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 (E2) secretion by inhibiting the expression of MAP3K8. These results help to characterise the pathogenesis of anovulation in PCOS, especially the regulation of E2 production.

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Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder in women and it 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 (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, Haozui 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, luteotropic hormone (LH), oestradiol ($E_2$), 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/m$^2$, 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 $E_2$ levels <30 pg/mL), the patients
subcutaneously received recombinant FSH (150–187.5 IU; Gonal-f, Follitropin Alfa, Serono) for COS. When two or more follicles were at least 18 mm in diameter and the serum E2 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 five PCOS and five control patients were used in miRNA and mRNA microarrays. The cumulus cells isolated from other 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 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 17α-hydroxylated steroids, dehydroepiandrosterone androstenedione, and the E2 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 E2 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 OD260/280 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 x 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 Cp-Cy3, and the labelled RNA was purified and hybridised to miRNA arrays.

miRNA profiling was performed using the Agilent human lncRNA+miRNA Array v2.0 (4 x 180K 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 hypergenomic 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, 1μg 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 reference. The 20μl reaction mixture contained 10μl of 2×QuantiTest SYBR Green PCR Master mix, 2μl of 10×miScript Universal Primer, 0.5μl specific miRNA primer, 1μl cDNA template and RNase-free water. The PCR program was as follows: 95°C for 10min, followed by 40 cycles at 95°C for 15s and 60°C for 1min.

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). Amplification reactions were conducted using SYBR Premix Ex Taq (Perfect Real Time; TaKaRa Bio) and an ABI PRISM 7300 system. The 20μl reaction mixture contained 10μl of 2×QuantiTest SYBR Green PCR Master mix, 1μl cDNA template, 1μl each primer and RNase-free water. The PCR reactions were performed at 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 60s. Each set of qRT-PCRs was repeated at least three times, and the fold change in the expression of each gene was calculated 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 1h, 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 restriction sites.

KGN cells were plated onto 24-well plates and allowed to grow for ~24h before transfection. The constructed reporter vectors (300ng) were transfected into cells together with the miRNA mimics and control mimics (100nM) in Lipofectamine 2000 (2μL). Cells were lysed after ~24h 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’-UGAUUGGUCAUGUGGGAG-3’; reverse: 5’-ACCCACAGCGAAGCAGA-3’) and the miRNA-509-3p inhibitor (5’-CUACCCAGACGACAAUCA-3’) were commercially synthesised by Gene Pharma (Shanghai, China). A stable negative control (nc mimic) (forward: 5’-UUCUCGGAAACGUGACGUTT-3’; reverse: 5’-ACCUGACGUUGGAGATTT-3’) and a nc inhibitor (5’-CAGUACUUUGUGUAGACAA-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 (miRNA 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 E2 secretion in the transfected KGN cells

Oestradiol secretion in the transfected KGN cells was determined by testing E2 accumulation in the culture medium. The transfected KGN cells were plated onto 24-well plates at a density of 1×105 viable cells per well and were incubated with 10−5 M of T (testosterone, substrate for E2) and hMG (500mIU/mL). Next, the media were collected after 48h, and the E2 concentrations were measured by means of the enzyme immunoassay (EIA) Kit (Amerlit 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±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 of 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...
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.
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 E₂ production in the KGN cells**

To further determine the molecular role of miRNA-509-3p in regulating MAP3K8, we determined the E₂ production in the medium of the KGN cells transfected

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

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<th>FC (P/N)</th>
<th>mRNA regulation</th>
<th>Target gene symbol</th>
<th>Genbank accession</th>
<th>FC (P/N)</th>
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<td>MAP3K8</td>
<td>NM_005204</td>
<td>1.615</td>
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<td>RND3</td>
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<td>NM_017488</td>
<td>1.777</td>
<td>Up</td>
</tr>
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**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.
with the miRNA-509-3p mimics or the inhibitor. E₂ 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 E₂ secretion (2.36-fold) compared with the KGN cells without transfection. Meanwhile, compared with the KGN cells without transfection, the E₂ 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 intro-ovarian paracrine interactions to provide the most appropriate microenvironment for 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
miRNA profiles of PCOS patients using cumulus cells as the study material. By comparing the lists of the differentially expressed miRNAs in the microarray 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), 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.

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 (miRNA or protein levels) and miRNA-509-3p in the transfected KGN cells was detected by qRT-PCR and western blotting analyses. The miRNA 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 the 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 ± 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.

Figure 6 Effect of miRNA-509-3p on E2 production in the KGN cells. The transfected KGN cells were plated onto 24-well plates at a density of 1 x 10⁴ viable cells per well and incubated with 10⁻⁵ M of T (substrate for E₂) and hMG (500 mIU/mL). Then, the media were collected after 48 h, and the concentration of E₂ 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 ± 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.
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 pathogenic 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 (Seger et al. 2001). Actually, granulosa cells from PCOS produce more E2 under basal FSH and insulin stimulation, and the enzymes for steroid 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 ovariulatory status of the patient rather than the polycystic morphology per se (Franks et al. 1998). To investigate whether miRNA-509-3p and MAP3K8 regulate E2 production, miRNA-509-3p mimics and an inhibitor were transfected into the KGN cells, respectively, to determine the E2 accumulation in the corresponding culture medium. The results indicate that miRNA-509-3p improves E2 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 (Hatziospostolou 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 E2 production via the MAPK signalling pathway in human granulosa cells (Deuura 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 E2 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 ovariectomy (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.

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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 Lijing 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.

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