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Oyster vasa-like gene as a marker of the germline cell development in Crassostrea gigas

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Abstract: The oyster vasa-like gene was previously demonstrated to be specifically expressed in germline cells of adult oysters Crassostrea gigas. In the present study, this gene was used as a molecular marker to establish the developmental pattern of germline cells during oyster ontogenesis, using whole-mount in situ hybridization and real-time PCR. The Oyvlg transcripts appeared to be localized to the vegetal pole of unfertilized oocytes and maternally transmitted to embryos. At early development, these maternal transcripts were observed to segregate into a single blastomere, from the CD macromere of 2-cell stage to the 4d mesentoblast of blastula. From late blastula stage, the mesentoblast divided into two cell clumps that migrated to both sides of the larvae body and that would correspond to primordial germ cells (PGCs). Based on these results, we postulate that the germline of C. gigas is specified at early development by maternal cytoplasmic determinants including Oyvlg mRNAs, in putative PGCs that would differentiate into germinal stem cells in juvenile oysters.

Author Keywords: Crassostrea gigas; Development; Germline; Oyster vasa-like gene; Primordial germ cells

Introduction

Germ cells represent a unique cell type, both highly specialized and totipotent since they transmit the genetic information between generations and give rise to an entire organism (Reviewed in 1). Germ cells arise from primordial germ cells (PGCs) that originate during early development of organisms. In many animals, the differentiation of PGCs depends on a specialized region of cytoplasm, called germ plasm (Reviewed in 2). It appears as an electron dense structure due to germinal granules containing many RNAs and proteins associated with clusters of mitochondria (1, 2). These granules were demonstrated to be germline–specific factors deposited in the eggs during oogenesis and maternally transmitted to embryos in organisms such as *Drosophila*, *Caenorhabditis*, zebrafish and *Xenopus* (3, 4). The germ plasm is localized in embryos in the region where the PGCs will form, and is incorporated into these cells as they form, usually around gastrulation. In most organisms, PGCs are formed outside the future location of the gonad and migrate to the somatic part of the gonad by passive morphogenetic movements during gastrulation and/or active migration, implying gene expression and cell interactions (1, 2). In *Drosophila*, germ plasm ("pole plasm") is situated in the posterior pole of embryos. It undergoes a series of synchronous nuclear divisions to produce a syncytium of nuclei that cellularize to form the pole cells (PGCs), 90-120 minutes after fertilization (2). In *Caenorhabditis*, the germinal granules are partitioned solely in a germline blastomere, the P cell, from the first division. The unique PGC (P_4), formed in 24-cell embryo, divides only once during embryogenesis into both Z_2 and Z_3 cells, the source of germ cells in adults.

The first germ plasm component to be identified was the *vasa* gene of *Drosophila* (5). This gene encodes a putative ATP-dependent RNA helicase in the DEAD-box protein family (6, 7). *Vasa*–related genes were isolated from many groups, including nematodes, sponges, cnidarians, amphibians, birds, fishes, and mammals (8-16). In all organisms examined, v*asa*

expression was restricted to the germ cell lineage and was detected throughout development from the embryo to the adult gonad. The role of *vasa* in germline formation in embryos was clearly demonstrated in *Drosophila*, *Caenorhabditis* and zebrafish by functional analysis of mutation or inactivation of the *vasa* gene (6, 17, 18).

In the oyster *Crassostrea gigas*, few data address the formation of the germline cells. Molluscs, like other protostomians such as annelids, follow a spiralian development characterized by an invariant cleavage pattern (19). The embryo is divided into blastomeres that receive a unique content of cytoplasm. Development may be tracked from the origin of blastomeres through entire development up to the formation of the definitive organs (20). However, the origin of germline is unclear in most mollusc species. In bivalves, the existence of PGCs was reported only in few species, such as *Cyclas cornea*, *Sphaerium japonicum* and *Lasea rubra* (21). In the clam *Sphaerium*, the PGCs were thought to arise from the 4d mesentoblast formed at the sixth cleavage. In *C. gigas*, the origin of the germline has not been established since germ cells can not be distinguished until juvenile stage (22). The orthologue of *vasa* gene (*Oyvlg*), previously characterized in *C. gigas* (23), could be a useful marker to study the germline development in oysters since it was demonstrated to be specifically expressed in germline cells in adult oysters both in males and females.

In the present study, we used the *Oyvlg* as a germline cell-specific marker to study the origin and the formation pattern of germ cells during *C. gigas* ontogenesis. We localized and assayed the expression of *Oyvlg* at different developmental stages using whole-mount *in situ* hybridization and real-time PCR techniques.

Materials and methods

Experimental reproduction and larval rearing

Wild mature oysters, collected on the Atlantic coast of Brittany (France), were used for experimental breeding. All steps of fertilization and rearing were realised with 0.2 µm-filtered seawater. The sex of oysters was determined by microscopic examination of gametes. Sperm and oocytes were collected by stripping the gonads and oocytes were counted using Malassez slides. Oocytes from 6 females (~ 40 million) and spermatozoids from 4 males were mixed in 5-L seawater cylinders (2.10^6 eggs/L), until the appearance of the second polar body. Fertilized eggs were then washed on a nylon screen (20 µm mesh) to eliminate excess sperm, and placed in 150 L tanks at a density of 40 embryos/L. Larval cultures were maintained at 25°C and aerated with air bubbling. The rearing density was progressively reduced to 5 larvae/L and 2 larvae/L at the D larval (2 days post-fertilization (dpf)) and pediveliger (18 dpf) stages, respectively. First fixations were observed 25 days after fertilization.

Various developmental stages, identified microscopically, were sampled: oocytes before fertilization, 4-cell and 8-cell embryos (1 and 2 hours post-fertilization, respectively), morulas (3 hpf), blastulas (5 hpf), gastrulas (7 hpf), trochophores (16 hpf) and D-larvae (2 dpf), early veliger larvae (7 dpf), later veligers (14 dpf), pediveligers (18 dpf), and larvae after fixation (27 dpf). One million oysters from each developmental stage from oocytes to trochophores, and 250 000 from later stages, were sampled for total RNA extraction. One million oysters from each stage from oocytes to trochophores, and 100 000 from later stages, were sampled for whole-mount *in situ* hybridization.

RNA extraction

Total RNA was extracted from the pool of eggs, embryos and larvae sampled for each developmental stage, using TRIzol reagent (GibcoBRL) and then treated with 0.1 unit/µL

DNase I (deoxyribonuclease) (Sigma) to prevent DNA contamination. RNA concentrations were measured, after DNase treatment, at 260 nm using the conversion factor 1OD = 40 µg RNA.

Reverse transcription

Reverse transcription (RT) was carried out as described in Huvet *et al*. (24) using oligo$(dT)_{23}$-anchored as a primer, 200 units Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Sigma) and 2 µg total RNA from each isolated developmental stage.

Real-time PCR analysis

The level of *Oyvlg* transcripts expressed during oyster ontogenesis was investigated by real-time PCR using an Icycler (Biorad). Amplification of oyster *Elongation Factor I* (**BQ426516**) cDNA was performed to confirm the steady-state expression of a housekeeping gene, allowing an internal control for *Oyvlg* gene expression. *Oyvlg*- and *Elongation Factor I*-specific primers were VS-S9 (5'-TCCCCGAGGAGATTCAGA-3'), VS-AS9 (5'-ACGTCAGTGCAAGCACCA-3') and qfElong I (5'-ACCACCCTGGTGAGATCAAG-3'), qrElong I (5'-ACGACGATCGCATTTCTCTT-3'), respectively. Real-time PCR assays were performed, in triplicate, with 5 µL cDNA (1/5 dilution) in a total volume of 15 µL with each primer at 0.33 µM, 1.5 µL fluorescein, and 1X "Quantitect SYBR Green PCR kit" (Qiagen). The cycling conditions consisted of Taq polymerase thermal activation for 15 min at 95°C, then 45 cycles of denaturation at 95°C for 30 seconds and annealing/elongation at 60°C for 1 min. For each individual sample, a melting curve program was carried out from 95°C to 70°C by decreasing temperature by 0.5°C each 10 seconds. Each run included the cDNA control, negative controls (total RNA treated with DNase I) and blank controls (water). PCR

efficiency (E) was determined for each primer pair by performing standard curves from serial dilutions to ensure that E ranged from 99% to 100%.

Calculation of relative *Oyvlg* mRNA levels was based on the comparative Ct method (25). The relative quantification value of the sample, normalized to the *Elongation Factor I* gene and relative to the control, is expressed as $2^{-\Delta\Delta Ct}$, where ΔCt = (Ct (*Oyvlg*) – Ct (*Elongation Factor I*)) and $\Delta\Delta Ct$ = ΔCt of cDNA sample - ΔCt of positive cDNA control.

Comparison of the level of *Oyvlg* mRNA (relative to *Elongation Factor I* mRNA) between developmental stages of oyster was performed by paired Student's *t*-test using SYSTAT 9.0 by SPCC.

Probe synthesis

Digoxigenin-labeled RNA sense and anti-sense probes were synthesised from the clone of an *Oyvlg* 675 bp fragment (**AY423380**), by amplification with VS-S1 (5'-TCCAGGAACCACAAGCCCTG-3') and VS-AS4 (5'-CTGGAGCTTTCCCAAGCTTG-3') specific primers using a DIG RNA labelling kit (Roche).

Whole-mount in situ *hybridization*

Fixation of samples and whole-mount *in situ* hybridization (ISH) were performed using the protocol described by Nederbragt *et al*. (26) for *Patella vulgata*. Prehybridization was done for 6 h at 65°C in hybridization buffer (5X SSC, 50% formamide, 100 µg/ml Torula RNA, 1.5% blocking reagent (Roche), 5 mM EDTA, 0.1% Tween 20). Sense or anti-sense probe was added (1 µl) and hybridized to target overnight at 65°C. Probes were washed away and antibody incubation was performed in a fresh solution of anti-digoxigenin antibody coupled to alkaline-phosphatase (AP) (diluted 1:5000) (Roche) for 1h. Samples were then washed and stained for 45 min in the dark with AP buffer containing 1% (w/v) polyvinyl

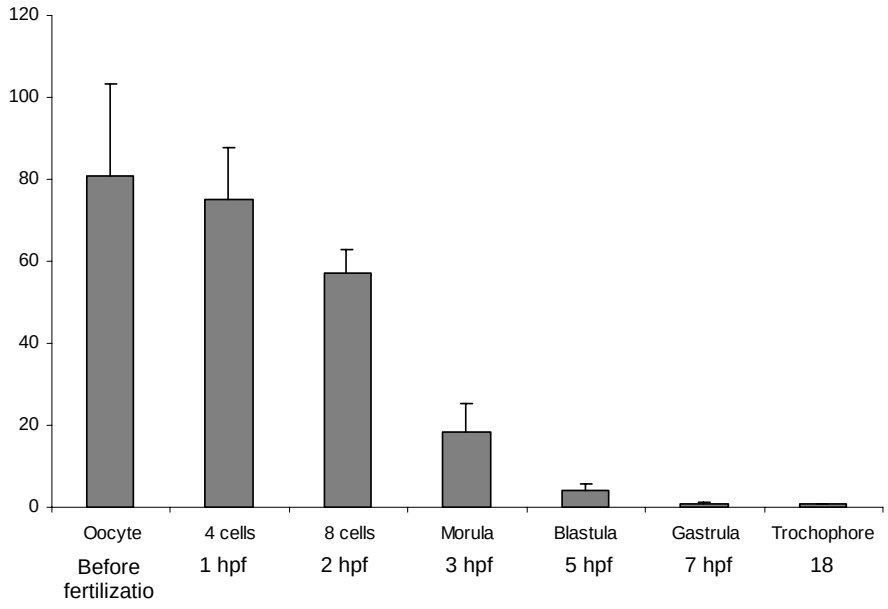
alcohol (PVA), 3.5 µl NBT (Nitro blue tetrazolium; 75 mg/ml) (Roche) and 3.5 µl BCIP (5-bromo-4-chloro-3-indolylphosphate; 50 mg/ml) (Roche) per ml of buffer. The reaction was stopped by washing twice in TBS-T (136 mM NaCl, 25 mM Tris, pH7.4, 2.7 mM KCl, 0.1% Tween). For photography, samples were dehydrated, washed twice in histoclear, and mounted in Canada balsam (Sigma). Pictures were taken with a Leitz DIAPLAN microscope with DIC optics (Nomarski) on 50 ASA Fuji Velvia film.

Results and Discussion

Oyvlg is maternally supplied to embryos

Real-time PCR analysis showed a maximum amount of *Oyvlg* transcripts in unfertilized oocytes (mean relative expression = 81.0 ± 22.4) and in early developmental stages, 4-cell (75.0 ± 12.8) and 8-cell embryos (57.2 ± 5.8) (Figure 1). The level of *Oyvlg* mRNA expression dropped significantly ($\alpha = 0.01$) in the morula (18.2 ± 7.2) relative to earlier stages. The transcription of molluscan embryonic genome begins in the gastrula, and early development is supported by transcripts from the maternal genome (27). Therefore, the large amount of *Oyvlg* mRNA observed in unfertilized oocytes and in first cleavage stage *C. gigas* embryos indicated that *Oyvlg* transcripts were maternally synthesized and transmitted to embryos to support early development, suggesting a role in precocious specification of germ cells. In *Drosophila*, *Hydra* and zebrafish, v*asa*-related genes were also shown to be maternally transmitted and implied in germline differentiation in embryos before the beginning of zygotic gene expression (5, 9, 18).

Figure 1: Level of *Oyvlg* transcripts relative to *Elongation Factor I* transcripts in unfertilized oocytes and in various developmental stages of *Crassostrea gigas* analysed by real-time PCR. Bars represent standard deviation at 5% level. hpf means hour post-fertilization.

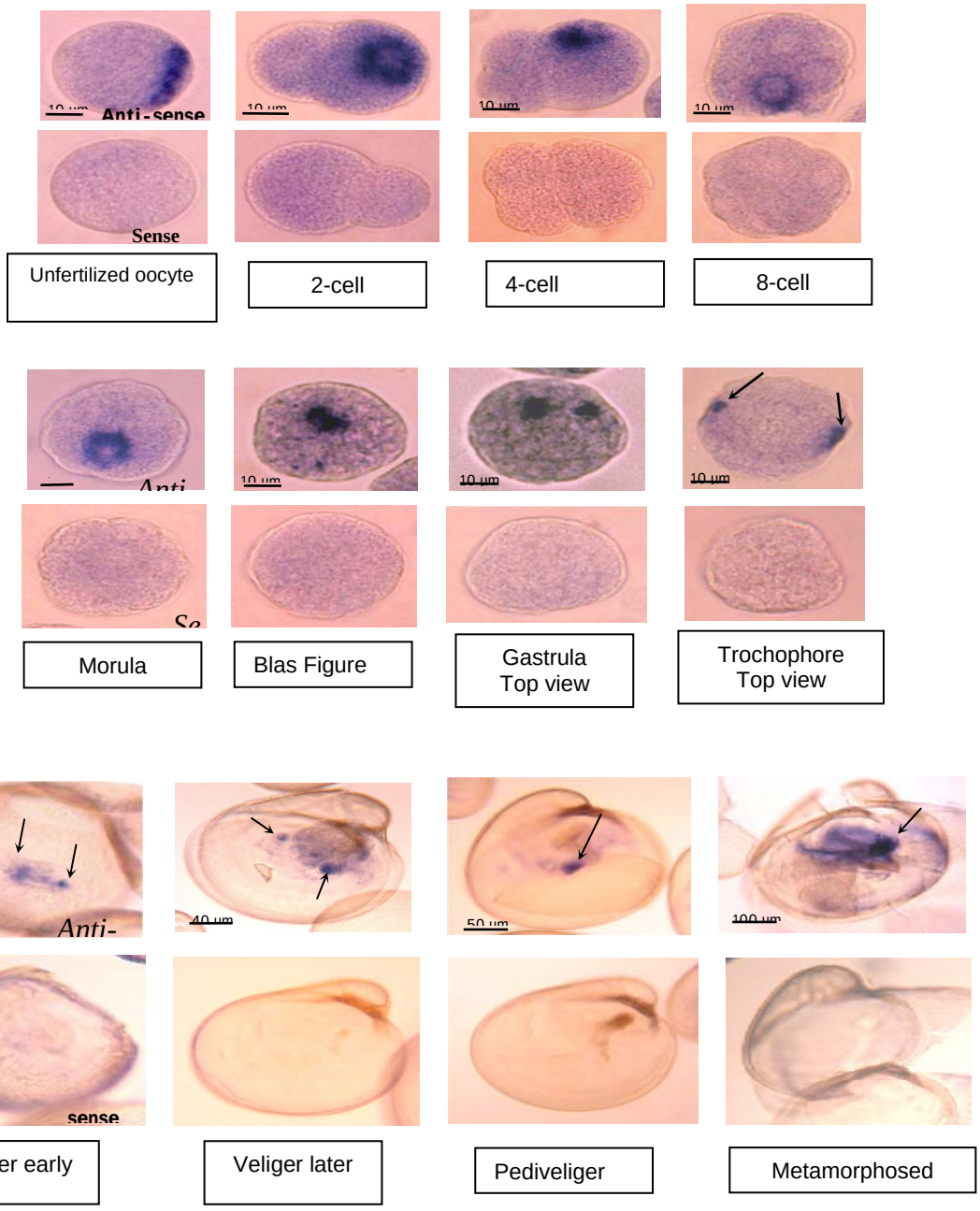


The asymmetrically localized cytoplasm could be a germ plasm

The localization of *Oyvlg* transcripts during embryogenesis was analysed by whole-mount ISH. In unfertilized oocytes, strong hybridization signal was localized in cytoplasmic granules at the vegetal pole of oocytes (Figure 2). The rest of each oocyte was not stained, nor was any sample hybridized to the sense probe (Figure 2). In early 2-cell and 4-cell-developmental stages a strong signal localized asymmetrically in the perinuclear cytoplasm of a single blastomere; no signal was detected in other parts of the embryos. In several organisms, including *Drosophila*, *Caeonorhabditis* and *Hydra*, v*asa-related* gene products were demonstrated to segregate in the germ plasm (5, 8, 9). The germ plasm is a specialized cytoplasmic region of oocytes and early embryos, containing dense cytoplasmic granules, rich in maternal mRNAs and proteins (4, 28). The specification of germline cells during embryogenesis was demonstrated to depend on the segregation of these maternal RNAs and

proteins in the germ plasm (2). The existence of germ plasm was postulated in the molluscs *Crepidula* and *Shpaerium* (21) based on morphological criteria, but it has never been mentioned in oyster. In the present study, the segregation of cytoplasmic granules containing *Oyvlg* transcripts successively in the vegetal pole of oocytes and in a single blastomere in early embryos indicate that the cytoplasmic region containing *Oyvlg* mRNAs could be the germ plasm.

Figure 2: Localization of *Oyvlg* transcripts in unfertilized oocytes and in various developmental stages of *Crassostrea gigas*. Whole-mount *in situ* hybridization was performed with *Oyvlg* sense and anti-sense riboprobes. The lower panels of each line represent negative controls hybridized with sense probes. *Oyvlg* transcripts are detected in unfertilized oocytes and in a unique blastomere of embryos from 2-cell stage to blastula. *Oyvlg* mRNAs distribute in two clumps from the gastrula stage and remain in this distribution until the metamorphosis. Scale bar 10 µm. Magnification X 500 for stages from unfertilized oocytes to gastrula and X 200 for later larvae stages. Arrows indicated hybridization signal.



Oyvlg mRNA: a determinant of oyster germline cell fate

The localization of *vasa* gene products in germ plasm appeared to occur either at a protein level or at a transcript level, depending on the organism. In *Drosophila*, *Caenorhabditis* and medaka, VASA protein is a determinant of the germline and is localized in the germ plasm, while the transcripts show no specific localization in early embryos (6, 8, 29). On the other hand, in zebrafish and *Ciona*, maternal *vasa* transcripts are germline determinants and localize in the cytoplasmic region where the PGCs will form at early developmental stages; in these species, VASA protein show no specific localization (13, 30). In molluscs, morphogenetic determinants of somatic lineage cells were found in the vegetal pole of uncleaved eggs and in restricted region of embryos (31). In *C. gigas*, we observed that *Oyvlg* transcripts were inherited by a single blastomere as early as the 2-cell embryo stage and segregated into a unique blastomere along all cleavage stages, while no ISH signal was detected in other cells (Figure 2). Based on these results, on the maternal transmission of *Oyvlg* transcripts, and on the specific expression of *Oyvlg* in germline cells in adult oysters (23), we postulated that *Oyvlg* mRNAs might be maternal determinants of germline cells in oysters as observed for zebrafish and *Ciona* (13, 30).

To definitively prove that *Oyvlg* mRNAs are germline determinants in oyster, we will need to further study the effect of inhibition of *Oyvlg* mRNA synthesis and the localization of OYVLG protein during early development.

Primordial germ cells in oyster embryos

In most organisms, PGCs form around gastrula stage in the region where the germ plasm is localized, and incorporate this germ plasm (1). Then, the small number of founder PGCs proliferate and migrate to the somatic part of the future gonad. In gastrula and trochophore oyster larvae, we observed two hybridization signals on the right and left of the midline of the

larvae (Figure 2, top view). From early veliger (7 dpf) to metamorphosed larvae (27 dpf), *Oyvlg* transcripts clearly localized in two stained clusters located in both sides of oyster larval body (Figure 2). This dual localization of *Oyvlg* transcripts in oyster larvae corresponded to the previous description of "germinal primordium" made by Lubet *et al*. (22) in mussel post-larvae. "Germinal primordium" were described as two cellular clusters, symmetrical about the midline of the mussel larval body, located between the kidney and the posterior part of the digestive gland. Likewise, our results suggest that these symmetrical clumps, stained by *Oyvlg* probe, are oyster primordial germ cells. These PGCs, the number of which was undetermined, appeared to form during gastrulation and were detected in both sides of veliger larvae body.

By whole-mount ISH, *Oyvlg* transcripts were detected throughout oyster development including in later stages, while by real-time PCR, *Oyvlg* expression steadily and significantly decreased through development, reaching a very low level in gastrula (0.8 ± 0.4) and trochophore stages (0.7 ± 0.3) ($P < 0.01$, Figure 1) and finally becoming essentially undetectable from D larval stage to metamorphosis (mean value < 0.1). This might be due to the very low number of PGCs compared to the total number of cells in oyster larvae, as observed in many organisms like *Caenorhabditis* and *Drosophila*, in which one and approximately 15 PGCs are formed during embryogenesis, respectively.

Primordial germ cells of oyster would arise from 4d mesentoblast

Development of molluscs, like other protostomians such as annelids, follows a spiral cleavage pattern (19). The egg is divided into blastomeres by extremely regular and invariant cleavages giving a precise cytoplasmic segregation (20, 21). On this basis, cell-lineage maps have been constructed, indicating the origin of most organs in some mollusc species, such as *Patella vulgata* (32). However, the origin of the germline remains unclear in most molluscs.

In *Sphaerium* and *Patella*, germ bands were assumed to arise from 4d micromere called mesentoblast, formed during the sixth cleavage (33, 34). The mesentoblast gives rise to endomesoderm that is thought to differentiate into heart, kidney, and gonad in adults. Based on these assessments and on the localization of *Oyvlg* transcripts during oyster development, we postulate on the developmental pattern of *C. gigas* germline from fertilization to metamorphosis using *Oyvlg* as a marker of germline (Figure 3). During the second cleavage, the *Oyvlg* transcripts appeared localized to the D quadrant, defined as the largest one (31). According to the disposition of blastomeres (32), we suggest that from third to sixth cleavages, the putative germ plasm we observed segregated successively in 1D, 2D and 3D macromeres and in the 4d micromere, respectively (Figure 3). In the gastrula, *Oyvlg* mRNAs were distributed in two clumps symmetric relative to the midline of larvae. These clumps could correspond to the Mr and Ml cells arising from the division of 4d mesentoblast, described in *Patella* and demonstrated to be the paired stem cells of mesodermal germ bands (32). The sinking of the Mr and Ml cells into blastocoel could explain the weak intensity of the hybridization signal observed in trochophore oyster larvae (Figure 3). These cells would then proliferate to give putative PGCs and migrate by undetermined ways to the anterior and the posterior sides of the body of later stage larvae and first post-larvae stage (Figure 3). In juvenile oysters, PGCs would differentiate into two groups of germinal stem cells (GSCs) that would correspond to the two mesodermal germinal buds situated in the pericardic region previously described in molluscs (35). When juvenile oyster reach sexual maturity, GSCs would proliferate intensively to form the gonadic tubules that develop from the pericardic region toward the anterior part of the oyster, symmetrically compared to the midline of the body (22). Gonadic tubules branch out in complex networks and invade the conjunctive tissue around the digestive gland to form the gonad (35). After spawning, gonad regresses and only small clusters of GSCs persist in few remaining tubules dispatched in conjunctive tissue

during the resting period (23). These GSCs will proliferate again at the beginning of the following reproductive cycle from remaining tubules to form the new gonad (23).

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Figure 3: Schematic summary of *Crassostrea gigas* germline development during embryogenesis, from unfertilized oocytes to metamorphosed larvae. Orange zones represent regions of cytoplasm containing germline determinants from oocyte to blastula stages and PGCs from gastrula stage. Arrowheads represent the direction of cell migation.

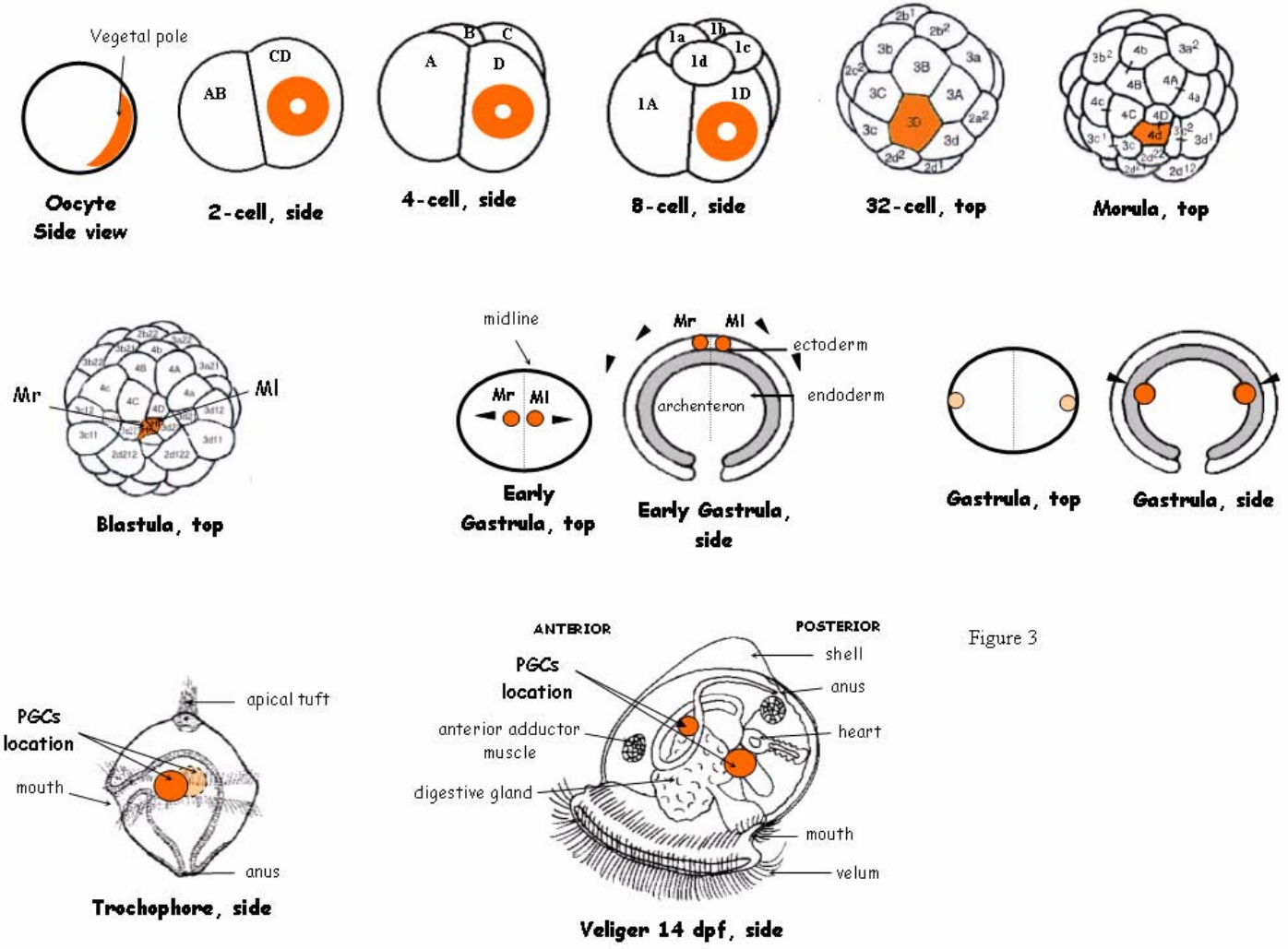

Figure 3

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