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Expression Profile of microRNAs and Their Targeted Pathways in Human Ovaries Detected by Next-Generation Small RNA Sequencing

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Recently, post-transcriptional gene regulation by microRNAs (miRNAs) has been reported to play a key role during ovary development and differentiation. However, there are no published studies identifying miRNA profiles of human ovarian tissues directly using next-generation sequencing technology. In the human ovary, a total of 762 known and 21 novel human miRNAs were detected, indicating that human ovaries have a complex population of small RNAs. To confirm the miRNA profile in human ovaries, quantitative real-time polymerase chain reaction was used to validate the expression of known miRNAs and novel miRNAs. The potential regulating roles of miRNA in physiological function of ovaries were analyzed by gene ontology and Kyoto encyclopedia of genes and genomes pathway annotation, and several important processes were identified to be targeted by the most abundantly expressed miRNAs, for example, antral ovarian follicle growth, ovarian follicle rupture, and fertilization. Our current findings extend the knowledge of the regulatory role of miRNAs and their targeted processes in human ovaries, suggesting that miRNAs play important roles in development and physiological function of ovaries. In this study, we provide a useful resource for further research of the regulatory role of miRNAs in the ovaries, which may also provide novel candidates for molecular biomarkers or treatment targets in the research of female infertility.

Introduction

The ovary is an oocyte-producing reproductive organ and is also responsible for synthesizing and secreting hormones, which are essential for the maintenance of the reproductive tract and menstrual cycle, and female secondary sex characteristics (Weghofer and Gleicher, 2009; Li *et al.*, 2015). Folliculogenesis in the ovary, for ovulation of a mature and viable oocyte and the development of a functional corpus luteum (CL), is the basic process and essential for establishment and maintenance of pregnancy (Hsueh *et al.*, 2015; Li *et al.*, 2015). It consists of several continual stages, including primordial follicle assembly, follicle growth and ovulation, and formation of CL, and is a dynamic and highly regulated process determining the female reproduction (Hsueh *et al.*, 2015). Any inappropriate development and coordination of folliculogenesis will result in serious ovarian pathologies such as premature ovarian failure and polycystic ovary syndrome (PCOS) (Hsueh *et al.*, 2015).

Recently, post-transcriptional gene regulation by microRNAs (miRNAs) has been reported to play a key role during ovary development and differentiation (Hawkins and Matzuk, 2010; Li *et al.*, 2015). miRNAs are small noncoding RNAs, average 21 nucleotides in size, and regulate gene expression through transcriptional repression or degradation based on direct base-pairing interactions with targeted mRNAs at the post-transcriptional level (Azuma-Mukai *et al.*, 2008). Most miRNAs are evolutionary, conserved in a large number of species, and function in most tissue development and differentiation (Azuma-Mukai *et al.*, 2008; Carletti and Christenson, 2009; Sorensen *et al.*, 2014).

The role of miRNAs in ovarian function has been investigated through *Dicer* knockout mouse models (Bernstein *et al.*, 2003). Dicer is an important RNase III enzyme required for mature miRNA production, and general knockout of Dicer in mice resulted in embryonic lethality (Bernstein *et al.*, 2003). Using conditional knockout mouse models of *Dicer* in ovarian granulose cells, these mice showed a reduction in ovarian weight and ovulation rates, abnormal estrous cycles, shorter estrus, and longer metestrus, paratubal cyst, and even infertility (Lei *et al.*, 2010). Oocytes with *Dicer*-specific knockout exhibited an impaired ability to extrude a polar body, multiple spindles, and chromatin condensation defects (Liu *et al.*, 2010; Yuan *et al.*, 2014). These results suggested that miRNAs are necessary for the maintenance of ovarian function and even fertility.

Mammalian ovaries exhibit spatiotemporal mRNA expression pattern as well as miRNAs (Lei *et al.*, 2010; Tong *et al.*, 2014; Zhang *et al.*, 2014). Moreover, miRNAs are the

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most abundant class of small RNAs in the ovary in different mammalian species (Carletti and Christenson, 2009; Ahn *et al.*, 2010; Chan and Ruohola-Baker, 2010; Hackl *et al.*, 2011). The miRNA expression profile in mouse, cattle, rat, and porcine was identified by microarray, high-throughput polymerase chain reaction (PCR), and next-generation sequencing techniques (Ahn *et al.*, 2010; Hackl *et al.*, 2011; Miretti *et al.*, 2011; Podolska *et al.*, 2011; Lin *et al.*, 2015). These results suggested that several miRNAs, for example, the let-7 family, miR-21, miR-99a, and miR-199b, expressed abundantly in these species and bioinformatic analysis revealed that these miRNAs might be involved in regulation of several biological processes (Hackl *et al.*, 2011; Miretti *et al.*, 2011; Podolska *et al.*, 2011; Shi *et al.*, 2014; Wagner *et al.*, 2014; Lin *et al.*, 2015).

For example, the expression of miRNAs in the let-7 family was required for mammalian developmental timing and tumor suppressor function (Lee *et al.*, 2015). MiR-21 plays crucial roles in inflammatory injury and carcinogenesis (Liu *et al.*, 2013; Yin *et al.*, 2015). MiR-99a could inhibit cell proliferation and regulate tumor progression and stem-like properties in cholangiocarcinoma (Huang *et al.*, 2015; Lin *et al.*, 2015). MiR-199b-5p could enhance the suppression on cell migration and could also participate in human erythropoiesis (Fang *et al.*, 2013; Li *et al.*, 2014).

Although the miRNA expression and function have been detected in mice and other mammalian models, the miRNA expression profile of human ovarian tissues was not documented in detail. In this study, we determined the miRNA expression profile of normal human ovaries and identified the abundantly expressed miRNAs and novel miRNAs using next-generation sequencing (NGS) technology. Moreover, bioinformatic analysis, gene ontology (GO) annotation, and pathway enrichment of the potential miRNA target genes for the abundantly expressed miRNAs indicated that many important processes related with ovarian function were enriched, such as antral ovarian follicle growth, ovarian follicle rupture, and fertilization. Interestingly, several similar biological processes were highlighted for the novel miRNA targets, for example, the MAPK signaling pathway. Our results suggested that miRNA in human ovaries plays important role in oogenesis, folliculogenesis, and other ovarian functions and this offers new insights for the treatment of reproductive and metabolic disorders associated with ovaries.

Materials and Methods

Ovary sample collection

The ovary tissues used for NGS were collected from three cadavers (aged 22, 27, and 31 years) from Anhui Provincial Hospital Affiliated with Anhui Medical University, Hefei, China. These samples with normal folliculogenesis enrolled in this study after histological examination. Detailed basic information of the three subjects is listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/dna). This study was approved by the Ethics Committees on Human Research of Anhui Provincial Hospital Affiliated with Anhui Medical University (Approve ID: 20131357). The family members of cadavers agreed to participate in this study and all samples were obtained with written informed consents.

Small library construction and sequencing

The small RNA sequencing for human ovaries was completed at BGI-Shenzhen (Tong *et al.*, 2014; Xu *et al.*, 2015). Total RNAs were extracted from ovary samples using TRIzol reagent (Invitrogen). The samples from three subjects were pooled homogeneously into one RNA sample. Thus, the total RNA samples were subjected to 15% (W/V) denaturing polyacrylamide gel electrophoresis and 18–22 nt small RNA fragments were isolated. These isolated small RNAs were ligated to the adaptors: 5′ adaptor-GTTCAGAGTTCTACAGTCCGACGATC, 3′ adaptor-TCGTATGCCGTCTTCTGCTTG, and then these RNAs were transcribed by reverse transcription PCR. After purification, the reverse transcription PCR (RT-PCR) products were sequenced by the Illumina Hiseq 2000 (Illumina) according to Illumina's protocol.

Computational analysis of sequencing data

The NGS data of human ovary were analyzed by our previously published tools, CPSS (Zhang *et al.*, 2012). Briefly, the adaptor sequences, low quality reads, and contaminated reads were first removed, and the remaining sequences were counted as the tags. These tags were mapped to the human genome using SOAP2.0 (Li *et al.*, 2009), and all the mapped tags were matched into several RNA databases, such as miRbase (Van Peer *et al.*, 2014), Rfam (Burge *et al.*, 2013), Genebank nonoding RNA database (Karolchik *et al.*, 2014), piRNA database (Li *et al.*, 2009), or repeats database (Tarailo-Graovac and Chen, 2009). Only the tags that matched with miRbase were classified as known miRNAs and submitted into further analysis. The tags were classified as unclassified tags if they were not assigned to any of the above databases, which were used for the prediction of novel miRNAs in human ovaries.

Prediction of novel miRNAs in human ovaries

To avoid false positive by discarding the candidates with low abundance, the unclassified tags with 45 reads were processed for novel miRNA prediction. MiRDeep (An *et al.*, 2013) and Mireap (http://sourceforge.jp/projects/sfnet_mireap/) were used to predict the novel miRNAs using following rules: (1) sequence length between 18–26 nt, (2) free energy for miRNA precursor ≤–18 kcal/mol, (3) space between miRNA and mRNA* ≤35nt, and (4) the bulge of miRNA and miRNA* ≥4. The secondary

Table 1. The Match Results of Clean Reads from Human Ovaries

Small RNA category	*No. of unique tags*	*Percentage*	*Total reads*	*Percentage*
miRNA	6045	2.13	5672362	67.50
piRNA	3926	1.38	198656	2.36
mRNA	73785	26.01	365156	4.35
rRNA	22140	7.81	342665	4.08
Repeat	106039	37.38	1351428	16.08
snRNA	2812	0.99	10035	0.12
snoRNA	2656	0.94	22948	0.27
tRNA	7159	2.52	79713	0.95
Others	14376	5.07	137127	1.63
Unannotated	44709	15.76	222990	2.65
Total	283647	100.00	8403080	100.00

TABLE 2. THE EXPRESSION PROFILE OF TOP 10 ABUNDANTLY KNOWN MICRORNAS IN HUMAN OVARIES

miRNA name	*Absolute count of miRNAs*	*Relative count of miRNAs (RPM)*	*Sequence*
hsa-let-7a-5p	1390772	165507	TGAGGTAGTAGGTTGTATAGTT
hsa-let-7c-5p	617576	518595	TGAGGTAGTAGGTTGTATGGTT
hsa-miR-140-3p	95693	11388	TACCACAGGGTAGAACCACGG
hsa-let-7e-5p	80962	9635	TGAGGTAGGAGGTTGTATAGTT
hsa-miR-199a-3p	80233	9548	ACAGTAGTCTGCACATTGGTTA
hsa-miR-199b-3p	76080	9054	ACAGTAGTCTGCACATTGGTTA
hsa-miR-103a-3p	58559	6969	AGCAGCATTGTACAGGGCTATGA
hsa-miR-320a	47006	5594	AAAAGCTGGGTTGAGAGGGCGA
hsa-miR-202-5p	45931	5466	TTCCTATGCATATACTTCTTTG
hsa-miR-26a-5p	43529	5180	TTCAAGTAATCCAGGATAGGCT

RPM, reads per million.

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structures of candidate novel miRNAs were predicted by RNA fold.

Bioinformatic analyses for known miRNAs in human ovaries

The bioinformatic analyses for known miRNAs were processed as our previous reports. In this study, the putative targets of the most abundantly known miRNAs in human ovaries were predicted by Targetscan (Lewis *et al.*, 2003), MicroCosm (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/#), and miRanda (Wang *et al.*, 2014), while the targets for the novel miRNAs in human ovaries were predicted by miRanda (Wang *et al.*, 2014). The predicted rules were also performed according to previous reports (Tong *et al.*, 2014; Xu *et al.*, 2015). After miRNA target prediction, these predicted genes were subjected to GO term analysis (Ashburner *et al.*, 2000). These targets were mapped into the GO annotation dataset, and the significantly enriched biological processes were identified by the hypergeometric test. When the enrichment ratio of GO term was >2 and the p-value was <0.05, this GO term was classified as a key term. For pathway analysis of these targets, the Kyoto encyclopedia of genes and genomes (KEGG) database was used for the signaling pathway annotation (Kanehisa *et al.*, 2010). Fisher's exact test was used to detect the enriched KEGG pathways. A relevant pathway was identified when the enrichment ratio was >1.5 and p-value was <0.05.

miRNA expression detection by quantitative real-time PCR

According to our previous reports (Zhang *et al.*, 2012, 2014), miRNA quantification was detected by real-time PCR by using the Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems) and an SYBR premix Ex Taq™ II kit (Takara) with the primers listed in the Supplementary Table S2. The snRNA level of U6 was used as an internal reference. In this study, the PCR reactions were performed at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 31 s.

Statistical analysis

In this study, the PCR experiments were run in triplicate for each of the three samples. Quantitative data from real-time PCR were compared using Student's t-tests. $p < 0.05$ was considered significant.

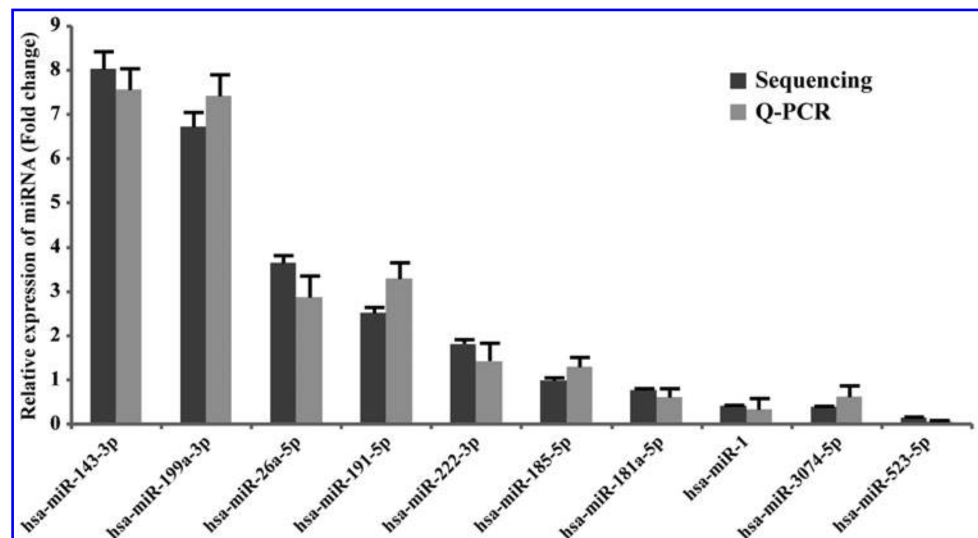

FIG. 1. Confirmation of the 10 most abundantly expressed miRNAs in human ovaries by quantitative real-time PCR. Validation of the expression of 10 most abundantly expressed miRNAs by quantitative real-time PCR. The quantitative real-time PCR data with bars represent mean ± SD from three independent experiments. The sequencing data with bars represent the 95% CI. CI, confidence interval; miRNAs, microRNAs; PCR, polymerase chain reaction; Q-PCR, quantitative real-time PCR.

Table 3. Gene Ontology Analysis for Predicted Targets of Top 10 Abundantly Known microRNAs in Human Ovaries

GO number	GO biological process	Targeted genes	Enrichment ratio	p
Biological process				
GO:0042990	Regulation of transcription factor import into nucleus	MBTPS1	97.92	0.0201
GO:0001547	Antral ovarian follicle growth	CCNE1, FOXO3	30.47	0.0037
GO:0045742	Positive regulation of epidermal growth factor receptor signaling pathway	AGT, FASLG	15.24	0.0107
GO:0001543	Ovarian follicle rupture	AGT, EDN2, NRIP1	9.78	0.0129
GO:0014065	Phosphatidylinositol 3-kinase cascade	LTK, NF1	9.38	0.0237
GO:0009566	Fertilization	PCSK4, PRDM14, PVRL2	7.31	0.0101
GO:0000902	Cell morphogenesis	IDUA, PRDM14, TFCP2L1, VHL	5.08	0.0098
GO:0008286	Insulin receptor signaling pathway	ATP6V1F, FOXO3, GSK3A, IRS2, STK11	3.52	0.0155
GO:0007156	Homophilic cell adhesion	CDH13, CDH20, CDHR5, CELSR2, ESAM, PCDH12, PCDH19, PCDHA1, PCDHA10, PCDHA11, PCDHA12, PCDHA13, PCDHA3, PCDHA4, PCDHA5, PCDHA7, PCDHA8, PCDHA9, PCDHAC2, PCDHGA1, PCDHGA10, PCDHGA11, PCDHGA12, PCDHGA2, PCDHGA3, PCDHGA5, PCDHGA6, PCDHGA7, PCDHGA8, PCDHGB1, PCDHGB2, PCDHGB4, PCDHGB6, PCDHGB7, PCDHGC3, PCDHGC4, PCDHGC5, PTPRT, PVRL2, ROBO2	2.92	0.0000
GO:0031295	T cell costimulation	BTLA, BX927235.1, CD274, CD4, CD5, CD80, CD86, CR788283.1, DPP4, HLA-DPA1, HLA-DQA1, HLA-DRB3, HLA-DRB4, HLA-DRB5, ICOS, ICOSLG, PIK3CA, PTPN6, SRC	2.51	0.0009
Molecular function				
GO:0031703	Type 2 angiotensin receptor binding	AGT	99.99	0.0197
GO:0042624	ATPase activity, uncoupled	ATP6V1F	99.99	0.0197
GO:0050080	Malonyl-CoA decarboxylase activity	MLYCD	63.42	0.0308
GO:0034647	Histone demethylase activity (H3-trimethyl-K4 specific)	KDM5B	63.42	0.0308
GO:0004633	Phosphopantothenoylcysteine decarboxylase activity	PPCDC	63.42	0.0308
GO:0008437	Thyrotropin-releasing hormone activity	TRH	63.42	0.0308
GO:0000831	Inositol hexakisphosphate 6-kinase activity	IP6K3	63.42	0.0308
GO:0000900	Translation repressor activity, nucleic acid binding	CELF1, CELF4, PAIP2B	5.89	0.0309
GO:0001784	Phosphotyrosine binding	LDLRAP1, MAPK3, PTPN3, PTPN6, SHC1	3.78	0.0206
GO:0004012	Phospholipid-translocating ATPase activity	ATP10A, ATP11A, ATP8B2, ATP8B4, ATP9B	3.27	0.0320
Cellular component				
GO:0030672	Synaptic vesicle membrane	SEMA4C, SV2A, SYN1	6.47	0.0129
GO:0033268	Node of Ranvier	CNTN2, NFASC, SCN1A, SCN2A	5.59	0.0143
GO:0008021	Synaptic vesicle	GIPC1, MT3, SV2A, SYN1	4.96	0.0099
GO:0030673	axolemma	ADORA2A, GABBR1, KCNC1, MAPK8IP3, ROBO2	4.89	0.0093
GO:0048188	Set1C/COMPASS complex	HCFC1, RBBP5, SETD1B, WDR82	4.35	0.0266
GO:0008021	synaptic vesicle	GIPC1, MT3, SV2A, SYN1, SYNGR2	3.91	0.0110
GO:0042613	MHC class II protein complex	BX927235.1, CR788283.1, HLA-DPA1, HLA-DQA1, HLA-DRB3, HLA-DRB4, HLA-DRB5	3.11	0.0147
GO:0009897	External side of plasma membrane	ATP6AP2, ENOX2, FASLG, ICOS, ITGAX, PDCD1, SELL	2.88	0.0131
GO:0009898	Internal side of plasma membrane	CACNB4, FGFR3, LDLRAP1, LOR, PGM5, PTP4A1, PTPN3, PTPN4, TRAF3, TRAF6	2.80	0.0071
GO:0016459	Myosin complex	CGN, CGNL1, DYNLL2, MYH10, MYH15, MYL12A, MYL2, MYL3, MYO19, MYO1A, MYO1G, MYO7B	2.17	0.0188

GO, gene ontology.

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Results

Overview of small RNA sequencing data

In this study, we obtained a total of 9,132,054 raw reads from human ovaries using Solexa NGS technology. After removal of 5′ and 3′ adaptor sequences, contaminations, and low quality reads, 8,403,080 clean reads, representing 283,647 unique tags, remained (Table 1). The majority size of these unique tags was 22 nt, varying between 18 and 26 (Supplementary Fig. S1). For detecting the miRNA expression profile from these reads, all the tags were matched into miRbase (V14.1). Thus, 762 miRNAs corresponding to 5,672,362 reads in human ovaries were identified as known miRNAs. In this study, after identification of known miRNAs, the rest of the reads were matched into other small RNA databases, including rRNAs, repeats, and snRNAs. The chromosome locations of the clean reads were analyzed and the distributions are shown in Supplementary Figure S2. In human ovaries, most reads are located in chromosome 9, followed by chromosome 22, 21, and 16, respectively.

The expression and enzymatic modification of known miRNAs

To identify known miRNA expression profile in human ovaries, the counts of each type of miRNA were normalized (normalized counts are displayed as reads per million) and 10 miRNAs with most abundant reads counts were listed (Table 2), indicating that they are highly expressed in human ovaries. To validate the miRNA expression detected by sequencing, 10 known miRNAs representing different expression levels were randomly chosen for quantitative real-time PCR. The expression pattern of these 10 miRNAs detected by quantitative real-time PCR was consistent with the results obtained from sequencing, and these results indicated that the miRNA expression identified by NGS technology was reliable (Fig. 1).

Recently, several reports suggested that miRNAs exhibit post-transcriptional enzymatic modification, such as 5′ or 3′ end trimming or additions of nucleotides and nucleotide changes of the mature miRNA sequence without a template, and these miRNA modifications may relate with miRNA stability or strengthen miRNA-mRNA interaction and even be involved in biological regulatory processes (Li *et al.*, 2005; Azuma-Mukai *et al.*, 2008; Morin *et al.*, 2008; Ebhardt *et al.*, 2009; Lu *et al.*, 2009; Fernandez-Valverde *et al.*, 2010). Therefore, we also analyzed the expression of miRNA isoform (including miRNA modification and editing) in human ovaries (Supplementary Tables S3 and S4). The miRNAs in the let-7 family showed the most abundant expression of miRNA modification and editing, which was in accordance with our previous reports (Tong *et al.*, 2014; Xu *et al.*, 2015), suggesting that the diversification of miRNA isoforms in let-7 family members in human ovaries might be involved in the processes of folliculogenesis and oogenesis (Miles *et al.*, 2012).

Prediction of miRNA-targeted genes and identification of miRNA-regulated biological processes

To better understand the role played by the most abundantly known miRNAs in human ovaries, we identified the targeted genes, signaling pathways, and biological processes that could be targeted by these miRNAs. The putative target genes of miRNAs were predicted using miRanda, Targetscan, and MicroCosm, and these predicted miRNA targets were performed for GO and KEGG pathway analysis to enrich the involved signaling pathways and biological processes. After GO analysis, we found that the putative target genes of known miRNAs appeared to be involved in a broad range of processes, such as folliculogenesis-related processes (e.g., GO:0001547, antral ovarian follicle growth, and GO:0001543, ovarian follicle rupture), ovarian function-related processes (e.g., GO:0009566, fertilization, and GO:0007156, homophilic cell adhesion), and several key cellular signaling pathways (e.g., GO:0042990, regulation of transcription factor import into nucleus, GO:0045742, positive regulation of epidermal growth factor receptor signaling pathway, and GO:0008286, insulin receptor signaling pathway) (Table 3). Moreover, the known miRNA-related regulating pathways were enriched by KEGG pathway analysis (Table 4). Many ovarian function and oncogenesis-related signaling pathways were found to be involved, including the ErbB signaling pathway and Toll-like receptor signaling pathway.

Identification of novel miRNAs and prediction of their targeted genes and pathways

Recently, the NGS techniques have greatly revolutionized the detection of novel small RNAs with high levels of sensitivity and accuracy. Thus, the unclassified reads from NGS data were analyzed by Mireap and miRDeep to identify novel miRNAs in human ovaries. In this study, Mireap and miRDeep predicted the novel miRNAs based on default parameters with the read count more than 45, which were defined as novel miRNAs in human ovaries. Therefore, 21 novel miRNAs were identified in human ovaries, and the top 10 most abundant novel miRNAs are listed in Table 5. To confirm these 10 novel miRNAs obtained from NGS data, we used quantitative real-time PCR to validate them

Table 4. Kyoto Encyclopedia of Genes and Genomes Pathway Analysis for Predicted Targets of Top 10 Abundantly Known microRNAs in Human Ovaries

Pathway name	*Targeted genes*	*Enrichment ratio*	p
Glycosaminoglycan biosynthesis–keratan sulfate	CHST1, CHST2	8.33	0.0286
α-linolenic acid metabolism	PLA2G2F, PLA2G3	6.58	0.0424
Carbohydrate digestion and absorption	ATP1B1, PLCB2, SLC5A1	4.36	0.0353
ErbB signaling pathway	BRAF, EGFR, EREG, HBEGF, HRAS, MAP2K2, MAPK3, MAPK8, PIK3CA, SHC1, SRC, STAT5B	2.40	0.0108
Toll-like receptor signaling pathway	CD80, CD86, FADD, IKBKG, IL6, MAP2K2, MAPK14, MAPK3, MAPK8, PIK3CA, TIRAP, TNF, TRAF3, TRAF6	2.39	0.0053

Table 5. Novel microRNAs Predicted from Small RNA Sequencing Data of Human Ovaries

miRNA name	*Mature sequence*	*Location of novel miRNA precursor*
Nov-0141	TAGCAGCGGGAACAGTTCTGCAG	chrX:133680351:133680433: –
Nov-0049	CACCCGTAGAACCGACCTTGCG	chr19:52195861:52195939: +
Nov-0089	TGAGGTAGTAGTTTGTACAGTTT	chr3:52302283:52302383: –
Nov-0024	TGTGACTGGTTGACCAGAGGGG	chr14:101521021:101521101: +
Nov-0022	TGGTTTACCGTCCCACATACAT	chr14:101490127:101490200: +
Nov-0014	AACCCGTAGATCCGAACTTGTGG	chr11:122022932:122023014: –
Nov-0138	TTATCAGAATCTCCAGGGGTAC	chrX:85158636:85158717: –
Nov-0137	TTATAATACAACCTGATAAGT	chrX:73507121:73507191: –
Nov-0011	TCTACAGTGCACGTGTCTCCAGT	chr11:72326100:72326178: –
Nov-0013	TCCCTGAGACCCTAACTTGTGATG	chr11:121970466:121970548: –

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(Fig. 2). We further predicted the targeted genes of these novel miRNAs by miRanda, and these predicted targets were also performed for GO and KEGG pathway analysis. GO annotation for the predicted genes of novel miRNAs revealed that they may be involved in a broad range of biological processes related with the ovarian function, such as GO:0043409, negative regulation of MAPKKK cascade, and GO:0051897, positive regulation of protein kinase B signaling cascade (Supplementary Table S5). Similarly, several key pathways were also enriched according to KEGG pathway analysis, including the MAPK signaling pathway (Supplementary Table S6).

Discussion

Development of the mammal ovary is the result of a series of complex biological processes, such as oocyte growth, development, and differentiation of ovarian somatic cells, and interaction of oocytes and follicular cells (Carletti and Christenson, 2009; Hawkins and Matzuk, 2010; Hsueh *et al.*, 2015; Li *et al.*, 2015). Among the small noncoding RNAs associated with various ovary-related biological processes, miRNAs are best characterized as playing a key role during ovarian growth and function (Ahn *et al.*, 2010; Lei *et al.*, 2010; Li *et al.*, 2015). In this study, for the first time, we analyzed the miRNA expression profile of human ovaries by NGS technology. Constructing expression profiles of small RNAs in human ovaries facilitates the understanding of their roles in the regulation of development and differentiation of ovaries.

Several known ovarian regulators or their receptors were reported to be targeted by miRNAs (Zhang *et al.*, 2014; Xu *et al.*, 2015). The let-7 miRNA family is expressed in the human, mouse, and cow ovaries and might be involved in regulation of oocyte maturation and oocyte–granulosa cell (GC) interaction in ovaries (Miles *et al.*, 2012; Tong *et al.*, 2014; Wagner *et al.*, 2014; Xu *et al.*, 2015), which were in accord with the has-let-7a-5p, has-let-7c-5p, and has-let-7e-5p and were the most abundant miRNAs expressed in human ovaries in this study. In previous reports, miR-199a and miR-140 were identified as the most downmodulated miRNAs in human ovarian cancer (Liu *et al.*, 2013; Lan *et al.*, 2015; Lian *et al.*, 2015). In our sequencing data, miR-199a, miR-199b, and miR-140 were expressed in high levels in human ovary, suggesting that the maintenance of expression level of miRNAs in ovaries could be associated with the cancer formation or development. Ovarian GCs, surrounding the oocytes, are responsible for the development, maturation, and release of mature egg for fertilization and also responsible for synthesizing and secreting hormones (Hawkins and Matzuk, 2010; Tong *et al.*, 2014; Zhang *et al.*, 2014; Xu *et al.*, 2015).

Recently, miRNAs have received widespread attention in ovarian GCs during folliculogenesis. For example, miR-320 was mainly expressed in GCs and oocytes of mouse ovarian follicles during folliculogenesis in a time-dependent manner (Feng *et al.*, 2015). MiR-320 inhibited proliferation of GCs by targeting *E2F1* and *SF-1* (Yin *et al.*, 2014). Moreover, miR-383 could promote the expression of miR-320 and

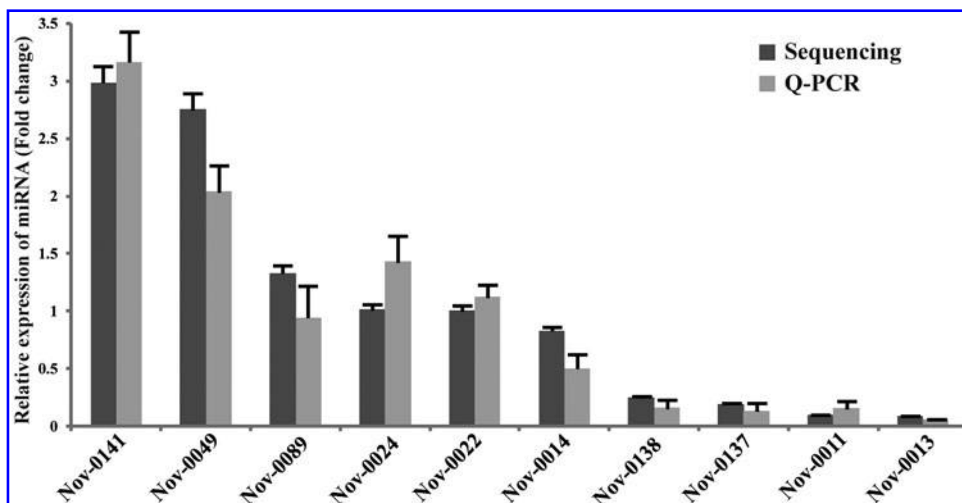

FIG. 2. Validation of the expression of 10 most abundantly expressed novel miRNAs in human ovaries by quantitative real-time PCR. In human ovaries, the 10 expression levels of novel miRNAs were validated by quantitative real-time PCR. The real-time PCR data with bars represent mean ± SD from three independent experiments. The sequencing data with bars represent the 95% CI.

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enhance miR-320-mediated suppression of GC proliferation (Yin *et al.*, 2014). In our study, the expression of miR-320a was also identified as one of the most abundant miRNAs in human ovaries. This suggested that the miRNAs, regulating the biological processes in GCs, take up a large slice of the miRNA expression profile of human ovary. Taken together, miRNAs and their function roles were generally involved in the physiological processes of ovaries.

In ovary life cycle, folliculogenesis is the most important process, including assembly of primordial follicles, follicle development, follicle rupture, and ovulation (Hsueh *et al.*, 2015; Li *et al.*, 2015). In this study, several folliculogenesis-related biological processes were identified for the ovary miRNA-targeted genes, such as antral ovarian follicle growth (GO:0001547), ovarian follicle rupture (GO:0001543), and fertilization (GO:0009566). Ovarian follicles are the basic units of female reproductive biology (Zhang *et al.*, 2014; Hsueh *et al.*, 2015). The formation, development, and maturation of follicles were also reported to be regulated by miRNAs (Lei *et al.*, 2010; Zhang *et al.*, 2014; Hsueh *et al.*, 2015; Li *et al.*, 2015). In mice, our previous report demonstrated that miR-376a regulates primordial follicle assembly by modulating the expression of *Pcna* (Zhang *et al.*, 2014). When the primordial follicle pool is established, the size of this initial pool, in part, determines the reproductive life span of the female. These primordial follicles eventually develop into primary, secondary, and tertiary vesicular follicles (Zhang *et al.*, 2014; Hsueh *et al.*, 2015). During these processes of follicle development, miRNAs were reported to be involved in GCs and oocytes (Carletti and Christenson, 2009; Lei *et al.*, 2010; Liu *et al.*, 2010). For example, miR-181a could regulate GC proliferation and ovarian follicle development in mice (Zhang *et al.*, 2013). Moreover, miR-224 is involved in the regulation of cumulus expansion and may affect ovulation and subsequent embryo development by targeting *Ptx3* (Yin *et al.*, 2014). Overall, folliculogenesis in human ovaries was regulated by miRNAs, and this study could provide a useful resource for further research of folliculogenesis in ovaries.

The ERK/MAPK pathway is involved in the regulation of various biological processes, including proliferation, differentiation, and cell cycle progression through several phosphorylation cascades (Mittal *et al.*, 2009; Yamashita *et al.*, 2013). In this study, the ERK/MAPK pathway was targeted by both the most abundantly known miRNAs and novel miRNAs in human ovaries, for example, GO:0045742, positive regulation of the epidermal growth factor receptor signaling pathway, and GO:0043409, negative regulation of MAPKKK cascade. Similarly, previous reports have demonstrated that the miRNAs participated in ovarian function through regulating the ERK/MAPK pathway. For example, overexpression of miR-15 reduced accumulation of proliferation- and apoptosis-related proteins, MAPK/ERK1, 2 and caspase 3, in GCs (Sirotkin *et al.*, 2014). Moreover, hCG-mediated miR-122 expression is mediated by the activation of ERK signaling pathways (Menon *et al.*, 2013).Our previous report also demonstrated that miR-485-5p could downregulate the expression of MAPK3 in PCOS cumulus GCs (Xu *et al.*, 2015). Taken together, ovarian miRNAs regulate ovary-related processes, for example, proliferation of GCs, through the ERK/MAPK pathway.

PCOS, one of the most common endocrine disorders, is a multifactorial and heterogeneous syndrome with a constellation of symptoms and signs in human ovaries, such as arrest of follicle growth, decreased GC proliferation, and ovulatory dysfunction (Fux Otta *et al.*, 2013). Although the underlying cellular mechanisms leading to PCOS remain unclear, PCOS is widely reported to be associated with increased risk of metabolic disorders, such as insulin resistance, diabetes, and obesity (Fux Otta *et al.*, 2013; Sorensen *et al.*, 2014). Our previous report has demonstrated that miRNAs and their targeted pathways play important roles in the etiology and pathophysiology of PCOS (Xu *et al.*, 2015). In this study, the insulin-related biological process (insulin receptor signaling pathway, GO:0008286) was also identified as the targeted pathway for the miRNAs in human ovaries. Compared with controls, miR-132 and miR-320 were identified that have decreased expression in the follicular fluid of women with PCOS (Onalan *et al.*, 2005). Moreover, miRNA-21, miRNA-27b, miRNA-103, and miRNA-155 were differentially expressed in PCOS serum (Murri *et al.*, 2013). These results suggested that miRNA in ovaries participates in regulation of not only physiological processes but also reproductive disorders, for example, PCOS.

In summary, for the first time, we have identified the known and novel expressed miRNA profiles of human ovarian tissues directly by high-throughput Solexa sequencing. Moreover, we validated the most abundantly expressed miRNAs and the predicted novel miRNAs in human cumulus GCs by quantitative real-time PCR. The GO term annotation and KEGG pathway analysis for the predicted miRNA targets further indicate that these miRNAs (most abundant) are involved in various biological processes, such as antral ovarian follicle growth, ovarian follicle rupture, and fertilization. Moreover, these miRNAs are also indentified as regulators in etiology and pathophysiology of PCOS. These results suggested that miRNAs play important roles in the development and physiological function of human ovary. Our work supports and further extends the knowledge of a regulatory role of miRNAs and their targeted processes in human ovaries and may also offer new insights for the treatment of reproductive and ovarian disorders.

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Disclosure Statement

No competing financial interests exist.

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