ORIGINAL ARTICLE

Endocrine Research

Identification of MicroRNAs in Human Follicular Fluid: Characterization of MicroRNAs That Govern Steroidogenesis in Vitro and Are Associated With Polycystic Ovary Syndrome in Vivo

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Context: Human follicular fluid is a combination of proteins, metabolites, and ionic compounds that is indicative of the general state of follicular metabolism and is associated with maturation and quality of oocytes. Deviations in these components are often associated with reproductive diseases. There has been no report of microRNAs (miRNAs) in human follicular fluids.

Objective: We hypothesized that human follicular fluid may contain miRNAs. We sought to identify cell-free miRNAs in human follicular fluid and to investigate the function of these miRNAs in vitro and any roles they play in polycystic ovary syndrome (PCOS).

Design: Genome-wide deep sequencing and TaqMan miRNA arrays were used to identify miRNAs, and the roles of the highly expressed miRNAs in steroidogenesis were investigated in KGN cells. Quantification of candidate miRNAs in follicular fluids of PCOS and controls was performed using TaqMan miRNA assays.

Results: We identified miRNAs in microvesicles and the supernatant of human follicular fluid. Bioinformatics analysis showed that the most highly expressed miRNAs targeted genes associated with reproductive, endocrine, and metabolic processes. We found that miR-132, miR-320, miR-520c-3p, miR-24, and miR-222 regulate estradiol concentrations and that miR-24, miR-193b, and miR-483-5p regulate progesterone concentrations. Finally, we showed that miR-132 and miR-320 are expressed at significantly lower levels in the follicular fluid of polycystic ovary patients than in healthy controls ($P = .005$ and $P = .0098$, respectively).

Conclusion: These results demonstrate that there are numerous miRNAs in human follicular fluids, some of which play important roles in steroidogenesis and PCOS. This study substantially revises our understanding of the content of human follicular fluid and lays the foundation for the future investigation of the role of miRNAs in PCOS. (***J Clin Endocrinol Metab* 98: 3068–3079, 2013)**

Follicle development in the ovary consists of the growth of competent follicles, the division of granulosa cells, the expansion of the follicular basal lamina, and finally, the formation of the follicular fluid in the follicular antrum (1). Human follicular fluid consists of secretions from the granulosa and thecal cells combined with plasma compo-

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Abbreviations: Ct, cycle threshold; EM, electron microscopy; GO, gene ontology; ICSI, intracytoplasmic sperm injection; miRNA, microRNA; PCOS, polycystic ovary syndrome.

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nents that cross the blood-follicular barrier via the thecal capillaries (2). Recent studies have shown that follicular fluid is a complex mixture of proteins, metabolites, and ionic compounds reflecting follicular metabolism (3–5). This fluid provides a very important microenvironment and contains regulatory molecules that are important for the maturation and quality of the oocytes (6). Certain lipids, proteins, vitamins, and metabolites in the follicular fluid have been found to be associated with reproductive diseases (7), oocyte quality (8), embryo quality, and the outcome of in vitro fertilization attempts (9, 10).

MicroRNAs (miRNAs) are small regulatory RNA molecules (typically around 22 nucleotides long) that modulate posttranscriptional gene regulation by binding to specific mRNA targets. These molecules play important roles in a wide range of physiological processes and have been implicated in the progression of numerous diseases (11, 12).

miRNAs have been found in human serum, plasma, and other body fluids (13–15), and the miRNA profiles of these fluids have been found to be associated with cancer and other diseases (16–19). miRNAs have also been identified in the ovarian tissues of other species, such as mouse (20), goat (21), and ruminant (22). Moreover, miRNAs have been found in equine follicular fluid (23). Therefore, we hypothesized that human follicular fluids might also contain miRNAs. In this study, we identified several miRNAs in human follicular fluids and determined their roles in steroidogenesis in vitro and polycystic ovary syndrome (PCOS) in vivo.

Materials and Methods

Follicular fluid samples

The donors of the follicular fluid used in this study were undergoing the first round of intracytoplasmic sperm injection (ICSI) treatment. Informed consent was obtained from each couple for the use of the follicular fluid sample that was obtained during oocyte retrieval for the ICSI treatment. Approval by the Institutional Review Committee of Fudan University was also obtained for this study.

Controls and PCOS patients were stimulated with recombinant FSH (Serono, Geneva, Switzerland) after pretreatment with GnRH agonists (Serono). FSH stimulation was initiated once down-regulation was confirmed via ultrasound and serum estradiol measurements. Real-time ultrasound scans were used to monitor follicular development from day 5 of recombinant FSH stimulation until the day of follicular puncture. When at least 1 ovarian follicle had grown to 18 mm in diameter, 10 000 IU human chorionic gonadotropin (Serono) was administered and the follicles were aspirated 34 to 36 hours later.

Follicular fluid was sampled by transvaginal ultrasound-guided puncture and aspiration of follicles 18 to 20 mm in diameter. Follicular fluid (4 μL) was carefully collected from the first aspirated follicle from each donor. The follicular fluid samples were centrifuged at 1300*g* for 10 minutes to remove the blood, and the remaining material was used in subsequent experiments.

Small RNA library construction

The small RNA library was constructed using the total RNA from 10 mL pooled follicular fluid from women undergoing ICSI. Small RNA cloning, sequencing, and analysis was carried out as described previously (14).

Vesicle preparation and electron microscopy visualization

Microvesicles were isolated from the follicular fluid by differential centrifugation according to previously published protocols (19, 24). After removing cells and other debris by centrifugation at 1300*g* for 10 minutes, the supernatant was centrifuged at 110 000*g* for 2 hours (all steps were performed at 4°C). The pelleted microvesicles were resuspended in serum-free medium.

The resuspended microvesicles were prepared for electron microscopy (EM) by fixing in an equal volume of 4% paraformaldehyde. A total of 50 μL fixed material was centrifugally deposited onto Formvar/carbon-coated EM grids with an Airfuge ultracentrifuge and an EM-90 rotor (Beckman Coulter, Inc, Brea, California) at 26 psi for 10 minutes. Karnovsky fixative was substituted for 1% glutaraldehyde to fix the sample-coated grids after washing.

RNA isolation, TaqMan miRNA array, and miRNA profiling

Total RNA was prepared using the miRNeasy kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's directions. In brief, 500 μL supernatant or microvesicle pellets resuspended in PBS were transferred to an Eppendorf tube and mixed thoroughly with 700 μL QIAzol reagent. After a brief incubation at room temperature, 140 μL chloroform was added and the solution was mixed vigorously. The samples were then centrifuged at 12 000 rpm for 15 minutes at 4°C. The upper aqueous phase was carefully transferred to a new tube and 1.5 volumes of ethanol were added. The samples were applied directly to RNA binding columns and washed. Total RNA was eluted in 25 μL nuclease-free H_2O, and 3 μL of this RNA solution was used as the input for each reverse transcription reaction. The human miRNAs hsa-miR-19b, hsa-miR-24, and hsa-miR-222 were detected in the follicular fluid with TaqMan miRNA Assays (Applied Biosystems, Foster City, California).

The expression profiles of the miRNAs in the supernatant and microvesicles were determined using the TaqMan Array Human MicroRNA A and B Cards Set version 3.0 (Applied Biosystems). The cards contain assays for 766 mature miRNAs present in the Sanger miRBase version 18.0. RT-PCRs were performed with Megaplex Primers Pool A with preamplification according to the manufacturer's instructions. PCRs were performed using 450 μL TaqMan Universal PCR Master Mix, No AmpErase UNG (2×), and 9 μL diluted preamplification product, and the reactions were brought to a final volume of 900 μL. One hundred microliters of the PCR mix were dispensed into each port of the TaqMan miRNA array,and the fluidic card was centrifuged and mechanically sealed. Quantitative RT-PCR was carried out on an Applied Biosystems 7900HT thermocycler using the manufac-

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turer's recommended program. Detailed analysis of the results was performed using the Real-Time Statminer software package (Applied Biosystems).

Gene ontology pathway analysis and miRNA target network analysis

Potential miRNA targets were identified from TargetScan, PicTar, miRBase, and Ingenuity Knowledge Base using the Ingenuity Pathway Analysis software package version 9.0 (Ingenuity Systems, http://www.ingenuity.com). Final target lists and relevant miRNAs were uploaded to the Ingenuity Pathway Analysis Server for core analysis with Fisher's exact test and a P value threshold set at .05. Only direct relationships between miRNAs and targets were considered.

Cell culture

A steroidogenic human granulosa-like tumor cell line, KGN, was kindly donated by Dr Fei Sun of the School of Life Sciences, University of Science and Technology of China. KGN cells are undifferentiated and maintain the physiological characteristics of ovarian cells, including the expression of functional FSH receptor and aromatase. The cells were cultured in DMEM/F12 medium (Sigma, St Louis, Missouri) supplemented with 10% fetal calf serum, 100 IU/mL penicillin (Sigma), and 100 mg/mL streptomycin (Sigma) in a 5% CO_2 atmosphere at 37°C.

Transient transfection and hormone analysis

KGN cells were transfected with either miRNA mimics or inhibitors and their controls using HiPerFect transfection reagent (QIAGEN) according to the manufacturer's instructions. The miRNA mimics were chemically synthesized, double-stranded RNAs that resemble mature endogenous miRNAs after transfection into cells, and the miRNA inhibitors were chemically modified antisense RNA oligonucleotides optimized to specifically target miRNA molecules in cells. The miRNA mimics and inhibitors were chemically synthesized by Shanghai GenePharma (Shanghai, China).

After a 24-hour transfection, the culture medium was replaced with serum-free medium and 10nM testosterone (for progesterone analysis) or 10 μM forskolin (for estradiol analysis) was added to each well. The cells were incubated for another 24 hours after which the culture medium was centrifuged at 5000 rpm for 5 minutes.

Concentrations of estradiol and progesterone in the supernatant were determined with the UniCel DxI 800 immunoassay system (Beckman Coulter), which is an automated random-access chemiluminescence-based assay. The intra- and interassay coefficients of variation were less than 10% and 15%, respectively.

Comparative miRNA analysis between controls and PCOS patients

The controls and PCOS patients were recruited from the reproduction center of Central South University (Changsha, China). The PCOS patients were diagnosed based on the criteria revised in 2006 by the Androgen Excess Society, including clinical and/or biochemical signs of hyperandrogenemia plus oligoamenorrhea or polycystic ovaries (25). The controls were from women without any genetic disorders who were undergoing in vitro fertilization because of male factors. Follicular fluids of both controls and PCOS patients are from stimulated cycles. Follicular fluids of controls and PCOS patients were collected from the largest follicles of the stimulated cycle. The follicles were similar in size in both groups, and the follicular fluid was collected as described above. Total RNA was extracted from 500 μL follicular fluid that had been obtained from each of the control and PCOS patients. Quantification of candidate mature miRNAs was performed using TaqMan miRNA Assays (Applied Biosystems) for human hsa-miR-132, hsa-miR-24, hsa-miR-520c-3p, hsa-miR-320, miRNA-193b, miRNA-483-5p, and U6 RNA. At present, there is no widely accepted endogenous control miRNA available for comparison with circulating miRNA levels. U6 RNA was used as a reference for circulating miRNA analysis in previous studies (26, 27). So this was used as the endogenous control in the current study. The fold change in expression of each miRNA was calculated as the mean difference between the $2^{-\Delta\Delta Ct}$ values of the PCOS group and the control group.

Statistical analysis

Data in this study are presented as the averages of at least 3 independent experiments ± SEM. Student's t test was used to determine the significance of any difference in the levels of miRNA expression between controls and PCOS patients. $P < .05$ was considered to be statistically significant. All statistical analyses were performed using SPSS software (version 16.0).

Results

Identification of miRNA-19b, miRNA-24, and miRNA-222 in human follicular fluid

miRNA-19b, miRNA-24, and miRNA-222 are expressed at high levels in plasma (13). Because there are similarities between the contents of follicular fluid and plasma, we first measured the expression level of these miRNAs in the follicular fluid. As shown in Figure 1, the

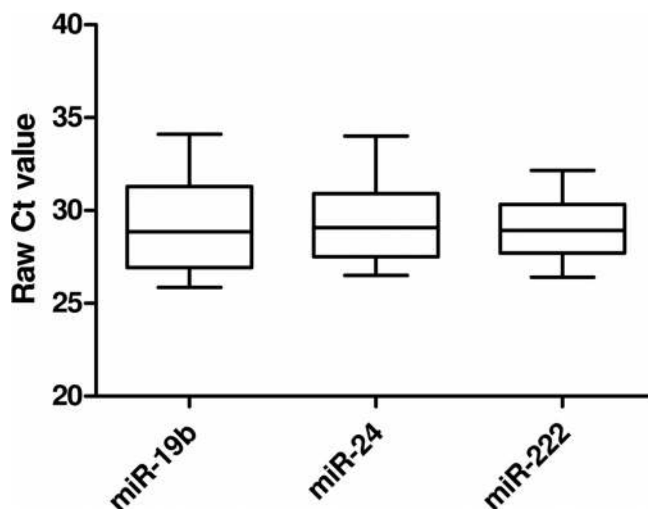

Figure 1. Expression levels of miR-19b, miR-24, and miR-222 in human follicular fluid (n = 20) are shown in raw Ct value. The bottom and top bars indicate the 10th and 90th percentiles. The bottom and top of each box indicate the 25th and 75th percentiles, and the line in the middle of each box is the median.

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3 miRNAs were found to be expressed in the follicular fluid.

Genome-wide analysis of circulating cell-free miRNAs from human follicular fluid

The small RNA library was constructed using the total RNA from 10 mL pooled follicular fluid from 20 women undergoing ICSI. Deep sequencing with an Illumina Hiseq2000 produced 7 519 079 clean reads of a total of 13 203 686 reads, and 15.37% of the total clean reads were aligned to known mature miRNAs in the miRBase version 18.0. These results provided direct evidence that mature miRNAs are present in the follicular fluid. A smaller proportion of the total clean data (4.8%) was aligned to other types of noncoding RNAs such as tRNA, rRNA, small nuclear RNA, and small nucleolar RNA. The length distribution of small RNAs between 15 and 28 nucleotides is summarized in Supplemental Figure 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org), and the distribution of reads is shown in Table 1.

To identify conserved miRNAs, all small RNA sequences were aligned with known nonredundant Magnolio-phyta miRNAs deposited in miRBase version 18.0. In total, 1 155 418 of the 7 519 079 clean reads were perfectly matched to 538 known miRNAs. The details of these miRNAs are listed in Supplemental Table 1.

miRNAs are present in both microvesicles and the supernatant of the follicular fluid

Studies have shown that there are microvesicles in serum, plasma, and saliva that serve as the carriers of circulating miRNAs (15, 28). Thus, we tested whether or not there are also membrane-bound microvesicles in follicular fluid. EM after differential ultracentrifugation showed spherical structures in the pellet with sizes ranging from 50 to 110 nm (Figure 2).

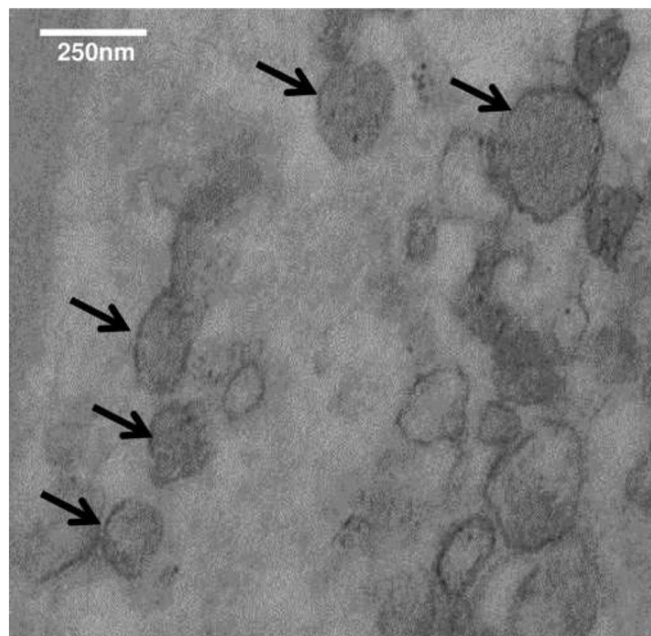

Figure 2. Circulating vesicles were purified from follicular fluid by differential ultracentrifugation. Recovery of vesicles consistent in size and morphology with exosomes and microvesicles was confirmed by EM.

Table 1. Categories of Small RNA in the Human Follicular Fluids Library

Category	Reads Number	Percentage of Total
miRNA	1155418	75.8
rRNA	296844	19.4
snRNA; splicing	63325	4.15
snRNA; snoRNA; CD-box	2946	0.19
snRNA; snoRNA; HACA-box	1276	0.08
snRNA; snoRNA; scaRNA	1061	0.07
Cis-reg; IRES	1012	0.07
Ribozyme	691	0.05
Cis-reg; riboswitch	659	0.04
snRNA	366	0.02
tRNA	74	0.004
Cis-reg; thermoregulator	18	0.001
CRISPR	6	0.0004

Abbreviations: snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; ScaRNA, Cajal body-specific RNAs; CRISPR, clustered regularly interspaced short palindromic repeats RNA.

To determine whether the miRNAs in the follicular fluid are contained within microvesicles or are free in solution, we separated the microvesicles and supernatant from fresh follicular fluids from women having ICSI. The RNA was then extracted independently from both the microvesicles and the supernatant. The TaqMan miRNA Array Card version 3.0, which includes 766 mature miRNAs, detected the presence of miRNAs in both the microvesicles and the supernatant. A total of 120 miRNAs (cycle threshold [Ct] <37) were found in the in microvesicles extracted from 8 mL fresh follicular fluid, and 82 miRNAs (Ct <37) were expressed in 500 μL supernatant (Table 2). Fourteen of the miRNAs were highly expressed (Ct <25) and most of these were found in both the microvesicles and supernatant. The 2 exceptions were miR518f and miR-618, which were detected only in the supernatant. The 14 miRNAs with the highest expression level (Ct <25) in either microvesicles or supernatant are shown in bold in Table 2.

Bioinformatics analysis reveals the role of miRNAs in targeting essential genes in metabolic pathways

According to the miRBase database (http://www.mirbase.org/), 2 of the highly expressed miRNAs (Ct <25) that we detected in the follicular fluid, miRNA-720 and miRNA-1274B, are probably fragments of tRNAs, and these were removed from subsequent analysis. Gene ontology

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Table 2. The miRNAs from TaqMan Low-Density Arrays Quantitative PCR Profiling in Human Follicular Fluid[a]

miRNA	Raw Ct MV	Raw Ct MV-Free Follicle Fluid
hsa-miR-483-5p	18.9909	19.9501
hsa-miR-574-3p	21.9409	23.9732
hsa-miR-518f		24.8455
hsa-miR-191	21.9955	24.9602
hsa-miR-193b	22.9488	24.9859
hsa-miR-1274B	22.9614	30.0201
hsa-miR-320	22.9677	25.9696
hsa-miR-720	23.0080	28.9727
hsa-miR-520c-3p	23.0502	28.0028
hsa-miR-24	23.9872	26.9982
hsa-miR-132	24.0027	27.9730
hsa-miR-146a	24.9564	28.9659
hsa-miR-222	24.9721	29.9727
hsa-miR-1290	24.9960	26.9614
hsa-miR-484	25.0131	25.9618
hsa-miR-19b	25.9811	28.9930
hsa-miR-106a	25.9836	30.0191
hsa-miR-212	25.9871	27.9673
hsa-miR-99b*	26.9664	27.9595
hsa-miR-342-3p	26.9783	
hsa-miR-210	26.9881	28.9561
hsa-miR-17	26.9890	29.9656
hsa-let-7b	27.1030	31.9815
hsa-miR-202	27.9558	30.9729
hsa-miR-186	27.9620	34.9590
hsa-miR-92a	27.9686	27.9645
hsa-miR-203	27.9693	32.9912
hsa-miR-21	27.9709	30.9143
hsa-miR-146b-5p	27.9862	30.9814
hsa-miR-197	28.9407	29.9511
hsa-miR-30a-5p	28.9439	32.9534
hsa-miR-1274A	28.9496	
hsa-miR-523[b]	28.9561	
hsa-miR-425*	28.9787	31.9793
hsa-miR-29a	28.9823	31.9741
hsa-miR-223	28.9893	32.9823
hsa-miR-30a-3p	28.9999	32.0859
hsa-miR-134	29.0047	29.9946
hsa-miR-214	29.0054	30.9377
hsa-miR-126	29.0371	32.0076
hsa-miR-345	29.9505	31.9640
hsa-miR-150	29.9585	
hsa-miR-629	29.9765	31.9946
hsa-miR-125b	29.9780	
hsa-miR-885-5p	29.9792	
hsa-miR-320B[b]	29.9806	
hsa-miR-339-3p	29.9825	
hsa-miR-30e-3p	29.9836	32.1037
hsa-miR-28-3p	29.9862	33.9551
hsa-miR-20a[b]	29.9862	
hsa-miR-324-5p	29.9929	32.9431
hsa-miR-10b*	30.0062	34.0041
hsa-miR-155	30.0178	32.9844
hsa-miR-409-5p	30.1960	
hsa-miR-423-5p	30.9451	34.9436
hsa-miR-503	30.9521	
hsa-miR-31	30.9545	
hsa-let-7c	30.9558	
hsa-miR-509-5p	30.9729	34.9708
hsa-miR-432	30.9824	
hsa-miR-1247	30.9957	32.9640
hsa-miR-424*	31.0091	31.0078
hsa-miR-193b*	31.0138	30.9757
hsa-miR-1271	31.0580	33.0009
hsa-miR-151-3p	31.9277	33.0058
hsa-miR-204	31.9385	
hsa-miR-103	31.9496	
hsa-miR-331-3p	31.9505	
hsa-miR-376a	31.9559	33.9734
hsa-miR-127-3p	31.9633	
hsa-miR-625*	31.9647	35.9580
hsa-miR-425	31.9660	
hsa-miR-34b	31.9754	
hsa-miR-590-5p	31.9772	
hsa-miR-886-5p	31.9829	35.9662
hsa-miR-663B	31.9975	31.0005
hsa-miR-30b	32.0028	
hsa-miR-125a-5p	32.0049	
hsa-miR-218	32.9370	
hsa-miR-130a	32.9473	35.9614
hsa-miR-140-3p	32.9654	35.9215
hsa-miR-31*	32.9714	
hsa-miR-1254	32.9741	33.9755
hsa-miR-30d	32.9778	
hsa-miR-489	32.9805	
hsa-let-7d	32.9890	
hsa-let-7g	32.9914	
hsa-miR-769-5p	32.9922	
hsa-miR-370	32.9949	32.0171
hsa-miR-1270	32.9981	
hsa-miR-532-5p	33.0037	32.0073
hsa-miR-185	33.0063	
rno-miR-7*	33.0413	
hsa-miR-199a-3p	33.9409	
hsa-miR-25*	33.9580	
hsa-miR-136*	33.9688	
hsa-miR-125a-3p	33.9934	
hsa-miR-195	33.9939	
hsa-miR-493	33.9964	
hsa-miR-376c	34.0063	
hsa-miR-93	34.9271	
hsa-miR-1227	34.9365	
hsa-miR-494	34.9546	33.9813
hsa-miR-410	34.9774	36.9512
hsa-miR-192	34.9781	
hsa-miR-548a-3p	34.9959	35.9483
hsa-miR-219-1-3p	35.0092	
hsa-miR-497	35.9607	
hsa-miR-19a	35.9780	
hsa-miR-20b	36.0031	36.0775
hsa-miR-93*	36.0046	32.9467
hsa-miR-411	36.0077	
hsa-miR-26b*	36.0085	
hsa-miR-603	36.0176	35.9976
hsa-miR-380-5p	36.0294	

(*Continued*)

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Table 2. Continued

miRNA	Raw Ct MV	Raw Ct MV-Free Follicle Fluid
hsa-miR-378	36.7518	
hsa-miR-374a	36.9594	
hsa-miR-618		25.0353
hsa-miR-409-3p		27.9661
hsa-miR-375		30.9703
hsa-miR-16		31.9699
hsa-miR-99b		32.0123
hsa-miR-323-3p		32.0155
hsa-miR-324-3p		32.9954
hsa-miR-145		32.9982
hsa-miR-1291		34.0002
hsa-miR-133a		34.0386
hsa-miR-27a		34.9667
hsa-miR-26b		34.9699
hsa-miR-328		34.9848
hsa-miR-224		36.0275
hsa-miR-99a*		36.0366

[a] These miRNAs were detected by TaqMan low-density arrays (Ct <37) in the microvesicles (MV) from 8 mL follicular fluid and in 500 μL MV-free follicular fluid. Highly expressed miRNAs (Ct <25) that were investigated in later bioinformatics and experimental analyses are in bold.

*, Mature miRNA expressed from the 3′ arm of a hairpin.

analysis showed that the 12 remaining highly expressed miRNAs primarily targeted genes in the pathways involved in a variety of cellular process, including reproduction, metabolism, immune system development, and cell proliferation (Figure 3A). The target genes also play key roles in reproductive, gastrointestinal, and hepatic diseases, cancer, endocrine disorders, and other ailments (Figure 3B).

The network of some important target genes and miRNAs is shown in Figure 3C. Among the target genes, estrogen receptor 1 (*ESR1*), which is the target of miR-222′miR-193b, and miR-520c-3p, plays an important role in reproductive steroidogenesis processes. *PTEN*, the target of miR-222′ is a tumor suppressor and a negative regulator of insulin signaling and glucose metabolism in adipose tissue. The ILs IL-1A, IL-10, IL-12B, IL-37, and IL-8 are key members of the immune system and are targets of miR-191, miR-483-5p, miR-146a, and miR-320. The *TGFβ1* and epidermal growth factor receptor (*EGFR*) genes, which are targets of miR-24, miR-574-3p, and miR-1290, mediate important signaling pathways that affect reproductive aging, cell proliferation, and metabolic diseases. The *HMGA2* gene, which is the target of miR-132, and the *RAB5B* gene, which is the target of miR-24 and miR-320, play key roles in the etiology of PCOS as determined in a recent genome-wide association study (29). Finally, PDK3, a major enzyme responsible for the regulation of glucose metabolism, is the target of miR-518a.

Identification of miRNAs affecting estradiol and progesterone secretion in KGN cells

Because the ovary regulates sex hormone concentrations and because several target genes of the miRNAs in the follicular fluid are clustered in reproduction and metabolism-related pathways, we tested whether the 12 highly expressed miRNAs affect steroidogenesis, and estradiol and progesterone secretion in particular, in vitro. We transfected corresponding miRNA mimics and inhibitors into the KGN cell line. As shown in Figure 4, we found that miRNA-24, miRNA-132, miRNA-320, miRNA-520c-3p, and miRNA-222 regulated estradiol secretion. The mimic of miRNA-24 decreased estradiol secretion, and its inhibitor increased estradiol secretion. miR-132, miR-320, miRNA-520c-3p, and miRNA-222 mimics increased estradiol secretion and their inhibitors decreased estradiol secretion, and other miRNAs did not regulate estradiol concentration (Figure 4 and Supplemental Figure 2). Among the 12 highly expressed miRNAs, miR-24, miR-193b, and miRNA-483-5p were found to regulate progesterone secretion. Mimics of these miRNAs decreased progesterone secretion and their inhibitors increased its secretion, and other miRNAs did not affect progesterone level (Figure 5 and Supplemental Figure 3).

Clinical characteristic of PCOS and controls

We recruited 22 controls and 22 PCOS patients, and the clinical characteristics of this cohort are shown in Table 3. Compared with controls, PCOS patients had significantly higher serum concentration of LH, and testosterone concentration in serum ($P < .001$). We also measured the estradiol and progesterone concentration in the follicular fluid from the PCOS patients and controls and found no difference in the levels of these 2 hormones (Supplemental Figure 4).

Association between the miRNAs in the follicular fluid and PCOS

Because some of the miRNAs we found appear to play important roles in steroidogenesis, we hypothesized that there would be differences in expression of these miRNAs between controls and PCOS patients. We measured the expression levels of miR-24, miR-132, miR-320, miR-222, miR-520c-3p, miRNA-193b, and miRNA-483-5p in PCOS and controls samples. As shown in Figure 6, the expression levels of miRNA-132 and miRNA-320 were significantly lower in controls than in PCOS patients ($P = .005$ and $P = .0098$, respectively).

Discussion

In this study, we used TaqMan miRNA arrays and deep sequencing to provide the first report of the presence of

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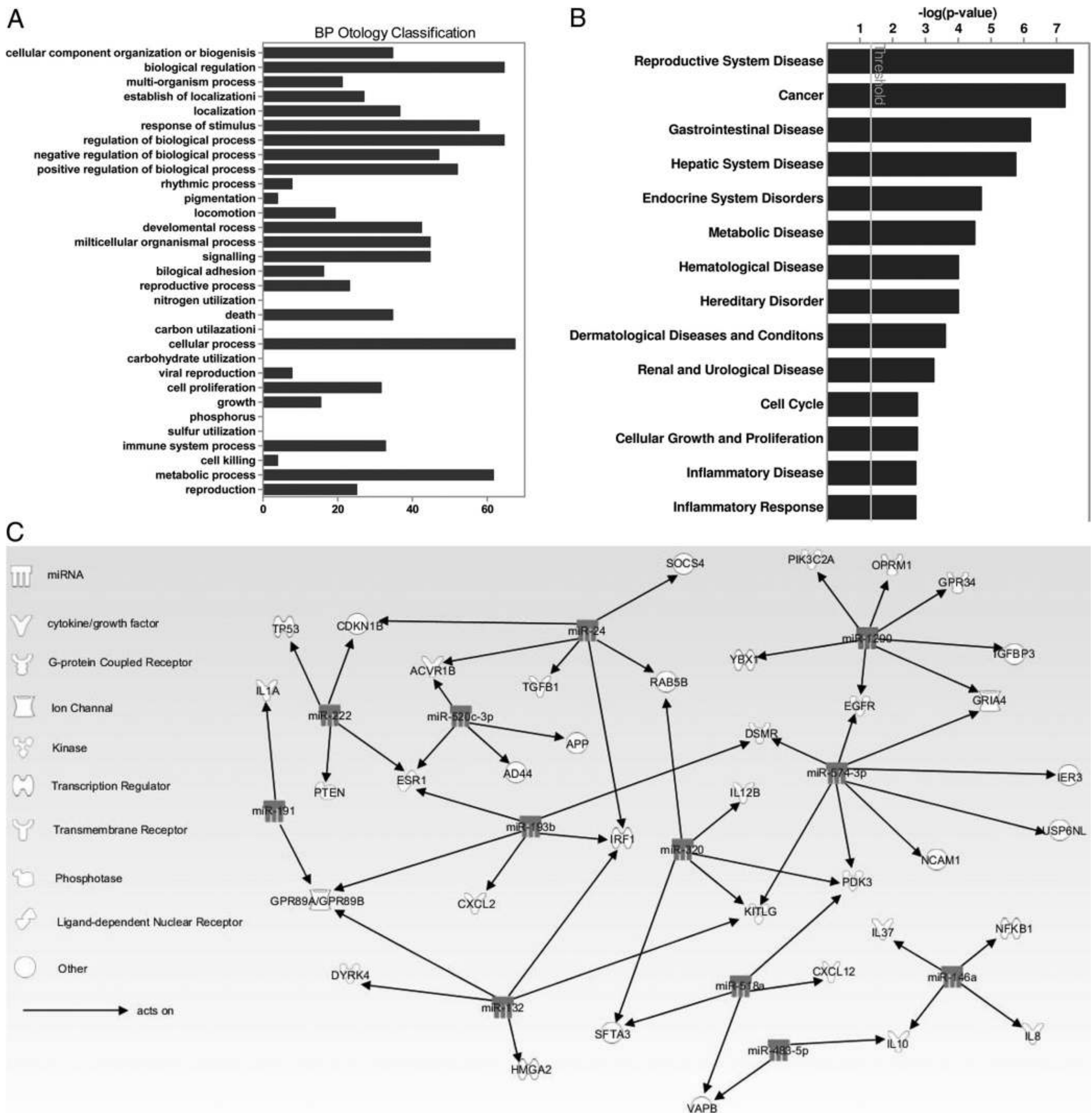

Figure 3. A, Gene ontology (GO) analysis of the biological processes of the target genes of the highly expressed miRNAs (Ct <25) in the follicular fluid. The vertical axis is the GO category, and the horizontal axis is the number of target genes. B, The target genes of the highly expressed miRNAs participate in a variety of cellular processes and in the progression of a variety of diseases. The *P* value was calculated using the right-tailed Fisher's exact test. Each bar represents the highest level of function for each category, each of which includes many sublevel functions. C, Key reproductive and metabolic genes are involved in the network of highly expressed miRNAs. BP, biological process.

miRNAs in both the supernatant and microvesicles from cell-free human follicular fluid. Functional analysis in KGN cells showed that miR-24, miR-132, miR-320, miR-222, miR-520c-3p, miRNA-193b, and miRNA-483-5p regulated hormone secretion. We also found that miRNA-132 and miRNA-320 were associated with PCOS. Thus, our study reveals the existence of miRNAs in human cell-free follicular fluid and demonstrates that some highly expressed miRNAs have key functions in steroidogenesis in vitro and are associated with PCOS in vivo.

The follicular fluid is rich in hormones, growth factors, cytokines, vitamins, proteins, and metabolites. Some studies have also shown that the follicular fluid contains essential substances involved in follicle growth, oocyte fer-

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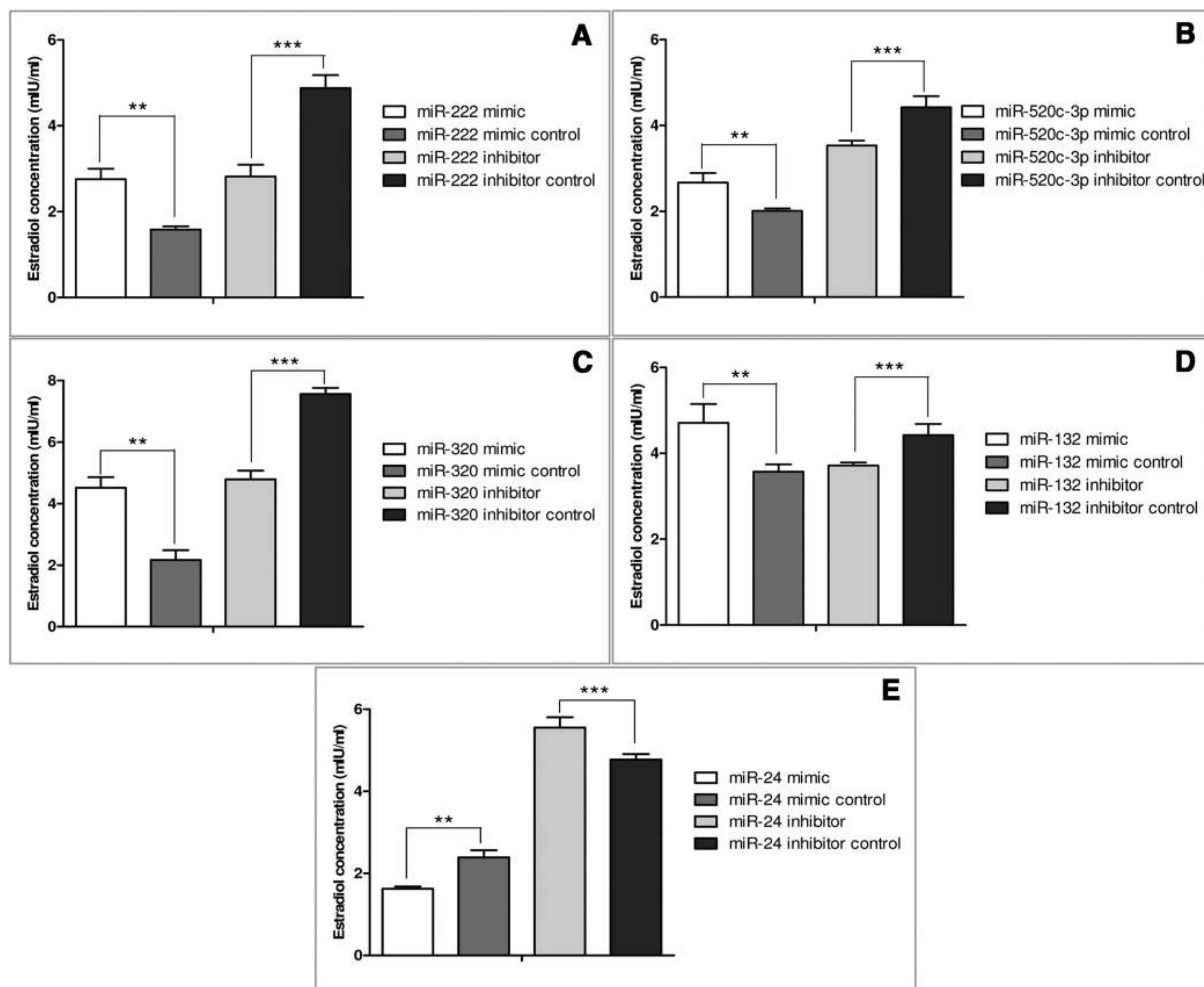

Figure 4. Effects of miRNAs on the release of estradiol by cultured KGN cell lines. A–D, The ectopic expression of miRNA-222 (A), miR-520c-3p (B), miR-320 (C), and miR-132 (D) stimulated estradiol release, and inhibition of these miRNAs inhibited estradiol release. E, The ectopic expression of miRNA-24 inhibited estradiol release and progesterone release, and inhibition of miRNA-24 stimulated estradiol release. **, $P < .01$; ***, $P < .001$.

tilization, spontaneous abortion, and PCOS. For example, increased ghrelin levels in the follicular fluid negatively affect human oocyte quality and in vitro embryo development (30). The metabolite composition of the follicular fluid is correlated with the developmental competence of the human oocyte (10), and some proteins in the follicular fluid that play roles in glucose metabolism, lipoprotein metabolism, cell proliferation, and insulin resistance have been found to be associated with PCOS (31). These studies provide evidence that there is information in the follicular fluid that could be used as a predictor and biomarker in human reproductive diseases.

Studies have shown that miRNAs exist in serum (13), urine (32), saliva (15), and semen (33) and that these circulating miRNAs can be used as biomarkers for various cancers (14, 34, 35), cardiovascular diseases (18, 36), and metabolic diseases (37, 38). Up until now, however, no studies have investigated miRNAs in human follicular fluid. In this study, we have used deep sequencing and TaqMan low-density cards to identify several miRNAs in the follicular fluid that appear to have important functions in a variety of cellular processes. Previous studies demonstrated that circulating miRNAs in serum and other body fluids were found to exist in both microvesicles and the supernatant (28, 39). Consistent with these studies, we also found microvesicles in the follicular fluid and miRNA profiling indicated that miRNAs were present in both the microvesicles and the supernatant of the follicular fluid.

Using the KGN cell line, we found that 8 of the 12 highly expressed miRNAs in the follicular fluid play significant roles in steroidogenesis. We found that miRNA-24 decreased estradiol secretion, but miRNA-

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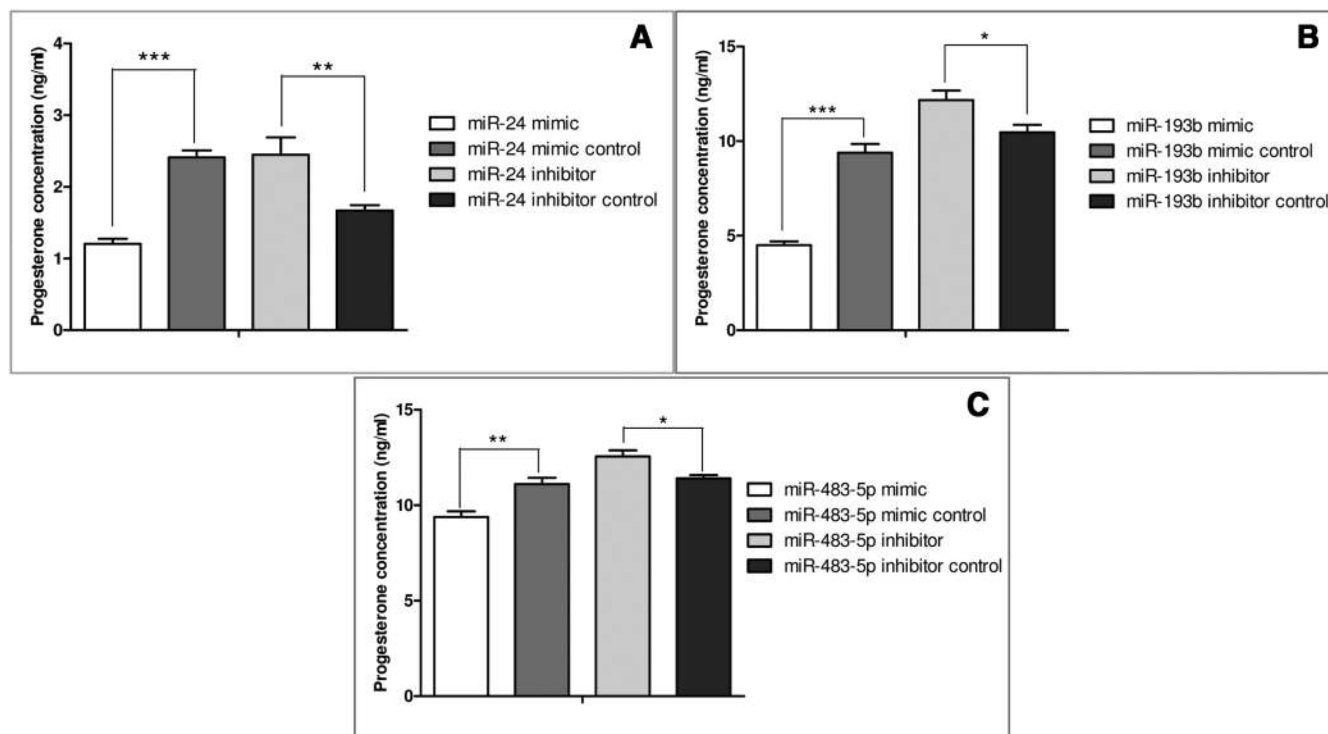

Figure 5. Effects of miRNAs on the release of progesterone by cultured KGN cell lines. A–C, The ectopic expression of miRNA-24 (A), miR-193b (B), and miR-483-5p (C) inhibited progesterone release, and inhibition of these miRNAs stimulated progesterone release. ***, $P < .001$; **, $P < .01$; *, $P < .05$.

132, miRNA-320, miRNA-520c-3p, and miRNA-222 promoted estradiol secretion. We also found that miR-24, miR-193b, and miRNA-483-5p regulated the progesterone concentration in the cell medium.

There are no previous studies showing that miRNA-24 regulates estradiol concentration, but there is evidence that miRNA-24 decreases TGFβ signaling by repressing the expression of Smad proteins (40). TGFβ signaling promotes estradiol release (41). So the miRNA-24 regulation of estradiol concentration can be explained by a mechanism in which overexpression of miRNA-24 will decrease TGFβ signaling, which in turn inhibits estradiol secretion. The mechanisms by which miRNA-132, miR-320, miR-222, and miRNA-520c-3p regulate estradiol secretion, and miRNA-24, miRNA-193b, and miRNA-483-5p regulate progesterone secretion, remain unknown and are worthy of investigation in the future.

Table 3. Clinical Characteristics of Control and PCOS Patients[a]

Variable	Control	PCOS	*P* Value
Age, y	30.83 ± 0.90	29.09 ± 0.70	.13
Height, cm	158.7 ± 0.82	158.0 ± 1.00	.69
Weight, kg	54.38 ± 1.63	58.75 ± 1.49	.06
LH, mIU/mL	4.69 ± 0.36	10.63 ± 1.37	**<.001**
FSH, mIU/mL	6.44 ± 0.42	5.45 ± 0.37	.08
E_2, pg/mL	42.34 ± 4.09	51.10 ± 7.76	.33
PRL, ng/mL	27.23 ± 3.33	18.63 ± 3.33	.29
T, ng/mL	0.17 ± 0.02	0.63 ± 0.09	**<.001**
BMI, kg/m^2	21.95 ± 0.51	23.38 ± 0.59	.07

Abbreviations: BMI, body mass index; E_2, estradiol; PRL, prolactin; T, testosterone.

[a] All results are presented as the mean ± SEM. Clinical indexes with significance differences ($P < .05$) are shown in bold.

Bioinformatics analysis revealed that several important target genes of the 12 highly expressed miRNAs are related to immune system development, cell proliferation, and metabolic and reproductive pathways (Figure 3C). Among these important target genes, *HMGA2* and *RAB5B* are 2 newly identified disease-related genes. A genome-wide association study in a large cohort of PCOS patients (8226 PCOS cases and 7578 controls) found a strong association between *HMGA2* and *RAB5B* and the etiology of PCOS (29). In our target gene network analysis, miRNA-132 is predicted to bind to *HMGA2*, and *RAB5B* is predicted to be the target of miR-24 and miR-320 (Figure 3C). As previously mentioned, miR-132, miR-24, miR-320, miR-520c-3p, miR-222, miRNA-193b, and miRNA-483-5p were found to regulate steroidogenesis in vitro. Because PCOS is a common endocrine disorder in women of reproductive age and is usually associated with abnormalities in steroidogenesis, we measured the expression levels of miR-24, miR-132, miR-520c-3p, miR-222, miR-320, miRNA-193b, and miRNA-483-5p in the follicular fluids of PCOS patients and controls. We found

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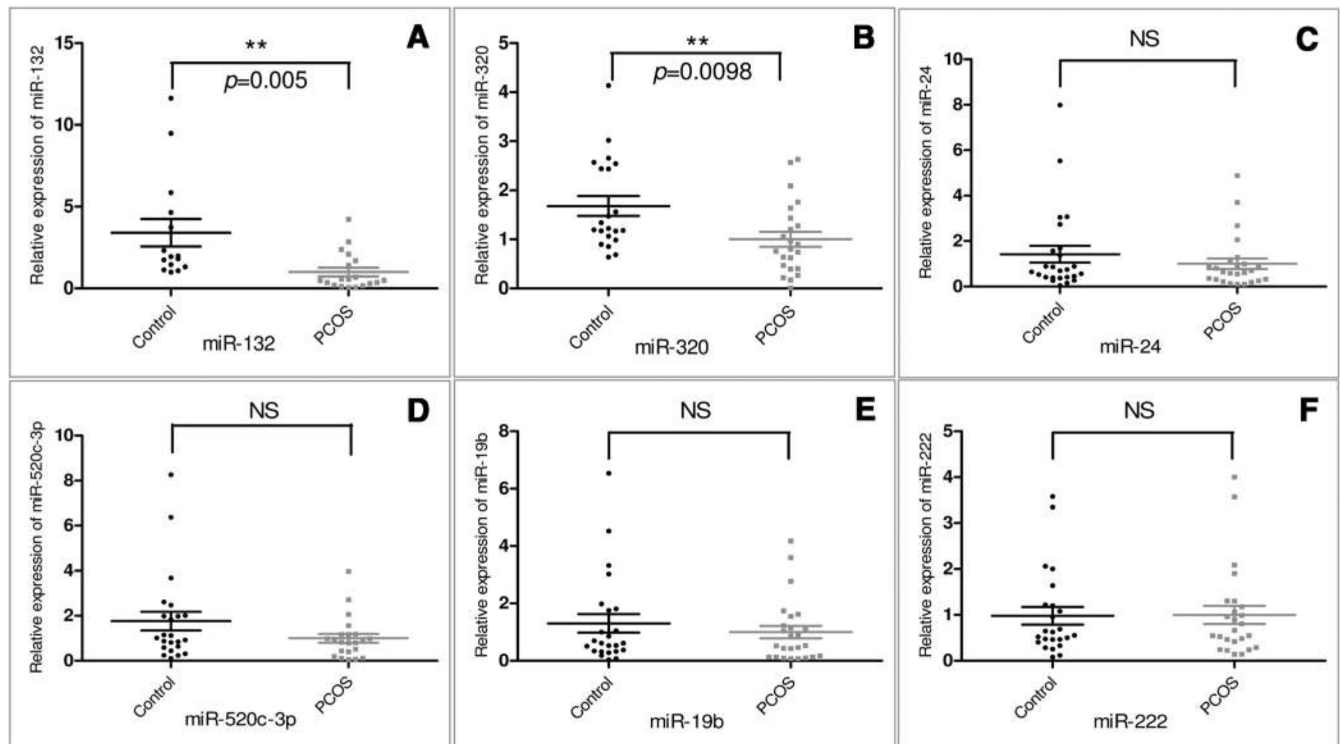

Figure 6. Comparison of miRNA levels in the follicular fluid from controls (n = 22) and PCOS patients (n = 22). A and B, miR-132 (A) and miR-320 (B) were significantly lower in PCOS patients than in controls ($P = .005$ and $P = .0098$, respectively). C–F, There were no significant difference in miR-24 (C), miR-520c-3p (D), miR-19b (E), or miR-222 (F) levels between PCOS patients and controls. All indicated *P* values were determined by 2-tailed Mann-Whitney *U* test. **, $P < .01$.

that both miRNA-132 and miRNA-320 were significantly associated with PCOS, and the expression levels of both of these were lower in PCOS patients than in controls.

A previous study has demonstrated that the absolute steroid levels in follicular fluid are associated with follicular size (42). In this study, we did not observe any differences in the follicular fluid concentration of progesterone or estradiol between PCOS patients and controls, and this suggests that the size of follicles in PCOS patients and controls are similar. Also, the hormone levels are the results of all factors in the follicular fluids, including all miRNAs, proteins, metabolites, ovarian stimulation treatment, etc, and some specific miRNAs that could regulate hormone levels in vitro might not determine final hormone levels in vivo.

miRNA-132 is known to lead to the activation of nuclear factor κB and to the transcription of *IL-8* and monocyte chemoattractant protein-1 (*MCP-1*) in both primary human preadipocytes and differentiated adipocytes (43). In addition, serum miRNA-132 levels are significantly decreased in gestational diabetes mellitus, suggesting that miRNA-132 may regulate insulin secretion (37). miRNA-132 is also known to have key roles in immune system development, metabolic pathways, carcinogenesis (44), neurodevelopment (45), and ischemia reperfusion injury (46). Likewise, the serum level of miRNA-320 has been found to be reduced in the diabetic population and in adipocytes, and miRNA-320 has been shown to play a key role in regulating insulin resistance (38, 47). These results suggest that miRNA-320 plays an important role in the signaling pathways of glucose metabolites. Because *HMGA2* and *RAB5B* have been newly identified as PCOS candidate genes in a genome-wide association study, and are predicted to be the target genes of miRNA-132 and miRNA-320, respectively, we postulate that lower miRNA-132 and miRNA-320 expression levels in the follicular fluid of PCOS patients may affect *HMGA2* and *RAB5B* gene expression.

In addition, previous studies have shown that miR-224, miR-378, and miR-383 are involved in regulating aromatase expression during follicle development (37, 48, 49), and miR-21 has been shown to promote follicular cell survival during ovulation (50). The aromatase expression and follicular cell activity are key to the etiology of PCOS. These previous data combined with our current results suggest that miRNAs may play key roles in the etiology and pathophysiology of PCOS, and elucidating the roles of miRNAs in PCOS should be the subject of future investigations. To our knowledge, this is the first report that miRNAs found in human follicular fluid affect steroidogenesis and are associated with PCOS.

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In conclusion, we show that human follicular fluid contains large amounts of miRNAs, some of which target genes that are important in metabolic and reproductive signaling pathways. In addition, we found that some of the highly expressed miRNAs affect steroidogenesis in vitro and are associated with PCOS in vivo. This study expands our knowledge of the contents of human follicular fluid and suggests a novel role for miRNAs in the etiology and pathophysiology of PCOS.

Acknowledgments

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