Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage

Celina E. Juliano, Ekaterina Voronin, Christie Stack, Maryanna Aldrich, Andrew R. Cameron, Gary M. Wessel

Abstract

Two distinct modes of germ line determination are used throughout the animal kingdom: conditional—an inductive mechanism, and autonomous—an inheritance of maternal factors in early development. This study identifies homologs of germ line determinants in the sea urchin Strongylocentrotus purpuratus to examine its mechanism of germ line determination. A list of conserved germ-line associated genes from diverse organisms was assembled to search the S. purpuratus genome for homologs, and the expression patterns of these genes were examined during embryogenesis by whole mount in situ RNA hybridization and QPCR. Of the 14 genes tested, all transcripts accumulate uniformly during oogenesis and Sp-pumilio, Sp-tudor, Sp-MSY, and Sp-CPEB1 transcripts are also uniformly distributed during embryonic development. Sp-nanos2, Sp-seawi, and Sp-ovo transcripts, however, are enriched in the vegetal plate of the mesenchyme blastula stage and Sp-vasa, Sp-nanos2, Sp-seawi, and Sp-SoxE transcripts are localized in small micromere descendents at the tip of the archenteron during gastrulation and are then enriched in the left coelomic pouch of larvae. The results of this screen suggest that sea urchins conditionally specify their germ line, and support the hypothesis that this mechanism is the basal mode of germ line determination amongst deuterostomes. Furthermore, accumulation of germ line determinants selectively in small micromere descendents supports the hypothesis that these cells contribute to the germ line.

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Keywords: Sea urchin; Germ line; Small micromeres; Deuterostome; Nanos; Vasa; DMRT; Mago nashi; Pumilio; Piwi; SoxE; Tudor; Boule; Germ cell-less; MSY; CPEB; Ovo; MORC

Introduction

The propagation of a species is dependent upon the segregation of the germ line from the surrounding soma during embryonic development and members of the animal kingdom use one of two distinct mechanisms to accomplish this task. In one mechanism, germ cells are specified autonomously as a result of maternally inherited factors. Animals such as D. melanogaster (Ephrussi and Lehmann, 1992; Illmensee and Mahowald, 1974; Illmensee et al., 1976) and C. elegans (Deppe et al., 1978; Kawasaki et al., 1998; Strome and Wood, 1982) exhibit this preformation mechanism by accumulating germ line determinants into a specific region of the oocyte during oogenesis. Any blastomere that inherits this so-called germ plasm is then specified to become a germ cell. In contrast, the germ line of mice is conditionally specified and depends on inductive signals amongst embryonic cells (Lawson and Hage, 1994; Tam and Zhou, 1996). Despite the sharp contrasts between autonomous and conditional mechanisms, similar molecular determinants, such as vasa and nanos, are required for germ line development regardless of how specification occurs.

A careful analysis of all organisms in which germ line determination has been examined revealed that conditional germ line specification is far more common in the animal kingdom and is likely the basal mechanism (Extavour and Akam, 2003). In support of this hypothesis, Xenopus laevis (Heasman et al., 1984; Ikenishi et al., 1986; Whittington and Dixon, 1975; Züst and Dixon, 1975) and Danio rerio (Braat et al., 1999; Yoon et al., 1997), two deuterostomes that have highly derived gastrulation movements (assessed by the origin
of the notochord), use autonomous mechanisms whereas animals that have retained basal characteristics of development, e.g. mouse and axolotl, use conditional mechanisms (Johnson et al., 2003a,b). Due to its position as a basal deuterostome, determining the mode of germ line specification used by the sea urchin, *Strongylocentrotus purpuratus*, would provide key information about the evolution of germ line determination.

The origin of the *S. purpuratus* germ line during embryogenesis has not been determined, although the small micromeres are candidate primordial germ cells (see below; Pehrson and Cohen, 1986). During early development in *S. purpuratus*, an unequal fourth cleavage division gives rise to a 16-cell embryo with 4 micromeres. A subsequent unequal division of the micromeres results in the formation of 3 small micromeres, which divide only once more until larval development. During gastrulation, these 8 cells reside at the tip of the archenteron and are then partitioned into both the right and left coelomic pouches. The left coelomic pouch of the larval pluteus is thought to give rise to ventral regions of the adult, including the somatic gonadal tissues, and the primordial germ cells (Houk and Hinegardner, 1980; MacBride, 1903; Ransick et al., 1996).

The slow cell cycle of the small micromere derivatives is consistent with a primordial germ cell identity, whereas other embryonic cells are highly proliferative through gastrulation (Tanaka and Dan, 1990). In addition, the small micromeres are the only cleavage-stage blastomeres that give rise solely to adult tissues (Pehrson and Cohen, 1986). To test the hypothesis that the small micromeres are the primordial germ cell lineage of the sea urchin, Ransick and coworkers (1996) examined the effect of micromere removal on adult fertility. Six of the seven adult sea urchins successfully raised from microsurgery experiments produced viable gametes supporting their conclusion that localized, obligatory germ cell determinants such as exist in *D. melanogaster* and *C. elegans*, are not present in the micromeres of *S. purpuratus*. Instead, Ransick and colleagues (1996) concluded that the germ line of this animal must be established following embryogenesis, or at least after the 16-cell stage. However, the origin of the sea urchin germ line remains undetermined as does the fate of the small micromeres.

The goal of this study is to address the origin of primordial germ cells in the sea urchin and the mechanism of their specification by examining the expression patterns of several conserved genes that have roles in germ line formation and maintenance in a variety of other animals (Table 1). Early, localized expression of germ line determinants would provide support for an autonomous specification mechanism whereas

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank accession</th>
<th>Domains and function</th>
<th>Orthologs (BLAST scorea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp-boule</td>
<td>XP786845</td>
<td>RNA recognition motif (RRM) and DAZ repeats, translational regulator involved in germ cell development and meiosis</td>
<td><em>S. oedipus</em> (1e–29)</td>
</tr>
<tr>
<td>Sp-CPEB1</td>
<td>XP786947</td>
<td>2 RRM domains, translational regulator during oogenesis (Chang et al., 1999; Gebauer and Richter, 1996; Hake and Richter, 1994)</td>
<td><em>H. sapiens</em> (4e–29)</td>
</tr>
<tr>
<td>Sp-germ cell-less</td>
<td>XP793954</td>
<td>BTB (Broad-Complex, Tramtrack and Bric a brac) protein interaction domain, conserved nuclear pore-associated protein required for germ cell formation in <em>Drosophila</em> (Leatherman et al., 2000)</td>
<td><em>M. musculus</em> (5e–113)</td>
</tr>
<tr>
<td>Sp-DMRT</td>
<td>XP790843</td>
<td>Doublesex DNA-binding motif (DM), transcription factor involved in sex determination</td>
<td><em>D. rerio</em> (1e–112)</td>
</tr>
<tr>
<td>Sp-mago nashi</td>
<td>XP791004</td>
<td>No defined domains, required for localization of oskar mRNA in <em>Drosophila</em> germ plasm assembly (Newmark and Boswell, 1994)</td>
<td><em>P. troglodytes</em> (7e–33)</td>
</tr>
<tr>
<td>Sp-MORC</td>
<td>XP790047</td>
<td>2 coiled-coil domains, nuclear protein required for spermatogenesis in mouse (Inoue et al., 1999; Watson et al., 1998)</td>
<td><em>H. sapiens</em> (7e–32)</td>
</tr>
<tr>
<td>Sp-MSY</td>
<td>XP785816</td>
<td>CSP or Y-box binding domain, translational repressor during mouse spermatogenesis (Giorgini et al., 2002)</td>
<td><em>R. norvegicus</em> (1e–126)</td>
</tr>
<tr>
<td>Sp-nanos2b</td>
<td>ABB89047</td>
<td>CCHC Zinc Fingers (2), translational regulator (with pumilio), conserved role in germ cell development</td>
<td><em>H. sapiens</em> (1e–126)</td>
</tr>
<tr>
<td>Sp-ovo</td>
<td>XP788176</td>
<td>C2H2 Zinc Finger (4) transcription factor, plays a role in <em>Drosophila</em> oogenesis and mouse spermatogenesis (Dai et al., 1998; Mevel-Ninio et al., 1991)</td>
<td><em>N. vectensis</em> (7e–21)</td>
</tr>
<tr>
<td>Sp-pumilio</td>
<td>XP794621</td>
<td>Pumilio-like repeats (3), translational regulator (with nanos), conserved role in germ cell development</td>
<td><em>H. sapiens</em> (6e–52)</td>
</tr>
<tr>
<td>Sp-seawi</td>
<td>AAG42534</td>
<td>PAZ (piwi, argonaute, zwille) domain, required for maintenance of germ line stem cells</td>
<td><em>D. melanogaster</em> (7e–52)</td>
</tr>
<tr>
<td>Sp-SoxE</td>
<td>XP786809</td>
<td>HMG box transcription factor involved in sex determination</td>
<td><em>N. vectensis</em> (0)</td>
</tr>
<tr>
<td>Sp-tudor</td>
<td>XP780689</td>
<td>Tudor domains (5), required for <em>Drosophila</em> pole plasm assembly and is expressed in mouse spermatocytes (Chuma et al., 2003; Thomson and Lasko, 2004)</td>
<td><em>C. elegans</em> (0)</td>
</tr>
<tr>
<td>Sp-vasa</td>
<td>XP781494</td>
<td>CCHC Zinc Fingers (3–4), DEAD box helicases, conserved marker of germ cells, function unknown</td>
<td><em>P. troglodytes</em> (0)</td>
</tr>
</tbody>
</table>

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a The first BLAST score listed for each gene is the best hit and the second BLAST score listed is the best hit amongst organisms whose germ lines have been intensively studied.

b An Sp-nanos1 homolog is present in the genome, but its expression was not detected in the ovary nor during embryogenesis by in situ RNA hybridization (data not shown).
late and/or diffuse expression would suggest a conditional mechanism.

**Experimental methods and procedures**

**Animals**

*Strongylocentrotus purpuratus* were obtained from Charles Hollahan (tidalflux@yahoo.com; Santa Barbara, CA) and housed in aquaria cooled to 16°C in artificial seawater (ASW; Coral Life Scientific Grade Marine Salt; Energy Savers Unlimited, Inc, Carson, CA). Females were shed by KCl (0.5 M) injection and eggs were collected in ASW; ovaries were removed and minced in ASW. To obtain embryos, fertilized eggs were cultured at 16°C in ASW supplemented with 1 mM 3-amino-triazol to remove fertilization envelopes, and collected at necessary developmental stages. Ovaries, eggs and embryos were fixed in paraformaldehyde and stored at −20°C (Arenas-Mena et al., 2000).

**Sea urchin germ line determinant homologs**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers used for QPCR</th>
<th>Primers used to make RNA in situ probe</th>
<th>Length of RNA in situ probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp-boule (08194)</td>
<td>F:aaacaccttggaggttgcc</td>
<td>F:gaacactacattcaaaacagga</td>
<td>295 nucleotides, includes RRM domain</td>
</tr>
<tr>
<td></td>
<td>R:ctgcgctcttgcctgta</td>
<td>R:ttggatggctggtgcttga</td>
<td></td>
</tr>
<tr>
<td>Sp-CPEB1 (15450)</td>
<td>F:aagttcgagcccaatcagaa</td>
<td>F:aaagtggccacattattag</td>
<td>1245 nucleotides, includes first RRM domain</td>
</tr>
<tr>
<td></td>
<td>R:ctttcctgctcaacatgcta</td>
<td>R:agtccttccattgccatc</td>
<td></td>
</tr>
<tr>
<td>Sp-germ cell-less (06481)</td>
<td>F:tctccaccaattttgctgctgct</td>
<td>F:agtccttccattgccatc</td>
<td>1181 nucleotides, includes BTB domain</td>
</tr>
<tr>
<td></td>
<td>R:ttgcgactttacacatgactc</td>
<td>R:aatctcctgctcttgag</td>
<td></td>
</tr>
<tr>
<td>Sp-DMRT (28120)</td>
<td>N/A</td>
<td>F:gttcctctgctcttcctc</td>
<td>314 nucleotides, includes DM domain</td>
</tr>
<tr>
<td>Sp-mago nashi (26559)</td>
<td>F:aaagttcgacagttccagctgagt</td>
<td>F:gttcctctgctcttcctc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R:ctctcctgctcaacatgcta</td>
<td>R:agtccttccattgccatc</td>
<td></td>
</tr>
<tr>
<td>Sp-MSY (27562)</td>
<td>F:agtccacttcaaaacagcag</td>
<td>R:aaagttcgagcccaatcagaa</td>
<td>452 nucleotides, includes N-terminus</td>
</tr>
<tr>
<td></td>
<td>R:ctttcctgctcaacatgcta</td>
<td>R:agtccttccattgccatc</td>
<td></td>
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<tr>
<td>Sp-nanos2 (03591)</td>
<td>F:gcagactcctacacactcctct</td>
<td>F:aaagttcgagcccaatcagaa</td>
<td>235 nucleotides, includes most of Y-box binding domain</td>
</tr>
<tr>
<td></td>
<td>R:ccgcaatagctgctgtgta</td>
<td>R:aaagttcgagcccaatcagaa</td>
<td></td>
</tr>
<tr>
<td>Sp-ovo (12448)</td>
<td>N/A</td>
<td>F:tgagctctgtgctgtgctgct</td>
<td>748 nucleotides, includes zinc fingers</td>
</tr>
<tr>
<td>Sp-pumilio (06847)</td>
<td>F:gttgcgctctgctgctgctctt</td>
<td>F:tgagctctgtgctgtgctgct</td>
<td>668 nucleotides, includes first 2 zinc fingers</td>
</tr>
<tr>
<td></td>
<td>R:atcttcccaggccagaggctga</td>
<td>R:gttgcgctctgctgctgctgct</td>
<td></td>
</tr>
<tr>
<td>Sp-soawi (16689)</td>
<td>F:ttgatgtgtgtgtgtgtgtgtgct</td>
<td>F:gttgcgctctgctgctgctgct</td>
<td>242 nucleotides, includes first 2 pumilio-like repeats</td>
</tr>
<tr>
<td></td>
<td>R:ttgtgtgtgtgtgtgtgtgct</td>
<td>R:gttgcgctctgctgctgctgct</td>
<td></td>
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<tr>
<td>Sp-SoXE (16881)</td>
<td>N/A</td>
<td>F:aaagttcgagcccaatcagaa</td>
<td>509 nucleotides, includes N-terminus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:aaagttcgagcccaatcagaa</td>
<td></td>
</tr>
<tr>
<td>Sp-tudor (08290)</td>
<td>F:ctcagactcctacacactcctct</td>
<td>F:aaagttcgagcccaatcagaa</td>
<td>301 nucleotides, includes 40% of HMG domain</td>
</tr>
<tr>
<td></td>
<td>R:aaacactacacactcctct</td>
<td>R:aaagttcgagcccaatcagaa</td>
<td></td>
</tr>
<tr>
<td>Sp-vasa (08908)</td>
<td>F:tctcagactcctacacactcctct</td>
<td>F:aaagttcgagcccaatcagaa</td>
<td>440 nucleotides, includes first tudor domain</td>
</tr>
<tr>
<td></td>
<td>R:aaacactacacactcctct</td>
<td>R:aaagttcgagcccaatcagaa</td>
<td></td>
</tr>
</tbody>
</table>

Quantitative PCR (QPCR)

RNA was extracted as previously described (Bruskin et al., 1981) from fresh *S. purpuratus* eggs, ovaries and embryos and the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster City) was used to prepare cDNA. QPCR was performed on the 7300 Real-Time PCR system (Applied Biosystems, Foster City) with the SYBER Green PCR Master Mix Kit (Applied Biosystems, Foster City). Primer sets were designed using primer3 (http://frodos.wi.mit.edu/cgi-bin/primer3/primer3...) to amplify products between 100 and 150 base pairs (Table 2). All QPCR experiments were run in triplicate and repeated at least once. Data for each gene was normalized against ubiquitin RNA levels and represented as a fold change relative to the amount of gene-specific RNA present in the ovary. GAPDH mRNA levels varied 2-fold through the developmental series and therefore did not make a good standard for these experiments (Fig. 1L).

**In situ RNA hybridization**

For Sp-mago nashi, Sp-nanos2, Sp-pumilio, Sp-tudor, and Sp-vasa, gene fragments were amplified by PCR from a genomic DNA template. These amplified gene fragments were then hybridized to an *S. purpuratus* ovary macroarray (http://supg.caltech.edu/resources/) and the resulting pSPORT clones provided a template to amplify fragments by PCR for cloning into pGEMT-EZ (Promega, Madison); primers (Table 2) were annealed at 55°C and extended at 72°C.

A DIG RNA Labeling Kit (Sp6/T7) (Roche, Indianapolis) was used to construct antisense DIG-labeled probes to each target gene from template cloned into pGEMT-EZ (described above). Whole-mount in situ RNA hybridization (WMISH) experiments were performed as previously described (Arenas-Mena et al., 2000). A non-specific DIG-labeled RNA probe complimentary to pSPT 18

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Ubq F: CACAGGCAAGACCGATCATCACAC; Ubq R: GAGGAGATGGCGGCACTCCTC.
GAPDH F: AGGCTTCTTCTAGCGGACAG; GAPDH R: TGCTAAGGCTTGGAAAGG.

* Primers are listed in the 5’ to 3’ direction.
was used as a negative control and images were taken at 40× magnification on a Zeiss Axiovert 200 M microscope.

Results and discussion

Identification of germ line homologs in *S. purpuratus*

A list of genes known to be essential for germ line determination and maintenance in several organisms was compiled and homologous sequences were found in the *S. purpuratus* genome using BLAST analysis (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/). This computational search resulted in the identification of the homologs listed in Table 1. For all of these genes, the relative levels of transcript present through development were determined by QPCR Table 2; (Fig. 1, see also Howard-Ashby et al., 2006; Materna et al., 2006) and the pattern of expression during development was determined by whole mount *in situ* RNA hybridization (Figs. 2–5).

Fig. 1. QPCR was used to measure the relative RNA levels of (A) Sp-boule, (B) Sp-CPEB1, (C) Sp-germ cell-less, (D) Sp-mago nashi, (E) Sp-MORC, (F) Sp-MSY, (G) Sp-nanos2, (H) Sp-pumilio, (I) Sp-seawi, (J) Sp-tudor, (K) Sp-vasa, and (L) Sp-GAPDH present in several developmental stages. All values were normalized against ubiquitin RNA and are represented as a fold change relative to the amount of RNA present in the ovary. *Indicates values that are too low to be determined.
**Transcript accumulation during oogenesis**

As shown by *in situ* RNA hybridization of whole-mount ovaries, developing *S. purpuratus* oocytes demonstrate uniform transcript accumulation of the 14 genes tested (Sp-boule, Sp-CPEB1, Sp-germ cell-less, Sp-DMRT, Sp-mago nashi, Sp-MORC, Sp-MSY, Sp-nanos2, Sp-ovo, Sp-pumilio, Sp-seawi, Sp-SoxE, Sp-tudor, and Sp-vasa) and no evidence of subcellular localization.

**Fig. 2.** Whole mount *in situ* RNA hybridizations of *S. purpuratus* ovaries and mature eggs (insets) were performed for germ-line gene homologs (Table 1). All transcripts accumulate uniformly in the oocytes throughout oogenesis and no subcellular localization is detected in the mature egg. In the Sp-vasa panel a dashed line circles an oocyte and the arrowhead indicates the germinal vesicle. Scale bars, 20 μM.
in the mature egg is seen (Fig. 2). The lack of localized cytoplasmic determinants during oogenesis is not compatible with a mechanism of autonomous specification in *S. purpuratus*. This is in strong contrast to the maternal deposition and localization of nanos mRNA in *D. melanogaster*, *C. elegans*, and *X. laevis* and *vasa* mRNA in *D. rerio* for subsequent incorporation into primordial germ cells (Kobayashi et al., 1996; MacArthur et al., 1999; Subramaniam and Seydoux, 1999; Yoon et al., 1997).

**Genes with uniform transcript accumulation during embryogenesis**

Transcript accumulation is not localized during embryogenesis for several of the genes tested. *Sp-boule*, *Sp-germ cell-less*, *Sp-mago nashi*, and *Sp-MORC* cannot be detected by *in situ* hybridization during development. However, this is likely due to the detection limit of this technique because *Sp-MORC* transcripts are detected using QPCR through early blastula and *Sp-boule*, *Sp-germ cell-less* and *Sp-mago nashi* transcripts are detected through gastrula (Figs. 1A, C–E). In contrast, *Sp-DMRT* transcript levels are high in blastula stage embryos and beyond, but transcripts do not accumulate in select cells of the larvae (data not shown). This *in situ* RNA hybridization expression pattern is consistent with the QPCR data of Howard-Ashby et al. (2006), which indicates an increase in *Sp-DMRT* RNA levels in mesenchyme blastulae. By *in situ* RNA hybridization, high levels of *Sp-pumilio*, *Sp-tudor*, *Sp-MSY*, and *Sp-CPEB1* transcripts accumulate uniformly early in embryogenesis (Fig. 3). *Sp-CPEB1* transcripts are undetectable in the blastula, whereas *Sp-pumilio*, *Sp-tudor*, and *Sp-MSY* transcripts are still enriched at this stage and beyond (Fig. 3). As shown by QPCR, *Sp-CPEB1* RNA levels sharply decline at early blastula (Fig. 1B), which is consistent with the *in situ* RNA hybridization expression pattern. *Sp-tudor*, *Sp-pumilio*, and *Sp-MSY* levels appear relatively consistent during embryogenesis by *in situ* RNA hybridization, but QPCR results indicate slight fluctuations in overall transcript abundance. Specifically, *Sp-tudor* RNA levels steadily increase through the

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Fig. 3. *Sp-pumilio*, *Sp-tudor*, *Sp-CPEB1*, and *Sp-MSY* whole mount *in situ* RNA hybridizations of *S. purpuratus* eggs and embryos at the indicated stages. All transcripts accumulate at high levels uniformly in cleavage stage embryos. *Sp-CPEB1* transcripts are undetectable in blastula stage embryos and beyond, whereas *Sp-pumilio*, *Sp-tudor*, and *Sp-MSY* remain uniformly distributed throughout embryogenesis. Scale bar, 20 μM.
4-cell stage and then decrease in the early blastula (Fig. 1J); Sp-pumilio RNA levels remain consistent early in development, peak at mesenchyme blastula and then decrease in the gastrula and pluteus (Fig. 1H); Sp-MSY RNA levels decrease in the mature egg as compared to the ovary, increase slightly in the early blastula and decrease in the gastrula (Fig. 1F). If any of these genes are involved in germ line determination in the sea urchin, their uniform expression early in development suggests a conditional mechanism of specification in the germ line. However, this set of genes does not give any insight into the origins of the sea urchin germ line. It is also important to note that uniform transcript accumulation may not accurately reflect the corresponding protein expression patterns which could be selective for certain cells or regions within cells.

**Genes with early, uniform transcript accumulation and late restriction during embryogenesis**

As shown by in situ RNA hybridization, Sp-ovo, Sp-vasa, and Sp-seawi transcripts accumulate uniformly through blastula formation and are then locally enriched at the vegetal plate of the mesenchyme blastula (Fig. 4). In the gastrula Sp-ovo transcripts are no longer detected whereas Sp-vasa and Sp-seawi transcripts remain, but progressively become restricted to the small micromeres at the tip of the archenteron (Fig. 4). In prism and early plutei, Sp-vasa and Sp-seawi transcripts are seen in both coelomic pouches, followed by restriction to the left pouch (Figs. 4B, C). As measured by QPCR, Sp-ovo RNA levels remain constant through mesenchyme blastula, and then decrease thereafter, which is consistent with the in situ RNA hybridization results presented here (Materna et al., 2006). Although Sp-vasa and Sp-seawi expression patterns are identical by in situ RNA hybridization, QPCR results indicate that these genes have unique patterns of RNA accumulation. Sp-vasa RNA levels decrease sharply from ovary to egg and then slowly increase throughout embryogenesis, whereas Sp-seawi RNA levels remain constant early in development, peak at mesenchyme blastula and then decrease sharply (Figs. 1I, K).

Sp-seawi homologs are required for germ line stem cell self-renewal in both Drosophila and C. elegans (Cox et al., 1998). Additionally, the mouse and zebrafish homologs, miwi and ziwi respectively, are expressed in PGCs in the embryonic gonad and may therefore also have a role in the PGC determination and

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**Fig. 4.** (A) Sp-ovo, (B) Sp-seawi, and (C) Sp-vasa whole mount in situ RNA hybridizations of *S. purpuratus* eggs and embryos at the indicated stages. All transcripts accumulate uniformly at high levels through the blastula stage and then become enriched at the vegetal plate of mesenchyme blastula embryos. Sp-ovo transcripts are undetectable in mesenchyme blastula stage embryos and beyond. Sp-seawi and Sp-vasa transcripts are locally enriched in the small micromeres at the tip of the archenteron in the gastrula and remain enriched in these cells as they are subsequently incorporated into both coelomic pouches of the prism. Initially Sp-seawi and Sp-vasa transcripts are enriched in both coelomic pouches of the pluteus, followed by restriction to the left pouch.
differentiation (Kuramochi-Miyagawa et al., 2001; Tan et al., 2002). Vasa is a conserved marker of early PGC development (Castrillon et al., 2000; Fujiwara et al., 1994; Gruidl et al., 1996; Hay et al., 1988; Komiyama et al., 1994; Yoon et al., 1997). The function of vasa is poorly understood in most animals, although it appears to act as a translational regulator during oogenesis in D. melanogaster (Styhler et al., 1998; Tomancak et al., 1998).

Therefore, localization of Sp-seawi and Sp-vasa transcripts in the small micromere lineage provides molecular evidence supporting the hypothesis that these cells contribute to the primordial germ cell population.

In Drosophila and mouse, ovo homologs are required for oogenesis and spermatogenesis respectively, but no role in PGC development is reported (Dai et al., 1998; Mevel-Ninio et al., 1991; Oliver et al., 1987). The presence of Sp-ovo transcripts in oocytes (Fig. 2) suggests that it also plays a role in sea urchin oogenesis. However, the enrichment of Sp-ovo transcripts at the vegetal plate of the mesenchyme blastula, including the small micromeres, suggests a previously undescribed role for this gene in PGC development.

Genes with restricted transcript accumulation late in embryonic development

Sp-nanos2 transcript accumulation first appears in several cells in the vegetal region of the blastula (Fig. 5A). Like Sp-vasa and Sp-seawi, Sp-nanos2 is locally enriched in the small micromere descendents at the tip of the archenteron during gastrulation and in the left coelomic pouch of the larva (Fig. 5A). However, Sp-nanos2 transcripts cannot be detected in larvae older than 4 days (Fig. 5A). The pattern of RNA accumulation determined by QPCR is consistent with the in situ RNA hybridization results; the ovary has the highest level of Sp-nanos2 RNA followed by a sharp decrease in the mature egg and a return to moderate levels in the early blastula which lasts through larval formation (Fig. 1G). A similar pattern of expression was recently reported for Hp-nanos in the sea urchin Hemicentrotus pulcherrimus (Fujii et al., 2006). Nanos homologs are consistently found expressed in the germ line of every animal examined, including hydra, Drosophila, C. elegans, Zebrafish, Xenopus, mice, and humans (Jaruzelska et al., 2003; Kobayashi et al., 1996; Koprunner et al., 2001; Mochizuki et al., 2000; Mosquera et al., 1993; Subramaniam and Seydoux, 1999; Tsuda et al., 2003). Therefore, the restricted accumulation of Sp-nanos2 transcripts in the small micromeres of S. purpuratus supports the hypothesis that these cells contribute to the PGC population.

Sp-SoxE transcripts accumulate at the tip of the gastrula and are then incorporated into the left coelomic pouch (Fig. 5B). These results are consistent with the QPCR data documented by Howard-Ashby et al. (2006) in that RNA levels increase in the gastrula. It is important to note that the localized pattern of transcript accumulation in the pluteus was observed in only 50% of the embryos whereas the remaining 50% had a broader distribution (data not shown). In the sea urchin Paracentrotus lividus, the transcript of Sox9, an Sp-SoxE ortholog (see Sox nomenclature in Howard-Ashby et al., 2006), also accumulates at the tip of the archenteron and in the left coelomic pouch (Duboc et al., 2005). Sp-SoxE is a member of the HMG-box containing transcription factor family and the mouse homologs, Sox9 and Sox8, are expressed in the embryonic male gonad and are required for male sexual differentiation (Chaboissier et al.,...
2004; Kent et al., 1996). Therefore, the dimorphic expression pattern of Sp-SoxE in *S. purpuratus* plutei suggests a conserved sex-specific role. Furthermore, the localization of *Sp-SoxE* transcript in the left coelomic pouch supports the hypothesis that this is the site of germ cell accumulation.

**Conclusions**

Pehrson and Cohen (1986) hypothesized that the small micromeres are the PGCs of the sea urchin. The small micromeres are good PGC candidates due to their slow cell cycle and their incorporation into the left coelomic pouch. However, little was known about the molecular array of the small micromeres. This study is the first comprehensive examination of the expression patterns of potential germ line determinants in the sea urchin. The enriched transcript accumulation of Sp-vasa, Sp-nanos2, Sp-seawi, and Sp-soxE in small micromeres descendents both before and after their incorporation into the left coelomic pouch supports the hypothesis that the small micromeres contribute to the germ line in the adult animal. Lineage tracing of these cells will be essential though to definitively test this premise.

This study does not provide any evidence of localized mRNA cytoplasmic determinants in the egg or early embryo, although the expression patterns of the encoded proteins have not been examined in most cases (see for example Rodríguez et al., 2005). It should be noted that in the sea urchin *Hemicentrotus pulcherrimus*, cytoplasmic mitochondrial rRNA is enriched at the vegetal pole of the unfertilized egg and is then segregated to the small micromeres during early development (Ogawa et al., 1999). Because cytoplasmic mitochondrial rRNA is enriched in both *D. melanogaster* and *X. laevis* germ plasm, this result indicates that some elements of the sea urchin germ line could be localized early (Kashikawa et al., 2001; Kashikawa et al., 1999), but are not essential. However, the evidence presented here, which is based on several molecular markers, suggests that conditional specification is the mode of germ line determination used by *S. purpuratus*, supporting the hypothesis that this is the basal mechanism of germ line determination amongst deuterostomes.

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**References**


