



Expression pattern of *Piwi*-like gene implies the potential role in germline development in the Pacific oyster *Crassostrea gigas*

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ARTICLE INFO

Keywords:

Piwi
Gonad development
Primordial germ cell
Molecular marker
Crassostrea gigas

ABSTRACT

Piwi is necessary for germ cell development in a diverse range of organisms and homologues have been commonly used to identify primordial germ cells (PGCs) during embryogenesis. Here, we isolated full-length cDNA of *Piwi* ortholog in the Pacific oyster *Crassostrea gigas* and characterized its expression patterns (*Cg-Piwi*-like) along with analyzing the expression alternation in the gonad of diploids compared with those sterile triploids. qPCR showed that the transcript of *Cg-Piwi*-like was mainly restricted to the gonad in diploids with ovarian tissues of triploids showing the highest expression. *in situ* hybridization revealed that *Cg-Piwi*-like was found in both female and male gonad where the strongest expression was shown in the germ cells at early stages with no signal in somatic cells. Whole-mount *in situ* hybridization suggested *Cg-Piwi*-like was maternally deposited and the localization of *Cg-Piwi*-like mRNA in mesodermal cells might be the putative PGCs in *C. gigas*. These results suggest that *Cg-Piwi*-like was involved in germ line formation, differentiation, and maintenance of germ cells in *C. gigas*. The obtained findings provide valuable evidences to further facilitate identification of the putative PGCs in the Pacific oyster using *Cg-Piwi*-like as a molecular marker.

1. Introduction

Primordial germ cells (PGCs) are critical for animal reproductive development, which are populations of undifferentiated stem cells among sexually reproducing animals. During embryogenesis, PGCs differentiate into the germ cells, either spermatocytes or oocytes, thereby, transferring genetic information from one generation to the next (Extavour and Akam, 2003). Until the advent of molecular techniques, PGCs were originally recognized by their morphologically characteristic large round nucleus, single large nucleolus, cytoplasm relatively clear of organelles, and granular cytoplasmic material (Extavour and Akam, 2003). Irrespective of the mode and timing of PGCs specification, the involvement of conserved genes in specifying PGCs has been shown to regulate germline development and many of these genes are commonly used as a germ cell, as well as stem cell marker (Ewen-Campen et al., 2010). These conserved genes, such as *Vasa* and *Nanos*, as the components of germline deposited during oogenesis, are required for germline formation (Asaoka et al., 1998; Deshpande et al., 1999; Draper et al., 2007; Fujiwara et al., 1994; Olsen et al., 1997; Castrillon et al., 2000). Along with *Vasa* and *Nanos*, two key germ cell specific genes, *Piwi* plays important roles in germline determination and

germline stem cell (GSC) maintenance to meiosis, spermiogenesis, and transposon silencing during germline development and gametogenesis of many metazoan species (Thomson and Lin, 2009).

The Argonaute family can be divided into Ago and Piwi subclasses based on amino acid sequence similarities (Peng et al., 2013). As a member of the Argonaute protein family, the important factors of RNA-induced silencing complex (RISC), occupying an important role in gene silencing by RNA interference (Seto et al., 2007), Piwi proteins are characterized by the two highly conserved domains: PAZ and PIWI. The PAZ domain is involved in the nucleic-acid binding process, which has shown to bind small non-coding RNAs (Lingel et al., 2003; Yan et al., 2003). The PIWI domain has an RNase H fold and performs an important role in RNA slicer activity that triggers target mRNA degradation in siRNA- and miRNA-induced gene-silencing pathways (Liu et al., 2004). In animals, unlike Argonaute, subdivided into Ago subfamily, which is expressed ubiquitously in all tissues, Piwi subfamily proteins are mainly limited in gametogenesis and early embryonic development, implying its potential roles in germline development (Kim, 2006).

Extensive genetic studies in *Drosophila*, *Caenorhabditis elegans*, *Danio rerio*, *Mus musculus* and *Homo sapiens* have indicated that *Piwi* genes are essential for germ line specification in model animals (Cox et al., 1998;

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<https://doi.org/10.1016/j.aqrep.2020.100486>

Received 14 April 2020; Received in revised form 6 September 2020; Accepted 18 September 2020

Available online 18 October 2020

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Harris and Macdonald, 2001; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007; Houwing et al., 2008; Gou et al., 2017). For instance, in *Drosophila*, *Piwi* is necessary for the maintenance of stem cells, along with controlling germline specification (Cox et al., 1998; Megosh et al., 2006). Similarly, in *D. rerio*, *Ziwi* mutants display germ cell apoptosis and loss of *Zili* function results in the failure of germ cells differentiation and meiosis (Houwing et al., 2007, 2008). There are three *Piwi* family proteins in *M. musculus*, namely, *Miwi*, *Mili*, and *Miwi2*, and all of which are required for male spermatogenesis process but not their female counterparts (Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). Suppression of any *Piwi*-related genes specifically imposes the male sterility (Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). Human genome encodes four *Piwi* paralogs, including *Hiwi*, *Hili*, *Hiwi2*, and *Piwil3* (Sasaki et al., 2003). A molecular study in patients with idiopathic non-obstructive azoospermia indicated that the genetic polymorphism in *Hiwi2* gene caused spermatogenesis defect and male infertility (Kamaliyan et al., 2017). *Hiwi* mutations were also detected in azoospermia patients (Gu et al., 2010). Thus, these well-studied organisms show that *Piwi* genes play an important role in reproductive system, though their functions are diverse in different animals.

Mollusca, as the second largest animal phylum, is an abundant and highly diverse group. Recent studies have showed significant advances in the understanding of the role of *Piwi* involved in germline development (Jehn et al., 2018; Huang et al., 2019; Ma et al., 2017). Knockdown of *Cf-piwi1* by double-stranded RNA (dsRNA)-mediated genetic interference (RNAi) led to marked apoptosis in *Chlamys farreri* gametogenesis and provoke defects in the development of germ cells in gonad, implying its important roles in the germ cell proliferation and differentiation (Ma et al., 2017). Recent studies also confirmed that mollusks utilize the PIWI/piRNA pathway as a defense against transposable elements in the germline (Jehn et al., 2018; Huang et al., 2019). In contrast to the detailed knowledge of gametogenesis for several mollusks, however, up to now, far less is known about the role of *Piwi* involved in maintenance and differentiation of PGCs. So far, expression of several conserved genes has been used to trace the embryological origin of the PGCs in mollusks, and to infer its mechanism of specification (Fabioux et al., 2004b; Xu et al., 2018; Swartz et al., 2008; Rabinowitz et al., 2008; Kakoi et al., 2008; Kranz et al., 2010).

Crossosrea gigas is a commercially important bivalve mollusk, and extensive studies have been focused on the germline development to monitor reproductive performance (Fabioux et al., 2004a, b; Fleury et al., 2008; Naimi et al., 2009a; Santerre et al., 2014; Meistertzheim et al., 2009; Naimi et al., 2009a, 2009b; Xu et al., 2018). Yet the origin of *C. gigas* germline has not been determined during embryogenesis, although the two cell clusters in the gastrulation are considered as candidate PGCs (Fabioux et al., 2004b; Xu et al., 2018). The maternal genes, such as *vasa*, *nanos*, and *piwi* involved in PGCs specification, are highly conserved in animals (Extavour and Akam, 2003). Therefore, those conserved PGCs-specific genes can be used as potential biomarkers to elucidate the origin of the PGCs. More importantly, as shown in the Pacific oyster, *vasa* and *nanos* expression were limited in the gonad, corresponding to the maternal materials for the specification of the germplasm during early embryogenesis (Fabioux et al., 2004a; Xu et al., 2018). Knockdown of *Vasa* by RNAi resulted in retarded gonad and germ cell apoptosis in the Pacific oyster (Fabioux et al., 2009). Thus, identification of target genes expressed in the gonad is a crucial step to develop a method to induce sterility in Pacific oyster since we successfully applied CRISPR/Cas9 genome editing technology in *C. gigas* (Yu et al., 2019). While the role of *Piwi* in *C. gigas* gametogenesis is relatively unknown though it has been well-studied in model organisms. In this study, full-length cDNA of *Piwi*-like was cloned, and *Piwi*-like expression profile during embryonic and larval development along with gametogenesis was examined to elucidate the role of *Piwi* and the mechanism of PGCs specification in *C. gigas*. We also investigated differentially expressed patterns of *Piwi* in sterile triploid oysters compared with

diploids to shed light on the role of *Piwi* gene in gonadal development and maintenance.

2. Materials and methods

2.1. Animals and treatment

Two-year-old diploid and sterile triploid Pacific oysters were obtained from a local oyster farm (from February to June 2017) in Rongcheng, China. Ploidy analysis of samples was analyzed with a flow cytometer. In each sampling time, oysters were dissected, and gonadal samples were taken and preserved in RNA Store solution at -20°C for spatial patterns of gene expression. Gonadal development stages (0: resting stage; 1: proliferative stage; 2: growing stage; 3: mature stage) and gonadal sex were determined by histological analysis of gonad as described in Jouaux et al. (2010) and Li et al. (2006). The ovary and testis were fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS (phosphate-buffered saline) at 4°C overnight. After fixation, the gonadal tissues were dehydrated through a graded series of PBST (phosphate-buffered saline plus 0.1 % Tween 20) into EtOH and submitted to ISH (*in situ* hybridization). For tissue distribution analysis, other tissues including gill, adductor muscle, labial palps, mantle, digestive gland, and hemolymph were sampled and frozen in liquid nitrogen and kept at -80°C .

To obtain embryo and larvae, oocytes and sperm were collected by dissecting gonoducts and *in vitro* fertilization was performed as described in Wang et al. (2012). Unfertilized oocytes, 2-cell, 4-cell, blastula, and gastrula embryos, and trochophores, D-shaped larvae, umbo larvae, and eyed-larvae were sampled and further processed for RNA extraction or WISH (whole mount *in situ* hybridization).

Nucleotide sequence homology analysis of *Cg-Piwi*-like was processed by DNAMAN version 8.0 (Lynnon BioSoft, USA). The conserved domains of *Cg-Piwi*-like protein were analyzed by the SMART program under default parameters (<http://smart.embl-heidelberg.de/>). Phylogenetic analysis was conducted using the neighbor-joining method in MEGA 7.0 Based on the 1000 bootstrap replicates (Kumar et al., 2016).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the tissue samples and embryo-larval samples using Trizol (Invitrogen) as the manufacturer's instructions. All RNA samples were initially tested for quality control to ensure there were less differences during sample processing. Samples were run on NanoDrop 2000 (Thermo Scientific) to confirm RNA concentration and integrity. The presence of 3 prominent bands by gel electrophoresis analysis suggested that RNA samples were of high quality. cDNA was synthesized with the PrimeScript™ reverse transcription kit (Takara) from 1000 ng of RNA samples according to manufacturer's instructions.

2.3. Molecular cloning of *C. Gigas piwi*-like cDNA

Full-length cDNA of *Cg-Piwi*-like was obtained by using the RT-PCR (reverse-transcription PCR) and RACE (rapid amplification of cDNA ends) procedures. All the primers used in the PCRs are listed in Table 1. First, the partial cDNA fragments of the *Cg-Piwi*-like were amplified by RT-PCR with two paired degenerate primers which were designed based on the *Cg-Piwi*-like sequence from the NCBI database with the accession number LOC105339049. The PCRs were performed with *Taq* DNA Polymerase (Takara) under the following conditions: 3 min at 94°C followed by 35 cycles of 30 s at 94°C , 1 min at 60°C , and 2 min at 72°C with a final extension step of 6 min at 72°C . To obtain a full-length *Cg-Piwi*-like cDNA, 5'-RACE and 3'-RACE were performed using the SMART RACE cDNA amplification kit (Clontech), using gene-specific primers (Table 1) designed based on the partial cDNA sequence obtained above. The PCRs were performed with *Tks Gflex™* DNA Polymerase (Takara) at 98°C for 1 min, 98°C for 10 s, 65°C for 30 s, 68°C for 30 s, for 35 cycles,

Table 1
Primers and their sequences used for PCR.

Primers	Primer sequences (5' to 3')	Usage
Piwi-ZF-1:	CATGAGAATTTACTGGGCGAA	RT-PCR
Piwi-ZR-1	CCTGCGAGTGAAGACAATGACC	RT-PCR
Piwi-ZF-2:	AATGCCAGCTGAGTTACCG	RT-PCR
Piwi-ZR-2	GCGACCTTTGGATTGTAGTCTT	RT-PCR
Piwi-3	GCCAAGACTACAATCCAAGGTCGCTA	RACE
Piwi-5	CGGAACGGATGATCTTGTGGCTGA	RACE
qCg-piwiF	ATGACACTGAATGCTAGAGTTGCG	RT-qPCR
qCg-piwiR	CCTGCGAGTGAAGACAATGACC	RT-qPCR
ISH-ACg-piwiF	GGTTTGTGCGGATGAAGCC	ISH
ISH-ACg-piwiR	GATCACTAATACGACTCACTATAGGGGTATCGAGTGAGTGCCTGGTT	ISH
ISH-SCg-piwiF	GATCACTAATACGACTCACTATAGGG TGGGATTCACTGACATAGCAT	ISH
ISH-SCg-piwiR	TATCGAGTGAGTGCTGGTT	ISH

and then the amplified products were treated with *Taq* DNA polymerase (Takara) in the presence of dATP to create complementary stick ends for TA clone (Marchuk et al., 1991). All the DNA fragments were subcloned into the pEASY-T1 vector (Transgen Biotech, China) and sequenced.

2.4. Expression analysis of Piwi-like mRNA in adult and embryo

Cg-Piwi-like expression was analyzed during the gametogenesis, embryogenesis, along with larval development and in different tissues (gill, adductor muscle, labial palps, mantle, digestive gland, hemolymph, and gonads). qPCR was performed using EvaGreen 2 × qPCR MasterMix-ROX (ABM) on a LightCycler® 480 real-time PCR system (Roche). Two parallel amplification of *C. gigas elongation factor1α (EF1α)* and *ribosomal protein S18 (RS18)* reference transcripts were carried out to normalize the expression level of *Cg-Piwi*-like transcript in the adult and larval samples, respectively. All primers used for the PCRs were listed in Table 1. The PCRs cycling conditions were as follows: 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 20 s. Relative *Cg-Piwi*-like mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. Data were presented as means ± SD with six samples in each group. The significant differences between the means were analyzed using the SPSS version 20 by two-tailed Mann-Whitney *U*-test. All statistical differences were considered significant when $P < 0.05$.

2.5. In situ hybridization

In situ hybridization on OTC (Leica) sections of adult gonad and whole embryos was performed according to protocols described in (Fabioux et al., 2004a, b) with some modifications. Briefly, oyster samples stored in methanol were rehydrated in descending methanol-concentration in PBST. After treatment with an age-dependent concentration of proteinase K (100 ng – 10 µg/mL), samples were incubated for 3 h at 65 °C in prehybridization solution (5 × SSC, 50 % formamide, 100 µg/mL yeast t-RNA, 1.5 % blocking reagent, 5 mM EDTA, 0.1 % Tween-20). The specific primers (Table 1) were used to synthesize sense and antisense digoxigenin-labeled RNA probes synthesized by the DIG RNA labeling kit (Roche) according to the manufacturer's instructions. Then samples were hybridized with 1 µg/mL DIG-labeled RNA probes overnight at 65 °C. Unbound probes were washed away, twice in 50 % formamide/2 × SSCT for 30 min, once in 2 × SSCT for 30 min, and twice in 0.2 × SSC with 0.1 % CHAPS for 30 min. The location of the *Cg-Piwi*-like mRNA was visualized with the DIG nucleic acid detection kit (Roche) by the binding site of the probes on the samples.

3. Results

3.1. Cloning and sequence analysis of *Cg-Piwi*-like gene

The full-length transcript of *Cg-Piwi*-like was 3465 bp long, containing an open reading frame (ORF) of 2622 bp that encoded putative

873 aa residues with a 5'-untranslated region (5'-UTR) of 131 bp, a 3'-untranslated region (3'-UTR) of 712 bp (Fig. 1). The *Cg-Piwi*-like protein contained two conserved domains: PIWI and PAZ. Phylogenetic analysis showed that the *Cg-Piwi*-like protein was most closely related to *Crasostrea virginica* and then clustered with *Mytilus galloprovincialis* (Fig. 2).

3.2. Expression profiles of *Cg-Piwi*-like gene in different tissues

The *Cg-Piwi*-like transcript was expressed almost exclusively in certain tissues. The expression level of *Cg-Piwi*-like in the testis was significantly higher than that of in the ovary, displaying a sexually dimorphic expression pattern (Fig. 3). In somatic tissues, *Cg-Piwi*-like gene had lower expression in gill and basically was not expressed in adductor muscle, labial palps, mantle, and digestive gland, whereas *Cg-Piwi*-like was highly prevalent in the hemolymph.

3.3. Temporal expression profile of *Cg-Piwi*-like gene

In *C. gigas* diploids, expression levels of *Cg-Piwi*-like in gonad increased along with gonadal development (Fig. 4). The *Cg-Piwi*-like transcript was expressed differentially in gonad during the reproductive cycle, with significantly higher expression in males from growing of gametes to ripeness stage. In the ovary of triploids, the expression level of *Cg-Piwi*-like mRNA significantly increased during the gametogenetic cycle, and was significantly higher than that of its corresponding diploids, whereas the expression was dramatically lower at the ripeness stage in males compared to those of diploids (Fig. 4).

qPCR results showed *Cg-Piwi*-like mRNA was maternally deposited in oocytes which showed the highest expression (Fig. 5A). The expression level of *Cg-Piwi*-like was decreased from the 2-cell stage to blastula stage. The expression level became lower at gastrulation and were barely detectable during the veliger stages (Fig. 5A).

3.4. Location of *Cg-Piwi*-like mRNA in adult gonad and embryos of *C. Gigas*

In the adult, *Cg-Piwi*-like was found in both female and male germ cells where expression appeared to be strongest in the germ cells at early stage with no detected signal in somatic cells (Fig. 6). *Cg-Piwi*-like signals were observed predominantly in early oocyte cytoplasm, while the positive signals became weaker at later stages during oogenesis. In testicular sections, *Cg-Piwi*-like mRNA was obviously detected in spermatogonia and spermatocytes. No signal was detected in mature spermatids.

Cg-Piwi-like was mainly located at the vegetal hemisphere of oocytes and evenly distributed to each micromere as the cells divided at early cleavage stages (Fig. 5B). The egg was divided into 2 cells where the *Cg-Piwi*-like was expressed in each cell. As the cleavage proceeds to the 4-cell stage, leading to the formation of 4 macromeres, *Cg-Piwi*-like mRNA was located to the macromeres. Subsequently, a cluster of the signals locally enriched at the vegetal region of multicellularity. The

A

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10      20      30      40      50      60      70      80      90      100     110     120
1      ACATGGGGCCAGTTTAAACCAAGAATCGAGACCTGGTGGATGCTGCACCAAGAAAGTGTAGACAACAAAACAGATTAAACAGACCAACGGTCTTAAAGAATTGCTTCTCAGCAGG
121     ATTAGATAAGTATGCTGGGAGAGGTAGAGCAAGACGAGGAGAGAGCCGGGGGGCCCTCGGAGGACCAAGCCGGGGCCAGGGGAGCAGCTGCCCCAGCAACAGCCCCCAGCAGC
1      M S G R G R A R A R G R A R G A S E D Q A R R P G E Q P A P Q Q Q P P Q
250     260     270     280     290     300     310     320     330     340     350     360
241     CTCTTCTGCCCTGCCCCGACCGCTGGTGGACCCAGCAGCCAGTGAAGACCTCTACAGGGTGGTCCAAGGAACCAGCCAGGGATGCCAGCGGGAGCGGTGATGTCC
37      P S S A P A P A P A G G P P A A S G R A S Y R G G A K E P R P G I A S G S G D V
370     380     390     400     410     420     430     440     450     460     470     480
361     CTGCTGATGCATCTCCAAGATGCCATAGGAACCAAGGACCGAGGAGAGAGGACAGCTGTCTATAGTGACCCAGAAATGAAGCCAGCATGGCTGCAGACAACAGGAACTT
77      P A D A L S K M S I G T K D A G E R R D R L F Y S D P E C K P A W L S D K R G T
490     500     510     520     530     540     550     560     570     580     590     600
481     CCGGCCGTCTTCCGGTGGTAAACCACTACTCAAGTGGAAATGACCCCGACTGGCACTTACCAATATGATGTGAAGTCAACCCACCCATTGACTCCAGGAAATGAGGATGG
117     S G R A L P V V T N Y F K L E M T P D W H L Y Q Y D V K F N P P I D S R K M R M
610     620     630     640     650     660     670     680     690     700     710     720
601     CACTGCTGATGACACATGAGAATTTACTGGGCGCAACAAAGCCGTTGATGGCATGATTGTATGCTCATCGTCCAGGACAGGTGACTGAGGTGCTCAGTGTGAGGAGACGG
157     A L L M T H E N L L G R T K A F D G M I L Y L P H R L Q E Q V T E V F S V R K T
730     740     750     760     770     780     790     800     810     820     830     840
721     ACGAGCTACAGATCCGGATCACAATCAGCTTAACCAATGAACCGCTCCCTCCCTCCCAAGTCATGAGGTCATAAATATCTTCCAGAGGGTGCCTGGCAATGATTGAGATGAAGC
197     D D V Q I R I T I T L T N E P P S S P Q V M Q V Y N I I F R R V L A M I E M K
850     860     870     880     890     900     910     920     930     940     950     960
841     AGATTGGTCTTAATCTCAACCCCTGCCTGTGTGGACATTCCCAAGCAAACTAACGGTATGCCCGGGTTGTGACGGCCATCGCCCGCTATGAGACAGACCCCTGCTATGGT
237     Q I G R N Y F N P A L S V D I P Q H K L T V M P G F V T A I A R Y E T D I L L C
970     980     990     1000    1010    1020    1030    1040    1050    1060    1070    1080
961     CGGACATCAGCCACAAGATCATCCGTTCCGACACACTGCTGGACCTGATGTCAGAACTGTACCAACAAGCCCGGGGAGCTTTTATGACGACTGCCTCAGGAAGTTTGGGCTCCA
277     A D I S H K I I R S D T L L D L M Y E L Y Q Q A R G D S F Y D D C V R K F V G S
1090    1100    1110    1120    1130    1140    1150    1160    1170    1180    1190    1200
1081    TTGTCCTGACCAAGTACAACAAGACGTCACCGAGTAGATGACTTTGATGGGACAAGAGCCGATCATTCTTCAAACCTCAGGAACGACACCACCATACCATCGCTGATGATTACA
317    I V L T R Y N N K T Y R V D D F D W D K R P D H S F K L R N D T T I T I A E Y Y
1210    1220    1230    1240    1250    1260    1270    1280    1290    1300    1310    1320
1201    AAAAGACTACAATATAGAGTGAAGACATGAACAGCCATGGTGTATCTCGACCCAAAAGAGGATATTAGAATGGGGCTACGGAGCCGATTTCCCTCCCCAGAATCTGTGA
357    K K S Y N I E V R D M N Q P L V S R P R K K D I R M G R T E P I F L L P E L C
1330    1340    1350    1360    1370    1380    1390    1400    1410    1420    1430    1440
1321    CCGTGCAGGTTTGTCCGATGAAGCAGAGCTGACTTGGCGTATGAAGATGTCGGTGTCACTCGAGTCCCCCTGAGGGCAGGAACAGGACTCTACAGGGCTTCATCAACAAA
397    I V T G L S D E A R A D F G V M K D V G A H T R V P P E G R N R T L Q G F I N Q
1450    1460    1470    1480    1490    1500    1510    1520    1530    1540    1550    1560
1441    TTAACCAAGTAAAAAGTGAAGCAGAAATGCAGGGTGGGACTAGCATTCTCAGACTCTGATGACACTGAATGCTAGAGTTGCCCCAGGAGATAATATCAAAAAGAACAACT
437    I N Q N E K V K A E M Q G W G L A F S Q T L M T L N A R V A P Q E N I Y Q K N N
1570    1580    1590    1600    1610    1620    1630    1640    1650    1660    1670    1680
1561    CCCAGCTGAGTTACCCAGAGGAGTGCAGACTGGAGCAGAGACATGAGGGTAAACAGCTGATCAGCCGGTCAACCTGGAGAACTGGGTCAATGTCTACTCGGAGAACAGTGGCC
477    A Q L S Y R Q E D A D W S R D M R G K Q L I T P V N L E N W V I V F T R R N S A
1690    1700    1710    1720    1730    1740    1750    1760    1770    1780    1790    1800
1681    AGGCTCAAGATCTGGTCCAGACCCCTGAGCAGAGTGGGGCCACCATGGGGATGCGTATCAATCCCCCAGATATGTGAACCTCAAGAGCAGCCGCAACCTTCTACACTGCTCTGA
517    Q A Q D L V Q T L S R V G P P M G M R I N S P T I C E L Q D D R N D S Y I T A L
1810    1820    1830    1840    1850    1860    1870    1880    1890    1900    1910    1920
1801    AGCAGTATGTCACCCCTCAAACCCAGCTAGCCCTGACCATCTCCCAACAACAGGAAGATCGATACCGTGAATCAAGAAGTCTGTGTGGTATCACCACCTTCCCTAGCCAGTGTG
557    K Q Y V T P Q T Q A A L L L P P T N R K D R Y D A I K K F C C Y D H P V P S Q
1930    1940    1950    1960    1970    1980    1990    2000    2010    2020    2030    2040
1921    TTGTCAGAGAATCTGTCAAAGAAGCAGATGTTGATGTCGGTGGCCCAAGATGCCCATTCAGCTCAACTGTAAGCTTGGGGAGAGGCTGGTGCCTGGACATCCCACTCAAGAATC
597    V Q R T L S K R Q M L M S V A T K I A I Q L N C K L G G E V V C L D I P L K
2050    2060    2070    2080    2090    2100    2110    2120    2130    2140    2150    2160
2041    TGGTGGTGGTGGGATAGATCTTACCATGATCTAGCAAGAAAGCCGTTCTGTGGGGGGATGATCGCTCCATGAACAGGCACTCACTCGATATTACTCGAGGTGCATCTCAAC
637    V Y Y G I D S Y H D S S K R G R S V G G Y I A S N Q A L I R Y S R C T P
2170    2180    2190    2200    2210    2220    2230    2240    2250    2260    2270    2280
2161    ATTCCATGCAGGATGTAGGATGGCCCAAAGTTTCATGAAAGGGCCCTGGAGAAGTACCATGAGGTGAATGGTGTTTACCAGGAGGAGATCATCGTGTCCGTCAGCGGTGCGGTG
677    I S N Q E L N D D L A T D R A G A L E K H E Y N G S L E P E R I I T P R D G V
2290    2300    2310    2320    2330    2340    2350    2360    2370    2380    2390    2400
2281    ATGGTCAGCTGCCAGCAGTGTGTAACCAAGTCCCTCAGCTCAACGAATGCTTCAAGAGGAGCGGAGCCAAAGACTACAATCCAAGGTCGCTATAGTAGTGTGGAAGAAAGGATCA
717    I G Q E L A S F P D R P P Q A N D C P R S N S Q N D I A P E A A T P A K N
2410    2420    2430    2440    2450    2460    2470    2480    2490    2500    2510    2520
2401    ACACCCGATCTTTCAGGAGGCGCCCTCCCTAAACAACCCGTCGCCAGGACATTTGTCGACAGCCGTCACCAGACCCATATGGTATGACTTCTTCTGGTGGCAGCTGTGA
757    I T R F F A R S R S L X N R C D G T I N D I T I T R P I R N D F L L Y S Q S
2530    2540    2550    2560    2570    2580    2590    2600    2610    2620    2630    2640
2521    GGCAGGAAACAGTGACCCCAACCACTACAACGCTGATCTGGGACACCCAGGACTGAAGCCTGACACATGCAGCCGCTGGCCCTACAAAATGTGCCACCTACTACAACCTGGCGGAA
797    R Q G T Y T T T H Y N Y L L D T T G L K P D H H Q R L A Y X M C H I Y Y X P S
2650    2660    2670    2680    2690    2700    2710    2720    2730    2740    2750    2760
2641    CCATCCGAGTCCCTGCTCCCTGCCAGTACGCCACAAGCTGGCCTTCTGGTGGGACAGTCCATTCACAAGGACCCGATATGGCTCTGCTGACCCGCTTACTTCTGTAAGCTAATG
837    I R Y P A P C Q Y A H K L A F L V G Q S I K D P H M A L S D R L Y F L *
2770    2780    2790    2800    2810    2820    2830    2840    2850    2860    2870    2880
2761    GCAGCAACTTGTGACCATAAACCAACCAATCAAGCAGAAGATCAGGTGATCACTGCCAGCCGGTACTGGACACTCAAGCCAGTCCAGGCCAGAAAACCTCCAGTAACCAACA
2881    CGTGTTCATACCAATTTTAAAGTAGTCAGTAGATTTTATAACCAATGTTTTTAAAGGTGAGAAATGAAGTGTACATTTAATGATGATCTGGTGTAAACATGAGTGTACATGAAAG
3001    TTTGTAGGTAATGAGAGATTCGCTTTTATACCTGGATGATGGTGTATTTTAAAGCAGCTAGATTTTAGTATTTTTTGTGTGTTGAGAAAGGATGTTGTTGGCAATCAGAT
3121    AATGCCAATAGTCGAAATAATGAGAAACGATAGTCACTCTTAAAAATATTAGTGTGAATGAATCAAAGGATCAAAAAAATAAGAAAAAACAAGATTTATACCGGTAATGATAT
3241    ACTTAGTACCAATAATGAGAAATTTAGTCACTTTTAACTCTTTTAAATGCCATATGTTTAAAAAAGTGAAGATGACAGCTTATTTTTTTTACACACTGTTATCATTAATA
3361    CATTTTGTTGGTCAATTTTGCAAAAATGTTATGTAAGTACTGATGACTAGAAAACCTGTTCAAAATAAAAGTATGAAAAAATAAAAAAAAAAAAAAAAAAAAAAAAAA

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Fig. 1. Nucleotide sequence of the *Cg-Piwi*-like cDNA and its predicted deduced amino acid along with domain structures of Piwi in *C. gigas*. A: The start (ATG) and stop (TGA) codons are underlined. PAZ and PIWI domains are highlighted in yellow and red, respectively. The polyadenylation signal (AATAAA) is marked in red. B: Two conserved domains of Piwi in *C. gigas*.

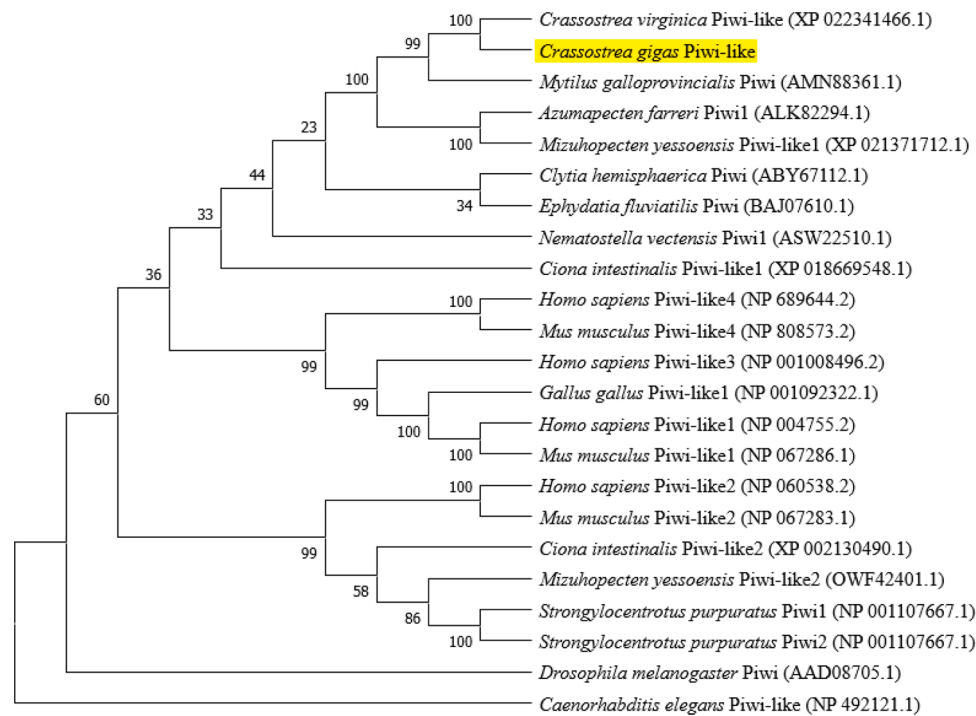


Fig. 2. Phylogenetic analysis of Piwi orthologs using the neighbor-joining method with 1000 bootstrap replications. Cg-Piwi-like protein was highlighted in yellow.

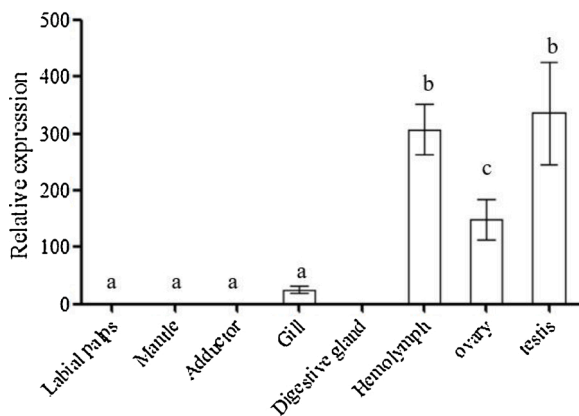


Fig. 3. Expression analysis of Cg-Piwi-like mRNA in various tissues. Bars with different letters differed at $p < 0.05$. Data are presented as means \pm SD with six samples in each group.

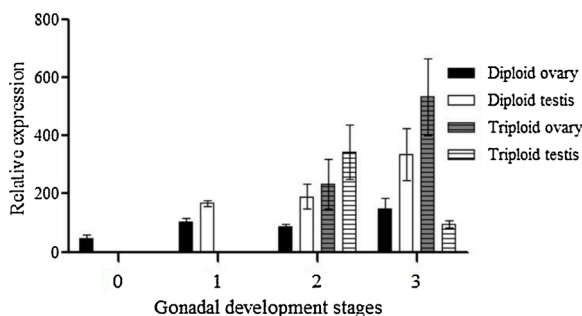


Fig. 4. Temporal expression profile of Cg-Piwi-like gene during gametogenesis. Data are presented as means \pm SD with six samples in each group.

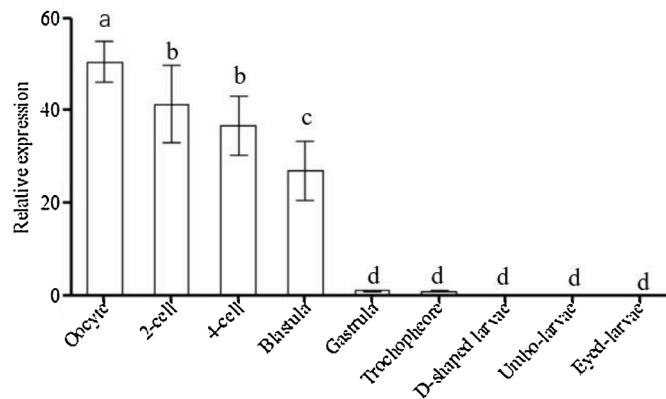
transcript abundance of Cg-Piwi-like slightly forward-scattered and back-scattered at the blastula stage, but progressively become restricted to two small descendent micromeres in mesoderm until the appearance of eyed-larvae (Fig. 5B). By the eyed-larvae stage, only one smaller spot could be detected.

4. Discussion

Gametogenesis and PGCs generation are the two essential components of animal reproduction. Previous research has shown that Piwi gene was involved in the process of germline fate specification, meiosis and maturation of gametes and germ stem cells self-renewal (Thomson and Lin, 2009). In this study, we identified the homologue of Piwi (Cg-Piwi-like), evolutionarily conserved germ line marker, and characterized its expression profile in embryo and adult C. gigas. The Cg-Piwi-like protein contained PIWI and PAZ conserved domains, which are the typical symbol of the Piwi-subfamily.

The expression of Cg-Piwi-like transcript was mainly restricted to the gonad as expected, which was in general agreement with the important role in the process of germline development (Houwing et al., 2007; Carmell, et al., 2007; Cox et al. 1998). Although Piwi is largely germline restricted, the expression patterns of Piwi in gonads differ from each other (Cox et al., 1998, 2000; Szakmary et al., 2005; Houwing et al., 2007; Carmell, et al., 2007). Most studies to date suggested that Piwi genes were specifically expressed in testis in mammals (Deng and Lin, 2002; Carmell, et al., 2007; Bao et al., 2014). Our results are similar to data for Piwi in zebrafish. The Piwi in zebrafish (Ziwi and Zili) can be detected in female and male germ cells (Houwing et al., 2007, 2008). Interestingly, besides high expression in gonadal tissues, Cg-Piwi-like transcript was also more abundant in the hemolymph, which indicates that it may has other functions. A latest study has demonstrated that several piRNA are ubiquitously expressed in gonadal tissues, eggs, and early embryo stages but also in hemolymph in the Pacific oyster (Jehn et al., 2018). It is well known that hemolymph is crucial in shellfish immune reactions and is involved in phagocytosis of microbes. Recent research also reported that the PIWI/piRNA pathway might be applied

A:



B:

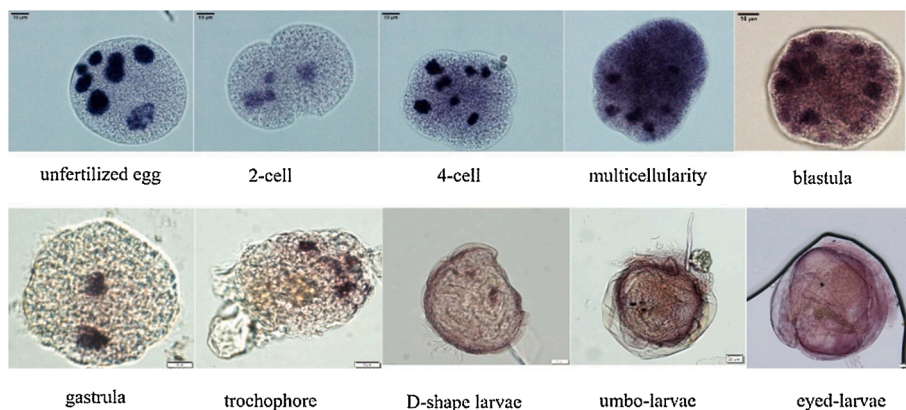


Fig. 5. Expression of *Cg-Piwi*-like gene in unfertilized oocytes and in various developmental stages of *C. gigas*. A: Quantitative real-time PCR (qPCR) results for *Cg-Piwi*-like. B: Location of *Cg-Piwi*-like in unfertilized egg and in various developmental stages by WISH.

to the antiviral defense of invertebrates (Léger et al., 2013; Hess et al., 2011).

In this study, *Cg-Piwi*-like mRNA expression levels showed a rising trend, and reached the highest level at mature stage during reproductive cycle of *C. gigas*. Recent research had indicated that the expression level of *Piwi* in testis was significantly higher than that in the ovary in fish (Tao et al., 2016; Zhang et al., 2014; Ni et al., 2019), which was consistent with our data. It has been proved that the expression of *Piwi* might be negatively regulated by DNA methylation in ovary (Zhang et al., 2014; Ni et al., 2019). These findings suggested that the *Cg-Piwi*-like gene was necessary for gonadal development, and was especially essential for the process of spermatogenesis in the testis. The conserved roles of *Piwi* is also strongly supported by ISH. *Cg-Piwi*-like mRNA was observed in all stages of oogenesis, and male germ cells were positive for *Cg-Piwi*-like RNA at stages from spermatogonia to spermatocytes. These expression patterns are consistent with the finding for fish (Ni et al., 2019; Wang et al., 2018; Zhao et al., 2012; Houwing et al., 2007; Wen et al., 2018) and were inconsistent with that mammals. In zebrafish, *Ziwi* is detected in both female and male germ cells, where expression appears strongest in the early differentiation of germ cells (Houwing et al., 2007). Loss of *Ziwi* leads to the failure of germ cells differentiation due to the appearance of germ cell apoptosis (Houwing et al., 2007). On the contrary, *Cg-Piwi*-like exhibited distinct expression patterns that were thematically different from those seen in mammal animals. The expression of the MIWI and MILI proteins in mouse is only testis restricted, and thus mutations in both *Piwi*-related genes caused male sterility with no defects in ovarian tissues (Wang et al., 2004; Carmell et al., 2007). The *Cg-Piwi*-like exhibited specific spatial expression

patterns correlated with germline development, indicating that *Cg-Piwi*-like was involved in differentiation, development, and maintenance of germ cells.

Of particular note here was the highest expression levels of *Cg-Piwi*-like in triploids during pre-ovulation and ovulation periods compared to diploids, which also could be seen obviously in other fish, such as crucian carp, half-smooth tongue sole (Zhang et al., 2014; Zhou et al., 2014). The *Piwi* pathway is well-recognized as a front-line defense against retrotransposon mobilization by binding with piRNA during gametogenesis development (Ku and Lin, 2014; Aravin et al., 2008; Reuter et al., 2011; Houwing et al., 2007). In crucian carp, five piRNAs showed significantly higher expression in the ovaries of sterile triploids than fertile diploids and tetraploids during ovulation (Zhou et al., 2014). In the latest studies, mollusks also utilize the PIWI/piRNA pathway as a defense against transposable elements in the germline (Jehn et al., 2018; Huang et al., 2019). Therefore, we speculate that PIWI/piRNA pathway might provide an adaptive defense in the transposon silencing in triploid oysters to ensure genomic stability. This silencing depends on the participation of the *Piwi* proteins and piRNA, and is essential for male fertility (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008).

Piwi is the conserved gene widely used as a molecular marker of PGCs (Cox et al., 1998; Houwing et al., 2008; Juliano et al., 2006; Sunanaga et al., 2010). *Cg-Piwi*-like mRNA was broadly expressed during early embryo development, and then gradually became enriched in the two cell clusters (presumptive PGCs) by the gastrula stage. The similar results from our previous work - were that, as levels of *Nanos* decline during gastrulation, its transcript became restricted specifically to the two putative germ cells during the same period (Xu et al., 2018). The

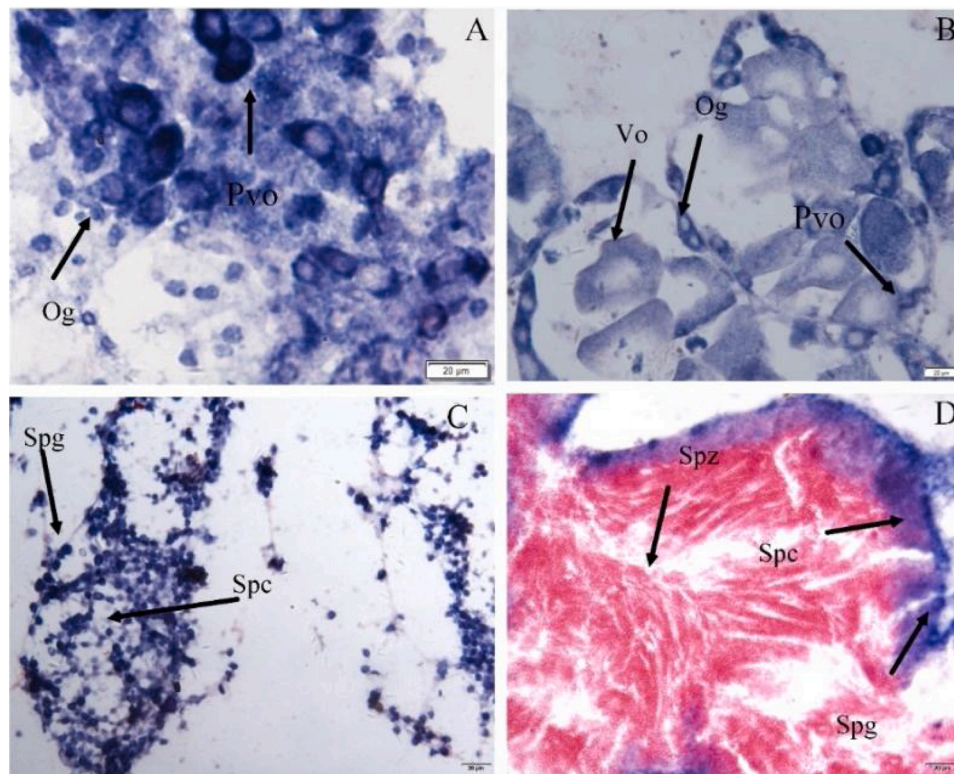


Fig. 6. Location of *Cg-Piwi*-like mRNA in *C. gigas* gonad during gametogenesis (A) and (C): Gametes proliferations in females and males; (B) and (D): mature stage in males and females. CT: Conjunctive tissues; Spg: Spermatogonia; Spc: Spermatocytes; Spz: Spermatozoa; Pro: Previtellogenic oocytes; Vo: Vitellogenic oocytes; Og: Oogonia.

characteristic of expression pattern was also seen in other mollusks (Swartz et al., 2008; Rabinowitz et al., 2008; Kakoi et al., 2008; Kranz et al., 2010). A common association between the putative PGCs and the gastrulation site might represent a conserved feature of molluscan development, although the fate map is opposite with germ cell formation involving preformation (Fabioux et al., 2004b). Consistent with other conserved germline genes, although uniformly distributed expression of *Cg-Piwi*-like was observed at earlier stages, by late larval stages, robust expression was specified in the putative PGCs and then was maintained lifelong in adult gonad tissue. The expression patterns reflected that both preformation and epigenesis of germ cell specification mechanisms might have co-existed in mollusks during embryonic development (Swartz et al., 2008; Rabinowitz et al., 2008; Kakoi et al., 2008; Kranz et al., 2010). This hypothesis would account for the extraordinary expression patterns of these conserved germline genes in a diverse and phylogenetically dispersed set of animals, such as *Clytia hemisphaerica*, *Capitella* sp., and *Nematostella vectensis* (Extavour et al., 2005; Leclère et al., 2012; Dill and Seaver, 2008). Besides, the overlapping sites for germ cell accumulation of these germ cell-associated genes in *C. gigas* suggest the conserved roles of these genes to mark putative PGCs, facilitating association of presumptive germ cells with mesodermally derived cells (Extavour and Akam, 2003). In other mollusks, such as in the *Crepidula fornicata* and *Sphaerium striatinum*, the PGCs resided in the embryonic mesoderm, demonstrating the cleavage pattern in PGCs specification in the embryonic mesodermal cells seems to constitute a molluscan specific trait (Lyons et al., 2012; Woods, 1931).

The recent advances of genome-editing technologies in the Pacific oyster, such as CRISPR, have enabled a new paradigm in which the editing of the genome can be precisely manipulated to study the function of any genes. The identification of candidate genes specifically expressed in the gonad (*vasa*, *nanos* and *piwi*) is the critical step for genetic engineering to induce complete sterility in the Pacific oyster.

In conclusion, *Cg-Piwi*-like was expressed throughout gametogenesis,

making it the ideal germ cell marker to study the molecular mechanism leading to the formation of the PGCs in *C. gigas*. Furthermore, the localization of *Cg-Piwi*-like transcript in the embryonic mesodermal cells supports the hypothesis that this is the site for germ cells accumulation.

Author contribution

Authors listed in the manuscript have made substantial contributions to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work.

CRedit authorship contribution statement

Rui Xu: Investigation, Methodology, Formal analysis, Writing - original draft. **Qi Li:** Methodology, Formal analysis, Writing - review & editing. **Hong Yu:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by the grants from National Natural Science Foundation of China (31972789 and 31672649), Fundamental Research Funds for the Central Universities (201762014), Shandong Province (2017LZGC009), and Guangxi Province (AA17204080-4).

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