells based on our and Xu's results (Xu *et al.* 2015). Previous research on laryngeal carcinoma reported that miRNA-423-3p directly interacts with AdipoR2 (adiponectin receptor 2) and promotes tumour progression (Guan *et al.* 2014). AdipoR2 is a receptor to adiponectin, which is a predominantly adipocyte-derived hormone that influences insulin sensitivity and energy homeostasis. In animal models, adiponectin may regulate ovarian steroidogenesis, folliculogenesis and ovulation (Chabrolle *et al.* 2007). Moreover, it has been demonstrated that a significantly lower proportion of theca cells express *AdipoR2* in polycystic ovaries than in normal ovaries, and the disruption of adiponectin and/or its receptors plays a key role in the pathogenesis of hyperandrogenism in PCOS (Comim *et al.* 2013) and impairs normal progesterone production (Wickham *et al.* 2013). In view of the results of the above reports, although the miRNA-423-3p potential target genes were not detected in our cDNA microarray, we also

determined the expression levels of *AdipoR2* in the PCOS or control cumulus cells by qRT-PCR. Consistent with the cDNA microarray data, the expression of *AdipoR2* showed no significant difference between the two groups (PCOS and control) (data not shown). The results may be associated with the fact that the adiponectin levels are not reduced in all PCOS patients. Dividing the patients (PCOS or control) into subgroups, such as obese or lean subgroups, according to their BMI may provide more information regarding the function of miRNA-423-3p and *AdipoR2*.

Another miRNA, miRNA-513b-5p, inhibits cell proliferation and migration and promotes apoptosis by targeting high-mobility group-box 3 protein (HMGB3) in gastric cancer (Chen *et al.* 2014). With regard to PCOS, the apoptosis level is increased in the granulosa cells (Onalan *et al.* 2005, Lin *et al.* 2012); thus, we considered that the up-regulated miRNA-513b-5p in the PCOS cumulus cells might participate in the regulation of apoptosis with an impact on the atretic process of the follicles, leading to follicle maturation disorder. However, the target genes or the regulation of miRNA-513b-5p in the occurrence of PCOS need to be further researched.

In summary, we isolated the differentially expressed miRNA and mRNA profiles in the cumulus cells from PCOS patients by microarrays. The expression levels of candidate miRNAs and their potential target genes were detected by qRT-PCR to confirm the microarray data. In this study, miRNA-509-3p, miRNA-513b-5p and miRNA-423-3p were up-regulated in the PCOS cumulus cells, which were also shown in previous data. In particular, as the potential target gene of miRNA-509-3p, *MAP3K8* was also detected in our cDNA microarray and showed a down-regulated expression in the PCOS cumulus cells. Next, we verified that miRNA-509-3p regulated *MAP3K8* expression by directly binding the 3'-UTR of mRNA based on the luciferase activity assay. The experiments transfecting the miRNA-509-3p mimics or the inhibitor in the KGN cells and the E₂ production determination in the corresponding medium further confirmed that miRNA-509-3p improves E₂ secretion by inhibiting *MAP3K8* expression. These results will help to characterise the pathogenesis of anovulation in PCOS, especially the regulation of oestradiol secretion.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0071>.

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.

Funding

This study was supported by the National Natural Science Foundation of China (Grant 81401172), the Natural Science Foundation of Shandong Province (Grant ZR2013HQ004) and the Yantai Science and Technology Development Project (Grant 2015YT06000550).

Author contribution statement

Xin Huang and Chang Liu involved in the design, experiments, analysis and interpretation; drafting; and final approval of the manuscript. Cuifang Hao contributed to the design, revisions to manuscript and final approval of the manuscript. Qianqing Tang involved in oestradiol determination and data acquisition. Riming Liu involved in oestradiol determination. Shaoxia Lin and Luping Zhang contributed to experiments (qRT-PCR, western blotting and luciferase activity assay), and Wei Yan contributed to cumulus cell collection and final approval of the manuscript.

Acknowledgements

The authors gratefully acknowledge the CapitalBio Corporation for conducting the RNA extractions and microarrays.

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Received 1 September 2015

First decision 1 October 2015

Revised manuscript received 13 March 2016

Accepted 21 March 2016