

Piwi is essential for gametogenesis in mollusk *Chlamys farreri*

Xiaoshi Ma^{*}, Aichang Ji^{*}, Zhifeng Zhang, Dandan Yang, Shaoshuai Liang, Yuhan Wang and Zhenkui Qin

Key Laboratory of Marine Genetics and Breeding (Ocean University of China), Ministry of Education, Ocean University of China, Qingdao, China

^{*}These authors contributed equally to this work.

ABSTRACT

Piwi (P-element induced wimpy testis) is an important gene involved in stem cell maintenance and gametogenesis in vertebrates. However, in most invertebrates, especially mollusks, the function of *Piwi* during gametogenesis remains largely unclear. To further understand the function of *Piwi* during gametogenesis, full-length cDNA of *Piwi1* from scallop *Chlamys farreri* (*Cf-Piwi1*) was characterized, which consisted of a 2,637 bp open reading frame encoding an 878-amino acid protein. *Cf-Piwi1* mRNA was mainly localized in the spermatogonia, spermatocytes, oogonia, oocytes of early development and intra-gonadal somatic cells. Additionally, the knockdown of *Cf-Piwi1* by injection of *Cf-Piwi1*-dsRNA (double-stranded RNA) into scallop adductor led to a loss of germ cells in *C. farreri* gonads. Apoptosis was observed mainly in spermatocytes and oocytes of early development, as well as in a small number of spermatogonia and oogonia. Our findings indicate that *Cf-Piwi1* is essential for gametogenesis in the scallop *C. farreri*.

Subjects Marine Biology, Molecular Biology

Keywords *Chlamys farreri*, *Cf-piwi1*, Germ cell, Gametogenesis, Testis, Ovary

INTRODUCTION

Gametogenesis is the basis of animal reproduction and mainly includes germ stem cell self-renewal, meiosis and maturation of gametes. Studies of gametogenesis will help improve the reproductive ability and preservation of economically important species. It is known that many genes are involved in this process for model organisms, but the roles of these genes remain largely unknown in non-model organisms.

Piwi (P-element induced wimpy testis), a PIWI subfamily member of the Argonaute superfamily, is identified based on two conserved domains, PAZ and PIWI (*Cerutti, Mian & Bateman, 2000*). The PAZ domain, at the center of the amino acid sequence, contains a typical single stranded nucleic acid binding motif that can bind to the 3' end of short RNA (*Lingel et al., 2003; Yan et al., 2003*). The PIWI domain, found in the C-terminal region, functions to maintain *Piwi*'s stability and is structurally similar to the RNase H catalytic domain (*Liu et al., 2004; Song et al., 2004*).

The *Piwi* gene was first identified in *Drosophila melanogaster* and demonstrated a potentially important role in maintaining germ cells (GCs) (*Lin & Spradling, 1997*). Subsequently, *Piwi* homologues were reported in a variety of species, including

Submitted 28 March 2017

Accepted 14 May 2017

Published 23 June 2017

Corresponding author

Zhenkui Qin, qinzk@ouc.edu.cn

Academic editor

Saverio Brogna

Additional Information and
Declarations can be found on
page 11

DOI 10.7717/peerj.3412

© Copyright
2017 Ma et al.

Distributed under
Creative Commons CC-BY 4.0

OPEN ACCESS

Caenorhabditis elegans, *Bombyx mori*, *Danio rerio*, *Coturnix coturnix* and *Homo sapiens* (Lau et al., 2001; Sasaki et al., 2003; Houwing, Berezikov & Ketting, 2008; Chen et al., 2012; Tatsuke et al., 2014). The expression of the *Piwi* gene is mostly restricted to gametogenesis and early embryonic development, but its expression pattern and functions are not consistent in different animals (Deng & Lin, 2002; Megosh et al., 2006; Carmell et al., 2007; Houwing, Berezikov & Ketting, 2008; Wang & Reinke, 2008). In *D. melanogaster*, *Piwi* mutants eliminate the self-renewing division of germ stem cells (GSCs), and overexpressing *Piwi* in the germarium somatic cells results in an increase in number of GSCs and the rate of mitosis (Cox et al., 1998). In the flatworm *Macrostomum lignano*, knockdown of *Piwi* results in a complete elimination of all stem cells, including GSCs and somatic stem cells (De et al., 2009). Tatsuke et al. (2014) suggested that Siwi (the silkworm homologue of the *Piwi* protein) recruits HP1 proteins to a target site guided by the *Piwi*-piRNA complex, and then the *Piwi*-HP1 complex functions as a rapid transcriptional repressor to regulate gene expression in *B. mori*.

Mollusks are one of the most abundant and biologically diverse groups in the animal kingdom. Identifying early GCs is beneficial to the study of gametogenesis. However, it is difficult in histological sections of the mollusk gonads to distinguish various types of germ cells accurately, especially for the early development stages, because some features, such as cell size and karyoplasmic ratio, are diverse in different sections. These problems limit the study on the molecular mechanism of gametogenesis in mollusks. In this study, we cloned full-length cDNA of *Piwi1* in the scallop *Chlamys farreri*, a commercially important bivalve mollusk in China, and revealed its expression characteristics in the gonads during gametogenesis. Furthermore, the role of *C. farreri Piwi1* (*Cf-Piwi1*) in the scallop during gametogenesis was examined using RNAi. Our aims are to demonstrate the function of *Cf-Piwi1* during gametogenesis and investigate its potential feasibility as a molecular marker to identify early GCs in the scallop gonads.

MATERIALS AND METHODS

Ethics statement

The collection and handling of the scallops *C. farreri* were performed in accordance with the Institutional Animal Care and Use Committee of the Ocean University of China and the local government.

Specimen collection and sampling

Adult *C. farreri* scallops with a mean shell height of 6.28 ± 0.43 cm were collected from Shazikou (Qingdao, China). Gonads were dissected into 0.2 cm^3 pieces. Some of these pieces were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 24 h, dehydrated through serial methanol dilutions (25, 50, 75 and 100%) and stored in pure methanol at -20 °C for *in situ* hybridization (ISH). Some other pieces were fixed in Bouin's solution (picric acid, saturated aqueous solution - 75 ml; formalin, 40% aqueous solution - 25 ml; acetic acid, glacial - 5 ml) for 24 h and then stored in 70% ethanol for histological observation. The remaining pieces were immediately frozen in liquid nitrogen and stored

at -80°C for total RNA isolation. All the reagents used without specific indication were provided by Sangon Biotech (Shanghai, China).

Histology

Gonads stored in 70% ethanol were dehydrated in an ethanol dilution series, cleared with xylene, and embedded in paraffin wax according to the description of [Liu et al. \(2014\)](#). Sections were made at $5\ \mu\text{m}$ thickness and stained with hematoxylin and eosin. Observations and digital images were taken with a Nikon E80i microscope (Nikon, Tokyo, Japan).

Gonads were divided into four stages according to previously described morphological characteristics ([Liu et al., 2012](#)). The gonadosomatic indices ($\text{GSI} = \text{gonad weight}/\text{soft tissue body weight} \times 100\%$) are defined as resting stage ($\text{GSI } 3.73\%$ for females and 3.49% for males), proliferative stage ($\text{GSI } 4.32\%$ for females and 4.38% for males), growing stage ($\text{GSI } 5.39\%$ for females and 5.42% for males) and mature stage ($\text{GSI } 14.29\%$ for females and 12.48% for males).

Total RNA extraction and reverse transcription

Total RNA was extracted using the thiocyanate–phenol–chloroform method according to [Chomczynski & Sacchi \(1987\)](#). Quality and quantity of the RNA were measured using agarose gel electrophoresis and spectrophotometry. Reverse transcription for full-length cDNA cloning and qRT-PCR were performed according to manufacturer instructions using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, USA) and Primescript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Otsu, Japan), respectively.

Cloning and sequence analysis of full-length cDNA

A *Piwi1* cDNA fragment of 311 bp was obtained from the *C. farreri* transcriptome ([Wang et al., 2013](#)) and compared to the National Center for Biotechnology Information (NCBI) database using BLASTX. Amplification of 5'- and 3'-RACE were conducted with scallop testis cDNA and two specific PCR primers (PR-5': 5'-GCAACAGACATCAA CATCTGTTTCTTGG-3', PR-3': 5'-ATGCTGATTGGAGCAGAGATCTTCGTGG-3') according to the SMART™ RACE cDNA Amplification Kit protocol (Clontech, Mountain View, USA). PCR products were gel-purified and cloned into the pMD18-T vector (Takara, Otsu, Japan) then transformed into *Escherichia coli* DH5 α competent cells (Takara, Otsu, Japan). Positive clones were selected and sequenced. The full-length cDNA sequence was assembled using DNASTAR, Lasergene version 7.1.

The identity and similarity of the deduced amino acid sequence were analyzed with other known PIWI1 (*Homo sapiens*, *Mus musculus*, *Sus scrofa*, *Gallus gallus*, *Caprimulgus carolinensis*, *Xenopus tropicalis*, *Danio rerio*, *Alitta virens*, *Lottia gigantea*, *Crossostrea gigas*, *Mytilus galloprovincialis*, *Caenorhabditis elegans*) in GenBank using the online BLASTX tool. Multiple alignments were performed using the software CLUSTALX version 1.81 and DNAMAN version 8.0. We conducted a phylogenetic analysis using the neighbor-joining method in MEGA 5.0 with 1,000 bootstrap replicates ([Koichiro et al., 2011](#)).

qRT-PCR analysis

qRT-PCR was conducted using SYBR Green Real-Time PCR Master Mix (TOYOBO, Osaka, Japan) and an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA). A parallel amplification of the *C. farreri elongation factor 1 α* (*EF-1 α*) reference transcript (GenBank accession no. [AEX08674.1](#)) was performed to normalize expression data of *Cf-Piwi1* transcripts. Two pairs of specific primers, *Piwi1* F-1: 5'-CGGAGGCGTTGTGTGTAGCA-3', *Piwi1* R-1: 5'-CTGTCCATCCCCAACACCATC-3' for amplifying a 193 bp of *Cf-Piwi1*, and *EF-1 α* F: 5'-ATCCTTCCCTCCATCTCGTCCT-3', *EF-1 α* R: 5'-GGCACAGTTCCAATACCTCCA-3' for amplifying an 86 bp of *Cf-EF-1 α* were designed. RT-PCR conditions consisted of an initial denaturation step at 95 °C for 30 s followed by 40 cycles of 5 s of denaturation at 95 °C and 30 s of annealing and extension at 60 °C. Gonads from five individuals at each developmental stage were sampled, and triplicate assays for each gonad cDNA were conducted. The data were analyzed using the ABI 7500 system SDS software version 1.4 with automatically set baseline and cycle threshold values. Relative *Cf-Piwi1* mRNA levels were calculated based on the $2^{-\Delta\Delta C_t}$ method.

All data are presented as the means \pm standard error of five samples with three parallel repetitions. Differences between the means were tested using one-way analysis of variance (ANOVA) followed by least significant difference tests with the significance level set at $P < 0.05$ in SPSS version 17.0.

Tissue ISH

DIG-labeled RNA sense and anti-sense probes were synthesized from a 557 bp fragment of *Cf-Piwi1* from sites 3,078 to 3,634 according to instructions with the DIG RNA Labeling kit (Roche, Basel, Switzerland). Gonads stored for tissue ISH were cleared in xylene and embedded in paraffin wax before sectioning at 5 μ m for testes and 7 μ m for ovaries. Sections were fixed to a slide with 0.1% polylysine for 10 h at 37 °C. Before washing three times with PBST (phosphate-buffered saline with 0.1% Tween 20) and digesting with proteinase K (2 μ g ml⁻¹) for 15 min at 37 °C, samples were dewaxed in xylene and rehydrated through a descending series of methanol dilutions. After fixing with 4% paraformaldehyde for 1 h and prehybridizing at 60 °C for 6 h in hybridization buffer (50% formamide, 5% SSC, 5 mM EDTA, 100 mg ml⁻¹ ribonucleic acid, 1.5% blocking reagent, 0.1% Tween 20), samples were hybridized with digoxigenin (DIG)-labeled probes at 1 mg ml⁻¹ in hybridization buffer for 16 h at 60 °C. Following hybridization, samples were washed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween 20, pH 7.5) and incubated with alkaline phosphatase-conjugated anti-DIG antibody from the DIG Nucleic Acid Detection Kit (Roche, Basel, Switzerland) at 4 °C. After staining with NBT/BCIP (Roche, Basel, Switzerland) for 3 h at room temperature, the sections were counterstained with 1% neutral red. Hybridization signals were detected and photographed using a Nikon E80i microscope (Nikon, Tokyo, Japan).

dsRNA synthesis

A 726 bp fragment of *Cf-Piwi1* cDNA from sites 11 to 736 was amplified using the primers *Piwi1* F-2: 5'-TAATACGACTCACTATAGGGTTGAGAGGCAAGAAGTAACA-3' and

Piwi1 R-2: 5'-TAATACGACTCACTATAGGGGTACAGATGAAGGCACTGTG-3' (T7 promoter sequence underlined) with *C. farreri* testis RNA as the template. The purified PCR fragment was transcribed, and the double-strand RNA (dsRNA) was synthesized *in vitro* using T7 MEGAscript RNAi Kits (Ambion, Austin, USA) according to manufacturer instructions. Quality and quantity of the *Piwi1*-dsRNA was measured by 1% agarose gel electrophoresis and spectrophotometry.

dsRNA injection and sampling

Scallops with a mean shell height of 6.13 ± 0.54 cm at the proliferative stage were collected from Shazikou (Qingdao, China) and maintained in aerated running filtered seawater and fed with single cell algae during the experiment. A total of 75 scallops were randomly assigned to three groups. Scallops from the dsRNA group and PBS group were injected with 25 μ g *Cf-Piwi1* dsRNA diluted in 100 μ l PBS and 100 μ l PBS only into adductor muscle, respectively. Scallops in the blank group were not injected with anything. Two injections were conducted during the experiment: at the beginning and at day 7 of the experiment. On the third day after injection, three scallops from each group were removed randomly, and their gonads were sampled as described above to estimate the *Cf-Piwi1* knockdown effect. On the 10th day, eight scallops (five males and three females) from each group were sampled again.

Types of germ cell quantification

Five- μ m gonadal sections were conducted from scallops of each group following the method mentioned in Histology. To determine the effect of *Cf-Piwi1* knockdown, five squares (6,400 μ m² for ovary and 1,600 μ m² for testis) of the sections were randomly picked to calculate the mean number and composition of germ cells in the gonads of each group. Differences between cell quantities of different groups were tested using one-way analysis of variance (ANOVA) followed by least significant difference tests with the significance level set at $P < 0.05$ in SPSS version 17.0.

TUNEL assay

Five- μ m sections of the gonads were prepared after the RNAi experiment. To detect *in situ* cell apoptosis, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using a TdT-mediated dUTP apoptosis detection kit (Promega, Madison, USA) with a hematoxylin counterstain. The sections were observed and photographed using a Nikon E80i microscope (Nikon, Tokyo, Japan).

RESULTS

Sequence and characteristics of the *Cf-Piwi1* full-length cDNA

The full length of the *Cf-Piwi1* cDNA was 4,986 bp (GenBank accession number: [KR869093](#)) with a 59 bp 5' untranslated region (UTR), a 2,290 bp 3' UTR and a 2,637 bp open reading frame (ORF), encoding a putative protein of 879 amino acids, with a predicted molecular weight of 99.34 kDa and theoretical isoelectric point of 9.16. Multiple alignment indicated that the predicted protein contained a PAZ domain and a PIWI domain ([Fig. S1](#)) and was

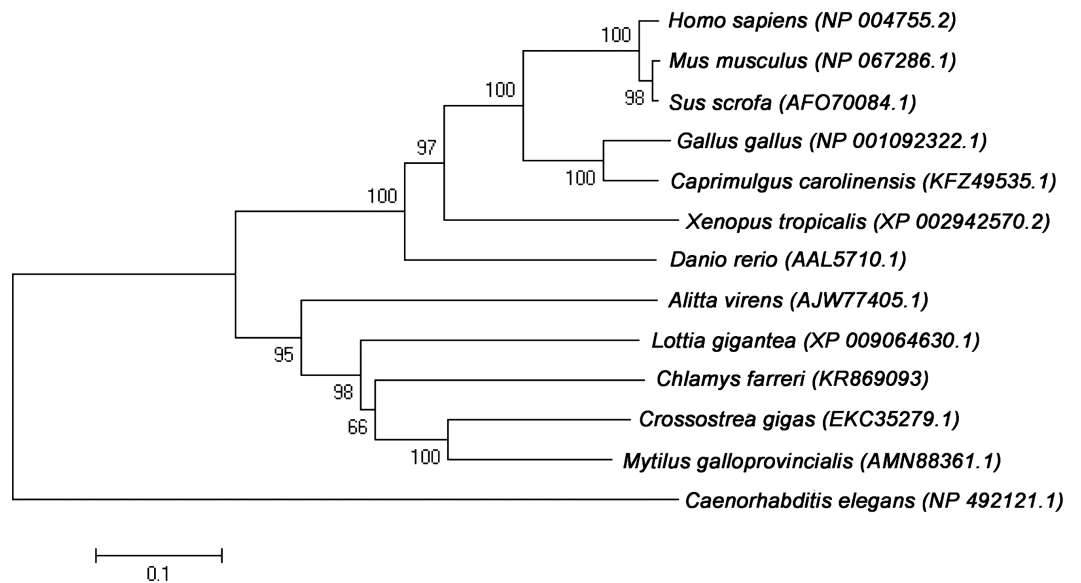


Figure 1 Phylogenetic analysis of *Piwi1* among various species based on the multiple sequence alignment.

highly homologous to other known PIWI1, with 57% identity to *Crassostrea gigas*, 51% to *Danio rerio* and 53% to *Homo sapiens*. Phylogenetic analysis showed that the predicted *Cf-Piwi1* first clustered with *C. gigas* and *M. galloprovincialis*, and then followed established evolutionary hypotheses (Fig. 1).

Quantitative expression of *Cf-Piwi1* mRNA in *C. farreri* gonads during the reproductive cycle

Expression levels of *Cf-Piwi1* in *C. farreri* gonads increased significantly from the resting to the mature stage (Fig. 2A and 2B). *Cf-Piwi1* expression in testes at the mature stage was approximately 2.5 times higher than that of the resting stage. In ovaries, it was about two-fold higher at the mature stage than the resting stage. No significant differences in *Cf-Piwi1* expression levels were observed between the ovary and testis at the same developmental stages ($P > 0.05$).

Cytolocation of *Cf-Piwi1* mRNA in *C. farreri* gonads during gametogenesis

Cf-Piwi1 mRNA was mainly located in GCs during early development. In ovaries, *Cf-Piwi1* transcripts were detected in oogonia, oocytes of early development and intra-gonadal somatic cells (ISCs) of germinal acini, but no positive signal was detected in mature oocytes (Figs. 2C–2H). In testes, obvious positive signals were observed in spermatogonia, spermatocytes and ISCs of germinal acini. However, no positive signal was visible in spermatids and spermatozoa (Figs. 2C, 2I–2N). Moreover, no positive signal was detected in gonads using sense probes (Figs. 2D, 2G and 2K).

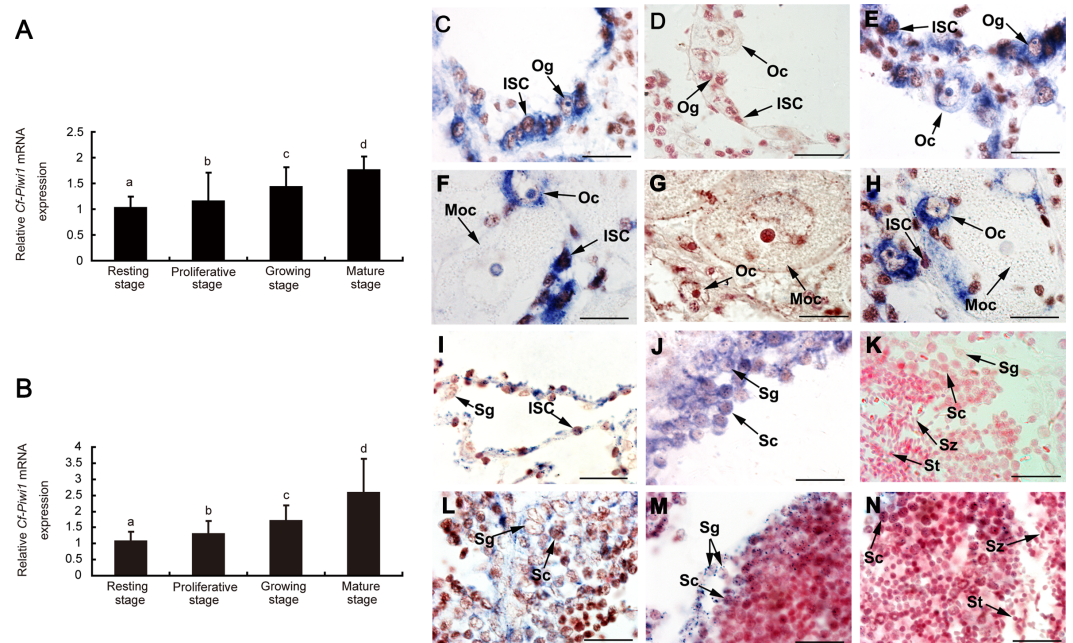


Figure 2 Relative abundance and location of *Cf-Piwi1* mRNA in *C. farreri* gonads. Relative abundance of *Cf-Piwi1* mRNA detected by qRT-PCR in (A) ovary and (B) testis. The expression level in gonads at the resting stage was set as 1.00; Values are the mean \pm SEM; $n = 3$; Different letters indicate statistically significant differences ($P < 0.05$). (C). Location of *Cf-Piwi1* mRNA detected by tissue ISH. Negative ISH using a sense probe (b, e, i). Positive signal from the anti-sense probe is stained in dark blue. (a), (c), (d) and (f), Ovaries at the resting, proliferative, growing, and mature stage, respectively; (g), (h), (j) and (k), Testes at the resting, proliferative, growing, and mature, respectively; (l), a different sight of the same section of (k) under microscope. ISC, Intragonadal somatic cell; Moc, Mature oocyte; Og, Oogonium; Oc, Oocyte; Sg, Spermatogonium; Sc, Spermatocyte; St, Spermatid; Sz, Spermatozoon. Magnification: Bar is 20 μ m.

Cf-Piwi1 knockdown led to abnormal development and apoptosis of GCs

qRT-PCR detected that levels of the *Cf-Piwi1* mRNA decreased significantly in the gonads of the dsRNA group than that of the PBS and Blank groups. The reduction of *Cf-Piwi1* expression levels in the *C. farreri* gonads between the first injection and the second injection in the dsRNA group was very similar, and the declines in the ovaries and testes were approximately 30% or 35% of that in the blank group, respectively (Fig. 3A and 3B).

To investigate the effects of *Cf-Piwi1* deficiency on gametogenesis, we performed a histological analysis. *Cf-Piwi1*-dsRNA provoked several defects in the development of GCs in both testes and ovaries. Compared with scallops from control groups, most oocytes in the ovaries of dsRNA scallops were at early developmental stage and many of them were stained darkly and presented abnormal morphological characteristics, implying that *Cf-Piwi1* downregulation might inhibit oocyte development (Figs. 3C–3H). In the testes of the *Cf-Piwi1* knockdown scallops, the arrangement of GCs in the germinal acini became loose, spermatids occurred only in few germinal acini and the number of spermatids was smaller compared visually to that of the blank and PBS groups (Figs. 3I–3N). Furthermore, we quantified the number and composition of germ cells in each group. In ovaries, more

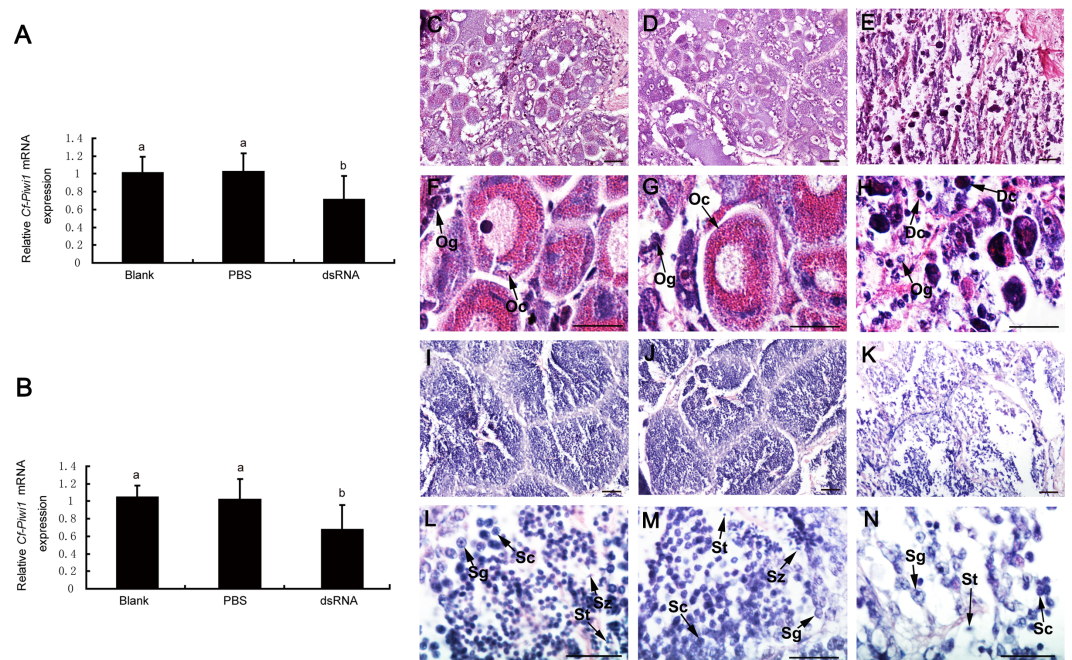


Figure 3 Expression of *Cf-Piwi1* mRNA and histology of scallop gonads after RNAi. Relative experiment level of *Cf-Piwi1* mRNA detected by qRT-PCR in (A) ovary and (B) testis on 10th day after RNAi. The expression level in gonads of the blank group was set as 1.00; Values are the mean \pm SEM; $n = 5$ in the testes; $n = 3$ in the ovaries; Different letters indicate statistically significant differences ($P < 0.05$). (C). Histological observation of scallop gonads on 10th day after RNAi. (a) and (d), ovary in the blank group; (b) and (e), ovary in the PBS group; (c) and (f), ovary in the dsRNA group; (g) and (j), testis in the blank group; (h) and (k), testis in the PBS group; (i) and (l), testis in the dsRNA group. Dc, Darkly stained cell; Og, Oogonium; Oc, Oocyte; Sc, Spermatoocyte; Sg, Spermatoogonium; St, Spermatoctid; Sz, Spermatozoan. Magnification: Bar is 40 μ m for (a), (b), (c), (g), (h) and (i); Bar is 20 μ m for (d), (e), (f), (j), (k) and (l).

than half the number of all kinds of germ cells decreased after knocking down of *Cf-Piwi1*, but the proportion of *Cf-Piwi1* expression cells (ISC 36.3%, oogonia 30.5% and oocyte 33.2%) increased when compared with control groups (ISC 32.1%, oogonia 35.7% and oocyte 18.2%) (Fig. 4A, Table S1). Similar results were obtained in testis with the percentage of spermatogonia and spermatoocyte changed from 11.4% and 67.2% of the control groups to that of 38.2% and 54.5% after RNAi (Fig. 4B, Table S2). Interestingly, we also found that in some germinal acini of the *Cf-Piwi1* knockdown scallops the number of spermatoocytes greatly decreased while spermatogonia and spermatoctids persisted (Figs. 3K and 3N).

The TUNEL assay results revealed that some of the oogonia and many oocytes were in the process of apoptosis in the ovaries of the dsRNA scallops, and that the majority of spermatoocytes and partial spermatogonia in *Cf-Piwi1* knockdown testes were undergoing apoptosis (Figs. 4E, 4H, 4K and 4N). Few apoptotic cells were found in the gonads of the blank and PBS groups (Figs. 4C, 4D, 4F, 4G, 4I, 4J, 4L and 4M).

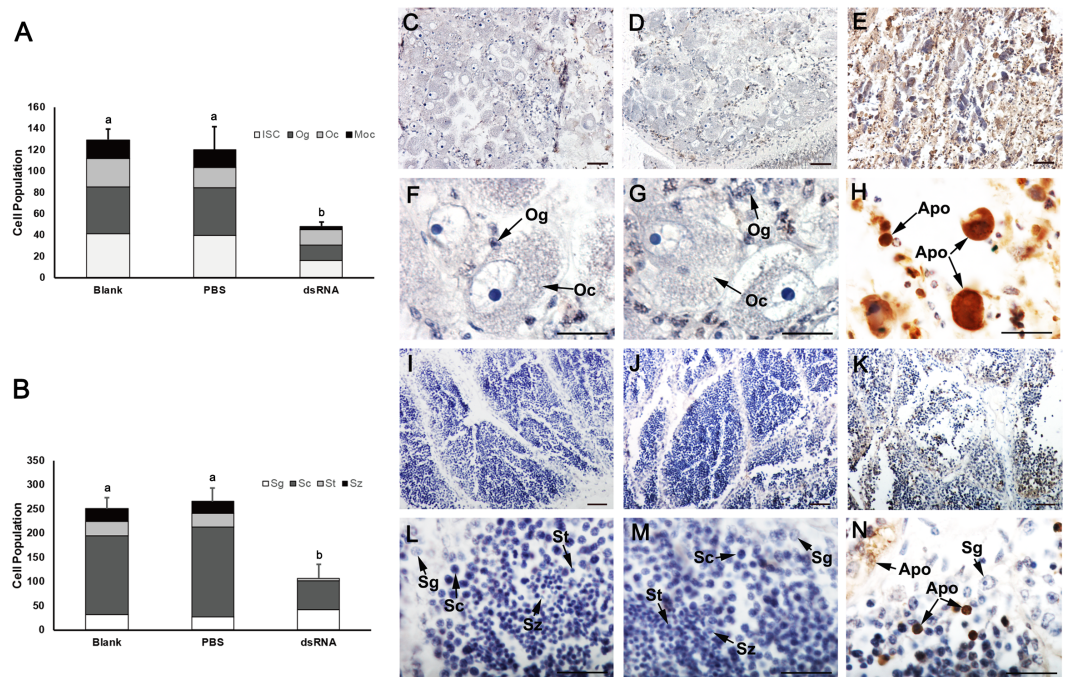


Figure 4 Quantification of the germ cells in scallop gonads after *Cf-Piwi1* knockdown and cell apoptosis analysis. Quantification of germ cells in (A) ovary, (B) testis and (C) cell apoptosis detection on 10th day after RNAi. (a) and (d), ovary in the blank group; (b) and (e), ovary in the PBS group; (c) and (f), ovary in the dsRNA group; (g) and (j), testis in the blank group; (h) and (k), testis in the PBS group; (i) and (l), testis in the dsRNA group. Apo, apoptosis cell; ISC, Intra-gonadal somatic cell; Moc, Mature oocyte; Og, Oogonium; Oc, Oocyte; Sg, Spermatogonium; Sc, Spermatocyte; St, Spermatid; Sz, Spermatozoon. The values are the mean \pm SEM; $n = 5$ in both testes and ovaries; different letters indicate statistically significant differences ($P < 0.05$). Magnification: Bar is 40 μm for (a), (b), (c), (g), (h) and (i); Bar is 20 μm for (d), (e), (f), (j), (k) and (l).

DISCUSSION

Cf-Piwi1 expression pattern in GCs is similar to that of fish

Localizations of *Piwi* transcripts are diverse in gonads of different species, although they are known to express mainly in GCs. In the planarian *Schmidtea mediterranea*, *Piwi* mRNA is visible in somatic stem cells and GCs (Reddien et al., 2005; Rossi et al., 2006; Palakodeti et al., 2008). In *D. melanogaster*, *Piwi* is expressed in all the cells of gonads (Cox, Chao & Lin, 2000). In *D. renio*, *Ziwi*, a *Piwi* homologue, is found only in GCs of gonads, where its expression appears to be the strongest in GCs at the mitotic and early meiotic stages (Houwling, Berezikov & Ketting, 2008). Similarly, in medaka (*Oryzias latipes*), *Piwi* is expressed in spermatogonia, spermatocytes and all ovarian GCs (Li et al., 2012). In *M. musculus*, *Miwi* expression appears to be restricted to the primary spermatocytes, secondary spermatocytes and the elongating spermatids, and no expression is observed in somatic cells of testis (Deng & Lin, 2002). In this study, *Cf-Piwi1* mRNA was expressed in male and female GCs of early development, which differs from that in mammals but is similar to that in fish.

***Cf-Piwi1* is possibly a molecular marker for early GCs**

Yano, Suzuki & Yoshizaki (2008) reported that *rtili*, a homolog of *Piwi* in the rainbow trout *Oncorhynchus mykiss*, is expressed specifically in spermatogonia and is used as a molecular marker to identify spermatogonia. In this study, we found that *Cf-Piwi1* expression was not only restricted to spermatogonia, but was also specifically visible in the GCs of early development, such as spermatogonia, spermatocytes, oogonia, and oocytes of early development. Thus, it can be potentially used to identify the GCs of early development in the testes and ovaries of *C. farreri*.

Cf-Piwi1* is essential for gametogenesis in *C. farreri

In model animals, the roles of *Piwi* on gametogenesis are diverse, but *Piwi* defects always result in the loss of GCs, reductions in nurse cell, poorly-developed egg chambers, and complete female sterility (*Lin & Spradling, 1997*). *Cox et al. (1998)* reported that *Piwi* mutations in *D. melanogaster* cause loss of GCs, but no dead cells were detected, which implies that the loss of *Piwi* can eliminate the self-renewing division of GSCs. Similarly, in *C. elegans*, decreasing *Piwi* expression by RNAi reduces the proliferation of GSC-equivalent cells (*Cox et al., 1998*). Moreover, in *Zili* (*Ziwi-like*) mutant zebrafish, almost all GCs are lost yet no apoptosis is present, suggesting that loss is possibly due to their inability to proliferate and differentiate (*Houwing, Berezikov & Ketting, 2008*). Mutation of a hypomorphic *Zili* allele blocks oogenesis in Meiosis I and induces terminal female sterility (*Houwing, Berezikov & Ketting, 2008*). However, *Houwing et al. (2007)* found that the reduction of *Ziwi* in *D. rerio* leads to various spermatogenic cell losses by apoptosis. In *M. musculus*, significant numbers of apoptotic cells were detected in spermatocyte layers due to the loss of *Mili* (*Kuramochi-Miyagawa et al., 2004*). *Miwi*-knockout mice display a drastic increase in apoptotic cell numbers of testes and spermatogenic arrest at the round spermatid stage (*Deng & Lin, 2002*). Additionally, *Miwi2* mutants exhibit spermatogenic cell apoptosis and predominantly arrest at the leptotene stage of meiosis (*Carmell et al., 2007*).

In this study, we found that knockdown of *Cf-piwi1* lead to significant cell number reduction and most of the remaining germ cells detained at the early development stages, implying its important role in the germ cell proliferation and differentiation. In the meantime, the proportion of *Cf-piwi1* expression cells increased after RNA interference also indicated that gametogenic arrest occurred at early development stages, which was accordant with the reported studies. Cell apoptosis assay presented that marked apoptosis occurred mainly in the spermatocytes of the testes and oocytes of the ovaries in *Cf-Piwi1* knockdown scallops, respectively, indicating that germ cells at earlier development stages gradually died after earlier accumulation. In addition, small numbers of spermatogonia and spermatids existed despite a great reduction of spermatocytes also demonstrating that spermatogenesis was blocked in spermatocytes. All these results suggest that *Cf-Piwi1* plays an important role in *C. farreri* gametogenesis.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the Scientific and Technological Innovation Project from Laboratory for Marine Fisheries and Aquaculture, Qingdao National Laboratory for Marine Science and Technology (2015ASKJ02) and National Natural Science Foundation of China (31602139). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

Laboratory for Marine Fisheries and Aquaculture, Qingdao National Laboratory for Marine Science and Technology: 2015ASKJ02.

National Natural Science Foundation of China: 31602139.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Xiaoshi Ma performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Aichang Ji performed the experiments, analyzed the data.
- Zhifeng Zhang conceived and designed the experiments, reviewed drafts of the paper.
- Dandan Yang performed the experiments, contributed reagents/materials/analysis tools.
- Shaoshuai Liang and Yuhuan Wang contributed reagents/materials/analysis tools.
- Zhenkui Qin reviewed drafts of the paper.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The *Cf-Piwi1* cDNA sequence described here are accessible via GenBank accession number [KR869093](#).

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as [Supplemental Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.3412#supplemental-information>.

REFERENCES

- Carmell MA, Girard A, Hj VDK, Bourc'His D, Bestor TH, De Rooij DG, Hannon GJ. 2007. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Developmental Cell* 12:503–514
[DOI 10.1016/j.devcel.2007.03.001](https://doi.org/10.1016/j.devcel.2007.03.001).

- Cerutti L, Mian N, Bateman A. 2000.** Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the *Piwi* domain. *Trends in Biochemical Sciences* 25:481–482 DOI [10.1016/S0968-0004\(00\)01641-8](https://doi.org/10.1016/S0968-0004(00)01641-8).
- Chen R, Chang G, Zhang Y, Dai A, Ma T, Li J, Zhai F, Hua D, Xia M, Chen G. 2012.** Cloning of the quail *PIWI* gene and characterization of PIWI binding to small RNAs. *PLOS ONE* 7:e51724 DOI [10.1371/journal.pone.0051724](https://doi.org/10.1371/journal.pone.0051724).
- Chomczynski P, Sacchi N. 1987.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analysis Biochemistry* 162:156–159.
- Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H. 1998.** A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. *Genes and Development* 12:3715–3727 DOI [10.1101/gad.12.23.3715](https://doi.org/10.1101/gad.12.23.3715).
- Cox DN, Chao A, Lin H. 2000.** *piwi* encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* 127:503–514.
- De MK, Pfister D, Kuaes G, Egger B, Salvenmoser W, Willems M, Steger J, Fauster K, Micura R, Borgonie G. 2009.** Stem cells are differentially regulated during development, regeneration and homeostasis in flatworms. *Developmental Biology* 334:198–212 DOI [10.1016/j.ydbio.2009.07.019](https://doi.org/10.1016/j.ydbio.2009.07.019).
- Deng W, Lin H. 2002.** *miwi*, a murine homolog of *piwi*, encodes a cytoplasmic protein essential for spermatogenesis. *Developmental Cell* 2:819–830 DOI [10.1016/S1534-5807\(02\)00165-X](https://doi.org/10.1016/S1534-5807(02)00165-X).
- Houwing S, Berezikov E, Ketting RF. 2008.** Zili is required for germ cell differentiation and meiosis in zebrafish. *EMBO Journal* 27:2702–2711 DOI [10.1038/emboj.2008.204](https://doi.org/10.1038/emboj.2008.204).
- Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, Van deH, Filippov DV, Blaser H, Raz E, Moens CB. 2007.** A role for *Piwi* and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell* 129:69–82 DOI [10.1016/j.cell.2007.03.026](https://doi.org/10.1016/j.cell.2007.03.026).
- Koichiro T, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011.** MEGA5: molecular evolutionary genetics analysis using likelihood, distance, and parsimony methods. *Molecular Biology and Evolution* 28:2731–2739 DOI [10.1093/molbev/msr121](https://doi.org/10.1093/molbev/msr121).
- Kuramochi-Miyagawa S, Kimura T, Ijiri TW, Isobe T, Asada N, Fujita Y, Ikawa M, Iwai N, Okabe M, Deng W. 2004.** *Mili*, a mammalian member of *piwi* family gene, is essential for spermatogenesis. *Development* 131:839–849 DOI [10.1242/dev.00973](https://doi.org/10.1242/dev.00973).
- Lau NC, Lim LP, Weinstein EG, Bartel DP. 2001.** An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* 294:858–862 DOI [10.1126/science.1065062](https://doi.org/10.1126/science.1065062).
- Li M, Hong N, Gui J, Hong Y. 2012.** Medaka *piwi* is essential for primordial germ cell migration. *Current Molecular Medicine* 12:1040–1049 DOI [10.2174/156652412802480853](https://doi.org/10.2174/156652412802480853).
- Lin H, Spradling AC. 1997.** A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* 124:2463–2476.

- Lingel A, Simon B, Izaurralde E, Sattler M. 2003. Structure and nucleic-acid binding of the *Drosophila* Argonaute 2 PAZ domain. *Nature* 426:465–469 DOI 10.1038/nature02123.
- Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshuator L, Hannon GJ. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. *Science* 305:1437–1441 DOI 10.1126/science.1102513.
- Liu J, Zhang Z, Zhang L, Liu X, Yang D, Ma X. 2014. Variations of estradiol-17 β and testosterone levels correlated with gametogenesis in the gonad of Zhikong scallop (*Chlamys farreri*) during annual reproductive cycle. *Canadian Journal of Zoology* 92:195–204 DOI 10.1139/cjz-2013-0202.
- Liu XL, Zhang ZF, Shao MY, Liu JG, Muhammad F. 2012. Sexually dimorphic expression of *foxl2* during gametogenesis in scallop *Chlamys farreri*, conserved with vertebrates. *Development Genes and Evolution* 222:279–286 DOI 10.1007/s00427-012-0410-z.
- Megosh HB, Cox DN, Campbell C, Lin H. 2006. The role of PIWI and the miRNA machinery in *Drosophila* germline determination. *Current Biology* 16:1884–1894 DOI 10.1016/j.cub.2006.08.051.
- Palakodeti D, Smielewska M, Lu YC, Yeo GW, Graveley BR. 2008. The PIWI proteins SMEDWI-2 and SMEDWI-3 are required for stem cell function and piRNA expression in planarians. *RNA* 14:1174–1186 DOI 10.1261/rna.1085008.
- Reddien PW, Oviedo NJ, Jennings JR, Jenkin JC, Sánchez AA. 2005. SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* 310:1327–1330 DOI 10.1126/science.1116110.
- Rossi L, Salvetti A, Lena A, Batistoni R, Deri P, Pugliesi C, Loreti E, Gremigni V. 2006. *DjPiwi-1*, a member of the *PAZ-Piwi* gene family, defines a subpopulation of planarian stem cells. *Development Genes and Evolution* 216:335–346 DOI 10.1007/s00427-006-0060-0.
- Sasaki T, Shiohama A, Minoshima S, Shimizu N. 2003. Identification of eight members of the Argonaute family in the human genome. *Genomics* 82:323–330 DOI 10.1016/S0888-7543(03)00129-0.
- Song JJ, Smith SK, Hannon GJ, Joshua-Tar L. 2004. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* 305:1434–1437 DOI 10.1126/science.1102514.
- Tatsuke T, Zhu L, Li Z, Mitsunobu H, Yoshimura K, Mon H, Lee JM, Kusakabe T. 2014. Roles of *Piwi* proteins in transcriptional regulation mediated by HP1s in cultured silkworm cells. *PLOS ONE* 9:e92313 DOI 10.1371/journal.pone.0092313.
- Wang G, Reinke V. 2008. A *C. elegans* Piwi, PRG-1, regulates 21U-RNAs during spermatogenesis. *Current Biology* 18:861–867 DOI 10.1016/j.cub.2008.05.009.
- Wang S, Hou R, Bao Z, Du H, He Y, Su H, Zhang Y, Fu X, Jiao W, Li Y. 2013. Transcriptome sequencing of zhikong scallop (*Chlamys farreri*) and comparative transcriptomic analysis with Yesso Scallop (*Patinopecten yessoensis*). *PLOS ONE* 8:e63927 DOI 10.1371/journal.pone.0063927.

- Yan KS, Yan S, Farooq A, Han A, Zeng L, Zhou MM. 2003.** Structure and conserved RNA binding of the PAZ domain. *Nature* **426**:469–474 DOI [10.1038/nature02129](https://doi.org/10.1038/nature02129).
- Yano A, Suzuki K, Yoshizaki G. 2008.** Flow-cytometric isolation of testicular germ cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout *vasa* regulatory regions. *Biology of Reproduction* **78**:151–158 DOI [10.1095/biolreprod.107.064667](https://doi.org/10.1095/biolreprod.107.064667).