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Association of exosomal microRNAs in human ovarian follicular fluid with oocyte quality

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ABSTRACT

The postponement of childbearing by women has led to an increase in infertility. The reproductive aging process leads to a decrease in both the quantity and quality of oocytes. The aim of this study was to investigate exosomal microRNAs in human ovarian follicular fluid and explore their potential association with oocyte quality. We collected ovarian follicle fluid from 68 patients and assigned the patients to A (superior oocyte quality) or B (poor oocyte quality) group according to their oocyte quality. Exosomal miRNAs were extracted, library constructed and sequenced using the Illumina HiSeq platform. Subsequently, we analyzed exosomal miRNA expression, predicted the miRNA target genes, and enriched Gene Ontology terms using GOSeq. Kyoto Encyclopedia of Genes and Genomes pathway analysis was performed using miRanda. A total of 47 miRNAs were found to be significantly differentially expressed between group A and group B ($p < 0.05$). Among nine differentially expressed miRNAs that were previously known, seven were upregulated in group B. *In silico* analysis indicated that several of these exosomal miRNAs were involved in pathways implicated in oocyte quality. Analysis of the expression of exosomal miRNAs in human ovarian follicular fluid showed that they were critical for maintaining oocyte quality. Our findings provide the basis for further investigations of the functions of exosomal miRNAs in the ovarian microenvironment and suggest that these exosomal miRNAs may be potential biomarkers for evaluating oocyte quality. The findings are potentially important to maintain oocyte quality in clinical settings.

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1. Introduction

In the past 40 years, females have tended to postpone their first childbearing owing to social and economic changes [1], and this trend continues. Studies have shown that oocyte quality is closely related to female aging; women older than 35 years are more prone to miscarriage, dysgenesis, and stillbirth than those younger than 35 [2,3]. However, some young women also experience the degradation of oocyte quality, based on two frequently used indicators, the anti-Müllerian hormone (AMH) and antral follicle count (AFC) [4]. However, the onset of reproductive decline varies among women and is difficult to determine.

Follicular development involves a complex network of interacting cellular signals between somatic cells and oocytes [5]. In particular, exosomes, which are nano-sized, membrane-bound vesicles, are released by different cell types and serve as important mediators of intercellular communication [6,7]. Exosomes contain proteins, DNA, and RNA and can directly influence cellular processes, causing intracellular changes [8,9]. Thus, exosomes are often used as indicators of oocyte quality and competence [10].

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by binding to the 3′-untranslated region of the

Abbreviations: microRNAs, miRNAs; AMH, anti-Müllerian hormone; AFC, antral follicle count; TEM, Transmission electron microscopy; NTA, Nanoparticle tracking analysis (NTA); GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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target mRNA, resulting in mRNA degradation or posttranslational silencing [11,12], thus playing a crucial role in the regulation of development. Exosomal miRNAs are present in ovarian follicular fluid and are involved in the exchange of genetic information between cells [13]. The lipid membrane structure of exosomes acts as a protective barrier against degradation of miRNAs by RNases, making exosomes a reliable source of miRNAs for potential clinical use. Exosomal miRNAs were also reported to affect embryonic potential and oocyte fertilization [14,15]. However, research on exosomal miRNAs in human ovarian follicular fluid and their association with oocyte quality is limited, and the mechanisms underlying their action remain unclear.

This study investigated the relationship between exosomal miRNAs present in human ovarian follicular fluid and oocyte quality. We analyzed exosomal miRNAs from ovarian follicular fluid of women with different oocyte qualities and explored the potential mechanisms underlying the action of miRNAs to provide support for further functional studies of exosomal miRNAs.

2. Materials and methods

2.1. Ethical statement

Written informed consent was provided by all patients who participated in this study. Ovarian follicular fluid was collected at the First Affiliated Hospital of Nanjing Medical University. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (Jiangsu Province Hospital; No: 2016-SR-056) and conducted according to the Declaration of Helsinki.

2.2. Study subjects

We recruited a total of 68 patients and allocated them to two groups according to the quality of their retrieved oocytes. Group A ($n = 33$) comprised patients with a high oocyte quality, and group B ($n = 35$) comprised patients with a poor oocyte quality. The oocyte quality was determined based on the AFC and AMH level. If the AFC was >10 and the AMH level was >1.5 ng/mL, the oocyte quality was considered high; if the AFC was ≤10 and the AMH level was ≤1.5 ng/mL, the oocyte quality was considered poor. This study was conducted at the Reproductive Department of the First Affiliated Hospital of Nanjing Medical University from May 2016 to July 2018.

2.3. Isolation of exosomes from ovarian follicular fluid

Ovarian follicular fluid was collected via transvaginal ultrasound-guided puncture. Immediately following oocyte retrieval, follicular fluid was collected and processed to remove the blood. Follicular fluid was then centrifuged at 2,000 *g* for 10 min to collect the supernatant. Thereafter, the supernatant was centrifuged twice at 100,000 *g* for 70 min (Optima™ MAX-XP tabletop ultracentrifuge; Beckman, Brea, CA, USA) to pellet the exosomes. All centrifugations were conducted at 4 °C. The pellets were resuspended in 50 mL of phosphate-buffered saline (PBS; pH 7.4) at 4 °C and used for analysis.

2.4. Exosome characterization

2.4.1. Transmission electron microscopy (TEM)

The morphology of exosomes was determined using a transmission electron microscope (HT7700; Hitachi, Fukuoka, Japan). Briefly, the isolated exosome preparations were fixed in 1% aqueous osmium tetroxide in cacodylate buffer overnight at 4 °C. The pellets were rinsed with sodium cacodylate (0.1 M, pH7.0) and dehydrated with 75% ethanol. Exosomes were placed on copper grids, stained with 2% uranyl acetate and lead citrate, and examined by TEM.

2.4.2. Nanoparticle tracking analysis (NTA)

Size distribution of the exosomes was determined by NTA (NanoSight NS300; Malvern, UK). Briefly, the samples were diluted with PBS and loaded into a NanoSight instrument, followed by recording videos, which were analyzed to obtain the mean particle size.

2.4.3. Western blotting

Exosomes were lysed with 1 × RIPA buffer at 25 °C. The protein concentrations were determined using a bicinchoninic acid assay (ThermoFisher Scientific, Waltham, MA, USA). Exosomal proteins (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes. The membranes were blocked and incubated with a primary mouse monoclonal CD63 antibody (dilution 1:500; #ab59479, Abcam, Cambridge, MA, USA) and a primary rabbit polyclonal TSG101 antibody (dilution 1:1,000; #14497-1-AP, ProteinTech, Rosemont, IL, USA) overnight at 4 °C. The membranes were subsequently washed with Tris buffer (pH 7.6) and incubated with a secondary horseradish peroxidase-conjugated antibody (Abcam) at 25 °C for 1 h. The signals were detected using a chemiluminescent substrate (ThermoFisher Scientific) and analyzed using a ChemiDoc® gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

2.4.4. miRNA extraction

Total RNA was extracted using the miRNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was dissolved in 30 μL of RNase-free water, and the RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The total RNA was electrophoresed in an agarose gel, and miRNAs were retrieved according to their size (18–50 nucleotides).

2.5. Library construction and high-throughput sequencing

miRNA libraries were prepared using the TruSeq small RNA sample prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, small RNAs were ligated with 3′ and 5′ adapters, followed by reverse transcription to produce single-stranded cDNA. cDNA was amplified by PCR for 11 cycles. The library was sequenced on a HiSeq 2500 platform using SE50 for 10 million clean reads per sample. Data from this study were deposited to the National Center for Biotechnology Information Sequence Read Archive under GEO accession number GSE154740.

2.6. miRNA expression analysis

miRNA expression profiles were analyzed using miRDeep2 [16]. Briefly, to obtain clean reads, sequences with a length between 18 and 26 nucleotides were selected and BLASTed against the miRNA database RFam (https://rfam.xfam.org/) and Repbase database (https://www.girinst.org/repbase/) to remove repeats, non-miRNA families (tRNAs, rRNAs, etc.), and low-quality sequences. Following quality control, the sequences were used for further miRNA analysis. Differential expression analysis was conducted using the DESeq/DESeq2 Bioconductor package (http://www.bioconductor.org/packages/2.12/bioc/vignettes/DESeq/inst/doc/DESeq.pdf). Following adjustment using the Benjamini–Hochberg approach to control the false discovery rate, the p-value was set at < 0.05 to identify differentially expressed miRNAs.

2.7. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

GOSeq (v1.34.1) was used to identify GO terms annotated to a list of enriched genes with a significance of $p < 0.05$. Significant differential gene expression was enriched in KEGG pathways, and miRNAs targeting mRNAs were analyzed using miRanda (v3.3a) (http://www.microrna.org).

3. Results

3.1. Exosome characterization

The average diameters of the exosomes isolated from follicular fluid were in the range of 150–200 nm, as determined by NTA (Fig. 1A), which was consistent with the characteristic sizes of exosomes [17]. The morphology of the isolated exosomes was confirmed by TEM (Fig. 1B). The isolated exosomes were also characterized using two protein biomarkers, CD63 and TSG101 (Fig. 2).

3.2. Differential miRNA expression in exosomes from follicular fluid

A total of 47 miRNAs were found to be significantly differentially expressed between the groups ($p < 0.05$). As shown in Fig. 3, there were distinct miRNA expression patterns between the A and B groups. Notably, nine miRNAs (Table 1) were previously known, and seven of these were upregulated in the bad group. The sequencing data was deposited in Gene Expression Omnibus portal of National Center for Biotechnology Information with a GEO accession number GEO154740.

3.3. GO analysis

Target genes of the significantly expressed miRNAs were annotated using GO analysis, and their functions were categorized.

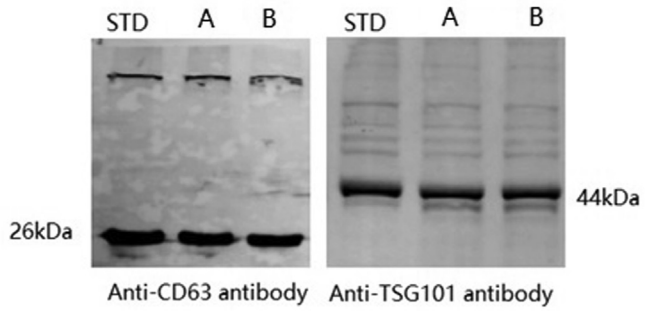

Fig. 2. Western blot analysis of exosomes isolated from ovarian follicular fluid of patients. STD: exosome standards; Group A: high oocyte quality; Group B: poor oocyte quality.

Among the most crucial biological processes involving target genes of the miRNAs that were significantly differentially expressed between the A and B groups were positive regulation of the transcription from the RNA polymerase II promoter, signal transduction, positive regulation of cell aging, and negative regulation of calcium-dependent cell–cell adhesion (Fig. 4).

3.4. KEGG pathway analysis

The KEGG pathway analysis showed that the most significant pathways were the Notch signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, steroid hormone biosynthesis, RNA degradation, gonadotropin-releasing hormone (GnRH) signaling pathway, insulin secretion, calcium, tumor necrosis factor (TNF), Toll-like receptor, PI3K/Akt, Ras, Wnt, ErbB, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and Hippo signaling pathways, with p-values between 7.17E−23 and 4.33E−03 (Table 2).

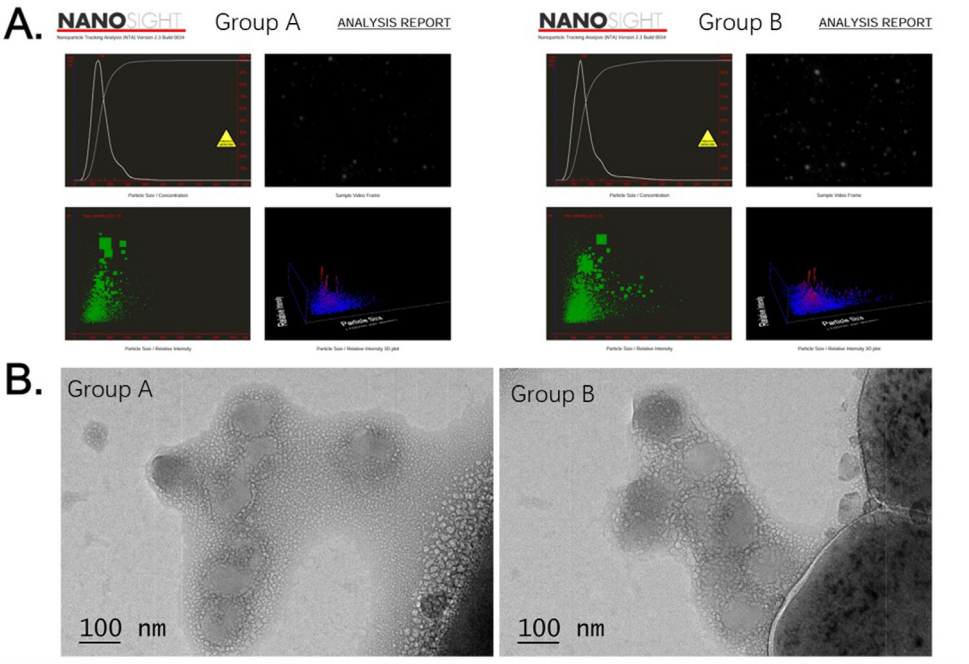

Fig. 1. Characterization of exosomes isolated from the ovarian follicular fluid of patients. (A) Size distribution of exosomes determined by nanoparticle tracking analysis. (B) Morphology of exosomes determined by transmission electron microscopy. Group A: high oocyte quality; Group B: poor oocyte quality.

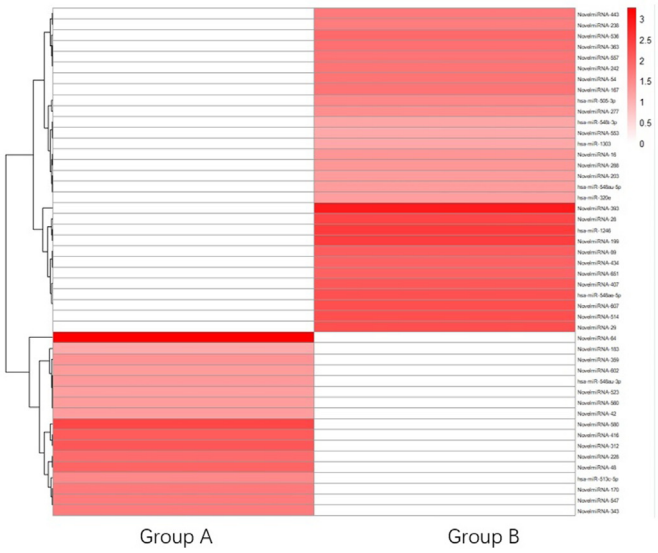

Fig. 3. Heatmap of significantly differentially expressed miRNAs in the A vs. B groups. $p < 0.05$. Group A: high oocyte quality; Group B: poor oocyte quality.

Table 1
Known significantly differentially upregulated and downregulated miRNAs in the B vs. A group ($p < 0.05$).

Gene_id	logFC	p-Value	Regulation
hsa-miR-1246	14.0243	0.001	Up
hsa-miR-548ae-5p	13.0886	0.002	Up
hsa-miR-505-3p	10.7255	0.018	Up
hsa-miR-548t-3p	9.0489	0.021	Up
hsa-miR-513c-5p	−10.5287	0.027	Down
hsa-miR-548au-5p	9.7746	0.037	Up
hsa-miR-320e	9.7407	0.039	Up
hsa-miR-548au-3p	−9.6736	0.041	Down
hsa-miR-1303	9.2770	0.049	Up

FC: fold change. Group A: high oocyte quality; Group B: poor oocyte quality.

4. Discussion

Oocyte degradation is a complex process, and ovarian follicular fluid provides a suitable microenvironment for maintaining oocyte quality. Exosomes present in ovarian follicular fluid are required for intercellular communication; they act as vectors carrying genetic information. However, the mechanism by which exosomal miRNAs affect oocyte quality is unclear. In this study, we investigated miRNA expression in exosomes collected from the ovarian follicular fluid of women with different oocyte qualities and found that there were nine mature significantly differentially expressed miRNAs between the A and B groups.

Four of the differentially expressed exosomal miRNAs that were detected in this study are known to target several pathways involved in the regulation of cell signaling, growth, secretion, and biosynthesis, which suggests that exosomal miRNAs found in ovarian follicular fluid are critical for transmitting signals that regulate cell growth and differentiation. GO analysis showed that signal transduction, cell aging, cell proliferation, metabolic processing, and cell–cell adhesion were among the most enriched terms.

The Notch signaling pathway is a highly conserved signaling mechanism; it is important for the processes of mammalian ovary development, such as differentiation and proliferation, and was shown to regulate steroidogenesis in mouse ovarian granulosa cells [18]. The Hippo signaling pathway, although not confirmed to play a role in mammalian ovary development, was reported to promote the classical development-associated Notch signaling pathway and play a critical role in regulating cell differentiation, proliferation, and oocyte polarity in *Drosophila* [19]. It was also reported that MAPK signaling pathway, which is regulated by GnRH signaling pathway activity, is essential for normal fertility [20]. The calcium signaling pathway potentially negatively affects the oocyte quality by disrupting sequential biochemical changes that initiate egg activation [21]. Kong et al. [22] found that TNF-α and a soluble Fas ligand released by mouse cumulus cells accelerate oocyte aging

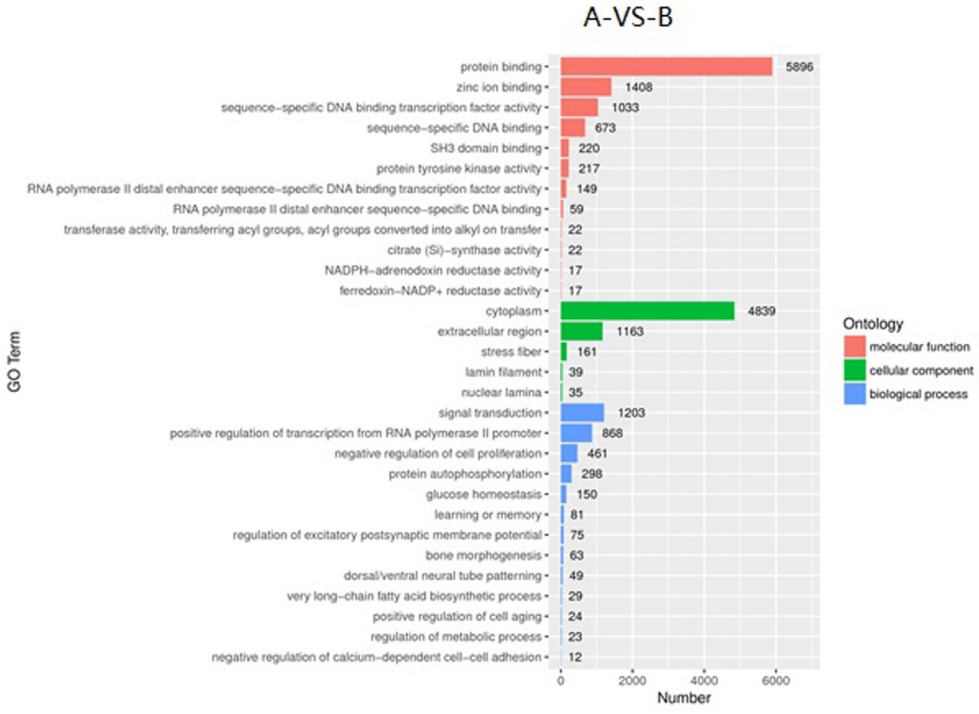

Fig. 4. GO analysis of target genes of significantly expressed miRNAs in follicular fluid exosomes from the A vs. B groups. Group A: high oocyte quality; Group B: poor oocyte quality.

Table 2
Selected list of KEGG pathways that showed significant gene enrichment in A vs B groups.

No.	Pathway ID	Pathway	p value
1	ko04330	Notch signaling pathway	7.17E-23
2	ko04010	MAPK signaling pathway	3.29E-15
3	ko00140	Steroid hormone biosynthesis	3.61E-12
4	ko03018	RNA degradation	5.97E-11
5	ko04912	GnRH signaling pathway	1.32E-09
6	ko04911	Insulin secretion	4.37E-09
7	ko04020	Calcium signaling pathway	2.06E-07
8	ko04668	TNF signaling pathway	3.42E-07
9	ko04620	Toll-like receptor signaling pathway	1.70E-06
10	ko04151	PI3K-Akt signaling pathway	6.07E-04
11	ko04014	Ras signaling pathway	8.35E-04
12	ko04310	Wnt signaling pathway	1.18E-03
13	ko04012	ErbB signaling pathway	2.48E-03
14	ko04064	NF-kappa B signaling pathway	2.61E-03
15	ko04390	Hippo signaling pathway	4.33E-03

$p < 0.05$. Group A: high oocyte quality; Group B: poor oocyte quality.

in vitro. According to Oad et al. [23], ErbB activation promotes signaling pathways such as MAPK and PI3K, thus playing a role in the stimulation of meiotic resumption by gonadotrophin. The NF-κB signaling pathway is also implicated in several processes, such as cell adhesion, proliferation, differentiation, and apoptosis [24].

The miRNA hsa-miR-1246 has been implicated in several diseases, especially cancer [25,26]. However, its functions in human reproduction remain unclear. The role of miR-1246 in bovine embryos was studied *in vitro* and *in vivo* by Ponsuksili et al. [27], who found that a number of differentially expressed endometrial miRNAs detected between days 3 and 7 of the bovine estrous cycle was associated with the state of pregnancy. In our study, hsa-miR-1246 was found to be upregulated in exosomes from the bad group, which suggests that it may be associated with the estrous cycle in humans and affect fertility.

The involvement of hsa-miR-513c-5p in human reproduction has not been investigated. However, miR-513-a-5p, miR-513b-5p, and miR-513c-5p were reported to be the most upregulated miRNAs in breast cancer, of which miR-513-a-5p acts as a risk factor for breast cancer by inhibiting the expression of the progesterone receptor [28]. The upregulation of hsa-miR-513c-5p in breast cancer may also be related to hormonal dysregulation. In our case, its downregulation in the bad group is expected to affect oocyte quality.

The miRNA hsa-miR-320e belongs to the miR-320 family, which includes miR-320a–e. In human follicular fluid, miR-320 has been reported to be associated with embryo quality *in vivo* [29]. Yin et al. [30] demonstrated that miRNA-320 was one of the most downregulated miRNAs in mouse ovarian granulosa cells following TGF-β1 treatment; miR-320 overexpression was shown to inhibit estradiol synthesis and the proliferation of mouse ovarian granulosa cells by targeting E2F1 and SF-1, which affected the follicular development in mice. In our study, hsa-miR-320e was upregulated in the bad group, and we speculated that this upregulation might affect the proliferation of ovarian granulosa cells and result in oocyte degradation. However, the exact function of hsa-miR-320e in this process requires further investigation.

Moreover, hsa-miR-1303 was upregulated in the bad group. It was demonstrated that miR-1303 is involved in tumorigenesis and the progression of several cancers, including prostate cancer [31]. In our study, the hsa-miR-1303-targeted gene was enriched in the Hippo signaling pathway. Hippo signaling is highly conserved and is crucial for cell signaling in growth and development. It is also responsible for the activation of primordial follicles in mice [32]. Li et al. [33] demonstrated that the Hippo signaling pathway and ovarian germline stem cells were associated with ovarian aging in mice. Therefore, the upregulation of hsa-miR-1303 in the bad group may explain, at least in part, the degradation of oocytes.

Furthermore, miR-505-3p represses the onset of puberty, and female mice overexpressing miR-505-3p in the hypothalamus present with delayed ovary maturation and lower fertility [34]. In our study, the expression of hsa-miR-505-3p was higher in the B group than in the A group, indicating that this miRNA possibly causes low fertility.

The miRNA hsa-miR-548 belongs to a poorly conserved family, which is widespread in all chromosomes, except chromosomes 19 and Y [35]. This miRNA gene family comprises 69 members, is primate-specific, and is believed to have originated from transposable elements. We detected the following miRNAs in this study: hsa-miR-548ae-5p, hsa-miR-548t-3p, hsa-miR-548au-5p, and hsa-miR-548au-3p. Three of these were upregulated and one (hsa-miR-548au-3p) was downregulated in the bad group, which indicates the complexity of their possible roles in human ovarian follicular fluid. Son et al. [36] reported that miRNA-548 might be involved in the regulation of high-mobility group box 1 and the pathogenesis of preterm birth. In the miR-548 family, nucleotide sequences are highly divergent, and the occurrence of “seed-shifting” events is not uncommon [35]. The various seed sequences play important and different roles in multiple regulatory networks and signaling pathways. Some miR-548 family members may interact with each other under certain circumstances, and this phenomenon further complicates studies of their exact functions. The exact roles of the miR-548 genes identified in our study are unclear. However, these genes were among the most significantly expressed miRNAs, which implies that they may be crucial for the maintenance of oocyte quality. It is thus necessary to explore their functions in future studies.

In conclusion, we investigated the association of exosomal miRNAs in human ovarian follicular fluid with oocyte quality. Further functional studies of these exosomal miRNAs and their mechanisms are warranted in the future. Our findings may pave a way in maintaining oocyte quality in the ovarian microenvironment and provide potential biomarkers for evaluating oocyte quality in clinical settings.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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