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Analysis of single-cell RNA sequencing in human oocytes with diminished ovarian reserve uncovers mitochondrial dysregulation and translation deficiency

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Abstract

Background Diminished ovarian reserve (DOR) is clinically characterized by a decrease in the number of available ovarian follicles and a decline in the quality of oocytes, accompanied by hormonal changes. Low quality of DOR oocyte leads to impaired embryo development, an increased risk of aneuploid pregnancies and miscarriages. However, the specific pathogenic mechanism remains unclear, posing a significant challenge for assisted reproductive technology.

Methods For the first time, our study employed single-cell RNA sequencing to reveal the altered transcriptomic landscape of DOR oocytes at GV stage after ovarian stimulation. Differentially expressed genes analysis (DEGs), functional enrichment analysis, weighted gene co-expression network analysis (WGCNA) and protein-protein interactions network analysis were performed.

Results We found 132 up-regulated genes and 466 down-regulated genes in DOR oocytes, with the down-regulated genes primarily enriched in mitochondrial function and translation. Hub genes, identified through integrated analysis of WGCNA and DEGs, were further validated in DOR and control oocytes using RT-qPCR. By utilizing hub genes and employing transcription factor enrichment tools, it had been predicted that pleomorphic adenoma gene 1 (*PLAG1*) played a crucial role as a transcriptional regulatory factor in DOR oocytes. Additionally, we conformed the *PLAG1-IGF2* axis was dysregulated in DOR oocytes.

Conclusions Transcriptome analysis revealed that DOR oocytes exhibited mitochondrial dysfunction and translational defects, and the *PLAG1-IGF2* axis might be a potential contributor for the low quality of DOR oocytes.

Keywords Oocyte, DOR, *PLAG1*, *IGF2*, Translation, Mitochondrial function

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Background

The worldwide prevalence of infertility among reproductive-aged couples ranges between 12.6% and 17.5% [1]. There are various factors that can contribute to infertility, and one such factor is DOR. According to the US Center for Disease Control and Prevention's national assisted reproductive technology data from 2014, approximately 30% of patients were diagnosed with DOR [2]. There is currently no universally agreed upon definition for DOR. The characteristics of DOR are the reduction in the number and quality of oocytes, a decrease in anti-mullerian hormone (AMH), and an increase in follicle-stimulating hormone (FSH) levels [3, 4]. The current etiological understanding of DOR primarily encompasses age, idiopathic, genetic mutations, autoimmunity, lifestyle, iatrogenic and metabolic disorder [5]. The majority of women with DOR who are experiencing infertility typically require assisted reproductive technology (ART) and generally exhibit reduced oocyte yield, lower rates of live birth, and higher rates of discontinuation of treatment compared to those with normal ovarian reserve (NOR) [6]. Unfortunately, there is no specific or gold-standard controlled ovarian stimulation (COS) strategy that can enhance ART outcomes in patients with DOR [7]. After adjusting for age, blastocysts from women with DOR are more likely to be aneuploid compared to those from women with NOR, as a proxy for oocyte quality [8]. Furthermore, studies have shown that these DOR patients may develop into premature ovarian failure (POF) within 1 to 6 years [9]. Oocyte donation may serve as the final resort for many patients with DOR because of insufficient follicle growth or oocyte quality [7].

Among all pathogenic factors contributing to DOR, genetic factors appear to be pivotal and have not been fully clarified. So far, it has been reported that genetic factors associated with DOR include gene mutations such as *FMR1*, *GPR84*, *EIF4ENIF1* and *MSH4* [10–13], polymorphisms including *GDF9*, *ESR1*, and *FSHR* [14–16], as well as differentially expressed genes including *AMH*, *LHCGR*, *IGF2*, *GPX4*, *FTH1*, *FTL*, *PRDX2*, *PRDX4*, *PHGDH*, *PSAT1*, and *AGER* [17–20]. Moreover, expression profiles of lncRNA and miRNA have been identified in granulosa cells and cumulus cells of patients with DOR [21, 22]. A recent study has shown that the expression of oxidative stress genes and inflammatory factors in follicular fluid of DOR cases is increased compared to those in NOR cases [23]. In addition to that, the mural granulosa cells of women with DOR exhibited a higher number of epimutations and greater variability in DNA methylation [24]. Due to the limitation of samples, the research mainly focuses on granulosa cells and follicular fluid, without directly exploring the cause of low quality through DOR oocytes.

The quality of oocytes relies on maternally produced components, such as mRNAs, proteins, and organelles essential for early embryogenesis. The transcription of oocytes ceases from the resumption of meiosis to zygotic genome activation, and the maturation of oocytes and the development of early embryos depend on the translation of maternal mRNAs [25]. The storage of a large number of activated ribosomes in oocytes is considered a feature of high-quality oocytes [26]. Previous study suggests that the ability of older human oocytes to carry out mRNA translation declines [27]. The decline in the mRNA translation of oocytes with low quality can be attributed to the underlying fact that ribosome levels and function decrease with advancing age [26]. Additionally, mitochondria play a crucial role as energy producers in oocytes, supporting the high-energy processes of oocyte maturation, fertilization, and embryonic development [28, 29]. The function of mitochondria in oocytes determines the quality of oocytes, and emerging evidence indicates that mitochondrial dysfunction is a crucial factor leading to the decline of oocyte quality and oocyte aging [29, 30]. Despite their important roles, it is currently unclear whether there are abnormalities in mitochondrial and ribosomal functions in DOR oocytes. In the present study, we aimed to assess the quality of oocytes by examining the expression of mitochondria- and translation-related genes in GV-stage oocytes from DOR and NOR groups through single-cell transcriptome profiling.

Materials and methods

Participants and sample collection

Patients with DOR ($n=14$) and a control group with normal ovarian reserve (NOR) ($n=9$) were recruited from the Reproductive Medicine Center of Jiangxi Maternal and Child Health Hospital as well as the Reproductive Medicine Center of Handan Central Hospital. These patients underwent intracytoplasmic sperm injection (ICSI) cycle using progestin-primed ovarian stimulation (PPOS) protocol for controlled ovarian hyperstimulation. The diagnostic criteria for DOR adopted a revised version of the Bologna standard, the patients were enrolled in DOR group based on the following diagnostic criteria: $AFC \leq 7$, $0.5 < AMH \leq 1.1 \text{ ng/mL}$, $FSH \geq 10 \text{ IU/L}$. The NOR patients were infertile due to male factor and had the $8 \leq AFC \leq 24$, serum $AMH \geq 1.5 \text{ ng/mL}$, $FSH < 10 \text{ IU/L}$. The participants had no history of diseases such as polycystic ovarian syndrome, endometriosis, chromosomal abnormalities, ovarian surgery, radiotherapy, diabetes, dysregulated thyroid disease or autoimmune diseases.

Retrieved oocytes were mechanically denuded of surrounding cumulus cells using flexible polycarbonate pipettes (G26712, Cook medical, USA) in combination with hyaluronidase (V900833, Thermo, USA) in G-MOPS media (10173, Vitrolife, Sweden). The identification of

GV oocytes was based on the presence of a well-defined germinal vesicle containing the characteristic prominent nucleolus. The GV oocytes were collected in DPBS (14190144, Gibco, USA) and washed three times. Subsequently, GV oocytes were aspirated and transferred to a 1.5-mL centrifuge tube (MCT-150-C, Axygen, USA) without RNase. The samples were quickly frozen in liquid nitrogen and stored at -80 °C for further analysis.

Library preparation and RNA sequencing

We extracted RNA from GV oocytes using the TRIzol reagent (15596018, Ambion, USA) and performed transcriptome amplification for cDNA amplification using the SMART-seq2 protocol [31]. The first strand cDNA was synthesized through reverse transcriptase and an oligo(dT) primer, with a special adapter sequence added to its 3' end for modification. Full-length cDNA enrichment was performed by PCR with 15 cycles of amplification. After purification with Agencourt Ampure XP beads (A63880, BECKMAN COULTER, USA), the size distribution and quantity of the product were assessed on Qsep400 (BIOPTIC, Italy) and Qubit4 (Q33238, Invitrogen, USA). For library preparation, we utilized 1 ng of amplified cDNA per sample employing the Nextera XT DNA library preparation Kit (FC-131-1096, Illumina, USA). The final libraries were subjected to paired-end (PE150) sequencing on an Illumina NovaSeq™ 6000 using library indexes corresponding to cell barcode.

Differentially expressed genes

The R package 'DESeq2' was used to conduct differentially expressed genes (DEGs) analysis within RStudio (Posit Software, USA). The DEGs were identified with a minimum 2-fold change and an adjusted P value of less than 0.05 between the DOR group and control group.

Gene ontology and pathway enrichment analysis

The biological process of Gene ontology (GO) enrichment analysis was annotated and generated using the annotation tools provided by database for Annotation, Visualization and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/tools.jsp>).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database-based pathway annotation was utilized for pathway enrichment analysis. The enriched KEGG pathways of the DEGs were also identified by DAVID database. All bubble plots were visualized using an online bioinformatics analysis platform (<http://www.bioinformatics.com.cn>).

Gene set enrichment analysis (GSEA)

Differentially expressed genes were used for GSEA analysis. The GO gene sets database within the Molecular Signatures Database (MSigDB) was utilized to identify

significantly enriched biological processes between the DOR and control groups. All plots were visualized using the ggplot2 package. The GSEA analysis incorporates four essential statistical measures: Enrichment Score (ES), Normalized Enrichment Score (NES), False Discovery Rate (FDR), and P-value.

Weighted correlation network analysis (WGCNA)

The WGCNA analysis was performed using the R package WGCNA (version 1.426), with FPKM values of all genes obtained from DOR and control samples. The genes exhibiting correlation were hierarchically clustered into a dendrogram. The detection of modules was performed using Dynamic Tree Cutting, and relevant modules were subsequently merged based on their highly correlated eigengenes. The eigengene values of the modules were correlated with the clinical traits to generate a module–trait heatmap. Hub genes were identified based on gene significance (GS)>0.7 and module membership (MM)>0.8.

Protein-protein interaction (PPI)

The construction of the PPI network was facilitated by utilizing the STRING database (version 12.0, <https://string-db.org>). The interaction score was set to high confidence (0.70). Subsequently, the downloaded TSV file of interactions was inputted into the Cytoscape software (Version 3.7.1). Hub genes were identified using the Molecular Complex Detection (MCODE) algorithm and the CytoHubba plugin.

Validation of real-time quantitative PCR

To validate the reliability of RNA-seq data, we selected hub genes associated with ribosome and mitochondrial functions, which were subsequently confirmed by RT-qPCR. The primers (see Supplemental Table I) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Total RNA was reverse-transcribed into cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (R211-01, Vazyme, China), following the manufacturer's instructions. Subsequently, RT-qPCR was performed using an ABI Prism 7500 system (Thermo Scientific™ Applied Biosystems, USA) with AceQ qPCR SYBR Green Master Mix (Q141-02, Vazyme, China) according to the manufacturer's instructions. The relative fold change ($2^{-\Delta\Delta Ct}$) was utilized to quantify the expression of genes in control and DOR oocytes.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (Version 9.5.1). The data were expressed as mean \pm SD unless otherwise specified. The unpaired Student's t-test was employed to determine statistically

significant differences. P values of <0.05 were considered statistically significant.

Results

Baseline characteristics of participants used for single-cell RNA sequencing

The baseline characteristics and laboratory results of the participants were detailed in Table 1. The DOR and control groups did not exhibit statistically significant differences in terms of Age, duration of infertility, and BMI. The serum AMH and AFC were significantly lower in the patients with DOR compared to the control group with normal ovarian reserve, while basal FSH levels were significantly increased in the DOR group compared with the control group. Participants with DOR had significantly fewer stimulation duration and total gonadotrophin dose. Additionally, the data showed that DOR was associated with the decreases in the number of oocytes retrieved, MII oocytes, 2PN embryos and available embryos.

Identification of genes and pathways significantly associated with DOR

To identify the genes and pathways altered in GV oocytes in DOR patients, total RNA was extracted from donated oocytes, followed by a comprehensive genome-wide transcriptomic analysis using Smart-seq2. The principal components analysis (PCA) revealed two distinct clusters between the DOR and control groups, indicating different gene expression profiles in the two groups (Fig. 1A). The genes with a $|\log_2(\text{fold change})| > 1$ and an adjusted P value <0.05 were considered as significant DEGs. A total of 598 DEGs were identified in the GV oocytes of DOR and control groups. Out of these DEGs, 132 genes were significantly upregulated, while 466 genes were significantly down-regulated (Fig. 1B). The top 20 most significant genes were highlighted on the volcano plot (Fig. 1B).

Table 1 Comparison of the baseline characteristics between the DOR group and control group used for single-cell sequencing analysis

	Control (n=9)	DOR (n=14)	P value
Age (years)	31±0.76	29.71±0.78	0.28
Duration of infertility (years)	4.556±0.69	4.071±0.55	0.5882
BMI (kg/m ²)	24.4±1.36	23.2±0.87	0.4422
AMH (ng/ml)	2.771±0.20	0.8007±0.03	<0.001
AFC	12.56±0.93	4.714±0.27	<0.001
Basal FSH (IU/L)	6.088±0.47	13.44±0.49	<0.001
Stimulation duration (days)	10.33±0.50	5.714±0.30	<0.001
Total gonadotrophin dose (IU)	2064±73.76	821.4±33.88	<0.001
Number of oocytes retrieved	12.22±1.0	4.929±0.35	<0.001
MII oocytes	9.556±0.87	3.071±0.22	<0.001
2PN fertilization	8.333±1.0	2.786±0.19	<0.001
Available embryos	6.444±0.8	1.875±0.23	<0.001

DOR: diminished ovarian reserve, BMI: body mass index, AMH: anti-Müllerian hormone, AFC: antral follicle count, FSH: Follicle-stimulating Hormone

The changes in expression of DEGs were clearly shown in the heatmap diagram (Fig. 1C).

To further uncover the potential functions of DEGs, gene ontology (GO) analysis and kyoto encyclopedia of genes and genomes (KEGG) analysis were conducted. The GO enrichment analysis indicated that the genes down-regulated in DOR oocytes were mainly enriched in biological processes such as cytoplasmic translation, translation, mitochondrial ATP synthesis coupled with proton transport, and aerobic respiration (Fig. 1D). Based on the top 10 most significantly enriched biological processes of down-regulated genes (Fig. 1D), it is suggested that there may be defects related to translation and mitochondrial function in DOR oocytes. While the upregulated genes were primarily involved in negative regulation of defense response to virus, cellular response to DNA damage stimulus, and protein ubiquitination (Fig. 1E). Moreover, the top 10 representative KEGG pathways were illustrated in Fig. 1F. The results demonstrated a significant enrichment of down-regulated genes in DOR oocytes within various pathways, including ribosome and oxidative phosphorylation. Therefore, the results of GO and KEGG analysis revealed that aberrations in ribosomal and mitochondrial functions were implicated in the pathogenesis of diminished ovarian reserve.

Analysis of pathways associated with translation and mitochondrial function

The pathway enrichment analysis can offer additional insights into the functions of genes, we further analyzed the pathways of translation and mitochondrial function that are mainly associated with downregulated genes. Translation is considered as house-keeping functions across different cell-types and developmental stages. The process of mRNA translation relies on ribosomes, which are initially assembled as two distinct subunits: a small 40 S subunit and a large 60 S subunit [26]. As shown in Fig. 2A, the expression of 17 genes in the large subunit of ribosomes was found to be decreased in the oocytes of DOR patients. Additionally, the expression of up to 26 genes composing the small subunit of ribosome also significantly declined. The mitochondria are another ubiquitous subcellular organelle and serve as the powerpacks of oocytes. Mitochondria has been reported to play central roles in improving oocyte quality and viability [28]. More than 30 down-regulated genes in DOR oocytes were identified to be involved in oxidative phosphorylation which occurred in mitochondria, such as NADH dehydrogenase, Cytochrome c oxidase and reductase, and F-type ATPase (Fig. 2B).

The Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether a pre-defined set of genes exhibits statistically significant and consistent differences between two biological states.

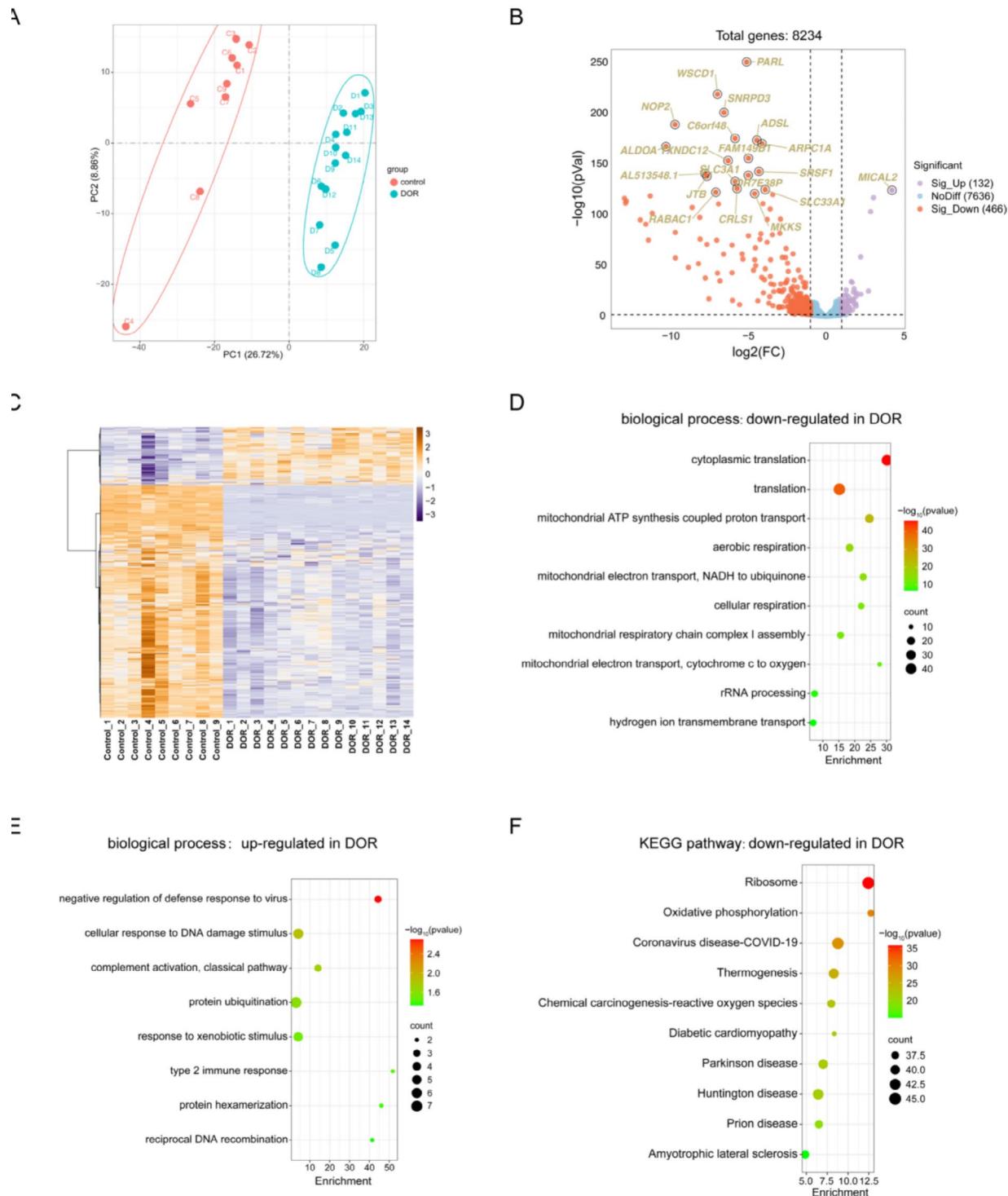


Fig. 1 The analysis of differential expression genes in germinal vesicle (GV) oocytes was conducted comparing control patients with normal ovarian reserve (NOR) and patients with diminished ovarian reserve. **(A)** Principal components analysis (PCA) plots for the single cell transcriptomes of GV oocytes. PC1 accounted for 26.72% of the total variability, while PC2 explained 8.86% of the total variability. The distinct colors assigned to individuals represent two clusters consisting of 23 samples. **(B)** A volcano plot depicted the distribution of differential expression genes (DEGs) in DOR oocytes compared to Control oocytes. The significant changes corresponded to a 2-fold change with a P-value less than 0.05. DEGs with the top 20 P-values were labeled. **(C)** The heatmap visually displayed the difference in gene expression between control group and DOR group. **(D)** Bubble plot showed the enriched Gene Ontology (GO) terms of down-regulated DEGs in DOR oocytes. **(E)** Bubble plot showed the enriched Gene Ontology (GO) terms of up-regulated DEGs in DOR oocytes. **(F)** Bubble plot showed the enriched KEGG Ontology (KO) terms of down-regulated DEGs in DOR oocytes

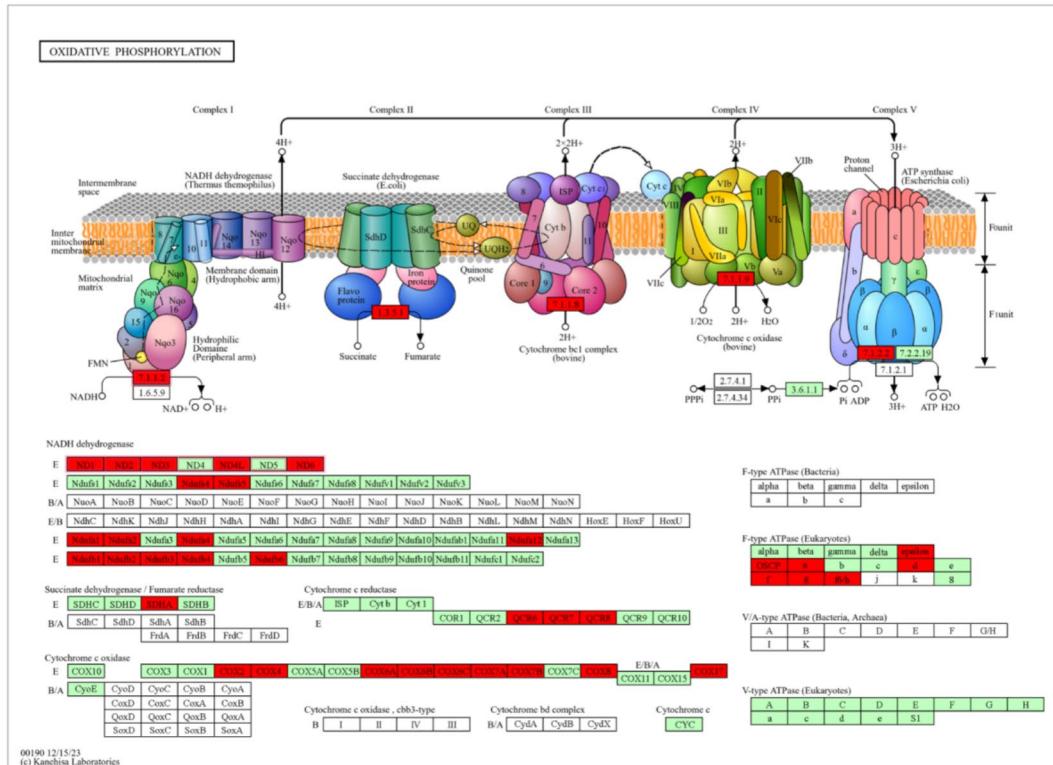
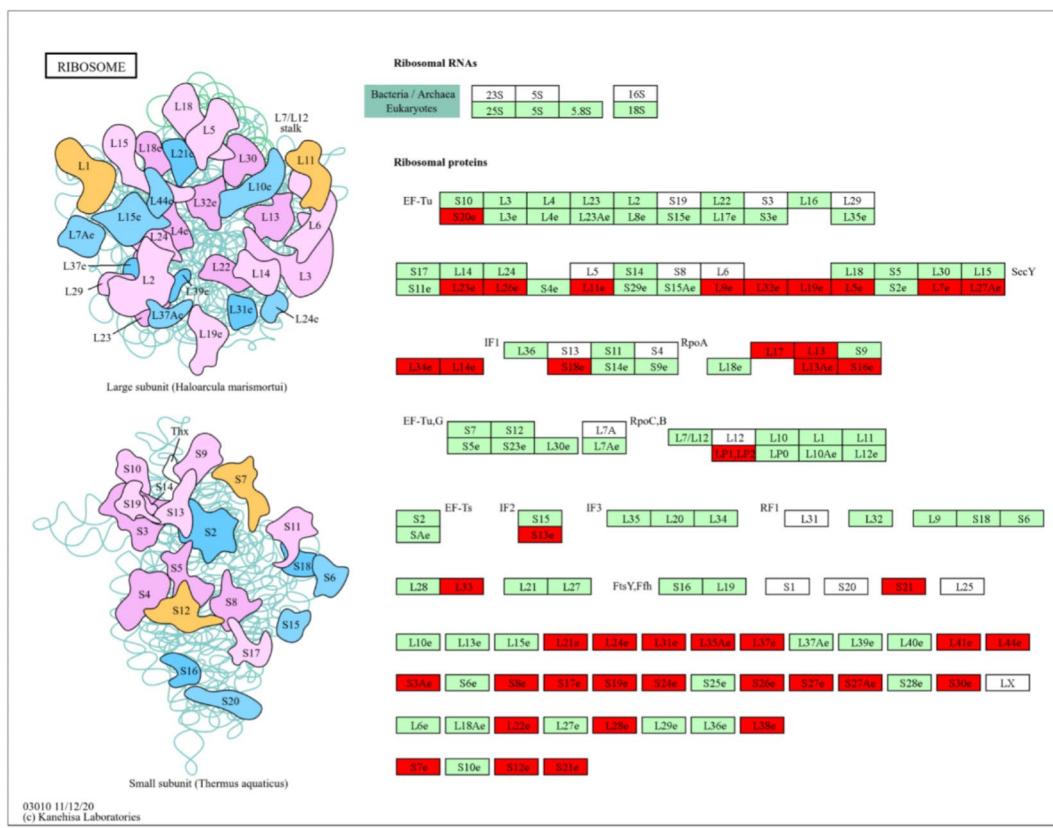


Fig. 2 KEGG pathway maps of ribosome and oxidative phosphorylation. The KEGG pathways analysis of ribosome (**A**) and oxidative phosphorylation (**B**) using KEGG Mapper (<https://www.genome.jp/kegg/mapper/>). Down-regulated DEGs in DOR oocytes were marker in red

GSEA analysis was performed on down-regulated genes in DOR oocytes. Consistent with GO and KEGG analyses, GSEA data confirmed that downregulated genes strongly enriched in pathways related to translation and mitochondrial function (Fig. 3), including cytoplasmic translation, mitochondrial respiratory chain complex assembly, cellular respiration, electron transport chain, NADH dehydrogenase complex assembly, ATP synthesis coupled electron transport, and oxidative phosphorylation. As expected, the low expression of genes in DOR oocytes was positively correlated with significant enrichment pathways (Fig. 3).

Weighted gene co-expression network analysis (WGCNA) and identification of key modules

To further understand the relationship between gene expression and DOR, WGCNA was performed on 8326 unique genes (fragments per kilobase of transcript per million fragments (FPKM) ≥ 1) and generated a co-expression network. We utilized the hierarchical average linkage clustering method to identify the gene modules within each gene network and then showed twelve gene modules were identified (Fig. 4A and B). The highest association in the module-feature relationship was between the midnight-blue module ($R^2 = -0.92$, $P = 3e-10$) and DOR, followed by the blue module ($R^2 = -0.75$, $P = 4e-5$) and red module ($R^2 = -0.73$, $P = 8e-5$) (Fig. 4C). The midnight-blue module contains 708 genes, while the blue module and red module contains 3409 and 1485 genes, respectively. All of these genes were shown in Supplemental Table II. Based on gene significance (GS) > 0.8 and module membership (MM) > 0.8 , the hub genes of the three key modules are identified and subjected to GO analysis. Genes in midnight-blue module were primarily involved in the biological processes of actin cytoskeleton organization, positive regulation of telomere

maintenance via telomerase, attachment of mitotic spindle microtubules to kinetochore (Fig. 4D and E). Moreover, the genes in red module were significant enriched in biological processes such as cytoplasmic translation, translation, mRNA splicing, and cellular response to oxidative stress (Fig. 4F and G). In addition to these two modules, genes in blue module were mainly associated with translation, cytoplasmic translation, mRNA splicing and so on (Fig. 4H and I). Therefore, these results indicated that the expression of genes involved in translation and mitochondrial function was highly negatively correlated with DOR.

Construction of protein-protein interaction (PPI) network and identification of hub genes through integrated analysis of DEGs and WGCNA

We further integrated WGCNA and DEGs analysis to screen for hub genes potentially associated with DOR. Firstly find the intersection between the key module genes and DEGs, and create a Venn diagram. A total of 104 genes were identified as the intersection between the midnight-blue module and DEGs, primarily involved in DNA replication and DNA duplex unwinding (Fig. 5A). In addition, 85 intersection genes were obtained between red module and DEGs, and these genes were significantly enriched in biological processes related to proton motive force-driven mitochondrial ATP synthesis, aerobic respiration, cellular respiration, cytoplasmic translation, and translation (Fig. 5B). After intersecting genes in the blue module with DEGs, we identified 276 intersection genes associated with cytoplasmic translation, translation, proton motive force-driven mitochondrial ATP synthesis, and mitochondrial respiratory chain complex I assembly, among others. (Fig. 5C). All the intersecting genes were listed in Supplemental Table III. Notably, a strong

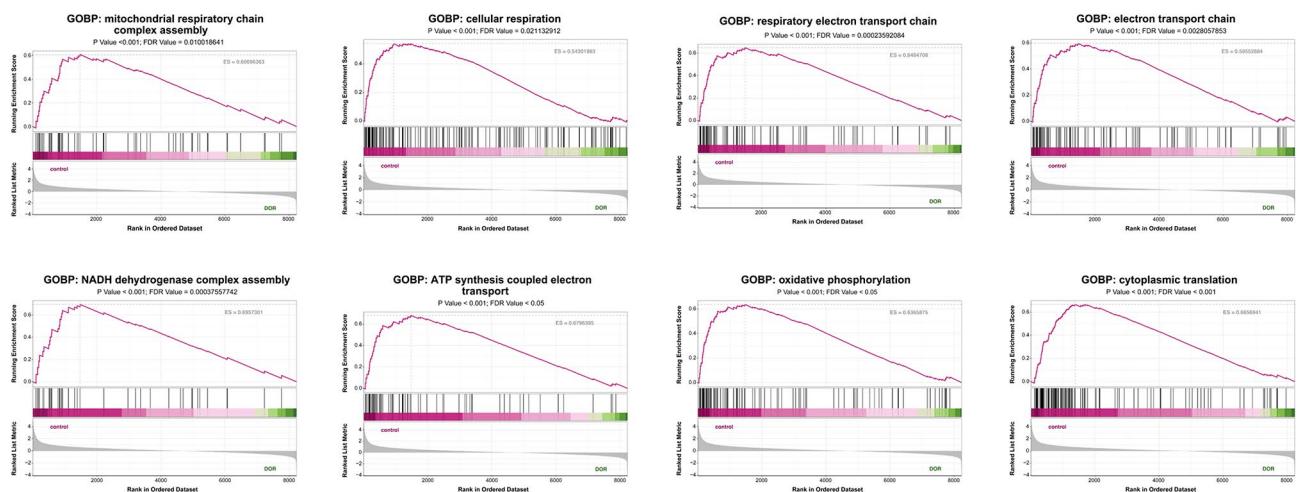


Fig. 3 Gene set enrichment analysis (GSEA). GSEA analysis for down-regulated DEGs in DOR group. Eight significantly enriched GO pathways were identified

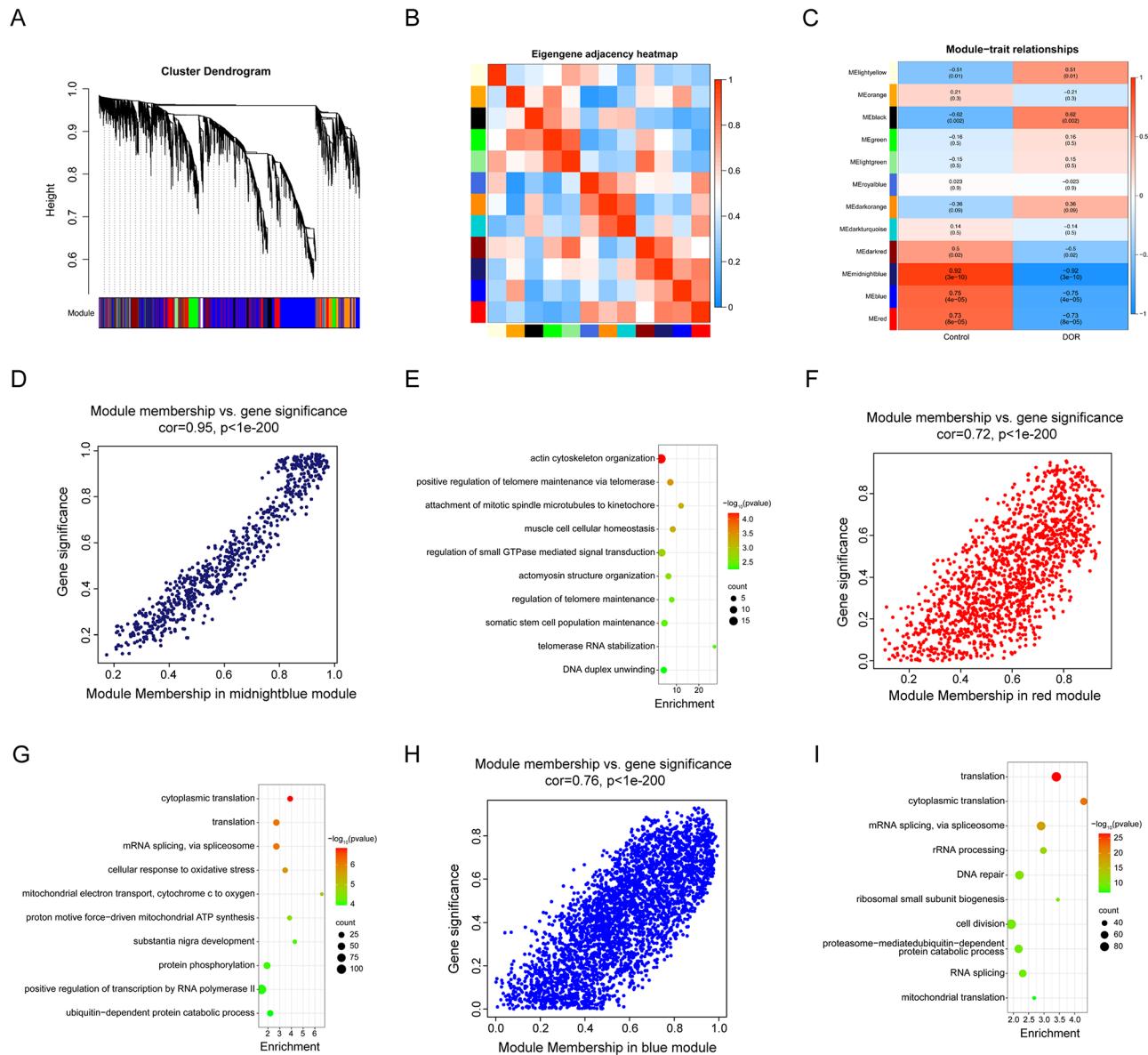


Fig. 4 Identification of co-expression modules using Weighted gene co-expression network analysis (WGCNA). **(A)** The hierarchical cluster tree illustrated the co-expression modules identified through WGCNA. The branches represented modules consisting of highly interconnected groups of genes. The modules were distinguished by various colors in the horizontal bar. **(B)** Eigengene adjacency analysis of different modules visualized with a heatmap. The color scheme indicated the correlation level, ranging from high to low with red, white, and blue. **(C)** The heatmap of module-trait relationships. The rows corresponded to distinct gene co-expression modules, while the columns represented different clinical phenotypes. Each cell contained the corresponding correlation coefficients and P-value given in parentheses. The color legend on the right showed the correlation level. ME, module eigengene. **(D)** A scatterplot of module membership (MM) vs. gene significance (GS) in midnightblue module. **(E)** The bubble plot showing the GO function enrichment of genes in midnightblue module. **(F)** A scatterplot of module membership (MM) vs. gene significance (GS) in red module. **(G)** The bubble plot showing the GO function enrichment of genes in red module. **(H)** A scatterplot of module membership (MM) vs. gene significance (GS) in blue module. **(I)** The bubble plot showing the GO function enrichment of genes in blue module

correlation between translation and mitochondrial function with DOR has once again been confirmed.

The PPI network was constructed to identify hub genes. Briefly, the translation-associated genes, generated by integrating WGCNA and DEG analyses, were imported into the STRING online database to predict the interactions between the proteins encoded by these genes, with

a high confidence score of >0.7. Based on the STRING analysis, we further analyzed these genes using the Cytoscape software platform. The top 20 hub genes were then screened by the 'cyto-Hubba' plugin in Cytoscape, based on a mixed character calculation that includes Degree, MCC, and MCODE algorithms (Fig. 5D). The expression of the top 20 hub genes associated with translation

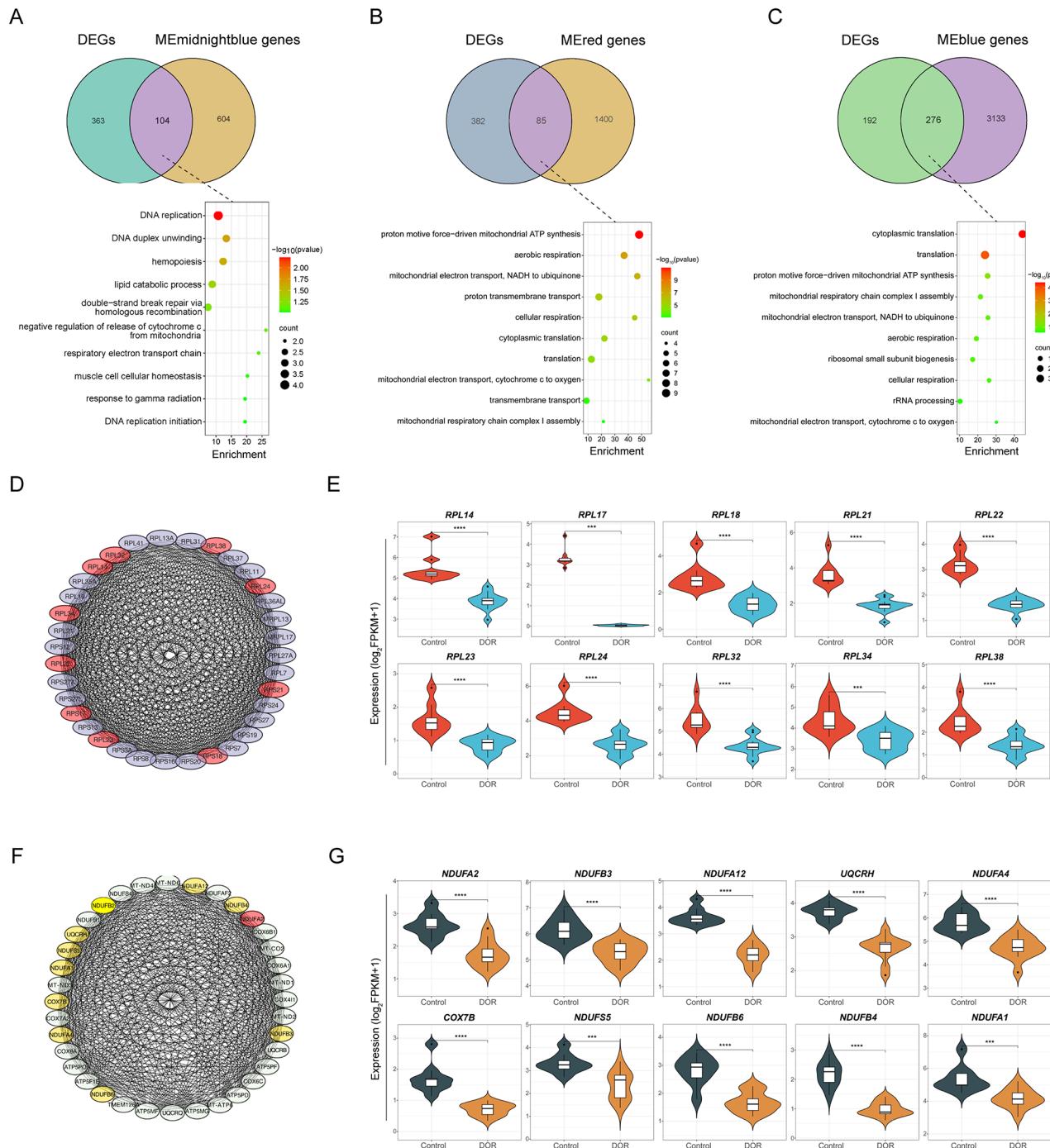


Fig. 5 Identification of hub genes through integrated analysis of DEGs and WGCNA. The Venn diagrams depicted the intersection of genes between the midnightblue (**A**), red (**B**), or blue (**C**) module and DEGs. Bubble plots displayed the top 10 GO terms of overlapped genes. The y-axis represented the GO-enriched terms, while the x-axis meant the fold of enrichment. The size of each dot corresponded to the number of genes associated with a specific term, and its color indicated the adjusted P-value. (**D**) The genes associated with translation from the integrated analysis of WGCNA and DEGs were used to construct PPI network using STRING online database. Hub genes were identified by cytoHubba plugin of cytoscape software. Color gradients represented the transition from higher to lower values, ranging from red to yellow. (**E**) The mRNA expression levels of the top 10 hub genes obtained through single cell RNA sequencing (scRNA-seq) were shown in violin plot. *** $P < 0.001$, **** $P < 0.0001$. (**F**) Identification of hub genes related to mitochondrial function using the cytoHubba plugin in cytoscape. Color gradients represented the transition from higher to lower values, ranging from red to yellow. (**G**) Violin plots of gene expression were generated for the hub genes associated with mitochondrial function based on scRNA-seq. *** for p -value < 0.001 , **** for p -value < 0.0001 .

was obviously decreased in DOR oocytes compared to control oocytes (Fig. 5E). In addition, a PPI network of genes related to mitochondrial function was constructed based on gene screening using the aforementioned methods, and the top 20 hub genes are shown in Fig. 5F. The expression of the top 10 hub genes related to mitochondrial function in transcriptomes of DOR and control oocytes was depicted, and all of these genes exhibited a significant reduction in DOR oocytes compared to control oocytes (Fig. 5G).

Validation of hub genes in human oocytes using RT-qPCR

The top 10 hub genes associated with translation (*RPL14*, *RPL17*, *RPL18*, *RPL21*, *RPL22*, *RPL23*, *RPL24*, *RPL32*, *RPL34*, *RPL38*) were verified in control and DOR oocytes

by RT-qPCR. The expression of these ribosomal genes was significantly lower in DOR oocytes compared to control (Fig. 6A). Additionally, the top 10 hub genes associated with mitochondrial function, namely *NDUFA2*, *NDUFB3*, *NDUFA12*, *UQCRH*, *NDUFA4*, *COX7B*, *NDUFS5*, *NDUFB6*, *NDUFB4*, and *NDUFA1* were also validated using RT-qPCR. The results revealed a significant decrease in the expression of these mitochondrial genes in DOR oocytes compared to control oocytes (Fig. 6B). *GDF9* was used as a negative control (Fig. 6). All the 20 hub genes were differentially expressed and consistent with RNA-seq results, suggesting the RNA-seq results are reliable and reproducible. Collectively, our data suggest that 20 hub genes are potential contributors to the pathogenesis of DOR.

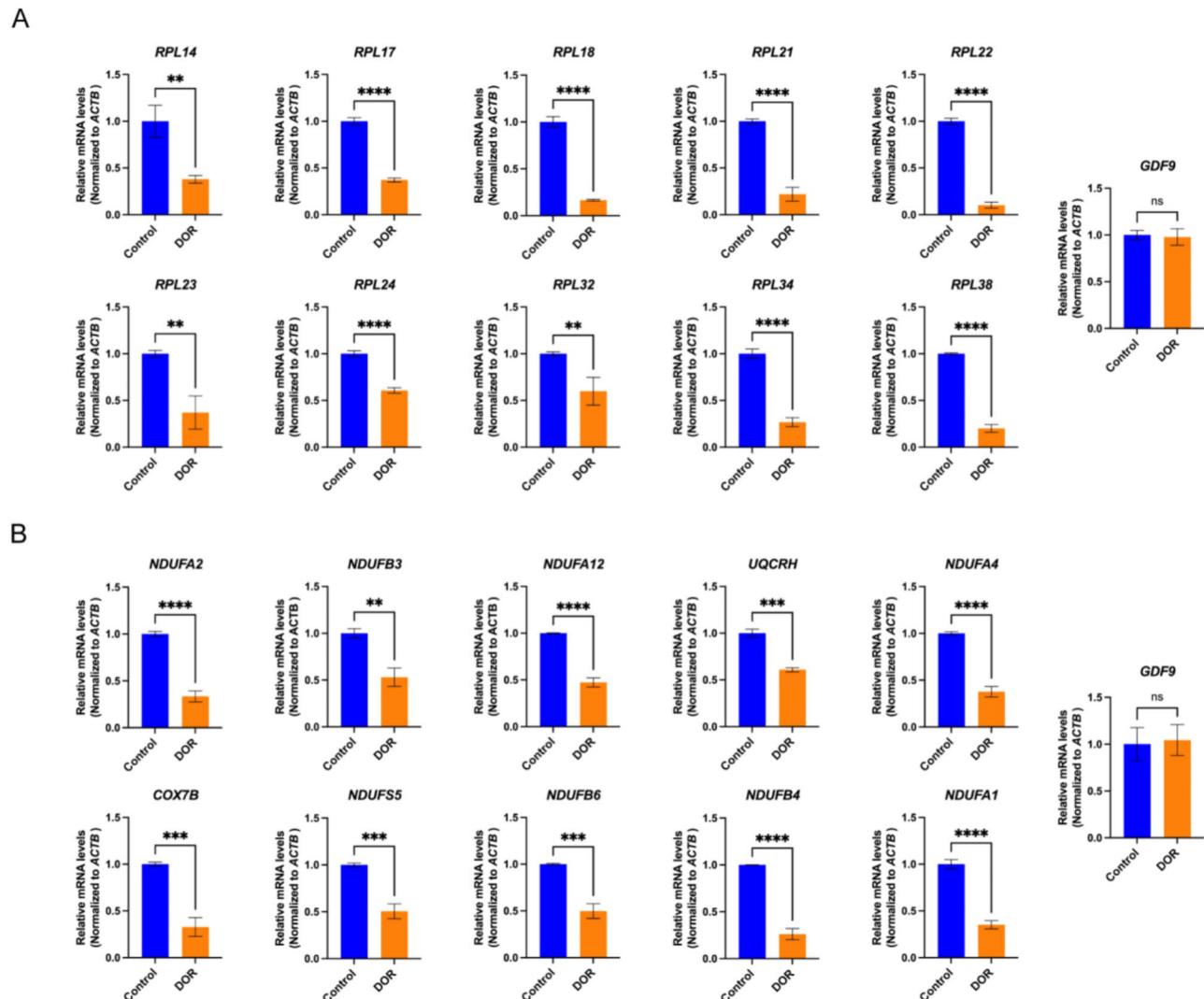


Fig. 6 Validation of scRNA-seq data with RT-qPCR in control and DOR oocytes. **(A)** RT-qPCR assays were performed to detect the relative expression profiles of hub genes associated with translation (**A**) and mitochondrial function (**B**) in control and DOR oocytes. *GDF9* was used as a negative control. Significance was indicated by *** for p -value < 0.001 and **** for p -value < 0.0001 . Data are presented as mean \pm SD

The *PLAG1-IGF2* axis is dysregulated in DOR oocytes

We have screened out translational and mitochondrial dysfunction as the primary characteristics of DOR oocytes, and the hub genes related to translation and mitochondrial function were further identified through integrated analysis of WGCNA and DEGs. We want to determine whether the down-regulation of these hub genes is caused by a disorder in some key transcription factors in DOR oocytes. We then utilized a transcript factor (TF) enrichment tool on KncokTF2.0 online database [32] to predict key TFs. We submitted a gene list

that includes all hub genes associated with translation or mitochondrial function to the KncokTF2.0 online database, and we set the *P*-value threshold at <0.05 for TF enrichment. The hub genes related to mitochondrial function were enriched in 9 transcription factors (Fig. 7A), while 55 transcription factors were predicted for the hub genes related to translation (Fig. 7B). The hub genes corresponding to each transcription factor are presented in Supplemental Table IV and V, respectively. We further identified 5 transcription factors, namely *PLAG1*, *ZEH2*, *FOXA1*, *PURA* and *SRPK2*, that were involved

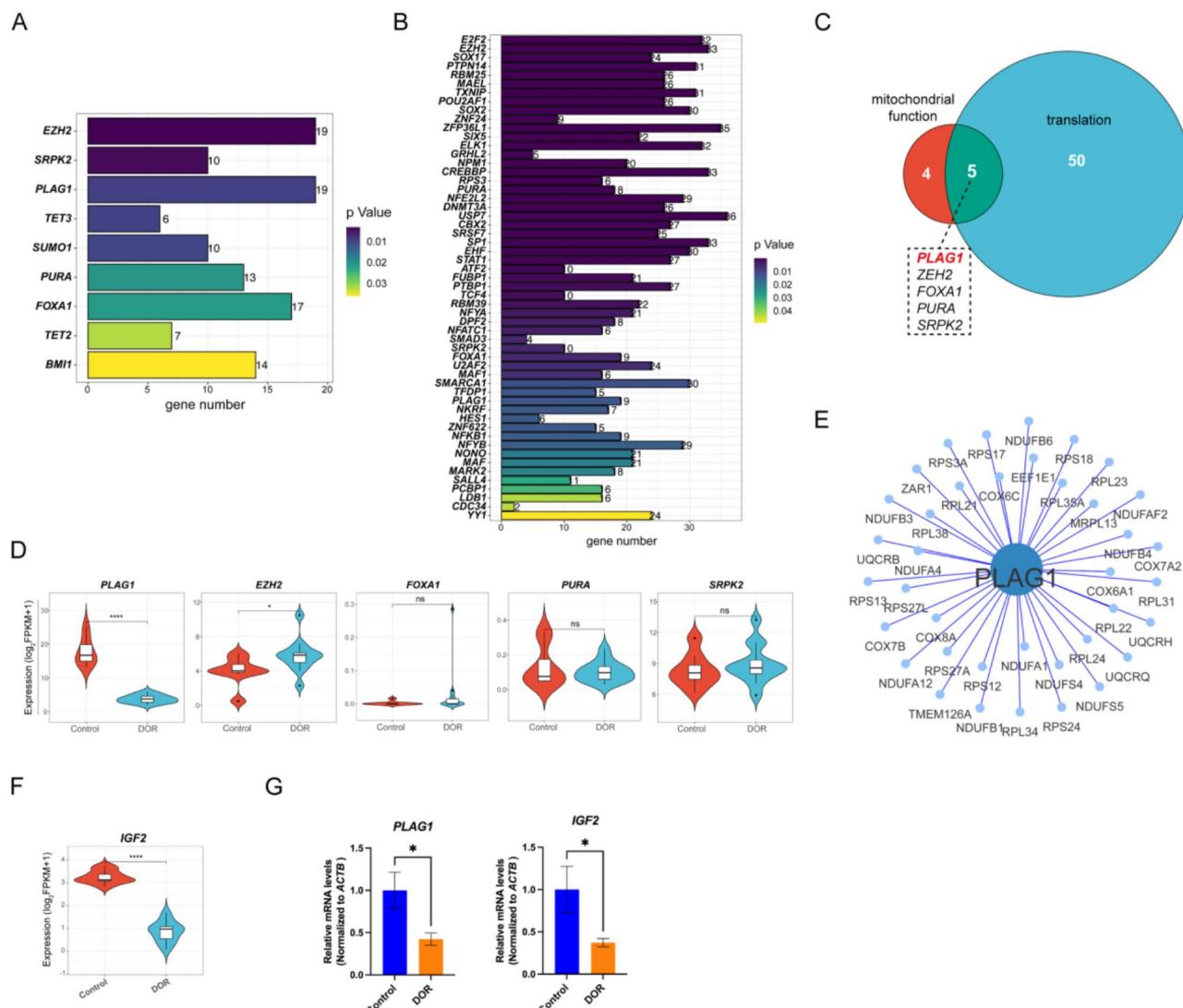


Fig. 7 Prediction of the potential transcriptional factors (TFs) of the hub genes. The KnockTF database (<https://biocilab.net/KnockTFv2/>) was used to predict the transcriptional factors associated with hub genes involved in mitochondrial function (A) and translation (B). The y-axis of the bar chart showed the predicted transcription factors, while the x-axis represented the number of hub genes regulated by each transcription factor. The color scheme indicated p-value. (C) Venn diagrams showed the intersection of TFs identified from (A) and (B). (D) The relative expression levels of five TFs were determined based on scRNA-seq. (E) The Venn network displayed the hub genes specifically targeted by PLAG1. (F) The violin plot displayed the expression of *IGF2* gene in control and DOR oocytes based on scRNA-seq. **** for *p*-value < 0.0001. (G) Expression of *PLAG1* and *IGF2* was determined by RT-qPCR to validate the scRNA-seq data. Significance was indicated by * for *p*-value < 0.05

in the transcriptional regulation of hub genes related to both translation and mitochondrial function (Fig. 7C). Subsequently, we detected the expression of 5 predicted transcription factors in DOR oocytes through RNA-seq data. Interestingly, only the expression of PLAG1 showed a significant decrease in DOR oocytes (Fig. 7D), and the expression of 38 hub genes was regulated by PLAG1 (Fig. 7E), suggesting that PLAG1 may play a crucial role as a transcriptional regulator in determining the quality of DOR oocytes.

The PLAG1-IGF2 axis has been well validated for its impact on ovarian follicles, oocytes, embryos, and fetuses [33, 34]. To uncover the role of PLAG1-IGF2 axis in DOR oocytes, we used oocyte transcriptome data to investigate the expression of IGF2 in DOR oocytes. In contrast to control oocytes, a decrease in the abundance of IGF2 mRNA was observed for DOR oocytes (Fig. 7F). Consistent with our RNA-seq data, the transcripts of PLAG1 and IGF2 were significantly decreased in DOR oocytes versus NOR oocytes using RT-qPCR (Fig. 7G).

Discussion

So far, research has failed to reveal the detailed transcriptomic landscape of human oocytes linked to young women diagnosed with DOR. In our study, we recruited infertile women with varying degrees of ovarian reserve who underwent ICSI cycles and then collected GV oocytes for single-cell RNA sequencing for the first time. Our results indicated that the down-regulated DEGs were primarily associated with mitochondrial function and translation, suggesting them as potential factors contributing to the low quality of DOR oocytes. Through a combined analysis of WGCNA and DEGs, we screened out hub genes that were highly relevant to DOR. We subsequently identified the transcription factor PLAG1, which was predicted to regulate mitochondrial and ribosomal genes, as being down-regulated in DOR oocytes. Furthermore, we validated that the PLAG1-IGF2 axis, which plays a crucial role in maintaining oocyte quality, exhibited abnormal expression in DOR.

Patients with diminished ovarian reserve are characterized by low levels of AMH, a decrease in the quantity and/or quality of eggs, and/or elevated basal levels of FSH among women of reproductive age [3, 4]. However, there are disagreements regarding whether the quality of oocytes in DOR patients is low. Some studies showed that there was no significant difference in the quality of oocytes between DOR and NOR patients, and the evaluation criteria were oocyte nuclear maturity [35], fertilization and miscarriage rate [36]. On the contrary, preimplantation genetic testing (PGT) indicated that DOR was associated with the increase of aneuploidy [8], and the results of a large-scale retrospective study showed that DOR was positively correlated with the

miscarriage rate [37], suggesting the decline of oocyte quality. The inconsistent definition of oocyte quality evaluation standards and the methodological differences in selecting surrogate indicators lead to the inability to objectively evaluate the quality of oocytes. For the first time, we performed single-cell transcriptome sequencing on DOR oocytes to directly reflect the molecular alterations of DOR oocytes, providing valuable evidence for assessing the quality of DOR oocytes. Differential gene analysis revealed changes in the expression of 598 genes, of which 466 genes were downregulated in DOR (Fig. 1B). These down-regulated genes are primarily related to mitochondrial function and translation (Fig. 1D), and considering that mitochondrial and ribosomal-associated proteins are key regulators of oocyte quality [26–30], this confirms the decline in the quality of DOR oocytes. It has been well demonstrated that mitochondrial dysfunction and translational defects can lead to oocyte aging [26, 29, 38–40]. Notably, we found that similar characteristics to aging oocytes also appeared in young DOR oocytes, suggesting that the oocytes of young DOR patients have already mismatched their actual age, which requires clinicians to pay more attention to young DOR patients.

To further investigate whether the alterations in DEGs are due to abnormal expression of specific transcription factors (TFs), we employed a TF enrichment tool to predict the potential transcription factors involved. We performed transcription factor enrichment analysis on the screened hub genes related to mitochondria and ribosomes, leading to the identification of PLAG1 (Fig. 7C). Pleomorphic adenoma gene 1 (PLAG1) is a member of the zinc finger transcription factor family, which is primarily known as an oncogene [33]. The phenotypic analysis of *Plag1* knockout mice unveiled a pivotal role of PLAG1 in the postnatal growth and reproductive processes of both male and female mice [33, 41, 42], as well as in the pituitary gland [43]. Moreover, genome-wide association studies in both humans and domestic animals provide further evidence of PLAG1's involvement in growth and reproduction [43]. Additionally, a role for PLAG1 in growth and reproduction was further corroborated by genome-wide association studies in humans and domestic animals [33, 44–46]. As a transcription factor, PLAG1 functions by binding to target genes and modifying their transcriptional efficiency. It has been demonstrated that PLAG1 can bind to the promoter of insulin-like growth factor 2 (IGF2), enhancing its activity and thereby promoting the expression of IGF2 [47]. Numerous studies conducted in both animal and human models have consistently provided robust evidence regarding the potential influence of IGF2 on the developmental competence of oocytes and embryos [48–50]. Using RT-qPCR, we validated that the expression of the PLAG1-IGF2 axis was downregulated in DOR oocytes

(Fig. 7G). It is noteworthy that studies have shown that IGF2 is capable of enhancing mitochondrial activity in neuronal culture-derived cell lines [48, 51]. Furthermore, the addition of IGF2 to the culture media enhances the developmental potential of oocytes and reduces structural abnormalities during meiosis [48, 52]. Collectively, our findings not only suggest that PLAG1-IGF2 can serve as a biological marker for DOR oocytes but also point to its potential as a novel therapeutic strategy for enhancing the quality of DOR oocytes.

Although we have presented the transcriptomic data of oocytes from DOR patients for the first time, our study also has some limitations. Firstly, due to the difficulty in obtaining samples, we selected GV-stage oocytes from ICSI cycles, but the representativeness of the transcriptomic data from GV oocytes in fully reflecting the quality of DOR oocytes is debatable. Furthermore, oocytes at the GV stage after ovarian stimulation exhibit insufficient maturity and poor developmental potential, making these samples somewhat limited as standards for assessing the quality of oocytes in cases of DOR. Secondly, Gonadotropins are key components in assisted reproductive therapy. Studies have shown that follicular stimulation has minimal impact on the transcriptome of bovine oocytes [53], and a recent study showed that using a higher dosage of gonadotropin did not lead to an increase in the number of retrieved MII oocytes [54]. However, a preponderance of scholars contends that gonadotropins have the capacity to enhance oocyte maturation and elevate oocyte developmental potential [55]. In the present study, variations observed in patients' baseline hormone levels resulted in discrepancies in the total administered gonadotropin dosages, which could potentially influence the molecular characteristics of oocytes. Consequently, this factor necessitates consideration in our analysis. Thirdly, the sample size used for single-cell RNA sequencing is relatively small, and thus the question of whether it can fully represent the transcriptomic characteristics of the DOR population requires careful consideration. We hope that more samples can be used to evaluate oocyte quality in DOR, in order to obtain more comprehensive, accurate, and reliable experimental results. Finally, we primarily relied on oocyte transcriptome to investigate the underlying mechanisms and changes associated with DOR. Moreover, our validation of the results was limited to the mRNA level. One significant limitation of focusing on gene expression is the disconnect that can exist between mRNA levels and actual protein abundance. This disconnect arises due to various post-transcriptional and post-translational processes that can regulate protein synthesis and degradation. In addition, changes in protein structure, activity, or localization may have profound effects on cellular function, even if the corresponding gene expression levels remain unchanged. Future studies

need to further validate the results of this research at the protein level, and integrate transcriptome and proteome data to provide a more holistic perspective to unveil the pathogenesis of DOR.

Conclusions

In this study, we provided the evidence for the poor quality of DOR oocytes through single-cell RNA sequencing of DOR oocytes. Our findings suggest that mitochondrial dysfunction and translational impairment are the primary defects present in DOR oocytes. Through bioinformatic analysis and experimental validation, we have demonstrated that the *PLAG1-IGF2* axis exhibits decreased expression in DOR oocytes, suggesting that the *PLAG1-IGF2* axis could serve as a potential target for the diagnosis and treatment of DOR. These findings enhance our comprehension of the fundamental genetic processes involved in DOR and may pave the way for innovative diagnostic approaches and treatment strategies aimed at addressing DOR.

Abbreviations

DEGs	Differentially Expressed Genes analysis
WGCNA	Weighted Gene Co-Expression Network Analysis
DOR	Diminished Ovarian Reserve
AMH	Anti-Müllerian Hormone
FSH	Follicle-Stimulating Hormone
ART	Assisted Reproductive Technology
NOR	Normal Ovarian Reserve
COS	Controlled Ovarian Stimulation
PPOS	Progestin-Primed Ovarian Stimulation
GSEA	Gene Set Enrichment Analysis
PPI	Protein-Protein Interaction
AFC	Antral Follicle Count
BMI	Body Mass Index
ICSI	Intracytoplasmic Sperm Injection

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12958-024-01321-8>.

Supplementary Material 1: Supplemental Table I: The primer sequences and primer information used in RT-qPCR for the study. Supplemental Table II: Gene list of the three modules showing high correlation with DOR identified through WGCNA. Supplemental Table III: Gene list of overlaps between genes in different WGCNA modules and DEGs. Supplemental Table IV: The predicted transcription factors for mitochondrial-related genes. Supplemental Table V: The predicted transcription factors for genes associated with translation.

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Author contributions

XL, XW, YZ and JT contributed to the study design. XW and HZ collected the oocyte samples from the patients. HZ, JL, PL and LX analyzed and interpreted the basic patient information. XL and NZ carried out the bioinformatics analysis. XL, HZ and LT jointly conducted validation experiments and analyzed the results. XL wrote the original manuscript and ZL, YZ, and JT were responsible for revising the manuscript. NZ and JT provide funding support for this project. JL, YZ and JT supervise the operation of the project and are

the joint corresponding authors for this manuscript. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by the Ethics Committee of Jiangxi Maternal and Child Health Hospital (Research license: EC-KT-202304). All experiments involving human were carried out in accordance with the guidelines for Ethical Review of Biomedical Research Involving Humans and ethical principles of the Declaration of Helsinki. Informed consent was obtained from all individual participants who were included in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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