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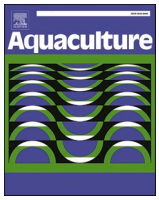

Investigation of the role of endogenous miRNAs in determining sterility in triploid Pacific oysters (*Crassostrea gigas*)

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ABSTRACT

Triploid oysters generate fewer gametes than diploid oysters and exhibit varying degrees of gonad development. The expression of related genes, such as those involved in gametogenesis and energy metabolism, may influence reproductive potential. In addition, microRNAs (miRNAs) are important regulators of gene expression. However, the contribution of miRNAs has not yet been assessed in sterile triploid Pacific oysters (*Crassostrea gigas*). We therefore obtained extensive miRNA and gene expression data to identify potentially critical miRNA-mRNA interactions that are linked to sterility in triploid *C. gigas*. Here, we found miR-263-x to be significantly downregulated in the gonads of sterile females. The upregulated target genes were associated with lipid droplet formation, which revealed the likely involvement of nutrient accumulation during gonadal development as regulated by miR-263-x in female sterile triploids. In males, the upregulated miRNAs (miR-1992-y and miR-2001-x) were shown to regulate spermatogenesis-related gene expression, including flagellar formation, and cell proliferation and migration. Our results suggest that energy redistribution regulated by miRNAs may be critical for gonad development in females, indicating that sterile triploids undergo a significant reduction in gamete number and show an increased accumulation of nutrients in the gonads. Our findings further indicate that decreased expression of genes related to spermatogenesis regulated by miRNAs is important in gonadal dysgenesis in sterile triploids. These results shed light on the epigenetic regulatory mechanisms governing sterility in triploid bivalves.

1. Introduction

Aquaculture is currently the fastest-growing food business worldwide, supplying approximately half of all blue food (Nascimento-Schulze et al., 2021). Marine bivalve aquaculture is a highly sustainable part of the industry. Bivalves are a diverse mollusk group with ecological and economic significance (Sousa et al., 2009) and are relevant to human nutrition as an important source of protein. Sexual reproduction is a prevailing and remarkable phenomenon in bivalves. These species generate gametes via meiosis to propagate offspring and maintain population stability. Improving our understanding of bivalve reproductive biology is vital for bivalve maintenance and stocking. Furthermore, control of gonadal development can also aid in achieving significant goals, including creating sterile cultured individuals to meet the needs of the market and preventing interbreeding between wild populations and cultured stocks in bivalve aquaculture. As a result, the mechanism of sterility during gonadal development and gametogenesis in bivalves has long been of interest to numerous aquatic scientists.

The Pacific oyster (*Crassostrea gigas*) is an economically important bivalve that has been introduced globally for aquaculture (Vendrami et al., 2019). Chromosomal ploidy manipulation is an important method for creating high quality germplasm. Genome polyploidization provides a favorable opportunity for the formation of new traits and new genes (Comai, 2005). Because diploid oysters are less marketable under spawning conditions, triploids were produced to enhance marketability due to their reduced gonadogenesis (Kang et al., 2013). Currently, commercial triploid oysters are normally created by mating diploid females with tetraploid males to avoid chemical toxicity (Guo et al., 1996). Because of the commercial benefits, such as limited reproductive potential, faster growth, year-round harvestability, and superior flesh flavor (Nell, 2002), triploid oysters have become an essential component of the global oyster aquaculture industry. Triploid oysters make up

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approximately 50% to 100% of hatchery seed production in the United States and France (Dégremont et al., 2016; Peachey and Allen Jr, 2016). Currently, the use of triploid oysters is becoming increasingly popular in China.

When compared to diploid Pacific oysters, triploids have much lower reproductive capacities. Specifically, triploids had lower gonadic occupation and a smaller number of mature gametes than diploid oysters (Normand et al., 2009; Jouaux et al., 2010). Furthermore, in triploid *C. gigas*, the effects of their low gamete quality reduce the genetic impact on natural populations (Suquet et al., 2016). Moreover, sterility in triploid oysters is often incomplete and varies considerably among individuals (Guo and Allen Jr, 1994). The partial fertility observed in triploid *C. gigas* greatly affects reproductive physiology. The complex reproductive properties of triploid oysters (the existence of both fertile and sterile types) make them important models for studying sterility, which drives the curiosity of biologists. Despite lists of sterile-related genes and proteins having been discovered via gonad transcriptome and proteome analysis in *C. gigas* (Dheilly et al., 2014; Chen et al., 2021), our understanding of the mechanisms underlying physiological differences in gametogenesis in triploid oysters is still incomplete, and the regulatory networks underlying gonadal development remain unclear.

MicroRNAs (miRNAs) are endogenous noncoding RNAs that regulate a variety of biological processes but do not code for proteins. They are 18 to 25 nucleotides (nt) in length and are key regulators of gene expression in metazoans. miRNAs regulate gene expression posttranscriptionally by inducing mRNA decay or translation suppression (Lu and Rothenberg, 2018). The miRNA-mRNA pairing is determined by the seed sequence of miRNA, nucleotides 2–8 at the 5′-end of mature miRNAs, which binds to the 3′-untranslated region (UTR) of the target mRNAs via sequence complementarity, resulting in translation suppression or mRNA degradation (Rosani et al., 2021). miRNAs play an important role in a variety of biological processes, such as cell apoptosis, proliferation, differentiation, and developmental timing (Nejad et al., 2018; Galagali and Kim, 2020). In addition, various expression profiling studies have found that miRNAs play a significant role in gonadal development in a variety of species. In mice, miR-21, miR-20, and miR-106a were implicated in controlling the self-renewal of spermatogonia stem cells. The number of germ cells experiencing apoptosis increased when miR-21 was inhibited (Niu et al., 2011), and the proliferation rate of spermatogonia stem cells was increased when miR-20 or miR-106a were overexpressed (He et al., 2013). In *Caenorhabditis elegans*, miR-35 was demonstrated to regulate germ cell proliferation. When there was a loss of miR-35, the number of nuclei in the distal mitotic gonad decreased (Liu et al., 2011). Such interactions between miRNA and mRNA in gonad development or germ cell proliferation have been widely studied in model animals, and in recent years, small RNA sequencing investigations have suggested that miRNAs participate in gonad development and triploid gonadal infertility in some aquatic polyploid species. In triploid cyprinids, the differentially expressed miRNAs (DEMs) were likely engaged in sperm activity and testicular development by targeting related functional genes (Tao et al., 2018). In triploid female rainbow trout, among DEMs, dre-miR-15a-5p_R + 1 was shown to target *ccne1*, which is involved in DNA repair and homologous pairing during the first meiotic division, suggesting a role for miRNAs in governing meiosis during gonad development (Huang et al., 2021).

Although the importance of miRNAs has already been extensively demonstrated, their role in overall gene expression control in the physiological function and development of both male and female gonads in triploid *C. gigas* is still unknown. Here, we investigated the interactome of both DEMs and their differentially expressed gene targets (DEGs) to determine the differences in the gonadal development and gametogenesis of fertile diploid, fertile triploid, and sterile triploid *C. gigas*. This study aims to determine the role of miRNA-mediated regulation in gametogenesis of sterile triploid Pacific oysters compared with fertile animals.

2. Materials and methods

2.1. Ethics statement

The Pacific oysters employed in this work were cultured animals, and all animal treatments followed the requirements of Ocean University of China's Animal Research and Ethics Committees (Permit Number: 20141201).

2.2. Sample collection and preparation

All *C. gigas* individuals used in this study were two years old and were collected in late June 2018 in Weihai, Shandong Province, China. Triploid oysters were obtained by mating male tetraploids with female diploids and were then maintained together with diploid oysters. CytoFLEX flow cytometry (Beckman Coulter, USA) was used to validate oyster ploidy by sampling gill filaments. Dissected gonad tissues were instantly frozen in liquid nitrogen before being stored at −80 °C for RNA extraction. Histological methods were used to identify the level of gonadal development and the sex of the oysters (Li et al., 2006). Eighteen gonad samples were selected according to whether the gonads displayed abundant gametes at stage III (maturation stage). In detail, biologic replicates (3 samples) of female diploids (F-2n-1, F-2n-2, F-2n-3), male diploids (M-2n-1, M-2n-2, M-2n-3), female fertile triploids (F-3nα-1, F-3nα-2, F-3nα-3), male fertile triploids (M-3nα-1, M-3nα-2, M-3nα-3), female sterile triploids (F-3nβ-1, F-3nβ-2, F-3nβ-3), and male sterile triploids (M-3nβ-1, M-3nβ-2, M-3nβ-3) were obtained.

2.3. Total RNA isolation, small RNA library preparation and sequencing

The RNA was prepared in the same way as in earlier RNA-seq work (Chen et al., 2021), with total RNA serving as the input material for sequencing. Polyacrylamide gel electrophoresis (PAGE) was utilized to isolate RNA with a size range of 18–30 nt from total RNA. Small RNA libraries were built using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) according to the manufacturer's instructions. The 5′ and 3′ adapters were first ligated sequentially to the RNAs. The ligation products were then reverse transcribed via PCR amplification, with the 140–160 bp PCR products being enriched to form cDNA libraries. Library quality was assessed on an Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. The small RNA libraries were sequenced on an Illumina NovaSeq 6000 platform.

2.4. Data processing and differential expression analysis

Raw reads from the sequencing machine were initially filtered for small RNA sequencing. The raw reads were then filtered to remove unclean reads containing low-quality bases or adapters that would affect the subsequent assembly and analysis. All clean tags were aligned in the GenBank and Rfam databases to detect and delete rRNA, snoRNA, scRNA, tRNA, and snRNA. High-quality clean tags were aligned to the *C. gigas* genome (unpublished) to examine their distribution and expression in the reference. Based on the results of mapping the genome and the positions of exons and introns in the genome, the tag sequences from mRNA degradation fragments were identified. To identify known miRNAs, the small RNA tags were aligned with miRNA sequences in other species using miRBase20.0. The genomic locations and secondary hairpin structures of miRNA precursors predicted by Mireap_v0.2 were used to identify novel miRNAs. The following order was used to determine the annotation results: rRNA > known miRNA > repeat > exon > novel miRNA > intron. Unannounced tags were those that could not be tagged with any of the aforementioned compounds. Transcripts per million (TPM) was used to assess miRNA expression levels. The miRNAs were regarded as significantly differentially expressed miRNAs (DEMs) if the absolute fold change (FC) ≥ 2 and $p < 0.05$.

2.5. Target mRNA prediction and functional analysis of miRNAs

The 3′-UTRs were thought to be potential binding sites between miRNAs and their target mRNAs. miRNA target prediction was performed with RNAhybrid (Version 2.1.2) + svm_light (Version 6.01), TargetScan (Version 7.0), and Miranda (Version 3.3a). The potential target genes were those identified by the intersection of the results from the three programs. The datasets in this investigation were evaluated by determining the negative correlation between DEMs and DEGs expression because most miRNAs have been found to induce mRNA degradation or repress mRNA translation.

OmicShare tools (https://www.omicshare.com/tools) were used to perform GO enrichment analysis and KEGG pathway analysis based on target DEGs of DEMs. Both GO terms and KEGG pathways were considered significantly enriched with a p value <0.05.

2.6. DEGs-DEMs integration and network analysis

To investigate the interactions between the DEGs and DEMs, the information, including the DEG-DEM relationships, was imported into Cytoscape (version 3.9.0). Based on sequencing data, the predicted core interaction networks between the DEGs and DEMs were constructed.

2.7. Validation of DEM and DEG expression using quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to validate the annotated miRNAs and mRNAs. A total of nine DEMs and ten DEGs were selected. First, small RNA (< 200 nt) extraction was performed using RNAiso for Small RNA (TaKaRa, Japan). First-strand cDNAs of the miRNAs were then synthesized utilizing a miScript II RT Kit (Qiagen, Germany). The reverse-transcribed reaction system consisted of 4.0 μL 5× miScript HiSpec Buffer, 2.0 μL 10× miScript Nucleics Mix, 2.0 μL miScript Reverse Transcriptase Mix, and 2 μg small RNA, and a suitable volume of RNase-free water was added to the cDNA synthesis reaction system (20 μL). The synthesis reaction was incubated at 37 °C for 60 min and terminated by heating at 95 °C for 5 min to inactivate the enzyme reaction. The combination of polyadenylation and oligo-dT primers with universal tags in the reverse transcription process ensures that genomic DNA is not detected during the subsequent amplification. The amplification was performed on a LightCycler 480 real-time PCR instrument (Roche, USA) using the miScript SYBR Green PCR Kit (Qiagen, Germany). The reactions took place in reaction samples with a total volume of 20 μL, including 2 μL 10 × miScript Universal Primer, 10 μL 2 × QuantiTect SYBR Green PCR Master Mix, 4 μL template cDNA, 2 μL miRNA Specific Primer, and 2 μL RNase-free water. Denaturation at 95 °C for 15 min was followed by 45 cycles at 95 °C for 15 s, 55 °C for 15 s, and 70 °C for 30 s. The biological replicates were carried out in a tree-independent manner. A no-template control served as a negative control, and deionized water was used as a blank control. To normalize miRNA expression, U6 snRNA was utilized as an internal control. Additionally, potential target gene expression patterns were investigated in the same way as in our previous work (Chen et al., 2021). In the female gonadal comparison group, *EF-1a*, *GAPDH*, *RS18*, *RO21*, and *RL7* were used for normalization. In the males, *RS18* and *RL7* were used for normalization. Supplementary Table S1 lists the primers for DEM and DEG qRT-PCR. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of each candidate DEM and DEG (Livak and Schmittgen, 2001). All data are shown as the mean ± SD. Statistical analysis was performed using GraphPad Prism software (version 8.0.1) and one-way ANOVA for multiple comparisons, and the statistical significance level was set at $p < 0.05$.

3. Results

3.1. Identification of miRNAs in the gonads of C. gigas

All raw data were uploaded to the SRA database at NCBI in Bioproject PRJNA791305, and the accession number list is presented in Supplementary Table S2. A total of 246,651,800 clean reads were obtained after initial filtering and were further trimmed to obtain small RNA clean tags for subsequent analysis (Supplementary Table S3). Of these 211,155,421 clean tags, approximately 12,959,534 rRNA, 121,637 snRNA, 3889 snoRNA and 564,753 tRNA reads were removed (Supplementary Table S4). Moreover, the tags from the mRNA degradation fragment were identified based on the position of the exons and introns in the genome. The length distribution of small RNAs is shown in Supplementary Fig. S1. Most of the small RNAs sequenced were 21–23 nt, and the highest peak appeared at 22 nt.

In the oyster gonad, 1467 tags were identified as potentially known miRNAs by aligning the small RNAs with all known miRNA precursors in animals, and 3754 tags were identified as potentially novel miRNAs. The number of mature miRNAs in each library differed. The M-3nα-1 library had the most conserved miRNAs (501 unique known miRNAs), whereas F-3nα-1 had the fewest (255 unique known miRNAs) (Table 1).

3.2. Differential expression of miRNAs between fertile (2n and 3nα) and sterile (3nβ) oysters

To determine the miRNAs and their target genes that play a regulatory role in triploid gonadal sterility, we obtained six groups: F-2n, M-2n, F-3nα, M-3nα, F-3nβ, and M-3nβ. The data processing and downstream analysis of miRNA and gene expression profiles are depicted as a workflow in Fig. 1. In brief, identification and statistical analysis of DEMs and DEGs were performed. miRNA data were submitted for analysis using a Venn diagram and heatmap to visualize the differences between sterile and fertile oysters. The list of DEMs and DEGs was also utilized for integration, network, and miRNA target analyses.

The TPM method was utilized to standardize and calculate the abundance of miRNAs. To obtain smaller but more biologically relevant sets of DEMs between fertile and sterile oysters, we applied a more stringent criterion and took the intersection of two comparison groups (3nα vs. 3nβ and 2n vs. 3nβ). Specifically, both the 2n and 3nα groups are fertile, and the 3nβ group is sterile; thus, we compared miRNA expression between two different groups, 3nβ vs. 2n and 3nβ vs. 3nα, and then took the intersection of the two groups to screen DEMs. In the female comparison, a total of 50 DEMs exhibited differential expression

Table 1
Summary of miRNA identification in Pacific oyster gonads.

Type	Known miRNA number	Novel miRNA number	Total miRNA number
All	1467	3754	5221
F-2n-1	294	1479	1773
F-2n-2	376	1668	2044
F-2n-3	337	1797	2134
M-2n-1	329	1777	2106
M-2n-2	454	2178	2632
M-2n-3	335	1486	1821
M-3nα-1	501	2354	2855
M-3nα-2	302	2266	2568
M-3nα-3	319	2264	2583
M-3nβ-1	323	1912	2235
M-3nβ-2	358	1433	1791
M-3nβ-3	439	2169	2608
F-3nα-1	255	1790	2045
F-3nα-2	296	2116	2412
F-3nα-3	326	1844	2170
F-3nβ-1	397	1174	1571
F-3nβ-2	301	1438	1739
F-3nβ-3	351	1489	1840

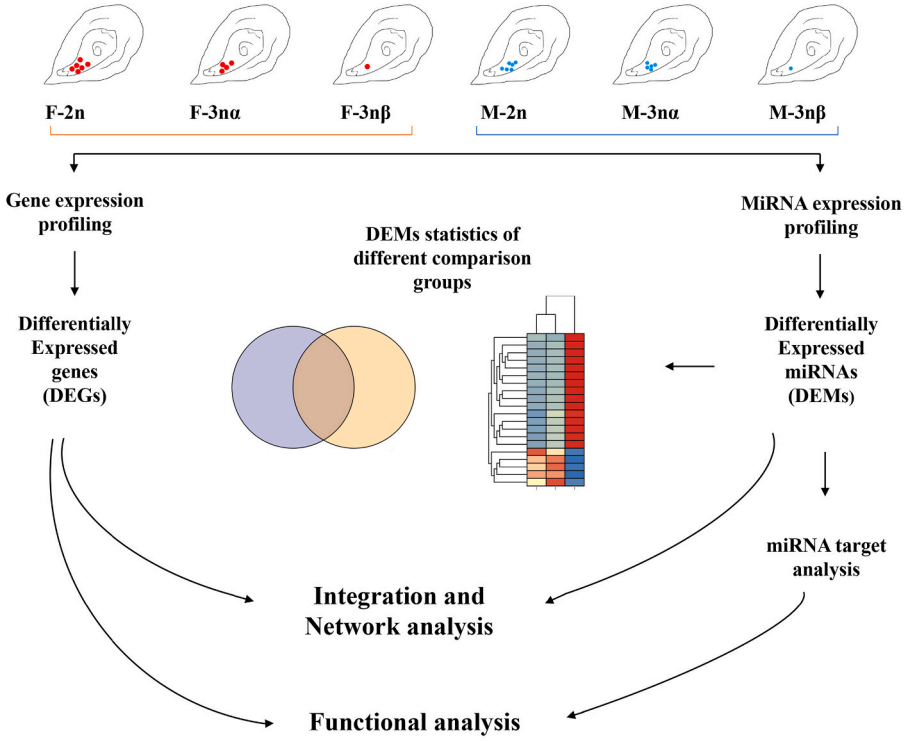

Fig. 1. Data processing and miRNA and gene expression profile analysis workflow.

patterns between fertile and sterile oysters (Fig. 2A), including nine known miRNAs (five upregulated and four downregulated in 3nβ) and 41 novel miRNAs (twelve upregulated and 29 downregulated in 3nβ) (Supplementary Table S5). In the male comparison, the abundance of 92 DEMs was significantly different between the fertile and sterile oysters (Fig. 2B). Of these miRNAs, ten were known miRNAs (five upregulated and five downregulated in 3nβ), and 82 were novel miRNAs (22 upregulated and 60 downregulated in 3nβ) (Supplementary Table S6).

Hierarchical clustering was used to describe the dynamic expression patterns of DEMs, and the entire expression spectrum of DEMs is displayed in the heatmap in Fig. 3. As shown in Fig. 3A and Fig. 3B, two distinct clusters were generated in the female and male comparison groups, respectively. The majority of the selected DEMs were decreased in sterile oysters, and a small proportion of DEMs showed a trend of elevated expression in sterile oysters. Moreover, in the intergroup cluster analysis results, the miRNA expression in diploids and fertile triploids was clustered into one team, in contrast to the sterile triploids.

3.3. Target gene prediction of DEMs and functional enrichment analysis

Compared with the mRNA differentially expressed in the same samples, the number of miRNA target genes determined was reduced. In the females, 44 of 50 DEMs were predicted to be involved in at least one miRNA-mRNA target pair. In the males, only one of the 92 DEMs had no predicted miRNA-mRNA target pair. In Fig. 4A, Venn diagrams demonstrate the number of unique and common target DEGs between fertile and sterile oysters. Altogether, 146 and 307 DEGs were characterized as target genes in the female comparison group and male comparison group, respectively (Supplementary Tables S7, 8). In addition, the two comparison groups of DEMs shared only twelve potential target DEGs.

The GO term distribution of the predicted target genes is displayed in Fig. 4B. For both the female and male comparison groups, targeted genes mainly participated in catalytic activity and binding in molecular function. Targeted genes in biological processes were mostly associated with the cellular process and single-organism process. In the cellular component category, target genes were mostly involved in cell and cell part.

To explore the target gene pathways that were significantly enriched during the sexually mature stage, we carried out functional KEGG analysis (Fig. 4C). In the female comparison group, the “PI3K-Akt signaling pathway” was the most enriched pathway, and the pathway with the largest number of involved genes was “RNA degradation”. Furthermore, twelve significantly enriched KEGG pathways were

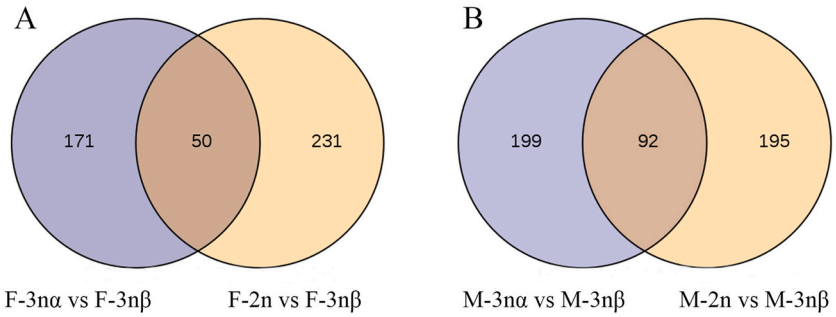

Fig. 2. Venn diagrams of differentially expressed miRNAs between fertile and sterile oysters. A: Female comparison group. B: Male comparison group.

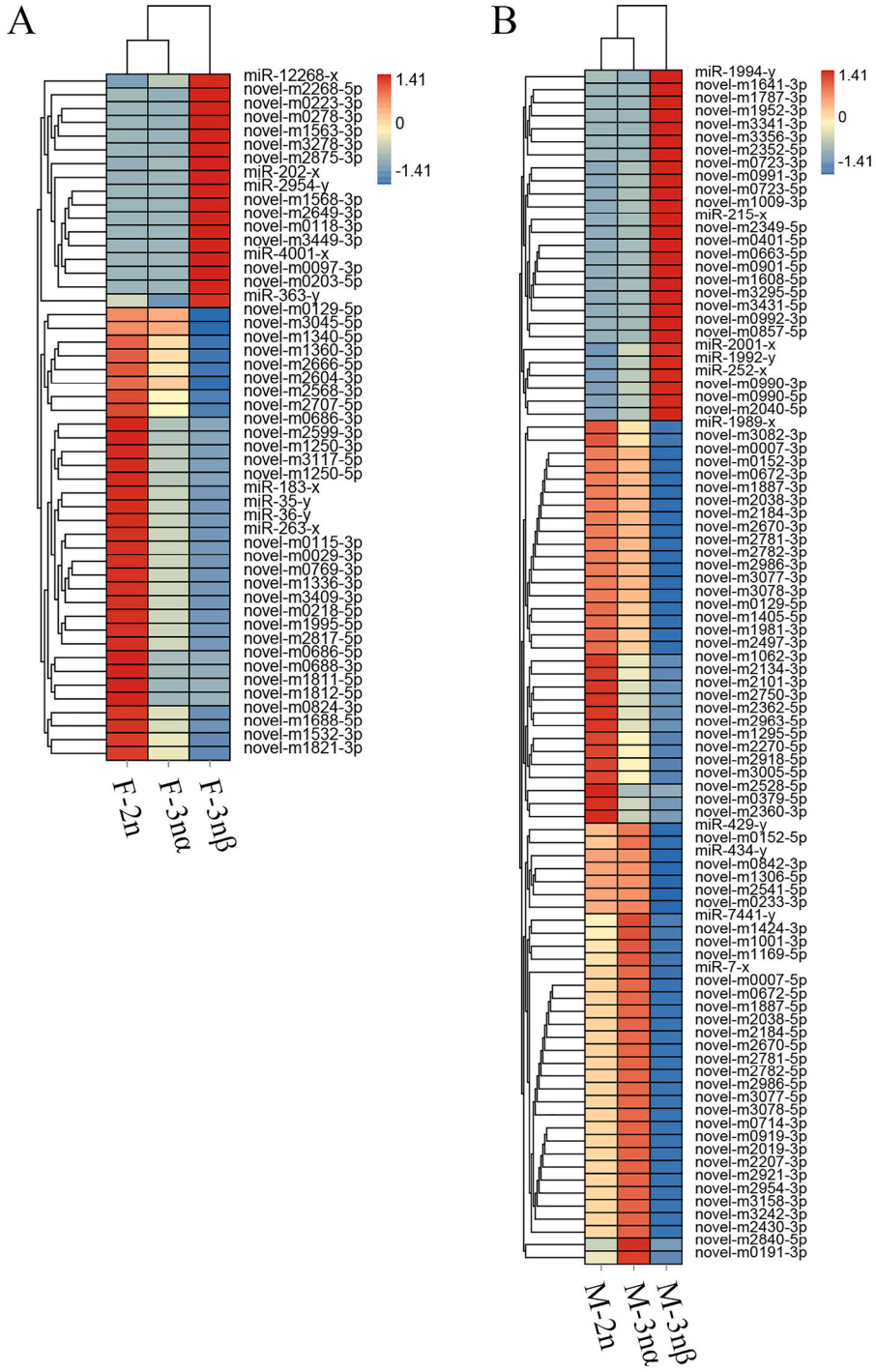

Fig. 3. Heatmap and hierarchical clustering were performed in all the groups. Each column represents one group, and each row represents one miRNA. miRNA with a lower expression level is shown in blue, while miRNA with a higher expression level is shown in red. A: Female comparison group. B: Male comparison group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detected in the male comparison group. Among those, the "Adrenergic signaling in cardiomyocytes" was the most enriched pathway, and the "RNA degradation" pathway included the largest number of genes.

3.4. Integrated miRNA-mRNA networks

DEM-DEG networks were built to study the potential roles of miRNAs in regulating critical DEGs in gonad development, with the expression of miRNAs higher than ten TPM (at least in one group of 2n, 3nα, and 3nβ). Using functional analysis of DEMs, we were able to anticipate the roles of miRNAs with focused relationships to mRNAs.

In the female comparison group, we only constructed networks of downregulated miRNAs and upregulated genes after removing DEMs with lower expression (TPM < 10 in at least two of the 2n, 3nα, and 3nβ groups). According to the constructed networks and prediction analysis (Fig. 5A), two miRNAs (miR-183-x and miR-263-x) and their target DEGs may have a role in controlling lipid droplet formation. The networks showed the potential targets for miR-183-x and miR-263-x: *Abhydrolase domain-containing protein 15-like* (*Abhd15*), *DnaJ homolog subfamily B member 6-like isoform X2* (*Dnajb3*), *putative nuclear hormone*

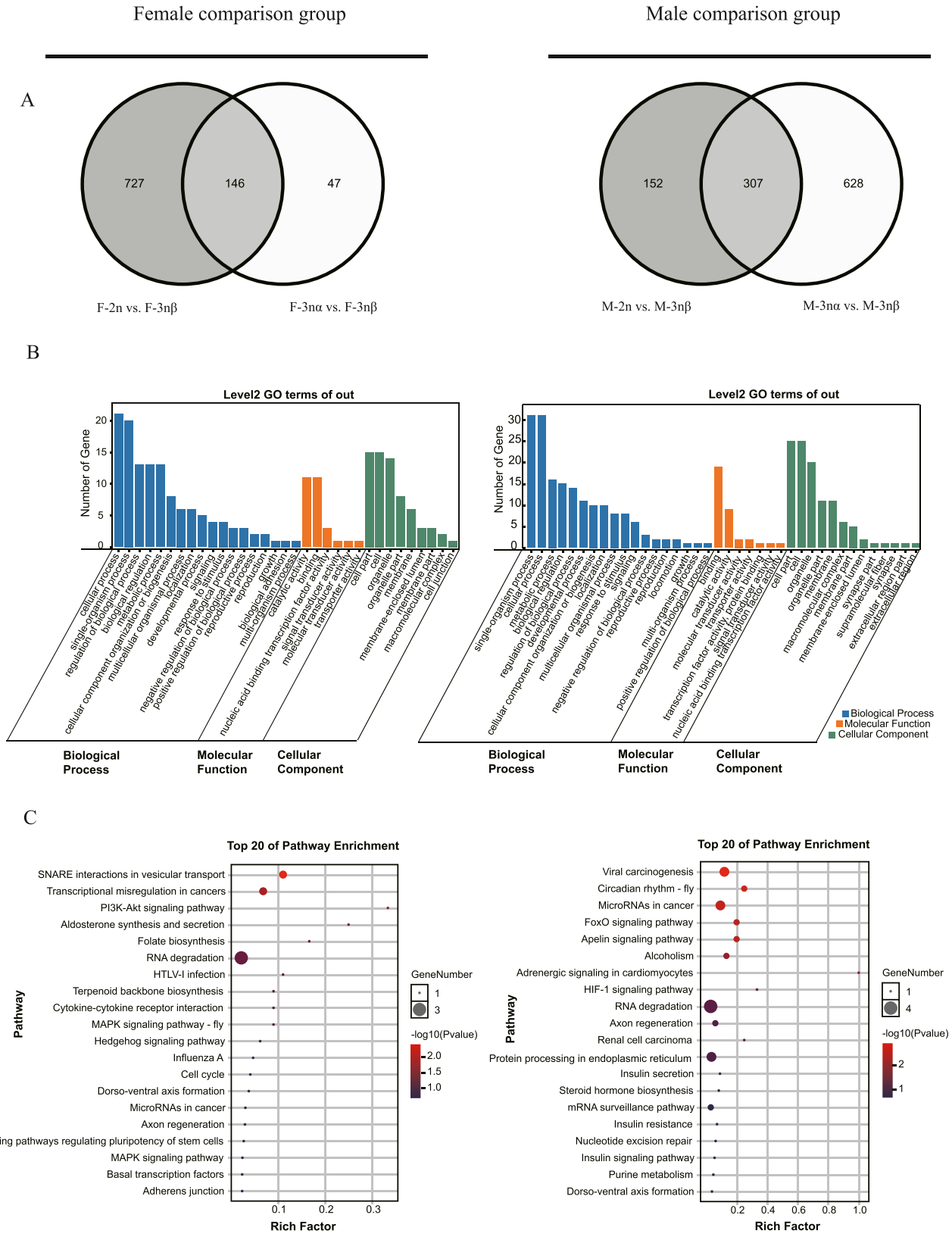

Fig. 4. Differentially expressed target genes and functional enrichment analysis during gametogenesis. (A) Venn diagram with intersected genes showing the number of differentially expressed target genes in 2n vs. 3nβ and 3nα vs. 3nβ. (B) Gene ontology distribution of the intersected target genes. (C) KEGG enrichment pathways of differentially expressed miRNA target genes in the fertile vs. sterile comparison. The KEGG enriched pathways are represented on the y-axis, and the enrichment factor is derived as the ratio of differentially expressed mRNAs divided by the number of annotated mRNAs in the pathway.

receptor HR38 (*Hr38*), *Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 2* (*ARAP2*), all connected to lipid metabolism.

In the male comparison group, we performed two analyses after removing DEMs with lower expression (TPM < 10 in at least two of the 2n, 3nα, and 3nβ groups), where we computed the networks of downregulated genes and upregulated miRNAs and, vice versa, the networks of downregulated miRNAs and upregulated genes (Fig. 5B). Integration analysis of the selected DEMs and DEGs showed two classes of regulatory networks in the male comparison group. Networks with seven and eleven nodes were obtained using the upregulated and downregulated DEMs in M-3nβ, respectively. Among the DEMs, there were five upregulated miRNAs (miR-1992-y, miR-1994-y, miR-2001-x, miR-252-x, and novel-m0990-5p) in M-3nβ. For the upregulated miRNAs, the target DEGs of miR-1992-y present in the network were *aarF domain-containing protein kinase 5-like isoform X1* (*ADCK5*), *LRP2-binding protein-like* (*lrp2bp*), *dynein beta chain, ciliary-like isoform X8* (*DNAH9*), and *axonemal dynein light chain domain-containing protein 1-like isoform X2* (*AXDND1*), involved in spermatogenesis and spermatozeugma migration. The target DEGs of miR-1994-y were *kinesin-like protein KIF20B* (*Kif20a*), *M-phase inducer phosphatase-like isoform X1* (*Cdc25b*), *rho GTPase-activating protein 11A-like* (*Arhgap11a*), *ciliogenesis-associated TTC17-interacting protein-like* (*catip*), *cilia- and flagella-associated protein 97-like* (*cfap97*), and *ADP-ribosylation factor-like protein 3* (*ARL3*), which are associated with the cell cycle and flagellar formation in spermiogenesis. The target DEGs of miR-2001-x were *abnormal spindle-like microcephaly-associated-like protein* (*ASPM*) and *kinesin-like protein KIF23 isoform X2* (*KIF23*), which are connected to spindle formation and the formation of intercellular bridges during spermatogenesis. The target DEGs of miR-252-x and novel-m0990-5p were *Kif20a*, *transcription factor E2F8-like isoform X1* (*E2F7*), *Lrp2bp*, *coiled-coil domain-containing protein 113-like* (*CCDC113*), and *SFI1-like protein* (*SFI1*), which are related to the cell cycle, cell migration, and ciliogenesis. miRNA-mRNA expression analysis revealed three DEMs (miR-1989-x, novel-m0129-5p, and novel-m0379-5p) with low expression in sterile males predicted to target genes associated with the restriction of cell proliferation (*E3 ubiquitin-protein ligase ZNRF3-like* (*znrf3*)) or promotion of glycogen synthesis (*protein phosphatase 1 regulatory subunit 3B-like isoform X2* (*PPP1R3B*)).

3.5. qRT-PCR verification of DEM and DEG expression

A total of nine core DEMs (miR-183-x, miR-263-x, miR-1992-y, miR-1994-y, miR-2001-x, miR-252-x, novel-m0990-5p, miR-1989-x, and novel-m0379-5p), which might be related to sterility in the constructed networks, were chosen for qRT-PCR to determine their expression levels. The expression levels of three DEMs (miR-263-x, miR-1992-y, and miR-2001-x) were found to be consistent with next-generation sequencing data (Fig. 6A). The other six DEMs showed the same expression trends as the sequencing results but were not significant. For qRT-PCR analysis, we selected ten sterile-related target genes (*Abhd15*, *Dnajb3*, *Hr38*, *ARAP2*, *ADCK5*, *lrp2bp*, *DNAH9*, *AXDND1*, *ASPM*, and *KIF23*) of the above three DEMs showing opposite expression patterns. The results demonstrated that the expression profiles of these target genes (except *lrp2bp* and *ASPM*) matched the sequencing results (Fig. 6B).

4. Discussion

The mechanism of sterility in triploid oysters is complex and is likely tightly regulated by genes from the transcriptome perspective: the downregulated genes related to gametogenesis are likely involved in limiting germ cell proliferation, whereas the expression of genes related to glycogen synthesis is upregulated, and the energy used for reproduction could conceivably be stored as glycogen in sterile triploid oysters (Chen et al., 2021). While extensive differences in the transcriptome are evident, the functional importance of gene expression regulation in germ cell development remains elusive. The purpose of our study was to combine miRNA and transcriptome data from diploid and triploid oysters to broaden our understanding and further understand the regulatory role of miRNAs in sterility. In summary, we constructed predicted interaction networks between miRNAs and mRNAs linked to sterility in triploid oysters. This work investigated the potential interplay between DEMs and DEGs during gametogenesis and germ cell development in triploid oysters.

miRNAs are fine tuners of a variety of biological activities, including fundamental cell proliferation and development, by posttranscriptionally controlling gene expression (Galagali and Kim, 2020). The importance of miRNAs in reproduction has been reported (Reza et al., 2019). We identified 50 DEMs and 91 DEMs that might be associated with gonadal dysgenesis in the female and male comparison groups, respectively. This result suggests that miRNAs might play an important role in the process of gametogenesis in the Pacific oyster. KEGG functional enrichment analysis of DEM target genes revealed that the pathways involved in RNA degradation might have pronounced impacts on the sterility of triploids. RNA degradation is required in the germline of multiple animal models for fertility (Nguyen-Chi and Morello, 2011; Blatt et al., 2021). Dysregulation of miRNA target genes associated with RNA degradation might lead to defects in gametogenesis in triploid oysters.

4.1. miR-263-x may regulate lipid droplet formation and glycogen storage in females

Our analysis revealed that miR-263-x (downregulated in sterile F-3nβ compared to fertile F-2n and F-3nα) might serve as a potential regulator of the target DEGs in females. In animals, miR-263a and miR-263b are thought to be members of a conserved family of miRNAs. Several studies have demonstrated that miR-263 can regulate ovary development in crabs and insects (Li et al., 2021; Zhou et al., 2020). In our study, several genes (*Abhd15*, *Dnajb3*, *Hr38*, and *ARAP2*) involved in lipid metabolism were estimated to be targeted by miR-263-x, showing that miR-263-x might regulate lipid synthesis. The four target DEGs showed upregulated expression in F-3nβ. In various murine and human cell lines, *Abhd15* has been shown to be a significant factor in the development of adipocytes and was found to be strongly upregulated during adipogenesis (Walenta et al., 2013). In 3T3-L1 adipocytes, *Dnajb3* appears to play a role in regulating glucose balance and insulin signaling and in promoting both basal and insulin-stimulated glucose uptake (Arredouani et al., 2019). *Hr38* is a ligand-regulated transcription factor that regulates carbohydrate metabolism and maintains glycogen storage during the developing larval stage in *Drosophila* (Ruaud et al., 2011). *ARAP2* is considered to be droplet-associated, and knockdown of *ARAP2* reduced triglyceride synthesis and lipid droplet formation in mouse fibroblasts (Chaudhari et al., 2016). The upregulation of these targets in F-3nβ illustrates that active fat synthesis and glycogen storage occurs in the gonads of sterile females. miR-263-x has potential targets involved in lipid synthesis and glycogen storage, according to our network analysis.

The success of gametogenesis depends on both exogenous and endogenous sources of nutrients and energy. In the gonadal tissues of Pacific oysters, the cells of vesicular connective tissue, also known as storage cells, are the predominant cell group, and they provide and accumulate nutrition for growing gametes. In the breeding season, the number of storage cells and developing germ cells demonstrate an inverse proportion of size dynamics: the smaller the number of gametes present, the more storage cells there are (Yurchenko and Kalachev, 2019). Accumulated nutrition allows oysters to produce mature gametes. Fertile oysters may use storage cell energy to produce many mature oocytes, whereas sterile F-3nβ triploids have very few follicles and oocytes. In our observations, sterile triploid females have large regions of connective tissue where they may have numerous lipid droplets and glycogen particles. There is also experimental evidence indicating that triploid oysters have a higher glycogen content than diploid oysters

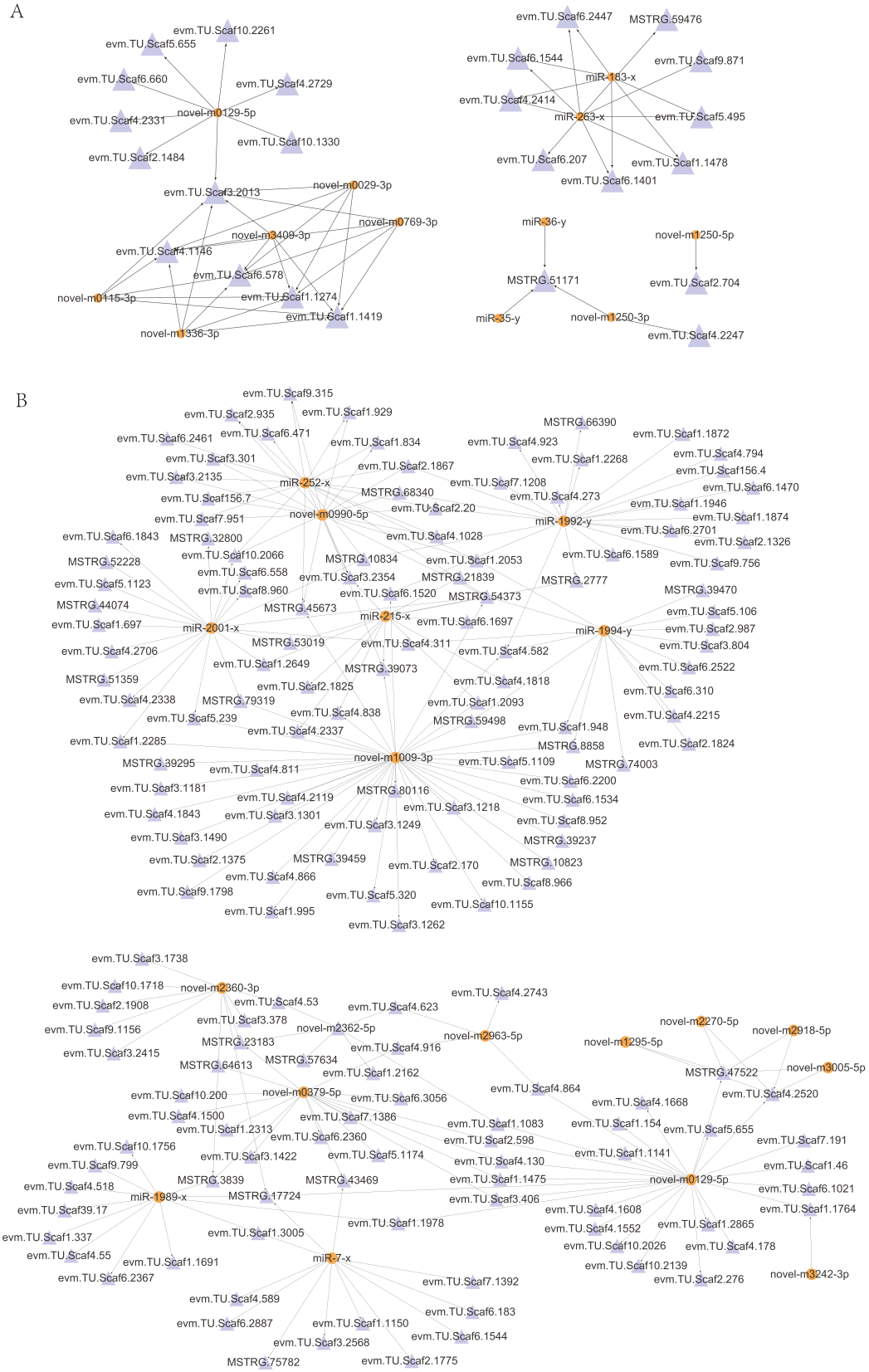

Fig. 5. Differentially expressed miRNAs and their target interactions in a network. (A) The interaction networks of miRNA with mRNA in the female comparison group. (B) The interaction networks of miRNA with mRNA in the male comparison group.

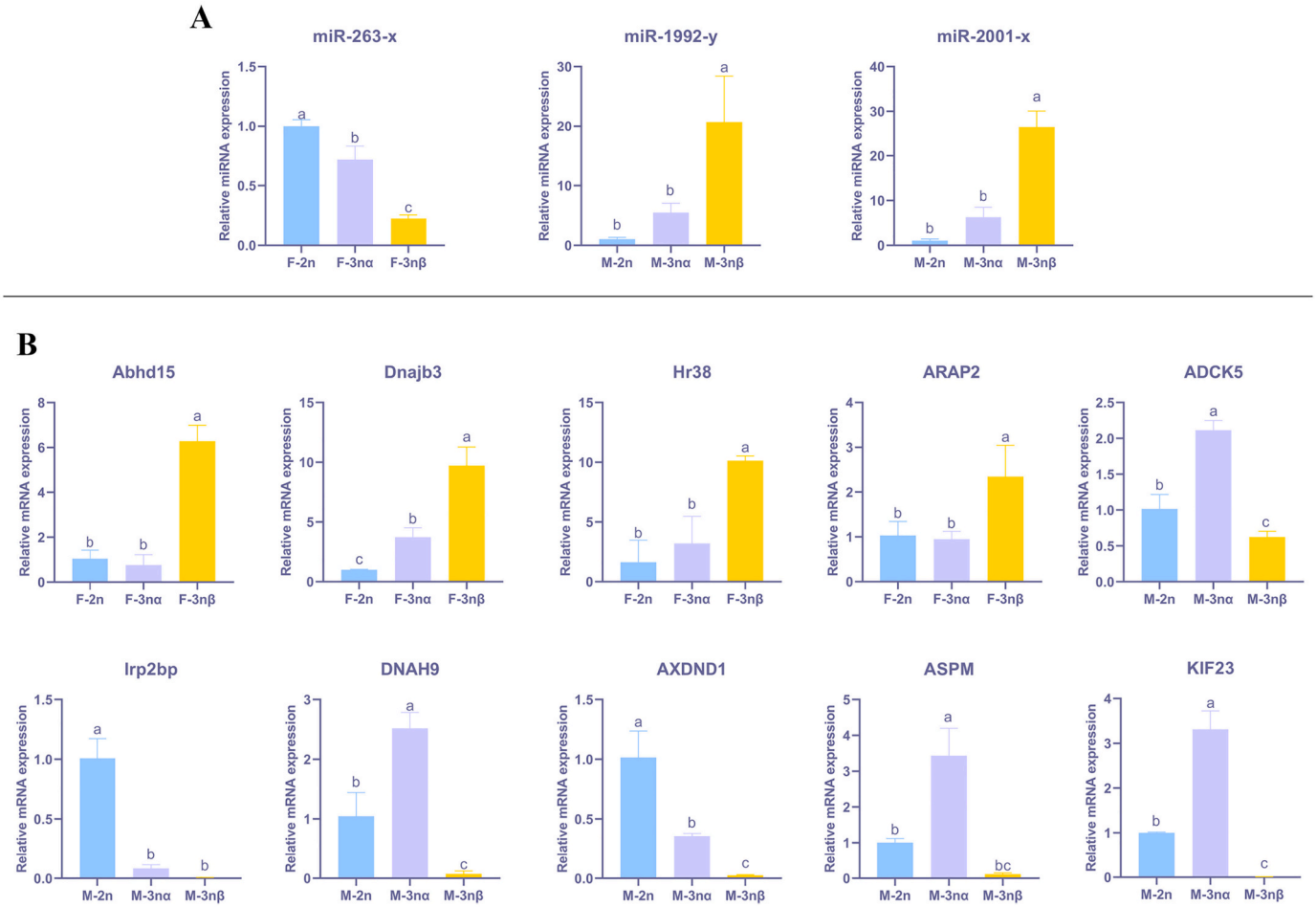

Fig. 6. Expression of miRNAs (A) and mRNAs (B) quantified with qRT-PCR. Values are shown as the mean ± SD ($n = 3$). Significant differences are denoted by distinct letters (a, b, and c), $p < 0.05$.

(Allen and Downing, 1986; Wang et al., 2021). The decreased expression of miR-263-x in sterile F-3nβ might result in lipid accumulation and glycogen storage in the storage cells of sterile females through targeting of genes associated with energy metabolism.

4.2. *miR-1992-y and miR-2001-x may play a regulatory function in the spermatogenesis process in males*

miR-1992-y has been identified in the gonads of *Crassostrea hongkongensis* (Wei et al., 2019), but few functional studies have been conducted. Among the target genes of miR-1992-y identified in our study, *ADCK5* belongs to the atypical kinase family (Manning et al., 2002) and is overexpressed in some cancer tissues and cells. Research has shown that *ADCK5* regulates the expression of the tumor oncogene human *pituitary tumor transforming gene-1* (*PTTG1*) by phosphorylating the transcription factor *SOX9*, allowing lung cancer cells to migrate and invade more effectively (Qiu et al., 2020). *PTTG1* is expressed specifically in the adult testis and may play a role in spermatogenesis (Pei, 1999). In our study, we found that *PTTG1* showed decreased expression in sterile M-3nβ compared to fertile M-2n and M-3nα, suggesting that the *ADCK5-SOX9-PTTG1* pathway mediated by miR-1992-y might exist in oysters to regulate spermatogenesis. Moreover, other targets of miR-1992-y, such as *DNAH9* and *AXDND1*, are essential for spermatogenesis, cell proliferation and migration, and sperm motility. *DNAH9*, a key component of outer dynein arms in flagellum and cilia, is expressed throughout the sperm tail and encodes a portion of the outer dynein arm heavy chain components (Fliegauf et al., 2005). Variants in *DNAH9* may lead to male infertility (Tang et al., 2021). *AXDND1* was found to be a novel testis-enriched gene that is necessary for spermiogenesis and male fertility (Ma et al., 2021). Cell migration is critical for development and a variety of biological processes. Spermatogenesis occurs in the gonadal follicles of oysters and culminates in the creation of spermatozeugma, which clump the spermatozoa together until they are exposed to ocean water. The downregulation of the target genes associated with cell migration may hinder spermatozeugma aggregation during spermatogenesis in M-3nβ. The cilia, or flagella, are a crucial part of the sperm tail structure. They play an important role in the sperm motility system. Flagellar or ciliary structural anomalies in the sperm tail directly impair sperm motility. The targets downregulated in M-3nβ may contribute significantly to a decline in sperm motility. Overall, these results suggest that miR-1992-y may suppress spermatogenesis and spermatozeugma migration and affect sperm motility by directly targeting the genes mentioned above.

miR-2001-x was recognized in the sea cucumber *Apostichopus japonicus* during intestine regeneration, and it was speculated that miR-2001-x may be involved in intestine regeneration (Sun et al., 2017). Several lines of evidence indicate a role for miR-2001-x as an essential regulator of spermiogenesis in lower animals. The creation of persistent intercellular bridges between daughter cells is a constant aspect of germ cell cytokinesis. Previous evidence has demonstrated that intercellular bridges play a significant role in postnatal spermatogenesis in mammals (Greenbaum et al., 2009). It is worth noting that *KIF23* has been discovered as a component of intercellular bridges during spermatogenesis (Greenbaum et al., 2007). Thus, decreased expression of *KIF23* targeted by miR-2001-x is frequently associated with the inability to form intercellular bridges during spermatogenesis, likely resulting in a

reduction in the number of sperm in triploid oysters.

In summary, the integrated analysis in this study revealed the roles of miRNAs in gonadal development in triploid *C. gigas*. Our results identified three miRNAs that may act as regulators of genes associated with gonadal dysgenesis in triploid oysters, providing valuable information for further studies exploring the molecular mechanisms of gonad development in oysters.

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CRediT authorship contribution statement

Chen Chen: Investigation, Formal analysis, Data curation, Writing – original draft. **Hong Yu:** Supervision, Resources, Writing – review & editing. **Qi Li:** Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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