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Original Article

Novel Hub genes co-expression network mediates dysfunction in a model of polycystic ovary syndrome

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Abstract: Background: This study aimed to integrate DNA methylation, miRNA, and mRNA microarray data to construct a gene co-expression network for polycystic ovarian syndrome (PCOS). Methods: The weighted gene co-expression network analysis (WGCNA) was conducted to construct a PCOS-related co-expression network by using the GEO public datasets. We performed Gene Ontology and KEGG pathway enrichment analyses for a further exploration of gene function in networks. Finally, the dysfunction module consisting of a co-expression network was mapped to the PCOS patients and tried to provide guidance to the PCOS phenotyping. Results: Three modules (Midnightbule, Pink, and Red) were identified to be PCOS-related by WGCNA analysis. These module-related genes were enriched in cell response to stimulus, PI3K-Akt signaling pathway, insulin biological process, signaling pathway, and cytokine-cytokine receptor interaction biological processes. The multiple-factor network, including miRNA-lncRNA and DNA methylation-mRNA interaction, was closely associated with PCOS dysfunction. Conclusion: Our study render a novel insight into the mechanisms and might provide candidate biomarkers and therapeutic targets for the classification of PCOS dysfunction.

Keywords: Polycystic ovarian syndrome, miRNA-lncRNA, DNA methylation-mRNA network, dysfunction phenotype, reproductive disorder

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine and metabolic disease affecting about 5-20% women of reproductive age worldwide, according to different diagnostic criteria for PCOS [1, 2]. It is one of the most common causes of infertility among reproductive-age women, which is frequently associated with reproductive co-morbidities (irregular menstruation, ovulatory dysfunction, and pregnancy complications), metabolic disorders (metabolic disorders, type 2 diabetes, and cardiovascular disease), and even psychological risk factors [1, 3].

Anovulation, hyperandrogenism, and polycystic ovarian morphology are known as the three major clinical symptoms of PCOS [4, 5]. The clinical phenotypes of individuals may show relatively diverse changes based on geographic location, ethnicity, and sociocultural background [6-8]. Notably, the occurrence of PCOS often clusters in family aggregation, which indicates that genetic factors play an important role in PCOS [9]. Although enormous efforts on genome-wide association studies have been conducted for decades, only a few genetic variants were identified in different populations, which accounts for nearly 10% of the heritability of PCOS [10-13]. In addition, many molecules associated with PCOS were previously identified using data from different omics. Based on these molecules, some gene co-expression network were constructed [3, 14]. However, these studies only involved transcriptome data, and the molecular genetic mechanisms of PCOS still remain unclear.

Accumulating evidence showed that DNA methylation and non-coding RNAs play an unexpected, critical role in regulating ovarian functions [15-19]. Few studies were available linking a hub genes network to characterize dysfunction module of PCOS [3, 20]. Our study integrated DNA methylation, miRNA, and mRNA microar-

Table 1. PCOS expression profile data set from GEO database

Type	Dataset ID	Platform	PCOS	Normal
mRNA	GSE34526	GPL570	7	3
mRNA	GSE5090	GPL96	9	8
mRNA	GSE124226	GPL570	4	4
Methylation	GSE80468	GPL13534	30	30
miRNA	GSE72274	GPL16543	5	5
lncRNA	GSE106724	GPL21096	8	4

ray data to reveal differential expression of miRNAs and corresponding target genes; furthermore, we also aimed to investigate the features of lncRNAs, miRNAs, and DNA methylation-mRNAs involved in a hub gene network of dysfunction module in PCOS phenotypes.

Materials and methods

Data collection and co-expression network of PCOS construction

Gene expression and DNA methylation datasets for 6 PCOS studies were downloaded (GSE34526, GSE5090, GSE124226, GSE80468, GSE72274, GSE106724) from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/gds), including 54 control (control group), 63 PCOS patients (case group), as shown in **Table 1**. The background correction, quantile normalization, and differential gene analysis were performed by limma package [21]. The differentially expressed genes (DEGs) of mRNA, miRNA and lncRNA were acquired when genes that had $|\log_2(FC)| > 1$ and a *P*-value <0.05 was considered statistically significant. PCOS-associated genes were screened in NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene) and OMIM database (https://omim.org). Only the shared DEGs in at least two of the included datasets were retained. Then, to optimize the set of potential PCOS-associated gene network, we identified possible genes interacting with the PCOS-related gene using STRING database (https://string-db.org/). We downloaded the DNA methylation data from GSE80468 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80468) generated with the Illumina Infinium HumanMethylation450 BeadChip array. The DNA methylation level of each probe was measured as the beta-value ranging from 0 to 1. To identify methylation-regulated downstream genes, we assigned the probes within the promoter region to that gene. The weighted gene co-expression network analysis was performed on the 3798 genes, which was associated with the composite PCOS signature using WGCNA package [22, 23]. A soft threshold of $\beta=7$ ($R^2>0.9$) and a minimum module size of 50 was selected to yield 17 modules. The Pearson's correlation coefficient was calculated between the samples and within each module.

Function enrichment analysis

The Gene Ontology (GO) annotation, and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted for the module-related genes in the PCOS-associated network using clusterProfiler package [24]. A *P*-value <0.05 under the hypergeometric test was considered significant.

Construction of a hub genes network for PCOS

The PCOS-related multi-factor regulatory network was constructed using the genes in the midnight blue, pink, and red modules. Then, we defined the hub genes as the top 10% of the genes screened using Cytohubba in Cytoscape (https://cytoscape.org/). The miRNA-mRNA interaction data in PCOS-related hub genes were obtained from starBase v3.0 (http://starbase.sysu.edu.cn/), while the miRNA-mRNA interactions were merely identified in at least four of the softwares including targetScan, picTar, RNA22, PITA and miRanda/mirSVR.

Dysfunction modules guide the PCOS phenotyping

The 37 hub genes associated with PCOS, which are screened by the Cytohubba plug-in, were mapped to GSE34526 dataset for unsupervised clustering. We used K-means unsupervised clustering method to classify all PCOS samples. The optimal K value was selected by using SSE method.

RNA quantification

We isolated total RNA of granulosa cells from 6 cases (3 PCOS samples and 3 control samples) by using the TRIzol reagent (Life Technologies, CA, USA), according to the manufac-

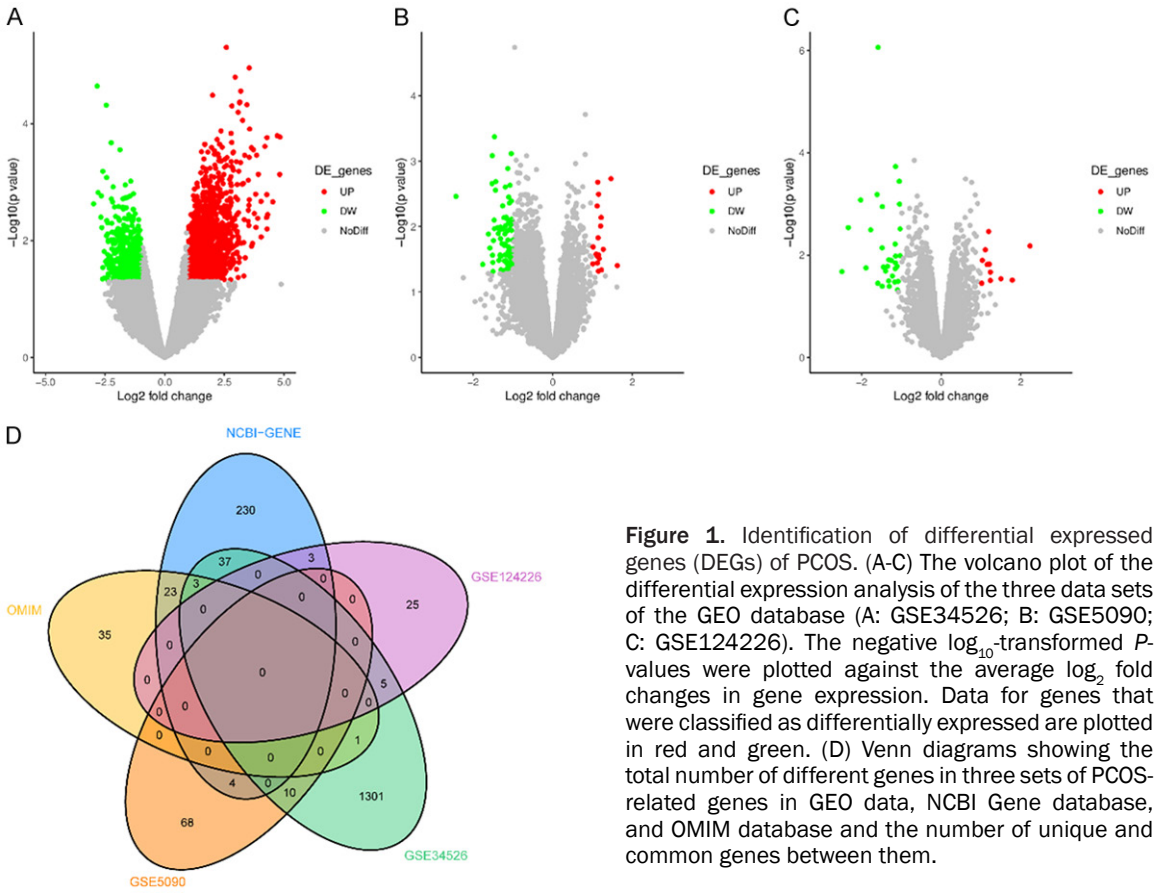

Figure 1. Identification of differential expressed genes (DEGs) of PCOS. (A-C) The volcano plot of the differential expression analysis of the three data sets of the GEO database (A: GSE34526; B: GSE5090; C: GSE124226). The negative $\log_{10}$-transformed *P*-values were plotted against the average $\log_2$ fold changes in gene expression. Data for genes that were classified as differentially expressed are plotted in red and green. (D) Venn diagrams showing the total number of different genes in three sets of PCOS-related genes in GEO data, NCBI Gene database, and OMIM database and the number of unique and common genes between them.

turer's protocol. Then, rRNA was removed using the MGIEasy rRNA Removal Kit. After library preparation, the library were sequenced on an DNBSEQ platform and 100 bp paired-end reads were generated. RNAseq raw read sequences were aligned against human genome assembly GRCh38.p13 by HISAT2 (v2.0.4). featureCounts (v1.5.0-p3) was used to count the reads numbers mapped to each gene. Differential expression analysis of two groups was performed using the DESeq2 R package (1.20.0). The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P*-value <0.05 found by DESeq2 were designated as differentially expressed.

Results

Identification of DEGs for PCOS co-expression network construction

A total of 86 PCOS-associated genes were identified as DEGs of PCOS (**Figure 1A-C**), which showed up at least twice in different databases (GSE34526, GSE5090, GSE124226) (**Figure 1D**). Then, the interacting genes with 86 PCOS-related genes were identified by STRING database (https://string-db.org/). A total of 4021 genes were defined as potentially related genes of PCOS, which share 3798 genes with expression profiles in GSE34626. These shared data were employed to construct co-expression network by WGCNA. Notably, these genes are allocated into 17 co-expression modules, as showed in **Figure 2A-C** and Table S1. Among them, the MEmidnightblue, MEpink, and MEred are three modules, which are considered as the most related to PCOS (**Figure 2B**).

The co-expression relationships among the three PCOS-related modules were analyzed and visualized in **Figure 3A**. In addition, we used Cytoscape to visualize 37 hub genes, which ranked the top 10% of the most connected co-expressed genes network in the significant modules (**Figure 3B**). Additionally, we fur-

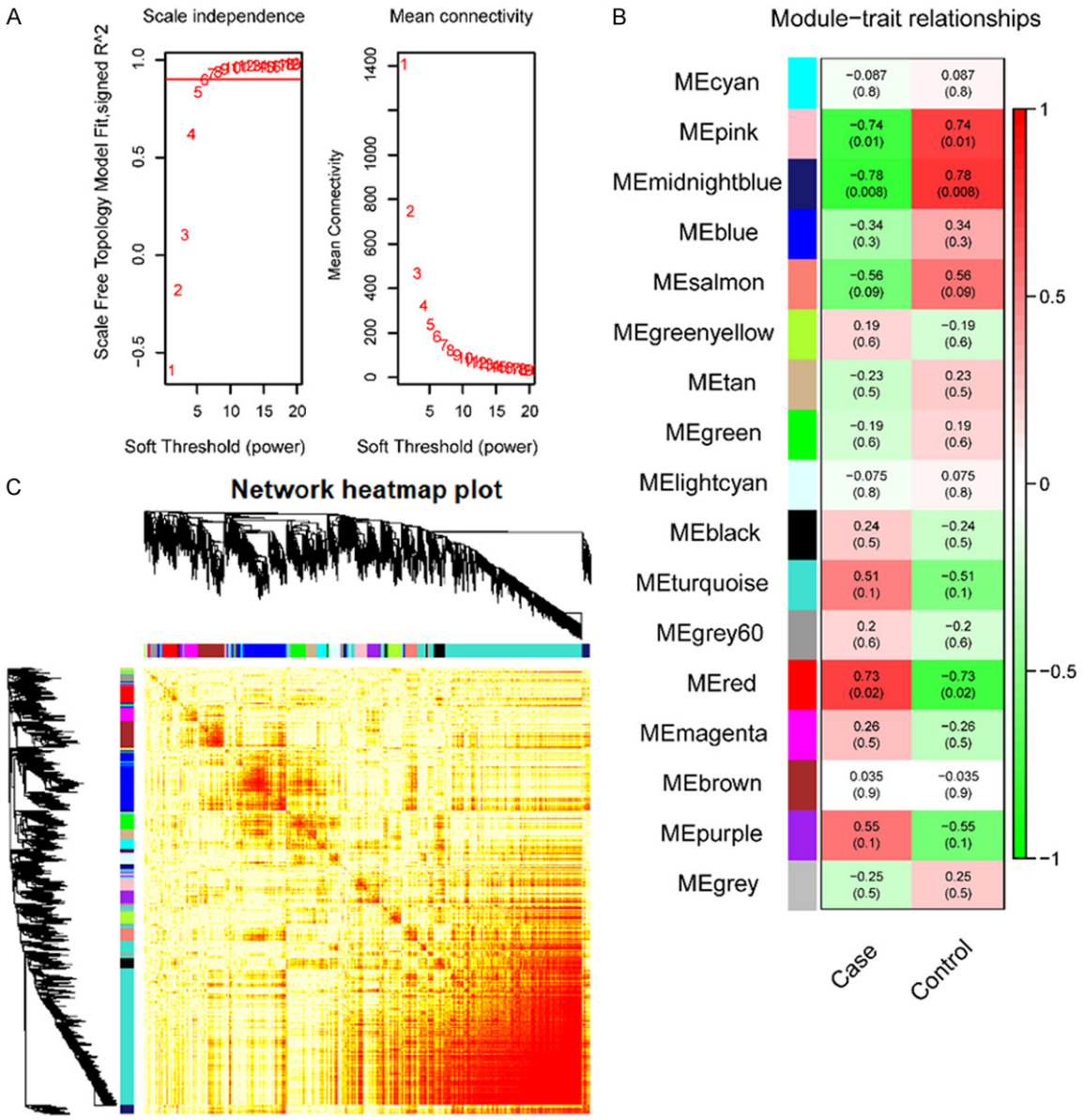

Figure 2. Weighted co-expression network of PCOS potential related gene sets. A. Left panel shows the analysis of the scale-free fit index for various soft-thresholding powers (β). The right panel shows the analysis of the mean connectivity for various soft-thresholding powers. B. Module-trait associations were evaluated by correlations between module eigengenes (MEs) and case-control groups. Each row corresponds to a ME, while each column corresponds to each group. C. Heatmap for topological overlap in the gene network. Each row and column correspond to a gene. The gene dendrogram and module assignment are shown along the left and top.

ther verified those co-expression genes using RNAseq data of granulosa cells obtained from 6 cases (3 PCOS samples and 3 control samples). As shown in **Figure 3C**, the PCOS-related module genes were verified in the RNAseq data. Notably, these DEGs between PCOS and control samples shared several key genes with three module-related genes, such as CHRDL2, FOXO6, CDH5, TAC3, MMP10 and INHBB. This suggests that these hub genes in three modules closely related to PCOS dysfunction.

Function enrichment analysis

To identify the module-related biologic processes, GO and KEGG enrichment analysis were performed. The results indicated that the biological process term 'plasma membrane pro-

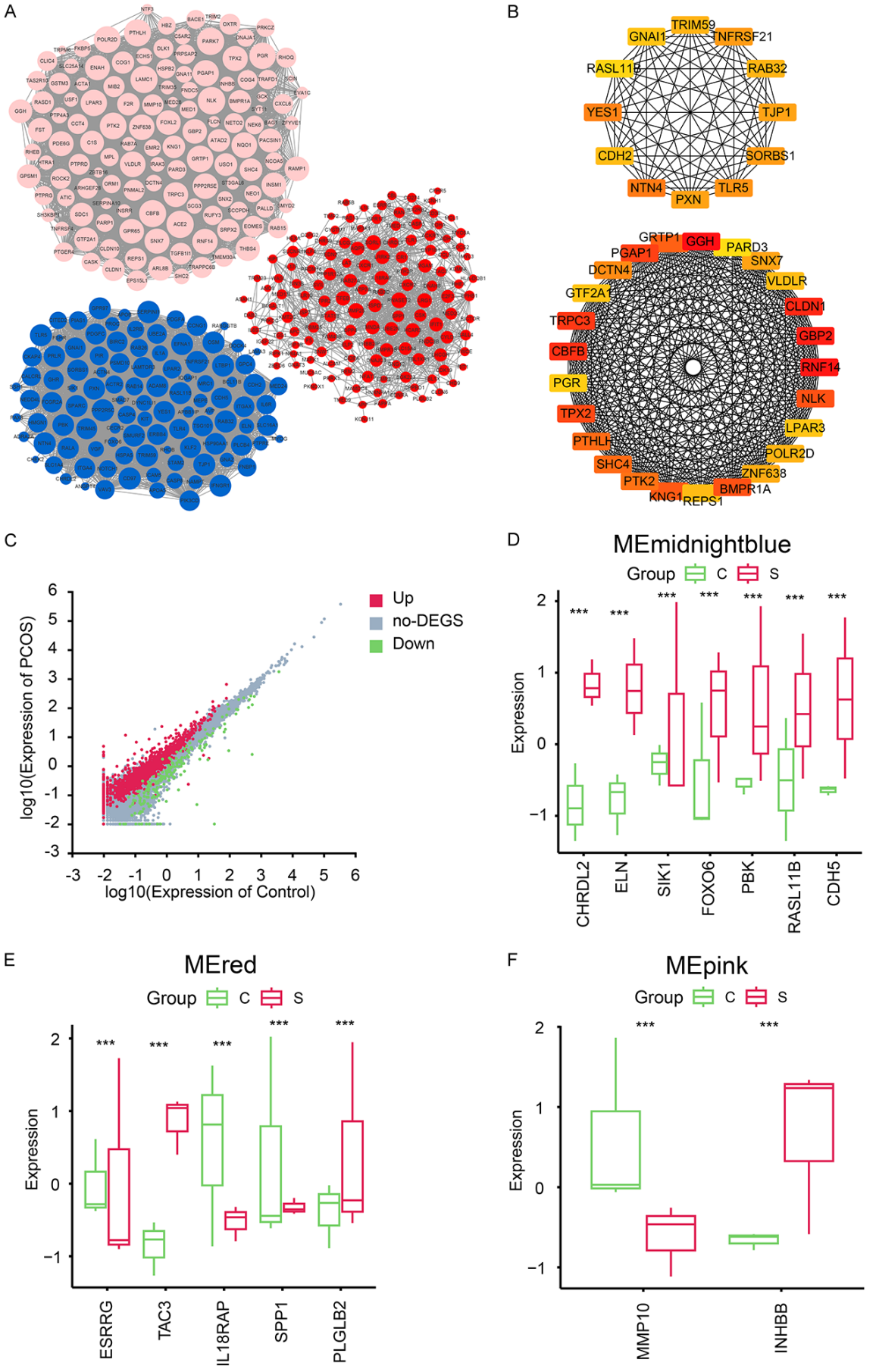
A
B
TRIM59
GNAI1
TNFRSF21
RASL11B
RAB32
YES1
TJP1
CDH2
SORBS1
NTN4
TLR5
PXN
GRTP1
GGH
PARD3
PGAP1
SNX7
DCTN4
VLDLR
GTF2A1
CLDN1
TRPC3
GBP2
CBFB
RNF14
PGR
NLK
TPX2
LPAR3
PTHLH
POLR2D
SHC4
ZNF638
PTK2
BMPR1A
KNG1
REPS1
C
Up
no-DEGS
Down
log10(Expression of PCOS)
log10(Expression of Control)
D
MEmidnightblue
Group C S
Expression
CHRDL2
ELN
SIK1
FOXO6
PBK
RASL11B
CDH5
E
MEred
Group C S
Expression
ESRRG
TAC3
IL18RAP
SPP1
PLGLB2
F
MEpink
Group C S
Expression
MMP10
INHBB

Figure 3. Co-expression network of PCOS related modules and PCOS core driver genes screened by Cytohubba. A. Sub-networks of module midnightblue, pink, and red were visulizated by cytoscape. B. Network analysis identified hub mRNAs using cytoHubba. C. Scatterplot showing gene expression levels between control and PCOS samples. Differentially expressed genes (DEGs) are marked in red or green. D-F. Boxplot showing expression levels of module-related genes between control and PCOS samples (***P*-value <0.001, **<0.01).

teins', the molecular function term 'response to stimulus', and the cellular component term 'endomembrane system', and the PI3K-Akt signaling pathway were mainly enriched in the MEmidnightbule module (**Figure 4A**); suggesting that this module was closely related to the cell stimulus and PI3K signaling pathway in the pathogenesis of PCOS. This suggests that some genes in our MEmidnightbule module may be closely related to polycystic ovary syndrome. The MEpink module was identified to be related to the insulin signaling pathway and insulin biological function, which is consistent with the accompanying of insulin resistance in mostly PCOS patients (**Figure 4B**). Moreover, the MEred module mainly participated in cytokine-cytokine receptor interactions (**Figure 4C**). Gonzalez et al. and Abdelhadi et al. found that the expression of TLR2, TLR8, and CD14 were up-regulated [25, 26], which indicates these interactions may play an notable role in the pathogenesis of PCOS.

Identification of DEMs and DMLs for PCOS

To identify the module-related non-coding genes, we performed a differential analysis in data set GSE72274 and GSE106724. We obtained 38 differentially expressed miRNAs, including 32 up-regulated miRNAs and six down-regulated miRNAs (**Figure 5A**) by using the limma package in R software to perform differential analysis on the expression data of miRNAs. Moreover, 87 differentially expressed lncRNAs were identified, including nine up-regulated lncRNAs and 78 down-regulated lncRNAs (**Figure 5B**). Based on the predicted target results from starBase v3.0, a multi-omics network was constructed, including miRNA-lncRNA, DNA methylation-mRNA interaction associated with PCOS dysfunction (**Figure 5C**).

Dysfunction modules guide the PCOS phenotyping

As being shown in **Figure 6A**, the optimal K value was three by K-means unsupervised clustering method to classify all PCOS samples in GSE34526. The PCOS patients could be divided into three different clusters when used R tSNE to reduce the dimensionality of gene expression profile data (**Figure 6B**). The expression of individual co-expression hub genes in all PCOS samples is also displayed in **Figure 6C**. Although no significant difference can be seen from the expression of a certain gene in classification, the combination of all co-expressed hub genes also shows a result that is highly consistent with the clustering in **Figure 6B**. Thus, we could speculate that the 37 co-expressed hub genes were necessary for the classification of PCOS phenotype. In order to identify differentially expressed genes in differential PCOS subtypes, we performed ANOVA analysis in three subtypes. **Figure 7** shows the expression of 19 significantly differentially expressed genes in 3 clusters of all PCOS samples ($P<0.05$), which indicates that these 19 genes might play a considerable role as marker genes for guiding PCOS subtypes.

Discussion

In the weighted correlation network analysis, we attempted to identify co-expression regulatory networks including DNA methylation, non-coding RNAs (both miRNAs and lncRNAs), and mRNA interactions in PCOS dysfunction modules. The co-expression network identifies the potential molecular mechanisms and biological processes for the pathogenesis as well as subtyping of PCOS.

Previous studies have tried to interpret the molecular mechanisms of PCOS by high-throughput sequencing or microarray data [15, 27-29]. However, there is a relatively high possibility of false-positive results due to all of them being mainly focused on a single dataset. Our study analyzed three mRNA expression profile datasets (GSE34526, GSE5090, GSE124226), one DNA methylation profile data (GSE80468), one miRNA (GSE72274), and one lncRNA profile data (GSE106724) using the limma package and selected only the DEGs that overlapped at least twice in the five datasets to prevent this shortcoming.

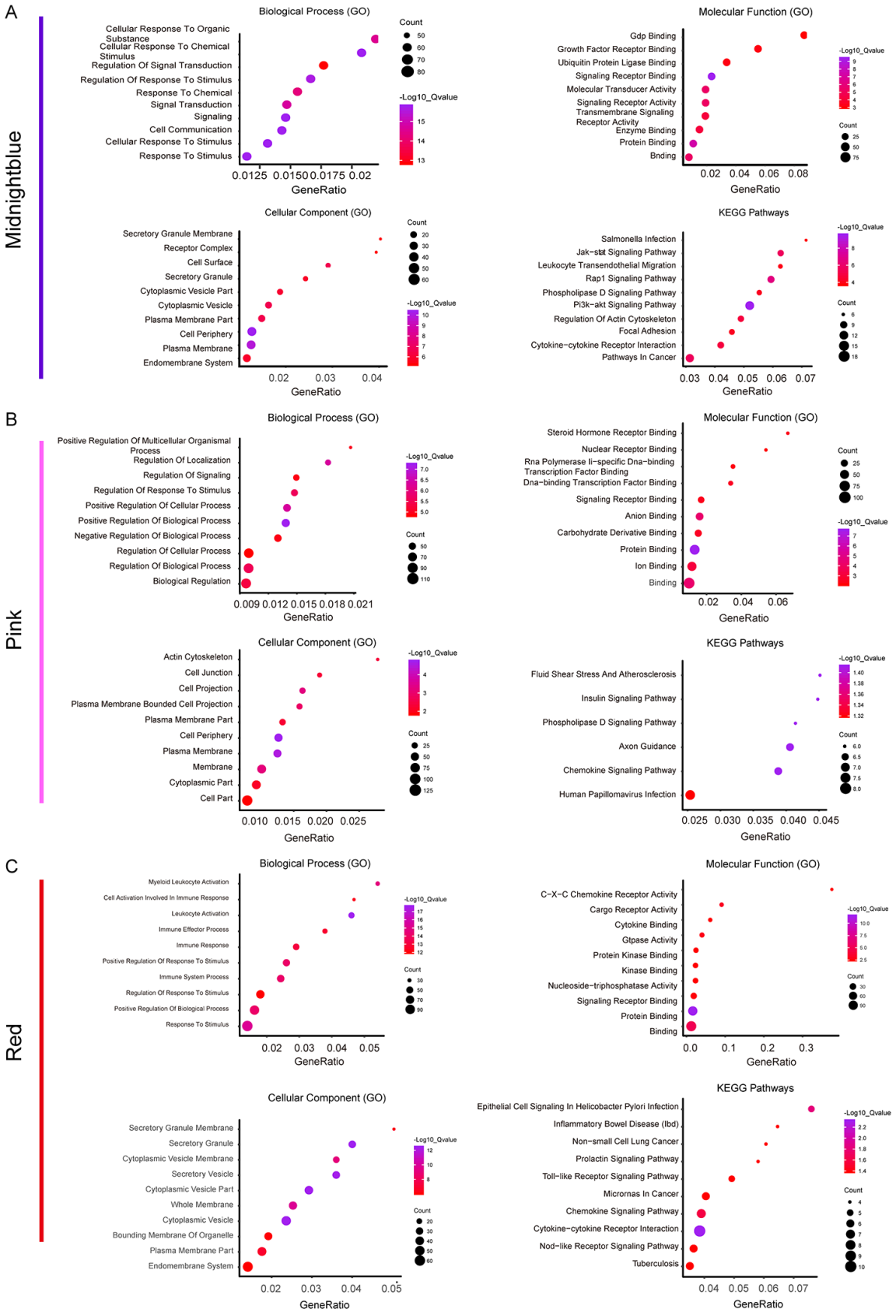

Figure 4. GO analysis and KEGG pathway enrichment analysis on genes in MEmidnightblue, MEpink and MEred modules. A-C. Dot plots show the results of enrichment analysis for genes in midnightblue module, pink module, and red module.

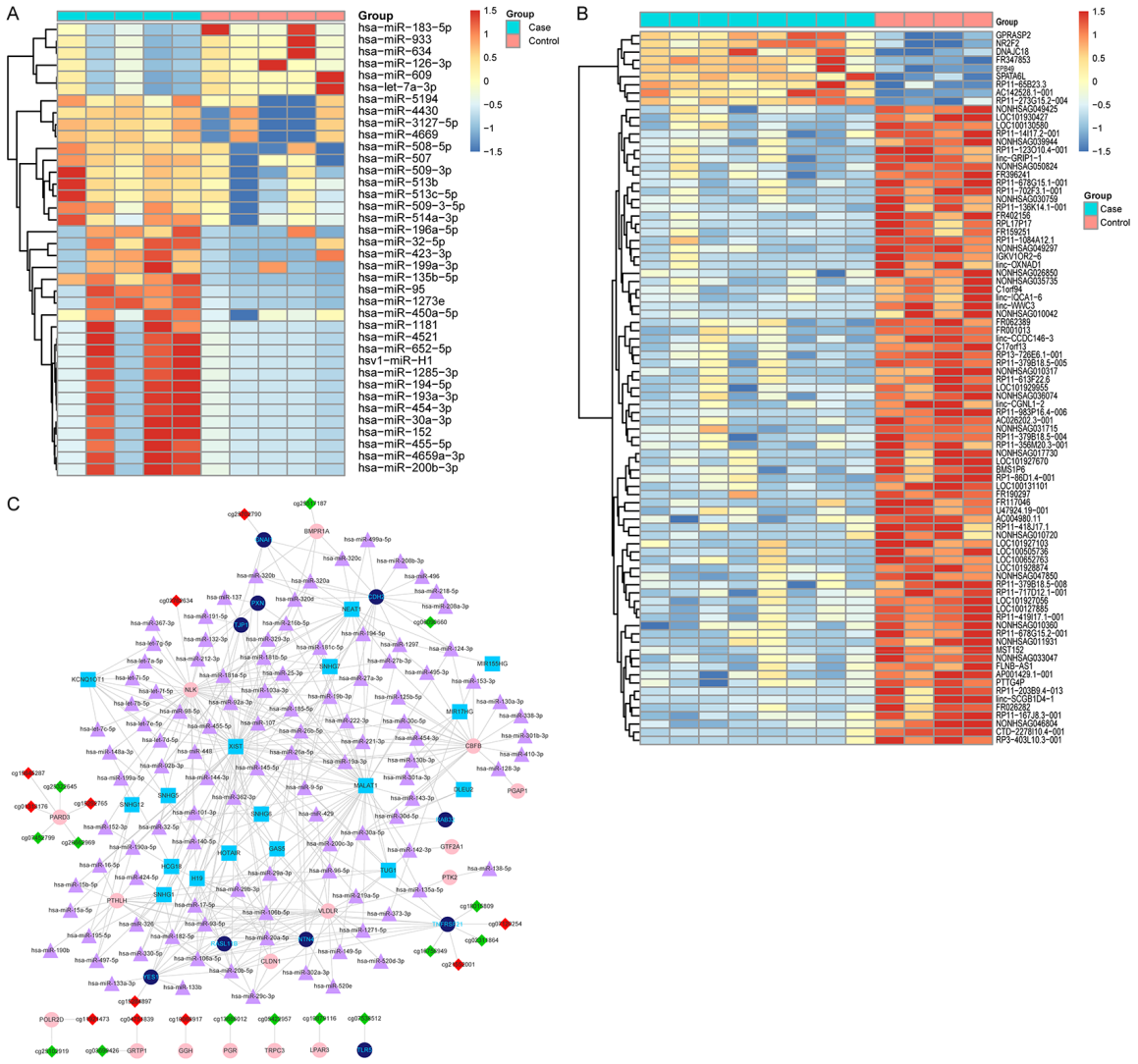

Figure 5. Heatmap of differentially expressed miRNAs (DEMs), lncRNA (DELs) levels and hub genes network mediated dysfunction module of polycystic ovary syndrome. A. A heatmap shows 38 differentially expressed miRNAs, including 32 up-regulated miRNAs and 6 down-regulated miRNAs. B. 87 differentially expressed lncRNAs were identified, including 9 up-regulated lncRNAs and 78 down-regulated lncRNAs. C. The network shows the methylation sites, lncRNAs, miRNAs, and mRNAs. Small rectangles indicate methylation sites and blue rectangles indicate lncRNAs. The circles represent hub mRNAs and triangles represent miRNAs.

The crucial DEGs were screened by WGCNA analysis which used GSE34526, GSE5090, GSE124226, GSE80468, and GSE72274 together with OMIM, NCBI-gene database as the training dataset, and GSE345268 as the validation datasets. Similarly, some key mRNAs were identified by previous studies (TLR5 [20], SHC4 [3]). Although no significant difference could be seen simply from the expression of a certain gene in classification, the 37 co-expressed hub genes are important for the classification of PCOS phenotype. These genes showed different expression levels in three clusters: LPAR3, GGH, BMPR1A, GTF2A1, RNF14, SNX7, GRTP1, SHC4, PGAP1, and PTHLH shared similar trends, while TJP1, GNAI1, YES1, CDH2, and NTN4 had a certain trend in common, and other genes, like PXN and GBP2, CBFB and CLDN1, shared similar characteristics with each other.

Interestingly, recent studies have reported that LPAR3 has phosphorylation activity of PPARγ, which might be involved in insulin signaling. Mohamad et al. have found that BMPR1A overexpression in PCOS women might be related to

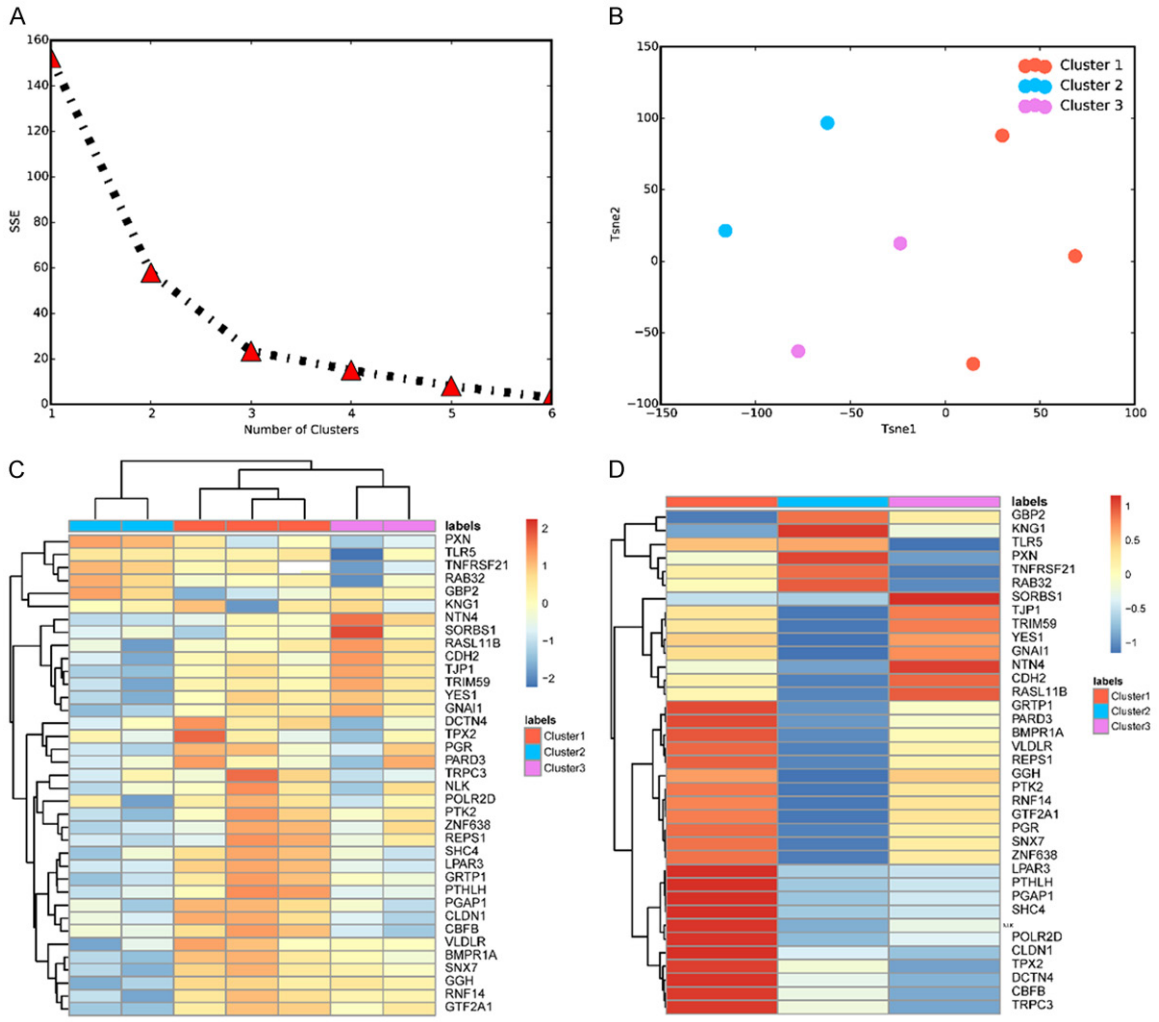

Figure 6. K-means clustering based on gene expression profile. A. Determining the optimal value of K in K-means clustering based on SSE to realize the selection of K value. B. Tsne diagram shows the clustering of PCOS samples. C. The expression of 37 co-expressed key genes in PCOS samples (log_2(exp+1) scale). D. The expression of co-expressed key genes under different Clusters. The color represents the expression value (log_2(exp+1)).

the pathogenesis of anovulation [30]. Besides, the increased expression of BMPR1A in granulosa cells may amplify the effect of AMH, which indicated that it may be involved in the process of recruiting dominant follicle development. The specific co-expression gene set might play a key role in polycystic ovarian syndrome (PCOS), which might provide candidate biomarkers and potential therapeutic targets for the classification of PCOS dysfunction. RNF14, also known as ARA54, was reported to be a coactivator of androgen receptors, indicating its vital role in hyperandrogenism in PCOS [31]. GBP2, one of the signaling proteins, and CLDN1 were related to endometrial receptivity [32]. Subsequently, these hub genes might have great potential to be a novel set of biomarkers in phenotyping PCOS, thus providing further references in the clinical management of these patients and optimizing their individualized therapy.

There are certain limitation in this study. First, the number of analyzed samples was limited, and the data were retrieved from different tissues or cells as well as different phenotypes, which could increase the heterogeneity of the analysis. Secondly, the validation of clinical samples is absent and requires a further experiment to confirm these results. Also, it is difficult to explore the correlation between the screened hub genes and specific clinical phe-

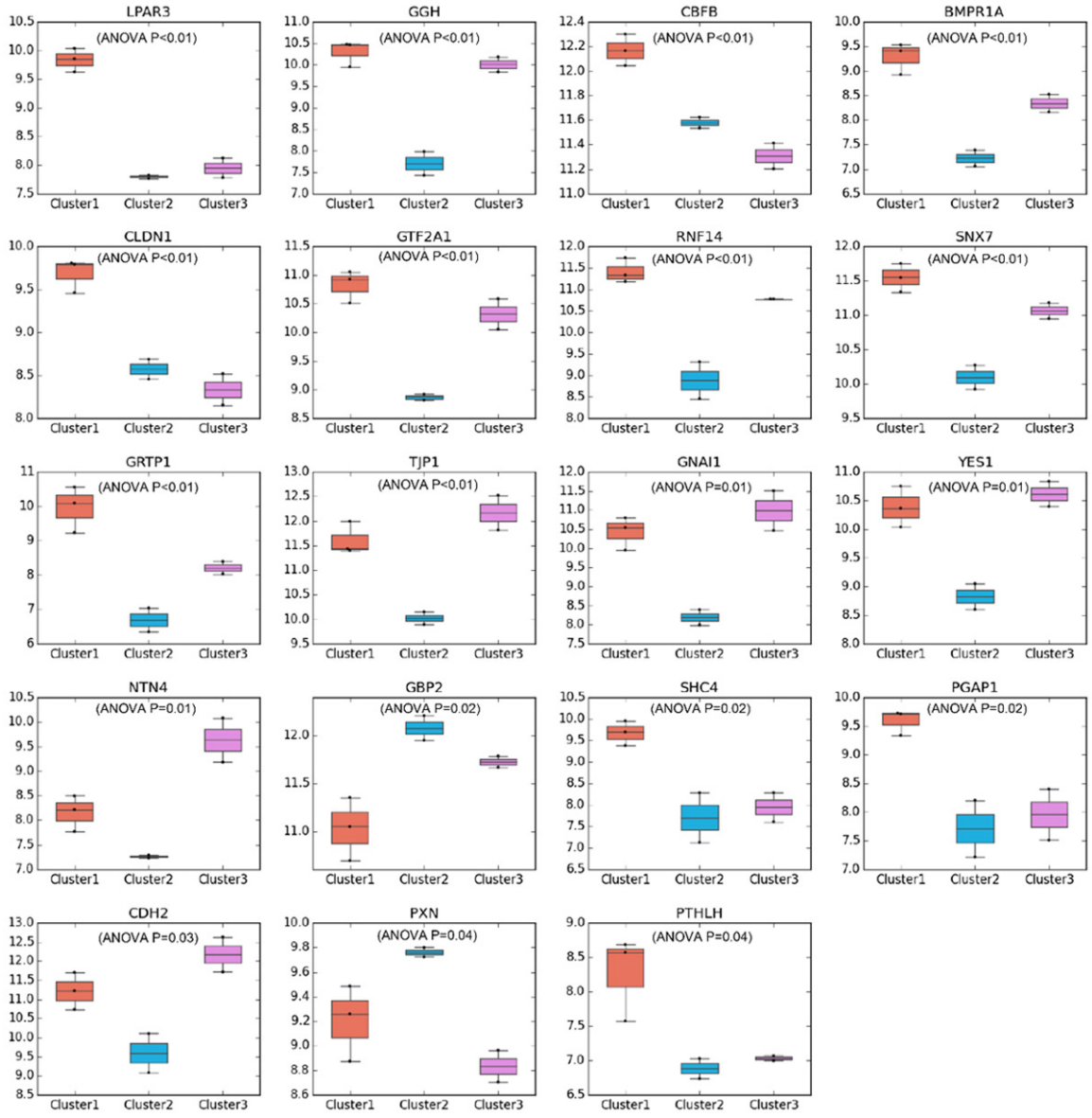

Figure 7. The expression of DEGs in different clusters. Box plot showing the mRNA expression of 19 genes in three clusters. Box plots: bar, median; box, 25th to 75th percentile (IQR); vertical/horizontal line across box, data within 1.5 times the IQR. The analysis of variance (ANOVA) was applied for statistical testing.

notypes of PCOS with the absence of this information which is also the reason why it is hard to associate the gene clusters obtained in **Figure 7** with PCOS phenotypes as it does not clarify the phenotypes and cell types from which the data were obtained.

In conclusion, the multiple-factor network, including miRNA-lncRNA and DNA methylation-mRNA interaction, was closely associated with PCOS dysfunction. WGCNA screened out key modules and Hub genes that might relate to clinical features and have biological significance. The obtained 37 co-expressed key genes provide adequate evidence to suggest clinical significance for PCOS typing.

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Disclosure of conflict of interest

None.

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pathogenesis of polycystic ovary syndrome. Mol Cell Endocrinol 2020; 506: 110760.
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Table S1. Statistics of the number of genes corresponding to each module

Module	No. of Genes
black	136
blue	508
brown	244
cyan	117
green	138
greenyellow	129
grey60	76
lightcyan	107
magenta	135
midnightblue	108
pink	135
purple	134
red	138
salmon	125
tan	126
turquoise	1439
grey	3