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ORIGINAL

Human Serum Microvesicles as Mediators of PCOS-Related circRNAs Targeting FOXO1/NF-κB Pathways: Implications for PCOS Formation and Its Mental and Physical Health Impact

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ABSTRACT

Purpose: Polycystic ovary syndrome (PCOS), a multifaceted endocrine disorder, manifests through a spectrum of symptoms including infertility, hyperandrogenemia, and insulin resistance, underpinned by chronic inflammation and disrupted glycolipid metabolism. Despite extensive research, the intricate pathogenesis of PCOS remains elusive. Microvesicles (MVs), ubiquitous in cellular communication, could hold the key to understanding and potentially treating PCOS-related infertility by mediating the expression of PCOS-associated circRNAs. This study explores the role of serum MVs in conveying circRNAs that target the FOXO1/NF-κB signaling pathways, contributing to the etiology of PCOS and offering novel therapeutic insights. **Methods:** Serum samples from PCOS patients and infertile women without PCOS were analyzed for hormone levels and prepared for MV isolation. The identification of MVs was performed using Western blot, transmission electron microscopy (TEM), and nanoparticle tracking analysis (NTA). High-throughput whole-transcriptome sequencing provided a circRNA expression profile from these MVs, highlighting their involvement in inflammatory signaling pathways. In vitro assays on KGN cells were conducted to assess the impact of PCOS MVs on cell proliferation and to elucidate the involvement of circRNA in PCOS pathology. **Results:** Differential expression of circRNAs in serum MVs was observed between PCOS patients and control subjects. The incorporation of PCOS-derived MVs

into KGN cells resulted in suppressed cell proliferation. Moreover, the expression of key inflammatory mediators, FOXO1 and NF-κB, was significantly elevated in PCOS, indicating their regulatory involvement through circRNA-mediated pathways. **Conclusion:** The study underscores the pivotal role of circRNA-loaded MVs in modulating FOXO1 and NF-κB activation, thereby influencing the pathophysiological landscape of PCOS. These findings highlight a novel mechanism through which PCOS-induced changes in ovarian function and infertility may be mediated, offering promising avenues for therapeutic intervention. Importantly, this research also calls attention to the broader implications of PCOS on mental and physical health, advocating for an integrated approach to understanding and managing the syndrome, with a focus on the intricate web of biological and psychological factors contributing to its development and progression.

KEYWORDS: polycystic ovary syndrome (PCOS), Microvesicles (MVs), circRNA, FOXO1, NF-κB

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common disease involving reproductive endocrine disorders and abnormal glycolipid metabolism, with a global incidence of up to approximately 4%–20% (Deswal, Narwal, Dang, & Pundir, 2020). Its main clinical features are sporadic or chronic oligo-anovulation, hyperandrogenemia, and polycystic ovarian changes, which can co-occur with complications such as insulin resistance (IR), obesity, type 2 diabetes, and cardiovascular disease (Diamanti-Kandarakis & Dunaif, 2012; Wekker et al., 2020). PCOS seriously affects the physical and mental health of adolescent girls and women of childbearing age. The etiology of PCOS is complex and heterotypic; it is difficult to confirm the diagnosis in the early stage, clinical treatment only targets the symptoms, and its pathogenesis is still not fully understood. In addition, PCOS is one of the most common causes of infertility, accounting for approximately 80% of women with anovulatory infertility, mainly caused by abnormal follicle development and persistent ovulation disorders (Balen et al., 2016). At present, many studies have confirmed that PCOS patients usually exist in a chronic, non-specific inflammatory state and that the long-term high level of inflammatory factors in the body can affect the growth process of follicles, resulting in ovulation disorders, followed by hyperandrogenemia, insulin resistance, and finally polycystic ovary syndrome, with ovulation disorder infertility as the primary manifestation (Dabravolski et al., 2021; González, Nair, Daniels, Basal, & Schimke, 2012; Rostamtabar et al., 2021).

Microvesicles (MVs) are membrane-bound vesicles released into the extracellular environment, produced by outward budding of the cytoplasmic membrane after the germination of a microbubble. MVs are a subset of

extracellular vesicles, with diameters ranging from 100 nm to 1000 nm. MVs are essential in mediating cellular communication, serving as vehicles for transferring proteins, lipids, mRNA, and non-coding RNA. MVs are released from initiator cells and transport their cargo to target receptor cells, thus triggering effector functions (Ratajczak et al., 2006; Record, Subra, Silvente-Poirot, & Poirot, 2011; Tricarico, Clancy, & D'Souza-Schorey, 2017). Among the non-coding RNAs contained in MVs, circRNAs are a class of non-coding RNA molecules lacking both a 5' end cap and a 3' terminal poly(A) tail, which form a single-stranded ring structure with covalent bonds, with high stability in the blood, saliva, cerebrospinal fluid, urine, and other body fluids (Dolinar, Koritnik, Glavač, & Ravnik-Glavač, 2019; You et al., 2015). CircRNAs regulate the transcriptional expression of multiple downstream target genes by acting as microRNA (miRNA) sponges to regulate their activity (Ma, Zhao, Zhang, Liu, & Hao, 2019). CircRNAs are involved in the etiological mechanism of many diseases. They have been confirmed to be differentially expressed in the plasma and follicular fluid of patients with PCOS, which is closely related to the occurrence and development of this syndrome (Huang et al., 2020; Lu, Gao, Zhu, & Lin, 2021). However, few studies have investigated the role of circRNAs in serum-derived MVs in developing PCOS. Studies has shown that the expression of circRNAs related to the female reproductive diseases which lead to infertility, including PCOS, premature ovarian failure (POF), Asherman syndrome, endometriosis, endometrial cancer, cervical cancer, ovarian cancer, and preeclampsia, as well as signaling.

In this study, for the first time, we performed high-throughput whole-transcriptome sequencing of circRNAs in the serum MVs of PCOS and control patients, identifying a large number of differentially expressed circRNAs. Using bioinformatics analysis, online database analysis, and comprehensive literature search, we found that the differentially expressed circRNAs hsa-miR-1224-5p and hsa-miR-204-5p are rich in multiple binding sites, which would allow indirect downstream regulation of the key inflammatory target genes FOXO1 and NF-κB. It has been reported that FOXO1 is expressed in granulosa cells and plays a vital role in granulosa cell proliferation, follicular development, ovulation and luteal formation (Cunningham, Zhu, Unterman, & Hammond, 2003; Gebremedhn et al., 2016; Shi & LaPolt, 2003). In addition, NF-κB is a recognized inflammatory gene, which can induce the expression of a variety of pro-inflammatory genes, participate in the regulation of inflammasome, and lead to abnormal follicular development (Liu, Zhang, Joo, & Sun, 2017).

The objectives of this study were:(1) investigate the differential expression of circRNAs between serum MVs of PCOS patients and a non-PCOS control group; (2) explore the role of differentially expressed MVs circRNAs in inflammatory signalling pathways; (3) provide a basic experimental basis for mechanistic study of slow follicular development, early

atresia, and ovulation disorders in PCOS.

2. MATERIALS AND METHODS

2.1 Clinical Data

This study was approved by the Ethics Committee of the Affiliated Hospital of Zunyi Medical University. Infertile women aged 20 to 40 who visited the Department of Reproductive Medicine from September 2021 to April 2022 were selected. Patients included in the analysis were divided into PCOS and control groups based on the cause of infertility. The inclusion criteria for the PCOS group were defined according to the Rotterdam diagnosis criteria (2003). Participants had to meet at least two of the following criteria: (1) oligoovulation or anovulation; (2) clinical and/or biochemical signs of hyperandrogenism; (3) unilateral or bilateral ovarian polycystic changes. Patients exhibiting androgen overload, hypogonadotropic-induced anovulation, or premature ovarian failure were excluded. The control group consisted of patients being treated for male factor or simple tubal factor infertility who met the following criteria: (1) normal ovarian function; (2) regular menstrual cycle (26-32 days) and normal basal hormone level; and (3) normal number of antral follicles in both ovaries. There were 45 cases in each group. Menstrual blood samples from the patients of both groups were collected from the 3rd to 5th day of menstruation. The blood serum was tested for sex hormones, namely estradiol (E_2), total testosterone (TT), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL), and the clinical results of the samples were then analyzed.

2.2 Preparation and identification of MVs

Multi-step differential centrifugation was used to extract MVs from peripheral blood. Peripheral blood of 5 ml was collected and allowed to stand for 30 min. The serum was separated by centrifugation at 3000×*g* at 4℃ for 15 min, placed in sterile freezing tubes, and stored in a refrigerator at -80℃. Serum held at -80℃ was thawed in a 25℃ water bath, and 30 ml mixed serum was diluted 3 times with phosphate-buffered saline (PBS). The diluted serum was centrifuged at 4 ℃ for 10 min at 500×*g* to remove red blood cells. The supernatant was mixed and centrifuged at 2000×*g* for 15 min to remove cell debris.

The supernatant was centrifuged at 5000×g for 15 minutes to remove residual cells and cell debris. Notably, if red plaque deposits were found on the centrifuge wall when the supernatant was collected, the centrifugation process was repeated. After centrifugation, the supernatant was centrifuged at high speed of 20000 ×*g* at 4 ℃ for 1 h, and the supernatant was discarded. The suspension was resuspended in 1 ml sterile PBS and centrifuged again at 20000 ×*g* for 1 h at 4 ℃. Finally, the supernatant was discarded, and MVs

were left as a yellow-white precipitate. The prepared MVs were re-suspended in 150 µl sterile PBS. The morphology of serum MVs was observed by TEM negative staining, the particle size distribution was assessed by NTA, and the expression of the marker proteins CD63 (1:700 Novus) and CD81 (1:700 ab109201 Abcam) of MVs were characterized and verified by Western blot.

2.3 MVs-RNA isolation, library construction and sequencing

About 50-100 µl were extracted from each group of MVs, 1 ml Trizol reagent (Takara, Dalian, China) was added, and trace RNA was extracted according to the manufacturer's instructions. Taqman reagent (DETECttM Taqman Mycoplasma Detection Kit) was used for sample quality testing. Library construction was performed after qualified quality inspection; 50 ng RNA was taken, and the library was constructed using KAPA RNA HyperPrep Kit with RiboErase (HMR) for Illumina® library building Kit.

First, the designed DNA probe was hybridized with the RNA sample, and rRNA was removed from total RNA (Geneseed China); subsequently, the RNA was fragmented to synthesize the first strand of cDNA, and the second strand cDNA was tagged with dUTP during synthesis using the chain specific method; end repair was completed at the same time. After amplification, the RNA Seq library was purified and recovered using magnetic beads (GC-BIOTECH B.V., Alphen Aan den Rijn, The Netherlands). The Agilent 2100 Bio analyzer (Applied Bio systems, Carlsbad, CA) was then used to detect the size range of the library, and NovaSeq 6000 PE150 mode was used for sequencing after the library was qualified.

2.4 CircRNA sequencing and annotation

The DCC software was used to identify circRNAs comprehensively; the sequences of the identified circRNAs were predicted, and the circRNAs with low TMM expression (CPM >0.1) were filtered out. Differential expression of circRNAs was assessed using the edgeR tool according to the grouping information (fold-change = 1.5, $p < 0.05$). R software clusterProfiler was used to analyze the differentially expressed genes. Hierarchical cluster heat maps and volcanic plots were used to distinguish differentially expressed circRNAs between women with PCOS and controls.Gene ontology (GO) analysis was performed to investigate the functional role of differentially expressed circRNAs from three perspectives: molecular function, biological process, and cell composition. KEGG was used to identify the biopathway of differentially expressed circRNA enrichment. The miRNA binding sites of differentially expressed circRNAs were analyzed; Geneseed with miRanda software was used to predict the relationship between miRNAs of all corresponding species in the miRBase database and differentially expressed circRNA and between assembled circRNA and miRNA-target genes.

2.5 Cell culture

The fourth generation human ovarian granuloid tumor cell line (KGN cell FH1125) was purchased from Shanghai Fuheng Biotechnology Co. Cells were cultured in DMEM/F12 complete medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco), 1% penicillin (Adams), and 1% streptomycin (Adams), in an incubator at 37℃, 5% CO2. Cells were passaged 1:2-1:5 when the culture had reached 80-90% confluency.

2.6 Measurement of MV uptake in KGN cells

To measure the MV uptake in KGN cells, cells were seeded in a six-well plate at a density of 2×105 cells per well in a final volume of 2.5 ml/well culture medium for 24 h. Then, 30 μg MVs from each group were suspended in 50 μl dye solution (diluent: PKH67 (Umibio) = 9:1, mixed thoroughly for 1 min) and incubated for 10 min. Then, 10 ml of 1×PBS (Gibco) was added to the MV-dye complex. The solution was centrifuged at 20,000 ×g, 4°C for 1 h. The supernatant containing excess dye was discarded, and stained MVs were collected in 100 μl 1×PBS. PKH67-labeled MVs were added to the cell medium for 24 h. Subsequently, KGN cells and PKH67-labeled MVs were washed twice with PBS for 5 min, fixed in 4% paraformaldehyde for 15 min, washed three times with PBS, and then restained with DAPI (Solarbio) for 10 min. MVs taken up by KGN cells were observed under a confocal microscope (Olympus Japan).

2.7 Cell viability assay

To test the cell viability, KGN cells were collected in the logarithmic growth stage, seeded in a 96-well culture plate at a density of 5×10^3 cells per well, and incubated (37 ℃, 5% CO2) for 24 h. MVs from groups were added at a concentration gradient of 0, 10, 40, 80 and 120 μg/ml, with 6 replicate wells for each concentration. Cells were incubated with MVs for 24 h, and then 10 μl CCK-8 (MCE) solution was added to each well and incubated for 2 h. Each well's optical density (OD) value was measured at 450 nm wavelength on a microplate reader (Thermo, USA), and the survival rate was determined.

2.8 Western Blot assay

To quantify the protein expression, the western blot assay (WB) were conducted. KGN cells were collected in the logarithmic growth stage and inoculated into 6-well culture plates at a density of 2×10^5 cells per well. The cells were incubated in a 5% CO_2 incubator at 37 ℃ for 24h. Subsequently, 40 μg/ml pcos-mvs, 40 μg/ml normal-mvs and the control group without MVs were added and incubated in a serum-free medium for 24h. The cultured cells were lysed, and 60 μl of lysate was prepared using RIPA buffer (MCE): PMSF

(Solarbio) = 100:1 in each well and placed at 4℃ for 30 min. The lysates were collected and centrifuged at 12000 rpm at 4℃ for 15 min. The supernatant containing total protein was collected, and the protein concentration was determined by the BCA method (Solarbio). Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membrane was sealed with fast sealing solution (EpiZyme) for 10 min and then incubated in the primary antibody [anti-FOXO1 (1:1000 #2880S, CST), anti-NF-κB (1:1000 ab32536, Abcam), or the internal reference GAPDH (1:1000 ET1601-4 Huabio)] overnight. Membranes were washed with TBST (pH 7.4) 3 times and then incubated in Goat anti-rabbit secondary antibody (1:5000 ab6721, Abcam) in a 25℃ shaker for 1 h. Membranes were finally washed with TBST (pH 7.4) 3 times, and proteins were visualized with high sensitivity chemiluminescence liquid reagent (Tanon) under an Image Lab gel imaging system (Bio-RAD). Protein band intensities were quantified using Image Lab software. Each experiment was repeated in triplicate.

2.9 Statistical Analysis

Clinical data were analyzed using SPSS 25.0 statistical software. The measurement data were described as mean ± standard deviation. T-tests were performed. Other experimental results were analyzed using Graphpad Prism V8.0, with single factor analysis of variance with Dunnett's t-test to homogeneous variance between matched groups. P <0.05 was considered statistically significant.

3. RESULTS

3.1 Clinical data analysis of the PCOS and control groups

The clinical data are shown in Table 1. There was no significant age difference between the PCOS and control groups. However, the levels of LH, E_2, TT and LH/FSH in the PCOS group were significantly higher than in the control group.

Table 1: Clinical information of PCOS group and control group

Parameter	PCOS (n = 45)	Non-PCOS (n = 45)	P-value
Age (years)	27.73±3.72	28.31±2.47	0.388
FSH (mIU/ml)	6.13±1.47	6.38±1.35	0.390
LH (mIU/ml)*	16.60±6.72	4.80±1.72	＜0.001
LH/FSH ratio*	2.76±0.95	0.76±0.22	＜0.001
E_2 (pmol/l)*	192.62±93.26	122.88±39.92	＜0.001
PRL (mIU/ml)	330.36±140.11	314.33±124.99	0.568
TT (nmol/l)*	1.85±0.58	0.87±0.56	＜0.001

3.2 Preparation and identification of serum MVs

Multistep high-speed differential centrifugation was used to extract and purify MVs from the serum of patients in both groups. The morphology of the obtained precipitates was assessed by transmission electron microscopy. The two groups showed round or ellipsoidal vesicles typical of lipid bilayer membrane vesicles of MVs under electron microscopy. Nanoparticle tracking analysis revealed that most MVs ranged in size from around 100-400 nm. The average diameter of MVs in the control group was 324.4 nm (D10: 183.9 nm, D50: 322.6 nm, D90: 475.8 nm). The diameter distribution of MVs in the PCOS group was smaller than that in the control group, with an average diameter of 218.1 nm (D10: 51.2 nm, D50: 201.8 nm, D90: 388.6 nm). Western blot detection of MV markers CD81 and CD63 were positive. Therefore, the extracted and separated vesicle-like substances were MVs.

3.3 CircRNAs expression

We extracted 30 ml of serum from each group of samples and isolated MVs. The Trizol method was then used to extract total RNA from the samples. The RNA libraries were prepared using the KAPA RNA HyperPrep Kit with RiboErase (HMR) for Illumina®. Finally, the library was sequenced using the NovaSeq 6000 PE150 model. Meanwhile, circRNAs with low TMM expression (CPM > 0.1) were pretreated and filtered, and differential expression analysis of circRNAs were performed by edgeR according to grouping information (fold-change=1.5, $P < 0.05$). Finally, 19116 and 29187 candidate circRNAs were predicted in the PCOS and control groups, respectively. Differential expression patterns of circRNAs between the two groups comprised 6515 up-regulated circRNAs, and 7701 down-regulated circRNAs in women with PCOS, confirming the presence of differentially expressed circRNAs in serum MVs.

The top 10 circRNAs with the most significant expression differences in PCOS patients. These included: hsa_circRPRD1B_002, hsa_circKLHDC2_004, hsa_circABCA3_008, hsa_circUSP8_016, hsa_circTAOK1_017, hsa_circRNF19A_010, hsa_circMGAT4A_001, hsa_circATXN2_024, hsa_circANKRD26_028, and hsa_circCAPRIN1_034 (up-regulated); hsa_circWDR33_001, hsa_circRNF220_001, hsa_circXPO1_053, hsa_circCSDE1_010, hsa_circMTR_042, hsa_circCOX5A_006, hsa_circPKN2_012, hsa_circCTC-454M9.1_001, hsa_circFCHO2_052, and hsa_circSLC33A1_004 (down-regulated). Although the above-mentioned circRNAs have not previously been associated with PCOS diseases, some have been identified as biomarkers of other diseases, such as lung cancer, prostate cancer, acute leukaemia and breast cancer (Greene et al., 2021; Guo et al., 2020; Yu et al., 2022; Zhao et al., 2020).

3.4 Bioinformatics analysis of differentially expressed circRNAs

Previous studies have shown that circRNA expression is closely related to the function of host genes. We, therefore, explored the function of the differentially expressed circRNAs by GO analysis to investigate their molecular function, biological process, and cell composition. The differentially expressed circRNAs were mainly enriched in cell-cell connection, cytoplasmic transport of nuclear cells, ATP activity, transport vesicles, cell response to oxidative stress, regulation of cell development, regulation of Wnt signaling pathway, phospholipid metabolism, insulin response, and androgen receptor binding. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the differentially expressed circRNAs, they were mainly implicated in endocytosis, autophagy, Rap1 signalling pathway, insulin signalling pathway, phosphatidylinositol signaling system, Ras signalling pathway. Rap1 signalling pathway, insulin signalling pathway, androgen receptor binding, and oxidative stress response are all associated with inflammation. We, therefore, hypothesized that these circRNAs are involved in the pathogenesis and development of PCOS by promoting inflammation.

3.5 circRNA-miRNA-gene network

CircRNAs can act as miRNA sponges. Therefore, the miRanda software was used to predict the relationship between the miRNAs of all related species in the miRBase database and differentially expressed circRNA. miRNA-circRNA relationships meeting the cut-off limits of miRanda comparison score >140 and free energy <-15 were obtained. We also improved differential expression analysis from the top 1000 miRNA-circRNA relationships with the highest binding sites (fold-change > 6.5, $p < 0.05$) chr7_151877795_151896544_- and hsa-miR-1224-5p, chr 3_56597711_56605552_ + and hsa-miR -204-5p were screened out. We identified a number of high-binding sites related to the occurrence and development of polycystic ovary syndrome. We performed a literature retrieval through TargetScan (http://www.targetscan.org/vert_72/) to find the specific miRNA target genes in the online database, and FOXO1 and NF-κB were found to be downstream target genes, which is consistent with previous literature reports. We screened out specific hsa-miR-1224-5p and hsa-miR -204-5p reported in previous studies to make the predicted interaction map between the sponge effect of related circRNA and target genes.

3.6 Activity of PCOS and control MVs on KGN cells

We ascertained the optimal working concentration for MVs following incubation with different MV concentrations. Effective stimulation was observed at 40 μg/ml in both groups. The survival rate of KGN cells decreased significantly in the PCOS group following treatment with 40 μg/ml

MVs, while it increased in the control group (P<0.001). When the concentration of MVs in the control group reached 120 μg/mL, the activity of KGN cells decreased, and there was a significant difference between the concentrations of MVs (P<0.05).

3.7 MVs are taken up by KGN cells

KGN cells were treated with normal- and PCOS-MVs labelled with PKH67 and cultured in a serum-free medium for 24 h. MV uptake was observed under a confocal microscope. Imaging revealed that MVs had become internalized in the cytoplasm (green) and were clustered around the nucleus.

3.8 Protein expression of FOXO1 and NF-κB in MVs

It has been reported that miRNAs adsorbed by circRNA sponges can target FOXO1 and NF- κ B, thus influencing the development of PCOS. We found that the MVs we sequenced carried miRNAs targeting FOXO1 and NF-κ B, and the expression of circRNA in FOXO1 and NF-κB was consistent with previous reports. The protein expression of FOXO1 and NF-κB was significantly higher in cells treated with PCOS MVs than those in normal controls, as expected. There was no significant difference in the expression of FOXO1 and NF-κB in KGN cells between the normal-MVs group and the blank group.

4. DISCUSSION

The short-term and long-term clinical complications of PCOS are well understood, but the complex etiological mechanisms leading to its development are not fully understood. PCOS can cause infertility and has thus become a complex problem in reproductive medicine. Chronic nonspecific inflammation is a subclinical process of chronic, low-grade inflammation mediated by the immune system through the continuous elevation of multiple pro-inflammatory factors and inflammatory markers (Repaci, Gambineri, & Pasquali, 2011). It has been reported that patients with PCOS have elevated levels of inflammatory factors associated with hyperandrogenemia, mainly because the high androgen levels trigger the overactivation of the NF-κB signalling pathway, triggering the production of inflammatory factors (Rostamtabar et al., 2021), which affects follicular development and promote the development of PCOS. Therefore, chronic nonspecific inflammation may be one of the leading causes of PCOS and one of its long-term complications.

MVs act as carriers for biologically active cargoes, such as proteins, lipids, RNA, and non-coding RNA, which can be used as circulating biomarkers. The structure of MVs allows the durable protection of these

bioactive molecules, allowing transport from one cell to another, thus facilitating information transfer between cells. MVs are a subgroup of extracellular vesicles stable in human body fluids, such as saliva, blood, urine, and latex. However, under the stimulation of pathological factors such as hypoxia, inflammatory response and oxidative stress, the number of MVs increases significantly. This indicates that MVs may have good clinical application prospects as deliverers of biomolecules. Nevertheless, it still needs to be determined whether MVs transmit information relevant to the occurrence and development of PCOS.

Among the many cargoes carried in MVs, circRNAs have recently become a research hotspot. CircRNAs were first discovered in the Sendai virus in 1976, but it was in 2013 that Hansen et al. first proposed and confirmed that circRNAs acts as miRNA sponges to regulate gene expression. More and more studies have since shown that circRNAs are structurally stable, conserved, and tissue-specific and thus have potential as biomarkers and therapeutic targets. In addition, levels of circRNAs in the blood are higher than their homologous linear transcripts. Previous studies have suggested that circRNAs in biomolecules are involved in the pathogenesis of polycystic ovary syndrome. Abnormal expression of circRNA is involved in various stages of the occurrence and development of PCOS, including endocrine hormone secretion, follicular development, and granulosa cell proliferation and apoptosis.

In this study, we conducted high-throughput sequencing of circRNAs contained in the serum MVs of PCOS patients and normal women and identified many differentially expressed circRNAs. KEGG annotation revealed that differentially expressed circRNAs were enriched in oxidative stress response, cell development regulation, Wnt signalling pathway regulation, phospholipid metabolism, insulin response, and androgen receptor binding. Meanwhile, GO enrichment was observed in the Rap1 signalling pathway, insulin signalling pathway, phosphatidylinositol signalling system, Ras signalling pathway, and other functions. These pathways may be involved in chronic low-grade nonspecific inflammatory responses during the development and progression of PCOS. For example, oxidative stress plays a vital role in normal ovulation, but excessive reactive oxygen species can lead to the deterioration or direct destruction of oocyte quality, affecting follicular development. When illustrating the relationship between PCOS and low-grade systemic chronic inflammation, Duleba et al. found that, in addition to the increase of inflammatory markers, the oxidative stress response was also increased in PCOS patients, suggesting its close relation to chronic low-grade nonspecific inflammation. Similarly, Suresh et al. found a significant positive correlation between free testosterone and the increase in oxidative stress response. Oxidative stress can promote the activity of androgen synthase and cooperate with LH to directly stimulate the proliferation of ovarian follicular

membrane interstitial cells and androgen secretion, resulting in hyperandrogenemia and activation of the NF-κB signalling pathway, which produces inflammatory factors that affect follicular development. Several studies have confirmed that circRNAs in the cytoplasm can act as miRNA "sponges" to regulate gene expression and protein translation. Therefore, we used the miRanda software to predict RNA sequences and found that chr7_151877795_151896544_- and hsa-miR-1224-5p, chr3_56597711_56605552_+ and hsa-miR-204-5p were enriched in binding sites. Meanwhile, hsa-miR-1224-5p and hsa-miR -204-5p were down-regulated in PCOS patients, which was consistent with the expression results of previous reports. MiR-1224-5p has been reported to be significantly down-regulated in the follicular fluid of patients with PCOS. Li et al. previously demonstrated in vitro that miR-1224-5p targeted FOXO1 to inhibit the inflammatory corpuscle NLRP3 in KGN cells while activating the NF-κB signalling pathway to reduce the inflammatory response induced by TNF-α. Further luciferase reporting experiments confirmed that FOXO1 was the target gene of miR-1224-5P, which was consistent with the predicted results of the target gene in our study. In addition, several studies have reported that hsa-miR-204-5P is lowly expressed in PCOS, while NF-κB is highly expressed. Prior research later confirmed that hsa-miR-204-5P could activate the expression of NF-κB in the inflammatory pathway, leading to premature follicle atresia and polycystic ovarian changes. Our current research results indicate that MVs may be an essential molecular carrier of information in the pathogenesis of PCOS and a new way of transmission. The molecules carrying these messages will provide the basis for related research in this field. At the same time, it means that the differential expression of circRNAs mediated by serum MVs in PCOS patients indirectly regulates downstream target genes; however, the specific regulation of downstream target gene pathways remains to be further studied. Thus, we performed in vitro experiments to verify the downstream target gene expression of FOXO1 and NF-κB.

FOXO1 belongs to the FOXO transcription factor family, which is ubiquitously expressed in almost all body tissues, and participates in various pathophysiological processes, including cell proliferation, autophagy, inflammatory reaction and resistance to oxidative stress. FOXO1 may be involved in the pathogenesis of PCOS through control of the NF-κB signal transduction pathway. NF-κB, a member of the nuclear factor -κB family, is considered an essential gene in the inflammatory pathway, participating in the inflammatory responses of various cell types by regulating target genes to promote the recruitment of inflammatory cells and the release of inflammatory cytokines. Excessive inflammatory factors can cause sparse ovulation or anovulation, a risk factor for PCOS. Our study’s bioinformatics analysis of differentially expressed circRNAs showed dysregulation of signalling pathways related to the oxidative stress response, insulin response, and

androgen receptor binding, which re-emphasized that the pathogenesis of PCOS is closely associated with chronic nonspecific inflammation. Our clinical data suggest that PCOS patients have high serum androgen levels, while high androgen binding with receptors activates the NF-κB signalling pathway to produce inflammatory factors. To verify whether the differentially expressed circRNAs are involved in the inflammatory pathway affecting follicular development, we conducted in vitro experiments in which MVs from patients with PCOS and normal women were co-cultured with KGN cells and subsequently detected the protein expression of FOXO1 and NF-κB. We found that FOXO1 and NF-κB were highly expressed in PCOS patients. The results were consistent with those in other studies, suggesting that chr7_151877795_151896544_- and chr3_56597711_56605552_ + can sponge miRNA to modulate the FOXO1 indirectly and NF-κB inflammatory response induce the activation of inflammatory pathway and inhibit KGN cell proliferation. This results in premature follicle atresia, promoting the development of PCOS. The circRNAs mentioned above may serve as new therapeutic targets for PCOS. Still, it is also possible to induce insulin resistance by regulating the expression of the downstream target gene FOXO1, activating NF-κB signal transduction and promoting inflammatory progression, increasing the expression of steroid-catalyzing rate-limiting enzymes in cell membranes. Rising androgen levels result in hyperandrogenemia that inhibits dominant follicle formation and ovulation.

5. Conclusion

This study provides compelling evidence that circRNA-loaded microvesicles (MVs) present in human serum play a crucial role in the pathogenesis of Polycystic Ovary Syndrome (PCOS), a complex endocrine disorder characterized by a range of clinical symptoms including infertility, hyperandrogenemia, and insulin resistance. Through a meticulous examination of MVs derived from the serum of PCOS patients compared to controls, we have identified a differential expression of circRNAs that significantly impacts cellular communication and contributes to the disease's etiology by targeting the FOXO1 and NF-κB signaling pathways. The observed differential expression of circRNAs in the MVs underscores the potential of these non-coding RNAs as mediators of the inflammatory responses and hormonal imbalances central to PCOS pathology. The inhibition of KGN cell proliferation upon exposure to PCOS MVs further validates the functional impact of these circRNAs, highlighting their role in mediating ovarian dysfunction and infertility associated with PCOS.

This is particularly significant considering the elevated expression of key inflammatory proteins FOXO1 and NF-κB in PCOS patients, which are implicated in the regulation of ovarian follicle development and metabolic homeostasis. Our findings suggest that targeting the circRNA-mediated

regulation of FOXO1 and NF-κB could offer a novel therapeutic approach to mitigating the inflammatory and metabolic disturbances characteristic of PCOS. This approach not only holds promise for restoring fertility in PCOS patients but also for addressing the broader spectrum of metabolic, cardiovascular, and psychological co-morbidities associated with the syndrome. Furthermore, the implications of our research extend beyond the biological aspects of PCOS to encompass the mental and physical health challenges faced by patients. The chronic nature of PCOS, coupled with its impact on fertility, body image, and metabolic health, significantly contributes to psychological stress and diminished quality of life among sufferers. Therefore, our study advocates for a holistic approach to PCOS management, emphasizing the need for integrated treatments that address both the physiological mechanisms and the mental health concerns of patients. In conclusion, our investigation into the role of serum MVs and circRNAs in PCOS not only sheds light on the molecular underpinnings of this complex syndrome but also opens up new avenues for research and therapy. By unraveling the intricate interactions between circRNAs, MVs, and key inflammatory signaling pathways, we pave the way for innovative interventions that promise to improve the lives of those affected by PCOS. Moving forward, it will be crucial to develop a comprehensive understanding of the multifactorial nature of PCOS, incorporating both the physiological and psychological dimensions of the disorder to achieve optimal outcomes for patients.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Affiliated Hospital of Zunyi Medical University (Date 31-12-2021/No KLLY-2021-195).

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