エゾアワビ卵母細胞および初期胚における母性ｍＲＮＡノックダウン法の開発

Doctoral Dissertation

DEVELOPMENT OF mRNA KNOCK-DOWN SYSTEM VIA dsRNA-MEDIATED RNA INTERFERENCE IN OOCYTES AND EARLY EMBRYOS OF PACIFIC ABALONE *Haliotis discus hannai*

September 2018

Graduate School of Marine Science and Technology

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CONTENTS

Chapter 1. Introduction and general background

1. Germ cells and their application to developmental biotechnology

Bisexually reproducing organisms have two major cell lineages, that is soma cells and germ cells. Somatic cells form the body responsible for the individual life and are mortal, whereas germ cells are unique in their ability to transfer genetic information and traits from generation to generation (Ikenishi et al., 1998; Xu et al., 2010). The proper development of germ cells and the integrity of their genome are paramount to the health of organisms and the survival of species.

Generally, germ cells are formed as primordial germ cells (PGCs) early in embryonic development that come from presumptive PGCs (pPGCs). The pPGCs are the germline precursor and undergo asymmetric cell division, producing two daughter cells with different fates: one cell is committed to the soma whereas the other is committed to pPGC. When they are committed to the germline, the parental cell is said to be a PGC. PGCs migrate through various somatic tissues to the developing gonad called the gonadal anlage. Upon arrival at the prospective gonad, PGCs coalesce with the somatic cells to form an intact gonad. Sometimes, in certain species such as in mice, PGCs may stop mitotic division and enter a period of mitotic quiescence in G0 (Braat et al., 1999). Such quiescent germ cells are now called prospermatogonia or gonocytes. During sexual maturation, gonocytes become oogonia and spermatogonia, and enter meiosis for ultimate differentiation respectively into gametes, sperm or eggs in the female and male.

PGCs, having a large size (about 20 μm in diameter), a large nuclei (6-10 μm) and relatively little cytoplasm, are characterized by the presence of nuage or germinal granules. Nuage is rich in mitochondria and is thus also called a mitochondrial cloud,

which is a membrane-less cytoplasmic organelle containing RNAs and proteins (Hamaguchi et al., 1982). Currently, many studies of germinal granules in several animal models have focus mainly on the asymmetric partitioning of the structures into prospective PGCs during early embryogenesis, indicating that putative pPGCs are fate-determined by maternally inherited factors. These maternal factors are usually concentrated in germinal granules in oocytes and cleavage embryos, and these factors directly involved in the germ cell fate decision are called germ plasm (GP). Germ plasm (GP) is a ribonucleoprotein (RNP) complex comprising dozens of different RNA and protein components. Their components assemble into a cytoplasmic architecture in a cytoskeleton-dependent manner. Although assembly and function of GP in diverse species and at different stages of germline development have not been fully understood, the GP of diverse species does share considerable similarities in morphology and molecular composition, suggesting a conserved role in germline development.

Germ cell markers have increasingly been characterized in aquaculture fishes. These genes include *vasa, nanos, dnd, dazl* and *cxcr4*, etc. Of them, the *vasa* gene was originally identified in *Drosophila* as maternal-effect gene required for the formation of the abdomen segments and for germ-cell specification (Santos and Lehmann, 2004). Following the isolation of *Drosophila vasa* gene, *vasa*-like DEAD-box RNA helicase genes that are expressed in germ cells were identified in many species, including zebrafish *Danio rerio*, medaka *Oryzias latipes*, rainbow trout *Oncorhynchus mykiss* (Raz, 2000). These researches suggested that *vasa* proteins show sequence conservation, allowing for profiling *vasa* protein expression via immunostaining by using an anti-*vasa*

antibody in diverse species. Importantly, the distribution of *vasa* RNA or protein was determined during different stages of development, thus providing information on the possible function of *vasa* during germ-cell development. Additionally, the loss-of-function phenotype in the fly *Drosophila*, the mouse and the nematode provided direct evidence for the role of *vasa* in the development of germ cells in these organisms (Lasko, 2013). Because the origin and precise route of germ-cell migration towards the gonad were unknown, it proved possible, using *vasa* as a molecular marker, to trace back the migration path and establish the position in which these cells originate (Li et al., 2009; Thorpe et al., 2004; Knaut et al., 2003; Kosaka et al., 2007). Continuing and expanding the work of other germ cell markers in different species is likely to contribute to our understanding of the molecular mechanisms of specification and differentiation of the germ cells across the animal kingdom.

The understanding of germ cell development provides a basis for germline engineering. One such approach is germ cell transplantation (GCT) (Xu et al., 2010). GCT was established initially in chickens *Gallus gallus* (Tajima et al., 1993) and mice (Brinster et al., 1994). Recently, GCT has been applied in a number of animal species including domestic mammals (Honaramooz et al., 2002) and teleosts (Yoshizaki et al., 2012). Because of the availability of transgenic fish (Yoshizaki et al., 2000) and its ease for *in vitro* fertilization and embryogenesis, GCT has been well used in aquaculture fish (Takeuchi et al., 2009). The first report is in the rainbow trout *Oncorhynchus mykiss* by grafting a testicular cell mixture into an isogeneic immature gonad with a low efficiency of donor-derived spermatogenesis (Nagler et al., 2001). Recently, successful

transplantation of spermatogonia into the testes of adult recipients has been performed in tilapia *Oreochromis niloticus* (Lacerda et al., 2006, 2010, 2012). Nowadays, the efficiency of germ cell transplantation has been improved by the development of the method for the in vitro culture of spermatogonia in several fish (Takeuchi et al., 2003; Saito et al., 2008; Okutsu et al., 2006). Other improvements include the identification of a cell surface protein that is predominantly expressed in rainbow trout type A spermatogonia (ASG), which could enable the enrichment of ASG from fish testes using specific antibody-mediated flow cytometry or magnetic cell sorting (Yoshizaki et al., 2012). Thus, these research efforts will lead to rapid advances in novel developmental biotechnology for aquaculture and conservation applications.

2. RNA interference and RNAi system

RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules. Historically, it was known by other names, including co-suppression, post-transcriptional gene silencing (PTGS), and quelling. Since the discovery of RNAi and its regulatory potentials, it has become evident that RNAi has immense potential in suppression of desired genes. RNAi is now known as precise, efficient, stable and better than antisense technology for gene suppression. Nowadays, RNAi has proved to be a very powerful tool in biomedicine for the investigation of gene function by gene silencing and for the control of virus by lowering mortality in various animals. Meanwhile, the mechanism of silencing genes by RNAi has allowed characterization of novel and unknown genes as

observed through loss-of-function experiments in a lot of organisms. For example, in tiger shrimp, silencing experiments has found that penaeidin acts as a pro-inflammatory cytokine (Li and Song, 2010). Generally, there are 3 common important features in RNAi. First, the inducer is dsRNA that may be produced locally or taken up by the cells through host receptors like *sid-1* (Labreuche et al., 2010) or scavenger receptors (Ulvila et al., 2006) and processed into small effector RNA duplexes. Second, the machinery requires a conserved set of proteins that unwinds RNA duplexes into siRNA and loads them as guide RNA. Finally, the target RNA is degraded in a homology-dependent fashion. The important role of the RNAi mechanism in the host is implicated by many studies in the regulation of endogenous gene expression and antiviral defense.

Relative to other animal groups, the studies of the RNAi in molluscs have been comparatively sparse and all the information known is probably based on a mosaic of about fourteen species. However, there appears to be a constant theme of homologous proteins and functioning similarities in the pathways rather than differences. The area needing most attention is the cephalopods for which there is a dearth of knowledge and may be the most likely place to find novel components (Owens and Malham, 2015). As we learn more about the mechanism of RNAi, it should become possible to design more efficient triggers and better assay conditions, and these parameters may be species-specific.

3. Background of abalone fisheries and aquaculture

Abalone, marine snails of the genus *Haliotis* and recalled as awabi in Japan, is a

popular and traditional food maintaining a good, consistent market value. Abalone are distributed along much of the world's coastline. They are found from the intertidal to depths of approximately 80-90 m, from tropical to cold waters (Hone and Fleming, 1998). Abalone species contain more than 15 subgenera comprising about 70 taxa and support an important marine fishery and aquaculture worldwide. In abalones, the so-called Pacific abalones includes *Haliotis discus hannai, H. discus discus, H. madaka,* and *H. gigantea,* among which *H. discus hannai* is the major abalone resource for coastal fisheries in Japan. In recent decades, the total quantity of abalone produced on farms worldwide has increased significantly while abalone obtained from fisheries has declined. Comparing current trends in supply and demand with those of the 1970s, when world abalone fisheries were at their height, is of limited value because, in those days, abalone fisheries were virtually unregulated, the illegal catch was insignificant, and abalone farming was only just beginning (Cook, 2014). Cook (2014) reported that legal landings from abalone fisheries have gradually decreased from almost 20,000 metric tons (mt) in the 1970s to only about 6,500 mt in 2015. In another way, over-exploitation, illegal harvesting, disease and habitat degradation have all contribution to production decline of abalone. Following such decline, several fisheries have suffered severe quota restrictions, or have been completely de-commercialized. So, as landings from legal fisheries were declining, farm production is rapidly increasing in several countries, especially in China and Korea.

Over the past decade, the major factor that affected the world abalone market has been a huge increase in illegal catches. In 2008, the worldwide illegal catch totaled about

5,300 mt, which representing over 65% of the total legal catch from fisheries (7,869 mt). Since 2008, there is some evidence that increased efforts to reduce poaching in countries such as South Africa and Australia, has resulted in slightly less illegal product reaching the market (Cook, 2014).

The total supply of abalone to the world market from all sources (fisheries, cultured and illegal catch), was 20,370 mt in 1970, 22,667 mt in 2002, 44,510 mt in 2008, 73,206 mt in 2010, and115,692 mt in 2013, 135,787 mt in 2015. The total supply in 2015 was more than three times that available in what has been considered the “heyday” of world abalone supply during the 1970’s. The largest increases have taken place as countries such as China and South Korea started farming abalone. Certainly, abalone production also shows changes in various countries between 2010 and 2015. While farm production in most countries, over this period, has either been stable or grown very slowly.

In China there are over 300 operating abalone farms in 2013, with the largest individual farm producing: over 1000 mt per year. Some of the newer Chinese farm are amongst the most efficient in the world. In the sea-based, abalone are housed in various types of rearing cages, suspended form long-lines. The abalone are generally fed a mixture of seaweeds, with some farms now favoring artificial feeds as the preferred diet. A major disadvantage of sea-based farms is that they are subject to bio-fouling that requires regular cleaning of cages. Even taking this into account, however, abalone in sea cages can be grown to market size at much lower costs than in the land-based farms.

Korea is now a major supplier of abalone to the world market. Farm production, which is estimated to have increased by over 60 times over the past 10 years, totaled

about 4,500 mt in 2007, and is estimated to reach over 10,000 mt by 2015. While, the majority of Korean production is consumed in domestic markets, the volume being exported to countries, such as Japan, China, the United State and Taiwan, has increased from about 70 mt in 2004 and 1,115 mt in 2014 (Park YB, personal communication). This production has had an important influence on the world market because the majority of Korean production is *Haliotis discus hannai*, the species that is most popular, and commands the highest price, in the Japanese market.

In the United State, farmed abalone production is about 362 mt in 2015. However, it is unlikely that total production along the Californian coast will increase much in the future because of very high land values and high compliance costs. In Europe, abalone farming is a very small, but growing, industry.

Significant quantities of farmed abalone are produced in Chile, Japan, South Africa, Australia, Taiwan, New Zealand, Mexico, Thailand and the Philippines and, in all of those cases, it seems likely that production will increase in the future (Korpov et al., 2000; Gordon et al., 2013).

The total supply of abalone available to the world market in 2015 was more than five times of that which was available in the 1970s (Cook, 2014). Certainly, this has resulted from huge increases in farm production. China is both a major producer and consumer of abalone, and while China has often been regarded as a huge marketing opportunity, overproduction in China could also represent a risk that may swamp world markets. Although it is expected that Chinese farm production will continue to increase, the rate of increase in production is expected to slow. Prices paid for farmed abalone on the world

market are affected by a number of different factors including the species cultured, the country of origin, the size at which the animals are marketed, and the quality of the meat. In Japanese and Chinese markets, *H. d. hannai* is the most valuable species and larger size animals command higher prices per kilogram. Recently, the fall in price together with the outbreak of several diseases reduced confidence in abalone farming in China and several farms ceased to operate, or turned to the production of alternative species. Fortunately, the most recent information suggests that the worst is now over and world abalone prices are beginning to move in a positive direction.

4. Objectives of the thesis

Despite these significant increases in abalone production, but several critical issues, such as slow growth rates and diseases, affect the abalone farming industry (Jiang et al., 2015). Importantly, gonad precocity of Pacific abalones has been observed but has not yet been well documented. The wild Pacific abalones reach sexual maturity as 3- or 4-year-olds, however, more than 50% of farmed abalone reach sexual maturity as 1-year-olds, leading to undesired smaller sized adults (Li et al., 2017). Thus, understanding of germ cells development in Pacific abalones is crucial for governing gonad development and precocity of this species. But, no germ cell related genes or germ cell marker could be used to identify germ cell development to date.

In present study, we aim to discover genes related to the gonadal development of Pacific abalone (Black) *H. d. discus*, especially as is required to identify genes specifically expressed in germ cells. To this end, functional analysis of the gonadal

transcriptome over multiple tissues from brain, ovary, testis and unfertilized eggs was conducted. A series of strategies, including different gene search methods and cloning method of target gene, RT-PCR and in situ hybridization (ISH) examination, were performed to discover and identify target genes. Finally, the role of *vasa* was also investigated in the oocytes and early embryos by knockdown of *vasa*-dsRNA. Our purpose is to demonstrate the function of target gene in germ cell development, investigate their feasibility as a germ cell molecular marker and evaluate the sterile potential of Pacific abalone *H. d. hannnai*.

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Chapter 2. Gonadal transcriptome analysis of Pacific Abalone (Black) *Haliotis discus discus*: identification of genes involved in germ cell development

1. Introduction

Abalones are one of the top-priced seafoods on the market owing to their delicate flavor and rich nutritional value. During the last decade, intensive harvest has led to depletion of their natural populations (Valenzuela-Miranda et al. 2015). High market prices for abalones has driven the development of its aquaculture. According to Cook (2014), annual global production of farmed abalone is currently on the rise, and most of the increases are in production of Pacific abalone (Black) *Haliotis discus discus* and a subspecies of Pacific abalone, Ezo abalone *Haliotis discus hannai*, which are cultured in China and Korea. Globally, the highest annual production of farmed abalone species is for Pacific abalone species (Di et al. 2015). However, despite these significant increases in production, several critical issues, such as slow growth rates and diseases, affect the abalone farming industry (Jiang et al. 2015).

Gonad precocity is one common issue that has been observed but has not yet been well documented. Pacific abalones in the wild reach sexual maturity as 3- or 4-year-olds; however, more than 50% of farmed abalone reach sexual maturity as 1-year-olds, leading to undesired smaller sized adults (Li et al. 2017, LY and YT unpublished). To combat this problem, sterilization may be an ideal strategy. Sterility of aquaculture-target fish has been achieved through traditional breeding techniques, such as triploidization, but high mortality caused by temperature- or chemical-shock treatments during the larval stages hinders commercial production of triploid abalones for use as aquaculture broodstock (Arai and Okumura 2013). In mollusks, commercial production of triploids has been achieved by crossing tetraploid x diploid Pacific oyster *Crassostrea gigas*, and this strategy appears to be ideal for the mass production of triploid artificial broodstock, as no

shock treatment is required (McCombie et al. 2005). However, no tetraploid broodstock has been produced in abalones to date. Further, production of abnormal aneuploid gametes was reported in sexually mature triploid Pacific abalones (Okumura 2007), which could pose many risks to the natural stock in the case of accidental release or escape.

Recently, an RNA knockdown technique was developed as an efficient method to induce sterility in aquatic animals. In teleost, such as medaka (Kurakawa et al. 2007), loach (Fujimoto et al. 2010) and sturgeon (Linhartova et al. 2015), germ cell-less phenotypes have been achieved experimentally by treatment with morpholino oligos that inactivate the mRNA essential for forming primordial germ cells (PGCs). In marine invertebrates, RNA interference (RNAi) treatment by injection of double-stranded RNA (dsRNA) against the gene of interest has been widely applied, such treatment to increase resistance to infection in marine shrimp *Litopenaeus vannamei* (Robalino et al. 2004). In mollusks, transient silencing of vasa mRNA has been shown to inhibit the development of germ cells in gonads of Pacific oyster (Fabioux et al. 2009). In Akoya pearl oyster *Pinctada fucata*, injection of vasa dsRNA into the ovary of fully matured females induced spawning 6 hours later, and the offspring harboring vasa dsRNA showed abnormal gonads that were significantly smaller than in the control group (Miura et al. 2013), suggesting that transient knockdown of maternal mRNA essential for germ cell development could induce sterile F1 offspring in mollusks. The implementation of these techniques requires identification of target genes related to germ cell development, especially genes involved in the establishment of PGCs. In mollusks, several genes

related to gonadal development have been identified, including *vasa* (Fabioux et al. 2004; Swartz et al. 2008; Obata et al. 2010) and *nanos* (Kranz et al. 2010). As shown in *Haliotis asinina* (Kranz et al. 2010), *vasa* expression was not limited to germ cells and might have a multifunctional role in other somatic tissues in abalones. Thus, identifying candidate genes that are specifically expressed in germ cells for RNAi treatment is a crucial step for developing a method to induce sterility in Pacific abalone.

Generally, a conventional approach for isolating genes in non-model organisms is PCR amplification based on conserved sequences of homologous genes. As this technology is time-consuming and not applicable for highly divergent target genes, the step of discovering genes represents a major bottleneck in the process (Bar et al. 2016). In the recent years, RNA-Sequencing (RNA-Seq) utilizing massively parallel DNA sequencing technology, including ABI SOLiD, Roche 454 and Illumina Solexa platforms, has opened opportunities in gene discovery (Nekrutenko et al. 2015; Zhang et al., 2015; Yang et al. 2017; Chatchaiphan et al. 2017). Currently, in addition to model species such as humans, RNA-Seq technologies are also being applied to an increasing number of marine species, including scallops (Miao et al. 2015), oysters (Li et al. 2017; Wang et al. 2018), marine snails (Gleason and Burton 2015), flounder (Shao et al. 2016; Fu et al. 2017) and deep sea mussels (Zheng et al. 2017). The technologies were widely used approach to address biological questions in all kingdoms (Song et al. 2017). In abalone species, RNA-seq techniques have also been used to explore the molecular mechanisms governing embryonic development and body growth (Franchini et al. 2011; Huang et al. 2012; Bester-van et al. 2013; Choi et al. 2015; Valenzuela-Miranda et al. 2015; Shiel et

al. 2015). For example, screening the expression of a batch of specific genes in different developmental stages of *Haliotis diversicolor* larvae would provide the fundamental resources for revealing the mechanisms of settlement and metamorphosis at the molecular level (Huang et al. 2012). In contrast, the current transcriptome resources of Pacific abalone *Haliotis discus discus* remain very scarce. Only 5,795 expressed sequence tag (EST) sequences were available in NCBI common databases, and this has been become a major obstacle to discovering the target genes of this species. In the present study, we aim to discover genes related to the gonadal development of Pacific abalone, especially as is required to identify genes specifically expressed in germ cells. To this end, functional analysis of the gonadal transcriptome over multiple tissues from brain, ovary, testis and unfertilized eggs was conducted. Then, a series of strategies, including different gene search methods, RT-PCR and in situ hybridization (ISH) examination, were performed to discover and identify target genes.

2. Materials and methods

2.1. Ethics statement, experimental fish and sample collection

All experiments were conducted at Tateyama Station (Banda), Field Science Center of the Tokyo University of Marine Science and Technology (Chiba, Japan) in accordance with the Guide for the Care and Use of Laboratory Animals of Tokyo University of Marine Science and Technology. All animals used in RNA-Seq study were 3-year-old Pacific abalone (male length: 57 mm and female length: 79 mm). The brain sample was a mixture of brain tissue from males and females. Unfertilized eggs, testis and ovaries were collected according to common procedures (Vicose et al. 2007). Additionally, various organs or tissues (brain, testis (mature), ovary (mature), immature gonad, unfertilized eggs, muscle, gill, kidney, mantle and foot) were excised and then used to examine the distribution of candidate genes in the tissues. Each collected sample was immediately preserved in RNAlater (Thermo Scientific, Waltham, MA, US) before storage at -80 °C.

2.2. RNA extraction, library construction and Illumina sequencing

Total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) following manufacturer instructions. The concentration and quality of each RNA sample were examined using a NanoDrop-2000 spectrophotometer (Thermo Scientific, Waltham, MA, US), and the RNA integrity was checked by ethidium bromide staining of 28S and 18S ribosomal bands on a 1% agarose gel. Equal amounts of high-quality RNA samples of each tissue were used to synthesize a cDNA library. Briefly, mRNA was purified from total RNA and used as templates to synthesize the first-strand and the second-strand

cDNA, according to the protocol of Super Script Double-Stranded cDNA Synthesis kit (Thermo Fisher Scientific, MA, USA). cDNA was cut into short fragments following the TruSeq RNA sample preparation guide. After end repair and the addition of poly(A), the short fragments were ligated with sequencing adapters and enriched by PCR amplification to construct the cDNA library templates. Finally, the library was loaded onto the channels of an Illumina HiSeq™ 2000 for sequencing.

2.3. Illumina read processing and assembly, functional annotation and classification

A Perl program was written to select clean reads. Low quality reads that were more than 50% bases with quality lower than 20 in one sequence, ambiguous reads containing more than 5% unknown bases and reads containing adaptor sequences were removed. Meanwhile, short sequences (< 200 bp) and partially overlapping sequences were filtered to obtain non-redundant sequences. Then, the clean reads of 4 libraries were assembled into contigs using the Trinity method to construct unique consensus sequences (Grabherr et al. 2011). TIGICL was used to assembly high quality, clean reads from hybrid libraries to form a single set of unigenes (Pertea et al. 2003).

All assembled unigenes were aligned against a protein database using Blastx (Altschul et al. 1990) with the priority order of NR, SwissProt, KEGG and COG database (E-value ≤ 1e-5). Protein sequences from the databases with the highest similarity scores were used as functional annotation for the related unigenes (Fig. 1A).

In the Gene Ontology (GO) database (http:// www.geneontology.org/), Blast2 GO was applied to identify annotated unigenes (Conesa et al. 2005). The WEGO software

(http:// wego.genomics.org.cn/cgibin/wego/index.pl), a statistics tool, was then used to perform GO functional classification of all unigenes. ESTScan software was used to determine the directions and CDS (coding sequence) of all unigenes that were not aligned to those in any of the databases mentioned above (Iseli et al. 1999).

2.4. Gene expression distribution and identification of candidate genes

Numbers of reads in RNA-Seq analysis were normalized with reads per kilobase of transcripts per million (RPKM) to compute gene expression levels (Mortazavi et al. 2008). The false discovery rate threshold (FDR < 0.05) was adopted for multiple testing corrections of the result (Benjamini et al. 2001; Lu et al. 2015). Genes were defined as specifically expressed genes (SEGs) in a single tissue by if FDR ≤ 0.05 and log2 ratio = 0 in a single tissue (RPKM value of the gene in one sample was at least two-fold that in another sample). Genes with FDR ≤ 0.05 and log2 ratio < 2 in multiple tissues were classified as co-expressed genes (CEGs). Venn diagrams were constructed according to gene expression distribution of annotated unigenes in single or multiple tissues.

The GO term database of unigenes was screened for 11 keywords related to reproduction including oocyte, sperm, fertilization, sex, meiosis, male, gonad, germ cell, gamete, female and egg to identify gonad-biased genes (Table S3). Fifty-two genes that were identified in other organisms (Matsumoto et al. 2013; Chen et al. 2014; Yue et al. 2015), including vasa, nanos, GnRH, dmc1, sox and dead end, were identified from the Venn diagram and searched in the unigenes database in order to obtain data on homologous genes (Fig. 1D, Table S3). To obtain more candidate genes in the co-

expression area of ovary and testis (i.e., zygotically expressed gonadal genes (ZEGG)) and the co-expression area of ovary, testis and unfertilized eggs (i.e., maternally expressed gonadal genes (MEGG)), these 11 keywords related to reproduction were used to identify candidate unigenes.

2.5. RT-PCR analysis

Isolated total RNA (3 μg) from all samples was reverse transcribed with Superscript III (Thermo Fisher Scientific) and an 18-nucleotide oligo d(T) primer according to manufacturer protocol. The resultant cDNA samples were respectively diluted 1:10 and used as templates. A series of PCR primers were designed in order to examine expression patterns for candidate genes, according to the sequences of corresponding unigenes (Table 1). Amplification reactions were performed with an initial denaturation step of 2 min at 95 °C followed by 27 cycles of denaturation at 94 °C for 30 sec, annealing between 55 °C to 60 °C for 30 sec and extension at 72 °C for 30 sec. PCR products were electrophoresed on 2.0% agarose gels. As a positive control for the RT-PCR analysis, amplification of β-actin (accession number: EF103363) and vasa (comp109508) were amplified to determine the concentration of the template and to provide an external control for PCR reaction efficiency under the same reaction conditions.

2.6. In situ hybridization

Digoxigenin-labeled sense and antisense RNA probes were individually synthesized from corresponding regions (Table 1): comp68131, nucleotides 742-1286 (545 bps);

comp124745, nucleotides 1790-2315 (526 bps); comp105243, nucleotides 375-1125 (751 bps); comp127344, nucleotides 980-1610 (631 bps); comp123498, nucleotides 516-1262 (747 bps); comp109508, nucleotides 1321-2004 (684 bps). These cDNA fragments of 7 candidate genes were subcloned into the pGEM T-easy vector. Sense and anti-sense probes were transcribed in vitro using DIG-labeled uridine triphosphate (UTP) (Roche, Mannheim, Germany) and T7 RNA polymerase (Promega). For the ISH, tissue samples from the central region of the gonads were fixed at 4 °C for 16 h in Bouin's solution. After dehydration in increasing concentrations of ethanol, a portion of each sample was embedded in paraffin wax and cut into 5-μm serial sections using a microtome. The paraffin sections were then mounted on Matsunami Adhesive Slides (MAS; Matsunami Glass Ind., Osaka, Japan), dewaxed and dehydrated by immersion in a xylene-ethanol series. The sections were stained with hematoxylin-eosin (HE) or processed for ISH with DIG-labeled RNA probes as previously described (Nagasawa et al., 2009).

Samples for identifying the localization of candidate genes were ovary from a 3-year-old (Shell length (SL): 69 mm) and testis from a 3-year-old (SL: 73.9 mm) that contained germ cells of different developmental stages. A sample from immature abalone was obtained from a 1-year-old abalone (SL: 39 mm). According to the criteria of germ cell type classification of Roux et al. (2013), oogenesis was divided into 9 stages and spermatogenesis was divided into 5 stages, which provides a standardized system for identifying cells in the gonad of Pacific abalone. The resulting sections of ISH were observed under an upright microscope (BX-50, Olympus, Tokyo, Japan) equipped with digital camera (DP-72, Olympus).

2.7. Identification of ZP domain proteins

The sequences and deduced amino acid sequences of comp68131 were analyzed by BLAST program (NCBI, http://blast.ncbi.nlm.nih.gov/blast.cgi). Amino acid sequence identity was performed using CLUSTAL W (http://www.ebi.ac.uk/Tools/ msa/clustalw2). Multiple alignments were performed with the MAP method at BCM Search Launcher web servers (http:// searchlauncher.bcm.tmc.edu/) and the output was shaded by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX-form.html). Phylogenetic analysis of 33 newly and previously identified ZP proteins of abalone (Aagaard et al., 2006; 2010), were determined from nucleotide alignments of the complete ZP domain using neighbor-joining of the Mega 7.0 molecular evolutionary genetic analysis software package.

3. Results

3.1. Transcriptome sequencing and assembly

For use as gonadal transcriptome resources of Pacific abalone four cDNA libraries were produced by applying stringent quality assessment and data filtering of sequences from brain, ovary, testis and unfertilized egg to obtain 50.3, 54.1, 55.5 and 51.8 million high quality clean reads, respectively. The raw transcriptome sequences in this study have been uploaded in the NCBI Sequence Read Archive (SRA) with the accession number SRP128760. Using the trinity assembly program, 234,353 unigenes were generated by hybrid de novo assembly having lengths ranging from 201 to 32,128 bp (average length, 939 bp and N50 length, 1,845 bp), and 14,873 unigenes had length >1,000 bp (Table 2).

3.2. Annotation, functional classification

To confirm the putative function of the obtained 234,353 unigenes, alignments were made to different protein databases in the following priority order: NCBI non-redundant (NR), SwissProt, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Cluster of Orthologous Groups (COG) with an E-value cut-off of 1e-5. The result showed that 24.9% (58,245) of unigenes were functionally annotated in these protein databases (Table 3). Owing to the absence of genome information of Pacific abalone, 75.1% of unigenes showed no homologs to known sequences in protein databases. As GO is a standardized system for gene functional classification, we carried out GO analysis of all of the unigenes (Fig. S1), and found that approximately 60% of annotated unigenes (36,820) had GO IDs and were categorized as coding for biological processes (45.9%), cellular

components (32.6%) or molecular functions (21.5%). In the biological processes, 2.2%, 1.3% and 1.1% of unigenes were annotated to developmental processes, reproduction, and reproductive processes, respectively (asterisks in Fig. 1S).

3.3. Identification of candidate genes related to reproduction

In order to identify candidate genes related to reproduction, the expression distribution of annotated unigenes in single and multiple tissues was analyzed against the reads per kilobase of transcripts per million (RPKM) to compute specifically expressed genes and co-expressed genes. Among the annotated unigenes, 48.6% (28,302) were presented in the Venn diagram identifying the genes having universal expression in all tissues and those with tissue-specific expression (Fig. 1B). As summarized in Fig. 2A, 1,383 unigenes were present in the area of zygotically expressed gonadal genes (ZEGG), i.e., genes co-expressed in the ovary and testis, and 1,315 unigenes were present in the area of maternally expressed gonadal genes (MEGG), i.e., co-expressed in ovary, testis and unfertilized eggs. In addition, 1,137 unigenes were co-expressed in the four tissues. Unigenes with tissue-specific expression were distributed as follows: 2,288 in brain, 861 in ovary, 1,963 in testis and 2,911 in unfertilized eggs.

Three kinds of search methods were carried out to identify candidate genes in the transcriptome resources shown on the Venn diagram. First, 23 genes with gonad-biased GO terms, such as genes specific to oocyte, sperm, fertilization, sex, meiosis, male, gonad, germ cell, gamete, female and egg, were obtained by querying the GO database (Fig. 1C) and grouped into 18 GO categories (Table S1). The distribution of these genes

is shown in the Venn diagram (Fig. 2B). Among the genes, *sphingosine-1-phosphate lyase 1-like* (*SGPL1*) and *beta-hexosaminidase subunit alpha* (*HEXB*) were found as co-expressed genes in the ovary and testis, and *mitochondrial inner membrane protease subunit* (*IMP*) was found as a co-expressed gene in the ovary, testis and unfertilized eggs.

Second, 36 genes were identified by screening of 52 known germ-cell and meitotic marker genes and genes related to gonadal development by querying the annotated unigene database (Fig. 1D). As shown in Fig. 2C and Table S2, 36 homologous genes were plotted on the Venn diagram, of which, 23 genes (*vasa*, *dmc1*, *nanos*, *dmrt*, *sox* family, etc.) were found to show co-expression with the four tissues (Fig. 2C). Three genes, *testis-specific serine/threonine-protein kinase1* (*TSSK1*), *TSSK4* and *TSSK5*, were expressed only in the testis. Two genes, *gonadotropin-releasing hormone receptor like* (*GnRHR*) and *Doublesex and mab-3 related transcription factor 1* (*Dmrt1*) were co-expressed in ovary and testis. Additionally, *gametocyte-specific factor 1* (*GSTF1*) was co-expressed in the ovary, testis and unfertilized eggs.

Finally, annotated unigenes present in the 2 areas of interest (ZEGG and MEGG) were queried using the same 11 keywords related to reproduction as in the GO term screening (Fig. 1E). In the area of ZEGG, 4 new genes (*meiosis-specific with OB domain-containing protein-like* (*MEIOB*), *gametocyte-specific factor 1* (*GSTF1a*), *Sperm protein 2*, *Meiosis I arrest protein-like*) were found in addition to the 4 genes (*SGPL1*, *HEXB*, *GnRHR* and *Dmrt1*) identified in previous searches (Fig. 2D). In the area of MEGG, 7 new genes (*SMAD1*, *vitelline envelope zona pellucida domain protein 12* (*ZP12*), *embryonic protein UVS.2*, *TSSK1b*, *Sperm protein*, *p-granule abnormal protein*

1-like and *Oocyte zinc finger protein XICOF6-like)* were found in addition to the 2 genes (*IMP* and *GTSF1*) identified in previous searches (Fig. 2D).

In total, 17 genes including 8 ZEGG genes that were co-expressed in the ovary and testis and 9 MEGG genes that were co-expressed in the ovary, testis and unfertilized eggs were found by these 3 methods.

3.4. Validation of target genes related to germ cell development

To identify and validate target genes potentially involved in germ cell development, structure prediction was carried out, and expression level statistics were determined based on RPKM values for each of 17 ZEGG and MEGG. The results showed that the full-length open reading frame (ORF) of these genes were predicted with the exception of *HEXB* (comp7069, length: 360 bp), *GnRHR* (comp76121, length: 622 bp) and *UVS.2* (comp105252, length: 504 bp). The identity of amino acid sequences of Pacific abalone unigenes with the first hit animals identified by Blastx showed a large variance (range: 25%~100%) (Table 4A and B). Among these 17 genes, the expression level of *HEXB* (comp7069) and *SGPL1* (comp129604) according to the RPKM values was very low (data no shown). Thus, expression patterns in gonads, unfertilized eggs and different somatic tissues of Pacific abalone were examined for 15 genes including 6 with ZEGG expression (Table 4A), 9 with MEGG expression (Table 4B) and a unigene encoding germ-cell marker, *vasa* (comp109508) using RT-PCR.

As shown in Fig. 3, *vasa* was expressed in immature and mature gonads, gill, brain, kidney and unfertilized eggs, while β-actin was expressed in all tissues. All 6 of the

ZEGG were shown here to be expressed in the ovary and/or testis. However, only *GTSF1* (comp124745) showed specific expression in gonads. Expression of the other 5 genes was observed in somatic tissues. All 9 MEGG were expressed in the ovary and/or testis. Interestingly, 8 of 9 genes, except *P granule abnormality protein 1-like* (comp119781), showed specific expression in gonads and most were also expressed in immature gonads and unfertilized eggs. In addition, *Dmrt1* (comp110091), *TSSK1b* (comp119405) and *sperm protein* (comp124732) were expressed in testis but not in ovary. *UVS.2* (comp105252) and *ZP12* (comp68131) were more dominantly expressed in ovary than in testis.

Based on RT-PCR results, 5 candidate genes (*ZP12*, *oocyte zinc finger protein XlCOF6-like*, *SMAD1*, *IMP* and *GSTF1*) and *vasa* were chosen for further analysis and determination of subcellular localization in gonads. ISH results revealed that hybridization signals using *vasa*- and *ZP12*-antisense probes were observed in germ cells of Pacific abalone (Fig. 4 and Fig. 5), whereas no signals were observed using antisense probes of the other 4 genes (Fig. S2). *vasa* mRNA was predominantly detected in oogonia and stage 1-3 oocytes of the ovary (Fig. 4B and E) and in spermatogonia and primary spermatocytes of the testis (Fig. 5B and E). No hybridization signal was observed in any germ cells using the *vasa*-sense probe (Fig. 4C and F and Fig. 5C and F). *ZP12* mRNA was detected in oogonia and stage1-3 oocytes of the ovary (Fig. 4H and K) while no expression was observed in testicular germ cells (Fig. 5H and K). Furthermore, *ZP12* mRNA was expressed specifically in the primordial germ cells (PGCs) of immature gonads of 1-year-old pacific abalone (Fig. 6B and E). No hybridization signals were

observed in any germ cells using the *ZP12*-sense probe (Fig. 4I and L, Fig. 5I and L, Fig. 6C and F). Positive signals in the hepatopancreas (HP) were observed using both anti-sense and sense *vasa* probes (Fig.4B and C), while the signals in germ cells was only observed when anti-sense prove was used. Similar positive signals in the HP were also observed using anti-sense and sense *ZP12* probes (Fig. 6B and C). Thus, we speculated that the signals found in the HP were background staining caused by endogenous alkaline phosphatase activity in the HP of abalone.

To confirm the cDNA sequence information of *ZP12* (comp68131) gene, sequence structure was predicted by the NCBI program and the relationships of the ZP protein family was analyzed against previous findings (34 homologous genes) in pink abalone *Haliotis corrugate* and red abalone *Haliotis rufescens* (Aagaard et al. 2006; 2010). The results showed that the *ZP12* gene of Pacific abalone encoded 360 amino acid residues, including the characteristic elements of the ZP protein family, such as the ZP domain and signal peptide at the N-terminus and the transmembrane at the C-terminus. Phylogenetic analysis revealed that the gene was clustered with *ZP12* gene of red abalone, suggesting that the comp68131 gene was Pacific abalone homolog of *ZP12* (Figure S3).

4. Discussion

RNA sequencing, based on recent next generation sequencing technologies, has provided a powerful, highly reproducible and cost-efficient tool for transcriptome research (Zhao et al. 2013). A total of over 200 million high-quality reads were obtained and assembled into 0.2 million unigenes. Among the genes, approximately 58,000 unigenes were functionally annotated in the protein databases. Compared with previous transcriptome results of other abalone species, the average length (939 bp) obtained in this study was much longer than that of other abalone species: 260 bp in *Haliotis midae* (Franchini et al. 2011) and 300 bp in *Haliotis diversicolor* (Huang et al. 2012). Importantly, number of annotated unigenes obtained in this study was more than that of *Haliotis midae* (3,841) and *Haliotis diversicolor* (9,527), indicating that the unigene database constructed in this study (58,245) contains abundant genomic resources for use in the discovery of genes, especially those related to the reproduction of Pacific abalone. Recently, Nam et al. (2017) constructed the first draft genome of Pacific abalone *Haliotis discus hannai* using Illumina and Pacbio platforms and identified 29,449 annotated genes. Our higher unigene number may be attributed to the differences of protein databases used for functional annotation or the criteria used to remove the short sequences and partially overlapping sequences to obtain non-redundant sequences.

The construction of Venn diagrams provides an effective way to query the tissue distribution of annotated genes and to identify candidate genes that are expressed in the area of interest. In this study, we conducted searches by three different methods on the Venn diagrams to screen for candidate genes that are specifically expressed in the germ

cells of Pacific abalone. First, based on gene function classification, genes were gonad-, reproduction- or sex-biased GO terms. Second, according to known genes related to germ-cell development, Pacific abalone homologous genes were obtained based on Pacific abalone having high similarity with other organisms at the sequence level. Finally, unigenes annotated with reproduction-related genes in the area of interest (genes co-expressed in ovary, testis, and/or unfertilized eggs) were identified. As the main purpose of this study was to identify genes involved in germ cell development, we had mainly focused on genes that were predominantly or specifically expressed in the gonads and eggs. First, second, and third approaches found 23 and 26 and 17 candidate genes, respectively, and the names of these genes found by each screening are summarized in Fig. 2. Of these candidate genes, *SGPL1*, *HEXB* and *IMP* were found in both of the first and third approaches, and the *GnRHRs*, *Dmrt1* and *GTSF1* were found both in the second and third approaches. No candidate genes were found by all these 3 approaches. We have not found any connections among candidate genes from 3 different approaches, however validation of spatial gene expression pattern by RT-PCR suggested that most of genes found in MEGG area by third approach showed specific gene expression in the gonads and eggs. This suggested that third approach was most effective to identify gonad-specific genes. Consequently, we found that genes showing different expression levels between mature testis and ovary (high in testis: *Dmrt1*, *TSSK1b*, and *Sperm protein*; high in ovary: *UVS.2* and *ZP12*) by RT-PCR analysis which could be useful to discriminate sex-specific gene expression patterns and molecular mechanisms of sex dependent germ-cell development in Pacific abalone. The involvement of brain in gonadal development,

such as gonadal sex differentiation, onset of puberty, and sexual maturation, has been studied in mammals, birds, fish, and insects (Plant 2015, Tsutsui and Ubuka 2014, Van Wielendaele et al. 2013, Zohar et al., 2010), but not been well studied in aquatic invertebrates. Although, we have not studied the sex- or gonadal development-related genes expressed in brain of Pacific abalone, it should be included in our transcriptome data deposited in NCBI Genbank SRA (accession # SRP128760) and would provide a useful information for the specific research fields such as the neuro-endocrine control of reproduction in abalones.

In a wide range of animals, the *vasa* gene has been identified and used as a reliable molecular marker for germ cell lineage (Raz 2000). However, the expression pattern of the *vasa* gene during gametogenesis shows large variations in different organisms. In mollusks, there have been reports of tissue distribution patterns and subcellular localization of *vasa* in gonads (Fabioux et al. 2004; Obata et al. 2010). These reports demonstrated that *vasa* mRNA is mainly expressed in early developmental stages of germ cells in both gonads but not in other tissues. In abalones, detailed expression patterns of *vasa* mRNA during embryogenesis were examined by whole mount ISH in *Haliotis diversicolor* (Kranz et al. 2010), but its expression in gonads was not examined. In this study, we first showed subcellular localization of *vasa* mRNA in mature gonads of Pacific abalone. The *vasa* mRNA expression pattern was similar to that found in other mollusks, indicating that the gene plays a crucial role in the development of germ cell lineage and can be used as a germ cell marker in this species. We also showed that the expression of *vasa* mRNA in somatic tissues of Pacific abalone suggests that *vasa* might

play a multifunctional role not confined to the germline, which is similar to the findings in teleosts (Yoshizaki et al. 2000; Huang et al. 2014).

Another gene showing germ cell-specific staining by ISH experiments was *ZP12*. The *ZP* gene family currently has hundreds of extracellular proteins with diversified functions in a wide variety of organisms (Wassarman et al. 2001; Barisone et al. 2003; Darie et al. 2004; Smith et al. 2005). The expression pattern of *ZP* genes was varied in organisms. In teleosts, such as gilthead seabream *Sparus aurata* (Modig et al. 2006), crucian carp *Carassius auratus* (Shi et al. 2013) and zebrafish *Danio rerio* (Liu et al. 2006), *ZP* mRNA was specifically expressed in the ovary. However, in medaka *Oryzias latipes* (Kanamori et al. 2003) and rainbow trout *Oncorhynchus mykiss* (Hyllner et al. 2001), these genes were expressed in ovary as well as in liver. In abalone species, at least 30 homologous genes in the *ZP* protein family were reported (Aagaard et al. 2010) but expression patterns were not studied. Since abalone lack liver tissue, the hepatopancreas plays a similar function as the liver in vertebrates. In the present study, we found, for the first time in mollusks, that *ZP12* mRNA was specifically synthesized in ovarian germ cells and PGCs of Pacific abalone, suggesting that the gene plays a functional role in the early developmental stage of germ cell lineage and can be used as a mitotic germ cell marker in Pacific abalone.

RNAi studies have been conducted in aquaculture-targeted mollusks. In Akoya pearl oyster, the F1 offspring from mature female oysters injected with *vasa* dsRNA prior to induction of spawning showed a significant reduction of gonadal size due to impaired germ cell development (Miura et al. 2013). This result suggests that “maternal RNA

knockdown", i.e., silencing specific maternal mRNAs involved in germ-cell development by intramuscular injection of dsRNA is an efficient strategy for inducing sterility in aquatic invertebrates. The final goal of this study is to establish a method to produce sterile abalone for improving growth during the spawning season and avoiding mortality caused by undesired spawning under the culture conditions. Here, we first searched for target genes related to germ cell development using a gonad-biased RNA-seq database and found a maternally expressed germ cell-specific gene, *ZP12*. In some cases, the maternal mRNA of genes involved in germ cell development, such as *vasa* and *nanos*, were localized not only in the cells having germ cell fate, suggesting that they could be influenced by early cell fate decisions of somatic tissues and are likely to control multiple early development events (David et al. 2012). Thus, ISH analysis of *ZP12* during embryonic and larval development of Pacific abalone will be studied in the future. Furthermore, maternal-RNA knockdown experiments using dsRNA designed for *vasa* and *ZP12* will be conducted to examine whether silencing of these genes could disturb PGCs differentiation and possibly result in sterilization in Pacific abalone.

The present study showed that RNA sequencing technology can be successfully applied to Pacific abalone to produce abundant genomic resources for studying gonad and germ cell development in this species. This more complete information will improve our understanding of the molecular mechanisms controlling gonadal development in commercially valuable abalone. Identification of target genes expressed in germ cells (*vasa* and *ZP12*) is the first key step for conducting maternal mRNA knockdown experiments by RNAi to induce sterility in Pacific abalone.

5. Conclusions

Present study showed that the RNA sequencing technology has been successfully applied in Pacific abalone. The data obtained in this study will provide abundant genomic resources for studying gonad and germ-cell development in this species and will also benefit to understand the molecular mechanisms controlling gonadal development of the commercially valuable abalone. Identification of target genes expressed in germ cells (*vasa* and *ZP12*) was a first key step for testing maternal mRNA knock-down experiment by RNAi to induce sterility in Pacific abalone.

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7. Figure Legends

Figure 1. Assembly and analysis pipeline of Pacific abalone *Haliotis discus discus* gonadal transcriptome. A: Workflow of transcriptome data analysis. B: Expression analysis and construction of Venn diagram. C: Identification of reproduction-related genes by searching GO term databases with 11 reproduction-related keywords. D: Identification of genes homologous to known germ cell and meiotic markers in annotated unigene database. E: Identification of reproduction-related genes in the areas of zygotically expressed gonadal genes (ZEGG) and maternally expressed gonadal genes (MEGG). uFe: unfertilized eggs. GO: gene ontology.

Figure 2. Distribution of all annotated unigenes and candidate genes in Venn diagrams. A: Numbers of annotated unigenes present in each area of Venn diagram. The areas of ZEGG and MEGG are indicated by yellow and blue dotted lines, respectively. B: Distribution of candidate genes found by screening reproduction-related GO terms (method shown in Fig. 1C). C: Distribution of homologous genes by screening with the known germ cell and meiotic markers (method shown in Fig. 1D). D: Distribution of candidate genes by screening with reproduction-, sex-, gonad-related keywords in the areas of ZEGG and MEGG. uFe: unfertilized eggs.

Figure 3. RT-PCR analysis of 15 candidate genes and *vasa* (comp109508) in unfertilized eggs, immature and mature gonads and somatic tissues of Pacific abalone. Tissues distribution patterns of candidate genes found in the areas of ZEGG and MEGG are

shown. *β-actin* (accession number: EF103363) was used as an internal control gene. M: DNA marker; uFe: unfertilized eggs; imM gonad: immauture gonad; DW: negative control without cDNA template. *: genes used for ISH examination.

Figure 4. Localization of *vasa* mRNA (A-F) and *ZP12* mRNA (G-L) in ovary of Pacific abalone. HE staining of ovary used for *in situ* hybridization (A, D, G, J). Anti-DIG signals obtained by *vasa*- (B, E) and *ZP12*-antisense probes (H, K) and *vasa*- (C, F) and *ZP12*-sense probes (I, L). D, E, F, J, K, L are high magnification images of areas enclosed in red boxes in the images to the left (A, B, C, G, H, I). OG: oogonia. St1: stage-1 oocytes. St2: stage-2 oocytes. St3: stage-3 oocytes. HP: hepatopancreas. Scale bars: 200 μm (A, B, C, G, H, I); 50 μm (D, E, F, J, K, L).

Figure 5. Localization of *vasa* mRNA (A-F) and *ZP12* mRNA (G-L) in testis of Pacific abalone. HE staining of testes used for *in situ* hybridization (A, D, G, J). Anti-DIG signals obtained by *vasa*- (B, E) and *ZP12*-antisense probes (H, K) and *vasa*- (C, F) and *ZP12*-sense probes (I, L). D, E, F, J, K, L are high magnification images of areas enclosed in red boxes in the images to the left (A, B, C, G, H, I). SG: spermatogonia, PS: primary spermatocytes. SS: secondary spermatocytes, ST: spermatids. SZ: spermatozoa. HP: hepatopancreas. Scale bars: 100 μm.

Figure 6. Localization of *ZP12* mRNA in an immature gonad. HE staining of immature gonad used for *in situ* hybridization (A, D). Anti-DIG signals obtained by *ZP12*-antisense

probe (B, E) and *ZP12*-sense probe (C, F). D, E, F are high magnification images of areas enclosed in red boxes in the images to the left (A, B, C). HP: hepatopancreas. PGC: primordial germ cell. Scale bars: 100 μm.

Supplementary Information

Figure S1. Gene ontology (GO) analysis of Pacific abalone *Haliotis discus discus* gonadal transcriptome. The subcategories are shown on the X-axis. The left and right Y-axes represent the percentage and number of unigenes belonging to each subcategory, respectively. Asterisks indicate GO terms related to development and reproduction.

Figure S2. ISH results of 4 candidate genes in adult ovary (A-I) and testis (J-R). HE staining (A, C, E, G, J, L, N, P), antisense probes for each candidate gene (B, D, F, H, K, M, O, Q) and sense probe for comp124745 (I, R). HP: hepatopancreas. Scale bars: 100 μm.

Figure S3. Molecular phylogenetic analysis of *ZP* genes among red abalone *Haliotis rufescens*, pink abalone *Haliotis corrugate,* and Pacific abalone *Haliotis discus discus*. Accession numbers of ZP protein family of red abalone were ZP12:GQ851903, ZP13:GQ851904, ZP14:GQ851905, ZP15:GQ851906, ZP16:GQ851907, ZP17:GQ851908, ZP18:GQ851909, ZP19:GQ851910, ZP20:GQ851911, ZP21:GQ851912, ZP22:GQ851913, ZP23:GQ851914, ZP24:GQ851915, ZP25:GQ851916, ZP26:GQ851917, ZP27:GQ851918, ZP28:GQ851919,

ZP29:GQ851920, ZP30:GQ851921, ZPB:GQ851922, ZPC:GQ851923, ZPD:GQ851924 and those of pink abalone were ZP2:DQ453710, ZP3:DQ453714, ZP4: DQ453718, ZP5: DQ453722, ZPA: DQ453726, ZP6: DQ453730, ZP7: DQ453734, ZP8: DQ453738, ZP9: DQ453742, ZP10: DQ453746, vitelline envelope receptor of lysin (VERL): DQ453750. ZP12 Hdd: ZP12 (comp68131) of Pacific abalone. Red dot indicates ZP12 of Pacific abalone. Scale bar shows distance as the numbers of amino acid substitutions per site.

8. Figures and tables

Figure 1

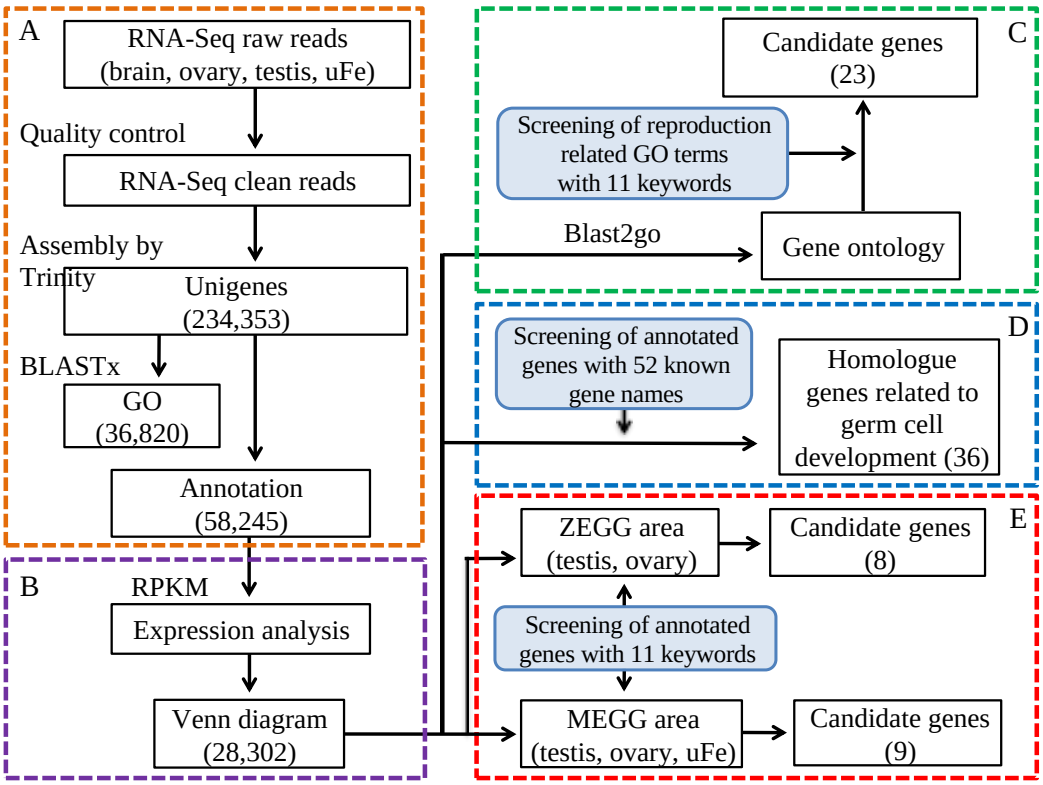
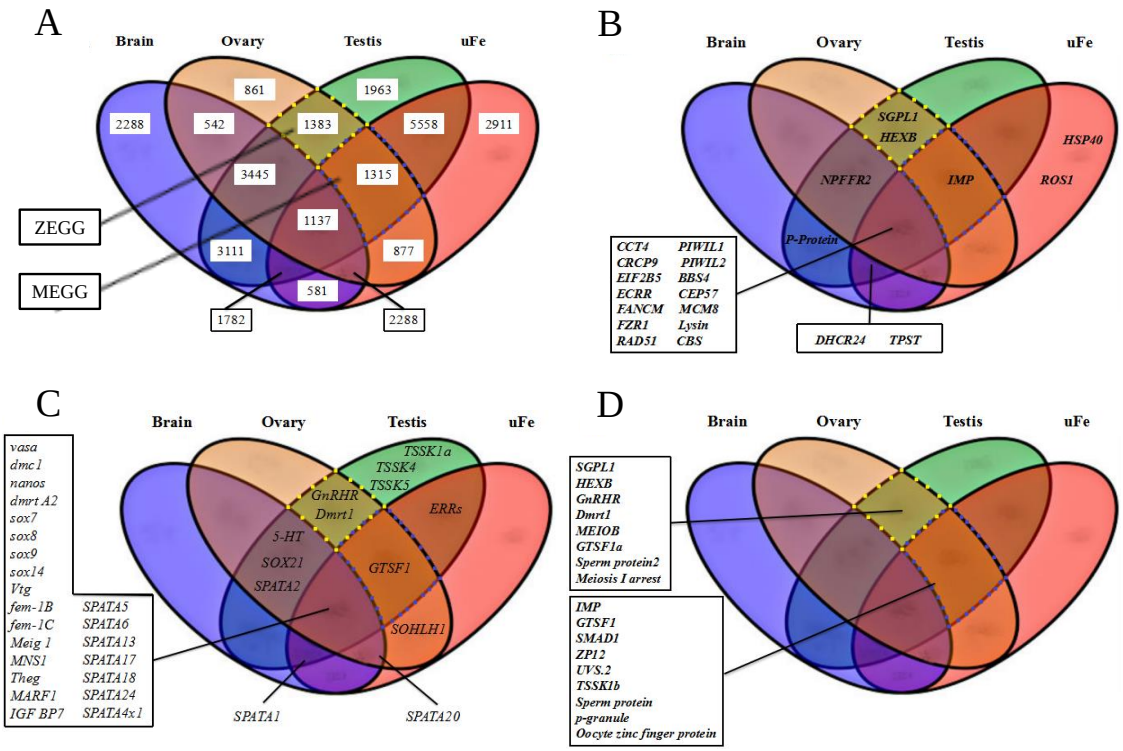


Figure 2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CRCP9 PIWIL2
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ECRR CEP57
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Theg SPATA18
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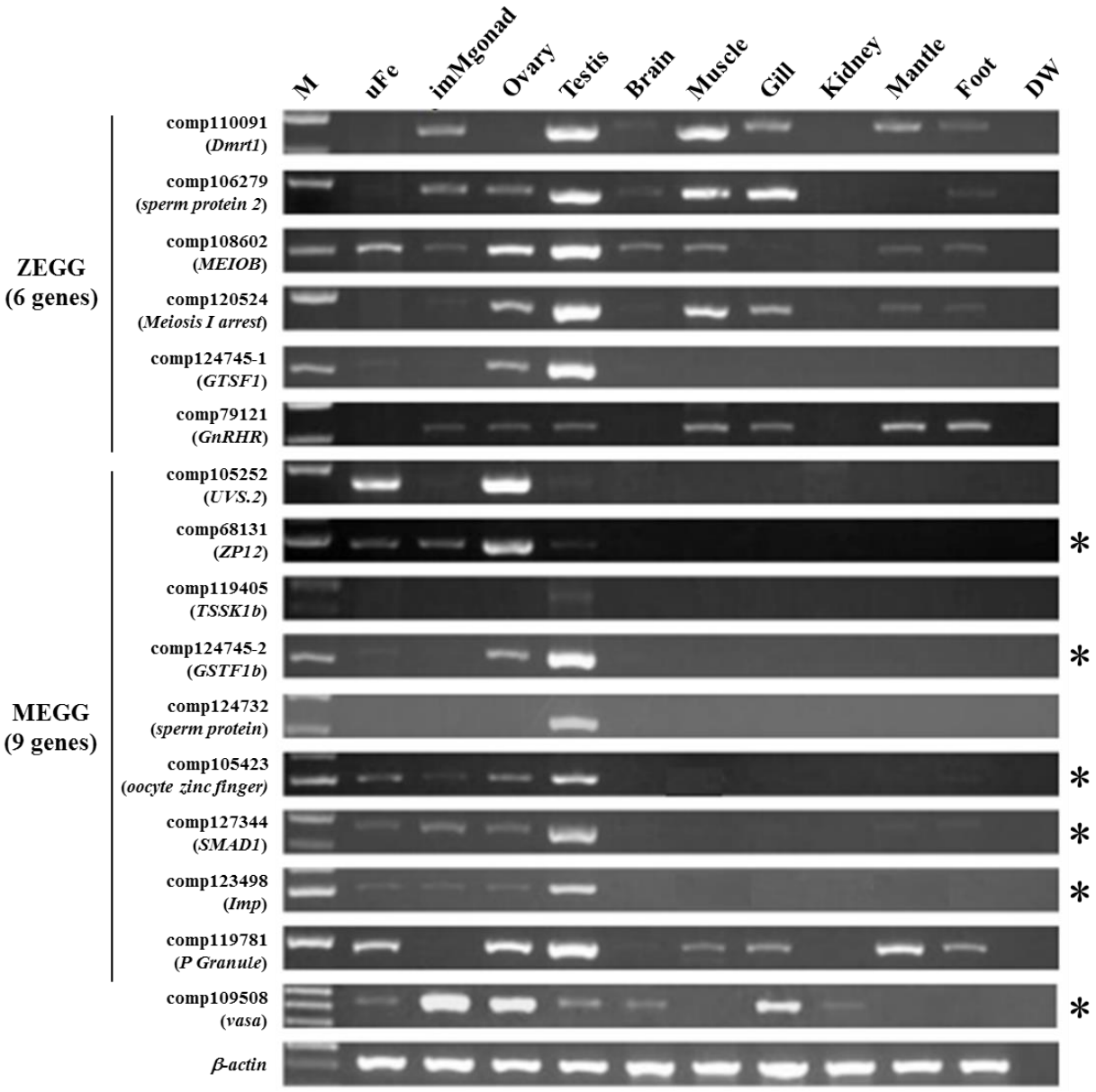
p-granule
Oocyte zinc finger protein

Figure 3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comp106279
(sperm protein 2)
comp108602
(MEIOB)
comp120524
(Meiosis I arrest)
comp124745-1
(GTSF1)
comp79121
(GnRHR)
comp105252
(UVS.2)
comp68131
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(oocyte zinc finger)
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Figure 4

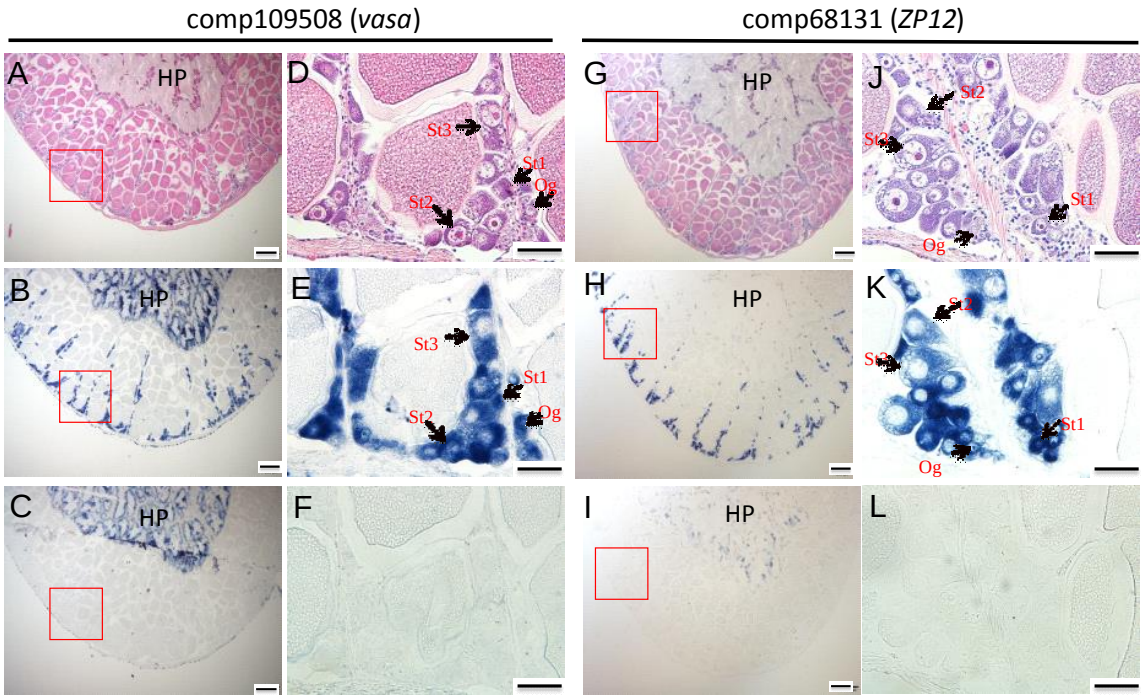
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Figure 5

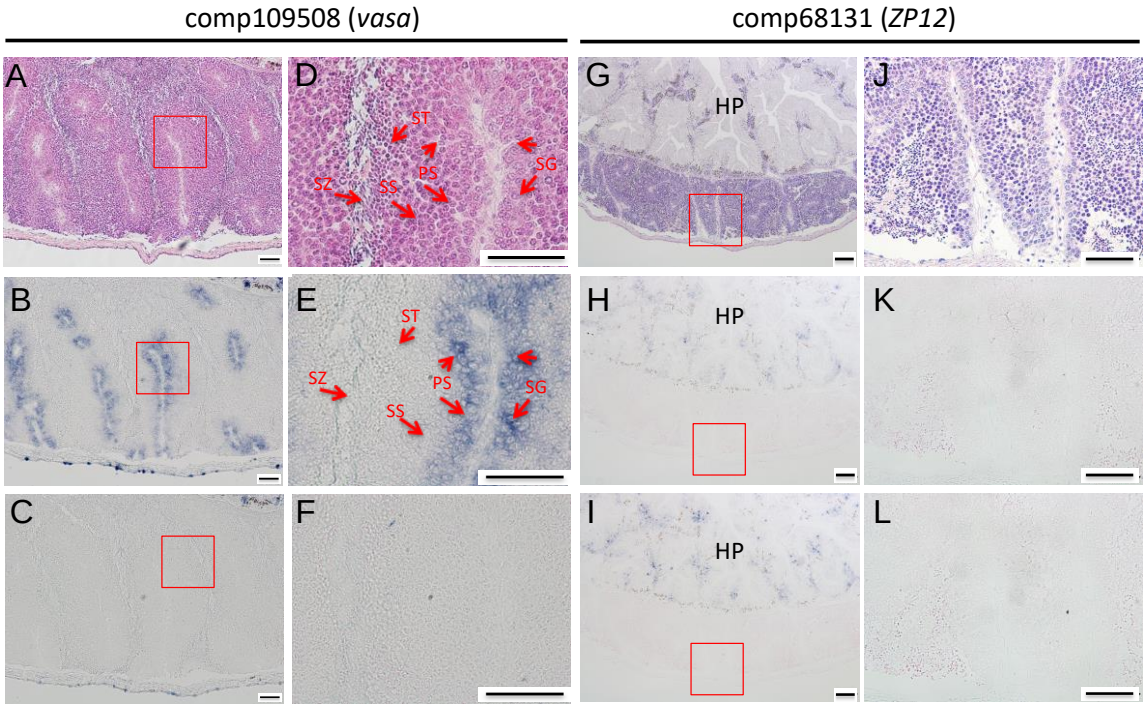
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Figure 6

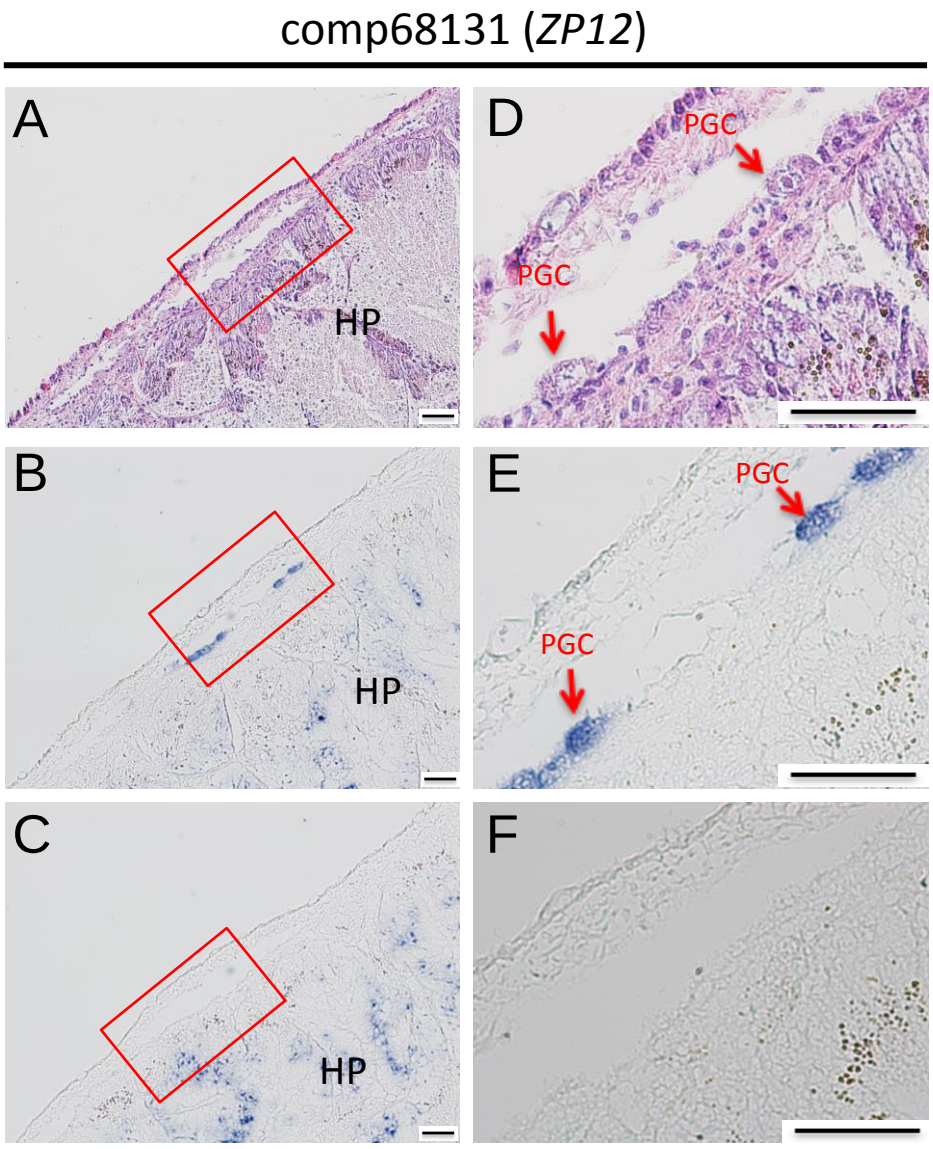


Table 1 Primers information for RT-PCR or ISH analysis

Gene ID	Sequence (5' to 3')	Region	Length (bp)	Purpose
comp110091_c0_seq1	CACCCCCGTGTCTGTTAGTT GACTTCAATGCCCACCTTGT	176-764	608	RT-PCR
comp106279_c0_seq1	GAAGATATGACCCCGGATGT CGTACCATTCCTCGATCGTT	105-537	452	RT-PCR
comp108602_c0_seq2	CCTGGGCTGTAAGGCTACTG TGGTCTTTGACCCATGATGA	578-1061	484	RT-PCR
comp120524_c0_seq10	TGACCAAATCAACCCTGACA TGCAACAACGTGACTTGTGA	1263-1932	669	RT-PCR
comp124745_c0_seq1	GCCGAAGAAACTAGCAGTGG CCTTCCCCCTTCTTACAAGC	1521-2046	526	RT-PCR
comp76121_c2_seq1	TAGGCCATTTGGACGATAGC AGCTTTTGTCGTCTGCTGGT	30-554	525	RT-PCR
comp105252_c1_seq1	AATGCCGCTCTCGTGTGTAT GCGTGCAAGTGGTTCATAGA	30-453	424	RT-PCR
comp68131_c0_seq1	AGCACGGATCTCCCAACTAA CACTTCTGAACCGGGAATGT	742-1286	545	RT-PCR ISH
comp119405-c0-seq1	GACGACTCGAAAGCAGGAAC GAGTTTGCTGCCACAAGTGA	1267-1736	470	RT-PCR
comp124745-c0-seq2	GCCGAAGAAACTAGCAGTGG CCTTCCCCCTTCTTACAAGC	1790-2315	526	RT-PCR ISH
comp124732_c1_seq10	TTGCCTCAGGAAGATGGTTC TTTGACCCAAACGATTCCTC	6-576	571	RT-PCR
comp105243_c0_seq1	TCACAGCAGCTTTGCCATAC CTGTGCGAGAAGTGGTTCAA	375-1125	751	RT-PCR ISH
comp127344_c4_seq3	TGCAGCCAGTGACCTATCAG GTCCAGCCACATCATGACAC	980-1610	631	RT-PCR ISH
comp123498_c1_seq2	GGAGGGAGACCATCACAAGA TGTTCAGCTGTGGTTGAAGG	516-1262	747	RT-PCR ISH
comp119781_c1_seq1	GGACAGCAACAACTGCTCAA CCACAGTCCTTGCGTCTACA	448-1126	679	RT-PCR
comp109508-c0-seq1 (*vasa*)	ACCCTGTTGGTTTCATCAGC CTCCACCTACACGACCCACT	1321-2004	684	RT-PCR ISH
β-actin	GAAGCGTACATGGTGGGACT GGTGACCTGCGAGATTCATT	447-866	420	RT-PCR

Table 2. Statistics summary of *H. discus discus* transcriptome data

Search Item	Number
Total number of clean reads in brain library	50,260,600
Total number of clean reads in testis library	54,079,800
Total number of clean reads in ovary library	55,498,812
Total number of clean reads in unfertilized egg library	51,839,694
Total nucleotides of 4 libraries (nt)	21,379,569,506
GC percentage of unigenes (%)	45.46
Total reads number of 4 libraries	211,678,906
Total number of unigenes	234,353
Mean length of unigenes (bp)	939
N50 length of unigenes (bp)	1845
N90 length of unignes (bp)	329
Max length of unigenes (bp)	32,128
Min length of unigenes (bp)	201

Table 3. Functional annotation of unigenes of *H. discus discus* transcriptome

Databases	Annotated transcripts
Total unigenes	234,353
NR	58,051
Swissprot	43,020
COG	17,290
KEGG	23,510
Annotated genes	58,245
Without annotated gene	176,108

Table 4.

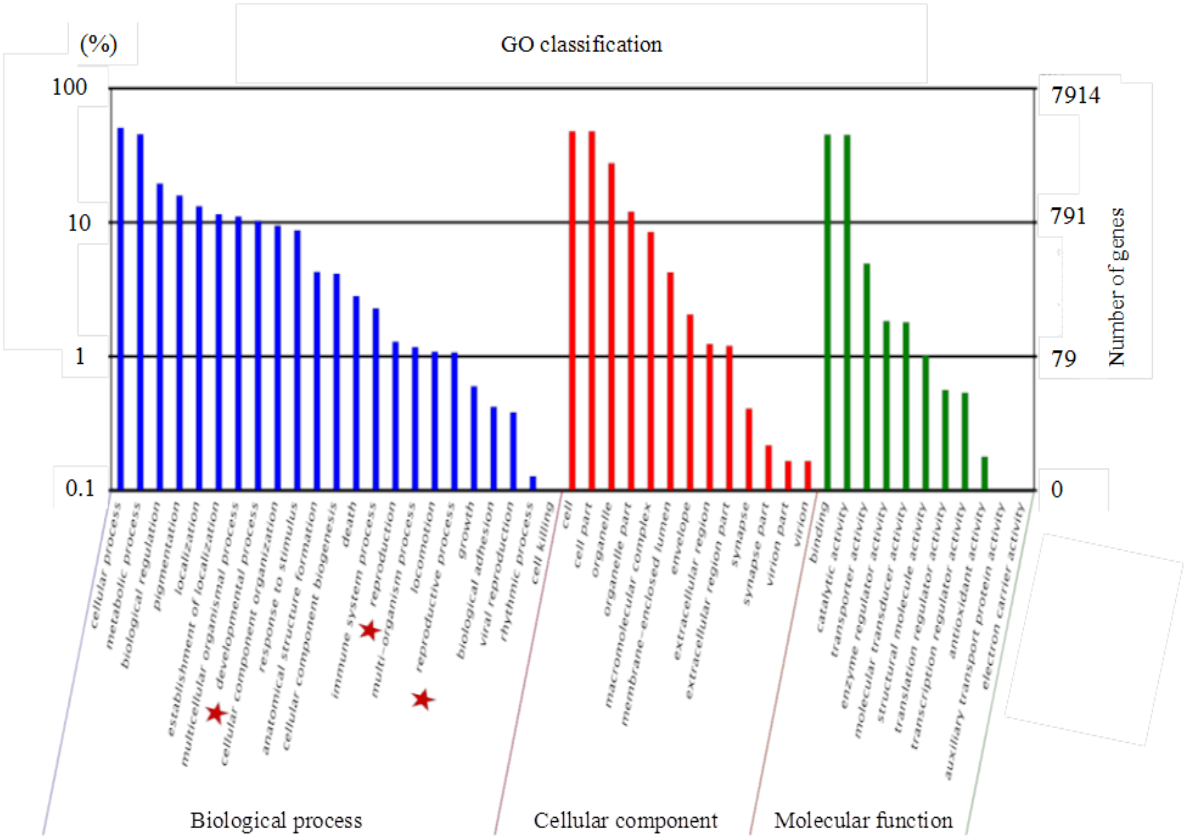
A. List of candidate genes in both ovary and testis

Gene ID	Length	Description	Hit species
comp110091_c0_seq1	1,715 bp	testis-specific DMRT1 (*Dmrt*1)	*Penaeus monodon*
comp106279_c0_seq1	1,657 bp	sperm protein2	*Haliotis rufescens*
comp108602_c0_seq2	1,282 bp	meiosis-specific with OB domain-containing protein-like (*MEIOB*)	*Aplysia alifornica*
comp120524_c0_seq10	4,298 bp	meiosis 1 arrest protein-like	*Aplysia alifornica*
comp124745_c0_seq1	2,544 bp	gametocyte-specific factor 1 (*GTSF1a*)	*Crassostrea gigas*
comp76121_c2_seq1	622 bp	gonadotropin-releasing hormone receptor-like (GnRHR)	*Aplysia californica*

Gene ID	Length	Description	Hit species
comp105252_c1_seq1	504 bp	embryonic protein UVS.2 (*UVS.2*)	*Crassostrea gigas*
comp68131_c0_seq1	1,604 bp	vitelline envelope zona pellucida domain protein 12 (*ZP12*)	*Haliotis rufescens*
comp119405_c0_seq1	1,416 bp	Testis-specific serine/ threonine-protein kinase 1 (TSSK1b)	*Columba livia*
comp124745_c0_seq2	2,813 bp	gametocyte-specific factor1 (*GTSF1*)	*Crassostrea igas*
comp124732_c1_seq10	749 bp	sperm protein (sperm protein)	*Haliotis discus*
comp105243_c0_seq1	2,298 bp	oocyte zinc finger protein XlCOF6-like	*Oryzias latipes*
comp127344_c4_seq3	6,660 bp	SMAD1 (*SMAD1*)	*Crepidula fornicata*
comp123498_c1_seq2	1,383 bp	mitochondrial inner membrane protease subunit (IMP)	*Papilio xuthus*
comp119781_c1_seq1	1,216 bp	P granule abnormality protein 1-like	*Aplysia californica*

B. List of candidate genes in gonad of both sexes and unfertilized eggs

Figure S1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developmental process
cellular component organization
response to stimulus
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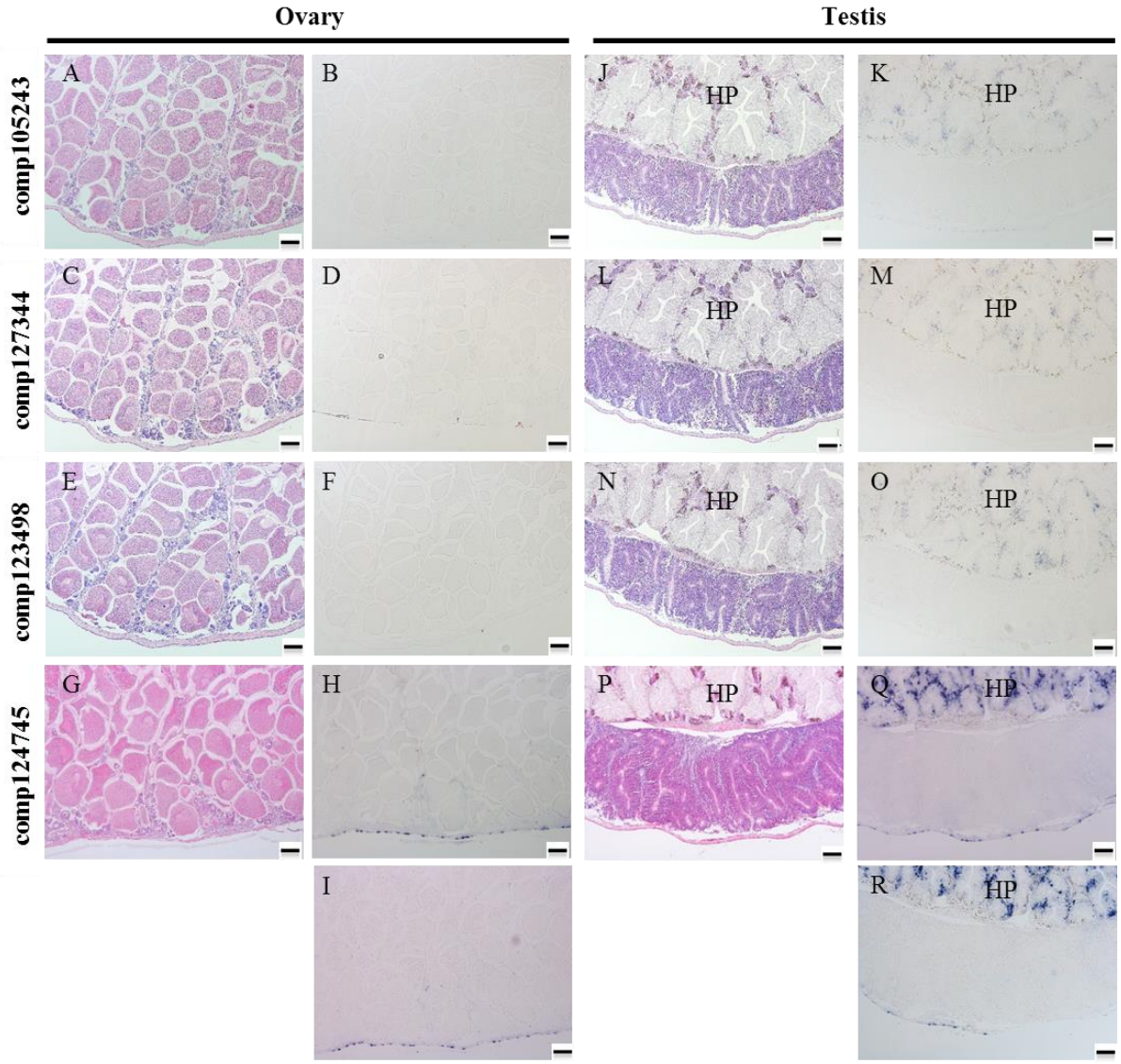
reproduction
multi-organism process
locomotion
reproductive process
growth
biological adhesion
viral reproduction
rhythmic process
cell killing
cell
cell part
organelle
organelle part
macromolecular complex
membrane-enclosed lumen
envelope
extracellular region
extracellular region part
synapse
synapse part
virion part
virion
binding
catalytic activity
transporter activity
enzyme regulator activity
molecular transducer activity
structural molecule activity
translation regulator activity
transcription regulator activity
antioxidant activity
auxiliary transport protein activity
electron carrier activity
Biological process
Cellular component
Molecular function

Figure S2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Testis
comp105243
comp127344
comp123498
comp124745
A
B
J
HP
K
HP
C
D
L
HP
M
HP
E
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O
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Q
HP
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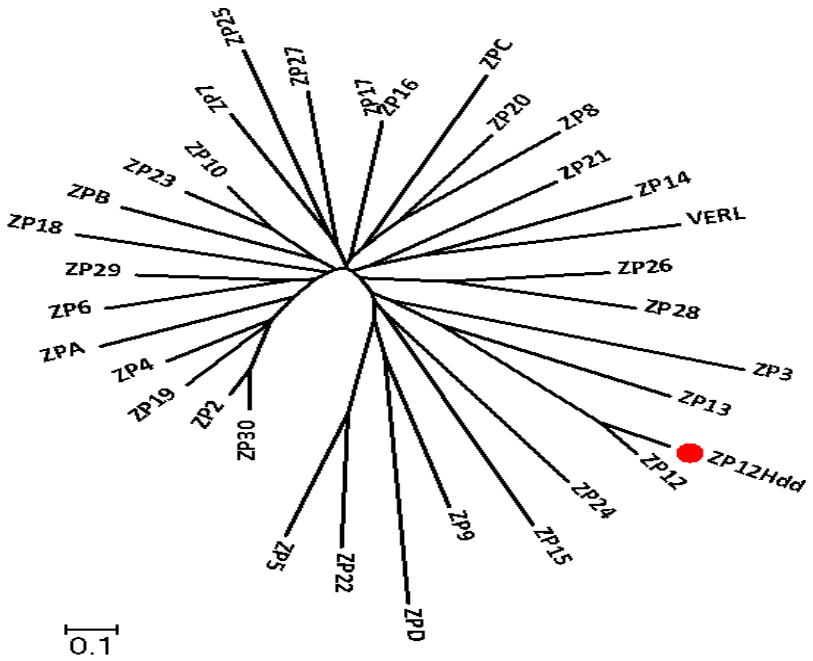
R
HP

Figure S3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ZP16
ZPC
ZP7
ZP20
ZP8
ZP10
ZP23
ZP21
ZPB
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ZP18
VERL
ZP29
ZP26
ZP6
ZP28
ZPA
ZP4
ZP3
ZP19
ZP13
ZP2
ZP12Hdd
ZP30
ZP12
ZP24
ZP5
ZP9
ZP15
ZP22
ZPD
0.1

Table S1. The statistics of reproduction-related genes found in GO term database

GO ID	Gonad-biased GO term	Sequence ID	Annotation	Gene name	Tissue expressing	Length (bp)
GO:0035036	sperm-egg recognition	comp127944_c0_seq1	T-complex protein 1 subunit delta-like isoform X3	*CCT4*	Brain, Ova, Tes, uFe	3,129
GO:0007338	single fertilization	comp127944_c0_seq1	T-complex protein 1 subunit delta-like isoform X3	*CCT4*	Brain, Ova, Tes, uFe	3,129
		comp123852_c1_seq1	protein-tyrosine sulfotransferase	*TPST*	Brain, Tes, uFe	1,714
		comp127314_c1_seq1	DNA-directed RNA polymerase III subunit RPC9	*CRCP9*	Brain, Ova, Tes, uFe	1,448
		comp7069_c0_seq1	beta-hexosaminidase subunit alpha	*HEXB*	Ova, Tes	360
GO:0007548	sex differentiation	comp129604_c3_seq1	sphingosine-1-phosphate lyase 1-like	*SGPL1*	Ova, Tes	505
		comp127951_c0_seq4	24-dehydrocholesterol reductase	*DHCR24*	Brain, Tes, uFe	2,380
		comp127242_c1_seq1	translation initiation factor eIF-2B subunit beta	*EIF2B5*	Brain, Ova, Tes, uFe	1,757
		comp128262_c0_seq1	DNA excision repair protein ERCC-1-like	*ERCC*	Brain, Ova, Tes, uFe	2,599
GO:0007127	meiosis I	comp12789_c0_seq1	Fanconi anemia group M protein	*FANCM*	Brain, Ova, Tes, uFe	2,444
GO:0007126	meiosis	comp123848_c0_seq3	fizzy-related protein homolog isoform X2	*FZR1*	Brain, Ova, Tes, uFe	3,202
		comp124777_c0_seq2	DNA repair protein RAD51-like protein	*RAD51*	Brain, Ova, Tes, uFe	1,497
		comp12789_c0_seq1	fanconi anemia group M protein	*FANCM*	Brain, Ova, Tes, uFe	2,444
GO:0046661	male sex differentiation	comp127951_c0_seq4	24-dehydrocholesterol reductase	*DHCR24*	Brain, Tes, uFe	2,380
GO:0048232	male gamete generation	comp30260_c1_seq1	heat shock protein 40	*HSP40*	uFe	451
		comp128262_c0_seq1	DNA excision repair protein ERCC-1-like	*ERCC*	Brain, Ova, Tes, uFe	2,599
		comp12541_c0_seq1	proto-oncogene tyrosine-protein kinase ROS	*ROS1*	uFe	204
GO:0008406	gonad development	comp127242_c1_seq1	translation initiation factor eIF-2B subunit beta	*EIF2B5*	Brain, Ova, Tes, uFe	1,757
		comp128262_c0_seq1	DNA excision repair protein ERCC-1-like	*ERCC*	Brain, Ova, Tes, uFe	2,599
		comp8210_c0_seq1	neuropeptide FF receptor 2	*NPFFR2*	Brain, Ova, Tes	402
GO:0007281	germ cell development	comp128765_c0_seq1	piwi-like protein 1	*PIWIL1*	Brain, Ova, Tes, uFe	7,017
		comp127420_c0_seq1	bardet-Biedl syndrome 4 protein	*BBS4*	Brain, Ova, Tes, uFe	2,576
		comp128654_c1_seq2	centrosomal protein of 57 kDa	*CEP57*	Brain, Ova, Tes, uFe	421
		comp4816_c0_seq1	P protein	*P protein*	Brain, Tes,	220
GO:0060293	germ plasm	comp126724_c0_seq1	piwi-like protein 2-like	*PIWIL2*	Brain, Ova, Tes, uFe	4,385
GO:0007276	gamete generation	comp128765_c0_seq1	piwi-like protein 1	*PIWIL1*	Brain, Ova, Tes, uFe	7,017
		comp124077_c0_seq1	DNA helicase MCM8-like isoform X1	*MCM8*	Brain, Ova, Tes, uFe	2,468
		comp123498_c1_seq1	mitochondrial inner membrane protease subunit	*IMP*	Ova, Tes, uFe	1,410
GO:0009566	fertilization	comp127314_c1_seq1	DNA-directed RNA polymerase III subunit RPC9	*CRCP9*	Brain, Ova, Tes, uFe	1,448
		comp44812_c0_seq1	sperm lysin	*sperm lysin*	Brain, Ova, Tes, uFe	889
		comp123852_c1_seq1	protein-tyrosine sulfotransferase	*TPST*	Brain,Tes, uFe	1,714
GO:0007565	female pregnancy	comp129157_c0_seq3	cystathionine beta-synthase	*CBS*	Brain, Ova, Tes, uFe	881
GO:0046660	female sex differentiation	comp129604_c3_seq1	sphingosine-1-phosphate lyase 1-like	*SGPL1*	Ova, Tes	505
		comp127242_c1_seq1	translation initiation factor eIF-2B subunit beta	*EIF2B5*	Brain, Ova, Tes, uFe	1,757
GO:0008585	female gonad development	comp129604_c3_seq1	sphingosine-1-phosphate lyase 1-like	*SGPL1*	Ova, Tes	505
		comp127242_c1_seq1	translation initiation factor eIF-2B subunit beta	*EIF2B5*	Brain, Ova, Tes, uFe	1,757
GO:0007292	female gamete generation	comp128262_c0_seq1	DNA excision repair protein ERCC-1-like	*ERCC*	Brain, Ova, Tes, uFe	2,599
GO:0046546	development of primary male sexual characteristics	comp127951_c0_seq4	24-dehydrocholesterol reductase	*DHCR24*	Brain, Tes, uFe	2,380
GO:0007343	Egg activation	comp123852_c1_seq1	Protein-tyrosine sulfotransferase	*TPST*	Brain, Tes, uFe	1,714

Abbreviations: Ova:ovary; Tes: testis; uFe: unfertilized eggs.

Table S2. The statistics of homologue genes of germ cell and meiotic marker genes found in annotated unigene database.

Candidate genes	Gene description	Longest sequence	Length (bp)	Tissue expressing	Hit species
Vasa	vasa-like protein	comp109508_c0_seq1	3,876	Brain, Ova, Tes, uFe	*Haliotis asinina*
Nanos	nanos-like protein	comp118188_c0_seq1	1,067	Brain, Ova, Tes, uFe	*Haliotis asinina*
GnRHR	gonadotropin-releasing hormone receptor-like	comp76121_c2_seq1	622	Ova, Tes	*Aplysia californica*
Dmrt A2		comp94900_c0_seq1	2,837	Brain, Ova, Tes, uFe	*Aplysia californica*
Vtg	vitellogenin	comp118352_c0_seq1	7,769	Brain, Ova, Tes, uFe	*Haliotis discus hannai*
ERRs	estrogen receptor-related receptor	comp128438_c0_seq1	9,233	Tes, uFe	*Marisa cornuarietis*
5-HT	serotonin receptor	comp106059_c0_seq1	5,608	Brain, Ova, Tes	*Aplysia kurodai*
IGF BP 7	insulin-like growth factor binding protein 7	comp107220_c0_seq1	1,955	Brain, Ova, Tes, uFe	*Haliotis diversicolor*
SOX-14	Transcription factor SOX-14	comp102304_c0_seq1	2,695	Brain, Ova, Tes, uFe	*Crassostrea gigas*
SOX-8	transcription factor SOX-8	comp119104_c0_seq1	3,417	Brain, Ova, Tes, uFe	*Crassostrea gigas*
SOX-9	transcription factor SOX-9-like	comp106895_c0_seq1	2,613	Brain, Ova, Tes, uFe	*Aplysia californica*
SOX7	transcription factor SOX -7	comp137107_c0_seq1	2,625	Brain, Ova, Tes, uFe	*Saccoglossus kowalevskii*
SOX-21	transcription factor SOX-21-like isoform X1	comp99568_c0_seq1	2,720	Brain, Ova, Tes	*Aplysia californica*
DMC1	DNA Meiotic Recombinase 1	comp97864_c0_seq2	1,514	Brain, Ova, Tes, uFe	*Nodipecten subnodosus*
DMRT1	testis-specific DMRT1	comp110091_c0_seq1	1,751	Ova, Tes	*Penaeus monodon*
Fem-1 C	fem-1 homolog A-like protein	comp124139_c1_seq1	3,756	Brain, Ova, Tes, uFe	*Crassostrea gigas*
Fem-1 B	fem-1 homolog B-like protein	comp118706_c0_seq1	2,395	Brain, Ova, Tes, uFe	*Locusta migratoria manilensis*
SPATA 18	spermatogenesis-associated protein 18	comp105325_c0_seq2	2,621	Brain, Ova, Tes, uFe	*Crassostrea gigas*
SPATA 24	spermatogenesis-associated protein 24	comp125335_c0_seq1	1,469	Brain, Ova, Tes, uFe	*Sarcophilus harrisii*
SPATA 1	spermatogenesis-associated protein 1	comp130320_c0_seq1	4,338	Brain, Tes, uFe	*Aplysia californica*
SPATA 2	spermatogenesis-associated protein 2	comp114565_c0_seq2	3,188	Brain, Ova, Tes	*Ovis aries*
SPATA 5	spermatogenesis-associated protein 5	comp128148_c0_seq3	2,968	Brain, Ova, Tes, uFe	*Crassostrea gigas*
SPATA 20 X1	spermatogenesis-associated protein 20	comp131253_c0_seq2	3,032	Brain, Ova, uFe	*Aplysia californica*
SPATA 6	spermatogenesis-associated protein 6	comp129396_c0_seq4	1,087	Brain, Ova, Tes, uFe	*Crassostrea gigas*
SPATA 13	spermatogenesis-associated protein 13	comp111950_c0_seq3	2,462	Brain, Ova, Tes, uFe	*Crassostrea gigas*
SPATA 17	spermatogenesis-associated protein 17	comp126723_c0_seq1	1,855	Brain, Ova, Tes, uFe	*Aplysia californica*
SPATA 4 X1	spermatogenesis-associated protein 4	comp117691_c0_seq1	1,366	Brain, Ova, Tes, uFe	*Aplysia californica*
MARF 1		comp111201_c0_seq1	5,718	Brain, Ova, Tes, uFe	*Aplysia californica*
Meig 1		comp114357_c0_seq1	1,181	Brain, Ova, Tes, uFe	*Aplysia californica*
MNS1		comp123299_c0_seq1	2,438	Brain, Ova, Tes, uFe	*Aplysia californica*
GTSF 1a	gametocyte-specific factor 1	comp124745_c0_seq2	2,813	Ova, Tes, uFe	*Crassostrea gigas*
TSSK 5	testis-specific serine/threonine-protein kinase5	comp118183_c0_seq2	2,241	Tes	*Crassostrea gigas*
TSSK 1	testis-specific serine/threonine-protein kinase1	comp37460_c0_seq1	1,708	Tes	*Crassostrea gigas*
TSSK 4	testis-specific serine/threonine-protein kinase4	comp98860_c0_seq1	1,892 bp	Tes	*Crassostrea gigas*
Theg	testicular haploid expressed protein	comp124689_c0_seq1	1,440 bp	Brain, Ova, Tes, uFe	*Scrobicularia plana*
SOHLH 1		comp124404_c0_seq3	2,870 bp	Ova, uFe	*Crassostrea gigas*

Abbreviations: *Dmrt A2*: doublesex- and mab-3-related transcription factor A2-like isoform X1; *MARF 1*: meiosis arrest female protein 1-like isoform X3; *Meig 1*: meiosis expressed gene 1 protein homolog isoform X1; *MNS1*: meiosis-specific nuclear structural protein 1-like; *SOHLH 1*: Spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 2; Ova: ovary; Tes: testis; uFe: unfertilized eggs.

Table S3. Eleven keywords and 52 genes known as germ-cell and meiotic markers

No	keywords
1	oocyte
2	sperm
3	fertilization
4	sex
5	meiosis
6	male
7	gonad
8	germ cell
9	gamete
10	female
11	Male

No	Genes	Gene description
1	vasa	vasa-like protein
2	nanos	nanos-like protein
3	GnRHR	gonadotropin-releasing hormone receptor-like
4	dmrt A2	doublesex- and mab-3-related transcription factor A2-like
5	vtg	vitellogenin
6	ERRs	estrogen receptor-related receptor
7	5-HT	serotonin receptor
8	IGF BP 7	insulin-like growth factor binding protein 7
9	SOX-14	transcription factor SOX-14
10	SOX-8	transcription factor SOX-8
11	SOX-9	transcription factor SOX-9-like
12	SOX7	transcription factor SOX -7
13	SOX-21	transcription factor SOX-21-like isoform X1
14	DMC1	DNA Meiotic Recombinase 1
15	DMRT1	testis-specific DMRT1
16	Fem-1 C	fem-1 homolog A-like protein
17	Fem-1 B	fem-1 homolog B-like protein
18	SPATA 18	spermatogenesis-associated protein 18
19	SPATA 24	spermatogenesis-associated protein 24
20	SPATA 1	spermatogenesis-associated protein 1
21	SPATA 2	spermatogenesis-associated protein 2
22	SPATA 5	spermatogenesis-associated protein 5
23	SPATA 20 X	spermatogenesis-associated protein 20
24	SPATA 6	spermatogenesis-associated protein 6
25	SPATA 13	spermatogenesis-associated protein 13
26	SPATA 17	spermatogenesis-associated protein 17
27	SPATA 4 X1	spermatogenesis-associated protein 4
28	MARF 1	meiosis arrest female protein 1-like isoform X3
29	Meig 1	meiosis expressed gene 1 protein homolog isoform X1
30	MNS1	meiosis-specific nuclear structural protein 1-like
31	GTSF 1a	gametocyte-specific factor 1
32	TSSK 5	testis-specific serine/threonine-protein kinase5
33	TSSK 1	testis-specific serine/threonine-protein kinase1
34	TSSK 4	testis-specific serine/threonine-protein kinase4
35	Theg	testicular haploid expressed protein
36	SOHLH 1	Spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 2
37	lhr	luteinizing hormone receptor
38	ar	androgen receptor
39	amh	anti-Mullerian hormone
40	Fhl3	Four and a half LIM domains protein
41	fshr	Follicle-stimulating hormone receptor
42	oct-4	octamer-binding transcription factor
43	gsdf	gonadal soma-derived factor
44	Spo11	Sporulation protein 11
45	atrx	X-linked α-thalassemia/mental retardation syndrome
46	ER	Estrogen receptor
47	Cyp19b	Cyp19b cytochrome P450 aromatase 19b
48	ARA-α	ARA-α androgen receptor alpha
49	Dax1	Dosage-sensitive sex reversal-adrenal hypophlasia congenital critical region on the X chromosome gene 1
50	WT1	Wilm's tumor 1
51	ELH	Egg-laying hormone
52	Bindin	Bindin

Chapter 3. Expression pattern of *vasa* in germ cells and *vasa* dsRNA-mediated RNA interference in oocytes and early embryos of Pacific abalone (Ezo) *Haliotis discus hannai*

1. Introduction

In sexually reproducing organisms, germ cells play important roles in gonadal differentiation and transmission of genetic information through the generations (Okutsu et al., 2007), as germ cell development is the basis of sexual reproduction and fertility (Xu et al., 2014). In fishes and many other organisms, germ cells form as primordial germ cells (PGCs) in early embryonic development. Presumptive PGCs (pPGCs), which are germline precursors, undergo asymmetric cell division, producing two daughter cells with different fates, one cell becoming a somatic cell and the other a pPGC. When both daughter cells are committed to the germline, the parental cell is said to be a PGC (Lin et al., 2012; Wylie, 1999).

Several germ cell-specific molecular markers that are useful for identifying germlines have been identified (Xu et al., 2010). Among these, *vasa*, the most well-documented germ cell marker in teleosts, encodes a DEAD (Asp-Glu-Ala-Asp) protein belonging to the family of putative ATP-dependent RNA helicases and is present in both polar granules at the posterior end of oocytes and nuage structure in Drosophila germ cells (Hay et al, 1988; Lasko and Ashburner, 1988). At present, *vasa* orthologs have been reported to exhibit highly conserved genes in a wide range of species, e.g., zebrafish (Yoon et al., 1997), medaka (Shinomiya et al., 2000) and trout (Yoshizaki et al.,2000). However, expression patterns are not always representative of germ cell lineages in gametogenesis. For example, in Asian seabass, *vasa* transcripts are restricted to the ovary and testis (Xu et al., 2014), but in half-smooth tongue sole, *vasa* mRNA is expressed in the gill, liver and heart as well as in the gonads (Huang et al.,2014). Similarly, during

embryogenesis, *vasa* mRNA was distributed in four cells considered to be germ cell precursors at the 32-cell stage in zebrafish (Yoon et al., 1997). In medaka, it was distributed uniformly in the blastomeres until late gastrulation (Shinomiya et al., 2000). Further, the *vasa* loss-of-function phenotype has also been demonstrated in various species, e.g., Drosophila (Ghabrial et al., 1999), mice (Tanaka et al., 2000) and Pacific oyster *Crassostrea gigas* (Fabioux et al., 2009), suggesting that the *vasa* gene is essential for germ cell development and could be applied to production of sterile species by transient knock-down. Thus, it will be valuable to understand expression patterns and the function of *vasa* in different organisms.

Abalone is a family of marine gastropods consisting of 55 currently described species with worldwide distribution in tropical and temperate waters (Geiger DL, 1999). High market prices for abalone has driven the development of its aquaculture, and according to Cook (2014), annual global production of farmed abalone is currently on the rise with most of the increases in production of cultured Pacific abalone (Ezo) *Haliotis discus hannai* in China and Korea. Despite significant increases in production, slow growth rates and disease continue to be critical issues affecting the abalone farming industry (Jiang et al., 2015). Importantly, gonad precocity of Pacific abalone (Ezo) has been observed but is not yet been well documented (Li et al., 2017). Wild Pacific abalone (Ezo) reach sexual maturity as 3- or 4-year-olds; however, more than 50% of farmed abalone reach sexual maturity as 1-year-olds, leading to undesired smaller sized adults (LY and YT unpublished). Thus, understanding germ cells development in Pacific abalone (Ezo) is crucial for controlling gonad development and precocity in this species.

However, no germ cell-related genes or germ cell markers have been available to date for identifying germ cell development.

In this study, we cloned and characterized cDNA sequences of the *vasa* gene in Pacific abalone (Ezo), a commercially important bivalve mollusk, studied its mRNA expression levels in different tissues and embryonic stages by real-time quantitative PCR (qPCR), and examined its germ cell-specific expression characteristics in adult gonads, juvenile and different embryonic developmental stages by in situ hybridization (ISH). In addition, the role of *vasa* was investigated in oocytes and early embryos by knockdown of *vasa* maternal RNA by injecting dsRNA designated against *vasa*. Our purpose is to demonstrate the function of the *vasa* gene in germ cell development, investigate the feasibility of using it as a germ cell molecular marker and for evaluating the potential of sterilization of Pacific abalone (Ezo) by RNA interference.

2. Materials and methods

2.1 Ethics statement, experimental fish and sample collection

All abalones were maintained at the Tateyama Station (Banda), Field Science Center of the Tokyo University of Marine Science and Technology (Chiba, Japan) in accordance with the Guide for the Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology. Abalones were kept in stable, controlled environments using tanks equipped with mechanical water flow-through and air systems. Abalones were reared in seawater at 20-25 °C with frequent water exchanges and fed on a specially formulated artificial diet.

2.2 Extraction of RNA and cloning of *vasa* homologue from Pacific abalone (Ezo)

Total RNA was extracted from the ovary of a 3 year-old abalone using Isogen reagent (Nippon Gene, Tokyo, Japan) according to manufacturer instructions. The RNA quality and purity were evaluated by Nanodrop 2000 (Thermo Scientific, USA). Purified total RNA was reverse transcribed with SUPERSCRIPT III (Life Technologies) using an oligo d(T) primer as described in the manufacturer protocol. Internal regions of *vasa* was amplified by RT-PCR with gene-specific and/ or degenerate primers that were designed against the conserved regions across mollusk orthologs (Table 1).

2.3 Cloning *vasa* cDNA and phylogenetic analysis

Amplified cDNA fragments were cloned into the pGEM T-Easy Vector (Promega, Madison, WI, USA) and sequenced. Following instructions for the SMARTer RACE

cDNA Amplification Kit (Clontech, USA), 5' rapid amplification of cDNA ends (RACE) was performed with the gene-specific primer (Vasa 5'race) and in combination with a 5' PCR anchor primer (5'-AP, Table 1). The 3'-end of *vasa* cDNA was amplified using gene-specific primers (Vasa 3'race) and a PCR anchor primer corresponding to the terminal anchor sequence of the cDNA (3'-AP, Table 1). All PCRs were performed on a PTC-200 thermal cycler (Bio Rad, USA). Denaturation at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by an additional extension at 72 °C for 10 min. The RACE products were electrophoresed on a 1.2% agarose gel and cloned into the pGEM T-Easy Vector (Promega, Madison, WI, USA). The full cDNA sequence and deduced amino acid sequences were analyzed via the BLAST program (NCBI, http://blast.ncbi.nlm.nih.gov/blast.cgi). Multiple amino acid sequence alignments were run on the Vector NTI Suit 8 software package (Life Technologies). Of which, the data of PL10 family sequence was shown in Fig.1. Phylogenetic analysis was constructed using the neighbor joining method in the Molecular Evolutionary Genetic Analysis by MEGA 4.1 software package (Life Technologies).

2.4 RT-PCR

Total RNA was extracted from different tissues (ovary, testis, unfertilized eggs, gills, hepatopancreas, mantle and muscle) using TRIzol reagent (Life technologies). Isolated total RNA (5 μg) were reverse transcribed with SUPERSCRIPT III (Life technologies) and an 18-nucleotide oligo d(T) primer, as described in the manufacturer's protocol. All generated cDNAs were diluted 1:10 and used as templates in RT-PCR targeting *vasa* and

β-actin genes with primers *Vasa* Fw and Rv and Actin RT Fw and Rv, respectively (Table 1). RT-PCR of the β-actin gene was conducted under the same reaction conditions to determine template concentration and to provide an external control for PCR reaction efficiency. Amplification reactions were performed with an initial denaturation step of 2 min at 95 °C followed by 28 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C for 30 sec and extension at 72 °C for 30 sec. PCR products were electrophoresed on 2.0% agarose gels.

2.5 Digoxigenin (DIG)-labeled dsRNA and dsRNA synthesis

A 747-bp cDNA fragment (nucleotides 1159-1906 of the *vasa* gene) was used as RNA probes, DIG-labeled dsRNA and dsRNA. Recombinant plasmids were synthesized and linearized as described above. The purified plasmids were transcribed in vivo on both strands using digoxigenin-labeled uridine triphosphate (Roche, Mannheim, Germany) and T7 or SP6 RNA polymerase (Ambion, Austin, TX, USA) to produce sense and antisense ssRNAs. Then, sense and antisense probes were extracted with phenol/chloroform, precipitated with ethanol, and suspended in RNase-free saline solution (10 mM Tris, 10 mM NaCl) at a final concentration of 0.5 μg μL-1 based on quantification by spectrophotometry (Nanodrop; Thermo Scientific, Villebon-sur-Yvette, France). Meanwhile, equimolar amounts of sense and antisense ssRNA were heated at 100 °C for 1 min, and allowed to cool at room temperature for 10 h for annealing. An aliquot (1 μg) of each dsDNA sample was analyzed by 1% agarose gel electrophoresis to ensure that it was a single band of 747 bp. Additionally, in the synthesis of DIG-labeled

dsRNA, single-stranded RNA was synthesized with DIG labeling using T7 RNA polymerase and DIG RNA-labeling mix (Roche). Sense and antisense DIG-labeled ssRNAs were annealed as described above. *vasa* probes, DIG-labeled dsRNA and dsRNA were stored at -80 °C.

2.6 dsRNA administration and sampling

To examine in vivo dispersion of *vasa* dsRNA in gonads, abalones were injected in the gonad with 150 μL of saline solutions containing 5 μg DIG-labeled *vasa*-dsRNA or saline solution for the control (weight: 119.6-175.2 mg). After 1, 3, 6 and 12 h post-injection (hpi), the gonads were cut into sagittal sections. Then, histological examination of samples was performed via DIG-labeled staining, the gonads were divided into 4 parts corresponding to areas A, B, C and D shown Fig. S1, and the population size of oocytes with hybridization signals was calculated and analyzed for each group.

To examine the effects of *vasa* dsRNA in oocytes of Pacific abalone (Ezo), different doses of *vasa* dsRNA (0 (control), 0.5, 5 and 50 μg) were injected into the female gonads of 6 abalones (weight: 154.6-413.2 mg) for each group. After 7 and 14 days post-injection (dpi), some samples were fixed in Bouin' solution at 4 °C for 12 h, dehydrated through serial ethanol dilutions (25%, 50%, 75% and 100%) and stored in 75% ethanol at 4 °C for ISH or histological observation. The remaining pieces were immediately placed in RNA Later solution (Sigma, St. Louis, MO) and stored in -80 °C until total RNA isolation using Isogen reagent (Nippon Gene).

To evaluate the effects of *vasa* dsRNA-mediated interference (dsRNAi) in early

embryos, 150 μL of saline solution with 100 μg *vasa* dsRNA or saline solution (control) was injected into mature female abalones. After 12 hpi, spawning was induced, and embryos at different developmental stages, including 0 (unfertilized eggs), 6, 12, 18, 24 and 48 hours post-fertilization (hpf) for both dsRNAi and control groups. Samples (about 1000 unfertilized eggs or embryos) were collected into 1.5 ml sterile tubes with three replicates per group and immediately stored at -20 °C in 70% ethanol and stored in RNA Later solution according the manufacturer (Ambion, RNAlater AM7020) for ISH and mRNA level detection.

2.7 Real-time quantitative PCR (qPCR)

qPCR was conducted using SYBR Green Real-Time PCR Master Mix (TOYOBO, Osaka, Japan) on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using primers *Vasa* RT QF and QR for *vasa* and Actin QFw and Rv forβ-actin (accession number, EF103363) as shown in Table 1. The qPCR conditions consisted of an initial denaturation step at 95 °C for 30 sec followed by 40 cycles of 5 sec of denaturation at 95 °C and 30 sec of annealing and extension at 60 °C. Assays for each individual were conducted in triplicate, and the relative mRNA levels of *vasa* gene were calculated against those of β-actin by the comparative Ct method (Fabioux et al.,2009). All data are presented as mean values±SEM. Data could be analyzed by ANOVA and post-hoc Tukey-Kramer's multiple comparisons test to identify significant differences between group means (GraphPad Prism 5). For all statistical tests, values were considered significantly different at $P < 0.05$.

2.8 In situ hybridization (ISH) analysis

Tissue samples were fixed in Bouin's solution at 4 °C for 6-16 h, embedded in paraffin wax, then sliced into 5 μm-thick serial sections using standard paraffin-embedding methods and stained with hematoxylin and eosin (HE). Samples of embryos at different developmental stages (unfertilized eggs, 32 cells, trochophore stage, veliger stage) were fixed in 4% PFA/PBS. ISH was performed as previously described (Nagasawa et al.,2009). Signals were detected by incubating the slides in detection buffer containing 200 μg/ml nitro-blue tetrazolium chloride (NBT) (Roche) and 175 μg/ml 5-bromo-chloro-3-indoxyl phosphate (BCIP) (Roche) and observing the sections under a BX-50 microscope (Olympus, Tokyo, Japan).

3. Results

3.1 Characterization of full-length *vasa* cDNA in Pacific abalone (Ezo)

The full-length Pacific abalone (Ezo) *vasa* was 3,866 bp and contained an open reading frame (ORF) of 2,406 bp, encoding 801 amino acid residues. The initiation ATG codon was at position 294 of the 5' untranslated region (UTR), and the ATT stop codon was at position 2,699. The 3' UTR was 1,167 bp with a poly-A tail (Fig. S2A). The polypeptide was predicted to contain consensus sequences characteristic of the DEAD-box motif, RGG motif, zinc-finger domain and RNA helicase domain (Fig. S2B). Phylogenetic analysis of DEAD-box protein family genes *vasa* and *PL10*, showed that *Haliotis discus hannai vasa* (*Hdhvasa*) is clustered together with the Vasa protein subfamily and separately from the cluster of PL10 for related family members (Fig. 1). Thus, we concluded that the clone isolated in this study encodes a gene homologous to Pacific abalone (Ezo) *vasa*.

3.2 Gonad- and embryo-specific expression of *vasa* mRNA

In adult Pacific abalone (Ezo), *vasa* mRNA expression was examined by RT-PCR in ovary, testis, unfertilized eggs, gills, hepatopancreas, mantle and muscle (Fig. 2A). Over the course of embryogenesis, the *vasa* transcript was detected at a relatively high expression level by qPCR in the early stages (1 to 6 hpf), followed by a decrease at 9 to 15 hpf, and reduced but detectable levels in the late stages to 24 hpf (Fig. 2B).

3.3 Expression pattern of *vasa* gene during early embryogenesis and in juveniles

In unfertilized eggs, *vasa* mRNA was detected in the animal hemisphere (Fig. 3A). During embryogenesis, it was strongly expressed in irregular patches of animal portion of 32-cell stage (12 hpf) (Fig. 3B). Later, it was restricted to cells of the ventral paraxial bands in the trochophore (24 hpf) (Fig. 3C) and the fields of visceral mass in veliger (48 hpf) (Fig. 3D). The positive hybridization signals of the *vasa* mRNA signal were not obvious in early stages of embryogenesis using whole mount in situ hybridization (Fig. 3E, F, G), while strong signal could be observed in the veliger stage (Fig. 3H). Additionally, no hybridization signals were observed with the sense probe of *vasa* at any of the developmental stages examined (data not shown). In juvenile (shell length, 4 mm) Pacific abalone (Ezo), the hybridization signals were observed at the peripheral cells of the visceral mass (Fig. 4C, G), while no signal was detected in other tissues.

3.4 Germ cell-specific expression of *vasa* mRNA in adult gonads

We observed gametogenesis in adult gonads of Pacific abalone (Ezo). In the female gonads, oogonia occurred in groups attached to the trabeculae and were usually surrounded by squamous-shaped follicular cells. Early oocytes had an obvious nucleus (Fig. 5A, D). Positive signals of *vasa* mRNA were predominantly detected in oogonia and stages 1-3 oocytes of the ovary (Fig. 5B, E). In the mature male gonads, spermatogonia, spermatocytes, spermatids and sperm were sequentially located from the trabeculae to the center (Fig. 5G, J). Hybridization signal was stronger in spermatogonia and in the primary spermatocytes in the testis (Fig. 5H, K). No hybridization signals were observed in any germ cells of gonads of both sexes using the sense probe (Fig. 5C, F, I,

L).

3.5 In vivo dispersion examination of *vasa*-dsRNA in female abalone gonads

Examination of the in vivo dispersion of DIG-labeled *vasa* dsRNA injected into female gonads by DIG-labeled staining of the hybridization signal of germ cells showed the progression of *vasa* dsRNA reaching germ cells (Fig. S3). The proportion of the oocytes with DIG-labeled signal in areas A-D (defined in Fig. S1) at different time points increased over time (shown by area in Fig. 6 and Table S1) with the following percent signal at each time point: 13.7% at 1 h, 23.1% at 3 h, 32.5% at 6 h and 62.4% at 12 h. The in vivo dispersion of DIG-labeled *vasa* dsRNA was highest at 12 h in this study.

3.6 Inhibiting effects of *vasa* dsRNA in oocytes of gonads and early embryos

In this study, *vasa* mRNA levels in gonads at 7 and 14 dpi were significantly decreased by *vasa* dsRNA treatment in all experimental groups (0.5, 5 and 50 μg dsRNA) compared to the control, but there were no significant differences between experimental groups (Fig. 7A). Histological examination revealed some abnormal germ cells in the experimental groups (Fig. 7B).

The *vasa* mRNA level in the dsRNAi group (100 μg *vasa* dsRNA) was significantly lower than in the control group at 0 and 6 hpf stages, but there were no differences at later embryo stages between the experimental and control groups (Fig.8). For eggs and embryos induced at 12 hpi, ISH findings were positive for *vasa* mRNA in unfertilized eggs, 4-cell stage and veliger stage embryos of the control group, and hybridization

signals were observed at different developmental stages of the dsRNAi groups but with relatively weaker signals than in the control groups (Fig. S4).

4. Discussion

All DEAD-box proteins are putative ATP-dependent RNA helicases (Hay et al., 1988). In the present study, the sequences included two ATPase motifs (AQTGSGKT, DEAD), three ATP binding and cleavage motifs (PTRELV, GG, TPGR), two RNA unwinding motifs (SAT, HRIGR) and the helicase C domain (ARGLD). These regulatory elements identified in this study are major characteristics of DEAD-box proteins and corroborate findings of previous studies (Rebscher et al., 2007; Obata et al., 2010). Aside from these typical features, the sequences of *Hdhvasa* gene had other motifs, including the multiple arginine-glycine-glycine (RGG) motifs and four zinc-finger domains at the N-terminal, which were also found in fish and mollusks (Yoshizaki et al., 2000; Fabioux et al., 2004). Phylogenetic analysis showed that the *Hdhvasa* gene was closely related to that in mollusk, especially the *H. asinina vasa* protein but not with other genes in the DEAD-box protein family, such *PL10*, indicating that Pacific abalone (Ezo) *vasa* sequence isolated here is an ortholog with ATP-dependent RNA helicase function.

In vertebrates and invertebrates, the *vasa* gene has been identified to play a crucial role in the development of germ cell lineage and is also the most reliable molecular marker for germ cell development (Raz E. 2000). The expression pattern of the *vasa* gene also shows large variation among different organisms. In the present study, we showed *Hdhvasa* gene expression patterns in different somatic tissues of Pacific abalone (Ezo), suggesting that *vasa* might play a multifunctional role rather than be confined to the germline, as in some other teleosts (Yoshizaki et al., 2000, Huang et al., 2014). There have been some reports of *vasa* subcellular localization in the gonads of mollusks (Obata

et al., 2010; Fabioux et al., 2004). The present experiments demonstrated that *vasa* mRNA is mainly expressed in the early developmental stages of germ cells in both male and female Pacific abalone (Ezo) gonads and that the subcellular localization of the *Hdhvasa* gene in adult gonads is similar with that found in other mollusks (Obata et al., 2010; Fabioux et al., 2004), indicating that the gene plays a crucial role in the development of the germ cell lineage and can be used as a germ cell marker in this species.

During the embryonic stage of Pacific abalone (Ezo), *vasa* mRNA is maternally inherited as it is abundant in early stages of embryos before zygotic transcription commences and declines to low levels. This expression pattern is consistent with that of many teleosts, such as zebrafish (Danio rerio) (Yoon et al., 1997), grass carp *Ctenopharyngodon idella* (Li et al., 2010) and Atlantic salmon *Salmo salar* (Nagasawa et al., 2013). In grass carp, the expression levels during early embryonic stages are high, even in fertilized eggs, and these high levels decrease gradually during cleavage and blastula stages and continue to decrease to lower levels until the gastrula stage (Li et al., 2010). A similar expression pattern has also been reported in medaka *Oryzias latipes* (Shinomiya et al., 2000). Results of ISH and whole-mount ISH confirmed that *vasa* mRNA expression persisted throughout all embryonic stages. In previous research on *H. asinina*, the *vasa* gene was found to be maternally expressed, a characteristic postulated to indicate the presence of germ cell-specific molecules, such *vasa* and *nanos*, in the unfertilized eggs of *H. asinina* and indicating that PGCs could potentially be specified by maternally-inherited determinates (preformation) (Kranz et al., 2010). Our expression

data are consistent with those in unfertilized eggs of *H. asinina*. At the 32-cell stage, the second quartet micromeres likely give rise both to trochoblasts and to the majority of the post-trochal ectoderm, as observed in *H. asinina* and *Patella vulgata* (Suzuki et al., 2009). All second quartet micromeres give rise to the shell field and mantle. The expression pattern that we observed in our study thus suggests that *Hdhvasa* plays a functional role in a range of somatic processes during Pacific abalone (Ezo) embryonic development. In later stages, *vasa* mRNA was expressed in the cells of ventral paraxial bands in the trochophore stage and was expressed in the fields of visceral mass in the veliger stage. Similarly, in juveniles of abalone, hybridization signals were also observed in peripheral cells of the visceral mass. Although cells indicated to be positive by hybridization were observed, these cells did not have the morphological characteristics of germ cells (PGCs) typically observed by HE staining. Therefore, we cannot determine whether these cells are putative PGCs or precursors of the PGCs. Our results indicate that *Hdhvasa* appears to become restricted to cell lineages from the trochophore stage of Pacific abalone (Ezo), which is consistent with findings in molluscan animals, such as *Crassostrea gigas* (Fabioux et al., 2004) and *Haliotis asinina* (Kranz et al., 2010). Further studies of germline progenitors during embryonic development using other molecular markers are needed.

In this era of wide availability of genomic sequence information, RNA-mediated genetic interference (RNAi) has become an increasingly important tool for investigating gene function and sterility establishment. In bivalve mollusks, dsRNA-mediated knock-down of specific genes has been demonstrated in several species, such as Japanese pearl

oyster *Pinctada fucata* (Suzuki et al., 2009; Fang et al., 2011), pearl oyster *Pinctada martensii* [33], Pacific oyster (Fabioux et al., 2009; Huvet et al., 2012) and Zhikong scallop *Chlamys farreri* (Wang et al., 2011). Various methods in various organisms have been used to produce experimental groups in dsRNAi studies; for example, microinjection in honeybees (disambiguation) (Beye et al., 2002), and soaking and feeding treatments in nematode *C. elegans* (Tabara et al., 1998; Timmons et al., 2001). Direct injection has been demonstrated for assessing the function of the *vasa* gene and investigating the potential of this methodology for sterility research of Pacific oyster (Fabioux et al., 2009; Huvet et al., 2012). In the present study, therefore, we first validated the dsRNA injection method by demonstrating in vivo dispersion of dsRNA in gonads by histological observation, and we showed that direct injection is both an efficient method for introducing dsRNA of the target gene into tissues of Pacific abalone (Ezo) and that dispersion efficiency of *vasa* dsRNA in the gonad was optimal at 12 hpi.

The *vasa* loss-of-function phenotype has been demonstrated in various organisms, which provides direct evidence for understanding the role of *vasa* in the development of germ cells and assessing sterility. For example, spawning induced 6 hpi of *vasa* dsRNA into the ovary of fully matured female in Akoya pearl oyster *Pinctada fucata* produced offspring with abnormal gonads with significant reduction in size compared to the control group (Miura et al., 2013). In Pacific oyster, transient silencing of *vasa* mRNA inhibited development of germ cells in gonads (Fabioux et al., 2009). In this study, *vasa* mRNA level in the oocytes of experimental groups was significantly decreased compared to the control groups, and the *vasa* dsRNA inhibiting effect lasted until 14 dpi. Further,

we found that knockdown of the *vasa* gene lead to abnormal germ cells in some female gonads using ether a low or high dose of *vasa* dsRNA. In these abnormal gonads, the ovary cavity of some abalones changed, and unattached and apoptotic cells were observed by HE and DAPI staining. Similarly, during embryonic development of Pacific abalone (Ezo), *vasa* mRNA levels in dsRNAi groups were also significantly downregulated compared to the control group by 6 hpf. All of these findings suggest that *vasa* is maternally inherited and plays an important role in germ cell development. Further, the method developed here may permit establishment of sterility in Pacific abalone (Ezo) for purposes of improving productivity in aquaculture.

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6. Figure legends

Figure 1 Molecular phylogenetic analysis of the amino acid sequences of DEAD-box protein family genes using the neighbor joining method. Sources (accession number) for *vasa* gene sequences: *Chlamys farreri japponensis* (DQ452383), *Crassostrea gigas* (AY423380), Haliotis asinia (GQ259890), *Haliotis discus hanai* (this study), *Danio rerio* (NM_131057), *Mus musculus* (NM_001145885), *Hydra vulgaris* (AB047382) and *Euphyllia ancora* (JQ968407). Sources (accession number) for *PL10* gene sequences: *Haliotis asinina* (GQ:259891), *Danio rerio* (NM_130941), *Chlamys farreri japponensis* (DQ452384) and *Mus musculus* (J04847.1)

Figure 2 Expression profile of *Hdhvasa* gene in somatic tissues and during embryogenesis. A): RT-PCR analysis of *vasa* in different adult tissues. Actin was used as an internal control. Gi: Gill; He: hepatopancreas; Ma: mantle; Mu: muscle; Ov: ovary; Te: testis; uFe, unfertilized eggs; RT(-): negative control without cDNA template. B): Temporal expression pattern of *vasa* during embryogenesis based on qPCR normalized to β-actin expression, and relative to the level at 0 h (unfertilized eggs). Error bars indicate mean standard error estimated from three independent replicates.

Figure 3 Spatial expression of *vasa* during embryogenesis. Section in situ hybridization (SISH, A-D); unfertilized egg (A, E, I), 32-cell (B, F, J), trochophore (C, G, K) and veliger (D, H, L) stages. Whole-mount in situ hybridization (WISH, E-H) and HE staining (I-L). Arrows indicate *Hdhvasa* expressing cells. Scale bars: 100 μm (A, B, C,

D); 50 μm (E, F, G, H, I, J, K, L).

Figure 4 Localization of *vasa* mRNA in juvenile by in situ hybridization. Live map of juvenile (A, E), HE staining (B, F), Hdhvasa antisense probe (C, G) and Hdhvasa sense probe (D, H). Arrows indicate germ cell-like. Scale bars: 1 mm (A, E); 200 μm (B, C, D); 50 μm (F, G, H). Abbreviations: gi, gill; mu, muscle; gu, gut; te, tentacle; mg, mid-gut gland.

Figure 5 Localization of *Hdhvasa* mRNA in adult gonads by in situ hybridization. HE staining of ovary (A, D) and testis (G, J) used for in situ hybridization. *Hdhvasa* antisense probe (B, E, H, K). *Hdhvasa* sense probe (C, F, I, L). E, K, F, L are high magnification images of regions enclosed in red dashed boxes in B, H, C, I, respectively. Scale bars: 100 μm. Abbreviations: OG, oogonia; OC1-OC5, oocytes in developmental stages 1-5; SG, spermatogonia; SC, spermatocyte; ST, spermatids; SZ, spermatozoa.

Figure 6 In vivo dispersion of *vasa* dsRNA to different areas of gonad. Abbreviation: hpi, hours post-injection.

Figure 7 Expression level of *Hdhvasa* mRNA between experimental and control groups and histological observation. (A) Expression level of *Hdhvasa* transcripts measured by qPCR normalized to actin transcripts and the control group. Error bars indicate standard error estimated from three independent replicates. Significant differences are indicated by

a and b (P<0.05). Abbreviation: dpi, days post-injection. (B) Histological observations in ovary. Arrows indicate apoptotic germ cells. Scale bars: 200 μm (A, D, G, J); 50 μm (B, C, E, F, H, I, K, L).

Figure 8 Expression levels of *Hdhvasa* transcripts relative to actin transcripts during embryogenesis between RNAi and control groups measured by qPCR. Expression level is normalized by actin expression as reference. Error bars are standard error estimated from three independent replicates. Significant differences are indicated by a and b (P<0.05). hpf: hours post-fertilization.

Table 1 Primers used in the different PCR amplifications

Supplemental Figures

Figure S1 Schematic representation of *vasa* dsRNA administration and designation of gonad areas for determination of the distribution of *vasa* dsRNA.

Figure S2 Nucleotide and amino acid sequences and schematic of *Hdhvasa*. (A) Complete nucleotide and amino acid sequences. Dotted underline, RGG motif; solid underline, zinc-finger domain; solid-line box, DEAD-box domain; broken-line box, RNA helicase domain. B: Schematic of *Hdhvasa* amino acids.

Figure S3 Histological observation of gonads using DIG-labeled staining. Histological

observation of gonads at 1, 3, 6 and 12 hpi in control group (A, B, C, D) and experimental group (E, F, G, H). I, J, K, L are higher magnifications of regions enclosed in red dashed boxes in E, F, G, H. Scale bars: 100 μm.

Figure S4 Expression profile of *Hdhvasa* mRNA in experimental and control groups using whole mount in situ hybridization. Red arrows indicate positive hybridization signal of *Hdhvasa* mRNA. Abbreviations: uFe, unfertilized eggs; Cont, control groups. Scale bars: 100 μm.

Supplemental Table

Table S1 Summary of number of cells and signals in areas of sagittal sections of gonads

7. Figures and tables

Figure 1

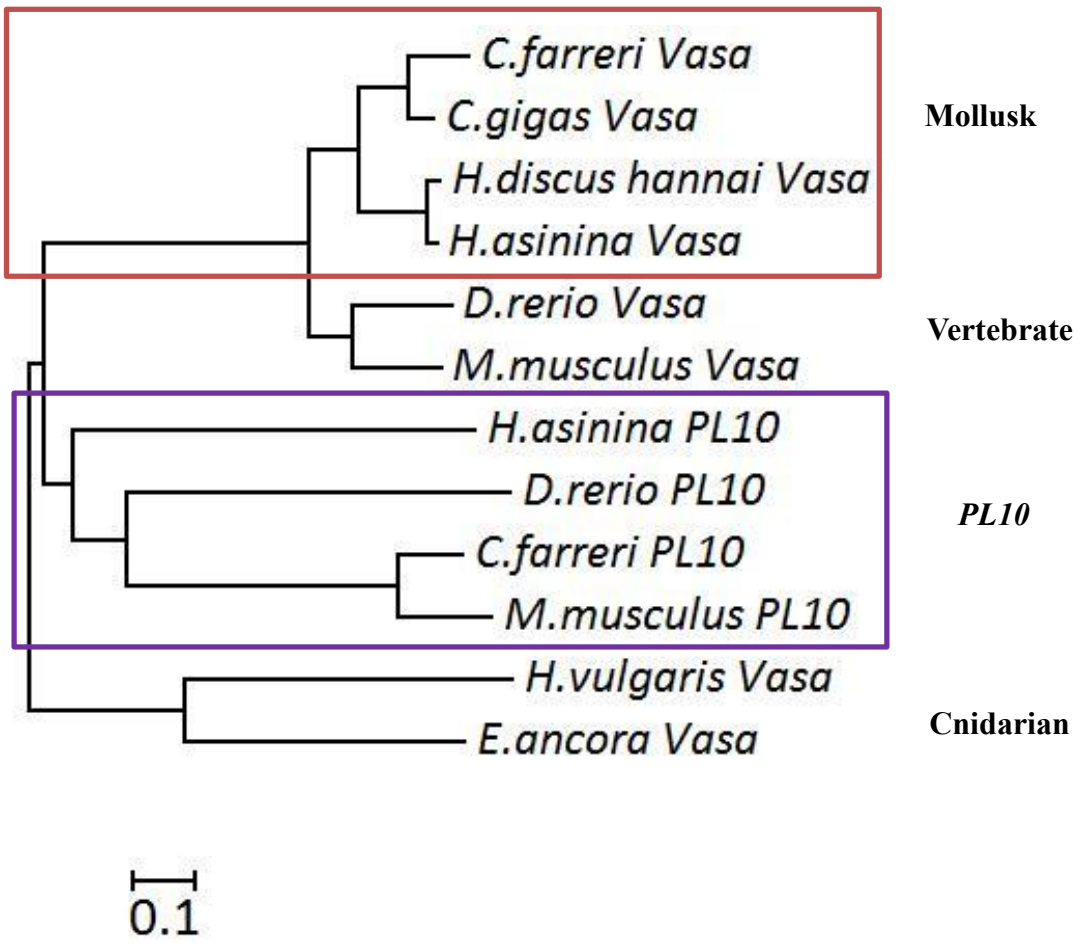
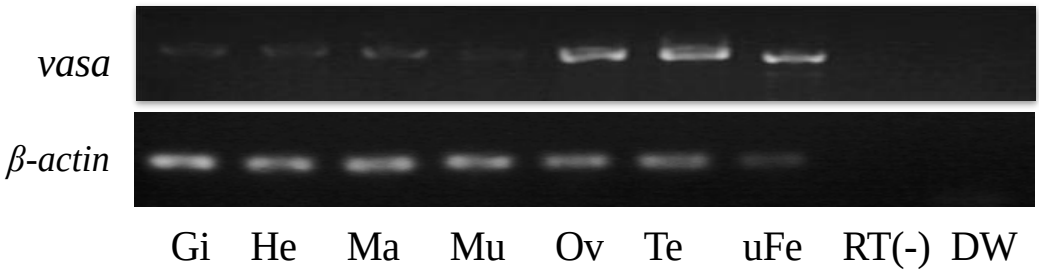


Figure 2

A


vasa
β-actin
Gi
He
Ma
Mu
Ov
Te
uFe
RT(-)
DW

B

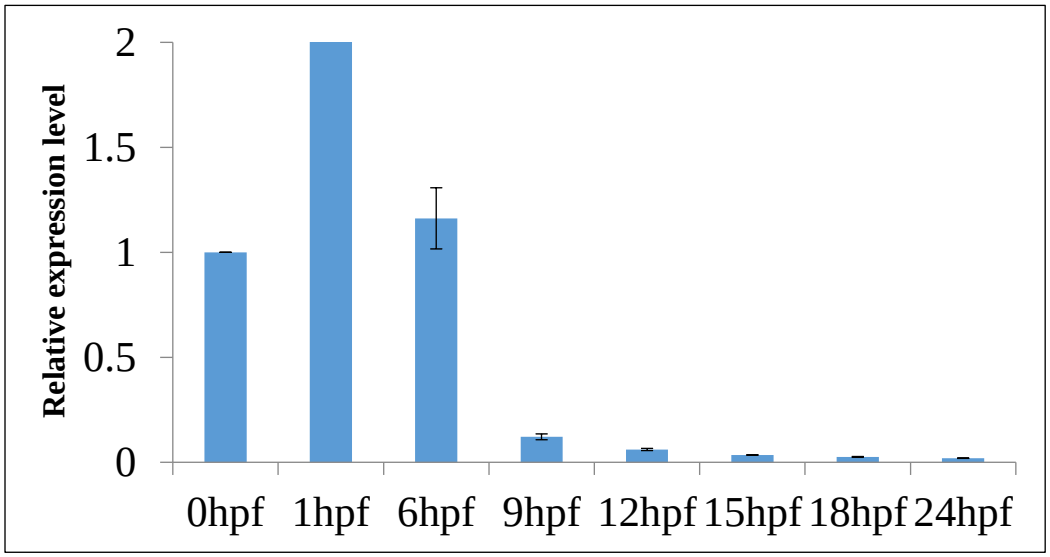

Relative expression level
2
1.5
1
0.5
0
0hpf
1hpf
6hpf
9hpf
12hpf
15hpf
18hpf
24hpf

Figure 3

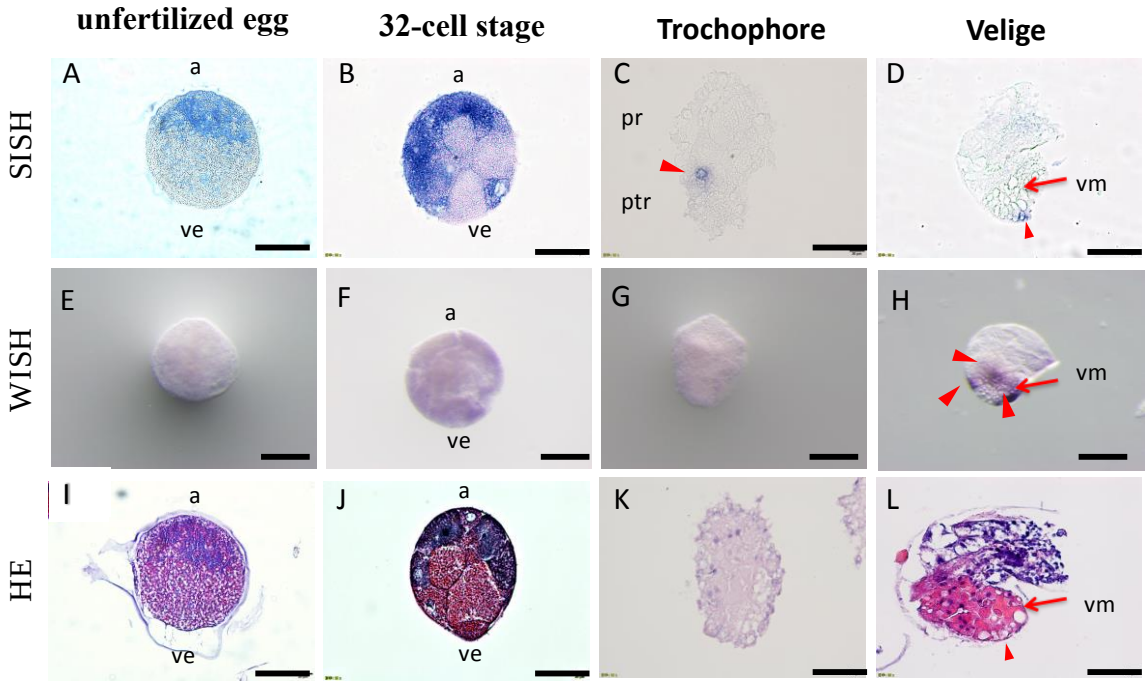

unfertilized egg
32-cell stage
Trochophore
Velige
SISH
WISH
HE
A
B
C
D
E
F
G
H
I
J
K
L
a
ve
pr
ptr
vm

Figure 4

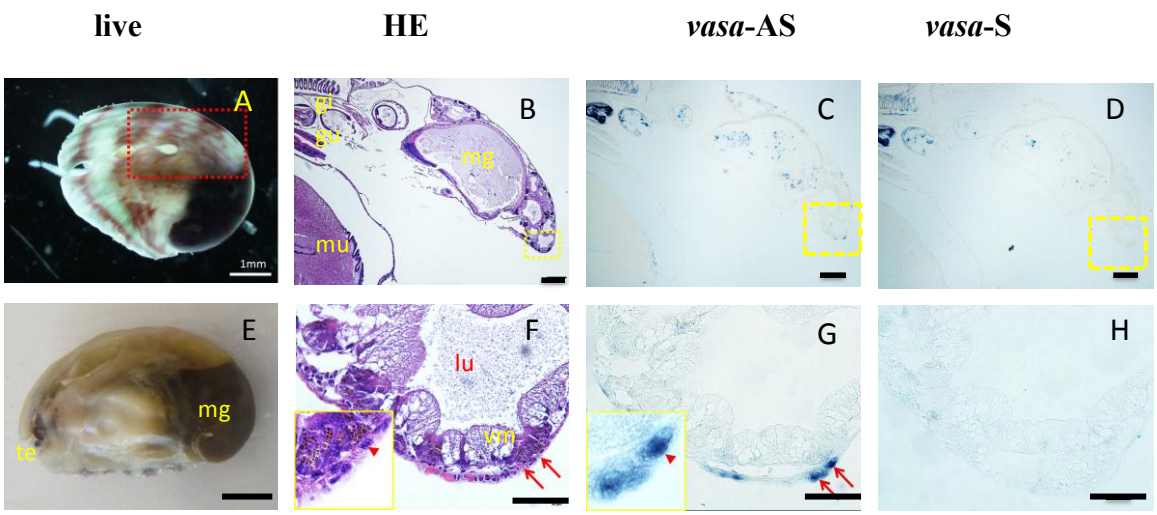


Figure 5

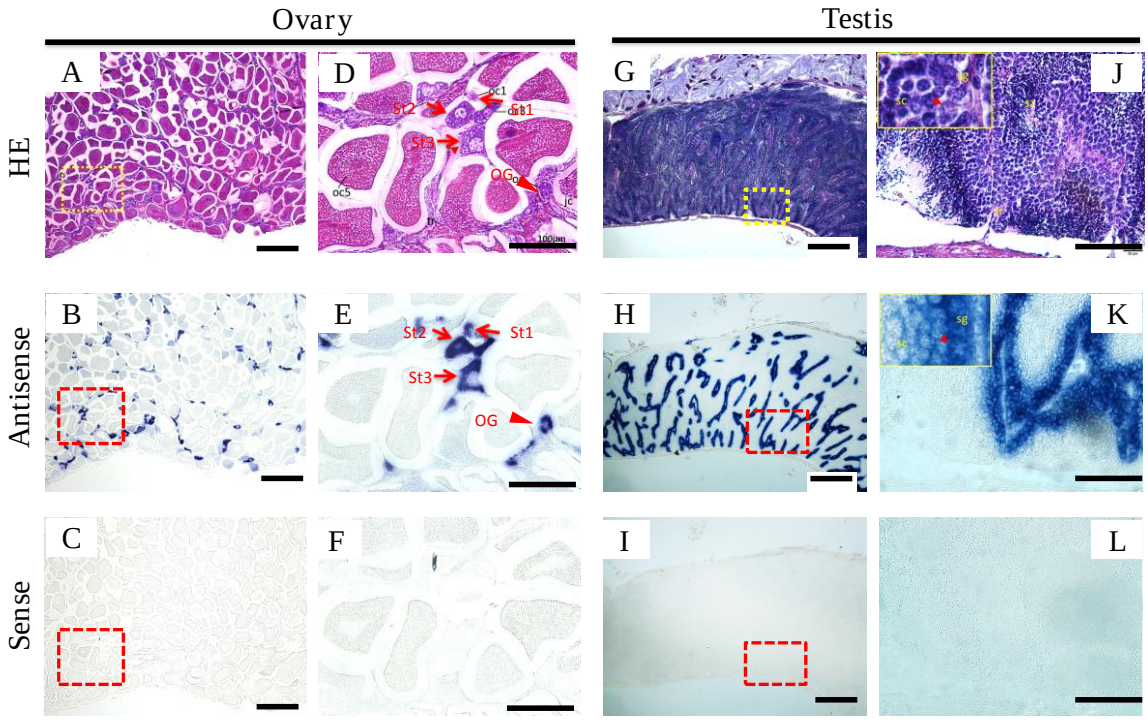
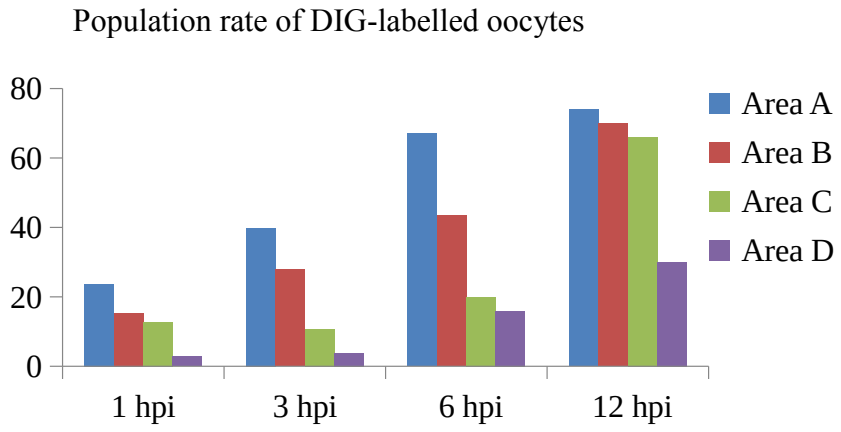

Ovary
Testis
HE
Antisense
Sense
A
D
G
J
B
E
H
K
C
F
I
L
St1
St2
St3
OG

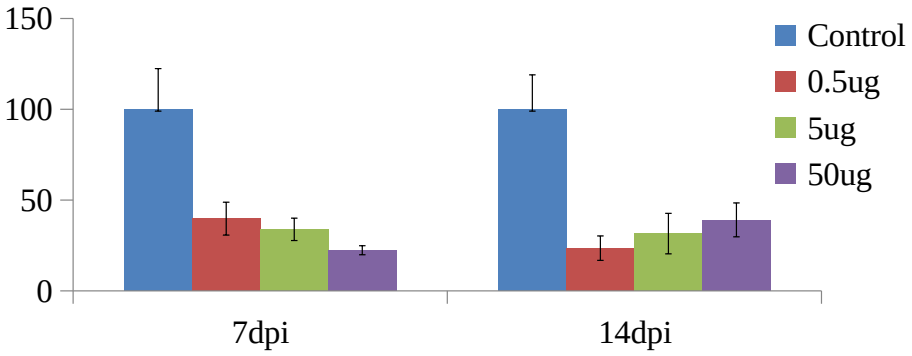
Figure 6



Average:	13.7%	23.1%	32.5%	62.4%

Figure 7

A



Population rate of abnormal gonads: 0% 17% 33% 33% 33% 0% 80% 77%

B

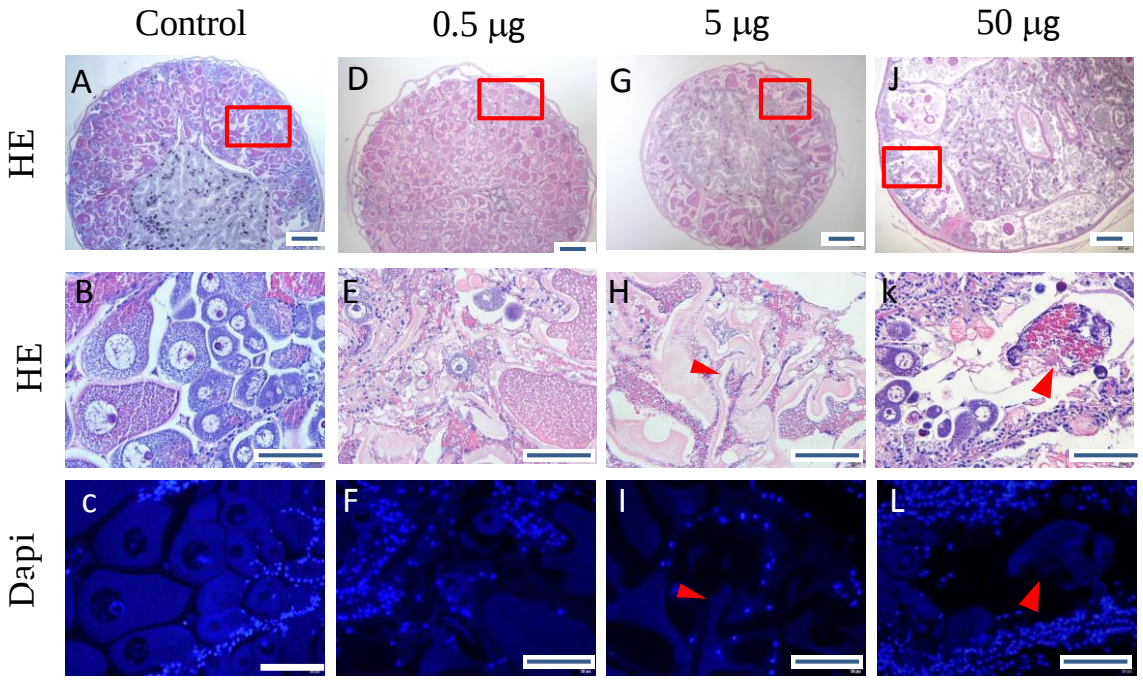


Figure 8

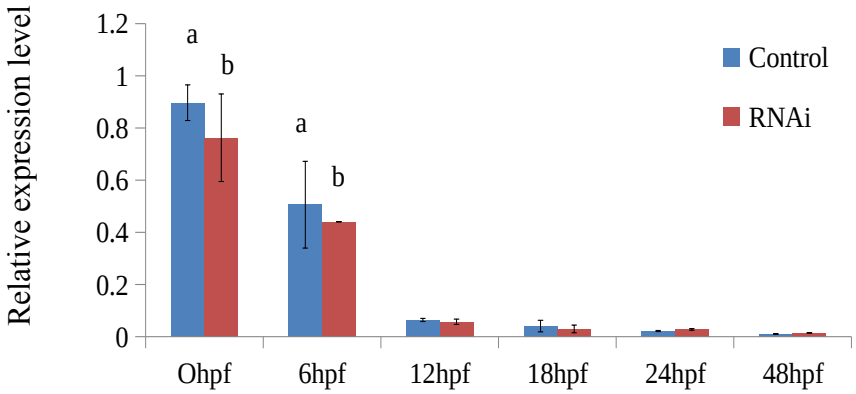

Relative expression level
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
b
Control
RNAi
Ohpf
6hpf
12hpf
18hpf
24hpf
48hpf

Table 1. Primers information for different PCR amplification

Gene ID	Sequence (5’ to 3’)	Length	TM(℃)	purpose
Vasa Fw	CAAGGGAATGTCCTAATGC	747bp	57	RT-PCR;
Vasa Rv	GTCTGTCTCTGGGTTTTAGG			ISH; RNAi
Vasa 5’race	CTCTTTGCCAGAGCTGTCTGCATTAGG		60	5’RACE
Vasa 3’race	TTGAGCCGTGTATACGCAAG		60	3’RACE
5'-AP	ATCAACGCAGAGTACGCGGG			RACE
3'-AP	GGTATCAACGCAGAGTACTT			RACE
Vasa RT QF	CATGGTTGAAGTGGACACAAGG	136bp	57	qPCR
Vasa RT QR	GGTACTAAGGCTGGAAGCACGA			
Actin RT Fw	GAAGCGTACATGGTGGGACT	420bp	57	RT-PCR
Actin RT Rv	GGTGACCTGCGAGATTCATT			
Actin QFw	GCAGAAGGAGATCACAGCCC	135bp	57	qPCR
Actin QRv	CTCCTGCTTGCTGATCCACA			

Figure S1

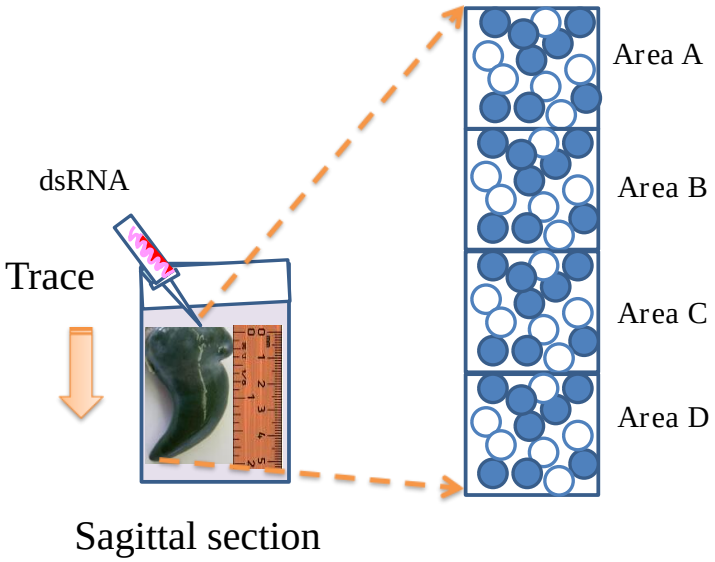


Figure S2

A

B

Figure S3

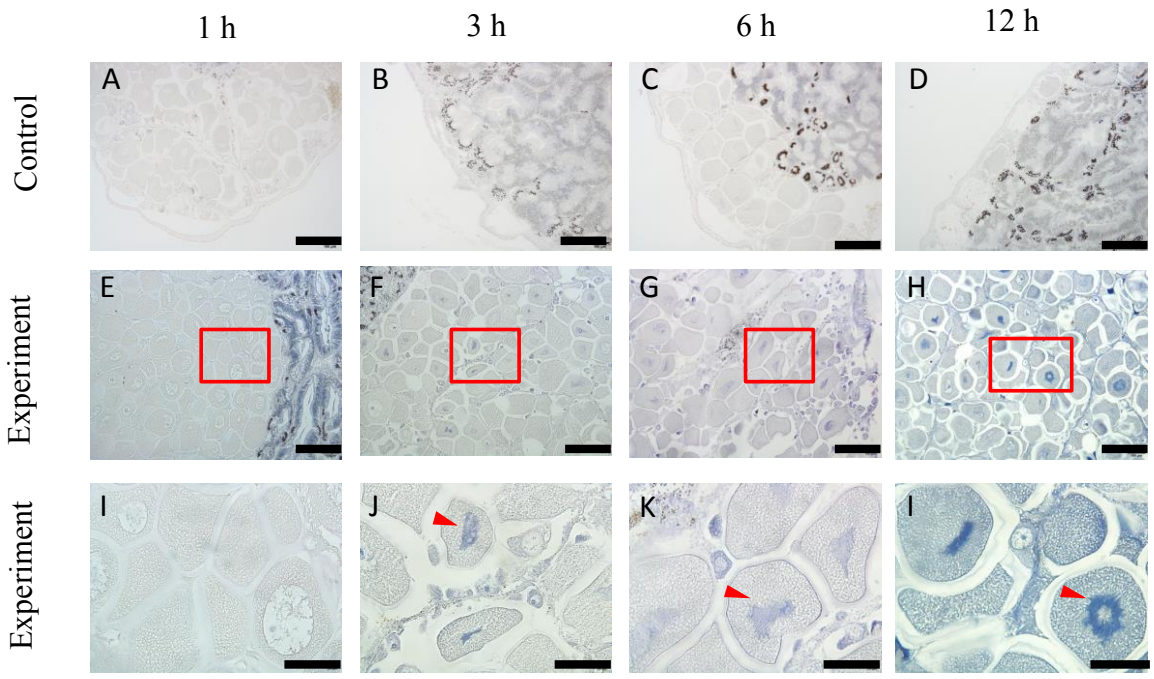


Figure S4

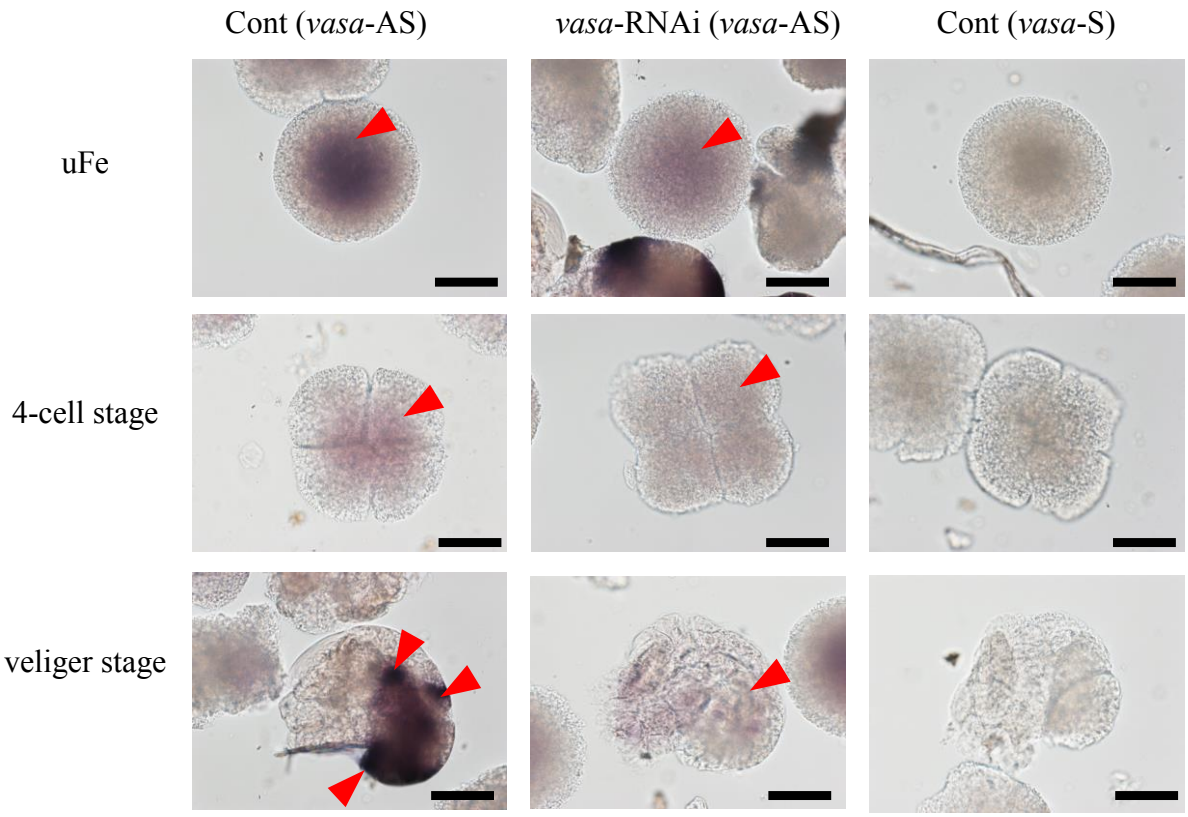

Cont (*vasa*-AS)
vasa-RNAi (*vasa*-AS)
Cont (*vasa*-S)
uFe
4-cell stage
veliger stage

Table S1

Table S1. Statistics summary of cells in sagittal section of gonads

5ug dsRNA	cells No.（1h）	Signal Cell No.(1h)	cells No.(3h）	Signal Cell No. (3h)	cells No.（6h）	Signal Cell No. (6h)	cells No.（12h）	Signal Cell No. (12h)
Area A	232	55	524	209	176	118	920	680
Area B	467	72	394	110	318	138	850	594
Area C	407	52	426	46	280	56	687	454
Area D	251	7	283	11	366	58	600	180
Total cell	1357	186	1627	376	1140	370	3057	1908

Chapter 4. General conclusions

Abalones are not only a tasty and important part of our diet but also a huge portion of our economy. The abalone industry is a fast growing market due to high international demands but several critical issues, such as slow growth rates and diseases, affect the abalone farming industry. To combat this problem, sterilization may be an ideal strategy by the RNA knockdown technique. In this dissertation, I aimed to develop a knock-down system via mRNA dsRNA-mediated RNA interference in Pacific abalone.

The implementation of the technique requires identification of target genes related to germ cell development. In first part, functional analysis of the gonadal transcriptome was conducted and a series of strategies were performed to discover and identify target genes involved in the germ cell development of abalone. Finally, 17 genes including 8 genes that were co-expressed in the ovary and testis and 9 genes that were co-expressed in the ovary, testis and unfertilized eggs were found. One of these candidate genes, *vitelline envelope zona pellucida domain protein 12* (*ZP12*), was expressed in the primordial germ cells of immature gonad and early developmental stages of germ cells of the adult female.

It is known that the *vasa* gene plays a crucial role in the development of germ cell lineage and is the most reliable molecular marker for studies on germ cell development in vertebrates and invertebrates. In this study, we cloned and characterized cDNA sequence of *vasa* gene in Pacific abalone, studied its mRNA expression levels in different tissues and embryonic stages, and examined its germ cell-specific expression characteristics in adult gonads, juvenile and different embryonic developmental stage. The cDNA sequence of *vasa* was 3,866 bp in length and encode a peptide of 801 amino acid residues. It was mainly expressed in both gonads, unfertilized eggs and somatic tissues. *vasa* mRNA was

predominantly localized in oogonia and stage 1-3 oocytes of ovary, in spermatogonia and primary spermatocytes of testis. In unfertilized egg, *vasa* was detected in animal hemisphere. During embryogenesis, it was strongly expressed in the irregular patches of animal portion of 32-cell stage. Later on, it was restricted to cells of ventral paraxial bands in trochophore and the fields of visceral mass in veliger.

To develop the mRNA knock-down system in the species, we evaluated the effects via *vasa* dsRNA-mediated RNA interference in oocytes and early embryos of Pacific abalone. The study shows that the dispersion efficiency of *vasa*-dsRNA in gonad with dsRNA treatment is optimal at the point of 12 hour post-injection. *vasa* mRNA level in the oocytes of experimental groups decreased significantly than that in control groups, and *vasa*-dsRNA inhibiting effect lasted until 14 dpi. Furthermore, we found that knockdown of *vasa* gene lead to abnormal germ cells in some female gonads using ether low or high dose of *vasa*-dsRNA. In these abnormal gonads, the ovary cavity of some abalones changed loose and apoptotic cells were observed in the gonads. Similarly, during embryonic development of Pacific abalone, *vasa* mRNA levels in RNAi groups were also significantly down-regulated than that of control group before 6 hpf. All these studies suggest that the knockdown of maternal mRNA via RNAi in Pacific abalone is feasible. Therefore, our research will support a base for the induction of sterile abalone strains to solve their problems.

Acknowledgements

I would like to express the deepest gratitude to my supervisor Prof. Goro Yoshizaki for providing me this precious opportunity to study and for his excellent guidance and encouragement. I would also like to thank my co-supervisors Prof. Yutaka Takeuchi for his kind help through my research period, and my dissertation committee Prof. Ryosuke Yazawa and Prof. Hidehiro Kondo for their pertinent advices. Their guidance helped me in all the time of research and writing of paper and thesis.

My deep appreciation goes to Dr. Dongdong Xu, Dr. Huan Ye, Dr. Huamei Yue, Dr. Xinxin Zhang, Dr. Qian Li, Dr. Fengyu Li, Dr. Kotaro Amezawa, Mr. Wataru Kawamura, Ms. Megumi Matsumoto and Mr. Hideaki Matsui for their valuable suggestions on the research and their help during Japan. I am also thankful to Qian Li, Fang Yang, Liping Xia, Hanlin Liu for their kindness and friendship during my study.

Finally, I would like to express my sincere and deepest gratitude to my family members. Special thanks to my wife Zhouju Wang for her generous support and understanding during my difficult times, and my lovely daughter Zixi Yu for giving me so much happy and memorable times. My deepest appreciation goes to my parents and parents-in-law for their constant support and encouragements for my academic careers.

Financial support was provided by the Japanese Ministry of Education, Culture, Sports, Science and Technology, and I wish to express my heartfelt gratitude for their help in financing my studies. Many thanks go to Tokyo University of Marine Science and Technology.