miR-15a-5p levels correlate with poor ovarian response in human follicular fluid

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Abstract

Poor ovarian response is a significant problem encountered during in vitro fertilization and embryo transfer procedures. Many infertile women may suffer from poor ovarian response and its incidence tends to be increasing in young patients nowadays. It is a major cause of maternal infertility because it is associated with low pregnancy and live birth rates. However, the cause of poor ovarian response is not clear. In this study, we extracted microRNAs from human follicular fluid and performed miRNA sequencing to investigate a potential posttranscriptional mechanism underlying poor ovarian response. The results showed that many miRNAs were obviously different between the poor ovarian response and non-poor ovarian response groups. We then performed quantitative polymerase chain reaction, Western blot analysis and used an in vitro culture system to verify the sequencing results and to study the mechanism. Notably, we found that miRNA-15a-5p was significantly elevated in the young poor ovarian response group. Furthermore, we demonstrated that high levels of miR-15a-5p in the young poor ovarian response group repressed granulosa cell proliferation by regulating the PI3K-AKT-mTOR signaling pathway and promoted apoptosis through BCL2 and BAD. This could explain the reduced oocyte retrieval number seen in poor ovarian response patients.

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Introduction

Poor ovarian response (POR) is a condition characterized by decreased follicular numbers and low estradiol levels following controlled ovarian hyperstimulation during in vitro fertilization and embryo transfer (IVF-ET) procedures. It has been reported that the incidence of POR is about 9%–24% among patients undergoing IVF (Tarlatzis et al. 2003). Advanced age is one of the most significant risk factors for POR. The prevalence of POR increases with age, and in women over 40 years of age, it is over 50% (Ferraretti et al. 2011). In POR patients undergoing IVF, the success rate is extremely low and the total cancelled cycles are quite high due to the loss of follicles from the ovary and to decreased oocyte quality.

Nevertheless, little is known about the molecular mechanisms underlying POR. Previous investigations have demonstrated that polymorphisms in the FSHR gene (Asn680Ser) and defects in the estrogen receptor beta may be associated with an altered ovarian response (Drummond & Fuller 2010, Sheikha et al. 2011). Estrogen receptor beta is critical for granulosa cell differentiation and for the ovulatory response to gonadotropins, and it acts by affecting aromatase activity, estradiol synthesis and the expression of the LH receptor (Couse et al. 2005). It has been reported that defects in estrogen receptor beta lead to a partial arrest of follicle genesis and ovulatory dysfunction in estrogen receptor beta knockout mice (Drummond & Fuller 2010). In other reports, some genes that may contribute to premature ovarian failure and be associated with POR have been identified, for example, Fmr1, Foxl2, Foxo3a and Gdf9 (Skillern & Rajkovic 2008).

Recently, it was reported that posttranscriptional alterations occurring in the germline were linked to defects in oocyte maturation (Espey & Richards 2002, Pangas & Rajkovic 2006, Murchison et al. 2007). In this regard, miRNAs execute key posttranscriptional functions by degrading target mRNAs or repressing their translation by binding to 3′UTR sequences and form RNA-induced silencing complexes to regulate gene expression (Benfey 2003). Through these mechanisms, miRNAs regulate many cellular processes including cell proliferation, differentiation and apoptosis (Bartel 2004).

We hypothesized that changes in miRNA patterns in follicular fluid could explain the pathological defects in oocyte maturation and the reduced numbers of retrieved
oocytes in POR patients. We recovered follicular fluid samples from POR and non-POR women to perform miRNA sequencing. The goals of this study are (i) to investigate possible changes in miRNA patterns in the follicular fluid of POR patients and (ii) to identify miRNAs and related biological processes that could explain the pathological defects in POR.

We divided POR patients into old and young groups. Based on the miRNA-sequencing results, we found that expressions of miR-15a-5p and miR-483-5p were clearly different. The expression of miRNA-15a-5p was significantly increased while miRNA-483-5p was significantly decreased in young POR patients when compared with non-POR in follicular fluid. It means miRNA-15a-5p and miRNA-483-5p might be associated with young POR. We then validated the sequencing results with additional samples by means of quantitative polymerase chain reaction (qPCR) and Western blot and studied the possible mechanisms through which these miRNAs exert their functions.

Materials and methods

Patients

In this study, we collected follicular fluid samples from 45 patients undergoing IVF and intracytoplasmic sperm injection. All patients were recruited from the Center for Reproductive Medicine, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, between April 2015 and April 2016. POR patients were diagnosed according to the Bologna Criteria (Ferraretti et al. 2011). Fifteen of them were young (about 25 to 35 years old) and fifteen were old (over 40 years of age). In order to define the presence of POR during IVF, at least two of the following three features had to be present: (i) advanced maternal age or any other risk factor for POR; (ii) previous POR or (iii) an abnormal ovarian reserve test. Two POR episodes after maximal stimulation were sufficient to define a patient as a poor responder in the absence of advanced maternal age or an abnormal ovarian reserve test. The other fifteen patients were non-POR with normal antral follicle counts whose infertility was caused by tubal or male infertility factors. All the patients recruited had regular menstrual cycles (26–35 days). We excluded patients suffering from other related disorders, such as polycystic ovary syndrome, Turner syndrome, thyrotoxicosis, hyperprolactinemia or recurrent spontaneous abortion, as well as patients who had undergone ovarian surgery or chemotherapy. All patients underwent controlled ovarian hyperstimulation protocols with different follicle-stimulating hormones (FSH/human menopausal gonadotropin starting doses depending on their age (Ferraretti et al. 2011). The Ethics Committee of the Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine approved the study and written consent was obtained from each patient included in the study.

Collection of human follicular fluid and granulosa cells

During IVF, patients received human chorionic gonadotrophin injection by transvaginal ultrasound-guided puncture and aspiration of follicles with a diameter of 18–20 mm. The follicular fluid (2 mL) from the first aspirated follicle, not contaminated by blood, was carefully collected from each donor, centrifuged at 2000 g for 20 min and stored at −80°C until miRNA isolation. At the same time, granulosa cells from the same patients undergoing IVF cycles were collected using the isolation protocol described previously (Matsubara et al. 2000). The granulosa cells were also stored at −80°C until RNA isolation.

MicroRNA extraction and purification

Total RNA from follicular fluid was extracted with the TRIzol LS Reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 0.75 mL of TRIzol reagent was added to 0.25 mL of follicular fluid. After incubating for 5 min, 0.2 mL of chloroform was added and the samples were mixed vigorously for 15 s. Following an additional 10-min incubation, samples were centrifuged at 12000 g for 15 min at 4°C. The upper aqueous phase was carefully transferred to a new tube and 0.5 mL of 100% isopropanol was added. After incubating at −20°C for 3 h, the samples were centrifuged at 12000 g for 20 min at 4°C. The RNA pellets were then washed with 1 mL of 75% ethanol, centrifuged at 12000 g for 5 min at 4°C and resuspended in 20 μL of RNase-free water. The RNA quality was checked by means of a Bioanalyzer 2200 instrument (Agilent). Then, the RNA (RIN>8.0) was purified with a miRNeasy Mini Kit (Qiagen) for miRNA and the purification was validated by gel electrophoresis. The microRNAs extracted from the follicular fluid of three young and three old POR patients (POR-Y, POR-O), as well as three non-POR patients, were sent for miRNA sequencing. The nine samples for miRNA sequencing were picked up randomly according to the Bologna Criteria.

Library construction and miRNA sequencing

Complementary DNA (cDNA) libraries for single-end sequencing were prepared using the Ion Total RNA-Seq Kit, v2.0 (Life Technologies) according to the manufacturer’s instructions. The cDNA library for miRNA sequencing was size-selected by means of PAGE gel electrophoresis. After enrichment, the mixed template-positive Ion ITM Ion Sphere particles were applied to the P1v2 Proton Chip (Life Technologies) and sequenced on Proton Sequencers. The EB-Seq algorithm was applied to identify differentially expressed genes after significance and false discovery rate (FDR) analysis, applying the following criteria: FDR<0.05 (Benjamini et al. 2001).

Gene ontology (GO) and pathway analysis

Targetscan (GO) and pathway analysis was utilized for predicting miRNA targets (Chiang et al. 2010, Agarwal et al. 2015). GO analysis was performed to elucidate the biological implications of unique genes present in the significant or representative profiles of differentially expressed miRNA target genes. GO annotations collected 36 h after human chorionic gonadotrophin injection by transvaginal ultrasound-guided puncture and aspiration of follicles with a diameter of 18–20 mm. The follicular fluid (2 mL) from the first aspirated follicle, not contaminated by blood, was carefully collected from each donor, centrifuged at 2000 g for 20 min and stored at −80°C until miRNA isolation. At the same time, granulosa cells from the same patients undergoing IVF cycles were collected using the isolation protocol described previously (Matsubara et al. 2000). The granulosa cells were also stored at −80°C until RNA isolation.

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were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/) and GO (http://www.geneontology.org/). Fisher’s exact test was applied to identify significant GO categories and FDR was used to correct the P values. Pathway analysis was used to identify significant pathways for the differentially expressed genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Fisher’s exact test was used to identify the significant pathways, and the threshold for significance was defined by the P value and the FDR (Draghici et al. 2007). Additionally, integrative miRNA–mRNA network analysis was carried out according to the significant GO categories and pathways.

**Verification of miRNA and mRNA expression**

Additional follicular fluid and granulosa cells from twelve POR-Y, twelve POR-O and twelve non-POR patients were used to validate the miRNA-sequencing results and to quantify mRNA expression. Quantitative PCR was performed after MicroRNA Reverse Transcription with the Mir-X miRNA qRT-PCR SYBR Kit (Toyobo, Osaka, Japan). Expression of these mRNAs was normalized to U6 levels. As for miRNA detection and quantification, qPCR of AKT, mTOR, S6K1, IGF2, BCL2 and BAD mRNA was carried out using the SYBR GREEN PCR Master Mix (Toyobo, Osaka, Japan). Expression of these miRNAs was normalized to GAPDH levels. The primer sequences are listed in Table 1. Amplification was carried out in an ABI PRISM7700 sequence detector system (Life Technologies, Inc.) using the following program: 5 min at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 59°C and 20 s at 72°C, ending with a 5-min extension at 72°C. All reactions were run in triplicate. The relative miRNA and mRNA levels were determined using the ΔΔCt method (Pfaffl 2001).

**Table 1  Primer list.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 1</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>5′-GTATCGAACGACCCCTCCAT-3′</td>
<td>218</td>
</tr>
<tr>
<td>Revers</td>
<td>5′-AGCCGCTTACCTAAGCATGCT-3′</td>
<td></td>
</tr>
<tr>
<td>mTOR</td>
<td>5′-TTGCTTTAGGCTGCTAATGAA-3′</td>
<td>183</td>
</tr>
<tr>
<td>S6K1</td>
<td>5′-TTTGGATCTACTCGGACTG-3′</td>
<td>196</td>
</tr>
<tr>
<td>IGF2</td>
<td>5′-CGATAGGACTGCTGATCAGTC-3′</td>
<td>122</td>
</tr>
<tr>
<td>BCL2</td>
<td>5′-GTGCGAGTTGAGGGGGATG-3′</td>
<td>130</td>
</tr>
<tr>
<td>BAD</td>
<td>5′-CGCAGGAGTGCAGCAGATG-3′</td>
<td>136</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GGAGGAGATCCCTCCAAAT-3′</td>
<td>197</td>
</tr>
</tbody>
</table>

**Cell culture and transfection**

The human granulosa-like tumor cell line KGN was maintained in phenol red-free DMEM/F12 (Gibco, Grand Island, NY) supplemented with 10% charcoal-stripped FBS (Blood Bioind Stem Origin: Israel) and incubated in 6-well dishes at a density of 1 x 10^5 cells/well at 37°C and 5% CO2, for 24 h. The miR-15a-5p mimic, miR-15a-5p inhibitor and their scrambled controls were purchased from Ribobio (Guangzhou, China). The miRNA mimics are double-stranded, chemically synthesized RNAs that resemble mature endogenous miRNAs, and the miRNA inhibitors are chemically modified antisense RNA oligonucleotides optimized to specifically target miRNA molecules in cells. Either miRNA mimics (50 nM/well) or inhibitors (100 nM/well) and their scrambled controls were transfected into KGN cells grown at 60% confluence by means of LipofectamineTM3000 (Invitrogen, Carlsbad, CA) (7.5 µL/well) diluted in Opti-MEM Medium (Gibco) according to the manufacturer’s protocol. The medium was replaced by fresh medium 6 h after transfection, and cells were cultured for another 48 h.

**Western blot analysis**

Aliquots of the cells harvested after transfection were mixed with sample buffer, consisting of 0.01% (w/v) bromophenol blue, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 25% (v/v) glycerol and 62.5 mM Tris–HCl (pH 6.8) and boiled for 10 min. Then, 10 µg of protein from the cell samples were loaded onto SDS polyacrylamide gels, electrophoresed and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk for 60 min at room temperature and then incubated overnight at 4°C with the primary antibodies. The primary antibodies were as follows: rabbit anti-Phospho-mTOR (1:1000 dilution; Ser2448, Cell Signaling), rabbit anti-mTOR (1:1000 dilution; Cell Signaling), rabbit anti-Phospho-p70 S6 Kinase (1:1000 dilution; Ser371, Cell Signaling), rabbit anti-Phospho-4E-BP1 (1:1000 dilution; Cell Signaling), rabbit anti-BCL2 (1:2000 dilution; Proteintech) and mouse monoclonal IgG1 antibody specific for β-actin (1:5000 dilution; Santa Cruz Biotechnology). After incubation with the primary antibodies, the membranes were washed four times with Tris-buffered saline containing 0.1% Tween-20 and incubated with the secondary antibodies (1:2000 dilution; Cell Signaling) for 90 min at room temperature. The ECL chemiluminescence kit (Millipore) was used to detect the protein bands.

**Statistical analysis**

All the results were expressed as mean±standard error of the mean (S.E.M.), and all the experiments were repeated at least three times except for miRNA mimic experiment, which was repeated 6 times starting from cell inoculation. To determine the differences between groups, we used the unpaired Student’s t-test or one-way ANOVA test followed by the Newman–Keuls multiple comparison test. All analyses were conducted using
SPSS 21.0 for Windows (IBM). Significance was defined as \( P < 0.05 \).

**Results**

**Clinical characteristics**

The clinical characteristics of the POR-Y, POR-O and non-POR patients are shown in Table 2. After comparing the POR-Y with the non-POR group, no statistical differences were found in terms of age, body mass index (BMI), baseline FSH or estradiol (E2) levels. Except for age \( (P = 0.00) \), no statistical differences were found between the BMI, baseline FSH and baseline E2 levels of the POR-O and non-POR groups. However, anti-Mullerian hormone (AMH) levels were much lower in the POR-Y \( (P = 0.004) \) and POR-O \( (P = 0.001) \) groups than those in the non-POR group. The number of retrieved oocytes was significantly different between the POR-Y and non-POR groups \( (P = 0.001) \) and between the POR-O and non-POR groups \( (P = 0.001) \). The average number of retrieved oocytes in the POR-Y and POR-O groups was 1.00, whereas for the non-POR group, the value was 10.67. In addition, the high-quality embryo rate was significantly lower for both the POR-Y \( (P = 0.0015) \) and POR-O \( (P = 0.0011) \) groups than that for the non-POR group. Furthermore, patients from both the POR-Y and POR-O groups underwent approximately 3–4 IVF cycles, significantly more than those in the non-POR group \( (P = 0.026 \text{ for POR-Y vs non-POR, } P = 0.039 \text{ for POR-O vs non-POR}) \). Nevertheless, the duration of infertility (years spent trying to get pregnant) between the three groups was not statistically different.

**Differential microRNA expression between the three groups**

MicroRNA sequencing was performed on the follicular fluid samples collected from nine women. After sequencing, approximately 5,900,000–23,700,000 clean reads were generated per sample. In order to control for sequencing quality, the Fast-QC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used, and the percentage of reads after filtering was above 70%. The length distribution for clean reads in each sample oscillated between 20 and 24bp. The EB-Seq algorithm was applied to identify differentially expressed genes after FDR analysis \( (\log_{2}FC > 1 \text{ or } < -1, \text{ FDR} < 0.05) \) (Benjamini et al. 2001). In total, 1258 miRNAs were identified. Seven upregulated miRNAs, including miRNA-15a-5p, miRNA-199a-5p, miR-374c-3p and miR-127-5p, as well as 32 downregulated miRNAs, including miR-483-5p, miR-483-3p, hsa-miR-4286 and miR-1180-3p were found when comparing the POR-Y group with the non-POR group (Table 3, FDR < 0.05). In addition, 24 upregulated miRNAs (including miR-885-5p,
miR-199a-5p, miR-3591-3p, miR-122-5p and miR-199a-3p) and 21 downregulated miRNAs (such as miR-513a-5p, miR-509-3p, miR-4433b-3p and miR-4433a-3p) were identified when comparing the POR-O group with respect to the non-POR group. In contrast, the main biological processes identified in the POR-Y group, many biological processes were found when the POR-Y group was compared with the non-POR group (Table 4, FDR < 0.05). We then analyzed and compared POR-Y with non-POR as well as POR-O with non-POR and presented our findings as Venn diagram (Fig. 1). The Venn diagram was showing the overlap of differentially expressed miRNAs among the groups. It has showed that there were 9 common miRNAs that were differentially expressed.

**GO and pathway analyses**

GO and pathway enrichment analyses were performed to identify significantly enriched biological processes. As shown in Supplementary Fig. 1 (see section on supplementary data given at the end of this article), when the POR-Y group was compared with the non-POR group, many biological processes were found to be significantly enriched, including the epidermal growth factor receptor signaling pathway, negative regulation of cell migration and the Wnt signaling pathway (Supplementary Fig. 1A). This indicates that the differential miRNA expression found in POR-Y patients may be associated with cell differentiation, proliferation and apoptotic processes that occur during follicular development and oocyte maturation.

In contrast, the main biological processes identified in the POR-O group with respect to the non-POR group were regulation of transcription, DNA-templated, nervous system development, etc (Supplementary Fig. 1B).

Pathway analysis was performed using the KEGG database. Enriched KEGG pathways are shown in Supplementary Fig. 2. These differentially regulated pathways were consistent with the results of the GO pathway analysis.
Table 4  Differential expressed miRNAs comparing the POR-O group with non-POR group (FDR <0.05).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>POR-O</th>
<th>NC</th>
<th>Log2FC</th>
<th>FDR</th>
<th>Style</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-194-5p</td>
<td>174.3865401</td>
<td>54.60975081</td>
<td>1.675058212</td>
<td>3.6997E-10</td>
<td>up</td>
</tr>
<tr>
<td>hsa-miR-679-5p</td>
<td>105.7298647</td>
<td>28.8821865</td>
<td>1.871829791</td>
<td>1.83924E-08</td>
<td>up</td>
</tr>
<tr>
<td>hsa-miR-351-5p</td>
<td>208.3542341</td>
<td>76.38851579</td>
<td>1.361891501</td>
<td>1.16585E-07</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-491-5p</td>
<td>62.3401532</td>
<td>12.37517564</td>
<td>2.337208465</td>
<td>1.5611E-05</td>
<td>up</td>
</tr>
</tbody>
</table>

FDR, false discovery rate; non-POR, non-poor ovarian response group; POR-O, old poor ovarian response group.

analysis. When the POR-Y group was compared with the non-POR group (Supplementary Fig. 2A), many of these signaling pathways, such as the Hippo signaling pathway, Wnt signaling pathway, pathways in cancer, Ras signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, mTOR signaling pathway and insulin signaling pathway, were all significantly enriched. The same phenomenon occurred in the POR-O group, as shown in Supplementary Fig. 2B, where pathways in cancer, the Hippo signaling pathway, MAPK signaling pathway and insulin signaling pathway were significantly different. However, the axon guidance pathway, related to the nervous system, was enriched the most. In addition, other previously unreported signaling pathways also seem to play a role in POR-O, such as the neurotrophin signaling pathway, focal adhesion and endocytosis pathways.

Network analysis

Figure 2 shows the results of the miRNA–mRNA network analysis, with the red box nodes representing overexpressed miRNAs and the green box nodes representing underexpressed miRNAs. The blue cycle nodes represent miRNAs. Their relationship is represented by one edge. One can see that the
microRNAs in the network regulated some common target genes, such as $FGF9$, $IPO7$, $TAOK1$ and $KPNA4$. Among them, miRNA-15a-5p was the one that regulated the most genes, including $BCL2$. To further explore the miR-15-5p-mRNA network, functional enrichment analysis of these predicted targets was performed (Fig. 3). This analysis shows that these genes correlate tightly with oocyte development, cell–cell signaling, apoptotic process, negative regulation of cell proliferation, phosphatidylinositol 3-kinase signaling, etc.

**Verification of miRNA expression**

Based on GO and pathway analysis of the miRNA sequencing, it can be concluded that the expressions of miR-15a-5p, miR-483-5p, miR-199a-5p and miR-885-5p were clearly different between the POR and non-POR groups (Fig. 4A). In order to validate the miRNA sequencing results, we collected additional follicular fluid and granulosa cells samples from another twelve POR-Y, twelve POR-O and twelve non-POR patients. The results from this analysis shows that the expression levels of miRNA-15a-5p and miRNA-483-5p in the follicular fluid were consistent with our high-throughput sequencing results: miRNA-15a-5p was significantly elevated in the POR-Y group ($P < 0.001$), but not in the POR-O group ($P < 0.05$) (Fig. 4B). As for miR-199a-5p and miR-885-5p, there were no significant differences between the three groups (Fig. 4D and E). Regarding the expression levels in granulosa cells, the tendency was the same as that seen in the follicular fluid: miRNA-15a-5p was significantly
upregulated ($P<0.01$) and miRNA-483-5p was significantly downregulated ($P<0.01$) in the POR-Y group but not in the POR-O group, as shown in Fig. 4 (F, G, H and I).

**Target mRNA expression in GCs**

After combining the qPCR results and the published studies (Cimmino et al. 2005, Guo et al. 2014, Kwon et al. 2014, Li et al. 2015, Long et al. 2016),
we decided to focus our attention on the possible biological effects of miRNA-15a-5p in POR-Y patients since it had been demonstrated to play critical roles in cell proliferation. We investigated the transcription of AKT, mTOR, S6K1, BCL2, BAD, IGF2 in the granulosa cells of POR-Y patients. The results are shown in Fig. 5 (A, B, C, D, E and F). When compared with the non-POR group, several genes promoting cell proliferation, such as AKT, mTOR and S6K1, were all greatly downregulated in POR-Y patients; interestingly, both AKT and S6K1 have binding sites for miRNA-15a-5p. The expression of BCL2, the target gene of miRNA-15a-5p (Cimmino et al. 2005, Li et al. 2015), was significantly downregulated, while BAD was highly upregulated in POR-Y patients (P<0.01). IGF2, which is the host gene of miRNA-483-5p and is co-expressed together with miRNA-483-5p (Shi et al. 2015), was shown to be downregulated in POR-Y patients, and this finding is consistent with our sequencing results.

miR-15a-5p regulates proliferation and apoptosis in the KGN cell line

The miR-15a-5p mimic, miR-15a-5p inhibitor and their scrambled controls were transfected into KGN cells for 48h, and qPCR and Western blot were performed to analyze the expression of related genes. The qPCR results, shown in Fig. 6, were entirely consistent with our previous results obtained with the granulosa cells of patients. Thus, after the miR-15a-5p mimic was transfected, we observed significantly decreased transcription of AKT, mTOR, S6K1 and BCL2 (P<0.001). In contrast, the expression of BAD was significantly increased after transfection (P<0.001). Importantly, following transfection of the miR-15a-5p inhibitor, the outcome was the opposite, with AKT, mTOR (P<0.05), S6K1 and BCL2 (P<0.01) showing significant upregulation when compared with the scrambled control. However, BAD expression levels were not significantly different from those of the scrambled control.

Regarding protein expression, following transfection of the miR-15a-5p mimic, BCL2 protein levels were markedly decreased (P<0.01), as were the levels of p-mTOR, p-S6K1 and p-4EBP1. Furthermore, the p-mTOR/ mTOR ratio was greatly decreased (P<0.001) (Fig. 7A and B). In contrast, after miR-15a-5p inhibitor transfection, only the levels of p-mTOR were significantly decreased.
increased \( (P < 0.05) \). The miR-15a-5p inhibitor had very limited effects on the KGN cell line (Fig. 7C and D).

**Discussion**

In this study, we performed miRNA sequencing to determine the miRNA profile of POR patients, explored the roles of miRNA-15a-5p and its possible relationship with the pathogenesis of POR in young patients. We demonstrated that miRNA-15a-5p was significantly increased in both the follicular fluid and granulosa cells of POR patients when compared with non-POR. Related genes, such as AKT, mTOR, S6K1, BCL2, BAD and IGF2 were significantly altered at the mRNA level in the granulosa cells of POR patients. *In vitro* culture experiments also showed similar results, with transfection of the miR-15a-5p mimic into KGN cells producing an outcome that was extremely consistent with that seen in the granulosa cells of POR-Y patients. In contrast, the miR-15a-5p inhibitor showed the opposite results. Furthermore, the Western blot results showed that the protein levels of p-mTOR, p-S6K1, p-4EBP1 and BCL2, as well as the p-mTOR/ mTOR ratio, were significantly decreased following miR-15a-5p mimic transfection. These findings strongly suggest that miRNA-15a-5p participates in the pathogenesis of POR in young patients.

POR is a significant challenge for both clinicians and patients. It is widely accepted that POR may be an early sign of ovarian aging and reduced ovarian reserve (Ferraretti et al. 2011). These patients show low responses to ovarian stimulation during IVF, produce low-quality embryos and are associated with high cancellation rates. So they undergo more IVF cycles. In our study, both the POR-Y and POR-O groups underwent approximately 3–4 IVF cycles, significantly more than that of non-POR group. Their AMH levels were significantly lower in both POR groups. AMH has the best sensitivity and specificity for predicting ovarian response (Ferraretti et al. 2011). This significantly lower levels of AMH in POR patients demonstrated their reduced ovarian reserve. Furthermore, the number of retrieved oocytes was significantly lower in the POR groups because POR patients have decreased follicular numbers even though following maximal stimulation. Many strategies have been attempted to try to improve this outcome, including high doses of gonadotrophins (Karande et al. 1990), a combination of clomiphene citrate and human menopausal gonadotropin (Saadat et al. 2003), human gonadotropin-releasing hormone antagonist and growth factors.
miRNAs and function in human follicular fluid

Hanrieder). Furthermore, in a study on chronic (et al., 2016 et al., 2014, Li et al., 2015). Alterations in miRNA expression (et al., 2014, Ma et al., 2014, Murchison et al., 2008), etc. However, the ovarian reserve decline in POR-O group is a normal physiological phenomenon mainly due to aging, POR-O women have poor ovarian response to FSH because their antral follicle has been used out, while it is pathological and abnormal for POR-Y patients, these females have abnormal antral follicle pool because of some unknown factors. We explored the reasons from the side of miRNA function, and further studies are needed to explore the mechanism underlying the disease.

The follicular fluid provides a life-sustaining microenvironment for oocytes and the surrounding granulosa cells. It contains a significant variety of molecules, including miRNAs, proteins, lipids and vitamins, which are vital for the maturation and quality of oocytes (Hannrieder et al., 2008, Revelli et al., 2009). It has been reported that the presence of miRNAs is in the supernatant, microvesicles or exosomes from cell-free human follicular fluid (Sang et al., 2013). Microvesicles and exosomes play an important role in cell communication by transferring proteins, RNA and/or miRNA molecules to target cells (Valadi et al., 2007, Yuan et al., 2009). These cell-secreted vesicles containing miRNAs and proteins were took up by surrounding granulosa cells (da Silveira et al., 2012).

miRNAs regulate gene expression post-transcriptionally (Cimmino et al., 2005, Wienholds & Plasterk 2005). Alterations in miRNA expression have been found to play a role in tumorigenesis and development (Ganepola et al., 2014, Guo et al., 2014, Song et al., 2015, Huan et al., 2016, Long et al., 2016). Moreover, miRNAs have been linked to female mouse infertility (Murchison et al., 2007, Nagaraja et al., 2008). MicroRNA-mediated gene regulation is critical for the normal development and function of reproductive somatic tissues and female fertility (Hong et al., 2008). MicroRNAs have also been shown to accelerate transcription turnover during oocyte maturation, which is essential for oocyte meiotic maturation and later ovulation (Murchison et al., 2007). Female mice in which Dicer, an essential factor for miRNA biogenesis, has been knocked out, become infertile (Murchison et al., 2007). It has been demonstrated that altered miRNA expression in cumulus cells is associated with POR, specifically with elevated expression of miR-21-5p, which is an anti-apoptotic factor (Ma et al., 2011, Karakaya et al., 2015). In our study, we found many significant changes in miRNAs through miRNA sequencing, especially in miR-15a-5p, strongly suggesting that it is involved in the pathogenesis of POR by regulating oocytes, granulosa cells proliferation and apoptosis.

In recent years, a number of reports have demonstrated that miR-15a-5p is associated with tumor proliferation and apoptosis (Cimmino et al., 2005, Guo et al., 2014, Li et al., 2015). Overexpression of miR-15a-5p reduced proliferation and induced cell cycle arrest in human hepatocellular carcinoma (Long et al., 2016). Furthermore, other studies indicate that miR-15a regulates biological processes through the insulin/Pi3K-AKT-mTOR signaling pathway (Kwon et al., 2014). In our study, by GO pathway and network enrichment analysis, we found that miR-15a-5p was closely linked to the Pi3K-AKT-mTOR signaling pathway, apoptosis, GnRH signaling pathway and the estrogen signaling pathway. All these biological processes are closely associated with female oocyte proliferation and maturation. Other investigations have also demonstrated that the oocyte PTEN-Pi3K-AKT pathway controls the initiation of oocyte growth and primordial follicle activation (Reddy et al., 2008, Adhikari et al., 2010, Zhang & Liu 2015). Furthermore, in a study on chronic lymphocytic leukemia, the mir15 family was shown to negatively regulate BCL2 expression and promote apoptosis (Cimmino et al., 2005), and BCL2 is one of the important targets of mir-15a-5p (Cimmino et al., 2005, Li et al., 2015).

Our results demonstrate that the levels of expression at the mRNA level of the related genes AKT, mTOR, S6K1 and BCL2 significantly decrease and of BAD significantly increase in the granulosa cells of POR-Y group compared to the non-POR group, and the in vitro culture experiments show similar results. In addition, the protein levels of p-mTOR, p-S6K1, p-4EBP1, BCL2 as well as the p-mTOR/ mTOR ratio significantly decrease after miR-15a-5p mimic transfection, p-mTOR, p-S6K1, p-4EBP1, as well as the p-mTOR/mTOR reflects the active status of PI3K-AKT-mTOR pathway, which is responsible for cell proliferation, their decrease indicates this signaling is repressed. Then how does miR-15a-5p influence the phosphorylation levels of these molecules? We hypothesized that miR-15a-5p might target some genes upstream and thus regulate the phosphorylation status of AKT-mTOR pathway indirectly by the affected genes as Bonci and his colleagues have reported. They have demonstrated that both miR-15a and miR-16 have direct interaction with the 3’UTRs of WNT3A (Bonci et al., 2008). WNT3A signaling has been demonstrated to promote increased β-catenin protein abundance and the activation of other survival
and proliferation pathways through phosphorylation of ERK and AKT (Almeida et al. 2005, Yun et al. 2005), when the levels of WNT3A decreased in the presence of miR-15a and miR-16, the expression of β-catenin and the phosphorylated AKT and ERK reduced (Bonci et al. 2008). Though the exact genes targeted by miR-15a-5p in our study needs to be further investigated.

In our results, we noticed the discrepancy between mRNA level and protein level of mTOR. This might be because gene expression is a complex process that is controlled at multiple steps such as transcriptional regulation, posttranscriptional regulation (RNA processing, RNA stability), translational regulation, post-translational regulation and protein stability. Due to these multiple regulatory checkpoints, RNA and protein levels are not always well correlated (Gupta et al. 2015). Abreu and coworkers also reported the correlation coefficients between mRNA and protein concentrations vary widely across organisms and are often surprisingly low. That is, while there is a clear and significant correspondence between the protein and mRNA concentrations in protein extracts from various organisms, more than half of the variation in protein concentrations cannot be explained by variation in mRNA concentrations. The protein-per-mRNA ratio is different for different genes, but it may also change for a given gene under different cellular conditions (de Sousa Abreu et al. 2009). The translation ‘rate’ of a protein is a composite of the rate of peptide bond formation between amino acids (Fraser et al. 2004), ribosome occupancy and density as well as tRNA availability (Arava et al. 2003). Therefore, the decrease of mRNA level does not necessarily mean the decrease of protein level at the same time.

Additionally, miR-483-5p was found to be significantly decreased in our sequencing and verification experiments. miR-483-5p is an intronic miRNA encoded within the second intron of the IGF2 gene (Landgraf et al. 2007, Ma et al. 2011). In a differential gene expression analysis of women with diminished ovarian reserve (closely associated with POR), it was shown that downregulation of IGFI and IGF2 ligands and their receptors may be associated with DOR pathogenesis. In our study, we also identified changes in the expression of IGF2 in the granulosa cells of POR-Y. We found that IGF2 is significantly downregulated in the granulosa cells of POR-Y patients, which is consistent with decreased expression of miR-483-5p in the follicular fluid of POR-Y patients. However, additional studies need to be performed to explore the possible mechanisms of miR-483-5p involvement in POR pathogenesis.

In conclusion, we found several differentially expressed miRNAs in human follicular fluid by means of miRNA sequencing. We demonstrate that high levels of miR-15a-5p in follicular fluid are associated with oocytes, granulosa cells proliferation and apoptosis. This is likely associated with the mechanisms that underlie POR pathogenesis, especially in the case of POR-Y patients. Furthermore, miR-15a-5p modulates the PI3K-AKT-mTOR signaling pathway and apoptosis, so it might be an important clinical parameter to predict POR. However, further studies are needed to explore its possible application.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0157.

Declaration of interest

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

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