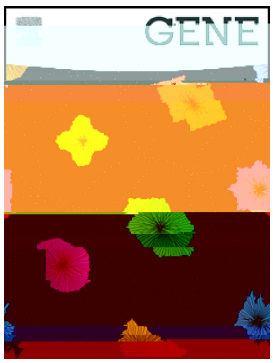

Oocyte maturation and origin of the germline as revealed by the expression of Nanos-like in the Pacific oyster Crassostrea gigas

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Oocyte maturation and origin of the germline as revealed by the expression of *Nanos*-like in the Pacific oyster *Crassostrea gigas*

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ABSTRACT

Nanos gene plays an important role in germline development in animals. However, the molecular mechanisms involved in germline development in Mollusca, the second largest animal phylum, are still poorly understood. Here we identified the *Nanos* orthologue from the Pacific oyster *Crassostrea gigas* (*Cg-Nanos*-like), and investigated the expression patterns of *Nanos* during gametogenesis and embryogenesis in *C. gigas.* Tissue expression analysis showed that *Cg-Nanos*-like was specifically expressed in female gonads. During the reproductive cycle, the expression of *Cg-Nanos*-like mRNA increased matching the seasonal development of the ovarian tissues in diploids, while the expression levels were significantly lower in the ovaries of sterile triploids compared to diploids. High expression of *Cg-Nanos*-like transcripts were detected in early embryonic stages, while the expression significantly dropped at gastrulation and was barely detectable in veliger stages. *In situ hybridization* showed that *Cg-Nanos*-like was expressed at different stages of developing oocytes, whereas positive signals were detected only in spermatogonia during the spermatogenic cycle. These findings indicated that *Cg-Nanos*-like was involved in the development of germ cells, and maintenance of oocyte maturation. In early embryogenesis, the transcripts were broadly expressed; following gastrulation, the expression was restricted to two cell clumps, which might be the putative primordial germ cells (PGCs) or their precursors. Based on the results, the formation of the PGCs in *C. gigas* was consistent with the model of transition from epigenesis to preformation.

Keywords: *Nanos*; germline; ovary; primordial germ cells; *Crassostrea gigas*

Abbreviations: PGCs: primordial germ cells; PBS: phosphate-buffered saline; PFA: paraformaldehyde; EF1- 7 bil kd fl k c l 1- 8 OP. 57 f l l j i nl bfk P. 58 M PQ7 phosphate-buffered saline plus 0.1% Tween 20.

1. Introduction

In all sexually reproducing organisms, gametes play an essential role in the reproductive process, since they transmit genetic materials from one generation to the next. Most animals generate their gametes from cells capable of producing all the differentiated cells, giving raised to an entire organism; these cells are called germ cells (Wylie, 1999). Germ cells derive from primordial germ cells (PGCs) that segregated from somatic cells at the beginning of embryogenesis (Extavour and Akam, 2003). In many animals, PGCs are specified in the embryonic development by maternally inherited cytoplasmic determinants, called germ plasm (Wylie, 1999). In *Drosophila*, several maternal components involved in PGCs formation of germ plasm have been identified (Hay et al., 1988; Cox et al., 1998; Lehmann and Nusslein-Volhard, 1991). As one of the components of germ plasm, *Nanos* gene is required for the formation of PGCs (For; 0 0 1 17( )-1i/0 0 1 19.39 c.63 Tm[ )]TJETB2tETBT1 n,f7nee98; 0 1 14Koba-111

In molluscs, the origins of the germline have not been unambiguously identified, and the PGCs are morphologically indistinguishable from the surrounding somatic cells during early developmental stages (Extavour and Akam, 2003). Until the advent of molecular techniques, the putative PGCs or their precursors are identified in several molluscs, such as *Ilyanassa obsolete*, *Saccostrea kegaki*, and *Haliotis asinine* based on *Vasa* and *Nanos* (Swartz et al., 2008; Rabinowitz et al., 2008; Kakoi et al., 2008; Kranz et al., 2010). In these organisms examined, the conserved germ line genes are uniformly expressed in early embryos, and become restricted to particular cells in the 4d lineages. The similar expression patterns of these genes during embryonic development indicated that the location of the germ line genes to 4d lineages might be conserved in mulluscs. These findings are consistent with the previous hypothesis that the PGCs arose from the 4d micromere and developed during the late larval stages from the mesodermal cells in molluscs (Extavour and Akam, 2003). However, the expression patterns of these genes during the gametogenesis in adults are currently unknown. In addition, it remains unclear whether these conserved germ line genes

Diploid and triploid oysters were supplied by a local oyster farm. The ploidy of samples was tested by flow cytometry. Diploid and triploid oysters at different developmental stages of the reproductive cycle were dissected and their gonads were collected. One part of the gonad c l j b e l b cf ba fk l fk l i fl k cl histological analysis. Another part was fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) over night at 4°C for *in situ hybridization* (ISH). The remaining gonads were preserved in RNA store solution (Dongsheng Biotech, China) until RNA extraction. The other adult tissues including gill, adductor muscle, labial palps, mantle, digestive gland, and hemolymph were sampled from mature oysters, frozen in liquid nitrogen and stored at -80 °C.

Embryonic and larval samples were obtained and cultured as described by Wang et al. (2012). Unfertilized oocytes, 2-cell, 4-cell, morula, blastula, and gastrula embryos, and trochophores, D-shaped larvae, umbo larvae, and eyed-larvae were sampled and treated the same as gonadal tissues procedure.

2.2 Histological analysis

The gonads of diploid and triploid oysters were fixed in l fk l i fl k cl /1 h, dehydrated through a graduated ethanol dilution series and embedded in paraffin wax. 5- j - ef h b fl k b b) ka fk ba f e E e bj l ifk-Eosin. Slides were examined using an Olympus BX53 microscope equipped with DP73 camera to determine sex and determined the sex and stages of the gonad based on the morphological criteria described by Jouaux et al. (2010), Li et al. (2006) and Enríquez-Díaz et al. (2009).

2.3 RNA extraction and cDNA preparation

Total RNAs were extracted from adult tissues and embryo-larval samples using Trizol reagent (Invitrogen) according to the manufacturer's protocols. RNA concentrations, integrity and quality were verified by NanoDrop 2000 (Thermo Scientific) and gel electrophoresis. For gene expression analysis, total RNA % d&b ba c l j b e j hib ba bj hi b for cDNA synthesis by PrimeScriptTM reverse transcription kit (Takara).

2.4 Cloning and sequence analysis of full-length *Nanos* cDNA

The full-length *Nanos* cDNA sequence was isolated by RACE PCR method with the SMARTer® O @B 2,0 Hf %@il k b e& ka eb dbkb .nb fcf .nfj b %K kl -0-.7 2-AGGACTTCAGACATAACGGACGGGACG-0 ka K kl -2-.7 2-TGTATGGAGGTTCGTGAGGCTGGGTG-0 &) ef e b b ab fdkba ba l k the sequence obtained from NCBI database with accession number LOC105348851. PCR cycling was fba l f e Qh Dcib DNA Polymerase (Takara) at 98°C for 1 min, 98°C for 10 s, 65°C for 20 s, 68°C for 30s, for 35 cycles. The purified PCR products were treated with Taq DNA polymerase (Takara) in the presence of dATP to create complementary stick ends for TA clone (Marchuk et al., 1991). Then products with treated sticky ends were cloned into pEASY-T1 vector (Transgen Biotech, China) and sequenced.

Sequence alignment of the CCHC zinc finger domains was performed using the DNAMAN version 8.0 (Lynnon BioSoft, USA). The percentage identity of the deduced amino acid sequence was calculated with other known *Nanos* proteins using the software Lasergene DNAStar Megalign version 8.1.2 (DNASTAR, USA). Phylogenetic tree was constructed using MEGA 7.0 (Kumar et al., 2016) based on the neighbor joining (NJ) method with 1000 bootstrap replicates. All the sequence data are available, with their GeneBank accession numbers are as follows: *Nematostella vectensis Nanos*1 (AAW29070), *Nematostella vectensis Nanos*2 (AAW29071), *Haliotis asinine Nanos*-like (ACT35656), *Ilyanassa obsolete Nanos*-like (ABV54788), *Ephydatia fluviatilis Nanos*-related protein (BAB19253), *Homo sapiens Nanos*1 (Q8WY41), *Homo sapiens Nanos*2 (P60321), *Homo sapiens Nanos*3 (P60323), *Hydra vulgaris Nanos*1 (BAB01491), *Hydra vulgaris* N*anos*2 (BAB01492) , *Drosophila melanogaster Nanos*1 (AAA28715), *Helobdella robusta Nanos* (AAB63111), *Danio rerio Nanos* (AAL15474), *Xenopus laevis Xcat*-2 (CAA51067), *Mus musculus Nanos*1 (BAC76003), *Mus musculus Nanos*2 (BAC82557), *Mus musculus Nanos*3 (BAC82558), *Mizuhopecten yessoensis Nanos*-like1 (OWF49530), *Mizuhopecten yessoensis Nanos*-like3 (OWF55054).

2.5 Real-time qPCR analysis

Real-

20 s/72°C) using EvaGreen 2× qPCR MasterMix-ROX (ABM) on a LightCycler® 480 real-time PCR system (Roche). Parallel amplifications of elongation factor 1- %BC. - & ka ribosomal protein S18 (RS18) reference genes were carried out in the adult and larval samples, respectively (Du et al., 2013; Jiang et al., 2017). Melting curves were constructed for each individual amplicon to ensure the accurate amplification. Relative expression levels of the target gene were calculated as 2^{-} @Q, and all data analyses were performed using two tailed

graded EtOH baths, then treated with an age-dependent concentration of proteinase K (100 ng -. - d,j I &cl /- j fk 04 @) ka b b b ba fk e b j b e b f b j .ni b ba above. Image were captured using a microscope.

3. Results

3.1 Sequence analysis *of Nanos*

The full-length sequence was 1257 bp, which l k fk ba 2 -untranslated region (UTR) of 81 bp, a predicted open reading frame (ORF) of 708 bp with an ATG start site and a TGA b j fk fl k l a l k) ka 0 -UTR of 468 bp (Fig. 1). A single poly (A) signal (AATAAA), was found 14 bp upstream of the poly (A) tail. *Cg-Nanos*-like

development of ovaries and testes (Fig. 4A), while triploid oysters showed retarded gonads with a limited number of gametes (Fig. 4B). Gonad of the triploid oysters were filled with connective tissues with undifferentiated germ cells at the initial of the reproductive cycle. While, a small number of previtellogenic oocytes and spermatocytes scattered with gonadal tissues in diploid oysters during the same period. Previtellogenic oocytes and vitellogenic oocytes predominated in the ovarian tissues and a large portion of spermatozoa were found in the testes in diploids in the maturation step. By contrast, the triploids were functionally sterile, with a few number of vitellogenic oocytes and spermatozoa generating. In ripe oysters, ovarian samples of diploids were occupied with a great amount of mature oocytes, with the same effect, the histology of testes showed that spermatozoa predominated. Compared to diploids, the triploids presented a diminished reproductive capability, and generated fewer gametes.

Expression levels of *Cg-Nanos*-like during the reproductive cycle in diploid and triploid oysters were shown in Fig. 5. In diploid oysters, *Cg-Nanos*-like j OK l iak be detected during the early gametogenetic cycle. At the maturation step, *Cg-Nanos*-like mRNA specifically expressed in female gonads and increased significantly; the expression level continued to dramatically rise in female gonads at the stage of ripeness. On the contrary, the expression of *Cg-Nanos*-lik*e* mRNA was barely detectable in the male gonads (Fig. 5A).

Cg-Nanos-like mRNA expression trend in triploid oysters during the gametogenetic cycle was similar to diploid oysters. *Cg-Nanos*-like expression in triploid ovaries in maturation stage was approximately five folds higher than that of the immature stage. The highest expression level was detected in female gonads at the ripeness stage. Whereas, the *Cg-Nanos*-like gene was expressed weakly in male gonads (Fig. 5B). Although the *Cg-Nanos*-like expression levels exhibited an upward trend in triploid ovaries during the gametogenetic cycle, the expression levels were dramatically lower compared to those of diploids at the late reproductive cycles (Fig. 5C).

The expression levels of *Cg-Nanos*-like mRNA during embryonic and larval stages of *C. gigas* showed that *Cg-Nanos*-like highly expressed in unfertilized oocytes, 2-cell, 4-cell, morula and blastula embryos. The level of its expression was decreased dramatically at the gastrula stage. During the veliger stages, *Cg-Nanos*-like mRNA was almost undetectable (Fig. 7A).

3.4 Localization of *Cg-Nanos*-like mRNA in *C. gigas* gonad during gametogenesis

In order to confirm whether *Cg-Nanos*-like function in adult germline development, cellular location of *Cg-Nanos*-

maturation (Fig. 6). *Cg-Nanos*-like was specially expressed in gonadal area, and no positive signals were detected in somatic cells. In ovaries, *Cg-Nanos*-like transcripts were expressed in all kinds of germinal cells, including oogonia, previtellogenic oocytes and vitellogenic oocytes. In contrast, the positive signals were detected exclusively in spermatogonia, but not in spermatocytes and spermatozoa. No positive signal was detected using sense probes (data not shown).

3.5 Localization of *Cg-Nanos*-like in *C. gigas* during the embryo-larval developmental stages

The location of *Cg-Nanos*-like transcripts was characterized in ten embryo-larval developmental stages (Fig. 7B). *Cg-Nanos*-like was first detected at the vegetal hemisphere of oocytes. After the first cleavage, *Cg-Nanos*-like expression was found in both cells of the 2-cell embryos. As cleavage proceeded to the 4-cell stages, the expression was detected in each cell. Following the morula stages, *Cg-Nanos*-like expression appeared to be more abundant and distributed uniformly in the micromeres. Interestingly, the transcripts abundance of *Cg-Nanos*-like slightly decreased at the blastula stage compared with the morula stage. Despite positive *Cg-Nanos*-like signals were localized uniformly at early cleavage stages; the ubiquitous expression was restricted to a pair of bilaterally symmetrical cells at gastrulation. In the subsequently cleavage stages from the D-shaped larvae to umbo-larvae, the *Cg-Nanos*-like mRNA was continuously detectable in two cell clusters. Finally, the transcripts were limited in single cluster at eyed-larval stage. No positive signal was detected using sense probes (data not shown).

4. Discussion

It has been reported that, in adult ovaries, *Nanos* and *Nanos*-related genes played a critical role in germ cells maintenance, and thus oocyte production (Mosquera et al., 1993; Kloc et al., 2000; Gilboa and Lehmann, 2004; Wang and Lin, 2004; Draper et al., 2007). Tissue expression analysis revealed that *Cg-Nanos*-like was predominantly expressed in the female gonads (ripeness stage), which agreed with the role in maintenance of oocyte production previously reported in other species (Mosquera et al., 1993; Kloc et al., 2000; Draper et al., 2007).

In the gonad of diploid oysters, *Cg-Nanos*-like expression level could be barely detected during the early reproductive cycle. Whereas the expression level increased linearly and abruptly as maturation proceeded to achieve the highest level in fully mature ovaries. In contrast, the expression of *Cg-Nanos*-like mRNA was almost undetectable in male gonads during the reproductive cycle. The dynamical expression patterns in adult developing ovaries indicated that *Cg-Nanos*-like

Nanos-like play critical roles not only in germ cells formation and regeneration, but also in the continued production of oocytes in adult oysters as demonstrated in *Drosophila* and *D. rerio* (Wang and Lin, 2004; Draper et al., 2007). Moreover, according to the expression profile in diploids, *Cg-Nanos*-like used as a molecular marker to elucidate the origination of germ cells.

To better understood the role of *Cg-Nanos*-like in the early embryonic differentiation, its expression was examined during ontogenesis in *C. gigas*. High expression level of *Cg-Nanos*-like were detected in unfertilized oocytes, and dropped significantly at the gastrula stages, then continued to decrease to an undetectable level by the stage of eyed-larvae. These results revealed that *Cg-Nanos*-like transcripts were maternally inherited. Such a conserved role of *Nanos* in transmitting the maternal mRNA to the early oyster embryo has also been reported in many species including fruit flies (Lehmann and Nusslein-Volhard, 1991), nematode worms (Subramaniam and Seydoux, 1999), zebrafish (Köprunner et al., 2001), frogs (Mosquera et al., 1993), and silkmoths (Nakao et al., 2008). The similar expression patterns of *Nanos* mRNA also have been reported in non-model organisms, such as *Branchiostoma floridae* (Wu et al., 2011), *Cynoglossus semilaevis* (Huang et al., 2017), and *H. asinine* (Kranz et al., 2010). Based on the analysis of ISH, *Cg-Nanos*-like mRNA was distributed uniformly at early cleavage. We postulated that *Cg-Nanos*-like might play a functional role in somatic embryogenesis process. An additional somatic role for *Nanos* has been demonstrated in other animals. For example, *Nanos* knockdown embryos exhibited defects in the behavior of the ectodermal and mesodermal germinal bands (Rabinowitz et al., 2008). However, from gastrula to umbo-larvae, the uniform distribution of the mRNA was eliminated, and specifically expressed in two bilaterally symmetrical cell clusters. Base on previous studies, the two cell clumps corresponding to the Mr and Ml cells, might be descendants of 4d, which could be putative PGCs or precursor cells for PGCs (Fabioux et al., 2004b). The specific localization of two cell clusters in *C. gigas* was also observed in *Vasa*

The previous work proposed that *C. gigas* PGCs were specified by maternally inherited determinants (preformation) for the asymmetrical distribution of maternal *Vasa* mRNA (Fabioux et al., 2004b). In contrast, our observations were clearly different from this conclusion. *Cg-Nanos*-like was ubiquitously expressed in early embryos and specified at the late embryogenesis, suggesting that *C. gigas* PGCs were not specified exclusively by inheritance of maternal determinants (preformation). Our findings were consistent with the model of transition from epigenesis to preformation. The inductive signals (epigenesis) might involve in specifying PGCs (Extavour, 2007). Similar findings were also detected in other molluscs (Swartz et al., 2008; Rabinowitz et al., 2008; Kranz et al., 2010; Kakoi et al., 2008; Rebscher, 2015). Therefore, we hypothesized that the germline specification for *C. gigas*, entailed a combination of inherited maternal determinants (preformation), which developed mesodermal bands at gastrulation, and followed by inductive signals to determine which cells of the mesodermal bands became the PGCs or their precursors.

In conclusion, our results suggested that *Cg-Nanos*-like was expressed and required not only in PGCs but also during gametogenesis in *C. gigas*. *Cg-Nanos*-like was involved in oocyte maturation and the formation of germ cells. In particular, *Cg-Nanos*-like could be a novel molecular marker to elucidate the origin of the PGCs in *C. gigas*.

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Figure Legends

Fig. 1. Nucleotide sequence of the *Cg-Nanos*-like cDNA and its predicted deduced amino acid. The start (ATG) and stop (TGA) codons are underlined. Zinc finger domains are highlighted in yellow. The polyadenylation signal (AATAAA) is marked in red.

Fig. 2. Sequence alignment and phylogenetic analysis. A: Sequence alignment of the CCHC zinc finger protein domains of *Nanos*-related proteins from vertebrates and invertebrates. Conserved C and H residues are marked by arrow, which can form two CCHC zinc-binding finger motif. Cg, *Crassostrea gigas*; Ch, *Crassostrea hongkongensis*; My, *Mizuhopecten yessoensis*; Nv, *Nematostella vectensis*; Ha, *Haliotis asinine*; Io, *Ilyanassa obsolete*; Ef, *Ephydatia fluviatilis*; Hs, *Homo sapiens*; Hv, *Hydra vulgaris*; Dm, *Drosophila melanogaster*; Hr, *Helobdella robusta*; Dr, *Danio rerio*; Xl, *Xenopus laevis*; Mm, *Mus musculus.* B: Phylogenetic analysis of *Nanos* based on multiple amino acid sequence alignment.

Fig. 3. Spatial expression of the *Cg-Nanos*-like. Expression pattern of *Cg-Nanos*-like in adult tissues in dioploids.

Fig. 4. Histological analysis of gonad in diploid and oysters during the reproductive cycle. A: Male and female stages of gametogenesis from 0 to 3 in diploid oysters. Stage 0: initiation of the reproductive cycle (a); stage 1: gametes proliferations in male (b) and female (e); stage 2: gametes maturation in males (c) and females (f); stage 3: ripeness stage in males (d) and female (g). B: Male and female stages of gametogenesis from 1 to 3 in triploid oysters. stage 1: gametes proliferations (a); stage 2: gametes maturation in males (b) and females (d); stage 3: ripeness stage in males (c) and females (e). UGC: undifferentiated germ cells; CT: conjunctive tissues; Spg: spermatogonia; Spc: spermatocytes; Spz: spermatozoa; Pvo: previtellogenic oocytes; Vo: vitellogenic oocytes; Og: Oogonia..

Fig. 5. Temporal expression of the *Cg-Nanos*-like. A and B: Expression levels of *Cg-Nanos*-like during reproductive cycle in diploid and triploid oysters, respectively. C: Comparing expression levels between diploid and triploid females at the same stage.

Fig. 6. *Cg-Nanos*-like expression in adult *C. gigas* gonad by ISH during gametogenesis. (A) and (C): Gametes proliferations in females and males; (B) and (D): Ripeness stage in males and females. CT: Conjunctive tissues; Spg: Spermatogonia; Spc: Spermatocytes; Spz: Spermatozoa; Pro: Previtellogenic oocytes; Vo: Vitellogenic oocytes; Og: Oogonia.

Fig. 7. Expression of *Cg-Nanos*-like during embryonic and larval stage. A: Quantitative real-time PCR (qRT-PCR) results for *Cg-Nanos*-like. B: Location of *Cg-Nanos*-like during embryo-larval developmental stages by WISH.

Fig. 1

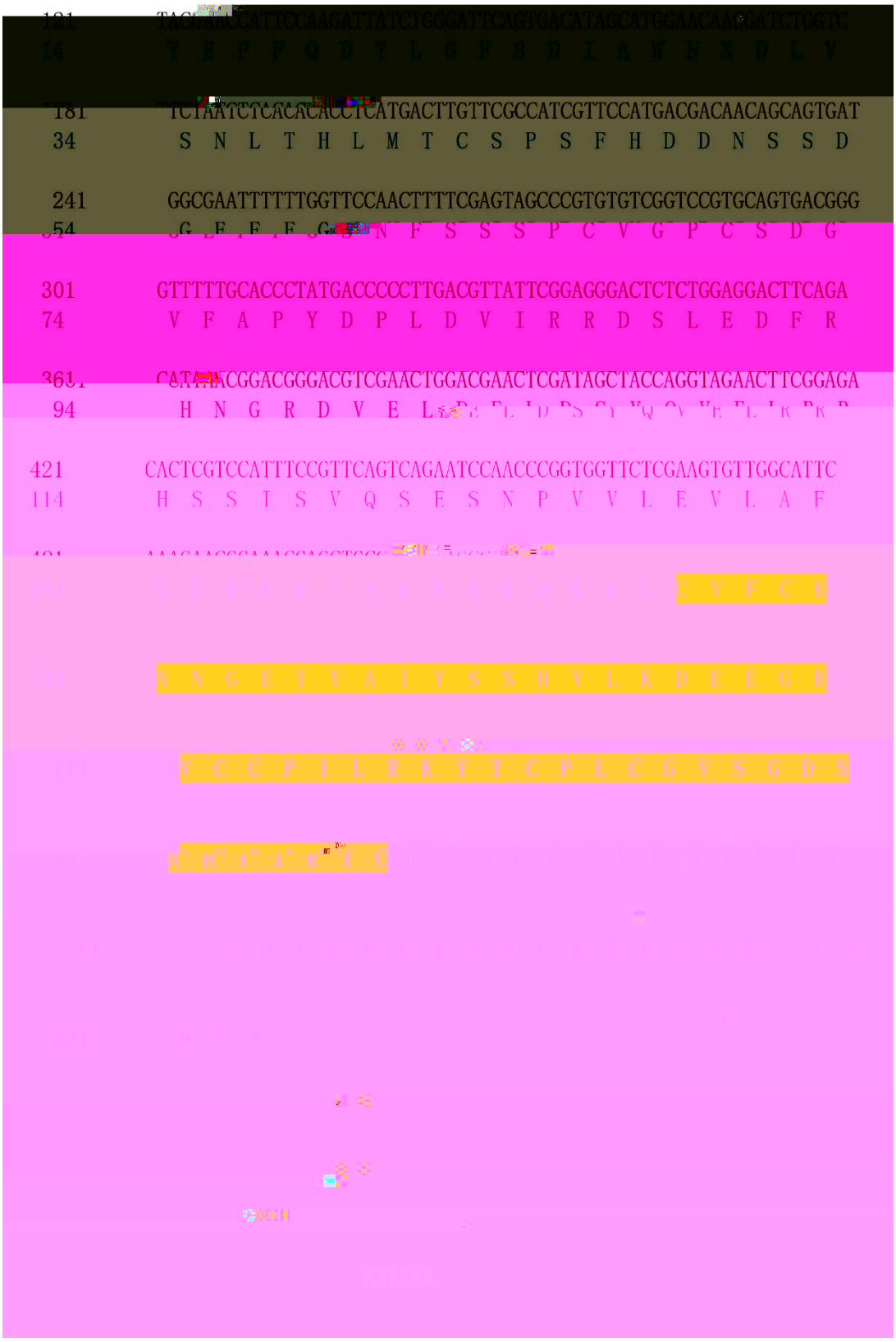

Fig. 2

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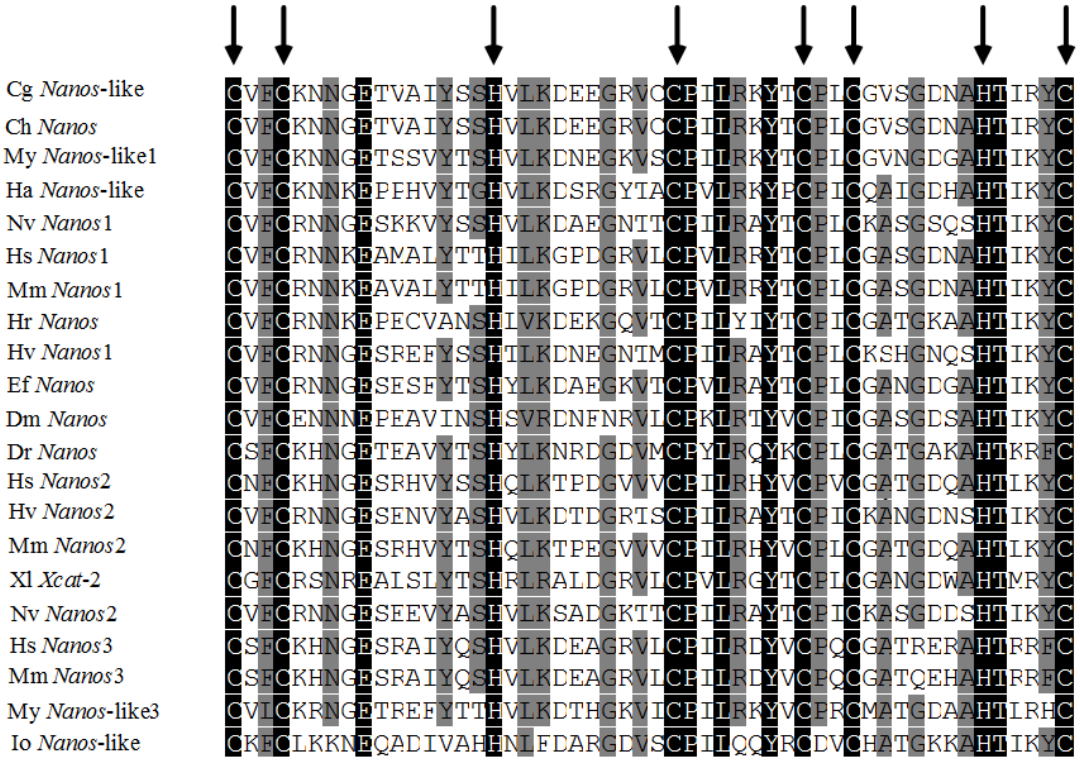

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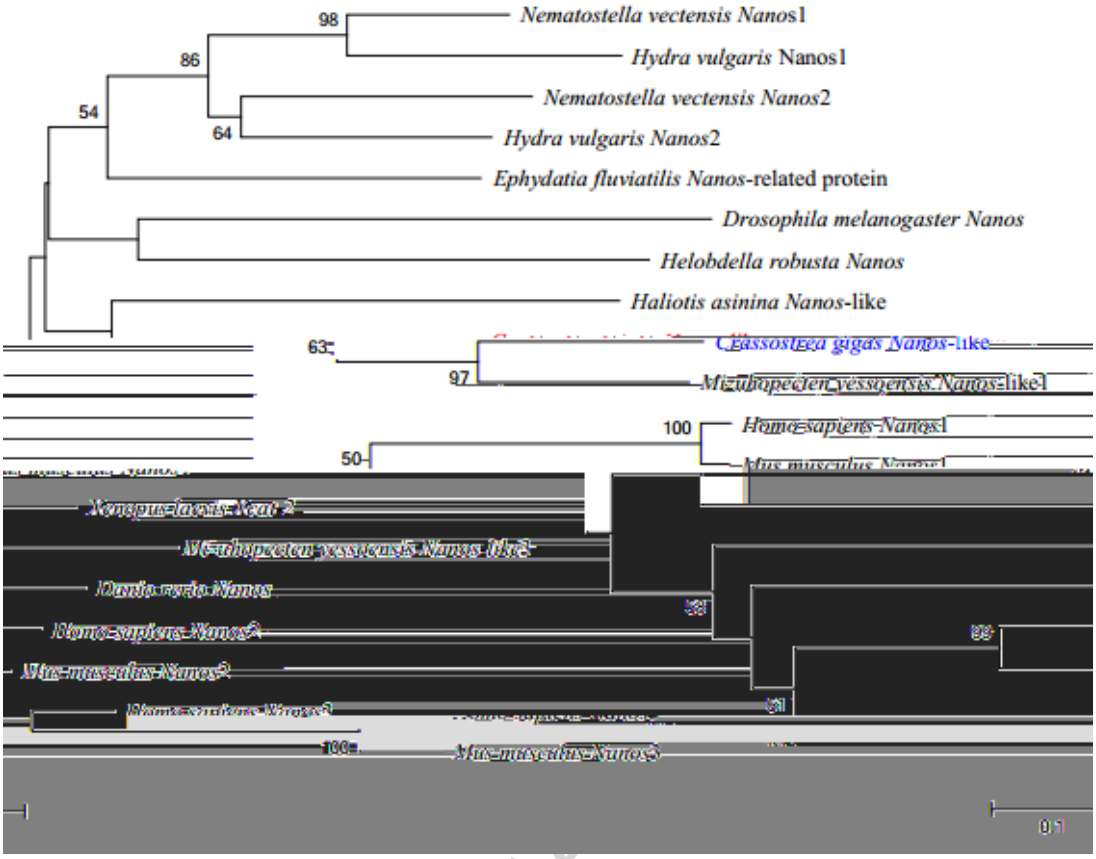

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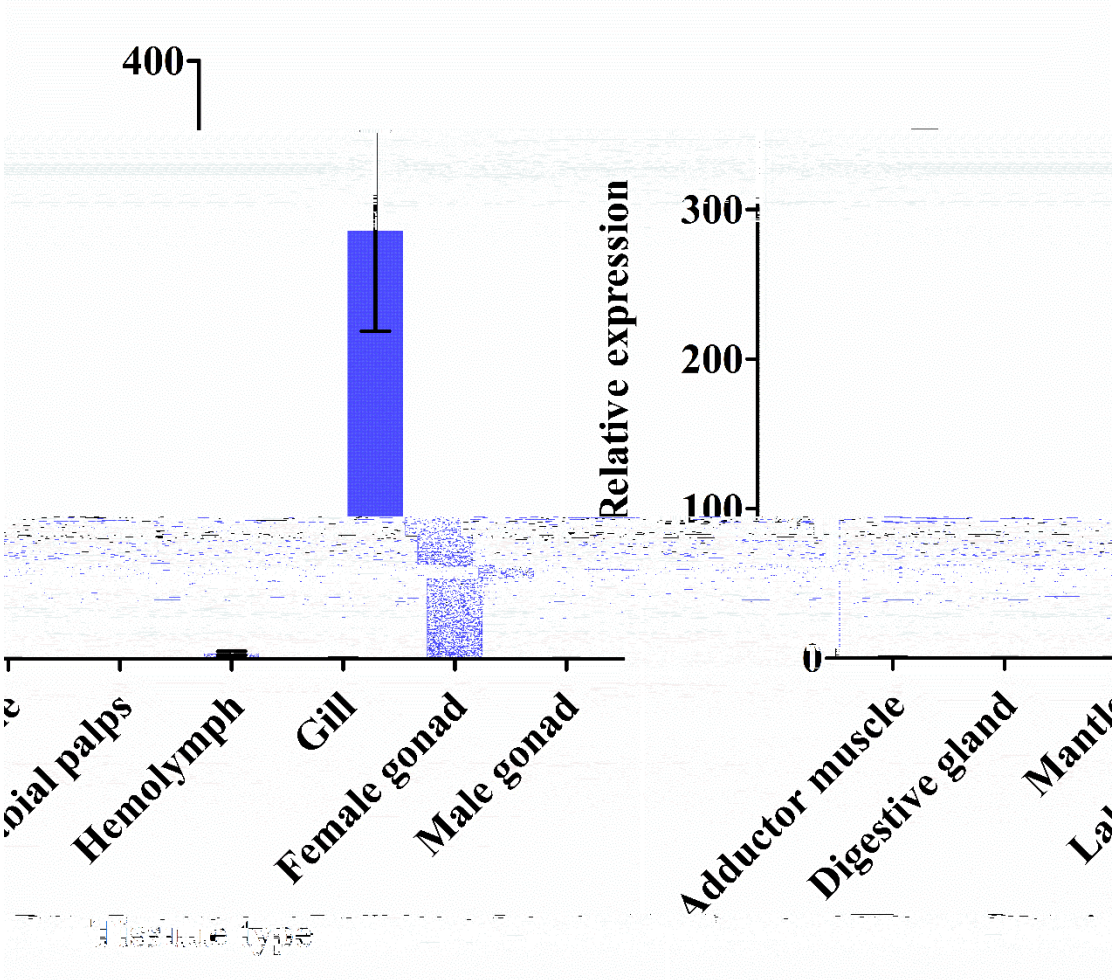

Fig. 4

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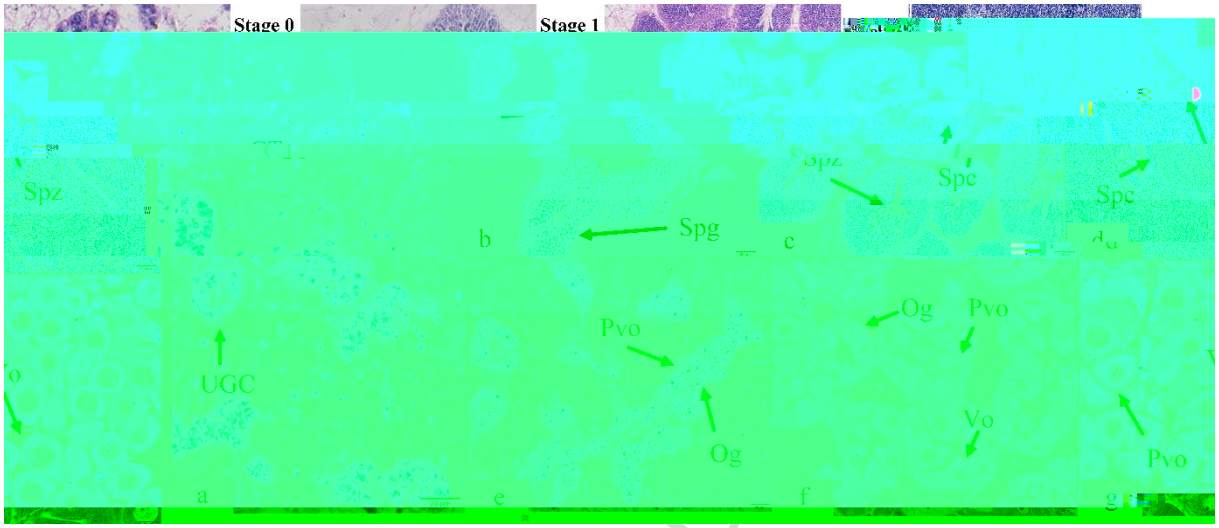

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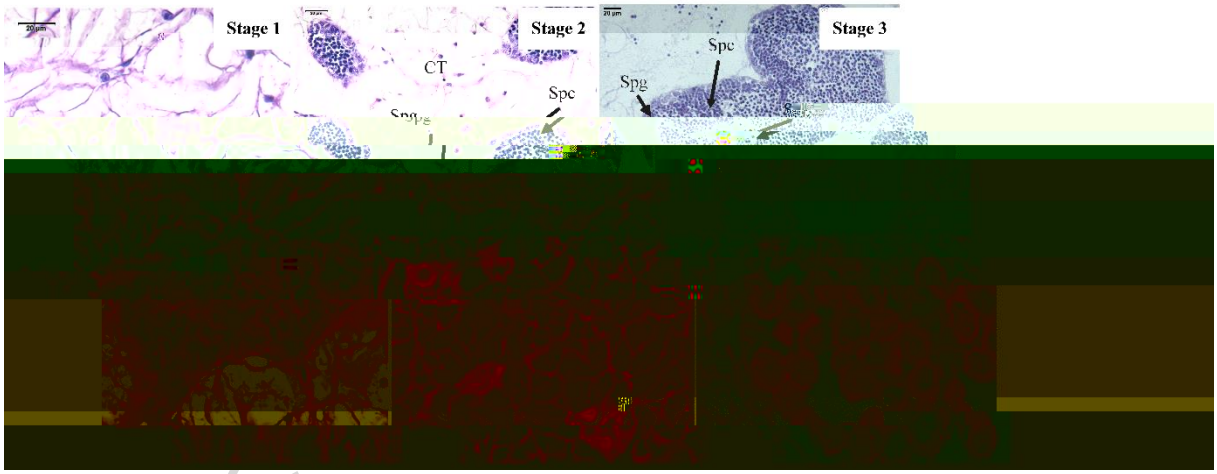


Fig. 5

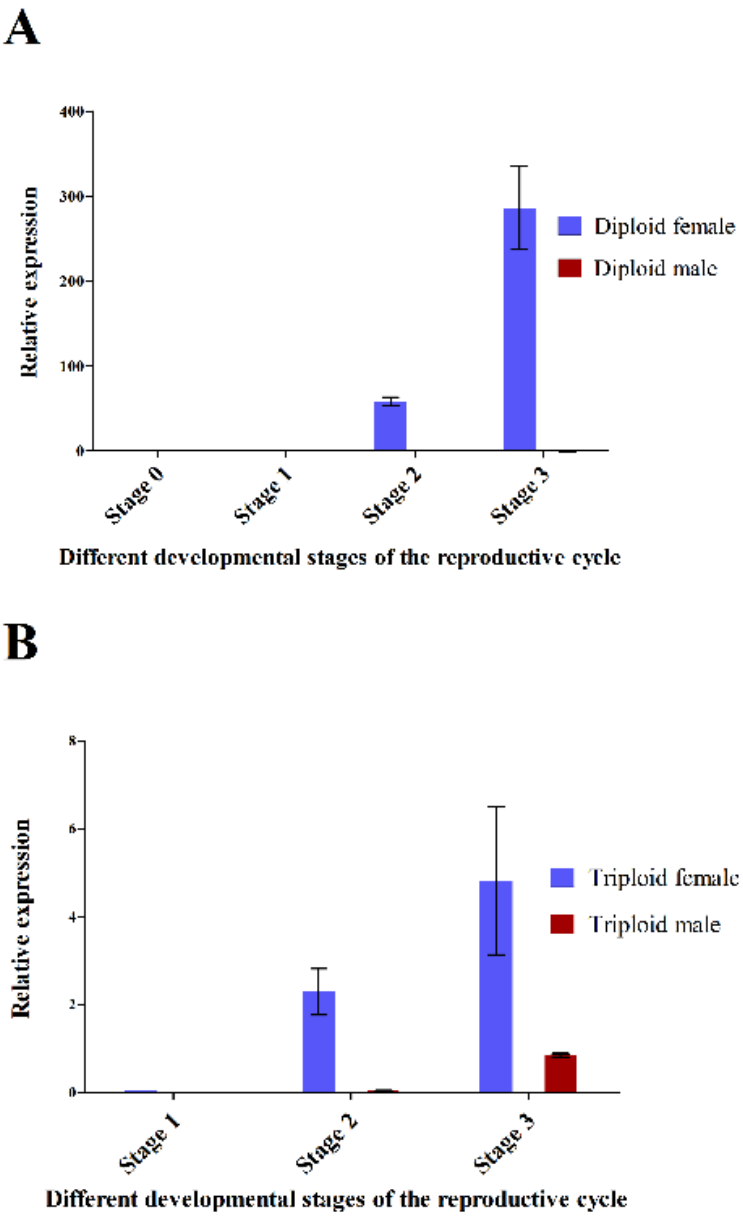


Fig. 6

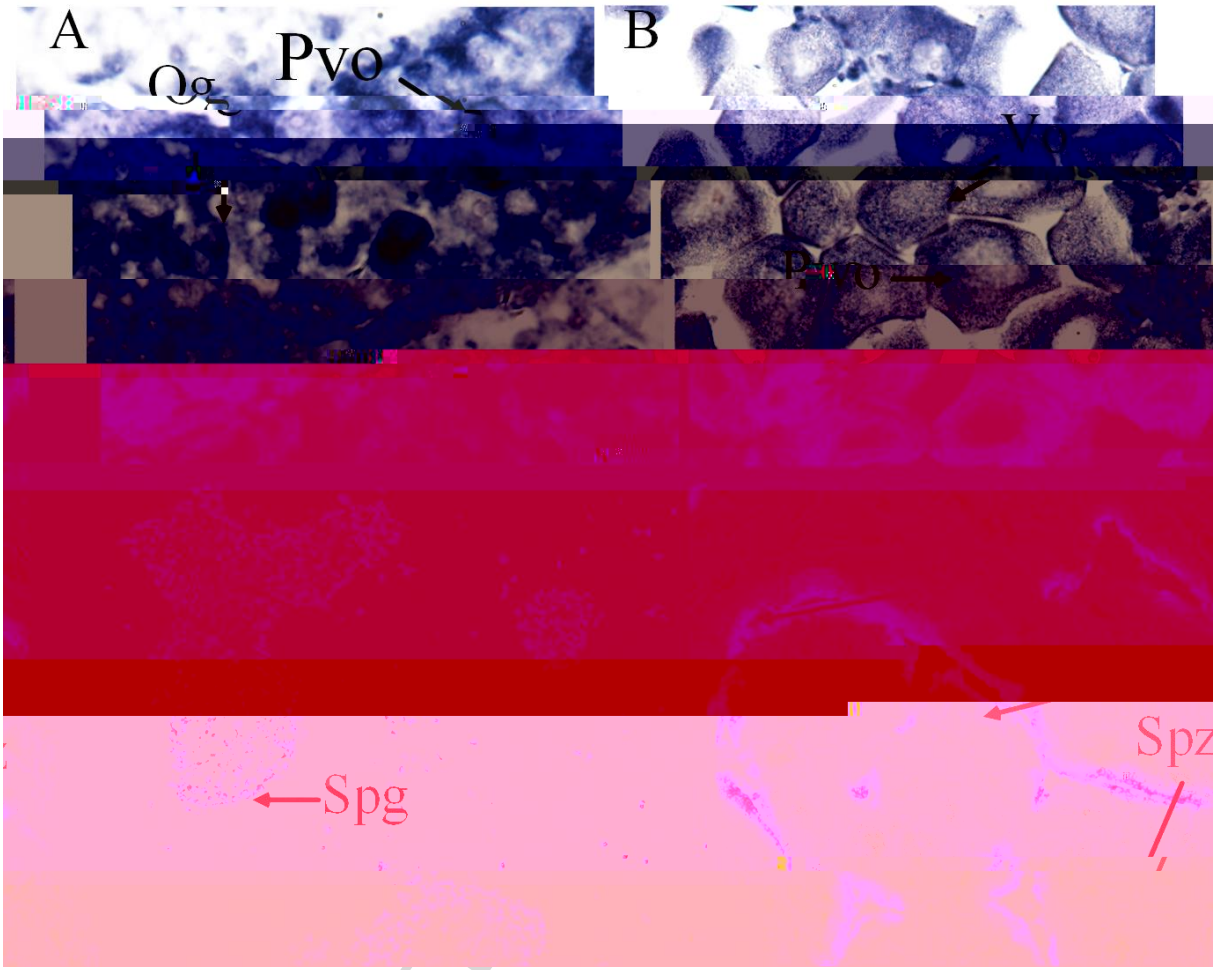
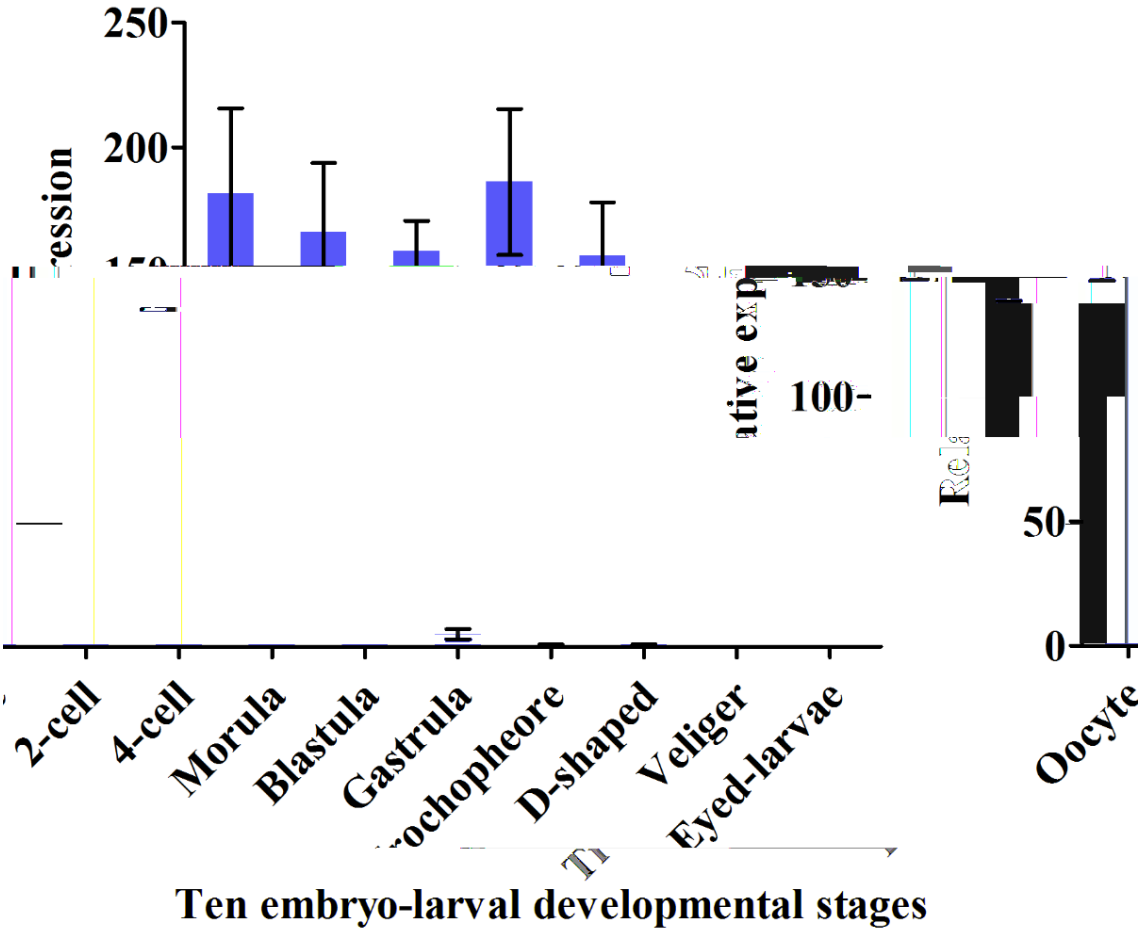


Fig. 7



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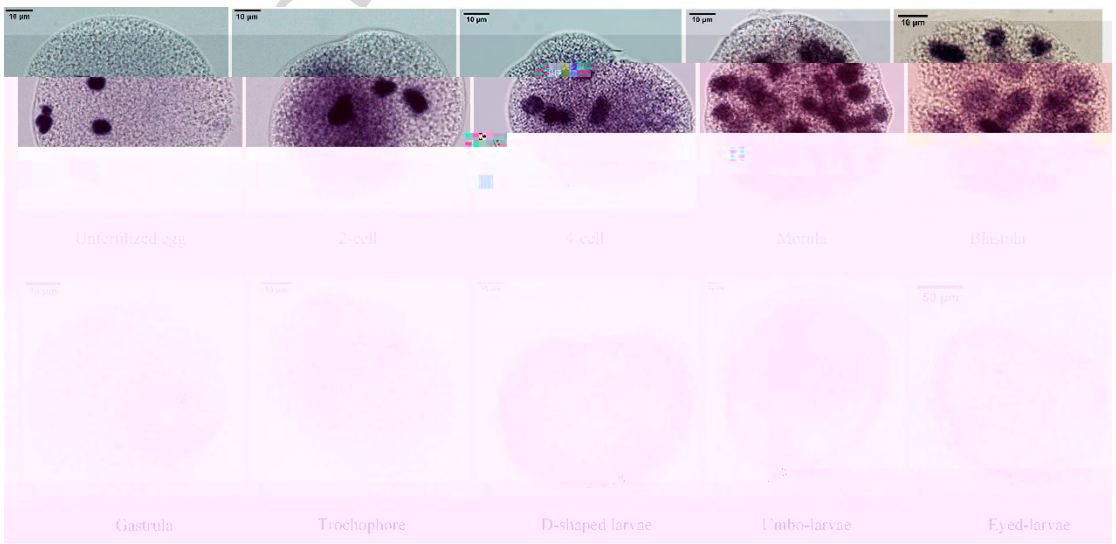

Highlights

1. A full-length cDNA (1257 bp) of *Nanos* from *Crassostrea gigas* was identified and characterized.
2. *Cg-Nanos*-like may play an important role in oocyte maturation in adult oysters.
3. *C. gigas* may developed their germline from the subpopulation of mesodermal cells.