Piwi1 is essential for gametogenesis in mollusk *Chlamys farreri*

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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, and 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 a small number of spermatogonia and oogonia. Our findings indicate that *Cf-Piwi1* is essential for gametogenesis in the scallop *C. farreri.*

1 ***Piwi1* is essential for gametogenesis in mollusk *Chlamys***

2 ***farreri***

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17 **Abstract**

18   *Piwi* (P-element induced wimpy testis) is an important gene involved in stem cell

19   maintenance and gametogenesis in vertebrates. However, in most invertebrates, especially

20   mollusks, the function of *Piwi* during gametogenesis remains largely unclear. To further

21   understand the function of *Piwi* during gametogenesis, full-length cDNA of *Piwi1* from scallop

22   *Chlamys farreri* (*Cf-Piwi1*) was characterized, which consisted of a 2,637 bp open reading frame

23   encoding an 878-amino acid protein. *Cf-Piwi1* mRNA was mainly localized in the

24   spermatogonia, spermatocytes, and oogonia, oocytes of early development and intra-gonadal

25   somatic cells. Additionally, the knockdown of *Cf-Piwi1* by injection of *Cf-Piwi1*-dsRNA

26   (double-stranded RNA) into scallop adductor led to a loss of germ cells in *C. farreri* gonads.

27   Apoptosis was observed mainly in spermatocytes and oocytes of early development, as well as a

28   small number of spermatogonia and oogonia. Our findings indicate that *Cf-Piwi1* is essential for

29   gametogenesis in the scallop *C. farreri.*

Introduction

30

31   Gametogenesis is the basis of animal reproduction and mainly includes germ stem cell self-

32 renewal, meiosis and maturation of gametes. Studies of gametogenesis will help improve the

33 reproductive ability and preservation of economically important species. It is known that many

34 genes are involved in this process for model organisms, but the roles of these genes remain

35 largely unknown in non-model organisms.

36   Piwi (P-element induced wimpy testis), a PIWI subfamily member of the Argonaute

37 superfamily, is identified based on two conserved domains, PAZ and PIWI (Cerutti et al. 2000).

38 The PAZ domain, at the center of the amino acid sequence, contains a typical single stranded

39 nucleic acid binding motif that can bind to the 3' end of short RNA (Lingel et al. 2003; Yan et al.

40 2003). The PIWI domain, found in the C-terminal region, functions to maintain Piwi's stability

41 and is structurally similar to the RNase H catalytic domain (Liu et al. 2004; Song et al. 2004).

42   The *Piwi* gene was first identified in *Drosophila melanogaster* and demonstrated a

43 potentially important role in maintaining germ cells (GCs) (Lin and Spradling 1997).

44 Subsequently, *Piwi* homologues were reported in a variety of species, including *Caenorhabditis*

45 *elegans*, *Bombyx mori*, *Danio rerio*, *Coturnix coturnix* and *Homo sapiens* (Lau et al. 2001;

46 Sasaki et al. 2003; Houwing et al. 2008; Chen et al. 2012; Tatsuke et al. 2014). The expression of

47 the *Piwi* gene is mostly restricted to gametogenesis and early embryonic development, but its

48 expression pattern and functions are not consistent in different animals (Deng and Lin 2002;

49 Megosh et al. 2006; Carmell et al. 2007; Houwing et al. 2008; Wang and Reinke 2008). In *D.*

50 *melanogaster*, *Piwi* mutants eliminate the self-renewing division of germ stem cells (GSCs), and

51 overexpressing *Piwi* in the germarium somatic cells results in an increase in number of GSCs

52 and the rate of mitosis (Cox et al. 1998). In the flatworm *Macrostomum lignano*, knockdown of

53 *Piwi* results in a complete elimination of all stem cells, including GSCs and somatic stem cells

54 (De Mulder et al. 2009). Tatsuke et al. (2014) suggested that Siwi (the silkworm homologue of

55 the Piwi protein) recruits HP1 proteins to a target site guided by the Piwi-piRNA complex, and

56 then the Piwi-HP1 complex functions as a rapid transcriptional repressor to regulate gene

57 expression in *B. mori*.

58   Mollusks are one of the most abundant and biologically diverse groups in the animal

59 kingdom. Identifying early GCs is beneficial to the study of gametogenesis. However, it is

60 difficult in histological sections of the mollusk gonads to distinguish various types of germ cells

61 accurately, especially for the early development stages, because some features, such as cell size

62 and karyoplasmic ratio, are diverse in different sections. These problems limit the study on the

63 molecular mechanism of gametogenesis in mollusk. In this study, we cloned full-length cDNA of

64 *Piwi1* in the scallop *Chlamys farreri*, a commercially important bivalve mollusk in China, and

65 revealed its expression characteristics in the gonads during gametogenesis. Furthermore, the role

66 of *C. farreri Piwi1* (*Cf-Piwi1*) in the scallop during gametogenesis was examined using RNAi.

67 Our aims are to demonstrate the function of *Cf-Piwi1* during gametogenesis and investigate its

68 potential feasibility as a molecular marker to identify early GCs in the scallop gonads.

69 ## Materials and methods

70 **Ethics Statement**

71   The collection and handing of the scallops *C. farreri* were performed in accordance with the

72 Institutional Animal Care and Use Committee of the Ocean University of China and the local

73 government.

74 **Specimen collection and sampling**

75   Adult *C. farreri* scallops with a mean shell height of 6.28 ± 0.43 cm were collected from

76 Shazikou (Qingdao, China). Gonads were dissected into 0.2 cm^3 pieces. Some of these pieces

77 were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h,

78 dehydrated through serial methanol dilutions (25, 50, 75 and 100%) and stored in pure methanol

79 at -20°C for *in situ* hybridization (ISH). Some other pieces were fixed in Bouin's solution (picric

80 acid, saturated aqueous solution - 75 ml; formalin, 40% aqueous solution - 25 ml; acetic acid,

81 glacial - 5 ml) for 24 h and then stored in 70% ethanol for histological observation. The

82   remaining pieces were immediately frozen in liquid nitrogen and stored at -80°C for total RNA

83   isolation. All the reagents used without specific indication were provided by Sangon Biotech

84   (Shanghai, China).

Histology

86   Gonads stored in 70% ethanol were dehydrated in an ethanol dilution series, cleared with

87   xylene, and embedded in paraffin wax according to the description of Liu et al. (2014). Sections

88   were made at 5 μm thickness and stained with hematoxylin and eosin. Observations and digital

89   images were taken with a Nikon E80i microscope (Nikon, Tokyo, Japan).

90   Gonads were divided into four stages according to previously described morphological

91   characteristics (Liu et al. 2012). The gonadosomatic indices (GSI = gonad weight/soft tissue

92   body weight × 100%) are defined as resting stage (GSI 3.73% for females and 3.49% for males),

93   proliferative stage (GSI 4.32% for females and 4.38% for males), growing stage (GSI 5.39% for

94   females and 5.42% for males) and mature stage (GSI 14.29% for females and 12.48% for males).

Total RNA extraction and reverse transcription

96   Total RNA was extracted using the thiocyanate–phenol–chloroform method according to

97   Chomczynski et al (1987) . Quality and quantity of the RNA were measured using agarose gel

98   electrophoresis and spectrophotometry. Reverse transcription for full-length cDNA cloning and

99   qRT-PCR were performed according to manufacturer instructions using the SMARTer™ RACE

100 cDNA Amplification Kit (Clontech, Mountain View, USA) and Primescript™ RT reagent Kit

101 with gDNA Eraser (Perfect Real Time) (Takara, Otsu, Japan), respectively.

Cloning and sequence analysis of full-length cDNA

103   A *Piwi1* cDNA fragment of 311 bp was obtained from the *C. farreri* transcriptome (Wang et

104 al., 2013) and compared to the National Center for Biotechnology Information (NCBI) database

105 using BLASTX. Amplification of 5'- and 3'-RACE were conducted with scallop testis cDNA

106 and two specific PCR primers (PR-5': 5'-GCAACAGACATCAACATCTGTTTCTTGG-3', PR-

107   3': 5'-ATGCTGATTGGAGCAGAGATCTTCGTGG-3') according to the SMART™ RACE

108   cDNA Amplification Kit protocol (Clontech, Mountain View, USA). PCR products were gel-

109   purified and cloned into the pMD18-T vector (Takara, Otsu, Japan) then transformed into

110   *Escherichia coli* DH5α competent cells (Takara, Otsu, Japan). Positive clones were selected and

111   sequenced. The full-length cDNA sequence was assembled using DNASTAR, Lasergene version

112   7.1.

113   The identity and similarity of the deduced amino acid sequence were analyzed with other

114   known PIWI1 (*Homo sapiens*, *Mus musculus*, *Sus scrofa*, *Gallus gallus*, *Caprimulgus*

115   *carolinensis*, *Xenopus tropicalis*, *Danio rerio*, *Alitta virens*, *Lottia gigantea*, *Crossostrea gigas*,

116   *Mytilus galloprovincialis*, *Caenorhabditis elegans*) in GenBank using the online BLASTX tool.

117   Multiple alignments were performed using the software CLUSTALX version 1.81 and

118   DNAMAN version 8.0. We conducted a phylogenetic analysis using the neighbor-joining

119   method in MEGA 5.0 with 1,000 bootstrap replicates (Koichiro et al. 2011).

120   **qRT-PCR analysis**

121   qRT-PCR was conducted using SYBR Green Real-Time PCR Master Mix (TOYOBO,

122   Osaka, Japan) and an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City,

123   USA). A parallel amplification of the *C. farreri elongation factor 1α* (*EF-1α*) reference transcript

124   (GenBank accession no. AEX08674.1) was performed to normalize expression data of *Cf-Piwi1*

125   transcripts. Two pairs of specific primers, *Piwi1*F-1: 5'-CGGAGGCGTTGTGTGTAGCA-3',

126   *Piwi1*R-1: 5'- CTGTCCATCCCCAACACCATC-3' for amplifying a 193 bp of *Cf-Piwi1*, and

127   *EF-1α*F: 5'-ATCCTTCCTCCATCTCGTCCT-3', *EF-1α*R: 5'-

128   GGCACAGTTCCAATACCTCCA-3' for amplifying an 86 bp of *Cf-EF-1α* were designed. RT-

129   PCR conditions consisted of an initial denaturation step at 95°C for 30 s followed by 40 cycles of

130   5 s of denaturation at 95°C and 30 s of annealing and extension at 60°C. Gonads from five

131   individuals at each developmental stage were sampled, and triplicate assays for each gonad

132   cDNA were conducted. The data were analyzed using the ABI 7500 system SDS software

133   version 1.4 with automatically set baseline and cycle threshold values. Relative *Cf-Piwi1* mRNA

134   levels were calculated based on the $2^{-\Delta\Delta Ct}$ method.

135   All data are presented as the means ± standard error of five samples with three parallel

136   repetitions. Differences between the means were tested using one-way analysis of variance

137   (ANOVA) followed by least significant difference tests with the significance level set at $P < 0.05$

138   in SPSS version 17.0.

139   **Tissue ISH**

140   DIG-labeled RNA sense and anti-sense probes were synthesized from a 557 bp fragment of

141   *Cf-Piwi1* from sites 3078 to 3634 according to instructions with the DIG RNA Labeling kit

142   (Roche, Basel, Switzerland). Gonads stored for tissue ISH were cleared in xylene and embedded

143   in paraffin wax before sectioning at 5 μm for testes and 7 μm for ovaries. Sections were fixed to

144   a slide with 0.1% polylysine for 10 h at 37°C. Before washing three times with PBST

145   (phosphate-buffered saline with 0.1% Tween 20) and digesting with proteinase K (2 μg ml^{-1}) for

146   15 min at 37°C, samples were dewaxed in xylene and rehydrated through a descending series of

147   methanol dilutions. After fixing with 4% paraformaldehyde for 1 h and prehybridizing at 60°C

148   for 6 h in hybridization buffer (50% formamide, 5% SSC, 5 mM EDTA, 100 mg ml^{-1} ribonucleic

149   acid, 1.5% blocking reagent, 0.1% Tween 20), samples were hybridized with digoxigenin (DIG)-

150   labeled probes at 1 mg ml^{-1} in hybridization buffer for 16 h at 60°C. Following hybridization,

151   samples were washed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween 20,

152   pH 7.5) and incubated with alkaline phosphatase-conjugated anti-DIG antibody from DIG

153   Nucleic Acid Detection Kit (Roche, Basel, Switzerland) at 4°C. After staining with NBT/BCIP

154   (Roche, Basel, Switzerland) for 3 h at room temperature, the sections were counterstained with

155   1% neutral red. Hybridization signals were detected and photographed using a Nikon E80i

156   microscope (Nikon, Tokyo, Japan).

157   **dsRNA synthesis**

158   A 726 bp fragment of *Cf-Piwi1* cDNA from sites 11 to 736 was amplified using the primers

159   *Piwi1*F-2: 5'-TAATACGACTCACTATAGGGTTGAGAGGCAAGAAGTAACA-3' and

160   *Piwi1*R-2: 5'-TAATACGACTCACTATAGGGGGTACAGATGAAGGCACTGTG-3' (T7

161   promoter sequence underlined) with *C. farreri* testis RNA as the template. The purified PCR

162   fragment was transcribed, and the double-strand RNA (dsRNA) was synthesized *in vitro* using

163   T7 MEGAscript RNAi Kits (Ambion, Austin, USA) according to manufacturer instructions.

164   Quality and quantity of the *Piwi1*-dsRNA was measured by 1% agarose gel electrophoresis and

165   spectrophotometry.

166   **dsRNA injection and sampling**

167   Scallops with a mean shell height of 6.13 ± 0.54 cm at the proliferative stage were collected

168   from Shazikou (Qingdao, China) and maintained in aerated running filtered seawater and fed

169   with single cell algae during the experiment. A total of 75 scallops were randomly assigned to 3

170   groups. Scallops from the dsRNA group and PBS group were injected with 25 μg *Cf-Piwi1*

171   dsRNA diluted in 100 μl PBS and 100 μl PBS only into adductor muscle, respectively. Scallops

172   in the blank group were not injected with anything. Two injections were conducted during the

173   experiment, at the beginning and at day 7 of the experiment. On the 3rd day after injection, 3

174   scallops from each group were removed randomly, and their gonads were sampled as described

175   above to estimate the *Cf-Piwi1* knockdown effect. On the 10th day, 8 scallops (5 males and 3

176   females) from each group were sampled again.

177   **Types of germ cells quantification**

178   Five-μm gonadal sections were conducted from scallops of each group following the

179   method mentioned in Histology. To determine the effect of *Cf-Piwi1* knockdown, five squares

180   (6400 μm^2 for ovary and 1600 μm^2 for testis) of the sections were randomly picked to calculate

181   the mean number and composition of germ cells in the gonads of each group. Differences

182   between cell quantities of different groups were tested using one-way analysis of variance

183   (ANOVA) followed by least significant difference tests with the significance level set at $P < 0.05$

184   in SPSS version 17.0.

185   **TUNEL assay**

186   Five-μm sections of the gonads were prepared after the RNAi experiment. To detect *in situ*

187   cell apoptosis, a TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end

188   labeling) assay was performed using a TdT-mediated dUTP apoptosis detection kit (Promega,

189   Madison, USA) with a hematoxylin counterstain. The sections were observed and photographed

190   using a Nikon E80i microscope (Nikon, Tokyo, Japan).

191   **Results**

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

193   The full length of the *Cf-Piwi1* cDNA was 4,986 bp (GenBank accession number:

194   KR869093) with a 59 bp 5' untranslated region (UTR), a 2,290 bp 3' UTR and a 2,637 bp open

195   reading frame (ORF), encoding a putative protein of 879 amino acids, with a predicted molecular

196   weight of 99.34 kDa and theoretical isoelectric point of 9.16. Multiple alignment indicated that

197   the predicted protein contained a PAZ domain and a PIWI domain (Fig. S1) and was highly

198   homologous to other known PIWI1, with 57% identity to *Crassostrea gigas*, 51% to *Danio rerio*

199   and 53% to *Homo sapiens*. Phylogenetic analysis showed that the predicted *Cf*-Piwi1 first

200   clustered with *C. gigas* and *M. galloprovincialis*, and then followed established evolutionary

201   hypotheses (Fig. 1).

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

203   **cycle**

204   Expression levels of *Cf-Piwi1* in *C. farreri* gonads increased significantly from the resting to

205   the mature stage (Fig. 2 Ⅰ and Ⅱ). *Cf-Piwi1* expression in testes at the mature stage was

206   approximately 2.5 times higher than that of the resting stage. In ovaries, it was about two-fold

207   higher at the mature stage than the resting stage. No significant differences in *Cf-Piwi1*

208   expression levels were observed between the ovary and testis at the same developmental stages

209   (*P* > 0.05).

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

211   *Cf-Piwi1* mRNA was mainly located in GCs during early development. In ovaries, *Cf-Piwi1*

212   transcripts were detected in oogonia, oocytes of early development and intra-gonadal somatic

213   cells (ISCs) of germinal acini, but no positive signal was detected in mature oocytes (Fig. 2Ⅲ a-

214   d). In testes, obvious positive signals were observed in spermatogonia, spermatocytes and ISCs

215   of germinal acini. However, no positive signal was visible in spermatids and spermatozoa (Fig.

216   2Ⅲ e-i). Moreover, no positive signal was detected in gonads using sense probes (Fig. 2Ⅲ b0,

217   d0, g0).

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

219   qRT-PCR detected that levels of the *Cf-Piwi1* mRNA decreased significantly in the gonads

220   of the dsRNA group than that of the PBS and Blank groups. The reduction of *Cf-Piwi1*

221   expression levels in the *C. farreri* gonads between the first injection and the second injection in

222   the dsRNA group was very similar, and the declines in the ovaries and testes were approximately

223   30% or 35% of that in the blank group, respectively (Fig. 3Ⅰ and Ⅱ).

224   To investigate the effects of *Cf-Piwi1* deficiency on gametogenesis, we performed a

225   histological analysis. *Cf-Piwi1*-dsRNA provoked several defects in development of GCs in both

226   testes and ovaries. Compared with scallops from control groups, most oocytes in the ovaries of

227   dsRNA scallops were at early developmental stage and many of them were stained darkly and

228   presented abnormal morphological characteristics, implying that *Cf-Piwi1* downregulation might

229   inhibit oocyte development (Fig. 3Ⅲ a-f). In the testes of the *Cf-Piwi1* knockdown scallops, the

230   arrangement of GCs in the germinal acini became loose, spermatids occurred only in few

231   germinal acini and the number of spermatids was smaller compared visually to that of the blank

232   and PBS groups (Fig. 3Ⅲ g-l). Furthermore, we quantified the number and composition of germ

233 cells in each group. In ovaries, more than half number of all kinds of germ cells decreased after

234 knocking down of *Cf-Piwi1*, but the proportion of *Cf-Piwi1* expression cells (ISC 36.3%,

235 oogonia 30.5% and oocyte 33.2%) increased when comparing with control groups (ISC 32.1%,

236 oogonia 35.7% and oocyte 18.2%) (Fig. 4 Ⅰ , Table S1). Similar results were obtained in testis

237 with the percentage of spermatogonia and spermatocyte changed from 11.4% and 67.2% of the

238 control groups to that of 38.2% and 54.5% after RNAi (Fig. 4 Ⅱ , Table S2). Interestingly, we

239 also found in some germinal acini of the *Cf-Piwi1* knockdown scallops, number of spermatocytes

240 greatly decreased while spermatogonia and spermatids persisted (Fig. 3 Ⅲ i&l).

241   The TUNEL assay results revealed that some of the oogonia and many oocytes were in the

242 process of apoptosis in the ovaries of the dsRNA scallops, and the majority of spermatocytes and

243 partial spermatogonia in *Cf-Piwi1* knockdown testes were undergoing apoptosis (Fig. 4 Ⅲ c, f, i,

244 l). Few apoptotic cells were found in the gonads of the blank and PBS groups (Fig. 4 Ⅲ a, b, d, e,

245 g, h, j, k).

Discussion

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

248   Localizations of *Piwi* transcripts are diverse in gonads of different species, although they are known

249 to express mainly in GCs. In the planarian *Schmidtea mediterranea*, *Piwi* mRNA is visible in somatic

250 stem cells and GCs (Reddien et al. 2005; Rossi et al. 2006; Palakodeti et al. 2008). In *D. melanogaster*,

251 *Piwi* is expressed in all the cells of gonads (Cox et al. 2000). In *D. renio*, *Ziwi*, a *Piwi* homologue, is

252 found only in GCs of gonads, where its expression appears to be the strongest in GCs at the mitotic and

253 early meiotic stages (Houwing et al. 2008). Similarly, in medaka (*Oryzias latipes*), *Piwi* is expressed in

254 spermatogonia, spermatocytes and all ovarian GCs (Li et al. 2012). In *M. musculus*, *Miwi* expression

255 appears to be restricted to the primary spermatocytes, secondary spermatocytes and the elongating

256 spermatids, and no expression is observed in somatic cells of testis (Deng and Lin 2002). In this study,

257   *Cf-Piwi1* mRNA was expressed in male and female GCs of early development, which differs from that in

258   mammals but is similar to that in fish.

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

260   Yano et al. (2008) reported that *rtili*, a homolog of *Piwi* in the rainbow trout *Oncorhynchus mykiss*, is

261   expressed specifically in spermatogonia, and is used as a molecular marker to identify spermatogonia. In

262   this study, we found that *Cf-Piwi1* expression was not only restricted to spermatogonia, but was also

263   specifically visible in the GCs of early development, such as spermatogonia, spermatocytes, oogonia, and

264   oocytes of early development. Thus, it can be potentially used to identify the GCs of early development in

265   the testes and ovaries of *C. farreri*.

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

267   In model animals, the roles of *Piwi* on gametogenesis are diverse, but *Piwi* defects always result in

268   the loss of GCs, reductions in nurse cell, poorly-developed egg chambers, and complete female sterility

269   (Lin and Spradling 1997). Cox et al. (1998) reported that *Piwi* mutations in *D. melanogaster* cause loss of

270   GCs, but no dead cells were detected, which implies that the loss of *Piwi* can eliminate the self-renewing

271   division of GSCs. Similarly, in *C. elegans*, decreasing *Piwi* expression by RNAi reduces the proliferation

272   of GSC-equivalent cells (Cox et al. 1998). Moreover, in *Zili* (*Ziwi-like*) mutant zebrafish, almost all GCs

273   are lost yet no apoptosis is present, suggesting that loss is possibly due to their inability to proliferate and

274   differentiate (Houwing et al. 2008). Mutation of a hypomorphic *Zili* allele blocks oogenesis in Meiosis I

275   and induces terminal female sterility (Houwing et al. 2008). However, Houwing et al. (2007) found that

276   the reduction of *Ziwi* in *D. renio* leads to various spermatogenic cell losses by apoptosis. In *M. musculus*,

277   significant numbers of apoptotic cells were detected in spermatocyte layers due to the loss of *Mili*

278   (Kuramochi-Miyagawa et al. 2004). *Miwi*-knockout mice display a drastic increase in apoptotic cell

279   numbers of testes and spermatogenic arrest at the round spermatid stage (Deng and Lin 2002).

280   Additionally, *Miwi2* mutants exhibit spermatogenic cell apoptosis and predominantly arrest at the

281   leptotene stage of meiosis (Carmell et al. 2007).

282   In this study, we found that knockdown of *Cf-piwi1* lead to significant cell number reduction and

283 most of the remaining germ cells detained at the early development stages, implying its important role in

284 the germ cell proliferation and differentiation. In the meantime, the proportion *Cf-piwi1* expression cells

285 increased after RNA interference also indicated the gametogenic arrest occurred at early development

286 stages, which was accordant with the reported studies. Cell apoptosis assay presented that marked

287 apoptosis occurred mainly in the spermatocytes of the testes and oocytes of the ovaries in *Cf-Piwi1*

288 knockdown scallops, respectively, indicating germ cells at earlier development stages gradually died after

289 earlier accumulation In addition, small numbers of spermatogonia and spermatids existed despite a great

290 reduction of spermatocytes also demonstrated that spermatogenesis was blocked in spermatocytes. All

291 these results suggest that *Cf-Piwi1* plays an important role in *C. farreri* gametogenesis.

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Figure 1

Phylogenetic analysis of Piwi1 among various species based on the multiple sequence alignment.
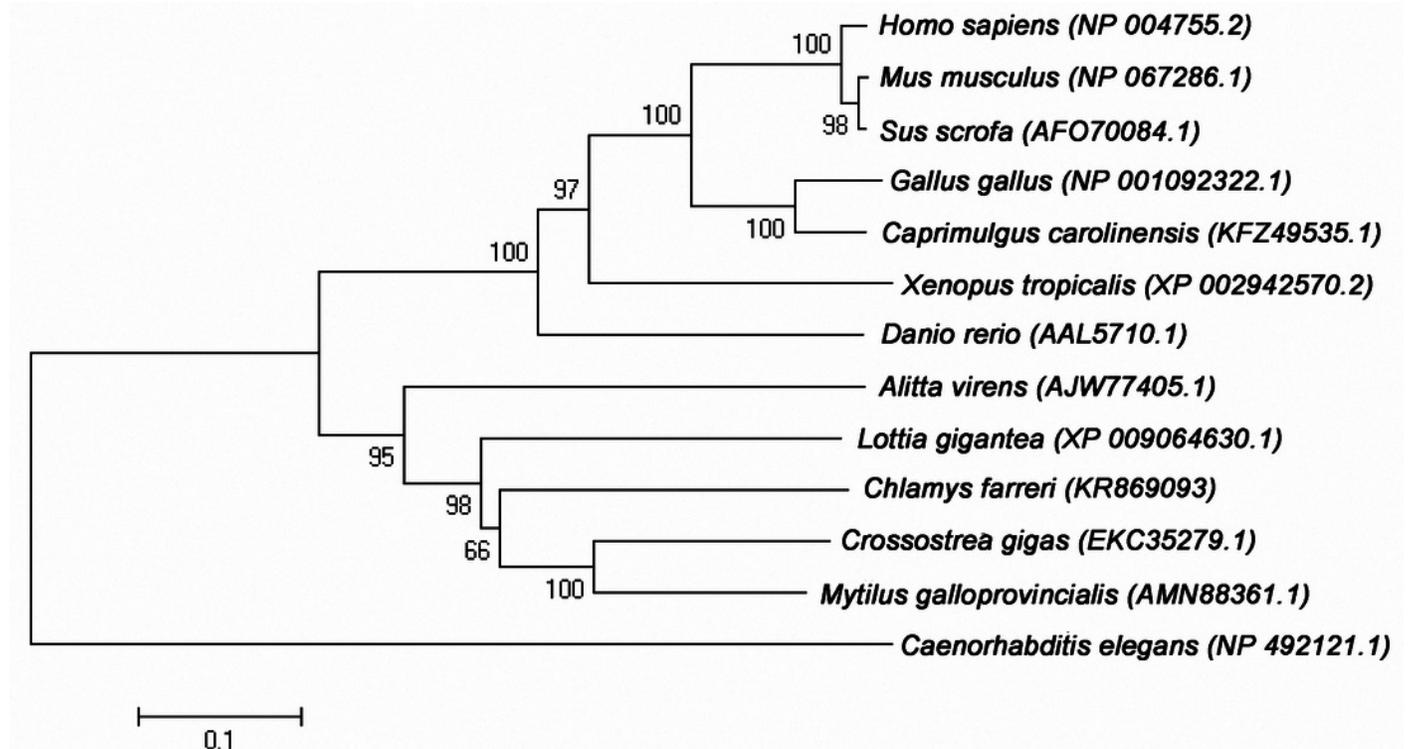
Figure 2

Relative abundance and location of *Cf-Piwi1* mRNA in *C. farreri* gonads.

Relative abundance of *Cf-Piwi1* mRNA detected by qRT-PCR in I) ovary and II) testis. The expression level in gonads at the resting stage was set as 1.00; Values are the mean ± SEM; n = 3; Different letters indicate statistically significant differences ($P < 0.05$). III). Location of *Cf-Piwi1* mRNA detected by tissue *ISH*. Negative *ISH* using a sense probe (b0, d0, g0). Positive signal from the anti-sense probe is stained in dark blue. (a), (b), (c) and (d), Ovaries at the resting, proliferative, growing, and mature stage, respectively; (e), (f), (g) and (h), Testes at the resting, proliferative, growing, and mature, respectively; (i), a different sight of the same section of (h) 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 µm.
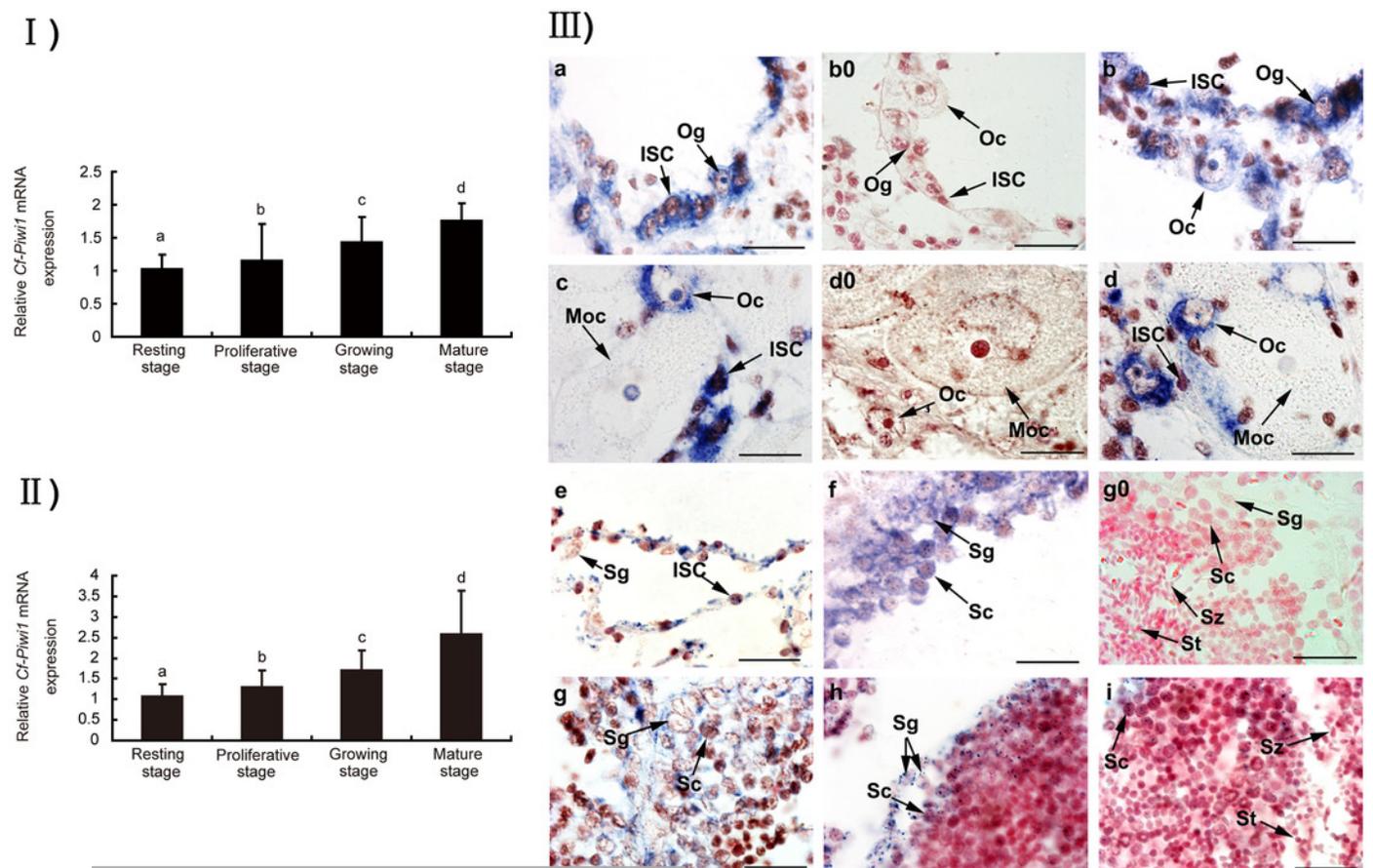
Figure 3

Expression of *Cf-Piwi1* mRNA and histology of scallop gonads after RNAi.

Relative experiment level of *Cf-Piwi1* mRNA detected by qRT-PCR in I) ovary and II) testis on 10th day after RNAi. The expression level in gonads of the blank group was set as 1.00; Values are the mean ± SEM; n = 5 in the testes; n = 3 in the ovaries; Different letters indicate statistically significant differences ($P < 0.05$). III). 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, Spermatocyte; Sg, Spermatogonium; St, Spermatid; Sz, Spermatozoon. 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).
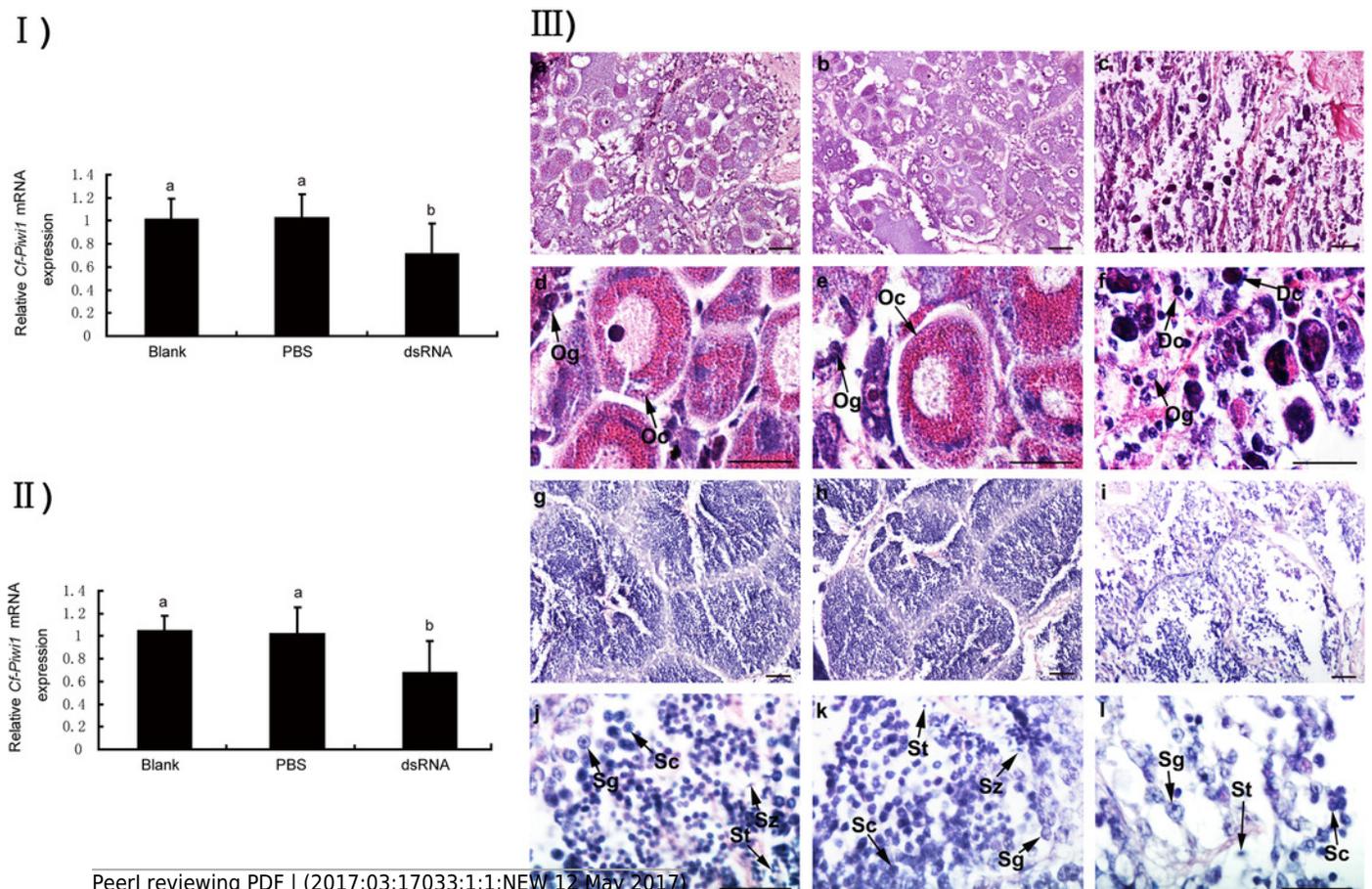
Figure 4

Quantification of the germ cells in scallop gonads after *Cf-Piwi1* knockdown and cell apoptosis analysis.

Quantification of germ cells in I) ovary and II) testis and cell apoptosis detection III) 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, Intragonadal somatic cell; Moc, Mature oocyte; Og, Oogonium; Oc, Oocyte; Sg, Spermatogonium; Sc, Spermatocyte; St, Spermatid; Sz, Spermatozoon. The values are the mean ± 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).