Role of Exosomal microRNAs and lncRNAs in the Follicular Fluid of Women With Polycystic Ovary Syndrome

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Research

Abstract

Polycystic ovary syndrome (PCOS) is a complex class of endocrine disorders with insulin resistance, compensatory hyperinsulinaemia and obesity. However, the pathogenesis and therapies of PCOS have not been fully elucidated. Exosomal miRNAs have the potential to serve as biomarkers and therapies for a wide range of medical conditions. In this study, we isolated exosomes from follicular fluid collected from 5 PCOS patients and 5 non-PCOS patients. miRNA cDNA library sequencing identified 124 miRNAs that were significantly upregulated nearly twofold, while 33 miRNAs were significantly downregulated nearly twofold in PCOS follicular fluid exosomes. These miRNA target genes were mainly involved in metabolic pathways, pathways in cancer, the PI3K-Akt signalling pathway, the MAPK signalling pathway, endocytosis, the Ras signalling pathway, the Hippo signalling pathway, and cellular senescence. According to the previously reported exosomal lncRNA data of PCOS follicular fluid, a miRNA and lncRNA coexpression network developed from data from starBase strictly screened 29 differentially expressed miRNAs. This network also helped to identify miRNA signatures associated with metabolic processes in PCOS. Collectively, these results demonstrate the potential pathogenesis of PCOS, and follicular fluid exosomal miRNAs may be efficient targets for the diagnosis and treatment of PCOS in long-term clinical studies.

Background

Polycystic ovary syndrome (PCOS) is a complex class of endocrine disorders with an overall incidence of approximately 5–20% and a prevalence of 5.61% in Chinese women aged 19–45 years [1, 2]. PCOS is defined by a combination of signs and symptoms of hyperandrogenaemia, ovarian dysfunction and polycystic ovaries (PCOs). In the majority of PCOS individuals, metabolic dysfunction, characterized by insulin resistance and compensatory hyperinsulinaemia, is obvious. PCOS is often complicated with hyperinsulinaemia, dyslipidaemia, and obesity, as well as hypertension, heart disease and endometrial cancer [3]. At present, the pathogenesis of PCOS has not been fully elucidated, and there is a lack of precise treatment. Insulin resistance and compensatory hyperinsulinism lead to metabolic dysfunction, which significantly contributes to the pathogenesis of PCOS [4]. Insulin tolerance is a metabolic state in which human physiological levels of insulin promote a decrease in the glucose utilization ability of tissues and cells. The body can maintain normal blood glucose levels only by compensatory increases in insulin secretion, thus inducing hyperinsulinaemia [5]. On the one hand, hyperinsulinaemia directly affects insulin receptors on ovarian theca cells, which affects the follicular development and pregnancy of PCOS patients; on the other hand, insulin selectively affects tissue-specific metabolism, increases the response sensitivity of ovarian theca cells to luteinizing hormone, and increases androgen secretion [6]. At the same time, higher insulin levels inhibit the synthesis of liver sex hormone binding globulin (SHBG), further increase the level of free androgen, and affect the pregnancy and embryo implantation of PCOS patients. High expression of androgen is also one of the causes of PCOS. The main cause of hyperandrogenesis is an increase in testosterone, androgen and dehydroepiandrosterone sulfate. Abnormal and immature oocytes exposed to high levels of androgen in the follicular fluid block the

development of dominant follicles, stop the growth of follicles and even block the growth of follicles. Furthermore, the endometrium is not resistant to progesterone due to the stimulation of oestrogen levels, which increases the risk of endometrial cancer [7]. Increasing evidence suggests that PCOS may be a complex polygenic disorder with a strong epigenetic influence. Eleven susceptibility loci were found in Chinese women with polycystic ovary syndrome. Some of these genes, such as INSR, FSHR and c9orf3, have been identified [8, 9]. Mendelian random analysis showed that single-nucleotide polymorphisms associated with the risk of polycystic ovary syndrome had a causal relationship with higher body mass index (BMI), insulin resistance and lower levels of sex hormone binding globulin (SHBG) in patients with polycystic ovary syndrome. Other previously reported gene mutations, namely, in YAP1, THADA and FSHB, have also been observed to have genome-wide significance [10]. It is worth noting that, to date, the heritability of PCOS may not exceed 10% [11]. Therefore, it is urgent to explore the characteristics and possible pathogenesis of PCOS from different aspects.

Exosomes are key mediators in different physiological and pathological processes and have played increasingly important roles [12]. S100 calcium binding protein A9 (s100-a9) is enriched in PCOS follicular fluid, and it can significantly enhance inflammation and destroy steroid production by activating the nuclear factor-κ B (NF-κ b) signalling pathway [13]. The expression of DENND1A variant 2 mRNA was significantly increased in urine exosomes from women with PCOS compared with normal cycling women [14]. Exosomal miR-323-3p from adipose mesenchymal stem cells promoted proliferation and inhibited the apoptosis of cumulus cells in a letrozole-induced PCOS mouse model [15]. During the cellular inflammatory response, the composition of exosomal miRNAs is different from that of normal exosomes [16]. It has been reported that the differential expression of plasma exosomal miRNAs may be related to the occurrence of PCOS and help to differentiate PCOS patients from controls. These results may contribute to the understanding of epigenetic modifications in PCOS pathophysiology [17]. However, there are few studies on miRNAs in PCOS follicular fluid exosomes. The main purpose of this study was to explore the expression profile of miRNAs in PCOS follicular fluid exosomes and to analyse their potentially important role in the development of PCOS.

Method

Exosomes Isolation and Characterization

Both the PCOS patients and non-PCOS patients consented for sample collection and molecular testing were approved by the the University of Hong Kong-Shenzhen Hospital Research Ethics Committee. All investigations were conducted in accordance with the Helsinki Declaration. Exosomes were isolated from 1ml follicular fluid using System Bioscience (SBI) ExoQuick™ Exosome Precipitation Kit, according to the supplier's protocols. Transmission electron microscopy were used to detect exosomes size and characterization. In short, a copper mesh was placed on a clean wax plate and 100μl of the exosome suspension was added. After 4 minutes, the copper mesh was removed and placed in 2% phosphotungstic acid for 5 min. The mesh was laid on the filter paper to dry and TEM was used to observe the morphological features of the exosomes [18]. The exosome pellet was dissolved in the

protein lysis buffer, and the protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Western blotting was used to check the marker of exosome via CD9 and TSG101 primary antibodies (Abcam, Cambridge, UK).

RNA isolation from exosomes

Total RNA was isolated from 200 µl of exosomes suspension using TRIzol reagents (Invitrogen, USA). RNA concentration was measured by Nanodrop 2000 spectrophotometer (Thermo Scientific) and stored at − 80°C. All solutions were prepared in RNase-free water and all methods were carried out in RNase-free conditions.

miRNA sequencing and bioinformatics analysis

miRNA sequencing libraries were constructed by TruSeq Small RNA Library Prep Kit (Illumina1) following manufacturer instructions. Sequencing libraries were sequenced with a NextSeq apparatus to generate ~ 16 million single-end 75 bp reads per sample. Afterwards, sequencing reads were obtained the final counts of miRNAs present in each sample. Briefly, adapter sequences were removed from sequencing reads and the remaining sequences were compared against the human mature miRNA from miRbase (release 22.1) (www. miRBase.org) using FANSe3 for miRNA identification, annotation and quantification. Differential miRNA expression analysis (P < 0.05, log2|FC|>1) between groups of interest was carried out with the R package EdgeR. To predict the genes targeted by differential miRNAs, miRTarBase (http://mirtarbase.cuhk.edu.cn/php/index.php) was used to identify miRNA binding sites. In addition, kyoto encyclopedia of genes and genomes (KEGG) pathway and gene ontology (GO) and pathway analyses were performed to identify miRNA-related genes, pathways and GO terms based on sequencing data sets. Cytoscape (www.cytoscape.org) was used to draw a miRNA-lncRNA network and the data output was received in Excel spreadsheets.

RT-qPCR analysis

Total miRNAs from the follicular fluid exosomes were extracted using the TRIpure Total RNA Extraction Reagent method (ELK Biotechnology, China). Real-time PCR was performed for validation using Mir-X miRNA qRT-PCR TB Green® Kit (Takara, Kyoto, Japan). In a simple, single-tube reaction, RNA molecules are polyadenylated and reverse transcribed using poly(A) polymerase. The relative microRNA levels were normalized to U6 expression for each sample. The miRNA primers used in the study are presented in Table 2. The reactions were performed with a Step One Plus Real-Time PCR System (Applied Biosystems) and Step One software v2.1. The PCR reaction included a fast start step of 10 min at 95 ∘C followed by 45 cycles of amplification where each cycle consisted of denaturation at 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. Analyses of gene expression was performed by the he delta-delta Ct method. Each experiment was repeated three times.

Statistical analysis

Data are shown as the means ± standard deviations. The statistical significance of the results from three independent assays was evaluated by Student's t-test. P < 0.05 was considered to indicate statistically significant differences.

Result

Comparison of clinical information between the PCOS group and the control group.

To ensure the reliability of the results of this study, the selected samples of this study were strictly screened according to the Rotterdam criteria (2003). There were two groups (PCOS patients = 5, control patients = 5) of patients, and their follicular fluid exosome samples were analysed. All enrolled participants were diagnosed with primary infertility and received the same ovulation induction treatment programme; they were between 26 and 36 years of age with a duration of infertility between 1 and 5 years. The results showed that there was no significant difference between the PCOS patients and the non-PCOS patients in age, infertility, body mass index (BMI) and fasting blood glucose (FBG) levels. In addition, the number of follicles and anti-Mullerian hormone (AMH) levels were clearly upregulated in the PCOS patients, and there were patients with an LH/FSH > 1 in the PCOS group. The general clinical data of the two groups of patients are shown in Table 1.

Table 1
Information of PCOS patients and non-PCOS donors

Parameter	PCOS group (n = 5)	Non-PCOS roup (n = 5)	P value
Age	33.2 ± 2.4	30.4 ± 2.9	0.17
Infertility	2.4 ± 1.6	2.6 ± 1.6	0.84
BMI	23.402 ± 2.7	21.984 ± 1.7	0.39
FBG (mmol/L	4.85 ± 0.4	4.632 ± 0.3	0.41
E2(pg/ml)	49.8 ± 13.3	45.6 ± 12.6	0.66
Progesterone (ng/mL)	0.332 ± 0.13	0.46 ± 0.34	0.51
Testosterone (ng/mL)	0.502 ± 0.12	0.454 ± 0.14	0.63
FSH	5.704 ± 1.03	7.114 ± 0.8	0.07
PRL	10.788 ± 1.6	12.278 ± 3.1	0.41
LH	7.784 ± 3.8	4.992 ± 2.1	0.23
Number of follicles	25 ± 5.9	12.4 ± 5.2	0.01
AMH (ng/ml)	5.278 ± 2.1	1.94 ± 0.5	0.02

Abbreviations: BMI: body mass index; FBG: fasting blood glucose; LH: luteinizing hormone; AMH：anti-Mullerian hormone; PRL: serum prolactin; E2: estradiol; FSH: follicle-stimulating hormone;

Table 2

Information of validated miRNAs.

miRNA	miRbase ID	Primers (5'— 3')
hsa-miR-200c-3p	MI0000650	TAATACTGCCGGGTAATGATGGA
hsa-miR-196a-3p	MI0000238	CGGCAACAAGAAACTGCCTGAG
hsa-miR-199a-5p	MI0000242	CCCAGTGTTCAGACTACCTGTTC
hsa-miR-143-5p	MI0000459	GGTGCAGTGCTGCATCTCTGGT
hsa-miR-483-3p	MI0002467	TCACTCCTCTCCTCCCGTCTT
hsa-miR-376a-3p	MI0000784	ATCATAGAGGAAAATCCACGT
hsa-miR-542-3p	MI0003686	TGTGACAGATTGATAACTGAAA
hsa-miR-21-5p	MI0000077	TAGCTTATCAGACTGATGTTGA
hsa-miR-4322	MI0015851	CTGTGGGCTCAGCGCGTGGGG
hsa-miR-132-3p	MI0000449	TAACAGTCTACAGCCATGGTCG

Differential expression of miRNA profiles in follicular fluid exosomes.

Exosomes were isolated from follicular fluid. Transmission electron microscopy (TEM) was used to detect exosomes approximately 50–200 nm in diameter from all samples (Fig. 1A). Western blot analysis was performed and revealed that two commonly used exosomal protein markers, namely, CD9 and TSG101, were highly enriched in the isolated exosomes relative to PBS (Fig. 1B). The results showed that exosomes from all follicular fluid samples were successfully purified. In total, 2457 miRNAs were identified in follicular fluid exosomes from both PCOS patients and controls in this study. Among them, 157 mature miRNAs in follicular fluid exosomes were significantly differentially expressed in the PCOS and control groups ($P < 0.05$, log2|FC|>1). The number of significantly upregulated miRNAs was 124, and the number of downregulated miRNAs was 33, as indicated by a volcano plot and a heatmap (Fig. 1C-D). To further validate the miRNA profiling results, ten miRNAs, hsa-miR-200c-3p, hsa-miR-196a-3p, hsa-miR-199a-5p, hsa-miR-143-5p, hsa-miR-483-3p, hsa-miR-376a-3p, hsa-miR-542-3p, hsa-miR-21-5p, hsa-miR-4322, and hsa-miR-132-3p, were randomly screened by RT-qPCR from PCOS patients and non-PCOS patients. According to the RT-qPCR results, the trends in the expression of the miRNAs determined by RT-qPCR were consistent with those obtained from RNA sequencing in PCOS and non-PCOS follicular fluid exosome samples (Fig. 1E-F).

Functional annotation and identification of the differentially expressed miRNA target genes.

KEGG pathway and GO analyses were performed to investigate the functions of 157 differentially expressed miRNA target genes. Furthermore, KEGG pathway enrichment revealed that the target genes were mainly involved in metabolic pathways, pathways in cancer, the PI3K-Akt signalling pathway, the MAPK signalling pathway, endocytosis, the Ras signalling pathway, the Hippo signalling pathway, and cellular senescence (Fig. 2A). GO enrichment analyses were also carried out to gain insight into the biological characteristics of the miRNAs. Metabolic processes were very prominent in both the significantly upregulated and downregulated miRNA target genes, including nucleic acid metabolic process, cellular macromolecule metabolic process, and heterocycle metabolic process (Fig. 2B-C). These results suggest that metabolic pathways possibly have great significance in the pathogenesis of PCOS.

Construction of the miRNA−lncRNA coexpression network

To further explore the epigenetic regulation of miRNAs, intersection analysis between miRNAs and lncRNAs was performed in follicular fluid exosomes from PCOS and non-PCOS patients (Fig. 3A). The differentially expressed lncRNAs in this study were strictly screened according to the previous research results of Liping Wang et al [19]. There were 29 differentially expressed miRNAs constructed with 2439 differentially expressed lncRNAs in the coexpression network via the data from starBase (Table 3). Subsequently, to further investigate the interconnections between the differentially expressed lncRNAs and miRNAs involved in the metabolic pathways (hsa01100) in PCOS, the network was represented, and the potential interaction was predicted (Fig. 3B). The results showed that the upregulated miRNAs, such as miR-369-3p, miR-139-5p, miR-371a-3p, miR-143-5p, miR-199a-5p, miR-196a-3p, and miR-26a-2-3p, reduced the expression of RDH10-AS1. In addition, NARF-IT1, AC090617.1, MZF1-AS1, AC009495.2, LINC01564, AQP4-AS1, L34079.3, OSBPL10-AS1, PIK3CD-AS2, LINC01181, LINC00907, and SP2-AS1 were regulated by the differentially expressed miRNAs. These findings suggest that these miRNAs and lncRNAs may play a role in the pathogenesis of PCOS.

Table 3
miRNAs in the miRNA−lncRNA coexpression network.

miRNA	logFC	PValue	DEG
hsa-miR-32-3p	6.254050282	5.30E-05	up_regulated
hsa-miR-200c-3p	-3.579123	0.000297	down_regulated
hsa-miR-196a-3p	4.452656099	0.000452	up_regulated
hsa-miR-199a-5p	3.455170178	0.000694	up_regulated
hsa-miR-143-5p	3.350954732	0.001784	up_regulated
hsa-miR-26a-2-3p	3.256636073	0.0036	up_regulated
hsa-miR-21-5p	2.052289441	0.008091	up_regulated
hsa-miR-106a-3p	5.883950978	0.010317	up_regulated
hsa-miR-132-3p	1.840853713	0.01079	up_regulated
hsa-miR-125b-1-3p	2.656313696	0.011578	up_regulated
hsa-miR-15a-3p	2.743713774	0.013771	up_regulated
hsa-miR-19a-3p	1.906020762	0.014684	up_regulated
hsa-miR-299-3p	2.111979855	0.017976	up_regulated
hsa-miR-24-1-5p	2.479764956	0.022368	up_regulated
hsa-miR-369-3p	2.247301418	0.023098	up_regulated
hsa-miR-30e-5p	1.467721548	0.023425	up_regulated
hsa-miR-139-5p	2.089759187	0.026395	up_regulated
hsa-miR-129-1-3p	3.7326463	0.028161	up_regulated
hsa-miR-34a-5p	1.607443155	0.028537	up_regulated
hsa-miR-369-5p	2.00127731	0.028573	up_regulated
hsa-miR-371a-3p	3.85268939	0.032162	up_regulated
hsa-miR-181b-2-3p	5.270233934	0.032403	up_regulated
hsa-miR-23a-5p	1.507630692	0.03562	up_regulated
hsa-miR-376c-3p	1.639249701	0.040714	up_regulated
hsa-miR-19b-3p	1.724336734	0.041056	up_regulated
hsa-miR-20a-5p	1.308580728	0.042476	up_regulated
hsa-miR-101-5p	1.6811069	0.045046	up_regulated

miRNA	logFC	PValue	DEG
hsa-miR-376a-5p	2.293758046	0.046278	up_regulated
hsa-miR-199a-3p	1.52738784	0.047854	up_regulated

Discussion

To date, a variety of factors have been reported to be involved in the pathogenesis and clinical phenotype of PCOS, such as excessive androgen synthesis, follicular atresia, and insulin resistance [20]. However, the causes of PCOS are still unclear. Human follicular fluid contains hormones, growth factors, cytokines, vitamins and cell metabolites. In addition, studies have shown that some molecular substances in follicular fluid are closely related to follicular growth, fertilization, spontaneous abortion and PCOS. For example, the content of orexin in follicular fluid is negatively correlated with follicular quality and embryo development after in vitro fertilization [21]. The composition of metabolites in follicular fluid is also different at all stages of follicular development [22]. Some proteins in the follicular fluid are closely involved in glucose metabolism, lipoprotein metabolism, cell proliferation, insulin resistance and other processes in PCOS patients [23]. These studies suggest that the information carried in follicular fluid is an important entry point for the study of PCOS. A large amount of evidence has shown that the release of membrane-sealed ventricular structures, such as exosomes and extracellular vesicles (EVSs), is an effective mechanism of intercellular communication under normal physiological and pathological conditions. Exosomes and extracellular vesicles in follicular fluid are considered carriers of information. These exosomes may also be involved in the progression of polycystic ovary syndrome and other diseases [24]. Therefore, this study explored the molecular characteristics of exosomal miRNAs in PCOS follicular fluid and elucidated the potential role of these miRNAs by using bioinformatics tools.

PCOS is a multifactorial disease caused by endocrine and metabolic dysfunction, and in recent years, the pathogenesis of polycystic ovary syndrome considered to be related to epigenetics. Many studies have shown that miRNAs in the follicular fluid of patients with polycystic ovary syndrome are altered [25]. It has been reported that the expression of hsa-miR-21 in PCOS plasma is upregulated, and hsa-miR-21 plays an important role in metabolic and immune system processes [26]. The results from the previous study in plasma were consistent with the results from this study that demonstrated that the expression of exosomal miR-21-5p in PCOS follicular fluid was upregulated. KEGG pathways and GO enrichment analyses revealed that the miRNA target genes were mainly involved in the MAPK signalling pathway and metabolic process. These biological functions have also been found to be associated with the activation of follicular development using lncRNA and mRNA profiles of follicular fluid from mature and immature ovarian follicles of PCOS patients [27]. Interestingly, the expression of exosomal miR-19 and miR-199 was increased in follicular fluid samples from patients with PCOS in this study. In addition, there is strong evidence that the activity and mRNA expression level of CYP19A1 and both miR-19 and miR-199 target genes were decreased in patients with PCOS, and this was associated with decreased follicle size [28, 29]. These findings suggest that upregulated miR-19 and miR-199 may be the cause of PCOS. LncRNAs are a

class of transcripts (> 200 nucleotides) lacking protein-coding capacity, and they function as competitive endogenous RNAs (ceRNAs) and are significantly correlated with some clinical phenotypes in PCOS [30]. Previous studies have found that the expression levels of RDH10-AS1, NARF-IT1, AC090617.1, MZF1-AS1, AC009495.2, LINC01564, AQP4-AS1, L34079.3, OSBPL10-AS1, PIK3CD-AS2, LINC01181, and LINC00907 were reduced in PCOS [19]. MZF1-AS1 has been reported to inhibit proline synthesis and neuroblastoma progression [31]. AC009495.2 was associated with acute myeloid leukaemia, and it could differentiate between acute myeloid leukaemia types and change the behaviour of acute myeloid leukaemia cells [32]. Energy stress-induced LINC01564 activated the serine synthesis pathway and facilitated hepatocellular carcinogenesis [33]. AQP4-AS1 plays a potential role in breast cancer [34]. The lncRNA PIK3CD-AS2 promoted lung adenocarcinoma progression via YBX1-mediated suppression of the p53 pathway [35]. In this study, these lncRNAs and differentially expressed miRNAs were used to construct a metabolic pathway-associated lncRNA-miRNA network, which indicated the key mechanisms of PCOS.

Conclusion

Taken together, our results indicated that exosomal miRNAs from PCOS follicular fluid were involved in the regulation of possible pathways, biological functions and cellular components of PCOS. Moreover, our study constructed miRNA−lncRNA regulatory networks in follicular fluid exosomes, which have crucial biological roles in the occurrence and development of PCOS. This study is of great significance in revealing new mechanisms of polycystic ovary syndrome and suggesting possible therapeutic targets.

Declarations

Funding

Authors' Contributions

Tianmin Ye conceived of the study, and participated in its design and coordination and helped to draft the manuscript. Suxia Lin and Shufang Ding collected samples, conducted experiments, and analyzed the results. Dandan Cao and Longdan Luo mainly completed the writing of the manuscript. William Shubiu Yeung initiated the research of the study, supervised the research progress, revised and finalized the manuscript. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

Ethics approval and consent to participate

Research that is performed on humans should follow international and national regulations in accordance with the Declaration of Helsinki, or any other relevant set of ethical principles. Informed consent for participation in the study or use of their tissue was obtained from all participants (or their parent or legal guardian in the case of children under).

Consent for publication

All the patients and all authors agreed to publish.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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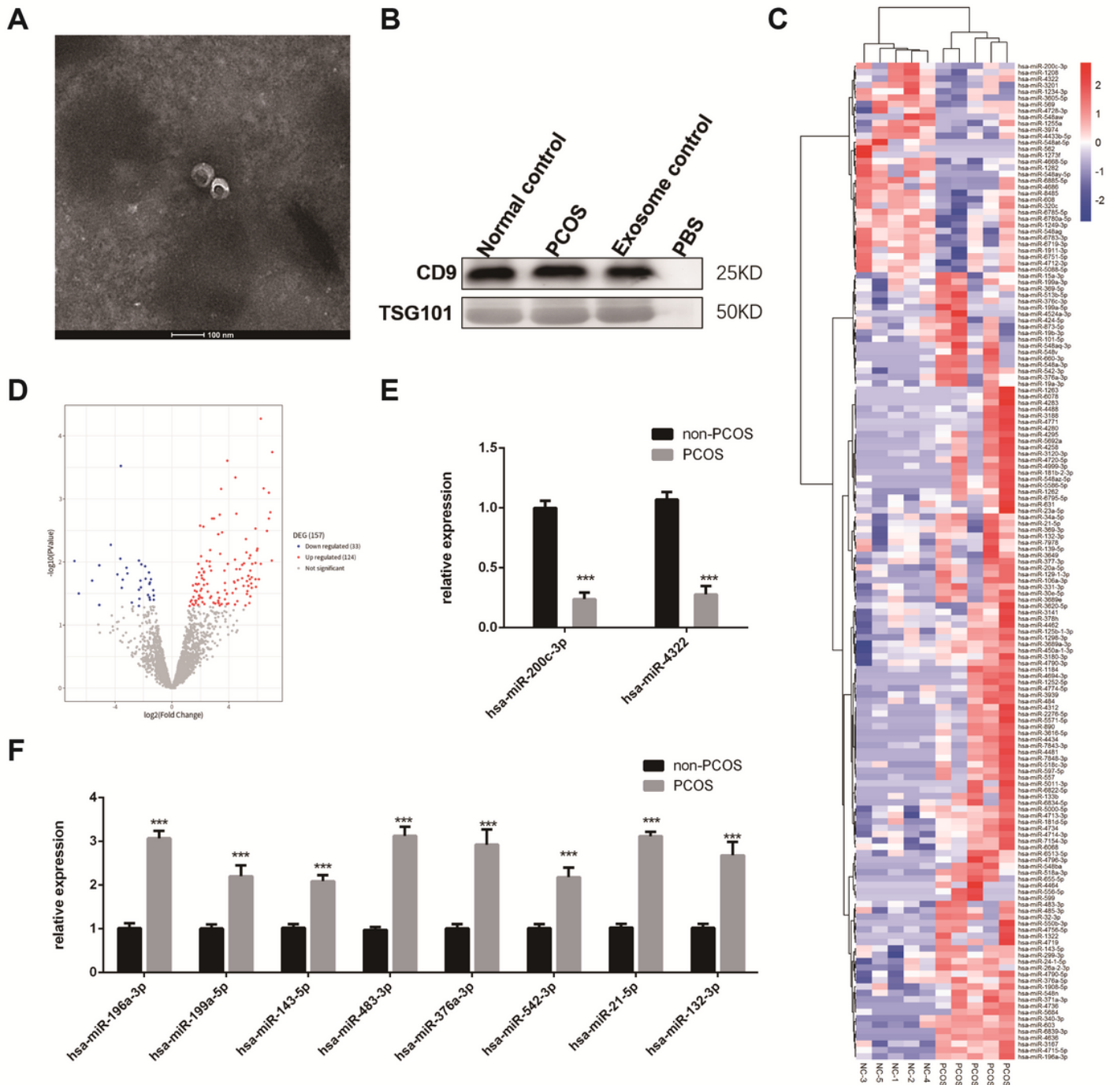
Figures

Figure 1

Characterization of exosomes and differential expression of miRNA profiling in the follicular fluid exosomes. A. Isolated exosomes micrograph of TEM. B. Exosome protein markers validation by western blotting. C. Volcano plot of diff-expressed miRNAs between PCOS and NC (non-PCOS). D. Heatmap of Volcano plot of diff-expressed miRNAs between PCOS and NC (non-PCOS). E-F. Validation the miRNA profiling by RT-qPCR. means ± standard deviations (***P < 0.001).
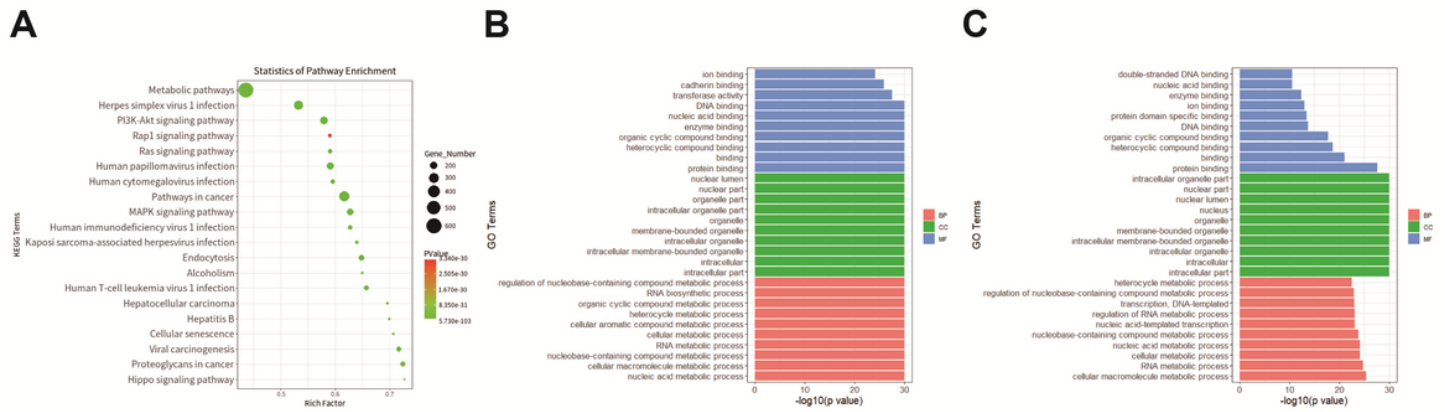
Figure 2

Enrichment analysis of the significantly diff-expressed miRNAs target genes. A. KEGG pathway enrichment analysis of the significantly diff-expressed miRNAs target genes. B. GO analysis of the significantly upregulated miRNAs target genes. C. GO analysis of the significantly downregulated miRNAs target genes.
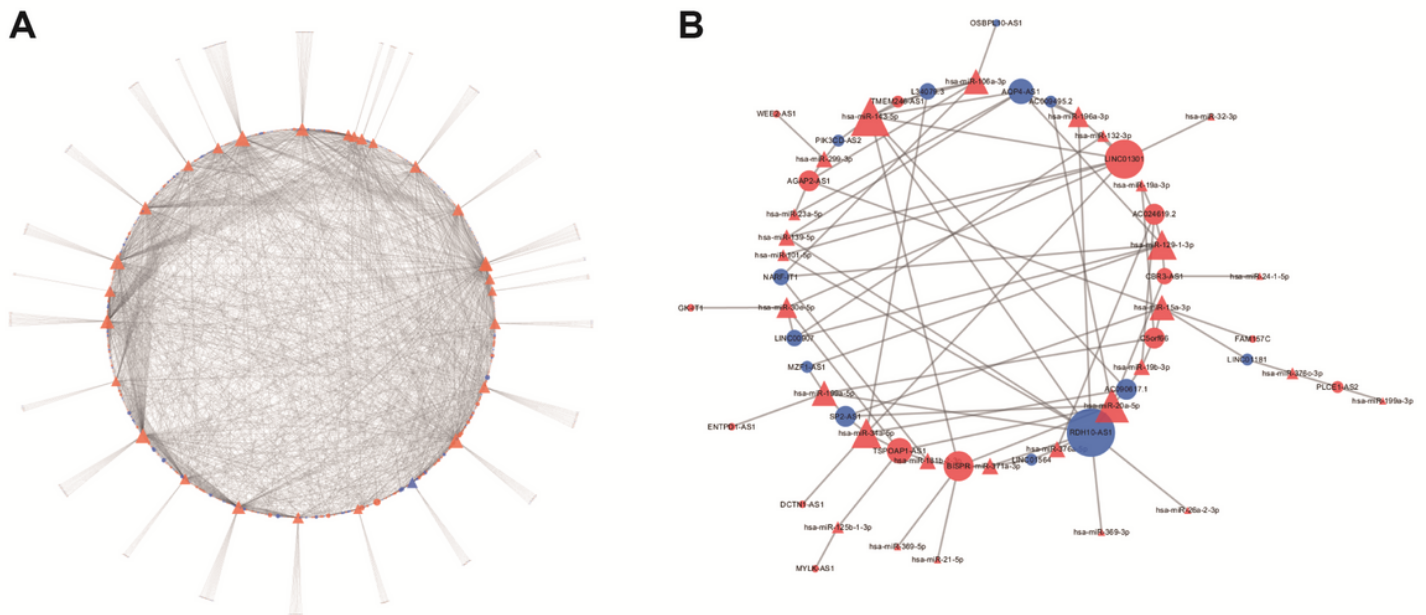

Figure 3

The miRNA−lncRNA co-expression network. A. A co-expression network with the significantly diff-expressed miRNAs and lncRNAs in follicular fluid exosomes. B. miRNA-lncRNA co-expression network related metabolic pathways (hsa01100) in follicular fluid exosomes.