Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 300 (2006) 406–415
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

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

Celina E. Juliano [a], Ekaterina Voronina [a], Christie Stack [a], Maryanna Aldrich [a], Andrew R. Cameron [b], Gary M. Wessel [a,*]

[a] *Department of Molecular and Cell Biology and Biochemistry, Brown University, 69 Brown Street, Providence, RI 02912, USA*
[b] *Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA*

Received for publication 10 May 2006; revised 20 July 2006; accepted 27 July 2006
Available online 4 August 2006

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.

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; Whitington and Dixon, 1975; Zust 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

* Corresponding author.
E-mail address: rhet@brown.edu (G.M. Wessel).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.07.035

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 4 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
S. purpuratus homologs of conserved genes involved in germ cell determination and differentiation

Gene	Genbank accession	Domains and function	Orthologs (BLAST score[a])
Sp-boule	XP786845	RNA recognition motif (RRM) and DAZ repeats, translational regulator involved in germ cell development and meiosis	*S. oedipus* (1e–29) *H. sapiens* (4e–29)
Sp-CPEB1	XP786947	2 RRM domains, translational regulator during oogenesis (Chang et al., 1999; Gebauer and Richter, 1996; Hake and Richter, 1994)	*M. glacialis* (2e–163) *H. sapiens* (1e–128)
Sp-germ cell-less	XP793954 XP784533	BTB (Broad-Complex, Tramtrack and Bric a brac) protein interaction domain, conserved nuclear pore-associated protein required for germ cell formation in *Drosophila* (Leatherman et al., 2000)	*M. musculus* (5e–113) *D. rerio* (1e–112)
Sp-DMRT	XP790843	Doublesex DNA-binding motif (DM), transcription factor involved in sex determination	*P. troglodytes* (7e–33) *H. sapiens* (7e–32)
Sp-mago nashi	XP791004	No defined domains, required for localization of oskar mRNA in *Drosophila* germ plasm assembly (Newmark and Boswell, 1994)	*X. laevis* (6e–72) *H. sapiens* (8e–72)
Sp-MORC	XP790047	2 coiled-coil domains, nuclear protein required for spermatogenesis in mouse (Inoue et al., 1999; Watson et al., 1998)	*R. norvegicus* (1e–126) *M. musculus* (1e–126)
Sp-MSY	XP785816	CSP or Y-box binding domain, translational repressor during mouse spermatogenesis (Giorgini et al., 2002)	*M.musculus* (6e–30) *H. sapiens* (1e–29)
Sp-nanos2[b]	ABB89047	CCHC Zinc Fingers (2), translational regulator (with pumilio), conserved role in germ cell development	*N. vectensis* (7e–21) *H. sapiens* (2e–17)
Sp-ovo	XP788176	C2H2 Zinc Finger (4) transcription factor, plays a role in *Drosophila* oogenesis and mouse spermatogenesis (Dai et al., 1998; Mevel-Ninio et al., 1991)	*H. sapiens* (6e-52) *D. melanogaster* (7e-52)
Sp-pumilio	XP794621	Pumilio-like repeats (3), translational regulator (with nanos), conserved role in germ cell development	*X. laevis* (0) *T.nigroviridis* (0)
Sp-seawi	AAG42534	PAZ (piwi, argonaute, zwille) domain, required for maintenance of germ line stem cells	*H. sapiens* (0) *M. musculus* (0)
Sp-SoxE	XP786809	HMG box transcription factor involved in sex determination	*P. lividus* (0) *X. tropicalis* (3e-45)
Sp-tudor	XP780689	Tudor domains (5), required for *Drosophila* pole plasm assembly and is expressed in mouse spermatocytes (Chuma et al., 2003; Thomson and Lasko, 2004)	*H. sapiens* (4e-45) *M. musculus* (8e-45)
Sp-vasa	XP781494	CCHC Zinc Fingers (3–4), DEAD box helicases, conserved marker of germ cells, function unknown	*C. savignyi* (0) *D. rerio* (5e-152)

[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

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* (Rodriguez et al., 2005), *Sp-SoxE*, *Sp-tudor*, and *Sp-vasa* sequences were identified in the *S. purpuratus* genome (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/) by BLAST analysis with germ-line associated gene sequences from *M. musculus*, *H. sapiens*, *D. melanogaster*, and *C. elegans*.

Quantitative PCR (QPCR)

RNA was extracted as previously described (Bruskin et al., 1981) from fresh *S. purpuratus* ovaries, eggs 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://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) 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://sugp.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.

Sp-boule, *Sp-CPEB1*, *Sp-germ cell-less*, *Sp-DMRT*, *Sp-MORC*, *Sp-MSY*, *Sp-ovo*, *Sp-seawi* and *Sp-SoxE* fragments were amplified by PCR from an *S. purpuratus* ovary cDNA library and cloned into pGEMT-EZ; primers (Table 2) were annealed between 43°C and 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

Table 2
Primers used for QPCR and construction of *in situ* RNA hybridization probes (Methods)

Gene (SPU_#)	Primers used for QPCR[a]	Primers used to make RNA *in situ probe*[a]	Length of RNA *in situ* probe
Sp-boule (08194)	F:accaacccttgagagtgcag R:ctgcgtcgcttgtctgatta	F:gaaccatcattccaaacagga R:ttgtggtacgtgtagctgtac	295 nucleotides, includes RRM domain
Sp-CPEB1 (15450)	F:aaggatggcaaacatccaag R:ctcctctgccactcatgtca	F:aaacggtacagactttatagc R:agtccttcagcattgagcatc	1245 nucleotides, includes first RRM domain
Sp-germ cell-less (06481)	F:tctcccaacctctttgtgct R:ttccgagcatcttggaatct	F:agtccacttccaagtacatct R:aatctcctcatctttgtgtag	1181 nucleotides, includes BTB domain
Sp-DMRT (28120)	N/A	F:cgatctcctcatcatcctcc R:tcttctgctgcttgctgtctc	314 nucleotides, includes DM domain
Sp-mago nashi (26559)	F:aggaggatgatgctttgtgg R:ggcatctctgctgttgttga	F:gctattccttttactccacag R:ccaatgagagagaagacgag	346 nucleotides, includes majority of gene
Sp-MORC (27562)	F:agcctagccccaagaagaag R:ctccttccagctctgtgtcc	F: cttcaagaagagatctga R: gagacttgagcaacaaac	452 nucleotides, includes N-terminus
Sp-MSY (03107)	F:gataccgtcccaaccagaga R:atcctcaccaccttccctct	F:ggaacagtcaagtggttcaac R:cgtacttgctccctactactg	235 nucleotides includes most of Y-box binding domain
Sp-nanos2 (03591)	F:gcaagaacaacggagagagc R:ccgcataatggacaggtgta	F:aaggtgatgaggggaggaag R:cgcaaatcacctgtacaaaaa	748 nucleotides, includes zinc fingers
Sp-ovo (12448)	N/A	F:tgcgacattaaacacaagcaa R:atgtctcttcaagtcaaaggt	668 nucleotides, includes first 2 zinc fingers
Sp-pumilio (06847)	F:gcctgatgaccgatgtcttt R:atctggagggctaggggtaa	F:gtcgcgcttcatacagcag R:cgagagctttctggatcacc	242 nucleotides, includes first 2 pumilio-like repeats
Sp-seawi (16689)	F:gtgatggtgttggtgacagc R:tattgatgcgcttcttgacg	F:caccaagcatggatcgtc R:gaaaagagaatacatggtgtcc	509 nucleotides, includes N-terminus
Sp-SoxE (16881)	N/A	F:agtgactcggacgatgcttcc R:ttgtggagctgtggatactgg	301 nucleotides, includes 40% of HMG domain
Sp-tudor (08290)	F:catccactgccaatgtgttc R:aaaccaatcagccgacattc	F:aggccctttcaagttctggt R:gcaaggtgaccgttaggtgt	440 nucleotides, includes first tudor domain
Sp-vasa (08908)	F:tcaactacgacctcccaagc R:tctcgcaatgttagcatcctt	F:ggacgatcaactagcttcta R:actccttcgtctttcttcat	511 nucleotides, includes C-terminus and 3′UTR

Ubq F: CACAGGCAAGACCATCACAC; Ubq R: GAGAGAGTGCGACCATCCTC.
GAPDH F: AGGCTTCTTCAGACGGACAG; GAPDH R: TGCTAAGGCTGTTGGAAAGG.
[a] 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).

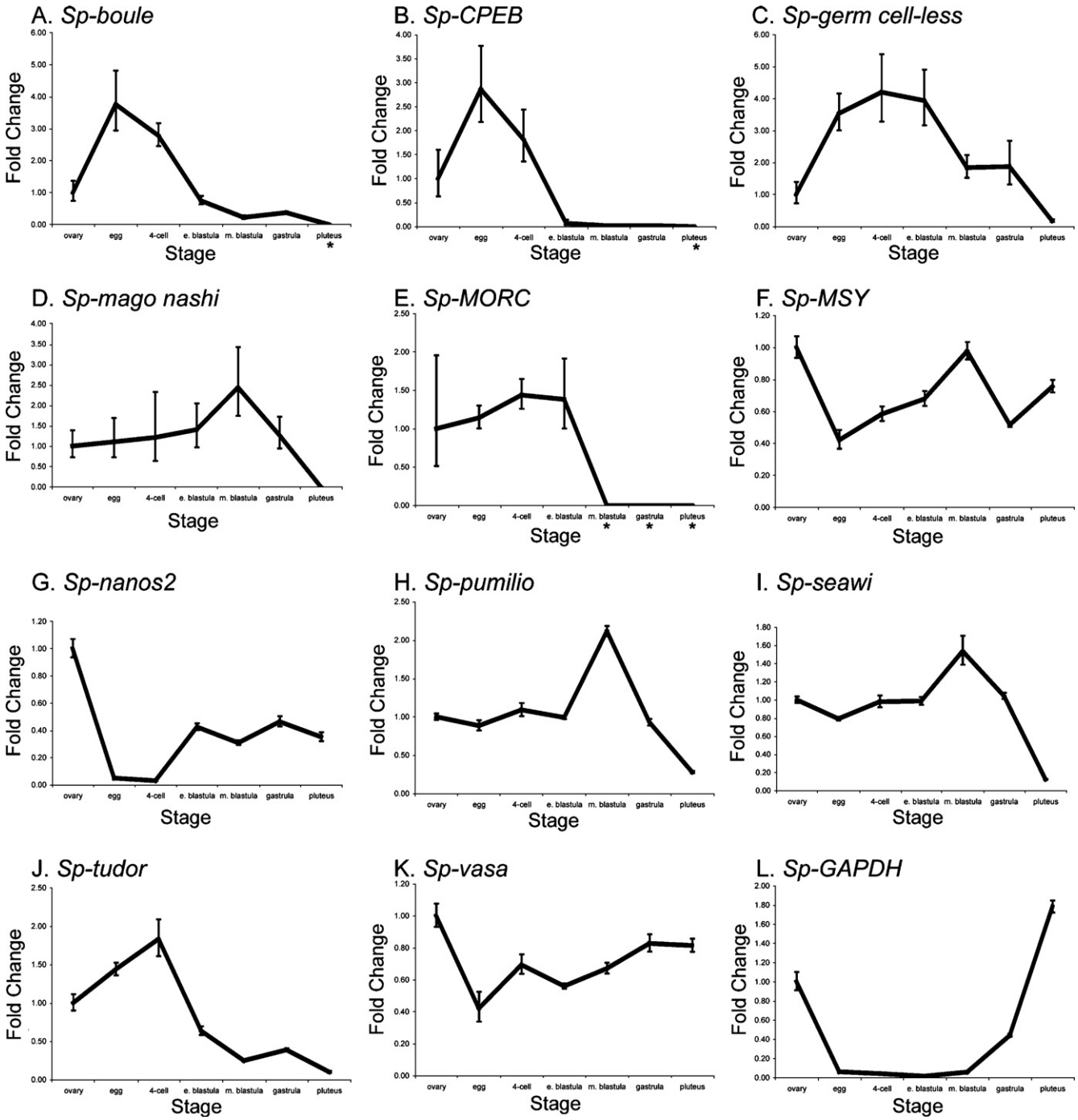

Fig. 1. QPCR was used to measure the relative RNA levels of (A) *Sp-boule*, (B) *Sp-CPEB1*, (C) *Sp-germ cell-less*, (D) *Sp-mago nashi*, (E) *Sp-MORC*, (F) *Sp-MSY*, (G) *Sp-nanos2*, (H) *Sp-pumilio*, (I) *Sp-seawi*, (J) *Sp-tudor*, (K) *Sp-vasa*, and (L) *Sp-GAPDH* present in several developmental 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

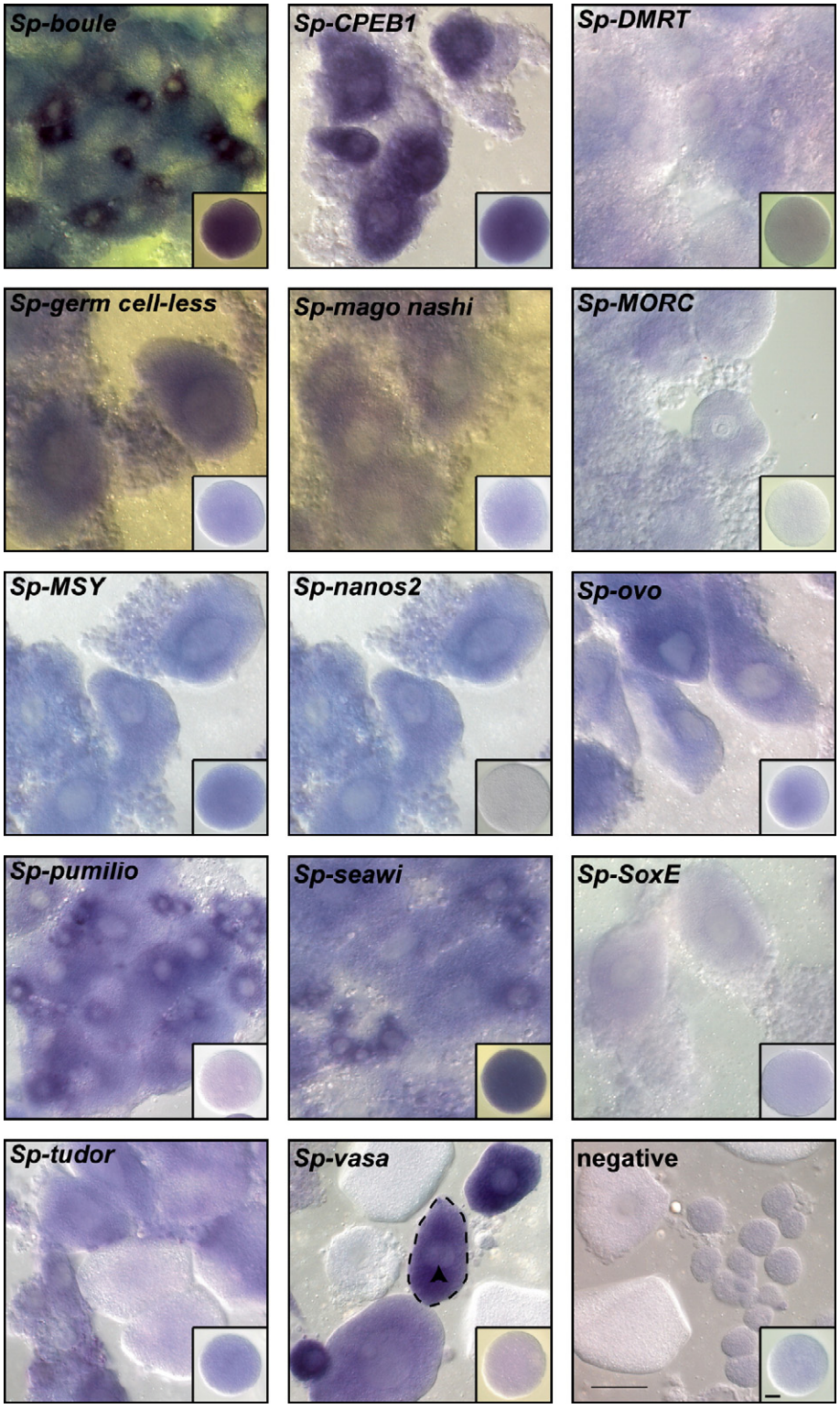

Fig. 2. Whole mount *in situ* RNA hybridizations of *S. purpuratus* ovaries and mature eggs (insets) were performed for germ-line gene homologs (Table 1). All transcripts accumulate uniformly in the oocytes throughout oogenesis and no subcellular localization is detected in the mature egg. In the Sp-vasa 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

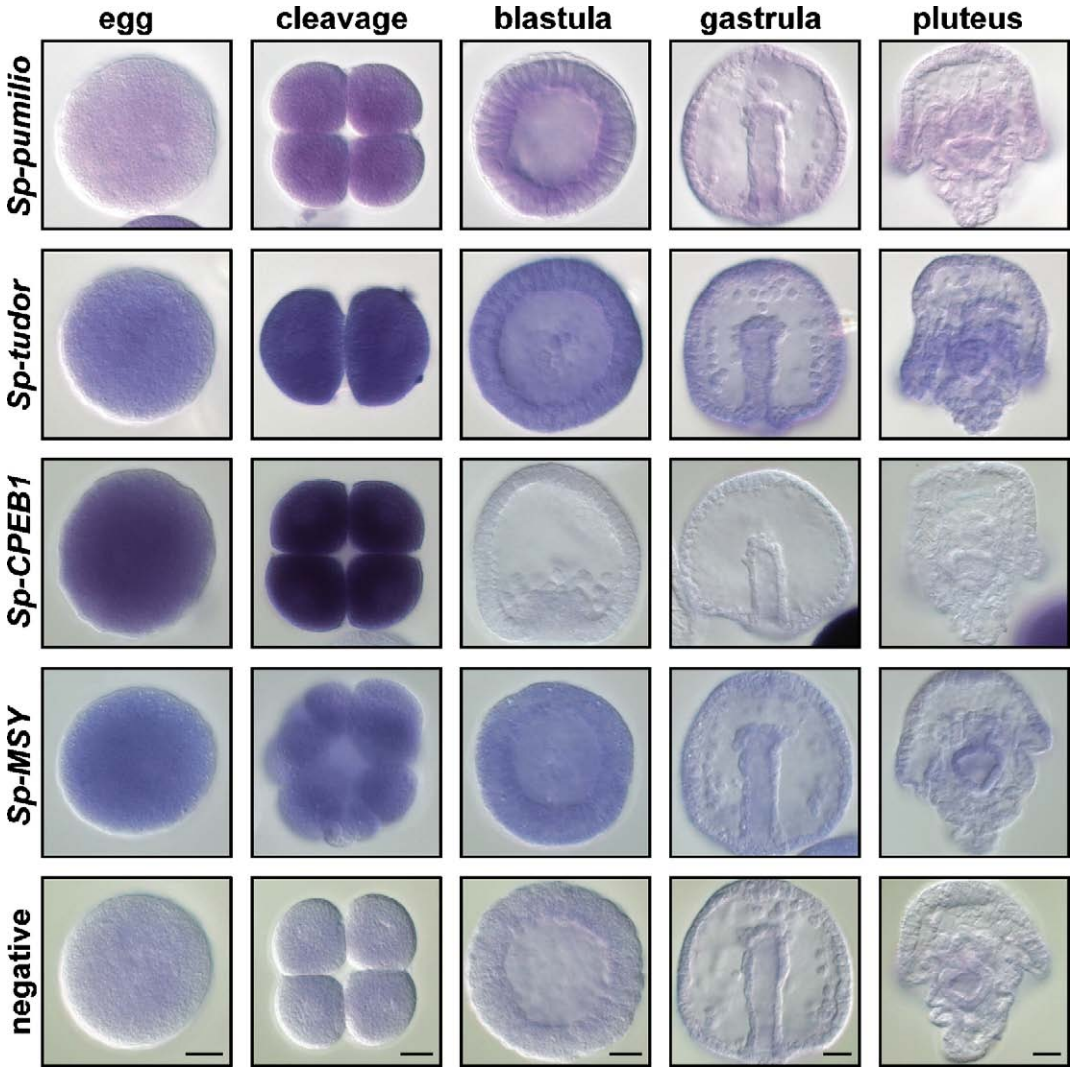

Fig. 3. *Sp-pumilio*, *Sp-tudor*, *Sp-CPEB1*, and *Sp-MSY* whole mount *in situ* RNA hybridizations of *S. purpuratus* eggs and embryos at the indicated stages. All transcripts accumulate at high levels uniformly in cleavage stage embryos. *Sp-CPEB1* transcripts are undetectable in blastula stage embryos 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

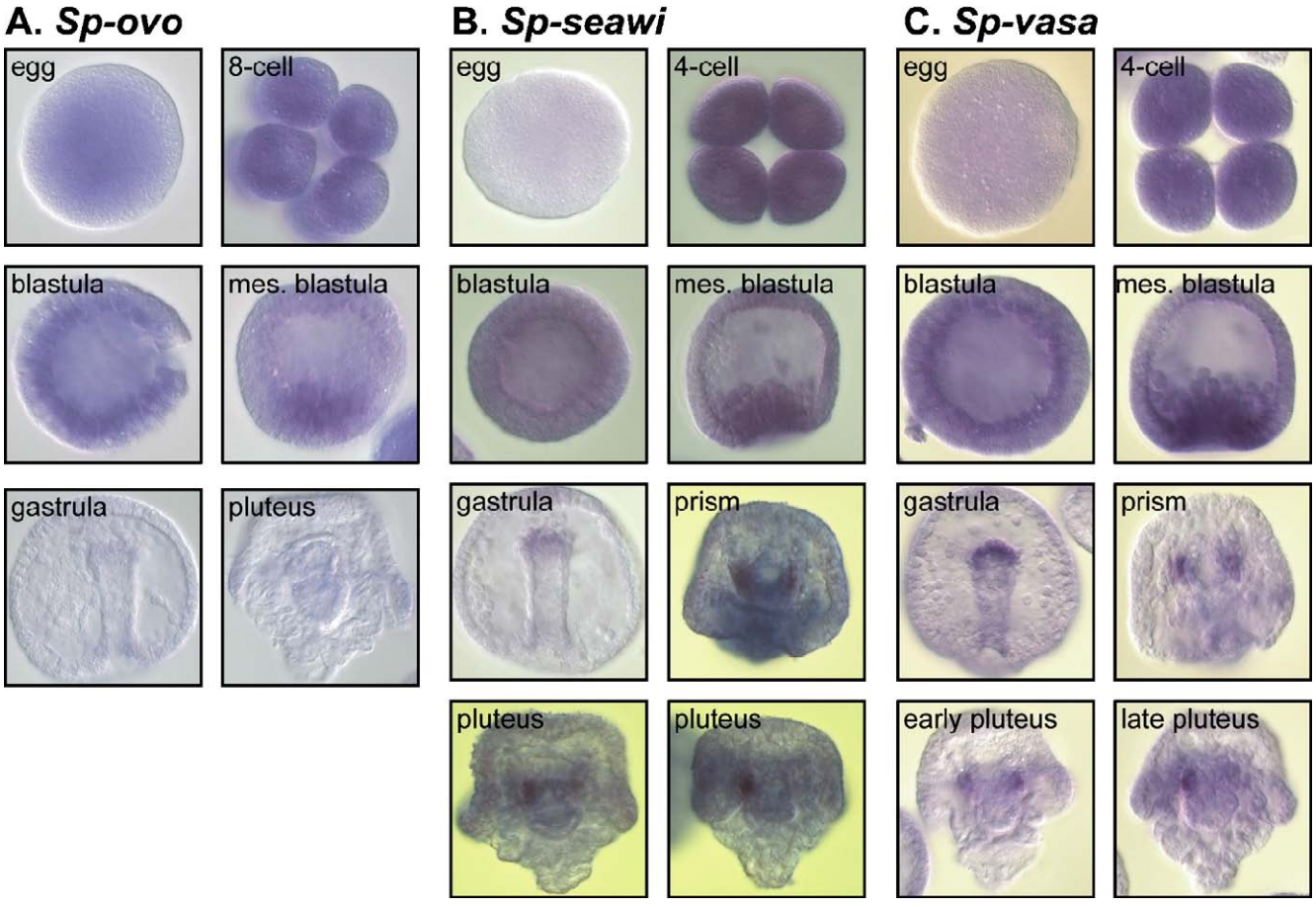

Fig. 4. (A) *Sp-ovo*, (B) *Sp-seawi*, and (C) *Sp-vasa* whole mount *in situ* RNA hybridizations of *S. purpuratus* eggs and embryos at the indicated stages. All transcripts accumulate uniformly at high levels through the blastula stage and then become enriched at the vegetal plate of mesenchyme blastula 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; Komiya 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.,

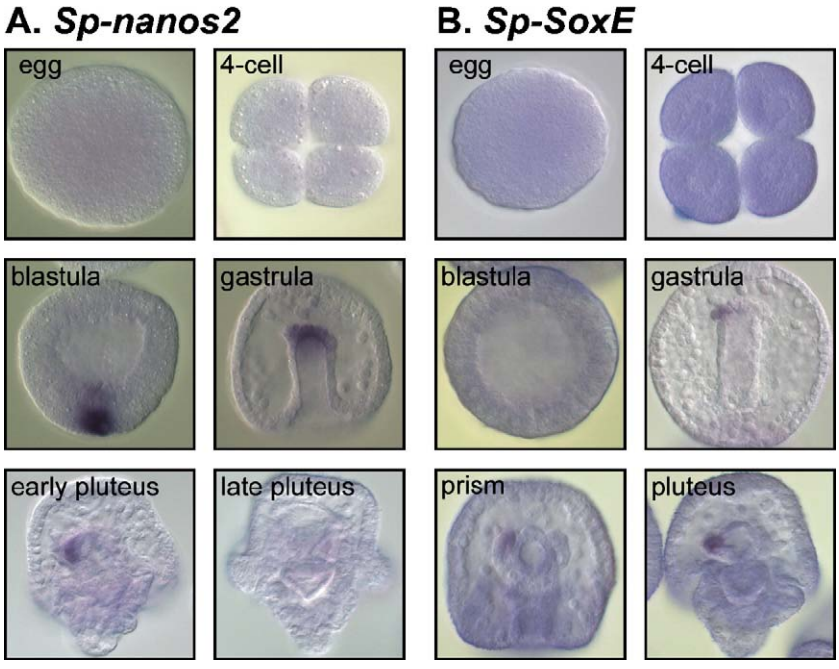

Fig. 5. (A) *Sp-nanos2* and (B) *Sp-SoxE* whole mount *in situ* RNA hybridizations of *S. purpuratus* eggs and embryos at the indicated stages. All transcripts are undetectable in cleavage stage embryos. *Sp-nanos2* transcripts are locally enriched in a subset of cells at the vegetal plate in blastula stage embryos, the small micromere descendents at the tip of the archenteron in gastrula stage embryos, and in the left coelomic pouch of the early pluteus. *Sp-SoxE* transcripts accumulate in the small micromere descendents at the tip of the archenteron in gastrula stage embryos and in the left coelomic pouch of the pluteus.

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 Rodriguez 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.

Acknowledgments

We thank Julian Wong for imaging assistance, and all current and past members of PRIMO for helpful discussions. This work was supported by grants from the NIH and the NSF.

References

Arenas-Mena, C., Cameron, A.R., Davidson, E.H., 2000. Spatial expression of Hox cluster genes in the ontogeny of a sea urchin. Development 127, 4631–4643.

Braat, A.K., Speksnijder, J.E., Zivkovic, D., 1999. Germ line development in fishes. Int. J. Dev. Biol. 43, 745–760.

Bruskin, A.M., Tyner, A.L., Wells, D.E., Showman, R.M., Klein, W.H., 1981. Accumulation in embryogenesis of five mRNAs enriched in the ectoderm of the sea urchin pluteus. Dev. Biol. 87, 308–318.

Castrillon, D.H., Quade, B.J., Wang, T.Y., Quigley, C., Crum, C.P., 2000. The human VASA gene is specifically expressed in the germ cell lineage. Proc. Natl. Acad. Sci. U. S. A. 97, 9585–9590.

Chaboissier, M.C., Kobayashi, A., Vidal, V.I., Lutzkendorf, S., van de Kant, H.J., Wegner, M., de Rooij, D.G., Behringer, R.R., Schedl, A., 2004. Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development 131, 1891–1901.

Chang, J.S., Tan, L., Schedl, P., 1999. The Drosophila CPEB homolog, orb, is required for oskar protein expression in oocytes. Dev. Biol. 215, 91–106.

Chuma, S., Hiyoshi, M., Yamamoto, A., Hosokawa, M., Takamune, K., Nakatsuji, N., 2003. Mouse Tudor Repeat-1 (MTR-1) is a novel component of chromatoid bodies/nuages in male germ cells and forms a complex with snRNPs. Mech. Dev. 120, 979–990.

Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., Lin, H., 1998. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. 12, 3715–3727.

Dai, X., Schonbaum, C., Degenstein, L., Bai, W., Mahowald, A., Fuchs, E., 1998. The ovo gene required for cuticle formation and oogenesis in flies is involved in hair formation and spermatogenesis in mice. Genes Dev. 12, 3452–3463.

Deppe, U., Schierenberg, E., Cole, T., Krieg, C., Schmitt, D., Yoder, B., von Ehrenstein, G., 1978. Cell lineages of the embryo of the nematode *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. 75, 376–380.

Duboc, V., Rottinger, E., Lapraz, F., Besnardeau, L., Lepage, T., 2005. Left–right asymmetry in the sea urchin embryo is regulated by nodal signaling on the right side. Dev. Cell 9, 147–158.

Ephrussi, A., Lehmann, R., 1992. Induction of germ cell formation by oskar. Nature 358, 387–392.

Extavour, C.G., Akam, M., 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 130, 5869–5884.

Fujii, T., Mitsunaga-Nakatsubo, K., Saito, I., Iida, H., Sakamoto, N., Akasaka, K., Yamamoto, T., 2006. Developmental expression of HpNanos, the *Hemicentrotus pulcherrimus* homologue of nanos. Gene Expr. Patterns 6, 572–577.

Fujiwara, Y., Komiya, T., Kawabata, H., Sato, M., Fujimoto, H., Furusawa, M., Noce, T., 1994. Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila vasa* and its specific expression in germ cell lineage. Proc. Natl. Acad. Sci. U. S. A. 91, 12258–12262.

Gebauer, F., Richter, J.D., 1996. Mouse cytoplasmic polyadenylylation element binding protein: an evolutionarily conserved protein that interacts with the cytoplasmic polyadenylylation elements of c-mos mRNA. Proc. Natl. Acad. Sci. U. S. A. 93, 14602–14607.

Giorgini, F., Davies, H.G., Braun, R.E., 2002. Translational repression by MSY4 inhibits spermatid differentiation in mice. Development 129, 3669–3679.

Gruidl, M.E., Smith, P.A., Kuznicki, K.A., McCrone, J.S., Kirchner, J., Roussell, D.L., Strome, S., Bennett, K.L., 1996. Multiple potential germ-line helicases are components of the germ-line-specific P granules of *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. 93, 13837–13842.

Hake, L.E., Richter, J.D., 1994. CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. Cell 79, 617–627.

Hay, B., Jan, L.Y., Jan, Y.N., 1988. A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell 55, 577–587.

Heasman, J., Quarmby, J., Wylie, C.C., 1984. The mitochondrial cloud of Xenopus oocytes: the source of germinal granule material. Dev. Biol. 105, 458–469.

Houk, M.S., Hinegardner, R.T., 1980. The formation and early differentiation of sea urchin gonads. Biol. Bull. 159, 280–294.

Ikenishi, K., Nakazato, S., Okuda, T., 1986. Direct evidence for the presence of germ cell determinant in vegetal pole cytoplasm of *Xenopus laevis* and in a subcellular fraction of it. Dev. Growth Differ. 28, 563–568.

Illmensee, K., Mahowald, A.P., 1974. Transplantation of posterior polar plasm in Drosophila. Induction of germ cells at the anterior pole of the egg. Proc. Natl. Acad. Sci. U. S. A. 71, 1016–1020.

Illmensee, K., Mahowald, A.P., Loomis, M.R., 1976. The ontogeny of germ plasm during oogenesis in Drosophila. Dev. Biol. 49, 40–65.

Inoue, N., Hess, K.D., Moreadith, R.W., Richardson, L.L., Handel, M.A., Watson, M.L., Zinn, A.R., 1999. New gene family defined by MORC, a nuclear protein required for mouse spermatogenesis. Hum. Mol. Genet. 8, 1201–1207.

Jaruzelska, J., Kotecki, M., Kusz, K., Spik, A., Firpo, M., Reijo Pera, R.A., 2003. Conservation of a Pumilio–Nanos complex from Drosophila germ plasm to human germ cells. Dev. Genes Evol. 213, 120–126.

Johnson, A.D., Crother, B., White, M.E., Patient, R., Bachvarova, R.F., Drum, M., Masi, T., 2003a. Regulative germ cell specification in axolotl embryos: A primitive trait conserved in the mammalian lineage. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 358, 1371–1379.

Johnson, A.D., Drum, M., Bachvarova, R.F., Masi, T., White, M.E., Crother, B.I., 2003b. Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution. Evolut. Develop. 5, 414–431.

Kashikawa, M., Amikura, R., Nakamura, A., Kobayashi, S., 1999. Mitochondrial small ribosomal RNA is present on polar granules in early cleavage embryos of *Drosophila melanogaster*. Dev. Growth Differ. 41, 495–502.

Kashikawa, M., Amikura, R., Kobayashi, S., 2001. Mitochondrial small ribosomal RNA is a component of germinal granules in Xenopus embryos. Mech. Dev. 101, 71–77.

Kawasaki, I., Shim, Y.H., Kirchner, J., Kaminker, J., Wood, W.B., Strome, S., 1998. PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans. Cell 94, 635–645.

Kent, J., Wheatley, S.C., Andrews, J.E., Sinclair, A.H., Koopman, P., 1996. A male-specific role for SOX9 in vertebrate sex determination. Development 122, 2813–2822.

Kobayashi, S., Yamada, M., Asaoka, M., Kitamura, T., 1996. Essential role of the posterior morphogen nanos for germline development in Drosophila. Nature 380, 708–711.

Komiya, T., Itoh, K., Ikenishi, K., Furusawa, M., 1994. Isolation and characterization of a novel gene of the DEAD box protein family which is specifically expressed in germ cells of *Xenopus laevis*. Dev. Biol. 162, 354–363.

Koprunner, M., Thisse, C., Thisse, B., Raz, E., 2001. A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes Dev. 15, 2877–2885.

Kuramochi-Miyagawa, S., Kimura, T., Yomogida, K., Kuroiwa, A., Tadokoro, Y., Fujita, Y., Sato, M., Matsuda, Y., Nakano, T., 2001. Two mouse piwi-related genes: miwi and mili. Mech. Dev. 108, 121–133.

Lawson, K.A., Hage, W.J., 1994. Clonal analysis of the origin of primordial germ cells in the mouse. Ciba Found Symp. 182, 68–84 (discussion 84–91).

Leatherman, J.L., Kaestner, K.H., Jongens, T.A., 2000. Identification of a mouse germ cell-less homologue with conserved activity in Drosophila. Mech. Dev. 92, 145–153.

MacArthur, H., Bubunenko, M., Houston, D.W., King, M.L., 1999. Xcat2 RNA is a translationally sequestered germ plasm component in Xenopus. Mech. Dev. 84, 75–88.

MacBride, E.W., 1903. The development of *Echinus esculentus*, together with some points in the development of *E. miliaris* and *E. acutus*. Philos. Trans. R. Soc. Lond., B Biol. Sci. 195, 285–327.

Mevel-Ninio, M., Terracol, R., Kafatos, F.C., 1991. The ovo gene of Drosophila encodes a zinc finger protein required for female germ line development. EMBO J. 10, 2259–2266.

Mochizuki, K., Sano, H., Kobayashi, S., Nishimiya-Fujisawa, C., Fujisawa, T., 2000. Expression and evolutionary conservation of nanos-related genes in Hydra. Dev. Genes Evol. 210, 591–602.

Mosquera, L., Forristall, C., Zhou, Y., King, M.L., 1993. A mRNA localized to the vegetal cortex of Xenopus oocytes encodes a protein with a nanos-like zinc finger domain. Development 117, 377–386.

Newmark, P.A., Boswell, R.E., 1994. The mago nashi locus encodes an essential product required for germ plasm assembly in Drosophila. Development 120, 1303–1313.

Ogawa, M., Amikura, R., Akasaka, K., Kinoshita, T., Kobayashi, S., Shimada, H., 1999. Asymmetrical distribution of mitochondrial rRNA into small micromeres of sea urchin embryos. Zool. Sci. 16, 445–451.

Oliver, B., Perrimon, N., Mahowald, A.P., 1987. The ovo locus is required for sex-specific germ line maintenance in Drosophila. Genes Dev. 1, 913–923.

Pehrson, J.R., Cohen, L.H., 1986. The fate of the small micromeres in sea urchin development. Dev. Biol. 113, 522–526.

Ransick, A., Cameron, R.A., Davidson, E.H., 1996. Postembryonic segregation of the germ line in sea urchins in relation to indirect development. Proc. Natl. Acad. Sci. U. S. A. 93, 6759–6763.

Rodriguez, A.J., Seipel, S.A., Hamill, D.R., Romancino, D.P., DiCarlo, M., Suprenant, K.A., Bonder, E.M., 2005. Seawi—A sea urchin piwi/argonaute family member is a component of MT-RNP complexes. RNA 11, 646–656.

Strome, S., Wood, W.B., 1982. Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. 79, 1558–1562.

Styhler, S., Nakamura, A., Swan, A., Suter, B., Lasko, P., 1998. vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development. Development 125, 1569–1578.

Subramaniam, K., Seydoux, G., 1999. nos-1 and nos-2, two genes related to Drosophila nanos, regulate primordial germ cell development and survival in *Caenorhabditis elegans*. Development 126, 4861–4871.

Tam, P.P., Zhou, S.X., 1996. The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. Dev. Biol. 178, 124–132.

Tan, C.H., Lee, T.C., Weeraratne, S.D., Korzh, V., Lim, T.M., Gong, Z., 2002. Ziwi, the zebrafish homologue of the Drosophila piwi: co-localization with vasa at the embryonic genital ridge and gonad-specific expression in the adults. Mech. Dev. 119 (Suppl. 1), S221–S224.

Tanaka, S., Dan, K., 1990. Study of the lineage and cell cycle of small micromeres in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. Dev. Growth Differ. 32, 145–156.

Thomson, T., Lasko, P., 2004. Drosophila tudor is essential for polar granule assembly and pole cell specification, but not for posterior patterning. Genesis 40, 164–170.

Tomancak, P., Guichet, A., Zavorszky, P., Ephrussi, A., 1998. Oocyte polarity depends on regulation of gurken by Vasa. Development 125, 1723–1732.

Tsuda, M., Sasaoka, Y., Kiso, M., Abe, K., Haraguchi, S., Kobayashi, S., Saga, Y., 2003. Conserved role of nanos proteins in germ cell development. Science 301, 1239–1241.

Watson, M.L., Zinn, A.R., Inoue, N., Hess, K.D., Cobb, J., Handel, M.A., Halaban, R., Duchene, C.C., Albright, G.M., Moreadith, R.W., 1998. Identification of morc (microrchidia), a mutation that results in arrest of spermatogenesis at an early meiotic stage in the mouse. Proc. Natl. Acad. Sci. U. S. A. 95, 14361–14366.

Whitington, P.M., Dixon, K.E., 1975. Quantitative studies of germ plasm and germ cells during early embryogenesis of *Xenopus laevis*. J. Embryol. Exp. Morphol. 33, 57–74.

Yoon, C., Kawakami, K., Hopkins, N., 1997. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 124, 3157–3165.

Zust, B., Dixon, K.E., 1975. The effect of u.v. irradiation of the vegetal pole of *Xenopus laevis* eggs on the presumptive primordial germ cells. J. Embryol. Exp. Morphol. 34, 209–220.