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Correlation between the follicular fluid extracellular-vesicle-derived microRNAs and signaling disturbance in the oocytes of women with polycystic ovary syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is a common reproductive disorder characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovaries. The quality of oocytes in PCOS patients remains poor, leading to poor pregnancy outcomes. The molecular mechanisms underlying the poor quality of oocytes in PCOS are not fully understood. This study aimed to explore the potential functional microRNAs (miRNAs) in follicular fluid (FF)-derived extracellular vesicles (FF-EVs) and their role in oocyte developmental competence in PCOS. We analyzed DEmiRNAs in FF-EVs and DEGs in oocytes from PCOS patients and controls using GEO database. We identified 14 potential functional DEmiRNAs in FF-EVs and predicted the target genes of 14 DEmiRNAs using TargetScan. We performed conjoint analyses between the target genes of these miRNAs and DEGs in oocytes, identifying 12 DEmiRNAs whose target genes overlap with oocyte DEGs. Thus, 12 functional DEmiRNAs were the hub miRNAs. These miRNAs were predicted to target genes involved in oocyte development and signaling pathways such as PI3K/Akt, Ras, and MAPK pathways. KEGG enrichment analysis suggested that these miRNAs might impair oocyte developmental competence in PCOS by dysregulating PI3K/Akt signaling pathway. qRT-PCR validated the increase of miR-93-3p and miR-152-3p, and the decrease of miR-625-5p and miR-17-5p in FF-EVs of PCOS patients. This study highlighted the significance of FF-EVs in the pathology of PCOS and revealed the potential role of the increase of miR-93-3p and miR-152-3p, and the decrease of miR-625-5p and miR-17-5p in impairing oocyte developmental competence in PCOS. Further research is needed to elucidate the specific mechanisms by which these miRNAs affect oocyte development and to explore the potential therapeutic implications.

Keywords Extracellular vesicle, Follicular fluid, Oocyte, Polycystic ovary syndrome, miRNA

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Introduction

Polycystic ovary syndrome (PCOS) is a highly prevalent endocrine and metabolic disturbance, characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovaries [1]. It affects up to 20% of women of reproductive age worldwide, occupying a significant position in the cause of infertility [2]. The diagnosis of PCOS is disputed, resulting from a wide range of clinical symptoms [3]. Currently, the Rotterdam criterion for diagnosing PCOS is preferred, which includes signs of androgen excess, oligo-ovulation or anovulation, or polycystic ovary morphology (PCOM) [4]. Additionally, patients with PCOS are more likely to experience menstrual irregularities, obesity, insulin resistance, etc [3]. Although the clinical characteristics of PCOS remain heterogeneous, disturbance in folliculogenesis is still a common characteristic leading to sub-fertility or infertility. Women with PCOS are more prone to develop low oocyte developmental competence, resulting in poor embryo development and pregnancy outcomes in some PCOS women [5]. Many factors, such as hyperandrogenism, hyperinsulinemia, and dysregulation of paracrine molecules, all disrupt the follicular micro-environment and further impair oocyte maturation [5]. However, the mechanism of poor oocyte development in PCOS remains to be elucidated.

Follicular fluid (FF) is the medium in the mature ovarian follicle for intercommunication among oocytes, granulosa cells (GCs), and other surrounding cells [6]. It de-rives from plasma or is secreted by GCs, theca cells, and oocytes [7]. There are proteins, steroids, and metabolites in FF, that support normal follicular growth and oocyte maturation [7]. Recently, exosomes extracellular vesicles(EVs) in FF have also attracted attention, as these carriers with key molecular cargos may exert function in steroidogenesis and follicular development [8]. EVs are lipid bilayer complexes, of which diameter is 50–150 nm [9]. Our research group's previous research found that EVs from PCOS follicular fluid disrupted oocyte mitochondria and spindles, hindering oocyte maturation through oxidative stress [10]. However, the specific molecular mechanism remains unclear.

FF-EVs can transport microRNAs (miRNAs), circular RNAs (circRNAs), long non-coding RNAs (lncRNAs), and proteins to recipient cells, which may target pathways associated with follicular development and oocyte maturation [11]. For instance, many miRNAs in human FF-EVs, such as miR-31, miR-95, and miR-99b-3p, are predicted to regulate the wingless (WNT), mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (ErbB), or transforming growth factor beta (TGFβ) signaling pathways [11]. Hence, the miRNA profile in FF-EVs is important for follicular development. MiRNAs are small non-coding single-stranded RNA molecules, of which the length is about 18–24 nucleotides [12]. It may suppress the expression of gene post-transcriptionally by binding the 3' untranslated regions of the target messenger RNAs (mRNAs) [12]. It has been reported that the miRNA profile in the FF differed between PCOS and normal control women [13]. Furthermore, Cao et al. revealed that the exosomal miR-143-3p/miR-155-5p derived from FF could regulate glycolysis in GCs, thus leading to follicular dysplasia in PCOS [14]. Therefore, changes in miRNA profile in FF-EVs may be relevant to the progression of this ovarian disease.

Currently, most of the articles reported that miRNA in FF-EVs acted on target genes in GCs to affect follicular development-related pathways [14, 15]. Considering EVs are capable of traversing the zona pellucida [16], we emphasized the FF-derived exosomal miRNAs could directly regulate signaling pathways in oocytes. In this study, we performed analyses of the FF-derived exosomal miRNA expression profiles and differentially expressed genes (DEGs) in oocytes between PCOS patients and control women. The target genes of differentially expressed miRNAs (DEmiRNAs) were analyzed by TargetScan. We further identify the hub miRNAs and mRNAs by analyzing the target genes and DEGs in oocytes. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, Gene Ontology (GO) enrichment, and Protein-Protein Interaction (PPI) networks were also constructed for a systematic understanding of PCOS at the molecular level. Finally, qRT-PCR was used to verify the differential expression of hub miRNAs in FF-EVs between PCOS patients and healthy fertile women. Identification of crucial exosomal miRNAs in the FF may provide new evidence for a better understanding of the pathology of PCOS.

Results

Identification of differentially expressed miRNAs in EVs from PCOS and Controls

The detailed information on GSE157037 was displayed in Table 1. 7 cases of PCOS and 8 controls were included in this study. The clustering analysis of DEmiRNAs was shown in Fig. 1A. A total of 14 DEmiRNAs (5 up-regulated and 9 down-regulated in PCOS patients compared with control women) were identified under the selection criteria of$|\log FC| > 1$ and $P < 0.05$ (Table 2). The DEmiRNAs between PCOS patients and control women were visualized in the volcano plot (Fig. 1B).

Oocytes derived DEGs between PCOS and controls

The detailed information on GSE155489 was demonstrated in Table 1 as well. In this analysis, 6 cases of PCOS and 8 controls were included. The clustering heat map and volcano plot were shown in Fig. 2A and B. A total of 976 genes (635 up-regulated and 341 down-regulated

Table 1 The detailed information of GEO datasets

GEO accession	Platforms	Samples	Ovarian stimulation	Ovum pick-up
GSE157037	GPL18573; Illumina NextSeq 500.	FF*-EVs* derived from 7 PCOS* and 8 Controls.	GnRH* antagonist protocols	≥ 2 follicles were ≥ 18 mm in diameter; 36 h after hCG* triggering.
GSE155489	GPL20795; HiSeq X Ten.	GV-stage oocytes derived from 6 PCOS* and 6 Controls.	GnRH* antagonist protocols	≥ 2 follicles were ≥ 12 mm in diameter; 36 h after hCG* triggering.

* FF, follicular fluid; EVs, EVsextracellular vesicles; PCOS, polycystic ovary syndrome; GnRH, gonadotro-pin-releasing hormone; hCG, human chorionic gonadotrophin

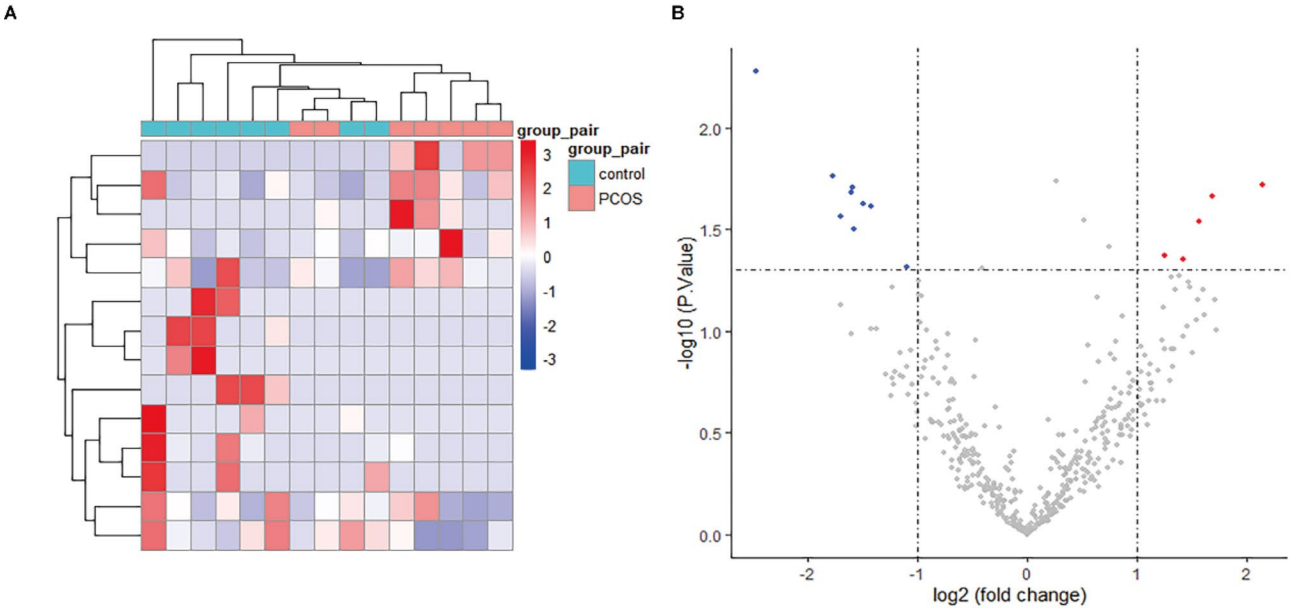

Fig. 1 Identification of differential miRNA profiles in FF-EVs between PCOS and control women. (**A**) Heat map revealed clustering analysis of differentially expressed miRNAs (DEmiRNAs) between PCOS and control women. Row and column represented DEmiRNAs and groups respectively. (**B**) The volcano plot showed the DEmiRNAs between PCOS and control women

Table 2 FF*-derived exosomal DEmiRNAs* of PCOS* patients compared with that of control women

Symbol	Log FC	*P* value
hsa-miR-145-5p_MIMAT0000437	2.15097	0.019
hsa-miR-93-3p_MIMAT0004509	1.68726	0.022
hsa-miR-152-3p_MIMAT0000438	1.57139	0.029
hsa-miR-191-3p_MIMAT0001618	1.42139	0.045
hsa-miR-146b-5p_MIMAT0002809	1.26057	0.042
hsa-miR-17-5p_MIMAT0000070	-2.46272	0.005
hsa-miR-508-3p_MIMAT0002880	-1.76261	0.017
hsa-miR-802_MIMAT0004185	-1.69716	0.027
hsa-miR-33a-5p_MIMAT0000091	-1.60194	0.020
hsa-miR-625-5p_MIMAT0003294	-1.58771	0.019
hsa-miR-507_MIMAT0002879	-1.57316	0.031
hsa-miR-1185-1-3p_MIMAT0022838	-1.49057	0.023
hsa-miR-374b-3p_MIMAT0004956	-1.41797	0.024
hsa-let-7c-3p_MIMAT0026472	-1.09097	0.048

* FF, follicular fluid; DEmiRNAs, differentially expressed miRNAs; PCOS, polycystic ovary syn-drome

in PCOS patients in comparison with control women) were regarded as DEGs. All DEGs were presented in Data sheet 1.

Afterward, analyses of GO and KEGG enrichment were performed to reveal the function of the above DEGs (Fig. 2C and D). These analyses revealed that DEGs were primarily enriched in 24 GO terms (Fig. 2C) and 8 KEGG pathways (Fig. 2D). For the BP, most of the genes were significantly enriched in the proteasomal protein catabolic process, non-coding RNA (ncRNA) metabolic process, and ribonucleoprotein complex biogenesis (Fig. 2C). For the CC, DEGs were significantly enriched in the mitochondrial matrix, mitochondrial inner membrane, microtubule, ubiquitin ligase complex, and so on (Fig. 2C). For the MF, these genes were enriched in transcription coregulator activity, ATPase activity, and protein serine/threonine kinase activity (Fig. 2C). As for the KEGG pathway, DEGs were found to be involved in ubiquitin-mediated proteolysis and nucleocytoplasmic transport pathway significantly (Fig. 2D).

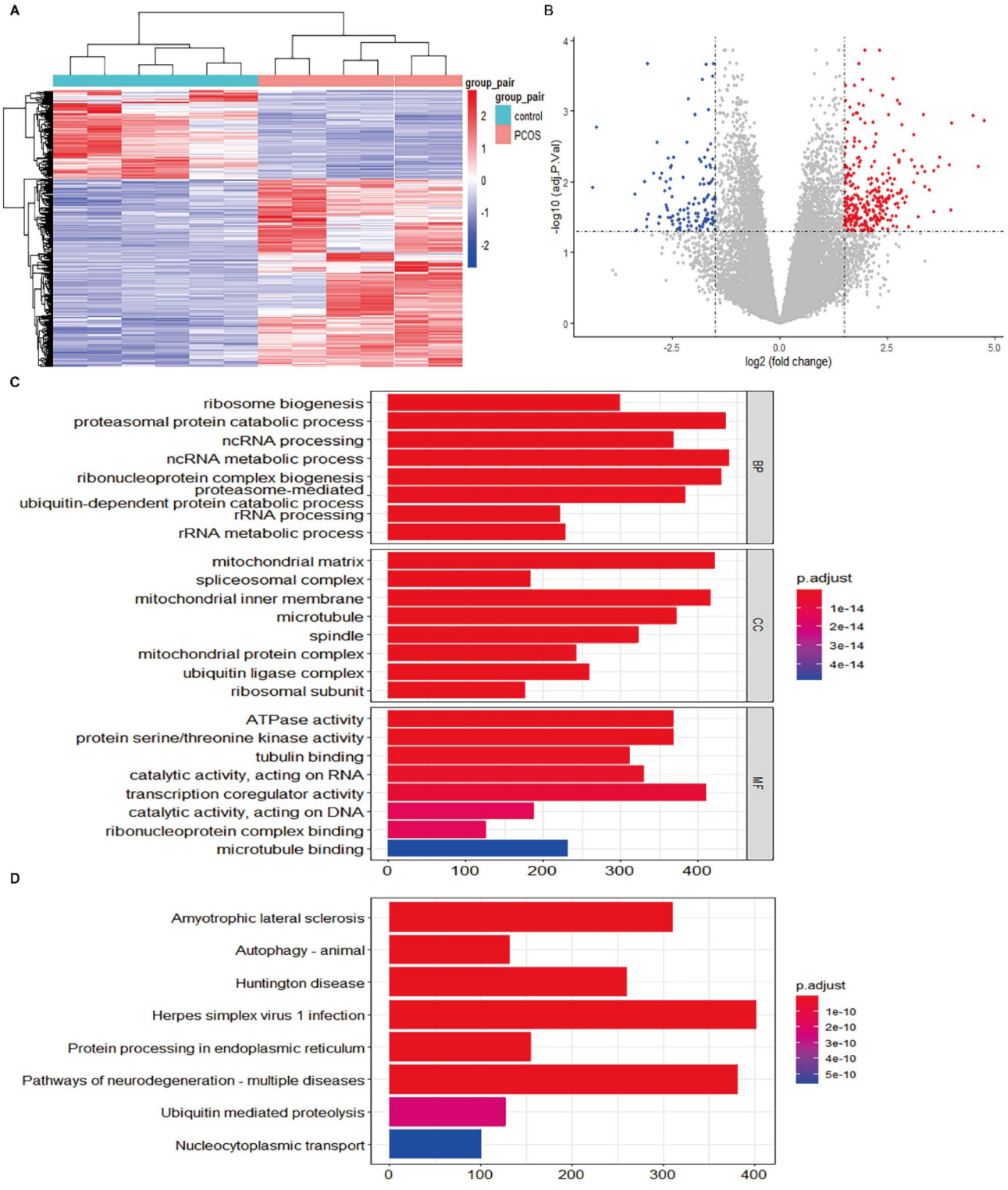

Fig. 2 Identification and functional analysis of differential mRNA transcriptional profiles in oocytes between PCOS and control women. (**A**) The heat map showed the clustering analysis of differentially expressed mRNAs (DEGs) between PCOS and control women. Row and column represented DEGs and groups respectively. (**B**) The volcano plot revealed DEGs between PCOS and control women. (**C**) The bar chart demonstrated the GO enrichment analysis of DEGs. BP: Biological process, CC: Cell component, MF: Molecular function. (**D**) The bar chart shows the KEGG pathway analysis of DEGs. The length of bars in (**C**) and (**D**) represented the number of DEGs that were involved. The color of the bars indicated the p values of pathways

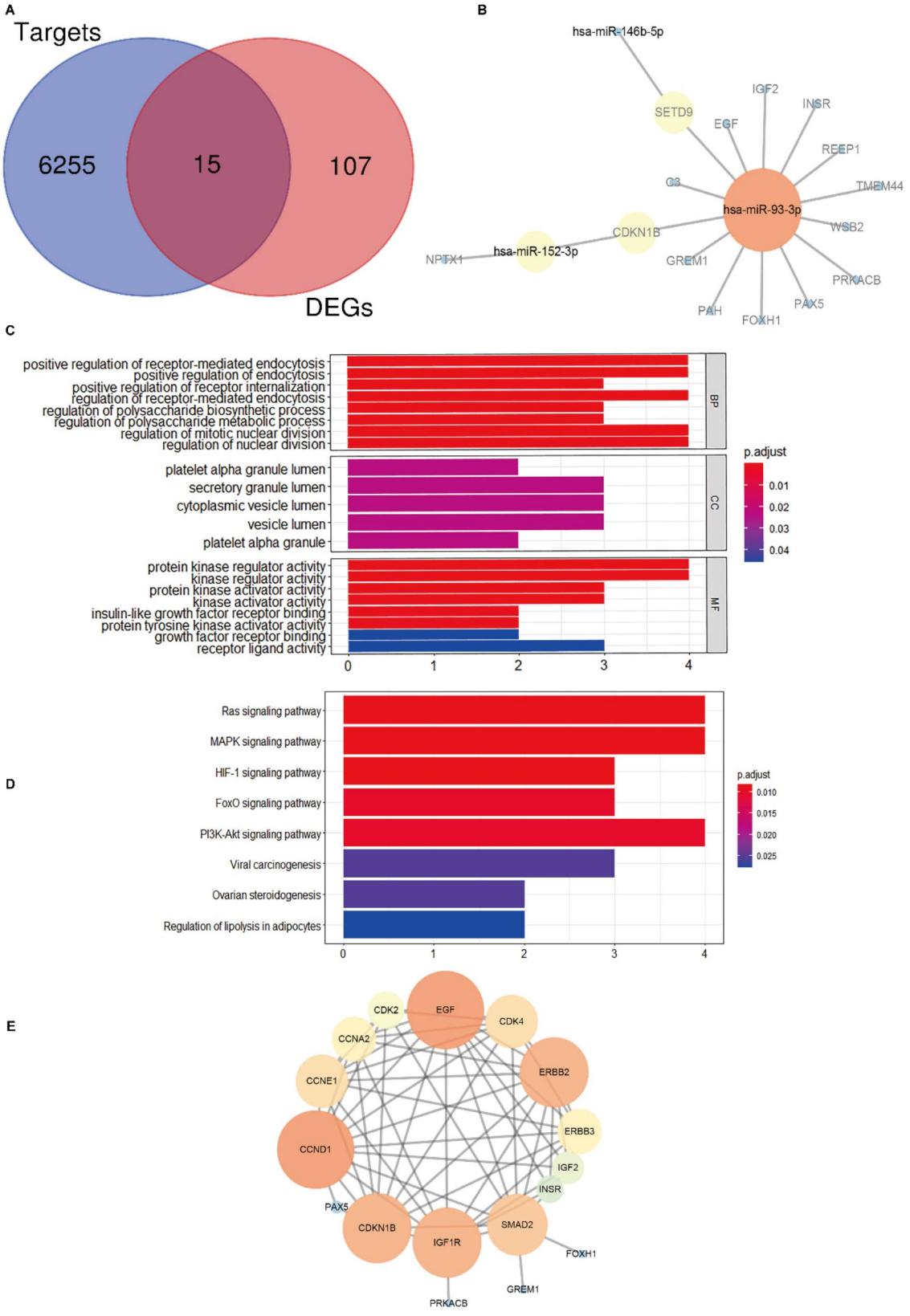

Fig. 3 (See legend on next page.)

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Fig. 3 Conjoint analyses of up-regulated DEmiRNAs in FF-EVs and down-regulated DEGs in oocytes. (**A**) The Venn diagram demonstrated the overlapping genes of the up-regulated DEmiRNAs' target genes and the down-regulated DEGs. (**B**) The co-expression network between the up-regulated DEmiRNAs and down-regulated DEGs. (**C**) The bar chart of GO enrichment analysis of overlapping genes. BP: Biological process, CC: Cell component, MF: Molecular function. (**D**) The bar chart showed KEGG pathway analysis of overlapping genes. The length of bars in (**C**) and (**D**) represented the number of overlapping genes that were involved. The color of bars indicated p values of pathways. (**E**) The protein-protein interaction network of overlapping genes. The size and color of node in (**B**) and (**E**) represented the number of interacting genes. Large sizes and dark colors indicated more genes interacted

Networks of DEmiRNAs-DEGs interaction

To reveal the effect of exosomal DEmiRNAs on oocytes, we did conjoint analyses of DEmiRNAs in FF-EVs and DEGs in oocytes. A total of 6270 genes were predicted to be target genes of significantly up-regulated miRNAs (Data sheet 2). 15 overlapping genes were identified between significantly up-regulated miRNAs' target genes and down-regulated DEGs (Fig. 3A). These overlapping genes were shown in Data sheet 3. The co-expression network of up-regulated miRNAs and above overlapping genes was constructed and demonstrated that 3 functional DEmiRNAs were the hub miRNAs, including hsa-miR-93-3p, hsa-miR-152-3p, and hsa-miR-146b-5p (Fig. 3B). Epidermal growth factor (EGF), insulin-like growth factor (IGF), cyclin-dependent kinase inhibitor 1B (CDKN1B), and SET domain-containing protein 9 (SETD9) could be silenced by hub miRNAs, which might impair the development of oocytes (Fig. 3B). To determine the function of the above overlapping genes, GO and KEGG analyses were also performed (Fig. 3C and D). The enriched GO functional terms were mostly involved in the positive regulation of receptor-mediated endocytosis, cytoplasmic vesicle lumen, protein kinase regulator activity, and growth factor receptor binding (Fig. 3C). Moreover, the enriched KEGG pathways were involved in the Ras signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, HIF-1 signaling pathway, FoxO signaling pathway, and ovarian steroidogenesis (Fig. 3D). The PPI network of these overlapping genes showed that there were hub genes involved in up-regulated hub miRNAs-mediated regulation in oocytes, such as EGF, IGF1R, and CDKN1B (Fig. 3E).

In addition, a total of 12,186 genes were identified as target genes of down-regulated miRNAs (Data sheet 4). Then, 93 overlapping genes were found between significantly down-regulated miRNAs' target genes and up-regulated DEGs (Fig. 4A). The overlapping genes were presented in Data sheet 5. The network of down-regulated DEmiRNAs and overlapping genes was built as well (Fig. 4B). It revealed that 9 functional DEmiRNAs were the hub miRNA and many ovarian function associated genes could be silenced including PIK3CA, FOXO4, and MAPT (Fig. 4B). For GO enrichment analysis, only microtubule was the associated (Fig. 4C). For KEGG pathways analysis, there were 3 pathways involved, including the Ras signaling pathway, PI3K-Akt signaling pathway, and HIF-1 signaling pathway (Fig. 4D). The PPI network of the overlapping genes revealed that there were hub genes involved in down-regulated hub miRNAs-mediated regulation in oocytes, such as PIK3CA, MAPT, GNB3, and FOXO4 (Fig. 4E).

Clinical characteristics of patients

To confirm the result of the bioinformatic analysis, a total of 5 PCOS patients and 5 control women were enrolled in this study. The clinical characteristics of these women were presented in Table 3. There was no significant difference in age, BMI, basal follicle-stimulating hormone (bFSH), or estradiol (E2) between the two groups (Table 3). In terms of antral follicle count (AFC) and number (No.) of oocytes retrieved, PCOS patients had higher levels compared with that of control women (Table 3).

Characterization of EVs

The protein markers of EVs, such as flotillin-1 (Flot-1) and CD9, were detected in the PCOS-EVs and Control-EVs (Fig. 5A). The results showed that these EVs markers were enriched in PCOS-EVs and Control-EVs, whereas the endoplasmic reticulum-specific protein calnexin was not detected in these EVs samples (Fig. 5A). The size and distribution of EVs between the two groups were evaluated using NTA, and the results revealed that these EVs were both around 100 nm in size (Fig. 5B). Our results show that PCOS patients have a certain extent of increase in exosome content compared to healthy controls. The morphology of these EVs was captured using TEM and showed that these EVs appeared as bilayers (Fig. 5C).

qRT-PCR validation of differentially expressed miRNAs in FF-EVs between PCOS and controls

The expression of hsa-miR-93-3p, hsa-miR-152-3p, hsa-miR-146-5p, hsa-miR-625-5p, hsa-miR-17-5p, hsa-miR-508-3p, hsa-miR-802, hsa-miR-33a-5p, hsa-miR-507, hsa-miR-1185-1-3p, hsa-miR-374b-3p, hsa-let-7c-3p in FF-EVs between PCOS patients and control women was validated using qRT-PCR (Fig. 6). The relative fold change of these miRNAs was normalized to hsa-miR-16-5p. The results demonstrated that hsa-miR-93-3p and hsa-miR-152-3p were significantly increased while hsa-miR-625-5p and hsa- let-7c-3p were significantly decreased in PCOS-EVs in comparison with that in control-EVs (Fig. 6A, B, D, and E). However, there was no

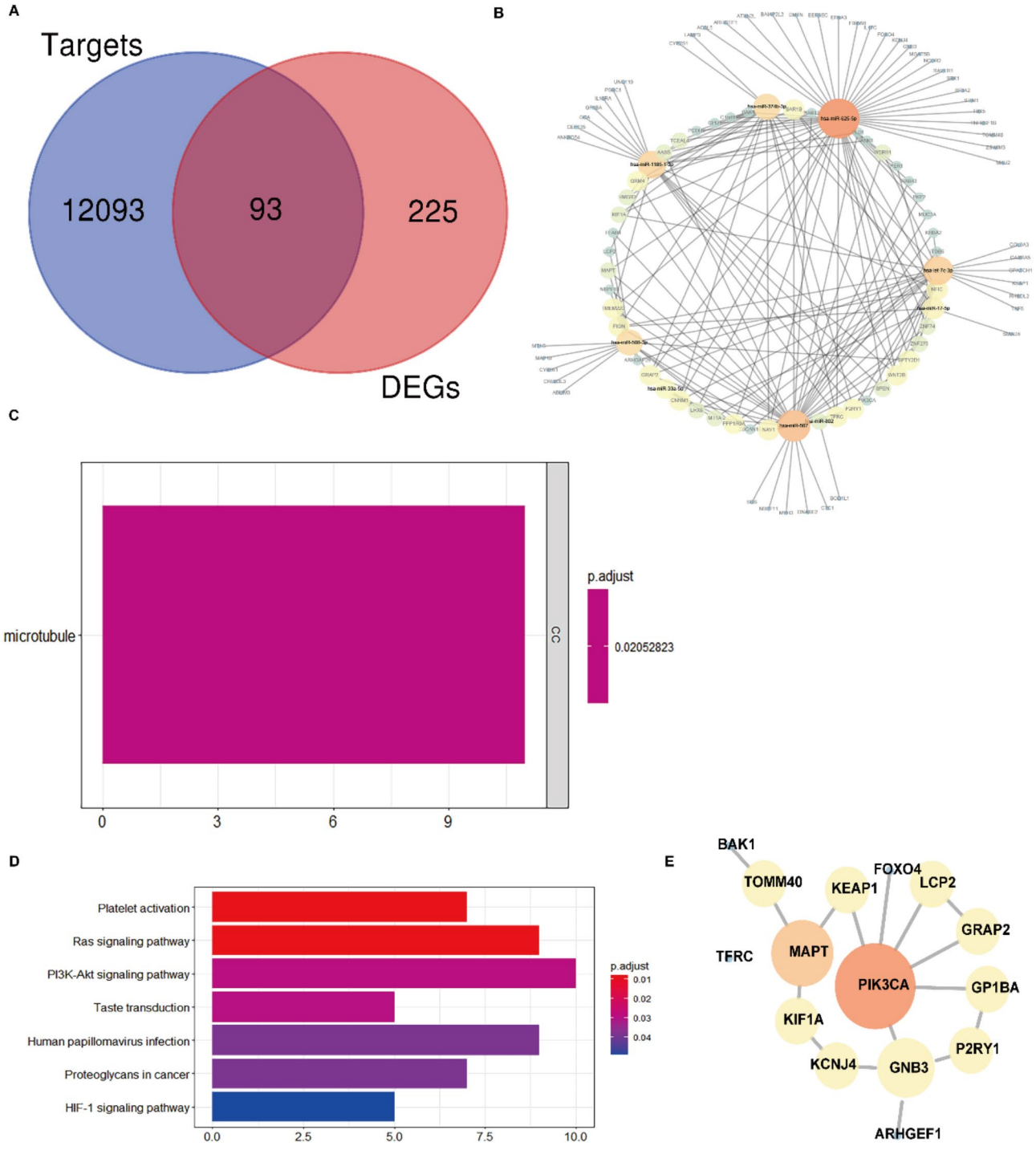

Fig. 4 Conjoint analyses of down-regulated DEmiRNAs in FF-EVs and up-regulated DEGs in oocytes. (**A**) The Venn diagram demonstrated the overlapping genes of the down-regulated DEmiRNAs' target genes and the up-regulated DEGs. (**B**) The co-expression network between the down-regulated DEmiRNAs and up-regulated DEGs. (**C**) The bar chart revealed GO enrichment analysis of overlapping genes. BP: Biological process, CC: Cell component, MF: Molecular function. (**D**) The bar chart showed KEGG pathway analysis of overlapping genes. The length of bars in (**C**) and (**D**) represented the number of overlapping genes that were involved. The color of bars indicated p values of pathways. (**E**) The protein-protein interaction network of overlapping genes. The size and color of node in (**B**) and (**E**) represented the number of interacting genes. Large sizes and dark colors indicated more genes interacted

Table 3 Baseline characteristics of PCOS patients and control women

Parameters	Ctrl $n=5$	PCOS* $n=5$	*P* value
Age	27.20 ± 3.19	27.20 ± 1.30	0.999
BMI*	23.17 ± 2.85	21.65 ± 2.44	0.392
bFSH*	7.55 ± 2.99	6.35 ± 4.60	0.636
E_2*	29.2 ± 8.57	28.15 ± 7.32	0.840
AFC*	13.60 ± 4.28	25.00 ± 2.83	0.001*
No. of oocytes retrieved	12.40 ± 4.22	20.80 ± 6.65	0.044*

* PCOS, polycystic ovary syndrome; BMI, body mass index; bFSH, basal follicle-stimulating hormone; E2, estradiol; AFC, antral follicle count

significant difference in other miRNAs between the two groups (Fig. 6C, F, G, H, I, J, K, and L).

Discussion

The human ovarian follicle is a dynamic complex, wherein FF represents an important microenvironment for the development of the oocytes [17]. FF provides a useful biological matrix not only for autocrine, paracrine, or endocrine signals but also for EVs communications [18]. Recently, emerging evidence has proved that there were EVs in FF of human follicles, and these carriers could transport ncRNAs or proteins to recipient cells in follicles [19]. Our research group previously discovered that EVs from PCOS follicular fluid interfered with oocyte mitochondria and spindles, impairing oocyte maturation via oxidative stress [1]. Notwithstanding, the molecular mechanism of FF-EVs on PCOS pathological oocytes' development has been still unknown. In this study, we identified and validated hub miRNAs of FF-EVs, including hsa-miR-93-3p, hsa-miR-152-3p, hsa-miR-625-5p, and hsa-miR-17-5p, that may potentially influence oocytes. The increase of hsa-miR-93-3p and hsa-miR-152-3p, and the decrease of hsa-miR-625-5p and hsa-miR-17-5pmight be associated with the disturbance of oocyte maturation in PCOS patients.

The result revealed that there were up-regulated miRNAs in FF-EVs of PCOS patients compared with that of control women, including hsa-miR-145-5p, hsa-miR-93-3p, hsa-miR-152-3p, hsa-miR-191-3p, and hsa-miR-146b-5p. Further con-joint analysis and qRT-PCR verified that hsa-miR-93-3p and hsa-miR-152-3p were the hub miRNAs. Butler et al. also demonstrated that the expression of hsa-miR-93-3p was elevated in FF of PCOS patients, which was consistent with our result [13]. They further found that hsa-miR-93-3p was correlated with C-reactive protein (CRP), which was a marker of inflammation [12]. It suggested that hsa-miR-93-3p might be involved in the regulation of inflammatory response to affect the follicular development of PCOS. Although the miRNAs studied above were non-EXO-bound RNAs, which de-rived from FF instead of FF-EVs, it still indicated that FF-derived hsa-miR-93-3p played an important role in impairing the follicular development. Moreover, miR-145-5P, miR-146b-5p, or miR-152-3p was reported to be associated with the biomarker of PCOS, the occurrence of PCOS, or abnormal follicle development, which was inconsistent with our study as well [20–22].

Our study also reported the FF-derived exosomal down-regulated miRNAs in PCOS patients, such as hsa-miR-17-5p, hsa-miR-508-3p, hsa-miR-802, hsa-miR-33a-5p, hsa-miR-625-5p, hsa-miR-507, hsa-miR-1185-1-3p, hsa-miR-374b-3p, and hsa-let-7c-3p. The conjoint analysis and qPCR validated that hsa-miR-625-5p and hsa-miR-17-5p were the hub miRNAs. A study described that miR-625-5p might suppress inflammatory response by targeting AKT2 and inhibiting the nuclear factor κB pathway [23]. Therefore, it indicated that the decrease of miR-625-5p in FF-EVs of PCOS might promote inflammation, resulting in the disturbance of oocyte growth. Besides, miR-17-5p was also reported to inhibit TXNIP/NLRP3 inflammasome

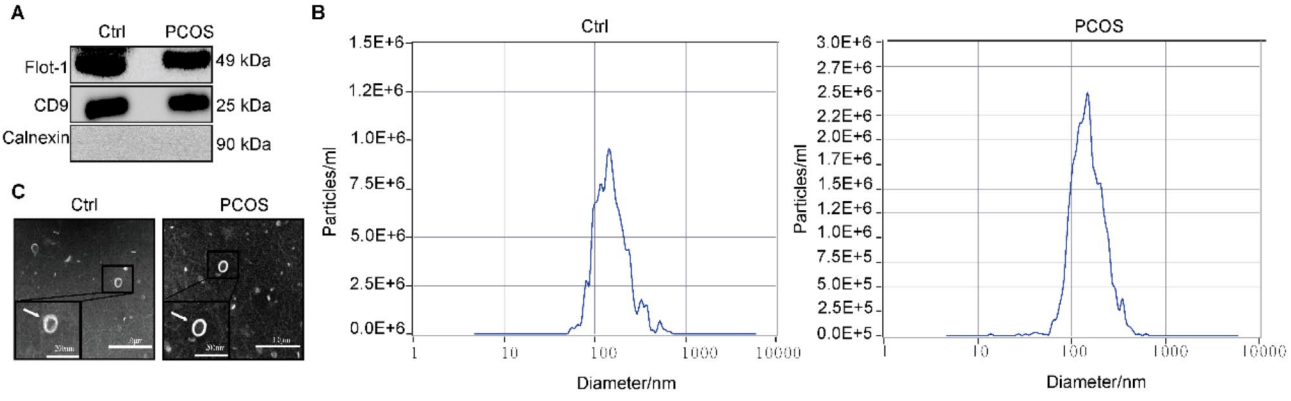

Fig. 5 Characterization of PCOS-EVs and Ctrl-EVs. (**A**) Western Blotting showed the expression of EV protein markers, Flot-1 and CD9, in PCOS-EVs and Control-EXO, but the endoplasmic reticulum-specific protein calnexin was not detected in EVs. Ctrl: Control. (**B**) The number and size of EVs were evaluated using NTA. The representative NTA images of PCOS-EVs and Ctrl-EVs were presented. (**C**) The representative TEM images of PCOS-EVs and Ctrl-EVs were shown

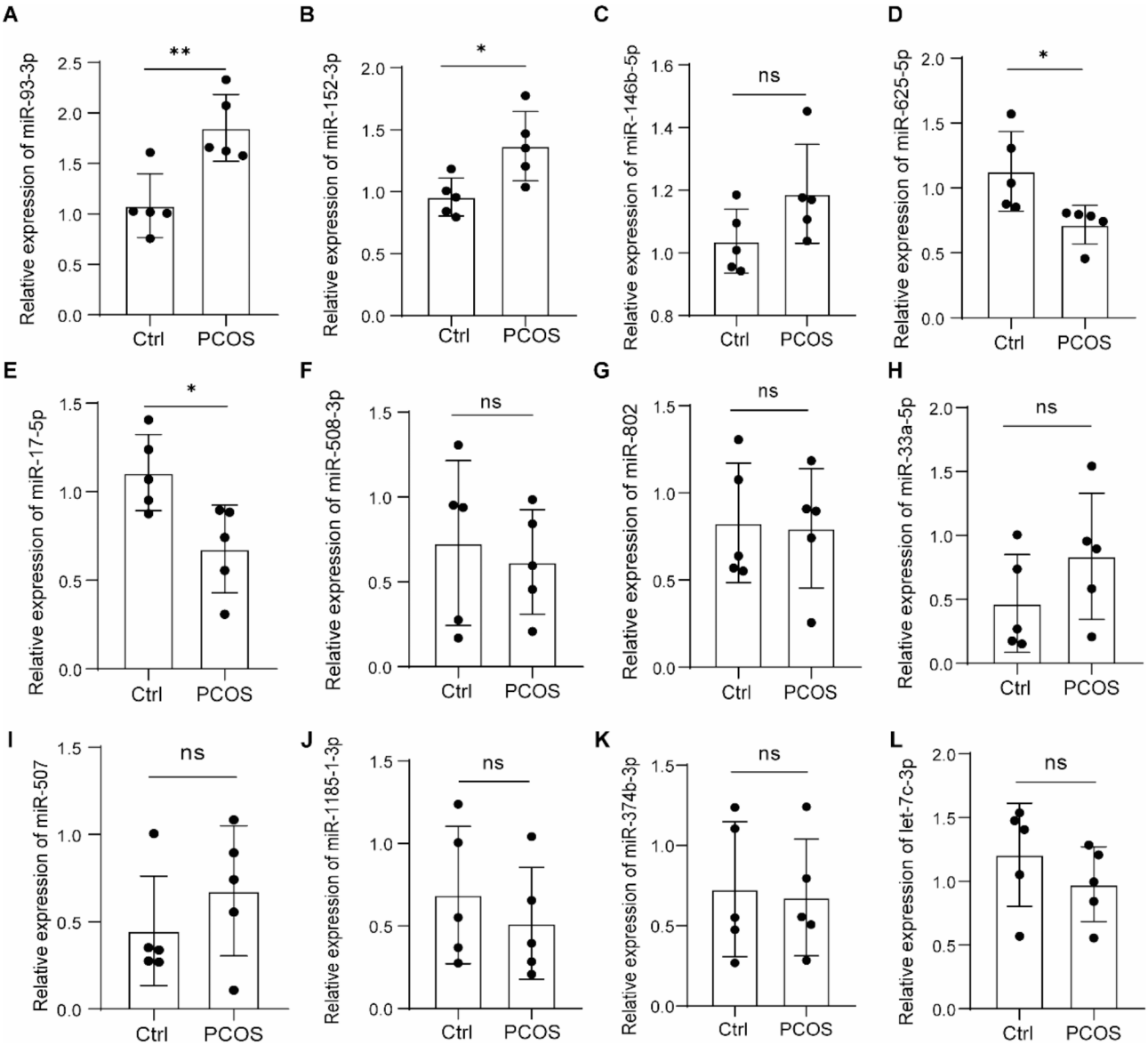

Fig. 6 Validation of the hub miRNAs in FF-EVs using qRT-PCR * $P < 0.05$, ** $P < 0.01$, ns means not significant

pathway in diabetes; thus, the down-regulation of miR-17-5p was related to the high level of inflammation as well [24].

Previous studies only focused on the effect of miRNAs on GCs [8, 25]. For example, many researches have proved the uptake of FF-EVs by GCs [8, 15, 25]. The FF-derived exosomal miRNAs of PCOS patients could target key elements in pathways in GCs, which were involved in the development of follicles, such as PI3K/Akt/mTOR signaling pathway [15] and Smad1/5/8 signaling pathway [25]. And here, to elucidate the mechanism of EVs directly affecting oocyte development, we conjointly analyzed the exosomal DEmiRNAs and DEGs of oocytes between PCOS and controls.

In this study, validated up-regulated EXO-miRNAs were predicted to directly target complement component 3 (C3), CDKN1B, EGF, forkhead box H1 (FOXH1), gremlin 1 (GREM1), IGF2, insulin receptor (INSR), phenylalanine hydroxylase (PAH), paired box 5 (PAX5), protein kinase cAMP-dependent catalytic beta (PRKACB), receptor accessory protein 1 (REEP1), SETD9, transmembrane protein 44 (TMEM44), and WD repeat and SOCS box containing 2 (WSB2) of oocytes. Thereinto, many genes were involved in oocyte developmental competence, such as EGF, IGF2, and INSR [25–27]. EGF can induce oocyte maturation, regulate the oocyte integrity during the meiotic maturation phase, and impact cumulus-oocyte complexes' metabolism [26]. Moreover, IGF2 may be a useful biomarker of meiotic resumption,

and the IGF2 level in human FF is also associated with oocyte maturation capability [28]. Besides, INSR-dependent signaling promotes oocyte maturation as well [29]. These three genes were enriched in PI3K/Akt pathway, Ras pathway, and MAPK pathway. PI3K/PTEN/Akt pathway is a critical regulator of oocyte meiotic maturation, and inhibition of this pathway may lead to pathological conditions of ovaries, such as premature ovarian insufficiency and infertility [30]. The Ras and MAPK pathways are also related to oocyte nuclear and cytoplasmic maturation [31, 32]. The above evidence indicated that the up-regulation of FF-derived exosomal hsa-miR-93-3p might inhibit the expression of EGF, IGF2, and INSR and affect the above pathways in oocytes to impair oocyte maturation.

As for the hub down-regulated miRNA, the predicted target genes were phospha-tidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), Ephrin-A3 (EFNA3), guanine nucleotide binding protein (G protein) beta polypeptide 3 (GNB3), forkhead box O4 (FoxO4), and so on. Although the direct effect of these genes on oocyte maturation has not been determined, they were involved in the Ras signaling pathway and PI3K-Akt signaling pathway as well [33, 34]. Thereinto, the decrease of EFNA3 might activate the PI3K/Akt pathway to promote vascularization [33]. Besides, the phosphorylation of the PI3K/Akt pathway could hinder the transcriptional activity of FoxO4 to suppress oxidative stress [34]. Hence, we speculated that the down-regulation of FF-derived exosomal hsa-miR-625-5p might promote the expression of EFNA3 and FoxO4, and dysregulate the above signaling pathways to affect oocytes competence or promote oxidative stress in oocytes.

Our study did an innovative conjoint analysis of FF-derived exosomal DEmiRNAs and DEGs in oocytes between PCOS patients and control women. This work might provide important evidence that FF-derived exosomal DEmiRNAs might directly im-pair oocyte maturation by affecting PI3K/Akt signaling pathway. However, the sample size needs to be expanded to validate the DEmiRNAs. Additionally, the specific mechanism by which the hub DEmiRNAs, such as hsa-miR-93-3p, hsa-miR-152-3p, hsa-miR-625-5p, and hsa-miR-17-5p, affect oocytes still needs to be further explored.

Materials and methods

Data collection

Data were obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). FF-derived exosomal miRNA expression profile was extracted from GSE157037 (PCOS: Control = 7: 8), while the gene expression profile in oocytes was extracted from GSE155489 (PCOS: Control = 6: 6). The above two expression profiles were obtained by high-throughput sequencing. Samples were collected from PCOS patients and control women. The diagnosis of PCOS was based on the Rotterdam criteria, which meets two of the three features as follows: oligo- or anovulation, signs of hyperandrogenism, or PCOM observed by ultrasound [35, 36]. Women in the control group had regular menstrual cycles without any sign of PCOS [35, 36].

As for sample information, in GSE157037, FF was collected from the first aspirated follicle, and isolation of EVs from FF samples was performed using Commercially available size exclusion chromatography column (qEVsingle/70 nm by Izon Sciences, UK) [36]. In GSE155489, oocytes were isolated by ultrasound-guided vaginal puncture, and germinal vesicles (GV)-stage oocytes were collected for sequencing [35]. The details of the platform and sample information were shown in Table 1.

Analysis of DEmiRNAs in FF-EVs

The raw data were preprocessed by TMM normalization, and clean data were analyzed by the "limma" package (version 3.50.0) in R software (version 4.1.2) to obtain the differential expression of exosomal miRNAs between PCOS patients and control women. The P value and log-2foldchange (log FC) were calculated. MiRNAs with|log FC| >1 and P value < 0.05 were selected as DEmiRNAs in our study.

Analysis of DEGs in oocytes

The data were also processed by TMM normalization. And then, the "limma" package (version 3.50.0) was utilized to identify DEGs in GC-stage oocytes between PCOS patients and control women. The threshold for identifying DEGs was set at|log FC| > 1 and adjust P value < 0.05.

Functional enrichment analyses

The biological process (BP), cell component (CC), and molecular function (MF) of DEGs were annotated by GO enrichment while signaling pathways involved in DEGs were analyzed by KEGG enrichment. The "clusterProfiler" package (version 4.2.1) was used for GO and KEGG analysis. Adjust P value < 0.05 was considered as the threshold for statistically significant enrichment.

Analysis of DEmiRNAs-DEGs co-expression

Target genes of differentially expressed miRNAs were predicted in TargetScan (https://www.targetscan.org/). Venn plots were drawn to determine the overlapping genes between DEGs in oocytes and target genes of exosomal DEmiRNAs. Given that miRNAs mainly played the role of silencing genes, we did conjoint analyses between down-regulated mRNAs in oocytes and target genes of exosomal up-regulated miRNAs to identify hub miRNAs, and vice versa. The miRNA-mRNA co-expression

network was built using Cytoscape software (version 3.7.2). The biological functions of those overlapping genes were further interpreted by GO and KEGG enrichment analysis.

Construction of PPI networks

The interactions of the above overlapping genes were analyzed by String online tools (https://www.string-db.org/). Only validated interactions with a combined score > 0.4 were regarded as significant. Then, the PPI networks among those overlapping genes were constructed using Cytoscape software (version 3.7.2).

Study population and sample collection

This study complied with the Declaration of Helsinki. All procedures were approved by the Ethics Committee and Academic Committee of Tongji Medical College, Huazhong University of Science and Technology (2022S095). Every human participant has signed their informed consent. In the Reproductive Medicine Center of Tongji Hospital, 5 PCOS patients and 5 control women were recruited for this study. According to the Rotterdam criteria, PCOS patients were diagnosed. The control group included women who were undergoing IVF/ICSI due to tubal obstruction or male infertility and without PCOS. The inclusion criteria were as follows: (a) age < 35 years, (b) body mass index (BMI) ranged from 18 to 28 kg/m2, (c) the protocol of ovarian stimulation was GnRH antagonist. We excluded women with chromosomal abnormalities, endometriosis, adenomyosis, tuberculosis of the reproductive system, abnormal sonographic appearance of ovaries, and other endocrine diseases, such as thyroid disease, hyperprolactinemia, diabetes mellitus, adrenal disease, etc. This study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

FF samples were collected when ovum pick-up was performed. All samples were stored in sterile centrifuge tubes and quickly transported to the laboratory on ice. Those samples were centrifuged 20 min at 2000 x g at 4℃ to remove cells. The cell-free FF samples were stored at -80℃ for further experiments.

Isolation of EVs from FF samples

The FF-derived EVs were isolated by ultracentrifugation. FF (25 ml) was diluted 1:1 with PBS to reduce the viscosity, followed by centrifugation at 2000 x g at 4℃ for 20 min. Then, the supernatant was centrifuged at 10,000 x g for 60 min at 4℃ to remove cell debris and apoptotic bodies and further filtrated through a 0.22-μm filter. The supernatant from the previous step was ultracentrifuged at 120,000 x g for 90 min at 4℃. The pellet was resuspended in PBS and further centrifuged at 120,000 x g for 90 min at 4℃ to collect EVs. Finally, the pellets were resuspended in 20–50 μl PBS and stored at -80℃ for further analysis.

Western blotting

The protein of EVs was extracted using RIPA lysis buffer (Servicebio, Wuhan, China) mixed with a proteinase inhibitor cocktail (Servicebio, Wuhan, China) for 30 min at 4℃. The resulting lysates were further centrifuged at 12,000 g for 30 min at 4℃. The protein concentration was measured using BCA Protein Assay Kit (Vazyme, Nanjing, China) according to the manufacturer's protocol. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). After blocking with 5% skimmed milk, the membrane was incubated with the antibodies CD9 (CST, 13174T, 1:1,000), Calnexin (Abcam, ab133615, 1:1000), Flotillin-1 (Flot-1, CST, 18634T, 1:1,000) at 4℃ overnight. After incubation with secondary antibodies (ant-rabbit IgG horseradish peroxidase-linked antibody 7074 S, CST, 1:5,000) for 1 h, membranes were then immersed in Super ECL Detection Reagent (YEASEN, Shanghai, China) and signals were detected using a Gene Gnome XRQ chemiluminescence imaging system (Syngene).

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was performed using NanoSight (LM10) according to the manufacturer's instructions. EVs samples were diluted to the appropriate concentration for the instrument, with the use of PBS at a ratio of 1:1000. The diluted samples were added to the sample pool, and detected the diameter and distribution of EXO samples.

Transmission electron microscopy

EVs (10 μl) suspension was deposited on a carbon-coated electron microscopy grid for 5–10 min, and the extra liquid was removed. After that, EV samples were stained with 2% uranyl acetate for 3–5 min and visualized using the HT7700 transmission electron microscope (TEM) (Hitachi, Japan).

RNA extraction and quantitative real-time polymerase chain reaction

Total RNAs of 5 PCOS-EVs and 5 Control-EVs were extracted with the use of Qiagen exoRNeasy Maxi Kit (77,164, Qiangen, Hilden, Germany) according to the manufacturer's instructions. Equal amounts of RNAs were converted to cDNA. The synthesis of cDNA and quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the use of the All-in-one miRNA qRT-PCR Detection Kit (QP015, GeneCopoeia, Guangzhou, China) according to the manufacturer's

instructions. The relative expression of miRNA was normalized to miR-16-5p, and calculated by the 2−ΔΔCt method.

Statistical analysis

All data were expressed as mean ± SD and statistical significance was evaluated by the Student t-test. Statistical significance was set at $P < 0.05$. The analyses were performed using GraphPad Prism 8.0 (GraphPad, USA) and R software (version 4.1.2).

Conclusions

Overall, our research identified and validated the hub miRNAs, including hsa-miR-93-3p, hsa-miR-152-3p, hsa-miR-625-5p, and hsa-miR-17-5p, by conjointly analyzing the DEmiRNAs in FF-EVs and DEGs in oocytes and qRT-PCR. And based on the KEGG enrichment, we found that the increase of hsa-miR-93-3p and hsa-miR-152-3p, and the decrease of hsa-miR-625-5p and hsa-miR-17-5p, might impair oocyte developmental competence in PCOS via dysregulating PI3K/Akt signaling pathway. Identification of these hub miRNAs in the FF-EVs can help us better understand the mechanism of low oocyte competence in PCOS.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-025-01619-0.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Author contributions

Z.L., Y.Z., and W.T. contributed to the design of study. Z.L., L.S. and H.Z. performed data search, data analyses, and experiments. Z.L. drafted the manuscript, which was revised by H.Z. Q.K. provided modifications for the response letter. All authors made revisions to this manuscript and gave approval of the submitted version.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This study complied with the Declaration of Helsinki. All procedures were approved by the Ethics Committee and Academic Committee of Tongji Medical College, Huazhong University of Science and Technology (2022S095). Every human participant has signed their informed consent.

Competing interests

The authors declare no competing interests.

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