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Integrated analysis of miRNA and mRNA expression profiles identifies potential regulatory interactions during sexual development of Pacific oyster *Crassostrea gigas*

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ABSTRACT

One of the most important physiological process in the life cycle of most bivalves is the sexual reproduction required for species propagation. Understanding the molecular regulatory mechanisms of gender determination and gonad development have potential application in bivalve aquaculture, but there is still limited knowledge about this issue. The Pacific oyster *Crassostrea gigas* is a commercially important bivalve in aquaculture worldwide, as well as a representative specie to study the sexual development in bivalves for its complicated sexuality, including dioecy, sex change and rare hermaphroditism. In this study, an integrated analysis of miRNA and mRNA expression profiles of oyster gonads at different gametogenesis stages was performed to explore their possibly regulatory patterns in sexual development of *C. gigas*. Based on small RNA sequencing, a total of 122 miRNAs were identified, including 53 conserved and 69 novel miRNAs. Six miRNAs exhibited sex-specific pattern that expressed exclusively from either male or female gonads with increasing abundance throughout the gonadal development. Moreover, 287 putative targets of the six sex-biased miRNAs were predicted by combining previous transcriptome data. GO and KEGG enrichment analysis of target genes revealed that these sex-biased miRNAs might participate in the regulation of gametogenesis. Further, potential miRNA-mRNA interactions based on six sex-biased miRNAs were collected. The homolog of a key participator in testis development, meiotic recombination protein SPO11 in *C. gigas* (CgSPO11), was predicted as a target of female-biased miRNA lgi-miR-96a. This negative miRNA-mRNA expression relationship was validated in additional samples by quantitative real-time PCR (qRT-PCR) and the regulation of CgSPO11 by lgi-miR-96a was preliminarily confirmed by a dual-luciferase reporter assay. This study provides information regarding the gonad miRNAs of *C. gigas* and leads to the discovery of miRNA-mRNA interactions in gonad development, which will facilitate further investigations in molecular mechanisms of bivalve sex differentiation.

1. Introduction

The bivalve aquaculture industry has been steadily increasing over the past decades (~2.7 million tons in 1970 to ~19.5 million tons in 2018, global production; FAO Global Aquaculture Production Statistical Database), and served as an important source of protein for millions of people. A better understanding of the reproductive biology of bivalve species is of crucial importance for their conservation and for maintaining and restocking populations (Breton et al., 2018). Further, control of gender and gonadal differentiation can help to reach important goals in bivalve aquaculture, such as producing monosexual or effectively sterile domesticated individuals to protect advances in genetic manipulation. Hence, the sex determination and differentiation of bivalve have long been of interest to researchers in aquatic science.

The Pacific oyster (*Crassostrea gigas*) is one of the most important commercial bivalves cultivated worldwide, as well as a typical model to understand the sexual development in bivalves. It is dioecious without secondary sex characteristics and can alternate sex between different reproductive seasons (Park et al., 2012; Yasuoka and Yusa, 2016). Furthermore, occasional hermaphroditism can also be observed (Coe,

Abbreviations: FOXL2, forkhead box L2; SOX9, SRY-box containing gene 9; SPO11, meiotic recombination protein SPO11; Tβ4, thymosin beta 4; GCs, granulosa cells.

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1943; Guo et al., 1998). Both genetic and environmental factors are probably responsible for the alternative sexuality in this species. Two genetic models, two-genotypes and three-genotypes, were proposed to explain the genetic impacts on sex ratios in factorial and nested crosses (Guo et al., 1998; Hedrick and Hedgecock, 2010). Exogenous steroids, temperature and food availability were also suggested as critical environmental factors that affect the sex ratios (Lango-Reynoso et al., 2006; Santerre et al., 2013). Recently, we performed a restriction site-associated DNA sequencing analysis based on observation of individual sex changes, and it suggested that *C. gigas* might have the ability to modify the activity of genes in response to changes in the internal or external environment, integrating genomic and environmental information to generate a particular phenotypic sex (Yue et al., 2020). In this developmental context, where many genes must be activated or suppressed in a tight spatial and temporal fashion, the regulations of gene expression in *C. gigas* sexual development become of critical importance as demonstrated across taxa (Barrionuevo et al., 2012; Piferrer, 2013). Though lists of sex-related genes have been identified in *C. gigas* by gonad transcriptome analysis (Zhang et al., 2014; Yue et al., 2018), the regulatory mechanisms in the process is still poorly understood.

MicroRNAs (miRNAs), non-coding RNAs that have approximately 22 nucleotides (nt), have emerged as key post-transcriptional regulators of gene expression in eukaryotes (Bartel and Chen, 2004). It has been reported that miRNAs play primary roles in regulation of sexual fate determination across taxa. In mice, a miRNA (mmu-miR-124) prevents the testis-determining factor SOX9 (SRY-box containing gene 9) expression in developing ovarian cells (Real et al., 2013), and the conserved gonadic microRNA miR-202-5p/3p is downstream of SOX9 in testis development (Wainwright et al., 2013). Research in *Caenorhabditis elegans* shows that the miR-35 family regulates the sex determination pathway at multiple levels, acting both upstream of and downstream from *her-1* to prevent aberrantly activated male developmental programs in hermaphrodite embryos (McJunkin and Ambros, 2017; Benner et al., 2019). Such miRNA-mRNA regulatory mechanisms in sex determination or gonadic development have been investigated precisely in model organisms, and recent small RNA sequencing studies pointed to miRNAs participation in sex differentiation of some aquatic species, such as tilapia and Amur sturgeon (Tao et al., 2016; Zhang et al., 2018). In tilapia, some miRNAs (*e.g.*, miR-96 and miR-737) were reported to target multiple genes involved in steroid synthesis, suggesting a role for miRNAs in regulating the biosynthesis of steroid hormones during tilapia early sex differentiation. (Tao et al., 2016).

The miRNA-mediated post-transcriptional regulation has been proved to be critical in sex determination and differentiation across taxa (Real et al., 2013; Wainwright et al., 2013; McJunkin and Ambros, 2017; Benner et al., 2019), while the possible role of miRNA in mollusk sex determination and differentiation is still not well understood. The advances of next generation sequencing technology provide opportunities to efficiently characterize small RNA transcriptomes in mollusks. In the present study, we investigated the miRNA profiles of *C. gigas* gonads at four development stages, including the resting, early gametogenesis, maturation, and spawning stage. Moreover, an integrated analysis of gonad miRNA and mRNA expression was performed by combing previous transcriptome data (Yue et al., 2018). These analyses might provide new information on the role of miRNA in oyster gonad function and help to clarify the regulatory network during sex determination and differentiation of *C. gigas*.

2. Materials and methods

2.1. Animal material sampling for small RNA sequencing

Twenty-one individuals investigated in our previous RNA-seq study were selected for small RNA sequencing (Yue et al., 2018). In brief, two-years-old individuals of *C. gigas* were collected monthly between December 2015 and July 2016 from Rushan, Shandong, China. Individual tissues were conserved in RNAwait (Solarbio) and stored at −80 °C. Gonad development stage and sex of the oysters were determined by histological methods (Enríquez-Díaz et al., 2008; Li et al., 2006). Twenty-one gonad samples were selected according to their representativeness of a gonadic category, with three individual per sex and stage except for stage II. For convenience, S0, S1F, S1M, S3F, S3M, S4F, and S4M stood for samples assigned to stage 0 (undifferentiated stage), stage I (early development stage), stage III (maturation stage), stage IV (spawning stage) with suffix by "F" or "M" to distinguish female or male respectively.

2.2. RNA extraction, libraries preparation and small RNA sequencing

RNA preparation was carried out in the same way as previous RNA-seq study (Yue et al., 2018) and total RNA was used as input material for the small RNA library. NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA.) was used for library construction. Firstly, small RNAs were ligated sequentially to 3′ and 5′ adapters. Then first strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H–) and the double-strand cDNA was performed followed by PCR amplification. PCR products corresponding to 140–160 bp (the length of small noncoding RNA plus the 3′ and 5′ adaptors) were recovered from 8% polyacrylamide gel. The library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. After cluster generation using TruSeq SR Cluster Kit v3-cBot-HS (Illumina), the libraries were sequenced on an Illumina Hiseq 2500 platform and 50 bp single-end reads were generated.

2.3. Filtering small RNA sequencing reads and small RNA identification

Clean reads of small RNA sequencing were obtained by trimming raw reads containing ploy-N, with 5′ adapter contaminants, without 3′ adapter or the insert tag, containing ploy A or T or G or C and low-quality reads. High quality clean reads ranging from 18 to 35 nt were mapped to *C. gigas* genome (oyster_v9) (ftp://ftp.ncbi.nlm.nih.gov/genomes/Crassostrea_gigas/) by Bowtie (Langmead et al., 2009) without mismatch. The mapped small RNA tags were aligned against the miRBase20.0 using modified software mirdeep2 (Friedländer et al., 2012) to obtain the known miRNA and the secondary structures of the conserved miRNA were drawn by srna-tools-cli. The unannotated reads were further filtered by annotated rRNA, tRNA, snRNA, snoRNA and repeat sequences in using RepeatMasker (open-4.0.3) software. Then, the available software miREvo (Wen et al., 2012) and mirdeep2 (Friedländer et al., 2012) were integrated to predict novel miRNA through exploring the secondary hairpin structure of miRNA precursor, the Dicer cleavage site and the minimum free energy of the small RNA tags unannotated in the former steps. To make every unique small RNA mapped to only one annotation, the priority rule was followed: known miRNA > rRNA > tRNA > snRNA > snoRNA > repeat > gene > NAT-siRNA > gene > novel miRNA > ta-siRNA.

2.4. Quantification and differential expression of miRNAs

The miRNAs expression levels were calculated by TPM (transcript per million) method, in which normalized expression = mapped read count/total reads × 1000000. Then paired differential miRNAs expression analysis was performed by comparing TPM value using DEseq R package (Anders and Huber, 2010). A false discovery rate $q < 0.05$ was set as the threshold for significantly differential expression. Hierarchical cluster analysis of differentially expressed miRNAs union was conducted to assess the expression pattern variations among different gonadic categories using pheatmap R package.

Table 1
The primer sequences used for miRNA real-time qPCR in this study.

Primer	Sequence (5′ to 3′)
lgi-miR-96a	CTTGGCACTGGCGGAATAATCA
lgi-miR-279	TGACTAGATCCACACTCATCCA
lgi-miR-263b	AATGGCACTGGTAGAATTCACGG
lgi-miR-8	TAATACTGTCAGGTAAAGATGTC
lgi-miR-29	CGGTAGCACCATTTGAAATCAGT
novel_167	TTTGGTAACCTAGCTTTATGAA
5S	TTGGATGGGTGACCGCCTG

2.5. Target mRNA prediction of miRNAs and GO and KEGG enrichment analysis

To predict mRNA targets of miRNAs, gene sequences were extracted from the annotation files of *C. gigas* genome and downstream 1000 bp sequences from the termination codon of genes without annotation information were obtained to determine complementary base-pairing relationships with known and novel miRNAs. Subsequently, miRanda-3.3a (Enright et al., 2003) was employed as the following command parameters: -sc 140, -en -10, -scale 4, -strict, -out. Based on the results of miRanda algorithm, the target genes of differentially expressed miRNAs were compared with the differentially expressed mRNA from transcriptome data. The target genes that were found to be differentially expressed in samples at the same gonadic category were considered as candidates.

Gene Ontology (GO) enrichment analysis based on target mRNAs of the differentially expressed miRNAs was implemented using the GOseq R package (Young et al., 2010), in which gene length bias was corrected and *C. gigas* genome was used as the reference set. Adjusted *P*-value 0.05 was set as the threshold for significantly enriched. KEGG (Kanehisa et al., 2007) is a database resource for understanding high-level functions and utilities of the biological system from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. The statistical enrichment of the target genes in KEGG pathways was tested using KOBAS software (Mao et al., 2005) under the entire genome background and KEGG pathways with corrected *P* value less than 0.05 were regarded as significantly enriched.

2.6. Quantitative real-time PCR assays

The miRNA expression pattern was examined using quantitative real-time PCR (qRT-PCR). Forty-two gonad samples, six biological replicates at per same gonadic category with samples for small RNA sequencing, were selected for RNA extraction. In brief, RNAiso for Small RNA (TaKaRa, Dalian, China) was firstly used for small RNA extraction, then the first-strand cDNA synthesis from miRNA was performed using the Mir-X™ miRNA First Strand Synthesis Kit (Takara, Dalian, China). The amplification was carried out on the LightCycler 480 real-time PCR instrument (Roche Diagnostics, Burgess Hill, UK) using Mir-X™ miRNA qRT-PCR TB Green™ Kit (TaKaRa). The primers for miRNA qRT-PCR are listed in Table 1. For each miRNA qRT-PCR reaction, combine the following reagents: cDNA, 2.0 μl; miRNA-specific primer (10 μM), 0.5 μl; mRQ 3' Primer (10 μM), 0.5 μl; ROX Dye (50×), 0.5 μl; TB Green Advantage Premix (2×), 12.5 μl; ddH_2O 9 μl. The reaction condition was denaturation of 95 °C for 10 s, then 40 cycles of 95 °C for 5 s, 60 °C for 20 s. The dissociation curves of PCR products (95 °C 60 s, 55 °C 30 s, and 95 °C 30 s) were performed to ensure the detection of a single specific product. Each microplate included negative control (template-free reaction) and blank control (sterile water). 5S rRNA expression was used to normalize miRNA expression. PCR efficiency and proper dilution of cDNA was determined for each primer pair by constructing a five points standard curve from 5-fold serial dilutions of a S3_F or S3_M individual template. In addition, potential target gene expression patterns were also investigated on samples for miRNA expression pattern validation as described in our previous study (Yue et al., 2018). The primers for target gene qRT-PCR were provided in Supplementary Table S1. Relative miRNA and mRNA expression levels was calculated by the $2^{-(\Delta\Delta Ct)}$ method (Schmittgen and Livak, 2008). After being examined for homogeneity of variances, data were analyzed by *t*-test using software SPSS 18.0 and levels were accepted as significant at P value < 0.05.

2.7. Dual-luciferase reporter assay

The psiCHECK-2 reporter vector (Promega, USA) was digested with restriction enzyme *Xho* I and *Not* I. DNA fragment containing putative miRNA binding sites and 200 bp 5′ and 200 bp 3′ flanking sequences was amplified using primers with restriction enzyme sites (Table 2). The digested and purified PCR product was ligated with linearized psiCHECK-2 to obtain wild type construct. The site-specific mutagenesis was performed in one-step PCR. Opposite pairs of primers (Table 2) were designed according to putative miRNA binding sites, and mismatches were introduced into seed sequences of binding sites. The wild type construct was used as template and PCR was performed by using Phanta@ Max Super-Fidelity DNA Polymerase (Vazyme, China). After digested by *Dpn* I, the amplified fragment was introduced into the *E. coli* DH 5α to obtain mutant construct. All constructs were verified by sanger sequencing using the conserved primer (5'-GAAGTTCCCTAACACCGAGT-3′). MiRNA mimics and negative control (NC) mimics were synthesized by Sangon Biotech (Shanghai, China). The day before transfection, HEK-293 T cells were seeded in 24-well plates. When cells were ~ 50% confluent, they were co-transfected with 500 ng recombinant vectors and 28 pmol of either miRNA mimics or NC mimics using Attractene transfection reagent (Qiagen, Germany). Each trial contains three parallel samples. The luciferase activity was measured 48 h after transfection using Dual-luciferase Reporter Assay System Kit (Promega, USA) and Synergy H1 Multi-Mode Microplate Reader (BioTek, USA). Firstly, growth medium was removed from the cultured cells and a sufficient volume of phosphate buffered saline (PBS) was gently applied to wash the surface of the culture vessel. PBS were completely removed and 100 μl 1× Passive Lysis Buffer (PLB) was dispensed into each culture well. The culture plate was gently rocked for 15 min at room temperature. Then, 20 μl cell lysates was dispensed into each well of a 96-well plate and 100 μl Luciferase Assay Reagent II was added for measurement of firefly luciferase activity with 2-s delay and a 10-s read. Next, 100 μl Stop & Glo® Reagent was dispensed into each cell followed by 2-s delay and a 10-s read time for Renilla luciferase activity. Relative

Table 2
Primers used for constructing luciferase reporters in this study.

Primer	Sequence (5′ to 3′)
CgFOXL2_psi_WF	CCGCTCGAGGATTAATGCGACCGTCTTC
CgFOXL2_psi_WR	AATGCGGCCGCAATACTGTTCTGCCAACCT
CgFOXL2_psi_MF	CGCCTAACGCTTAGTGGAATATCGCTCTTATTATCAACAAATTTCCG
CgFOXL2_psi_MR	CGGAAATTTGTTGATAATAAGAGCGATATTCCACTAAGCGTTAGGCG
CgSPO11_psi_WF	CCGCTCGAGCAATGACGCCATGAAGATGACGT
CgSPO11_psi_WR	ATAAGAATGCGGCCGCGTAACCGTTGAAATATGGCATC
CgSPO11_psi_MF	TACAGGTAGTAAGATGGCGTCTGCACGTGCTTGCTAC
CgSPO11_psi_MR	CCATCTTACTACCTGTATCATACAGGACACATTTTCC

Table 3
Summary of miRNA identification in gonads in different stages of the Pacific oyster.

	Known miRNA	Precursor of known miRNA	Novel miRNA	Star strand of novel miRNA	Precursor of novel miRNA
Total	53	52	69	53	73
S0_1	51	50	57	27	61
S0_2	51	50	58	32	65
S0_3	47	46	65	33	72
S1F_1	50	48	62	31	66
S1F_2	48	46	56	30	61
S1F_3	49	48	52	29	59
S1M_1	50	50	55	28	60
S1M_2	51	51	57	32	66
S1M_3	51	49	58	31	64
S3F_1	49	47	52	17	55
S3F_2	40	41	45	22	52
S3F_3	39	38	38	14	44
S3M_1	46	44	57	25	62
S3M_2	45	44	45	19	52
S3M_3	49	49	54	23	60
S4F_1	48	47	51	24	56
S4F_2	45	46	47	17	56
S4F_3	50	49	42	24	49
S4M_1	49	48	56	26	61
S4M_2	45	45	49	23	54
S4M_3	49	47	47	23	57

luciferase activity (Renilla luciferase signal: firefly luciferase signal) was analyzed by *t*-test using software SPSS 18.0 and levels were accepted as significant at P value < 0.05.

3. Results

3.1. Small RNA sequencing statistics

Pacific oyster gonads from seven stages of development were sampled and used to prepare a total of seven small RNA libraries. Sequencing of these libraries generated 281,831,815 single-end raw reads (Supplementary Table S2). All raw data were submitted to the Short Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra/) at the National Center for Biotechnology Information (NCBI), in Bioproject PRJNA679138 under the accession number list in Supplementary Table S3. After trimming, 273,874,212 clean reads remained. Majority of small RNA reads in all libraries was between 28 and 31 nucleotides (nt) long (Supplementary Fig. S1). Moreover, libraries from stage 0 and stage I gonads had additional weaker peaks at 22 nt (Supplementary Fig. S1). A total of 204,223,313 clean reads ranging from 18 to 35 nt were mapped to the *C. gigas* genome. The mapping rate varied among libraries, ranging from 72.72% to 88.85% (Supplementary Table S2).

3.2. Identification of miRNAs in the gonad of C. gigas

The sequences mapped above were used for small RNA identification. After compared with known mature miRNAs that included in the miRbase (http://www.mirbase.org/), 53 known mature miRNAs were identified. Of these, 35 known mature miRNAs were expressed in all gonads. The numbers of mature miRNAs varied among libraries. Libraries from the S3F_3 gonad accounted for the lowest number of conserved miRNAs (39 unique known miRNAs), whereas those from S0_1, S0_2, S1M_2 and S1M_3 gonads accounted for the highest 51 (Table 3). Meanwhile, 52 precursor sequences of known miRNAs were also acquired. The mapped small RNA reads without annotation information were collected for novel miRNA identification using miREvo and mirdeep2. Finally, 69 novel miRNAs, 53 novel miRNA star strands and 73 novel precursor sequences were identified (Table 3).

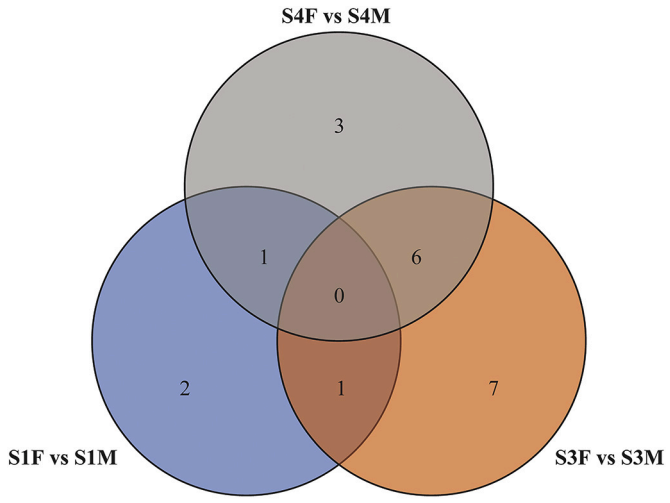

Fig. 1. Venn diagram of shared differentially expressed miRNAs between ovaries and testes at different developmental stages.

3.3. Dynamics of miRNA abundance during gonadal development of C. gigas

Based on TPM method and DEGseq R package, differential expression analysis of the miRNAs over the course of gonadal development revealed significant changes in abundance over time. Of the 122 miRNAs, 78 had at least one significant abundance change among the seven gonadic categories of *C. gigas*. Abundance of 46 miRNAs in the ovary and 45 miRNAs in the testis was significantly different from undifferentiated gonad in at least one sampling point (Supplementary Fig. S2A, B). Moreover, 43 and 40 miRNAs had significant changes in abundance over time within the ovary and testis, respectively (Supplementary Fig. S2C, D). The differential expression of miRNAs between ovary and testis was also demonstrated. The abundance of 20 miRNAs was significantly different between the ovary and testis in at least one developmental stage. Among them, eight miRNAs had significant changes in abundance between the ovary and testis in two developmental stages (Fig. 1).

For describing the dynamic expression patterns of the differentially expressed miRNAs, hierarchical clustering was carried out and finally six distinct clusters were generated. As shown in Fig. 2, most miRNAs in cluster 1 were abundant in gonads at early development stage and spawning stage (stage 0, I and IV). While, majority of miRNAs in cluster 3 was abundant in mature gonads. Most miRNAs in cluster 2 steadily decreased over time, and in contrast, most miRNAs in cluster 6 showed increase in abundance over time. Moreover, most miRNAs in cluster 4 and 5 had significant changes in abundance between the ovaries and testes at maturation and/or spawning stages. The abundance of most miRNAs in cluster 4 was increasing in testes, whereas that most of miRNAs in cluster 5 was gradually increasing in ovaries over time. For identifying potential miRNAs involved in oyster sex differentiation, we directed our attention to the sex-biased miRNAs that were identified exclusively from either male or female gonads. Six most differently expressed miRNAs, including three female-biased miRNAs (lgi-miR-263b in cluster 5, lgi-miR-96a and lgi-miR-279 in cluster 6) and three male-biased miRNAs (lgi-miR-8, lgi-miR-29 and novel_167 in cluster 4) were selected for further investigation.

3.4. Target prediction of sex-biased miRNA and function enrichment analysis

To better elucidate the biological function of the six sex-biased miRNAs, their potential targets were predicted using the genome of *C. gigas* by the miRanda software. Combined with the differentially expressed mRNA of the same samples (Yue et al., 2018), the number of

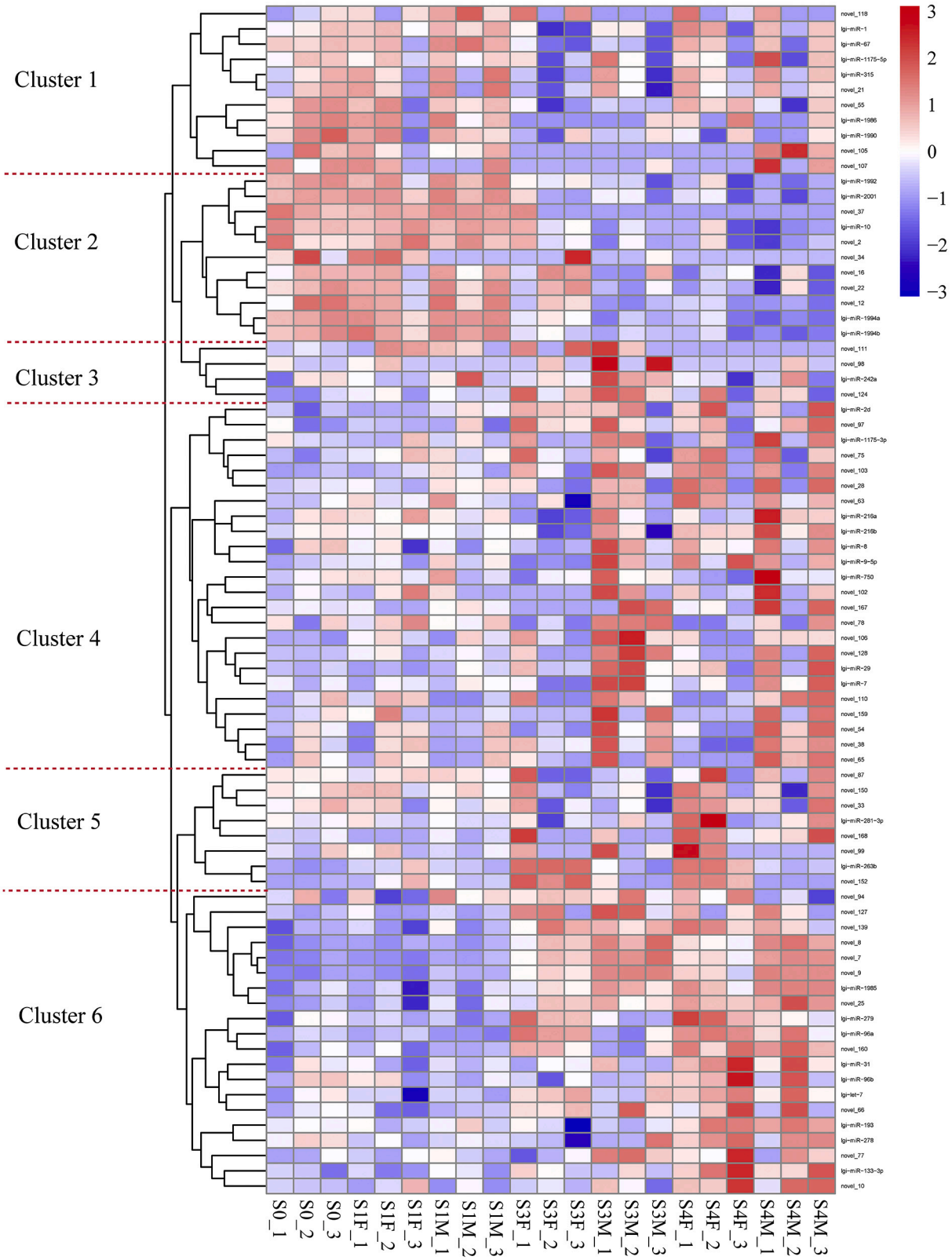

Fig. 2. Hierarchical clustering of differentially expressed miRNAs among the seven different gonadic categories of *C. gigas*. In the hierarchical clustering analysis, miRNAs showing similar expression profiles on samples clustered together. Expression levels are depicted with a colour scale, in which shades of red represent higher expression and shades of blue represent lower expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
GO terms enriched in putative target genes of the six sex-biased miRNAs.

Gene sets	GO accession	Description	Term type	*P* Value
Targets of ovary-biased miRNAs	GO:0007017	microtubule-based process	Biological process	2.50E-04
	GO:0005874	microtubule	Cellular component	1.72E-06
	GO:0099080	supramolecular complex	Cellular component	2.57E-06
	GO:0099081	supramolecular polymer	Cellular component	2.57E-06
	GO:0099512	supramolecular fiber	Cellular component	2.57E-06
	GO:0099513	polymeric cytoskeletal fiber	Cellular component	2.57E-06
	GO:0015630	microtubule cytoskeleton	Cellular component	7.63E-06
Targets of testis-biased miRNAs	GO:0044281	small molecule metabolic process	Biological process	2.23E-04
	GO:0019752	carboxylic acid metabolic process	Biological process	2.32E-03
	GO:0043436	oxoacid metabolic process	Biological process	2.32E-03
	GO:0044430	cytoskeletal part	Cellular component	5.69E-03
	GO:0005874	microtubule	Cellular component	8.72E-03
	GO:0000166	nucleotide binding	Molecular function	2.06E-03
	GO:1901265	nucleoside phosphate binding	Molecular function	2.06E-03

predicted target genes of miRNAs was narrowed down. For the three female-biased miRNAs (lgi-miR-96a, lgi-miR-279 and lgi-miR-263b), 52 target genes were identified. For the three male-biased miRNAs (lgi-miR-8, lgi-miR-29 and novel_167), 245 target genes were identified. Besides, the two types of sex-biased miRNAs shared 10 potential target genes.

Function enrichment analysis was performed based on 287 target genes of sex-biased miRNAs to identify GO and signaling pathways that regulated by miRNAs in oyster gonad development. GO enrichment analysis on 52 differentially expressed target genes of three female-biased miRNAs suggested that the most enriched biological process and cellular component terms were “microtubule-based process” and “microtubule”, respectively (Table 4). There were no cellular component terms significantly enriched with target genes of the three female-biased miRNAs. The GO enrichment analysis on 245 target genes of three male-biased miRNAs revealed that the most enriched biological process, cellular component and molecular function terms were “small molecule metabolic process”, “cytoskeletal part” and “nucleotide binding”, respectively (Table 4). The KEGG pathway analysis of the differentially expressed target genes of three female-biased miRNAs suggested that “phagosome” was significantly enriched (Fig. 3A). Furthermore, the KEGG pathway analysis revealed that the target genes of three male-biased miRNAs might function in “glutathione metabolism” (Fig. 3B).

3.5. *Integrated expression analysis of the sex-biased miRNAs and targeted mRNAs*

To understand the possible miRNA-mRNA networks involved in gonad differentiation, the interacting relationships between the six sex-biased miRNAs and 287 putative targets were further collected. A total of 304 putative miRNA-mRNA interaction pairs were predicted (Supplementary Table S4) and 25.66% of them (78) presented a negatively correlated expression pattern. Here the potential negative regulation networks were drawn among the six sex-biased miRNAs and their potential targets (Fig. 4). Interestingly, the miRNA novel_167 was predicted to bind the complementary regions in the coding region of a reported sex-related gene CgFOXL2. The miRNA lgi-miR-96a was predicted to bind the complementary regions in the coding region of a male-biased gene CgSPO11 (meiotic recombination protein SPO11). To

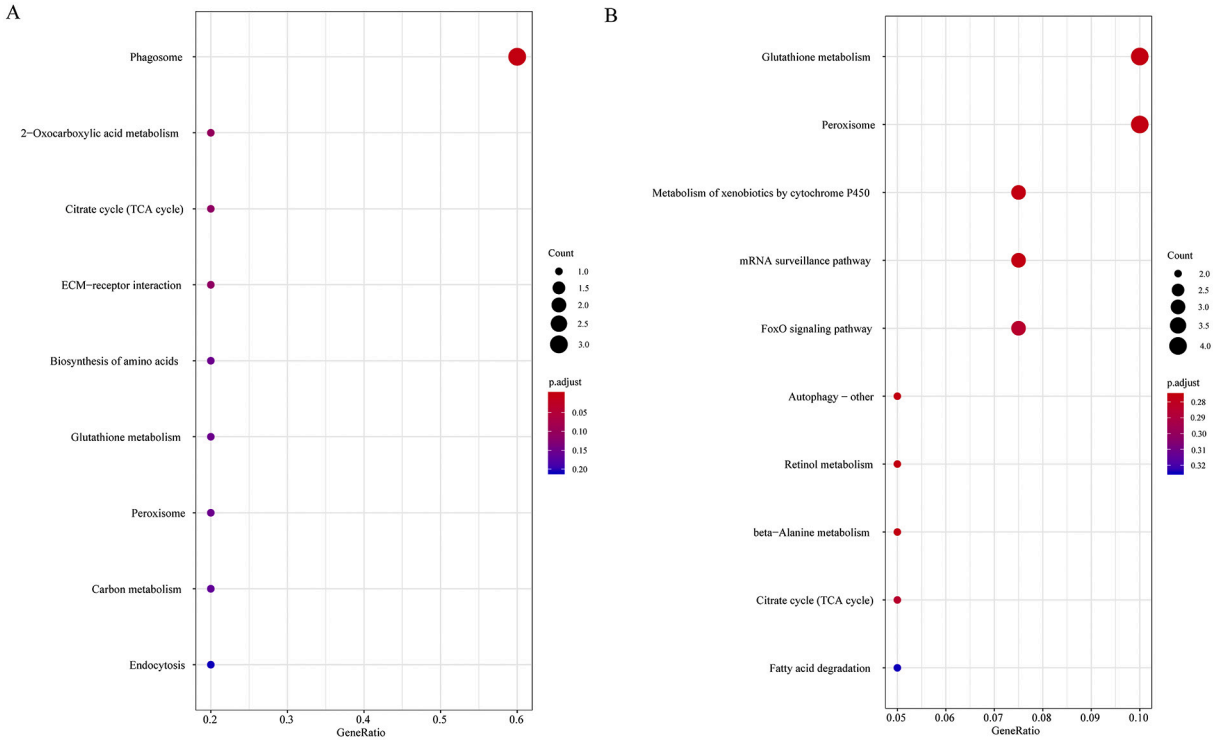

Fig. 3. KEGG annotation of target genes of six sex-biased miRNAs. Fig. 3A and B showed KEGG pathway enriched in target genes of female-biased and male-biased miRNAs, respectively. “Gene ration” means that the ratio of the number of the genes in the specific set and the number of genes annotated in this pathway. The greater of the gene ratio, the greater the degree of enrichment.

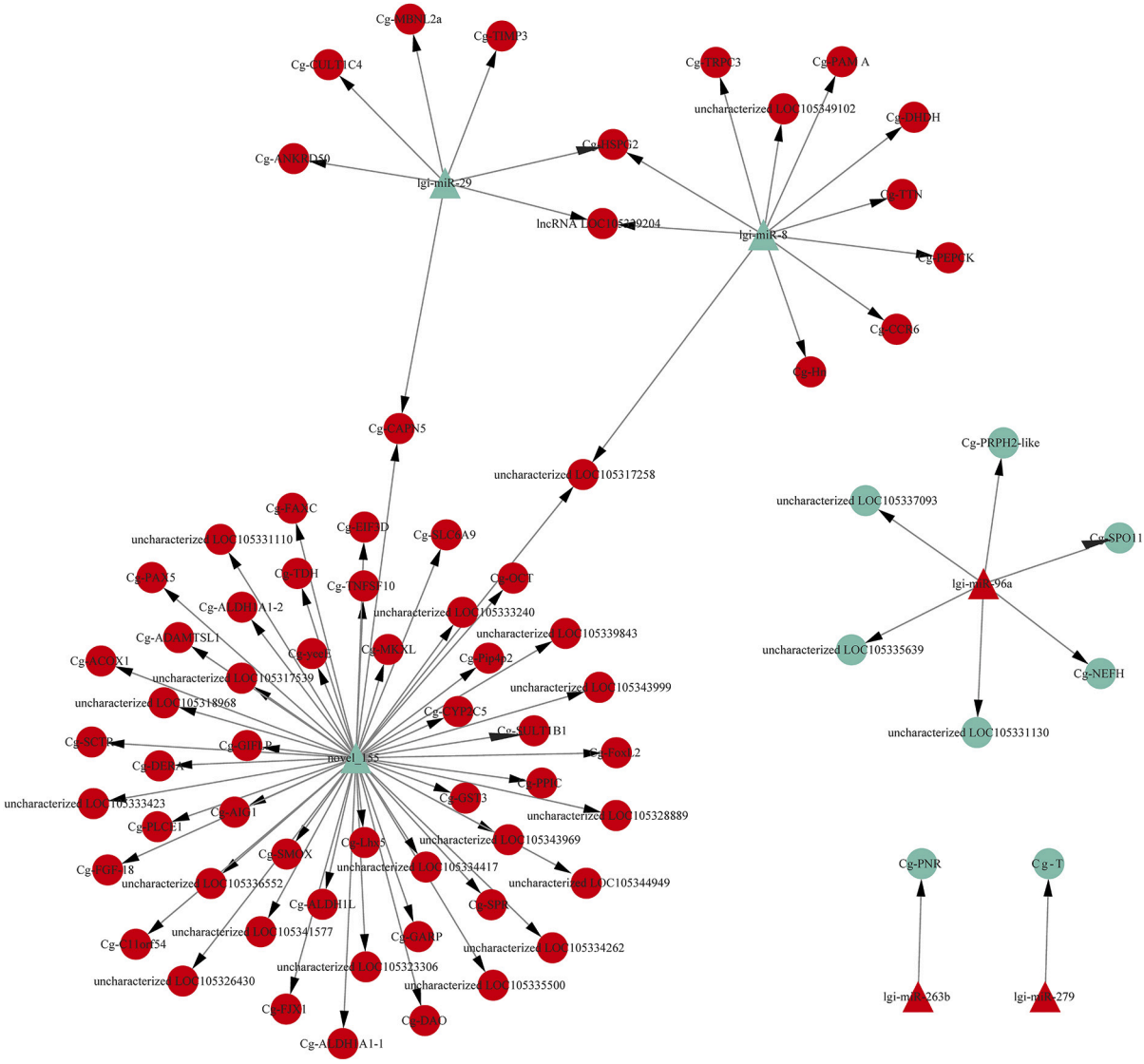

Fig. 4. Interaction network of six sex-biased miRNAs and some of their target genes. The network of eight miRNAs and their target genes that with opposite expression pattern was shown. Green and red triangle represents miRNAs highly expressed in testis and ovary, respectively. Green and red circle represents putative sex-related targets highly expressed in testis and ovary, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

validate the negative expression patterns between sex-biased miRNAs and their targets, real-time qRT-PCR was performed on the six miRNAs firstly. From the qRT-PCR, the expression profiles of four miRNAs were confirmed on additional samples. The female-biased lgi-miR-96a, lgi-miR-279 and lgi-miR-263b were confirmed with increasing expression over the course of ovary development (Fig. 5A, B, C). The male-biased novel_167 exhibited increasing expression over the course of testis development and its abundance was significantly higher in testis at stages III and stage IV compared with gonads at other categories (Fig. 5D). Moreover, the expression patterns of CgFOXL2 and CgSPO11 were also investigated using primers in Supplementary Table S1. The expression of CgFOXL2 was increasing over the course of ovary development and significantly higher in ovary than in testis (Supplementary Fig. S3A), showing a negative relation with novel_167. In contrast, the expression of CgSPO11 was increasing over the course of testis development and significantly higher in testis than in ovary (Supplementary Fig. S3B), showing a negative relation with lgi-miR-96a.

Dual-luciferase reporter assays were performed to further validate the prediction about the novel_167-CgFOXL2 and lgi-miR-96a-CgSPO11. Reporter vectors were constructed based on primers in Table 2 and co-transfected with NC mimics or miRNA mimics in HEK293T cells. As a result, the predicted interaction between novel_167 and CgFOXL2 was considered as false positive as there was no difference in relative luciferase activity between experimental and control groups (data not shown). In comparison, the predicted interaction between lgi-miR-96a and CgSPO11 was verified *in vitro*. For CgSPO11 wild type vector containing putative binding site of lgi-miR-96a (Fig. 6A), the relative luciferase activity decreased significantly in cells co-transfected with lgi-miR-96a mimics in comparison of that in other groups (Fig. 6B). Additionally, the base-pairing interaction was reduced when specific mutations on the target site were introduced according to the relative luciferase activity of CgSPO11 mutant type vector in cells (Fig. 6).

4. Discussion

The small RNA libraries of oyster gonads in this study displayed interesting length distribution of reads. The most abundant small RNAs were 28–31 nt long in all libraries, which were in the size range of PIWI-

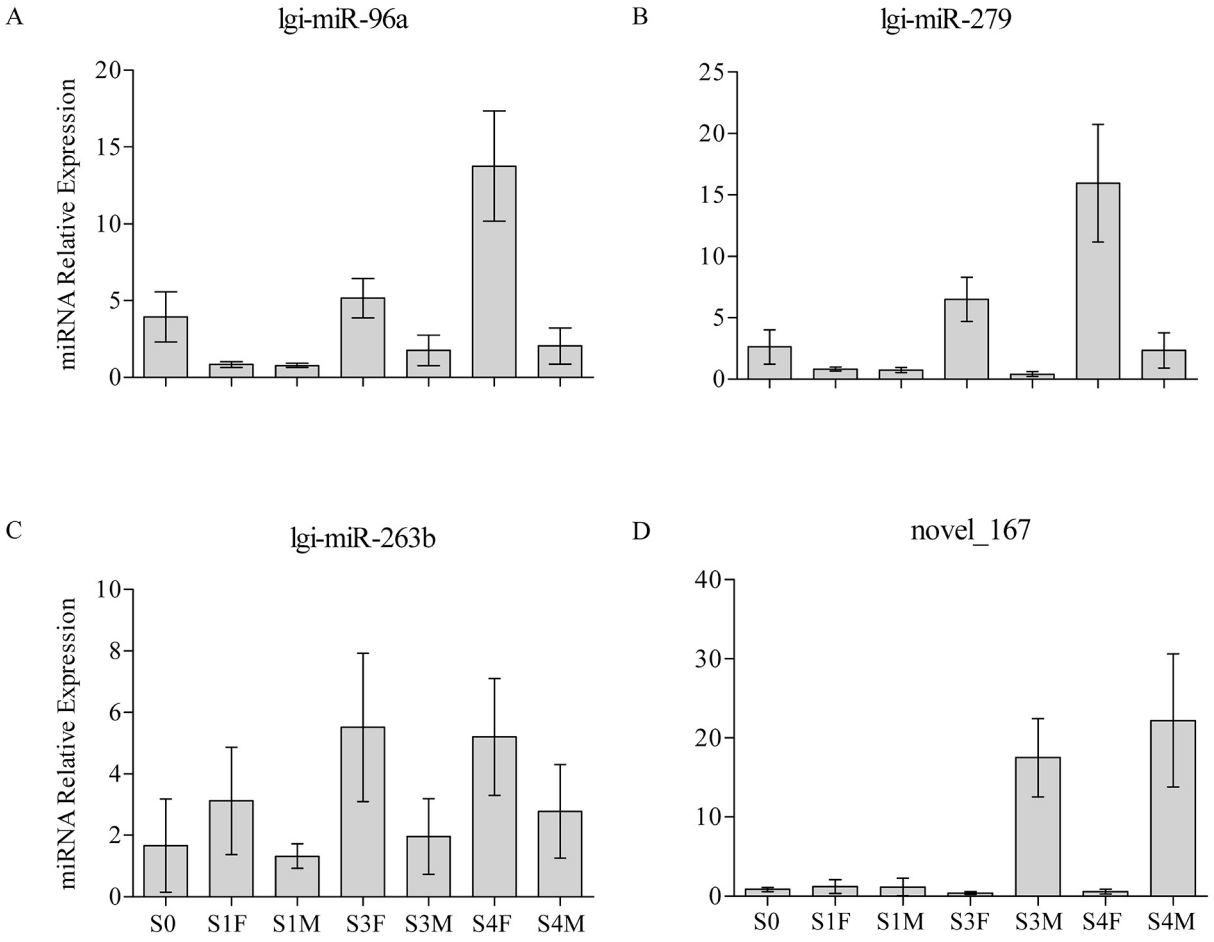

Fig. 5. Expression profiles of the four sex-biased miRNAs. The expression of four miRNAs was validated by qRT-PCR. These histograms A, B, C and D represent mean transcript expression during different gonadic categories. Development stage and sex are indicated at the bottom of corresponding figure. Bars represent standard error.

interacting RNAs (piRNAs; 26–32 nt) (Aravin et al., 2006; Girard et al., 2006). This was consistent with the enrichment of piRNAs in gonads that found across taxa (Aravin et al., 2006; Girard et al., 2006; Klattenhoff and Theurkauf, 2008; Thomson and Lin, 2009). Moreover, there was an additional typical miRNA peak of 22 nt in undifferentiated gonad and gonads at early stage. Similarly, there are both typical miRNA and piRNA peaks in the miRNA transcriptome of Nile Tilapia by Solexa sequencing (Flynt et al., 2009). Complex length distributions of reads that with majority of 26–31 nt were also reported in zebrafish gonads throughout gonadal development (Presslauer et al., 2017). Together, the reads in small RNA libraries of animal gonads, especially the mature gonads, often peaked at 26–32 nt long that represents the piRNAs. Additionally, length distribution of small reads was fluctuant during gonadal development and this indicated the dynamics of small RNA majority.

In order to understand the role of miRNAs in Pacific oyster sex determination and differentiation, the expression profile of 122 miRNAs in gonad was analyzed. There were six miRNAs, three female-biased miRNAs (lgi-miR-183, lgi-miR-96a and lgi-miR-279) and three male-biased miRNAs (lgi-miR-8, lgi-miR-29 and novel_167), exhibiting sex-specific pattern that expressed exclusively from either male or female gonads with increasing abundance throughout the gonadal development. Some of these miRNAs also show differential expression between testis and ovary in other species and their functions have been investigated, such as miR-279 in *Drosophila melanogaster* (Yoon et al., 2011; Marco, 2014), which may direct the function analysis of miRNA in oyster for function consistency. However, most of these miRNAs were rarely reported with sex-biased expression pattern in previous studies and functions of novel miRNAs remain unknown.

Subsequent enrichment analysis based on target genes of the six sex-biased miRNAs provided some clues to their potential functions. GO enrichment of the targets of the three female-biased miRNAs displayed overrepresentation of microtubule motor activity, and this was expected to be relevant to the development and function of flagella through which sperm cells move. In accordance with GO enrichment analysis, KEGG enrichment indicated some targets were involved in Citrate cycle (TCA cycle), which may be the dominant source of ATP during the hyperactivation of sperms (Miki et al., 2004). This suggests that these female-biased miRNAs might participate in downstream regulation of ovary differentiation by inhibiting the initiation of male gametogenesis. KEGG pathway analysis based on targets of three male-biased miRNAs revealed the enrichment of glutathione metabolism that pivotal in ovarian cellular processes such as cell differentiation, proliferation and apoptosis (Sies, 1999; Nunes and Serpa, 2018). From this result, these male-biased miRNAs may function as a regulator of genes involved testis development by repressing genes in the ovary differentiation pathway.

Three sex-biased miRNAs in small RNA sequencing libraries were considered as potential key post-transcriptional regulators in oyster gonad development after qRT-PCR validation in additional samples. The expression profile of miR-279 in *D. melanogaster* was similar with that of female-biased lgi-miR-279 in *C. gigas* and *Crassostrea hongkongensis* (Wei et al., 2019). In the *Drosophila* ovary, miR-279 mediates feedback inhibition of JAK/STAT morphogen signaling to establish a cell fate threshold for differentiating as border cells or follicle cells (Yoon et al.,

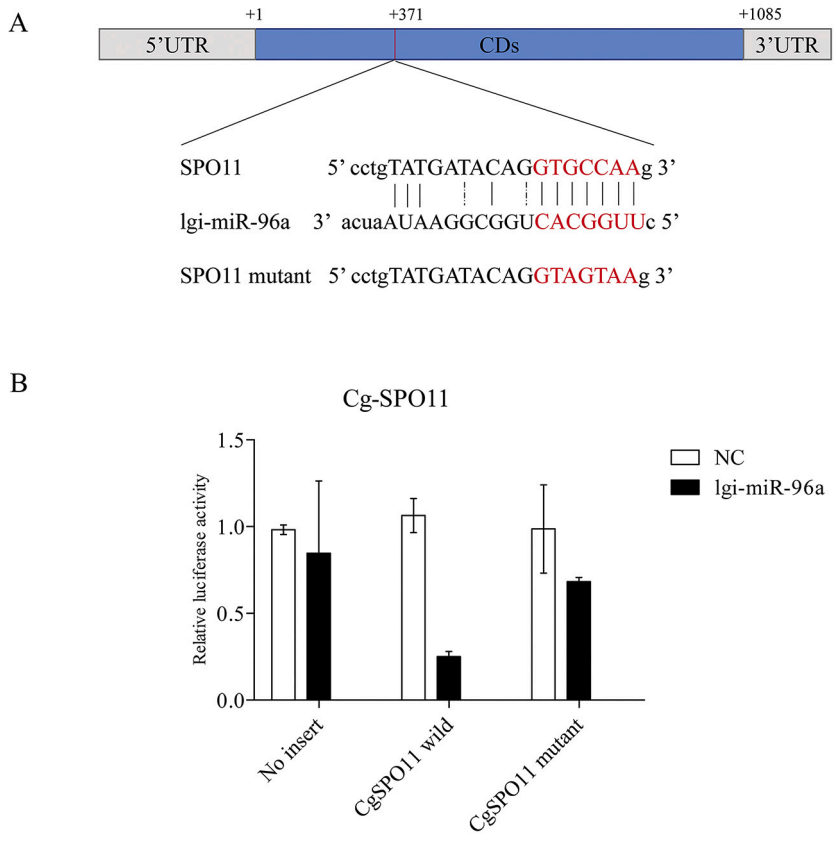

Fig. 6. Interaction between lgi-miR-96a and CgSPO11 *in vitro*. (A) Putative binding sites of lgi-miR-96a on the CDs region of CgSPO11 and mutations were written in red. (B) The relative luminescence ratio in CgSPO11 CDs luciferase reporter assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2011). Currently, little is known about its functions in the gonad development of non-model species. There were dozens of targets of male-biased novel_167 according to our integrated analysis and what aroused our interest was the homolog of conserved female determining FOXL2 (forkhead box L2) in *C. gigas* (Ottolenghi et al., 2005; Uhlenhaut and Treier, 2006; Georges et al., 2014; Zhang et al., 2014). But the interaction between novel_167 and CgFOXL2 could not be validated *in vitro*. Together, the role of miR-279 and the species-specific novel_167 in oyster are preliminarily investigated and post-transcriptional regulation mediated by them needs more evidences at experimental level.

The lgi-miR-96a was expressed highly in ovaries of *C. gigas* and might be related to gonad development. Interestingly, miR-96 was expressed highly in XY gonad of tilapia and targeted multiple genes involved in steroid synthesis (Tao et al., 2016). This is inconsistent with lgi-miR-96a expression in oyster gonads and we speculated that different miRNAs even within one family may have different functions. In this study, female-biased lgi-miR-96a was predicted as a negative regulator of multiple genes that significantly downregulated in oyster ovaries at maturation and spawning stages. Among these targets, the CgSPO11 (meiotic recombination protein SPO11) was identified as a homolog of gene that required for meiotic synapsis. The SPO11 protein initiates meiotic recombination by generating DNA double-strand breaks (DSBs) in *Saccharomyces cerevisiae* (Keeney et al., 1997), and in the absence of SPO11 protein, DSBs are not formed and homologous chromosomes do not synapse at wild-type levels (Giroux et al., 1989). Disruption of mouse SPO11 results in infertility as spermatocytes arrest prior to pachytene with little or no synapsis and undergo apoptosis (Romanienko and Camerini-Otero, 2000). From our dual-luciferase reporter assays, CgSPO11 could be inhibited by lgi-miR-96a by directly binding to their CDs region. Previous studies reported that miRNAs binding to the CDs mainly led to translation inhibition (Rigoutsos, 2009; Brümmer and Hausser, 2014) and thus the negative relationship between lgi-miR-96a and CgSPO11 mRNA abundance needs more investigation in future studies. Combined with the oyster oogenesis without meiotic synapsis before the release of the primary oocyte, it is speculated that lgi-miR-96a might inhibit CgSPO11 in *C. gigas* ovarian cells to maintain the primary oocyte preparatory to the first meiotic division. Of course, the potential role of this interaction could not be excluded from repressing initiation of testis differentiation pathway during the ovarian development.

Potential miRNA-mediated post-transcriptional regulation in oyster gonads is a clue to the gonad differentiation mechanisms of bivalves. Better understanding about gonad differentiation could be beneficial to artificial control of bivalve reproduction or gonad development, though there is certainly lots of work need to be done before application in bivalve aquaculture. Further investigation on the influence of key miRNA overexpression or silencing (*e.g.*, lgi-miR-96a) in oyster gonad differentiation, especially *in vivo*, is required in follow-up studies.

5. Conclusions

The objective of this study was to discover gonad miRNAs in *C. gigas* and to further reveal potential miRNA-mRNA regulatory relationships in sex determination and differentiation. From the small RNA sequencing result, six miRNAs were regarded as potential post-transcriptional regulators in oyster sexual development because they were most differentially expressed between ovaries and testes. Subsequent function analysis of targets suggested that these sex-biased miRNAs might function as regulators of genes involved in gametogenesis. After validation by qRT-PCR and dual-luciferase reporter assay, the negatively regulation between lgi-miR-96a and CgSPO11 appeared to be interesting starting point for future research to understand miRNA-mRNA interaction in oyster gonad differentiation. Taken together, the discovery of

oyster gonad miRNAs provides a useful information for gonad development researches and the miRNA-mRNA interactions identified in this study will help to reveal the post-transcriptional regulation mechanisms in bivalve sexual development.

Authors' contributions

CY carried out the sample collection, histological analysis, data curation, formal analysis and the original draft. QL conceived of the study, participated in experimental design and coordination, and contributed to the manuscript review and editing. HY participated in the formal analysis. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737294.

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